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13. ABSTRACT (Maximum 200) Cyclophosphamide is widely used to kill breast cancer cells that detach from primary tumors prior to the surgical removal of the latter and that relocate in various parts of the body where they usually cannot be detected and where they multiply. Unfortunately, even though cyclophosphamide is amongst the best available drugs for this purpose, its use rarely results in cures because, amongst these relocated cancer cells, there are, or soon are, some that can defend themselves against cytoxan and therefore are not killed by it. The objective of our studies is to find out how such cells do this and, then, to develop strategies that would eliminate, negate or circumvent the defensive measures used by them to survive. Thus far, our investigations suggest/support the following: 1) prognostic determinants that can be used to identify patients for whom cyclophosphamide therapy would prove to be futile include cellular levels of ALDH-1 and, perhaps, ALDH-3, 2) clinical ALDH-3-mediated breast cancer cell resistance to cyclophosphamide may be overcome by the co-administration of gossypol or other drugs that inhibit ALDH-3, and 3) consistent with existing information, but yet to be unequivocally established, it may be prudent to avoid certain dietary constituents when taking cyclophosphamide because they may decrease the sensitivity of breast cancer cells to it by inducing ALDH-3 in these cells.				
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ABBREVIATIONS

- ALDH-1: human cytosolic class 1 aldehyde dehydrogenase
rALDH-1: recombinant ALDH-1
ALDH-2: human mitochondrial class 2 aldehyde dehydrogenase
rALDH-2: recombinant ALDH-2
ALDH-3: human cytosolic class 3 aldehyde dehydrogenase
nALDH-3: "normal" ALDH-3, viz., ALDH-3 purified from human normal stomach mucosa
tALDH-3 "tumor" ALDH-3, viz., ALDH-3 purified from human breast adenocarcinoma MCF-7/0/CAT cells
- pan-GST: pan-glutathione S-transferase
GST α : glutathione S-transferase α
GST μ : glutathione S-transferase μ
GST π : glutathione S-transferase π
- DT-D: DT-diaphorase; NAD(P)H:quinone oxidoreductase
UDP-GT: UDP-glucuronosyl transferase
CYP 1A1: cytochrome P450 1A1
- GSH: glutathione
TCDD: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin
- MCF-7/0 cells: cultured human breast adenocarcinoma cells
MCF-7/0/MC cells: MCF-7/0 cells cultured in the presence of 3 μ M 3-methylcholanthrene for 5 days to transiently induce ALDH-3
MCF-7/0/CAT cells: MCF-7/0 cells cultured in the presence of 30 μ M catechol for 5 days to transiently induce ALDH-3
MCF-7/0/BP cells: MCF-7/0 cells surviving continuous exposure to increasing concentrations of benz(a)pyrene over a period of several months and stably overexpressing ALDH-3
MCF-7/0/MAF cells: MCF-7/0 cells surviving exposure to 1 mM mafosfamide for 30 minutes and stably overexpressing ALDH-3
MCF-7/OAP cells: MCF-7/0 cells surviving continuous exposure to increasing concentrations of 4-hydroxycyclophosphamide over a period of several months and stably overexpressing ALDH-3
- ARE: antioxidant responsive element
ARE-B: battery of enzymes that have in common an ARE in the 5'-upstream regions of the genes that codes for them
XRE: xenobiotic responsive element
XRE-B: battery of enzymes that have in common an XRE in the 5'-upstream regions of the genes that codes for them
- AhR: aromatic hydrocarbon receptor
AhR⁺: AhR-positive
ER: estrogen receptor
ER⁺: ER-positive
ER⁻: ER-negative
- API-2: 4-chloro-*N*-[(propylamino)carbonyl]benzenesulfonamide
NPI-2: (acetyloxy)[(4-chlorophenyl)sulfonyl]carbamic acid 1,1-dimethylethyl ester
NPI-4: 4-chloro-*N,O*-bis(ethoxycarbonyl)-*N*-hydroxybenzenesulfonamide
NPI-5: *N,O*-bis(methoxycarbonyl)-*N*-hydroxymethanesulfonamide
NPI-6: 2-[(ethoxycarbonyl)oxy]-1,2-benzisothiazol-3(2*H*)-one 1,1-dioxide

INTRODUCTION

Cyclophosphamide, mafosfamide and 4-hydroperoxycyclophosphamide are antineoplastic agents collectively referred to as oxazaphosphorines [Sladek, 1994]. Each of these is a prodrug, i.e., per se, without cytotoxic activity. Salient features of the metabolic activation of oxazaphosphorines are presented in Figure 1. Oxazaphosphorines are clinically effective; they play a lead role in the treatment of breast cancer until resistant subpopulations become the dominant population. An understanding of how resistance to these agents is effected would likely be of value because measures may then become apparent as to how to reverse and/or prevent it. It is this understanding which is the overall objective of our first-generation investigations.

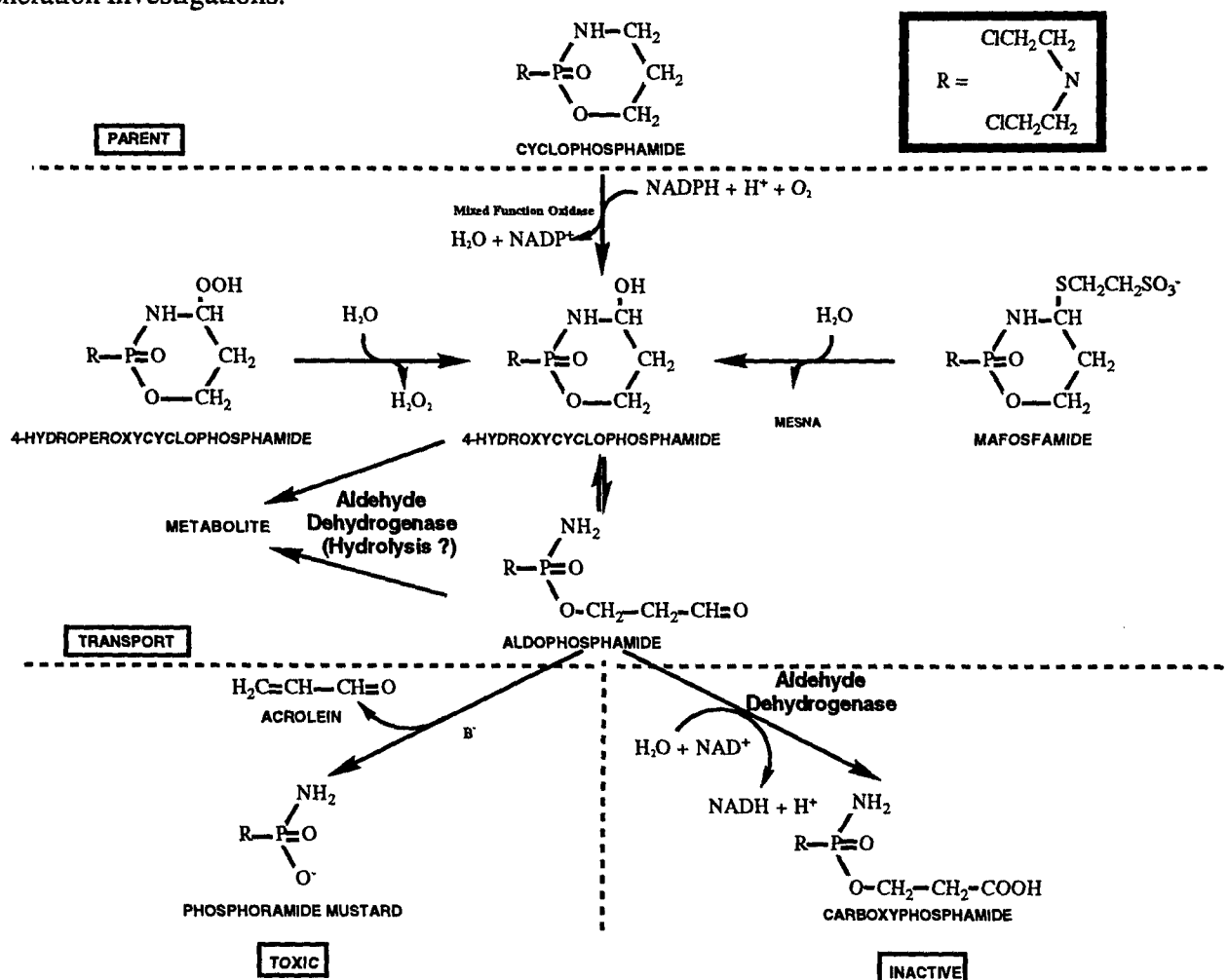


Figure 1. Salient features of oxazaphosphorine metabolism. The prodrugs, cyclophosphamide, mafosfamide and 4-hydroperoxycyclophosphamide, each give rise to 4-hydroxycyclophosphamide which exists in equilibrium with its ring-opened tautomer, aldophosphamide. 4-Hydroxycyclophosphamide and aldophosphamide are, themselves, also without cytotoxic activity. However, aldophosphamide gives rise to acrolein and phosphoramidate mustard, each of which is cytotoxic; the latter effects the bulk of the therapeutic action effected by the oxazaphosphorines [Sladek, 1994]. Alternatively, aldophosphamide can be further oxidized to carboxyphosphamide by certain aldehyde dehydrogenases [Manthey et al., 1990; Dockham et al., 1992; Sreerama and Sladek, 1993a, 1994; Sladek, 1994]. Carboxyphosphamide is without cytotoxic activity nor does it give rise to a cytotoxic metabolite. Aldehyde dehydrogenase-catalyzed oxidation of aldophosphamide to carboxyphosphamide is, therefore, properly viewed as an enzyme-catalyzed detoxification of the oxazaphosphorines. Aldehyde dehydrogenase-catalyzed hydrolysis of 4-hydroxycyclophosphamide and/or aldophosphamide to an inactive metabolite is also shown but is only a possibility, i.e., it is yet to be demonstrated.

Most pertinent to these investigations is the irreversible detoxification that occurs when NAD(P)-dependent aldehyde dehydrogenases catalyze the oxidation of a pivotal metabolite, viz., aldophosphamide, to carboxyphosphamide, Figure 1. Human class-1, -2 and -3 aldehyde dehydrogenases, viz., ALDH-1, ALDH-2 and ALDH-3, respectively, as well as succinic semialdehyde dehydrogenase, all catalyze the oxidation of aldophosphamide to carboxyphosphamide, but not equally well [Dockham et al., 1992; Sladek, 1993, 1994; Sreerama and Sladek, 1993a, 1994].

Aldehyde dehydrogenases are bifunctional enzymes in that they catalyze not only the oxidation of aldehydes, but also the hydrolysis of ester bonds. Several such bonds are present in 4-hydroxycyclophosphamide and aldophosphamide. Whether aldehyde dehydrogenases catalyze the hydrolysis of either of these intermediates to an irreversibly inactive metabolite is not known.

Using cultured human breast adenocarcinoma MCF-7/0 cells and two oxazaphosphorine-resistant sublines derived therefrom, viz., MCF-7/OAP (stable resistance achieved by growing the parent MCF-7/0 cells in the presence of continuously increasing concentrations of 4-hydroperoxycyclophosphamide for many months [Frei et al., 1988]) and MCF-7/0/MC (transient resistance achieved by growing the parent MCF-7/0 cells in the presence of a polycyclic aromatic hydrocarbon, e.g., 3 μ M 3-methylcholanthrene, for 5 days [Sreerama and Sladek, 1993b, 1994]), we have demonstrated that ALDH-3 is an important determinant of cellular sensitivity to the oxazaphosphorines [Sreerama and Sladek 1993a,b, 1994]. MCF-7 cells do not contain any of the mixed function oxidases that activate cyclophosphamide, Figure 1. Thus, we used mafosfamide and/or 4-hydroperoxycyclophosphamide rather than cyclophosphamide in all of these and other experiments with cultured MCF-7 cells because these agents, like cyclophosphamide, give rise to 4-hydroxycyclophosphamide, but they do so in the absence of any enzyme involvement, Figure 1.

The above investigations led us to hypothesize that 1) clinical breast cancer cellular resistance to cyclophosphamide and other oxazaphosphorines is the consequence of elevated ALDH-3 levels, 2) ALDH-3 mediates cellular resistance to oxazaphosphorines by catalyzing the oxidative and/or hydrolytic detoxification of these agents, 3) inhibitors of the detoxifying reaction can be identified and utilized to reverse the resistance, 4) hypomethylation of ALDH-3 genomic DNA accounts for oxazaphosphorine- and activated Ah receptor-induced ALDH-3 overexpression, 5) activated Ah receptor-induced ALDH-3 overexpression can only occur in cells that are estrogen receptor-positive, and 6) agents known to induce xenobiotic-metabolizing enzymes via the antioxidant responsive element (ARE) will also induce ALDH-3 overexpression, since the ARE consensus sequence is present in the 5'-flanking region of the ALDH-3 gene. Testing of these hypotheses was divided into seven tasks (statement of work):

Task # 1: Quantify cellular ALDH-3 levels in surgically removed human breast tumor samples.

Task # 2: Ascertain the ability of ALDH-3s to catalyze the oxidative and/or hydrolytic detoxification of cyclophosphamide (aldophosphamide) at a rate sufficient to account for the oxazaphosphorine-specific acquired resistance exhibited in our model systems.

- Task # 3: Synthesize and identify agents that inhibit the ALDH-3-catalyzed oxidative and/or hydrolytic detoxification of cyclophosphamide (aldophosphamide).
- Task # 4: Evaluate identified inhibitors of the relevant ALDH-3 activity with respect to their ability to sensitize our oxazaphosphorine-resistant models to the oxazaphosphorines.
- Task # 5: Identify the molecular basis for the apparent overexpression of ALDH-3s in our model systems.
- Task # 6: Ascertain the ability of Ah receptor ligands to induce ALDH-3 overexpression and oxazaphosphorine-specific acquired resistance in estrogen receptor-positive and -negative breast cancer cell lines that lack and express Ah receptors.
- Task # 7: Ascertain the ability of ligands for ARE to induce ALDH-3 activity and oxazaphosphorine-specific acquired resistance in our model system.

Repository breast tumor samples and culture models, viz., MCF-7/0, MCF-7/OAP and MCF-7/0/MC, were chosen to test the hypotheses delineated above. Methods/technology to be used in testing the above-listed hypotheses include immunocytochemistry, ELISA, ultracentrifugation, density-gradient centrifugation, column and thin-layer chromatography, HPLC, spectrophotometry to monitor catalytic rates, synthetic organic chemistry, cell culture and colony-forming assays, RT-PCR, Northern and Southern blot analysis, methylation-sensitive restriction enzyme diagnosis and receptor binding assays.

Results of investigations conducted in months 25 through 36 and directed towards completing the seven (original) tasks are summarized in the text that follows. They and summaries of findings made in months 1 through 24 (details presented in the two previous annual reports) will support the following:

Original Tasks	Original Timetable (months)	Progress
1	1 - 48	Nearly completed
2	1 - 24	Completed
3	1 - 24	Completed
4	6 - 30	Completed
5	24 - 48	Partially completed
6	12 - 42	Nearly completed
7	36 - 48	Completed

Additionally, several new tasks were identified and undertaken. Each was either an extension of, or complementary to, one of the old tasks, or was prompted by the findings of one of the old tasks. Results

of these investigations are also included in the text under the following headings (numbering is according to the original task that gave rise to the new task):

- Task # 1a: Ascertain whether glutathione levels predict the clinical outcome of cyclophosphamide therapy.
- Task # 1b: Ascertain whether simultaneous elevation of ALDH-3, glutathione S-transferase and DT-diaphorase, or of these three enzymes and cytochrome P-450 1A1, occurred in any of the breast tumor samples, and, if so, whether this was due to chance alone or, perhaps, to coordinated induction of these enzymes.
- Task # 1c: Ascertain whether breast tumor ALDH-3 levels parallel salivary ALDH-3 levels.
- Task # 7a: Ascertain whether limonene and/or any of several of its metabolites induce ALDH-3 levels in human breast adenocarcinoma MCF-7/0 cells.
- Task # 7b: Generate a stably cyclophosphamide-resistant MCF-7/0 subline by exposing MCF-7/0 cells to gradually increasing concentrations of benzpyrene.
- Task # 7c: Generate a stably cyclophosphamide-resistant MCF-7/0 subline by exposing MCF-7/0 cells once to a high concentration of mafosfamide for 30 minutes.

Results of investigations conducted in months 25 through 36 and directed towards completing the six added tasks are also summarized in the text that follows. They will support the following:

Added Tasks	Progress
1a	Nearly completed
1b	Completed
1c	Partially completed
7a	Completed
7b	Completed
7c	Completed

BODY

Task # 1: Quantify cellular ALDH-3 levels in surgically removed human breast tumor samples.

Cellular levels of ALDH-3 in 171 surgically removed primary (122) and metastatic (49) human breast tumor samples were semiquantified with the aid of immunocytochemical staining methodology. ALDH-1 is another known determinant of cellular sensitivity to the oxazaphosphorines [reviewed in Sladek, 1993]. Thus, ALDH-1 levels in breast tumor tissues, together with those of ALDH-3, are more likely to correlate with clinical outcome than are ALDH-3 levels alone. Hence, semiquantification of ALDH-1 levels was added to the original task.

The immunocytochemical staining methodology utilized to visualize and semiquantify ALDH-3 and ALDH-1 present in breast tumor tissue is described and illustrated in Figure 2. It was developed and standardized with the aid of frozen human liver and stomach mucosa samples, and several cultured cell lines, that contained known amounts of ALDH-3 and/or ALDH-1 activities. The optimized method proved to be highly sensitive and reproducible when used on frozen breast tumor tissue sections which usually contained significantly lower amounts of aldehyde dehydrogenase as compared to that contained by human liver and stomach mucosa.

Results of these measurements were presented in the year-2 progress report.

Briefly, mean \pm SD values for ALDH-3 and ALDH-1 values were 0.84 ± 0.82 and 1.40 ± 0.87 , respectively, in the primary breast cancer samples ($n = 122$). They were 1.31 ± 0.74 and 1.53 ± 0.79 , respectively, in the metastatic breast cancer samples ($n = 49$). Metastatic breast cancer ALDH-3 levels were statistically greater ($P = 0.006$) than primary breast cancer levels; metastatic breast cancer ALDH-1 levels were not ($P = 0.18$).

Further analysis of the data is presented herein.

Available to us were the archived medical records that had been generated for 20 of the 122 primary tumor sample donors and for all 49 of the metastatic tumor sample donors. Patient and tumor characteristics, treatments, and treatment outcomes were obtained from these records.

There was no correlation between ALDH-3 and ALDH-1 levels, nor between either enzyme level and the patient's age or estrogen or progesterone receptor status.

Only seven of the 20 primary tumor samples for which the corresponding medical records were available were obtained from patients who were subsequently treated with a chemotherapeutic regimen that included cyclophosphamide. Only four of these seven were disease-free for at least two years. Mean ALDH-3 and ALDH-1 levels in the primary breast tumor samples obtained from these patients were 0.75 and 0.75, respectively. They were 1.33 and 1.33, respectively, in the three primary breast tumor samples obtained from patients that did not remain disease-free for at least two years.

Most of the metastatic tumors were obtained from patients who had been, and/or were going to be, treated with chemotherapeutic agents, most commonly, cyclophosphamide, adriamycin, methotrexate, 5-fluorouracil and/or vincristine. Usually, these agents were given in one of several combinations.

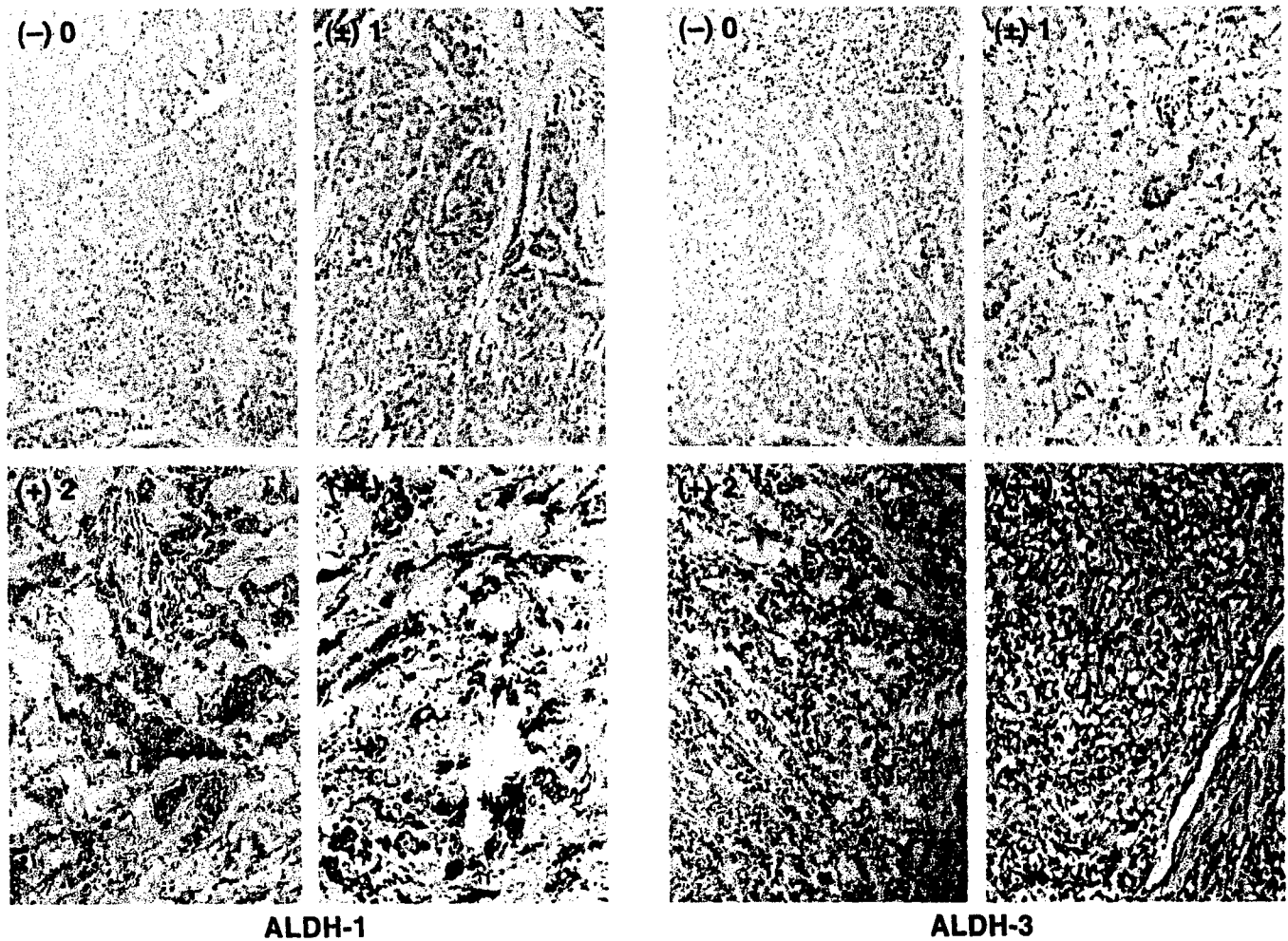


Figure 2. Immunocytochemical visualization/quantification of aldehyde dehydrogenase in breast malignancies. Human breast cancer tissue samples had been stored in liquid nitrogen from the time of biopsy; in many cases, associated medical charts were available for review. Immunocytochemical staining was of formalin-fixed 4 μ m sections. Blocking was with, successively, hydrogen peroxide, goat serum, avidin, biotin and BSA. Primary antibodies were chicken anti-ALDH-1 IgY and anti-ALDH-3 IgY. The secondary antibody was biotinylated goat anti-chicken IgG. Binding to the secondary antibody was with an avidin/biotinylated peroxidase complex. Peroxidase-catalyzed oxidation of diaminobenzidine tetrahydrochloride to an insoluble, intensely brown, metabolite was used to visualize the enzymes of interest. Tissue samples were lightly counter stained with hematoxylin to ensure visualization of all cells. Dehydration was with ethanol and xylene. Mounting was with Permount. Scoring intensities were rated on a 0 to 3 scale: no visible staining was scored as 0; borderline, faint staining was scored as 1; and clearly visible, progressively intense, staining was scored as 2 and 3. Medical charts were reviewed only after staining intensities had been scored. Microscope magnification was 100x.

Mean ALDH-3 and ALDH-1 levels were somewhat higher in metastatic tumor cells that survived exposure to cyclophosphamide-based chemotherapeutic regimens than were ALDH-3 and ALDH-1 levels in metastatic tumor cells that survived treatment strategies that did not include cyclophosphamide; the difference was not statistically significant in the case of ALDH-3 whereas it was in the case of ALDH-1, Figure 3. Enzyme levels were not altered by the inclusion of adriamycin, tamoxifen, or radiation in the therapeutic regimen.

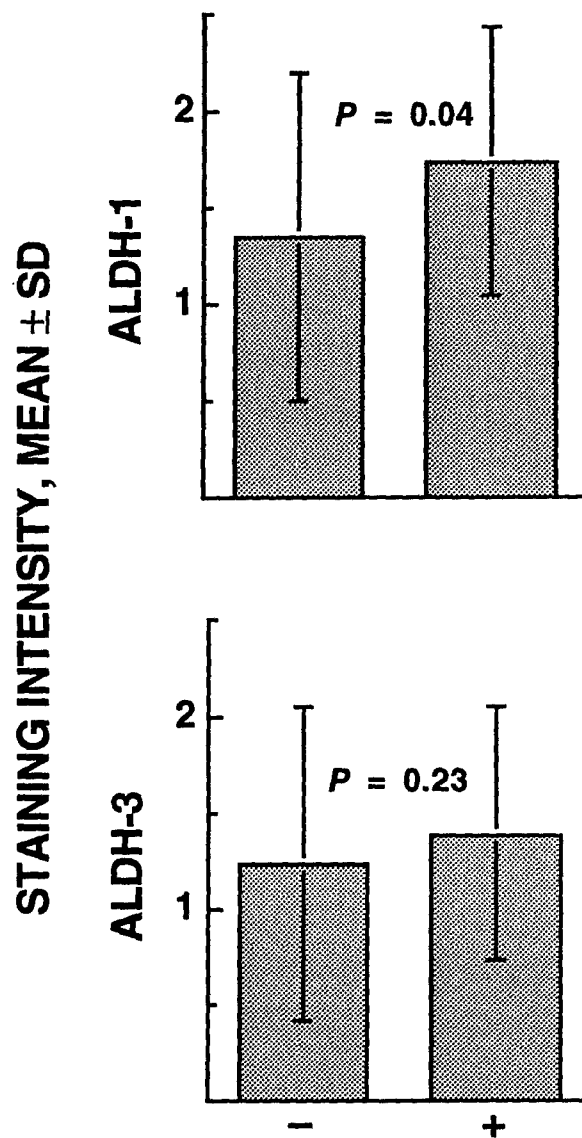


Figure 3. ALDH levels in human metastatic breast tumors obtained from patients that, earlier, had not (-; n = 26) and had (+; n = 23) been treated with cyclophosphamide-based chemotherapeutic regimens. Staining intensities were rated on a 0 to 3 scale: no visible staining was scored as 0; borderline, faint staining was scored as 1; and clearly visible, progressively intense, staining was scored as 2 and 3.

Mean ALDH-3 levels were higher in metastatic tumors that, upon subsequent treatment, "did not respond" (tumor size did not decrease or even increase) to cyclophosphamide-based chemotherapeutic regimens than were ALDH-3 levels in metastatic tumors that, upon subsequent treatment, "did respond" (tumor size decreased) to these regimens, but the difference was not statistically significant, Figure 4. Moreover, mean ALDH-3 levels were also higher by about the same amount in metastatic tumors that, upon subsequent treatment, "did not respond" to treatment strategies that did not include cyclophosphamide than were ALDH-3 levels in metastatic tumors that, upon subsequent treatment, "did respond" to these strategies, although this difference, too, was not statistically significant, Figure 4.

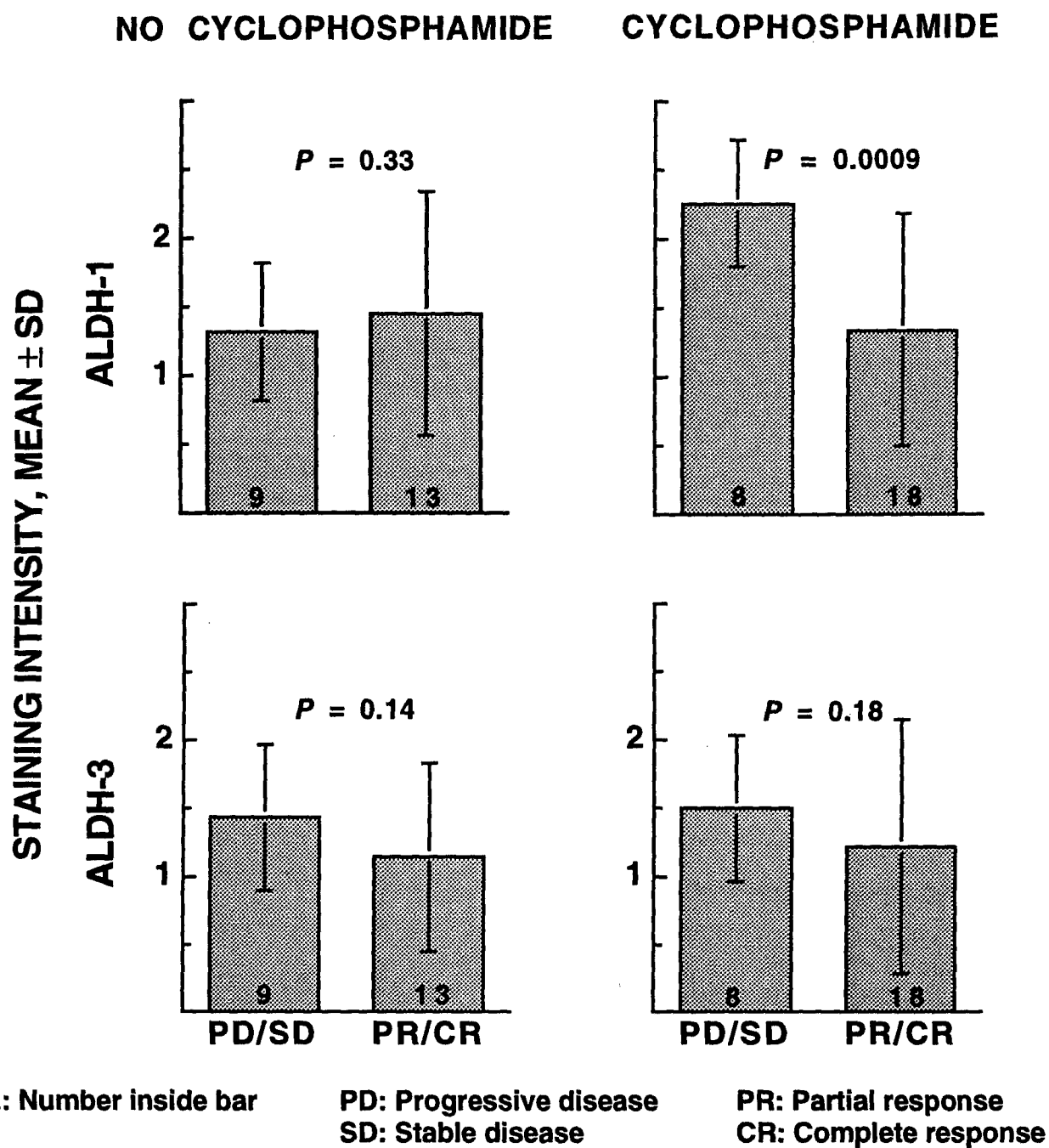


Figure 4. Relationship between ALDH levels in human metastatic breast tumors and response to subsequent treatment with cyclophosphamide-based chemotherapeutic regimens. Staining intensities were rated on a 0 to 3 scale: no visible staining was scored as 0; borderline, faint staining was scored as 1; and clearly visible, progressively intense, staining was scored as 2 and 3.

Mean ALDH-1 levels were statistically higher in metastatic tumors that, upon subsequent treatment, "did not respond" to cyclophosphamide-based chemotherapeutic regimens than were ALDH-1 levels in metastatic tumors that, upon subsequent treatment, "did respond" to these regimens, Figure 4. Mean ALDH-1 levels in metastatic tumors that, upon subsequent treatment, "did not respond" to treatment strategies that did not include cyclophosphamide were not statistically different from ALDH-1 levels in metastatic tumors that, upon subsequent treatment, "did respond" to these strategies, Figure 4.

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We also quantified ALDH-3 and ALDH-1, as well as pan-glutathione S-transferase (pan-GST), glutathione S-transferases α , μ and π (GSTs α , μ and π), and DT-diaphorase (DT-D), levels in normal (26) and malignant (90 primary and 22 metastatic) breast tissue samples procured from the Cooperative Human Tissue Network, Midwestern Division, Columbus, Ohio. Catalytic assays (spectrophotometric) and enzyme-linked immunosorbent assays (ELISAs) were used for this purpose [Sreerama and Sladek, 1997].

Results of these measurements were presented in the year-2 progress report.

Briefly, pan-GST, GSTs α , μ and π , and DT-D levels varied widely (56 - 8880, 0 - 2500, 0 - 3400, 100 - 6500 and 10 - 6250 mIU/g breast tissue, respectively), and, as in the investigations reported above, ALDH-3 and ALDH-1, each also varied widely (1 - 247 and 1 - 287 mIU/g breast tissue, respectively [Sreerama and Sladek, 1997]).

ALDH-3 and ALDH-1 levels in normal breast tissue samples predicted the respective levels of these enzymes in paired primary, as well as metastatic, breast tumor tissue samples, Figure 5. We did not have enough paired samples to ascertain whether cellular levels of ALDH-3 or ALDH-1 in primary breast malignancies predicted cellular levels of these enzymes in metastatic breast malignancies.

The Cooperative Human Tissue Network, Midwestern Division, has yet to collect and provide us with information as to how the specimen donors were subsequently treated and the clinical responses thereto. If and when they do, clinical responses to cyclophosphamide as a function of ALDH-3 and ALDH-1 levels will be evaluated.

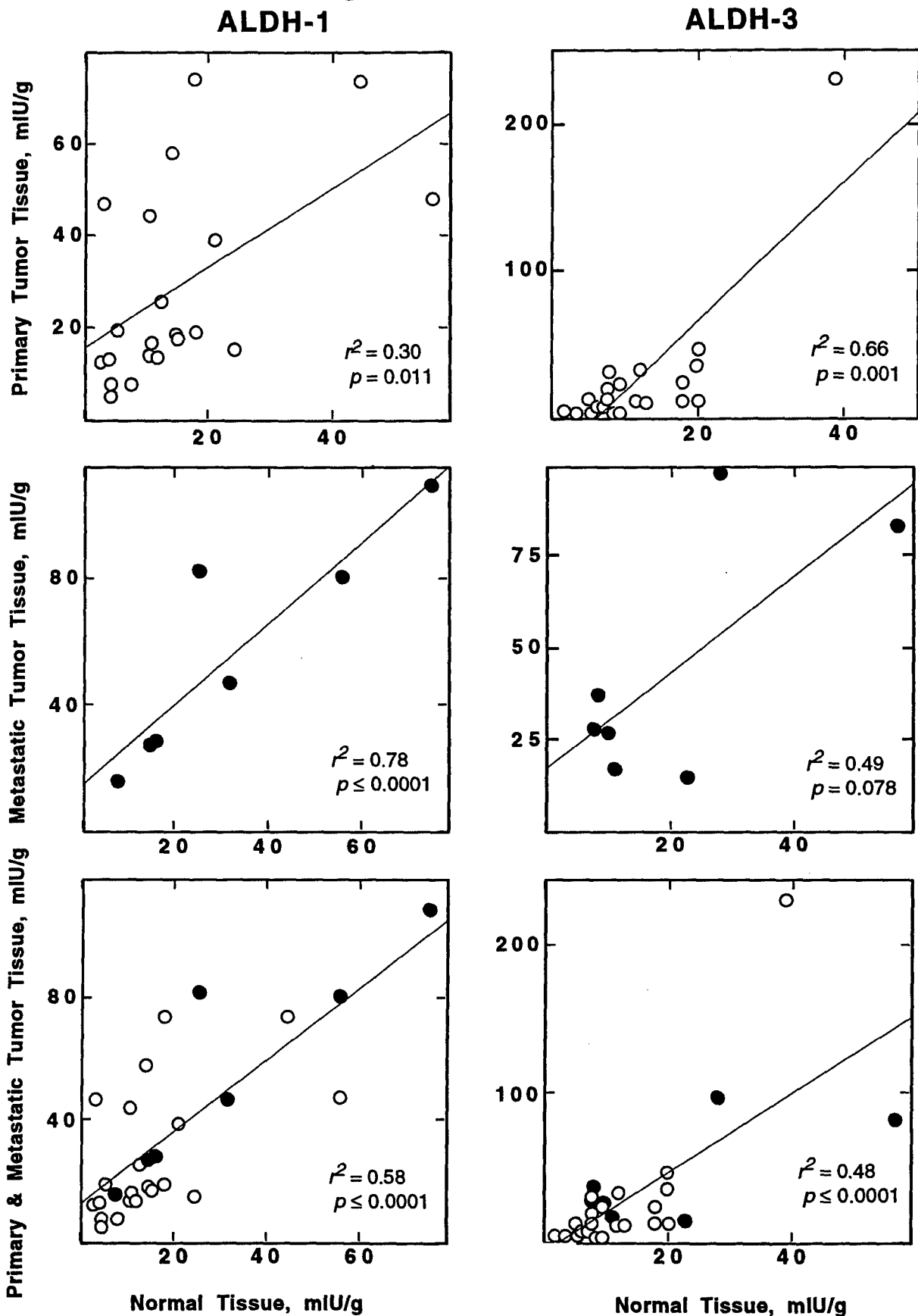


Figure 5. ALDH-1 and ALDH-3 levels in paired human normal and primary tumor (n = 21; O), and normal and metastatic tumor (n = 7; ●), breast tissues. ALDH-1 (NAD [4 mM] -linked oxidation of acetaldehyde [4 mM]) and ALDH-3 (NADP [4 mM] -linked oxidation of benzaldehyde [4 mM]) catalytic activities were indirectly quantified by ELISAs. Points are means of duplicate determinations made on single normal and malignant breast tissue samples taken from each of 26 patients.

Task # 1a: Ascertain whether glutathione levels predict the clinical outcome of cyclophosphamide therapy.

Knowledge of glutathione (GSH) levels in metastatic breast tumor tissue could also be of value in the rational use of cyclophosphamide in breast cancer patients with metastatic disease because cellular sensitivity to cyclophosphamide decreases as cellular levels of GSH increase [reviewed in Sladek 1993 & O'Brien and Tew, 1996], and the levels of GSH in metastatic breast tissue reportedly vary widely [El-Sharabasy et al., 1993; Perry et al., 1993]. Hence, we also quantified GSH levels in most of the normal and malignant (primary and metastatic) breast tissue samples that we had procured from the Cooperative Human Tissue Network, Midwestern Division. Quantification was spectrophotometrically as described by Anderson [Anderson, 1985]. Confirming and extending the observations of others [El-Sharabasy et al., 1993; Perry et al., 1993] GSH levels also varied widely in normal and malignant breast tissues, Figure 6.

GSH levels in normal breast tissue samples did not predict ($P > 0.1$) for the corresponding GSH levels in paired, primary, or metastatic breast tumor tissue samples (linear regression analyses of data not shown). We did not have enough paired samples to ascertain whether cellular levels of GSH in primary breast malignancies predicted cellular levels of this tripeptide in metastatic breast malignancies.

As in the cases of ALDH-3 and ALDH-1, if and when the Cooperative Human Tissue Network, Midwestern Division, collects and provides us with information as to how the specimen donors were subsequently treated and the clinical responses thereto, clinical responses to cyclophosphamide as a function of GSH levels will be evaluated.

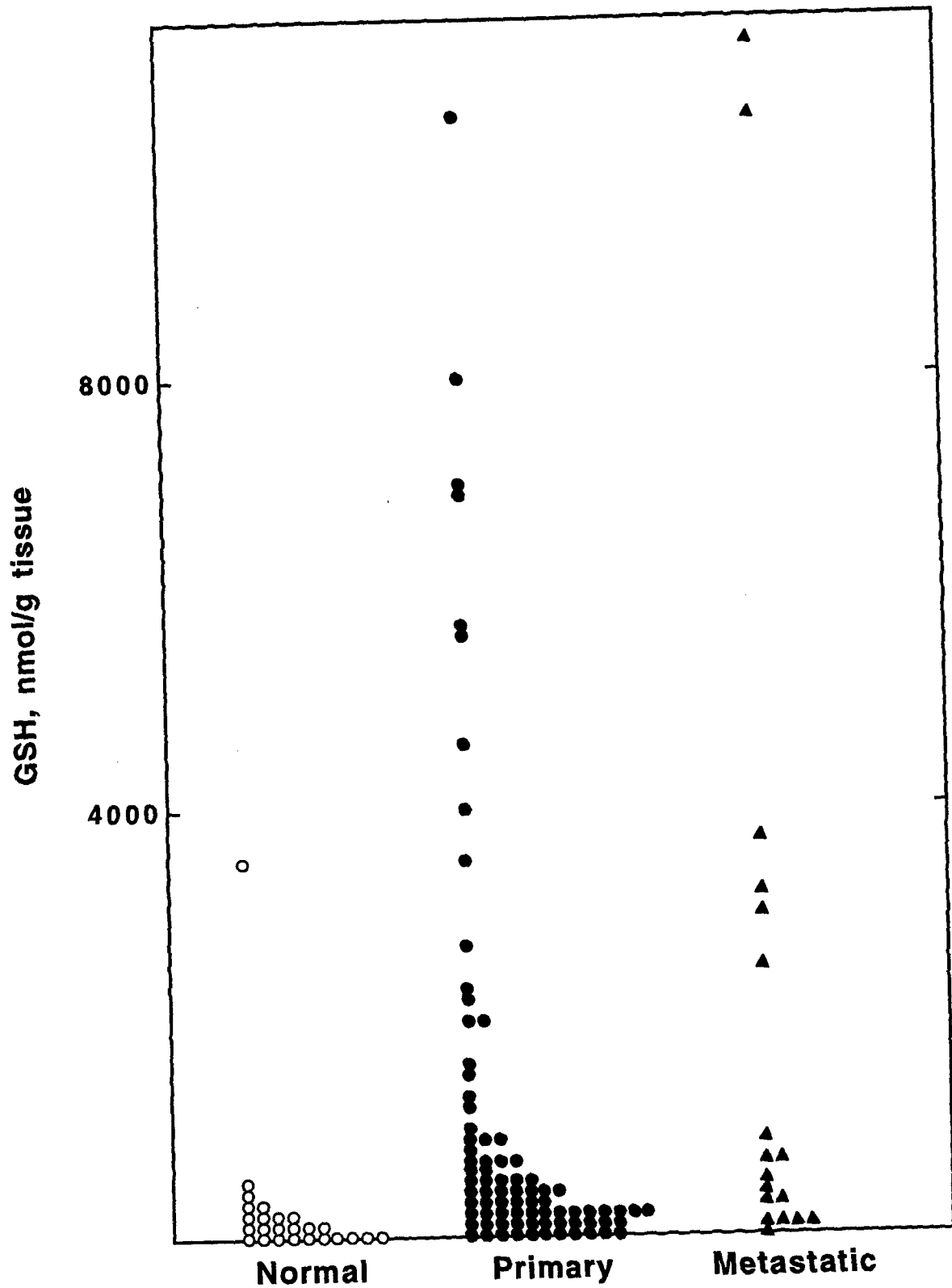


Figure 6. GSH levels in human normal breast (n = 25) and primary (n = 82) and metastatic (n = 18) breast tumor tissue samples. Points are means (rounded off for clarity of presentation to zero if they were < 50 mIU/g, and to 100 mIU/g or the nearest multiple thereof if they were \geq 50 mIU/g) of duplicate determinations made on single normal and malignant, or just malignant, tissue samples taken from each of 100 patients.

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Task # 1b: Ascertain whether simultaneous elevation of ALDH-3, glutathione S-transferase and DT-diaphorase, or of these three enzymes and cytochrome P-450 1A1, occurred in any of the breast tumor samples, and, if so, whether this was due to chance alone or, perhaps, to coordinated induction of these enzymes.

Xenobiotics that are abundantly present in the diet/environment, e.g., 3-methylcholanthrene and catechol, rapidly, coordinately, and reversibly induce ALDH-3, DT-D, GSTs, UDP-glucuronosyl transferase (UDP-GT) and, in some cases, cytochrome P-450 1A1 (CYP 1A1) in cultured human breast cancer models, *vide infra* [Sreerama and Sladek, 1994; Sladek et al., 1995, Sreerama et al., 1995a; Rekha and Sladek, 1997a]. Consequently, reversible multienzyme-mediated multidrug resistance/collateral sensitivity to cyclophosphamide and certain other anticancer drugs is rapidly effected [Rekha and Sladek, 1997a]. Some of the latter are also already used, e.g., mitoxantrone [reviewed in Hainsworth, 1995], or show promise, e.g., EO9 [Smitskamp-Wilms et al., 1996], in the treatment of breast cancer. Ingestion of certain dietary substances, viz., coffee and broccoli, has been shown to result in the coordinated elevation of ALDH-3, DT-D, and the GSTs in human saliva [Sreerama et al., 1995b]. Stable (irreversible) intrinsic as well as acquired phenotypes of this sort have also been observed in cultured human cancer models [Rekha et al., 1994; Sladek et al., 1995; Rekha and Sladek, 1997a]. Not known is whether coordinated elevation of these enzymes effected either by 1) a relevant mutation in, ostensibly, one of the two signaling pathways schematically presented in Figure 12, *vide infra* (enzyme levels are stably elevated), or 2) by the introduction of certain dietary or pharmacological agents that transiently induce the expression of these enzymes by, ostensibly, one of the two signaling pathways schematically presented in Figure 12, *vide infra* (enzyme levels return to basal levels within days upon cessation of inducer intake), ever occurs *in vivo* in human normal and/or malignant breast tissue. Thus, in addition to ALDH-1, ALDH-3, pan-GST, GSTs α , μ and π , and DT-D levels (year-2 progress report), CYP 1A1 levels were quantified in most of the normal and malignant (primary and metastatic) breast tissue samples procured from the Cooperative Human Tissue Network, Midwestern Division, Figure 7, and the combined data was analyzed with this question in mind in a first attempt to address it.

Evidence (levels that are each more than one standard deviation above normal breast tissue mean levels) for the coordinated induction of ALDH-3, DT-D, pan-GST and CYP 1A1 (induced gene expression effected by transactivation of a xenobiotic responsive element (XRE), a cis-acting DNA element present in the 5'-upstream regions of the genes coding for these enzymes [Figure 12, *vide infra*]) was observed in only three samples, viz., two primary, and one metastatic, breast tumors, Table 1, an observed frequency of 0.030 (3/99). As judged by the same criteria, coordinated induction of ALDH-3, DT-D, and pan-GST, but not of CYP 1A1 (induced gene expression effected by transactivation of an ARE, a cis-acting DNA element present in the 5'-upstream regions of the genes coding for these enzymes [Figure 12, *vide infra*]) was observed in only seven additional samples, viz., four primary, and three metastatic, breast tumors, Table 1, an observed frequency of 0.091. In one case, sample 6, a normal breast sample obtained from the same patient was available. ALDH-3 and DT-D levels in this sample were each more than two standard deviations, and pan-GST and GST π levels were nearly (~0.86) one standard

deviation, above corresponding normal breast tissue mean values; CYP 1A1 was not detected in this sample (data not shown).

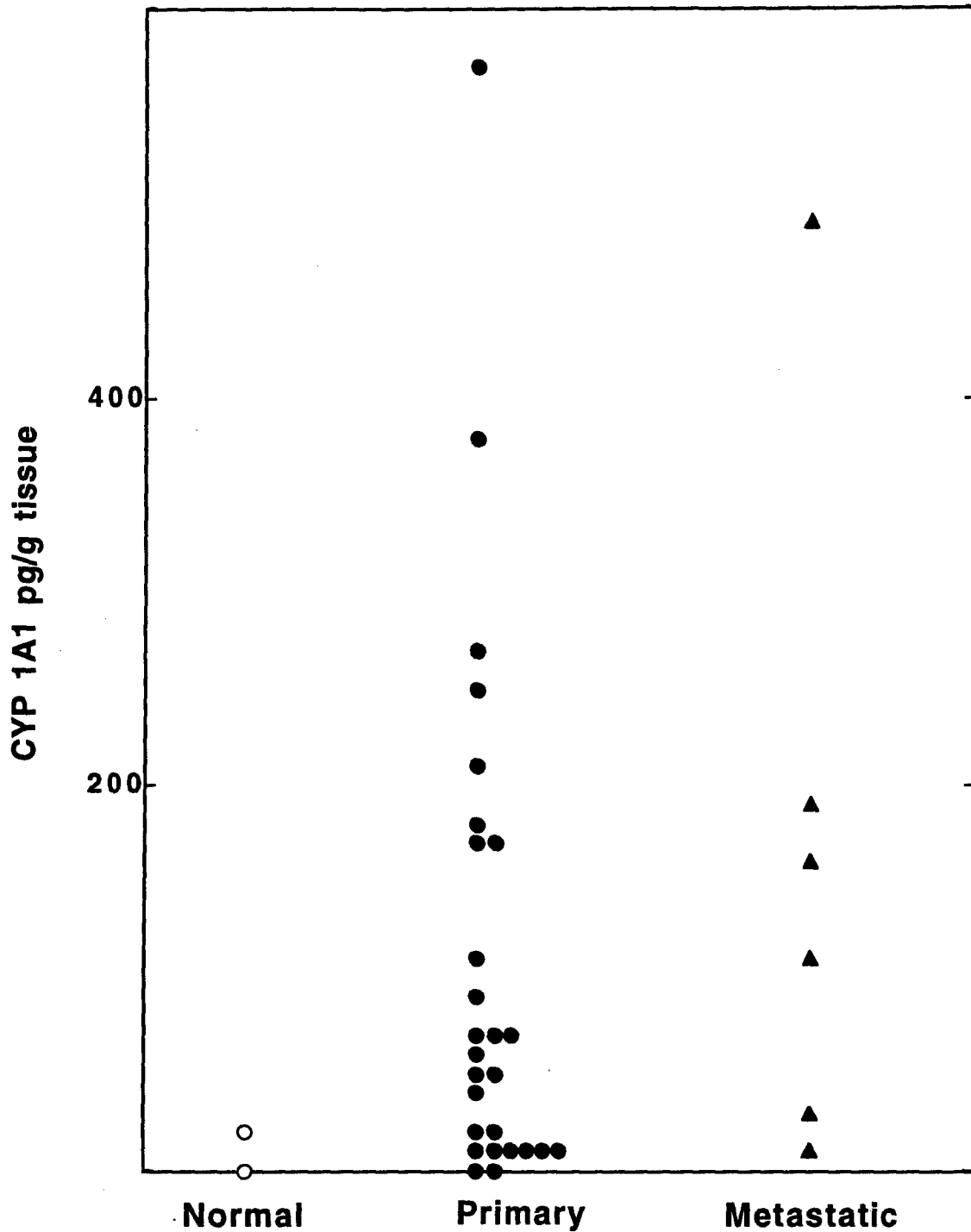


Figure 7. CYP 1A1 levels in human normal breast (n = 21) and primary (n = 80) and metastatic (n = 19) breast tumor tissue samples. CYP 1A1 levels were quantified by an ELISA. Points are means (rounded off for clarity of presentation to zero if they were > 0 and < 5 pg/g, and to 10 pg/g or the nearest multiple thereof if they were ≥ 5 mIU/g) of duplicate determinations made on single normal and malignant, or just malignant, tissue samples taken from each of 99 patients. CYP 1A1 was not detected in 19 of 21 (90%) normal breast tissue samples, 53 of 80 (66%) primary breast tumor samples, and 13 of 19 (68%) metastatic breast tumors samples. These zero values are not shown in this figure.

Table 1. Malignant (primary and metastatic) breast tissue samples exhibiting markedly elevated levels of ALDH-3, DT-D, pan-GST and GST π (each > 1 SD above their respective normal breast tissue mean values)^a

Sample	Malignancy	ALDH-3	DT-D	pan-GST	GST π	CYP 1A1
1		28	5520	2630	2100	0
2		35	4310	7280	6200	177 ^b
3	Primary	39	6250	4870	1900	5
4		45	1410	3000	2900	206 ^b
5 ^c		171	1890	4450	3600	5
6 ^{c, d}		231	2950	5180	4150	0
7		28	1730	5650	3850	ND ^e
8	Metastatic	53	1510	6430	3800	0
9		64	1600	4630	3900	485 ^b
10 ^{c, f}		97	2540	7600	6500	0

^aIncluded in this table are the 10 malignant samples, out of 112, in which ALDH-3, DT-D, pan-GST and GST π levels were each > 1 SD above their respective normal breast tissue mean values. ALDH-3, GST π and CYP 1A1 levels were quantified by ELISAs, and those of DT-D and pan-GST were quantified by spectrophotometric assays. Values are means of duplicate determinations made on single malignant tissue samples taken from each of 10 patients. Units are mIU/g tissue except in the case of CYP 1A1 where they are pg/g. There were no normal breast tissue samples (n = 26) in which all four levels were > 1 SD above their respective normal breast tissue mean values. There were no samples excluded from this listing because only pan-GST or only GST π failed to meet the criteria of levels > 1 SD above their respective normal tissue mean values.

^bValue is > 1 SD above the normal breast tissue mean value.

^cSamples in which ALDH-3, DT-D, pan-GST and GST π levels were each > 2 SD above their respective normal breast tissue mean values.

^dSamples in which ALDH-3, DT-D, pan-GST and GST π levels were each > 1 SD above their respective primary breast tumor tissue mean values.

^eNot determined

^fSamples in which ALDH-3, DT-D, pan-GST and GST π levels were each > 1 SD above their respective metastatic breast tumor tissue mean values.

Expected frequencies, based on the assumption that elevated expression (level greater than one standard deviation above normal breast tissue mean level) of these enzymes is the consequence of independent events, were 0.011 (ALDH-3, DT-D, GST [pan-GST and GST π] and CYP 1A1 levels were > 1 SD above their respective normal breast tissue mean values in 30, 28, 43 and 29 of 99 samples, respectively) and 0.048 (ALDH-3, DT-D and GST [pan-GST and GST π] levels were > 1 SD above their respective normal breast tissue mean values in 32, 38 and 53 of 110 samples, respectively). As judged by

chi-squared analysis, observed frequencies did not differ significantly ($p = 0.09$ and 0.13 , respectively) from expected frequencies. The reader is advised that the choice of one standard deviation above normal breast tissue mean values as indication of coordinated induction was entirely arbitrary. The clinical ramifications of coordinated enzyme induction by pharmacological and/or dietary/environmental agents are potentially substantial, especially with regard to chemotherapeutic strategies. These have been detailed elsewhere [Sreerama et al., 1995b; Rekha and Sladek, 1997a].

Task # 1c: Ascertain whether breast tumor ALDH-3 levels parallel salivary ALDH-3 levels.

Given that 1) ALDH-3 is a demonstrated molecular determinant of cellular sensitivity to the oxazaphosphorines [reviewed in Sladek et al., 1995], 2) it is transiently induced in model systems by agents widely present in the diet or elsewhere in the environment [Sladek et al., 1995; Sreerama et al., 1995a], 3) it is constitutively present in the saliva and that salivary ALDH-3 levels are elevated following the ingestion of broccoli or other dietary materials, e.g., coffee, known to contain agents that induce ALDH-3 [Sreerama et al., 1995b], and 4) cellular levels of ALDH-3 vary widely in normal and malignant breast tissues [task # 1; year-2 progress report; Sreerama and Sladek, 1997], it follows that salivary levels of ALDH-3 may reflect normal and tumor tissue, e.g., breast, levels of this enzyme, and, thus, that salivary levels of ALDH-3 would be a prognostic indicator of tumor cell sensitivity to the oxazaphosphorines. Attractive is the non-invasiveness of sample collection. Accordingly, an investigation designed to test this notion has been initiated. With the caveat that only a small number of samples have been evaluated thus far, our findings to date indicate that breast (normal, benign or primary malignant) tissue ALDH-3 levels do not parallel salivary ALDH-3 levels, Figure 8.

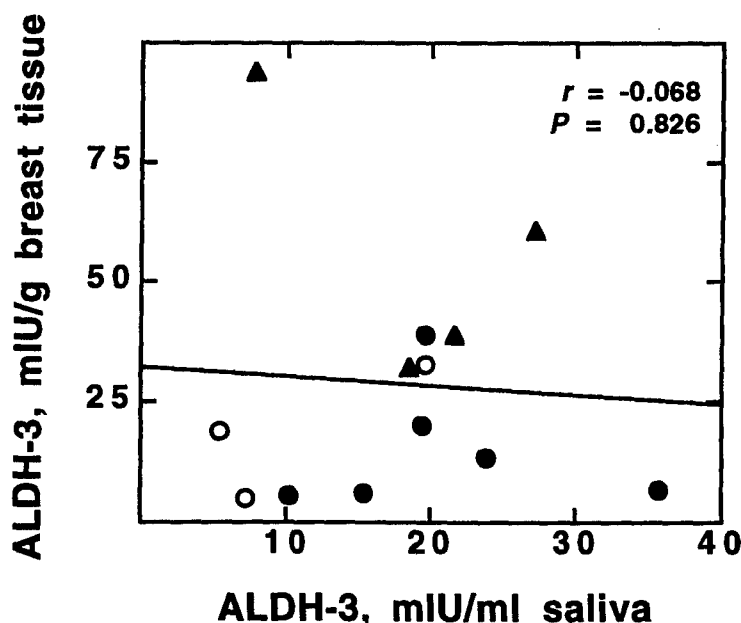


Figure 8. ALDH-3 levels in presurgery saliva samples ($n = 13$), and in subsequently surgically removed normal ($n = 3$; O), benign ($n = 6$; ●) and primary malignant ($n = 4$; ▲) breast tissues. Saliva and surgically removed breast tissues (normal, benign and primary malignant) were obtained through the Tissue Procurement Facility at University of Minnesota Cancer Center, Minneapolis. Processing of saliva and breast tissues, and quantification of ALDH-3 activity (NADP [4 mM]-linked oxidation of benzaldehyde [4 mM]) by ELISA were as described previously [Sreerama et al., 1995b and Sreerama and Sladek, 1997].

Task # 2: Ascertain the ability of class 3 aldehyde dehydrogenases to catalyze the oxidative and/or hydrolytic detoxification of cyclophosphamide (aldophosphamide) at a rate sufficient to account for the oxazaphosphorine-specific acquired resistance exhibited in our model systems.

This task was essentially completed last year. A brief summary of our findings follows. Several years ago we demonstrated that, as judged by the formation of NAD, cytosolic class 3 aldehyde dehydrogenases purified from MCF-7/OAP and MCF-7/0/MC cells catalyze the oxidation of aldophosphamide to carboxyphosphamide, and, moreover, that cytosolic fractions prepared from these cells, predictably, catalyze this reaction as well, albeit seemingly not very rapidly, viz., 0.28 and 0.2 $\mu\text{mol}/\text{min}/10^9$ cells, respectively [Sreerama and Sladek, 1993a, 1994]. Subsequently, we found MCF-7/0 cells to exhibit oxazaphosphorine-specific resistance when purified ALDH-3 was electroporated into them [Sreerama and Sladek, 1995]. More recently, we ascertained that, as judged by the formation of carboxyphosphamide itself, purified tALDH-3, as well as cytosolic fractions prepared from MCF-7/0/CAT cells (MCF-7/0 cells grown in the presence of catechol for five days to transiently induce ALDH-3 and the associated oxazaphosphorine-specific resistance [Sreerama et al., 1995a]), catalyze the oxidation of aldophosphamide to carboxyphosphamide (year-2 progress report). No unidentified NBP-positive metabolites were present. Amounts of 4-hydroxycyclophosphamide/aldophosphamide and carboxyphosphamide present in MCF-7/0/CAT cells after 30 min exposure to mafosfamide were approximately equal. Thus it would appear that although the reaction rate is seemingly slow, ALDH-3-mediated oxazaphosphorine-specific resistance results solely from ALDH-3-catalyzed oxidation of aldophosphamide to carboxyphosphamide, the latter being without cytotoxic activity.

Task # 3: Synthesize and identify agents that inhibit the ALDH-3-catalyzed oxidative and/or hydrolytic detoxification of cyclophosphamide (aldophosphamide).

Eleven compounds were evaluated during the first two years of the granting period. Five of these were synthesized in the laboratory of Dr. H. T. Nagasawa.

The ALDH-3 present in human tumor cells/tissues (tALDH-3), e.g., cultured breast adenocarcinoma MCF-7 cells, colon carcinoma C cells, and salivary gland Warthin tumors and mucoepidermoid carcinomas, although otherwise seemingly identical to the ALDH-3 present in human normal tissues/fluids (nALDH-3), e.g., stomach mucosa and saliva, differs from the latter in that it exhibits a much greater ability to catalyze the oxidative detoxification of the oxazaphosphorines [Sladek et al., 1995; Sreerama and Sladek, 1996]. Hence, both tALDH-3 and nALDH-3 were included in our investigations.

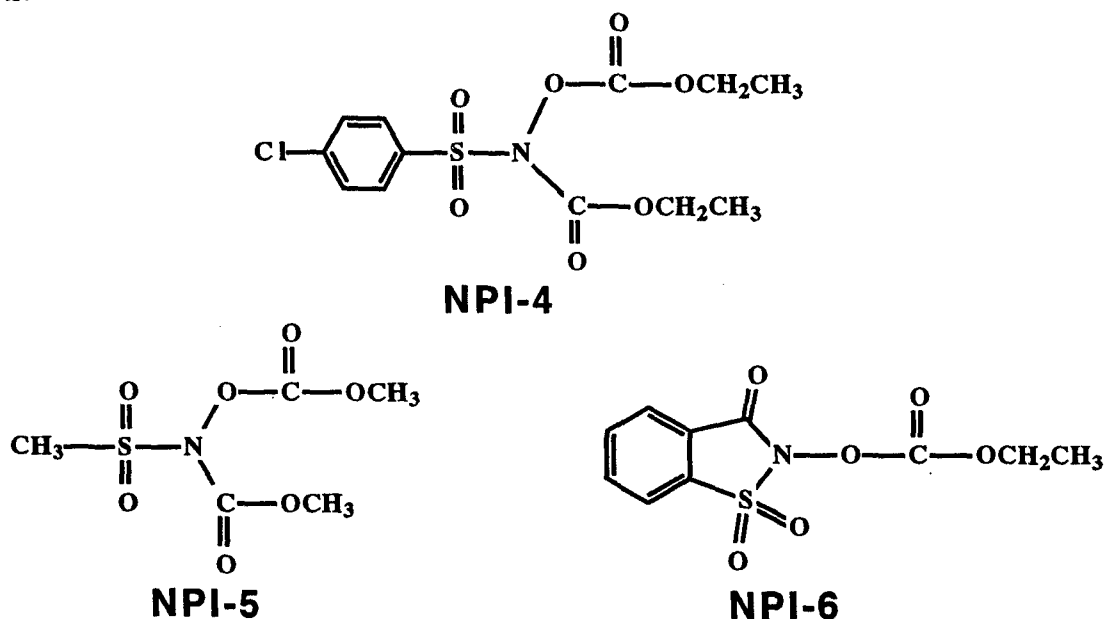
Human ALDH-1, known to also catalyze the irreversible oxidation (detoxification of aldophosphamide [Dockham et al., 1992]), and human ALDH-2 were included in our investigations so that the relative specificity, if any, of the inhibitory effect of these agents towards each of the three classes of aldehyde dehydrogenases could be ascertained.

Results of these investigations have been detailed in the year-1 and year-2 progress reports.

Briefly, one, ethylphenyl(2-formylethyl)phosphinate did not inhibit tALDH-3 or any of the other three aldehyde dehydrogenases (rALDH-1 [recombinant ALDH-1], rALDH-2 [recombinant ALDH-2], nALDH-3) tested, although it was found to be a relatively good substrate for rALDH-1; none of four cinnamic acid derivatives proved to be a very potent inhibitor of the human ALDH-3s, nor were either of these enzymes differentially more sensitive to the inhibitory action of these agents; gossypol proved to be a very potent irreversible inhibitor of the human ALDH-3s and they were differentially more sensitive to it, thus this agent was submitted to further testing [task # 4; Rekha and Sladek, 1997b]; and, whereas all five chlorpropamide analogues inhibited tALDH-3, only two, 4-chloro-*N*-[(propylamino)carbonyl]benzenesulfonamide (API-2; reversibly) and (acetyloxy)[(4-chlorophenyl)sulfonyl]carbamic acid 1,1-dimethylethyl ester (NPI-2; irreversibly), were very potent and/or selective in that regard, thus, of the five chlorpropamide analogues, only they were submitted to further testing [task # 4; Devaraj et al., 1997; Rekha et al., 1997].

Three additional chlorpropamide analogues (Figure 9) were synthesized and evaluated during the past year with respect to their ability to inhibit rALDH-1-, rALDH-2-, nALDH-3- and tALDH-3-catalyzed oxidations. The rationale for the design of these and several other analogues of chlorpropamide was given in the year-1 progress report. Synthesis of the new analogues was by the laboratory of Dr. H. T. Nagasawa. Generation and purification of human rALDH-1 and rALDH-2 were as described elsewhere [Devaraj et al., 1997]. Purification of nALDH-3 and tALDH-3 was as described previously [Sreerama and Sladek, 1993b; Sreerama et al., 1995a].

A summary of our findings is presented in Table 2. Although NPI-4 and NPI-5 were potent inhibitors of tALDH-3, they and NPI-6 did not selectively inhibit this enzyme, thus aborting further investigation of these agents.



NPI-4: 4-chloro-*N,O*-bis(ethoxycarbonyl)-*N*-hydroxybenzenesulfonamide
 NPI-5: *N,O*-bis(methoxycarbonyl)-*N*-hydroxymethanesulfonamide
 NPI-6: 2-[(ethoxycarbonyl)oxy]-1,2-benzisothiazol-3(2*H*)-one 1,1-dioxide

Figure 9. Structures of chlorpropamide analogues NPI-4, NPI-5 and NPI-6.

Table 2. Inhibition by chlorpropamide analogues of human aldehyde dehydrogenase-catalyzed oxidation: IC₅₀ values^a

Inhibitor	IC ₅₀ , μM			
	rALDH-1	rALDH-2	nALDH-3	tALDH-3
NPI-4	1.0	4.0	2.0	1.9
NPI-5	0.7	ND	3.2	2.3
NPI-6	5.0	ND	>400	>400

^aEnzymes were incubated with vehicle or 4 - 8 different concentrations of one of the putative inhibitors for 20 min at 37°C and pH 8.1, substrate was added, and initial catalytic rates were quantified spectrophotometrically as described previously [Devaraj et al., 1997; Rekha and Sladek, 1997b; Rekha et al., 1997]. Substrate was acetaldehyde (4 mM and 2 mM, respectively) for rALDH-1 and rALDH-2, and benzaldehyde (4 mM) for ALDH-3s. Cofactor was NAD (4 mM for rALDH-1 and rALDH-2; 1 mM for ALDH-3s). Uninhibited catalytic rates (mean; n = 2) were 0.60, 2.0, 31, 33 IU/mg protein for rALDH-1, rALDH-2, nALDH-3 and tALDH-3, respectively. Computer-assisted unweighted nonlinear regression analysis effected by the STATview statistical program [Brainpower, Inc., Calabas, CA] was used to generate the curves that best-fit plots of enzyme activities (% control) as a function of inhibitor concentrations and, subsequently, to estimate the concentration of inhibitor that effected a 50% decrease in catalytic activity (IC₅₀).

ND = Not Determined.

Task # 4: Evaluate identified inhibitors of the relevant class 3 aldehyde dehydrogenase activity with respect to their ability to sensitize our oxazaphosphorine-resistant models to the oxazaphosphorines.

None of the three chlorpropamide derivatives evaluated in the past year with respect to their ability to selectively inhibit ALDH-3 showed promise in that regard (task # 3). Thus, none were evaluated with respect to task # 4.

Results of our previous task # 4 investigations have been detailed in the year-1 and year-2 progress reports.

Briefly, the ability of two chlorpropamide analogues, viz., NPI-2 and API-2, and gossypol to negate the influence of relatively high cellular levels of ALDH-3 on the cellular sensitivity of cultured human breast adenocarcinoma MCF-7/0/CAT cells to oxazaphosphorines was evaluated [Rekha et al., 1997]. Each was found to sensitize this, otherwise mafosfamide-insensitive, cell line to this and other oxazaphosphorines. These findings establish the therapeutic potential of combining NPI-2, API-2 or gossypol with an oxazaphosphorine in the treatment of certain cancers.

Uncertain is whether these agents will inhibit tALDH-3 *in vivo* at doses that do not cause untoward effects since this possibility remains essentially untested. Experiments with a limited number of animals

showed that NPI-2, 1 mmol/kg, ip, did not inhibit aldehyde dehydrogenase-catalyzed oxidation of acetaldehyde in rats, as judged by its failure to cause elevated plasma levels of acetaldehyde in animals given ethanol [Lee et al., 1992a]. The aldehyde dehydrogenases that are thought to catalyze the bulk of acetaldehyde oxidation *in vivo*, viz., class 2, and to a lesser extent class 1, aldehyde dehydrogenases, are not very sensitive to the inhibitory action of NPI-2. In contrast, the class 3 aldehyde dehydrogenases, especially tALDH-3, are. Thus, the possibility that tolerated doses of NPI-2 will inhibit tALDH-3 *in vivo* remains viable. API-2, 1 mmol/kg, ip, on the other hand, markedly inhibited aldehyde dehydrogenase-catalyzed oxidation of acetaldehyde in rats, as judged by the markedly elevated plasma levels of acetaldehyde that were observed when the animals were treated with this agent prior to being given ethanol [Lee et al., 1992b].

Preclinical investigations demonstrating the cancer chemotherapeutic potential of gossypol have already prompted phase 1, and, subsequently, phase 2, clinical trials of this agent for the treatment of certain cancers, including metastatic breast cancers [Seidman, 1996]. Thus, much is known about the pharmacokinetic behavior and toxicity of gossypol in humans [Wu, 1989]. Whether doses of gossypol sufficient to inhibit tALDH-3 *in vivo* can be given to humans safely remains to be determined but seems probable given the foregoing and that the concentration of gossypol needed to inhibit tALDH-3 *in vitro* is much less than that needed to inhibit tumor cell proliferation in our *in vitro* model, viz., cultured human breast adenocarcinoma MCF-7/O/CAT cells. Given the antitumor activity that gossypol itself exhibits, it can be envisaged that in the case of some cancers, gossypol could be of dual therapeutic value when combined with an oxazaphosphorine in the therapeutic protocol.

Task # 5: Identify the molecular basis for the apparent overexpression of class 3 aldehyde dehydrogenases (ALDH-3s) in our model systems.

RT-PCR and Southern blot analysis were used to ascertain whether the elevated levels of ALDH-3 that we observed in MCF-7/OAP and MCF-7/O/MC cells [Sreerama and Sladek, 1993a, 1994; Sladek et al., 1995] and in MCF-7/O/CAT cells [task # 7; year-1 progress report; Sreerama et al., 1995a; Sladek et al., 1995], were the consequence of transcriptional activation, DNA amplification or some other mechanism. Two oligonucleotide primers specific to human stomach mucosa ALDH-3 cDNA, and two specific to β -actin cDNA (internal control), were designed with the aid of a Macintosh-based software program, viz., Oligo 4.0, for use in RT-PCR. Primer design was based on published human stomach mucosa ALDH-3 and human β -actin cDNA sequences, respectively [Nakajima-Iijima et al., 1985; Hsu et al., 1992]. Thus designed ALDH-3 primers were 5'-ACTGGGCGTGGTCCTCGTCATTGG-3' (5'-end; corresponds to bases 312-335 of the sense strand) and 5'-GTGAGGATGGTGGGGGCTATGTAG-3' (3'-end; corresponds to bases 942-965 of the antisense strand). β -Actin primers were 5'-TGACGGGGTCACCCACACTGTGCCCATCTA-3' (5'-end; corresponds to bases 1038-1067 of the sense strand) and 5'-CTAGAAGCATTGCGGTGGACGATGGAGGG-3' (3'-end; corresponds to bases 1876-1905 of the antisense strand). ³²P-labeled human stomach mucosa ALDH-3 full-length cDNA (successfully cloned in our laboratory) was used as the probe for Southern blot analysis.

ALDH-3 mRNA levels were elevated in MCF-7/OAP (~5-fold), MCF-7/0/MC (~5-fold) and MCF-7/0/CAT (~8-fold) cells as compared to the ALDH-3 mRNA level in MCF-7/0 cells, Figure 10. The latter was in agreement with a previous observation, viz., as judged by Northern blot analysis, ALDH-3 mRNA levels were elevated in MCF-7/0/CAT cells [task # 7; year-1 progress report; Sreerama et al., 1995a].

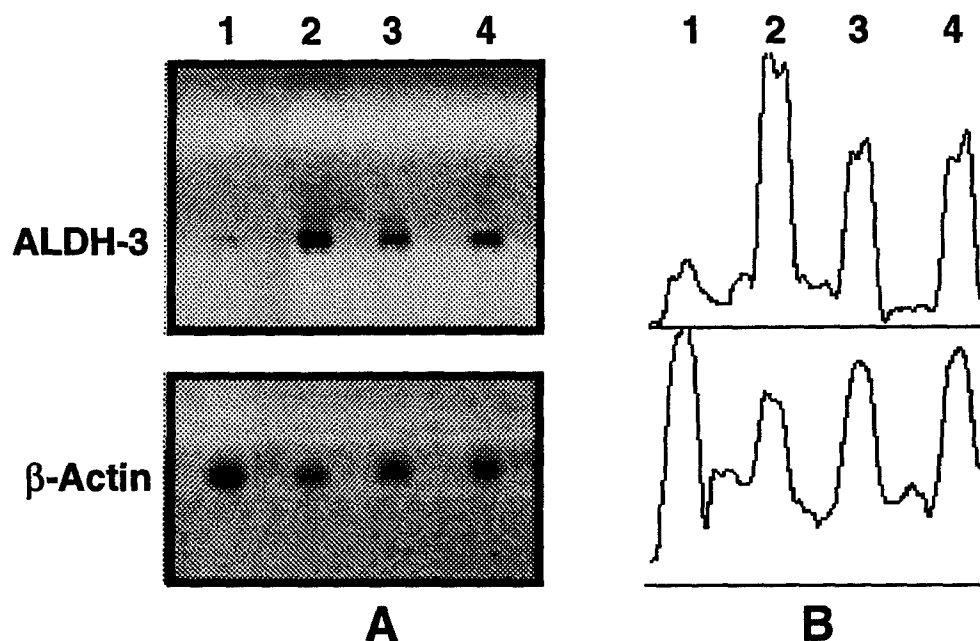


Figure 10. ALDH-3 mRNA levels in MCF-7/0 (lane 1), MCF-7/0/CAT (lane 2), MCF-7/0/MC (lane 3) and MCF-7/OAP (lane 4) cells as judged by RT-PCR. Isolation of total RNA from MCF-7/0, MCF-7/0/CAT, MCF-7/0/MC and MCF-7/OAP cells was with the aid of a RNA isolation kit (Gentra Systems, Inc., Minneapolis, MN). Synthesis of first-strand cDNA catalyzed by reverse transcriptase, and the subsequent PCR catalyzed by Vent DNA polymerase (New England Biolabs, Beverly, MA), were with the aid of a RT-PCR kit (Stratagene, Inc., La Jolla, CA). Briefly, first cDNA was synthesized by mixing 10 μ g of the isolated total cellular RNA with 300 ng of oligo(dT) primer in 40 μ l of diethylpyrocarbonate-treated sterile water, incubating the resultant mixture at 65°C for 5 min (denaturation of RNA) and then at 25°C for 10 min (primer annealing to mRNA), adding 10 μ l of freshly-prepared RT mixture [5 μ l of a buffer solution (500 mM Tris-HCl, 50 mM dithiothreitol, 50 mM MgCl₂, 500 mM KCl and 0.5 mg/ml BSA), 1 μ l of RNase block (ribonuclease inhibitor; 40 IU/ μ l), 3 μ l of deoxyribonucleotide (dNTP) mixture (100 mM each of dATP, dGTP, dCTP and dTTP) and 1 μ l of Moloney murine leukemia virus reverse transcriptase (50 IU/ μ l)], and incubating at 37°C for 60 min. The reaction was stopped by heat inactivation (90°C for 2 min). An aliquot (1-5 μ l) of thus synthesized cDNA was mixed with 5 μ l of a buffer solution [200 mM Tris-HCl, 100 mM KCl, 20 mM MgSO₄, 100 mM (NH₄)₂SO₄ and 0.1% (w/v) Triton X-100], 1 μ l of dNTP mixture (100 mM each), 1 μ l of 5'-end primer (10 μ M), 1 μ l of 3'-end primer (10 μ M), and sufficient sterile water to give a final volume of 49 μ l in a 500 μ l thin-wall PCR tube. Samples were first denatured at 94°C for 5 min in a thermocycler (Techne, Inc., Princeton, NJ) and then the amplification reaction was started by adding 1 μ l of Vent DNA polymerase (2 IU/ μ l). Cycling parameters were 1) 10 cycles of denaturation, annealing and chain extension at 94°C, 52°C and 72°C, respectively, for 1 min at each temperature, 2) 19 cycles of denaturation, annealing and chain extension at 94°C, 55°C and 72°C, respectively, for 1 min at each temperature, and 3) 1 cycle of denaturation and annealing, 1 min each, at 94°C and 55°C, respectively, and chain extension at 72°C for 15 min. Control experiments were with primers for β -actin. The PCR products were separated on 1% (w/v) agarose gels and visualized by ethidium bromide staining (Panel A). The visualized gel was photographed and the photograph was digitized with the aid of a Color OneScanner connected to a Power Macintosh computer equipped with "Ofoto 2.0" scanning software (Apple Computer, Inc., Cupertino, CA). Semiquantification (Panel B) of the PCR products by densitometry was with the aid of Image 1.6 software (NIH, Bethesda, MD).

Southern blot analysis of genomic DNA isolated from MCF-7/OAP cells showed no increase in genomic *ALDH-3* DNA copies, thus demonstrating that increased ALDH-3 mRNA levels in these cells was not due to gene amplification, Figure 11. As expected given that methylcholanthrene- and catechol-induced elevations of ALDH-3 levels were transient [Sreerama and Sladek, 1994; Sreerama et al., 1995a], Southern blot analysis of genomic DNA isolated from MCF-7/0/MC and MCF-7/0/CAT cells showed no

increase in genomic *ALDH-3* DNA copies, Figure 11. Elevated levels of *ALDH-3* mRNA in MCF-7/OAP, MCF-7/0/MC and MCF-7/0/CAT cells were not the consequence of hypomethylation of a relevant *ALDH-3* DNA regulatory element because neither the *ALDH-3* mRNA level nor the *ALDH-3* catalytic activity was increased when MCF-7/0 cells were cultured in the presence of 1, 5 or 10 μ M 5'-azacytidine, a known hypomethylating agent (task # 6, Table 5).

Given the foregoing, elevated levels of *ALDH-3* in MCF-7/OAP, MCF-7/0/MC and MCF-7/0/CAT cells appears to be the consequence of transcriptional activation mediated by relevant trans-acting factors, although mRNA stabilization cannot be ruled out by these experiments.

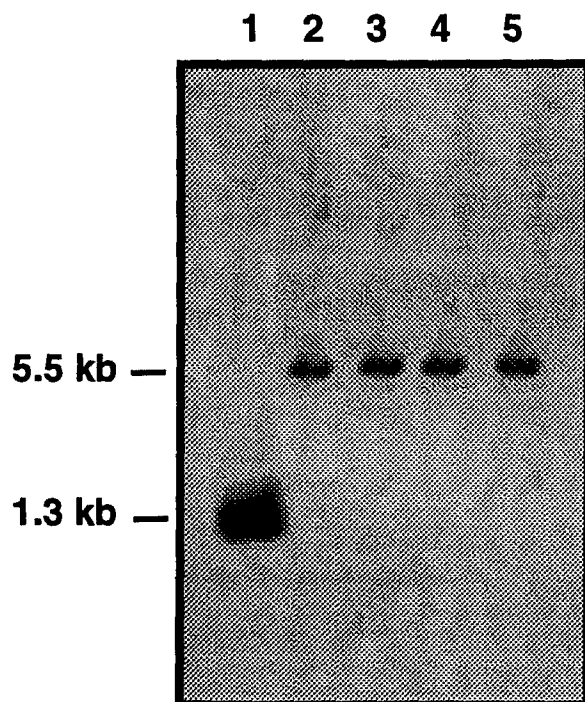


Figure 11. Southern blot analysis of genomic DNA isolated from MCF-7/0 (lane 2), MCF-7/0/CAT (lane 3), MCF-7/0/MC (lane 4) and MCF-7/OAP (lane 5) cells. Southern blot analysis of genomic DNA was essentially as described by Hsu et al. [1992]. Briefly, isolation of genomic DNA from MCF-7/0, MCF-7/0/CAT, MCF-7/0/MC and MCF-7/OAP cells was with the aid of a DNA isolation kit (Gentra Systems, Inc., Minneapolis, MN). Thus isolated genomic DNA (30 μ g in each case) was first digested with *Eco*RI (37°C for 2 hrs), and the digest was separated on 1% agarose gels and then transferred onto a Zeta Probe membrane (Bio-Rad Laboratories, Hercules, CA). Membrane-bound DNA was then denatured by exposure to UV light and hybridized with 32 P labeled full-length stomach mucosa *ALDH-3* cDNA. Finally, an X-ray film was exposed to the hybridized membrane-bound product at -70°C for 36 to 48 hrs and developed. A plasmid, viz., pCR 3.1, (50 ng) into which the full-length stomach mucosa *ALDH-3* cDNA (lane 1) was inserted at the *Eco*RI site was used as the reference.

Stably elevated levels of *ALDH-3* in MCF-7/OAP cells are probably the consequence of stable upregulation of the rate-limiting event in the ARE signaling pathway, Figure 12. This notion is based on the facts that 1) *ALDH-3*, pan-GST, DT-D and UDP-GT levels, but not those of CYP 1A1, are coordinately elevated when induced expression is effected via the ARE signaling pathway, 2) cellular levels of all five enzymes are coordinately elevated when induced expression is effected via the XRE signaling pathway, and 3) *ALDH-3*, GST, DT-DT-D and UDP-GT levels, but not those of CYP 1A1, are coordinately elevated in MCF-7/OAP cells, Figure 12 and Table 3.

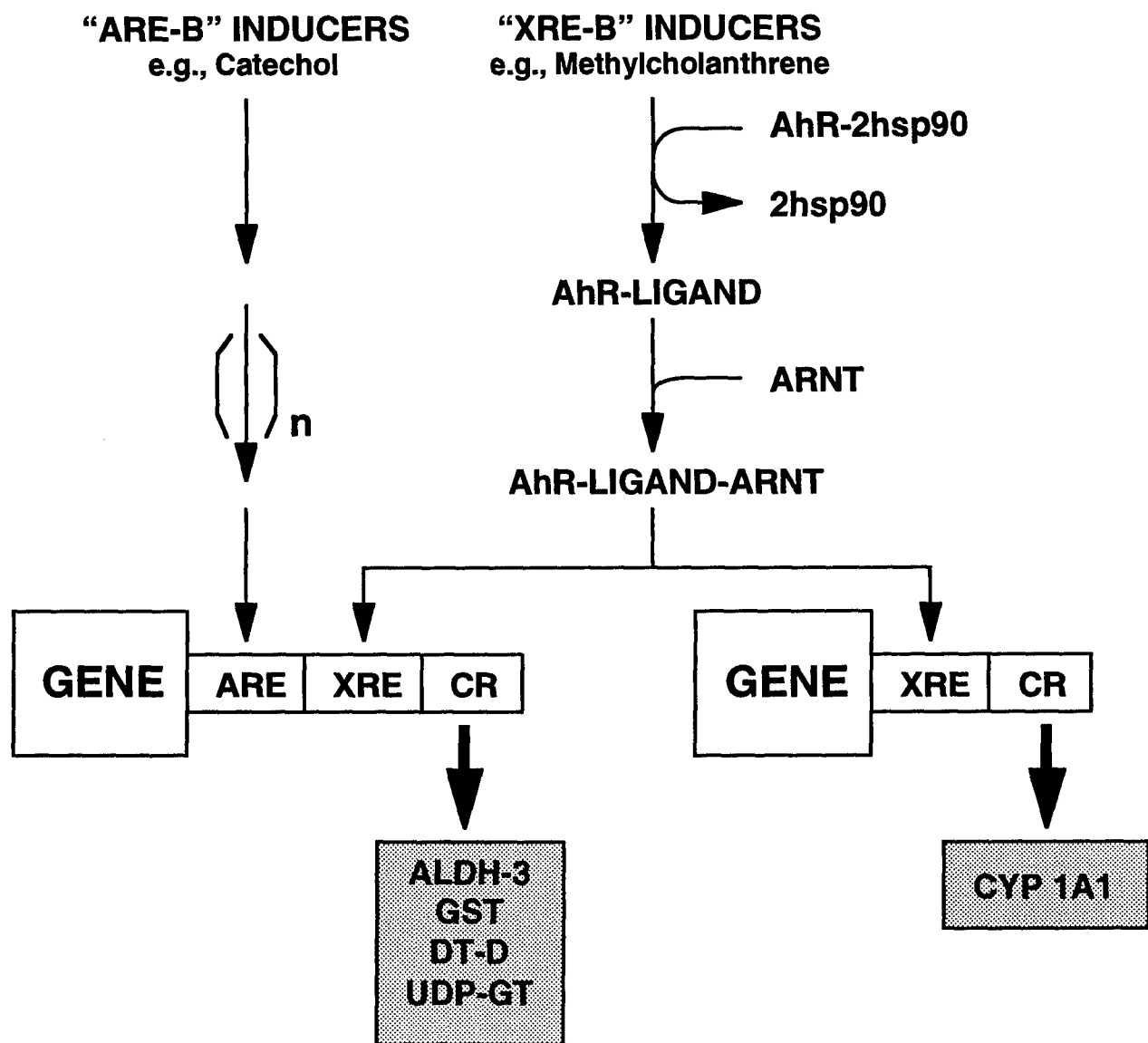


Figure 12. Induction of ALDH-3 and other drug metabolizing enzymes: signaling pathways. ARE-B inducers: agents, e.g., phenolic antioxidants such as catechol, that induce the transcription of a battery (B) of genes having in common an antioxidant responsive element (ARE) in their promoter (5'-upstream) region. XRE-B inducers: agents, e.g., 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and polycyclic aromatic hydrocarbons such as methylcholanthrene, that induce the transcription of a battery (B) of genes having in common a xenobiotic responsive element (XRE) in their promoter (5'-upstream) region. Although details of the signaling pathway by which ARE-B inducers effect increases in enzyme levels is largely unknown, a putative trans-acting factor has been identified [Wasserman and Fahl, 1997]. On the other hand, details of the signaling pathway by which XRE-B inducers effect increases in enzyme levels is largely known and is as depicted in this schematic. Further details may be found in Nebert and Jones, 1989; Belinsky and Jaiswal, 1993; Nebert 1994; Sladek et al., 1995 and Wasserman and Fahl, 1997. AhR, aromatic hydrocarbon receptor; hsp90, heat shock protein 90 kDa; ARNT, aromatic hydrocarbon receptor nuclear translocator; CR, coding region; GST, glutathione S-transferase; DT-D, DT-diaphorase; UDP-GT, UDP-glucuronosyl transferase; CYP 1A1, cytochrome P450 1A1.

Table 3. ALDH-3 and other enzyme activities in MCF-7/0, MCF-7/0/CAT, MCF-7/0/MC and MCF-7/OAP cells^a

Cells	Enzyme Activity, mIU/10 ⁷ Cells							
	ALDH-3	GST				DT-D	UDP-GT	CYP 1A1
		pan	α	μ	π			
MCF-7/0	2	25	9	13	11	82	0.04	0.03
MCF-7/0/CAT	768	250	29	121	62	6395	0.07	0.03
MCF-7/0/MC	310	150	20	113	34	495	0.78	0.48
MCF-7/OAP	254	157	27	81	32	340	0.16	0.03

^aALDH-3, pan-glutathione S-transferase (GST), GSTs α, μ and π, DT-diaphorase (DT-D), UDP-glucuronosyl transferase (UDP-GT) and cytochrome P450 1A1 (CYP 1A1) enzyme activities were quantified as described elsewhere [Sreerama and Sladek, 1993a; 1994; Sreerama et al., 1995a; Rekha and Sladek, 1997a].

Task # 6: Ascertain the ability of Ah receptor ligands to induce class 3 aldehyde dehydrogenase overexpression and oxazaphosphorine-specific acquired resistance in estrogen receptor-positive and -negative breast cancer cell lines that lack and express Ah receptors.

We previously noted [Sreerama and Sladek, 1994; Sladek et al., 1995] that polycyclic aromatic hydrocarbons such as 3-methylcholanthrene induced ALDH-3 and oxazaphosphorine-specific resistance in breast cancer cells that were, reportedly, estrogen receptor-positive (ER⁺), e.g., MCF-7/0, T-47D and ZR-75-1 (all, reportedly, Ah receptor-positive [AhR⁺]), but not in those that were, reportedly, estrogen receptor-negative (ER⁻), e.g., MDA-MB-231 (reportedly, AhR⁺) and SK-BR-3 (Ah receptor status unknown) [Engel and Young, 1978; Vickers et al., 1989; Safe et al., 1991; Taylor-Papadimitriou et al., 1993]. In contrast, phenolic antioxidants induced ALDH-3 and oxazaphosphorine-specific resistance in both ER⁺ and ER⁻ cells [Sladek et al., 1995; Sreerama et al., 1995a and unpublished observations].

Updating the above, we have now confirmed/established that each of the eight human breast (adeno)carcinoma cell lines that we carry are AhR⁺, that the MCF-7/0, MCF-7/OAP, T-47D and ZR-75-1 cell lines that we carry are ER⁺, and that the MDA-MB-231, SK-BR-3, HS-578-T and MDA-MB-435 cell lines that we carry are ER⁻, Table 4. Finally, we have shown that polycyclic aromatic hydrocarbons, e.g., 3-methylcholanthrene, as well as phenolic antioxidants, e.g., catechol, induce ALDH-3 in ER⁺ cell lines, but that only the latter induce ALDH-3 in ER⁻ cells. Thus far, then, our findings are consistent with our original hypothesis, viz., Ah receptor ligands, e.g., 3-methylcholanthrene, will, via XREs present in the 5'-upstream region of the ALDH-3 gene, induce ALDH-3 overexpression in ER⁺ breast cancer cells but

not in ER⁻ breast cancer cells, whereas, induction of ALDH-3 effected by agents that cause the activation of AREs present in the 5'-upstream region of the ALDH-3 gene is estrogen receptor-independent.

Table 4. Estrogen receptor status and the ability of 3-methylcholanthrene and catechol to induce ALDH-3 and resistance to mafosfamide in human breast (adeno)carcinoma cell lines^a

Estrogen Receptor	Cell Line	ALDH-3 & Resistance To Mafosfamide Induced By	
		3-Methylcholanthrene	Catechol
Positive	MCF-7/0		
	MCF-7/OAP	Yes	Yes
	T-47D		
	ZR-75-1		
Negative	MDA-MB-231		
	SK-BR-3	No	Yes
	HS-578-T		
	MDA-MB-435		

^aCells were grown (monolayer) in the presence of 3 μM methylcholanthrene or 30 μM catechol for 5 days and harvested. ALDH-3 activity (NADP-linked enzyme-catalyzed oxidation of benzaldehyde by Lubrol-treated whole homogenates) was quantified spectrophotometrically as described previously [Sreerama and Sladek, 1993a]. Semiquantification of estrogen receptor levels was by an enzyme-linked immunosorbent assay (ELISA) as described by Hornbeck et al. [1991]. Semiquantification of Ah receptor levels was essentially as described by Harris et al. [1989] except that ³H-labeled benzpyrene, instead of ³H-labeled 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), was used as the receptor ligand. Each of the cell lines was Ah receptor-positive. A colony-forming assay was used to quantify cellular sensitivity to mafosfamide [Sreerama and Sladek 1993a].

Unknown is whether estrogen receptor (ER) expression is merely associated with AhR-agonist responsiveness in human breast cancer cells, or if ER expression is somehow an absolute requirement for AhR-agonist responsiveness to occur. Investigations in that regard showed that AhR⁺, ER⁻, otherwise AhR-agonist nonresponsive MDA-MB-231 cells become AhR-agonist responsive (TCDD induction of chloramphenicol acetyltransferase activity) when transiently co-transfected with pRNH11c (an AhR-agonist responsive plasmid containing the regulatory/XRE human CYP 1A1 region fused to the bacterial chloramphenicol acetyltransferase reporter gene) and a human ER expression plasmid [Thomsen et al., 1994], suggesting the latter to be the case.

The lack of ER expression in human breast cancer cell lines such as MDA-MB-231 could be due to hypermethylation of the gene coding for ER. Supporting this notion are the reports that the ER gene is hypermethylated and DNA methyltransferase activity is relatively elevated in several ER⁻ human breast tumor cell lines including MDA-MB-231 [Ottaviano et al., 1994], and that treatment of MDA-MB-231 cells with the DNA demethylating agent 5'-azacytidine resulted in expression of ER mRNA and protein [Ferguson et al., 1995].

Therefore, we hypothesized that MDA-MB-231 cells would become AhR-agonist responsive, as judged by 3-methylcholanthrene induction of ALDH-3 and other relevant enzymes, upon treatment with the DNA demethylating agent 5'-azacytidine.

Although we have yet to test for the presence of ER, our hypothesis appears to be correct, Table 5. Also as expected, 5'-azacytidine-induced AhR-agonist responsiveness was transient; it was completely lost at about 10 days after 5'-azacytidine removal (data not shown). These observations, too, support the notion that ER is an absolute requirement for AhR-agonist responsiveness.

Disagreeing with this notion is the very recent report of Wang et al., [1997]. They found that an AhR-agonist, viz., TCDD, induced CYP 1A1 in the ER⁻ MDA-MB-468 cell line. We have recently obtained MDA-MB-468 cells and intend to ascertain whether we can reproduce their results.

Table 5. ALDH-3, pan-GST and DT-D induction by 3-methylcholanthrene in constitutively ER⁻ human breast adenocarcinoma MDA-MB-231 cells treated with 5'-azacytidine^a.

5'-Azacytidine (μM)	Methylcholanthrene (μM)	Enzyme Activity, mIU/ 10^7 cells		
		ALDH-3 ^b	pan-GST ^c	DT-D ^d
0	0	2	29	65
0	3	2	32	63
1	0	2	28	64
5	0	2	30	61
10	0	2	30	64
1	3	5	44	95
5	3	19	81	159
10	3	40	158	249

^aMDA-MB-231 cells (1×10^5 /plate) were plated and incubated at 37°C for 168 hours. 5'-Azacytidine and 3-methylcholanthrene were added 24 and 72 hours, respectively, after the initiation of incubation. Cells were harvested at the end of the 168 hour incubation period and enzyme activities were quantified in 105,000 g supernatant fractions prepared therefrom as described elsewhere [Sreerama and Sladek, 1994].

^bClass-3 aldehyde dehydrogenase; NADP (4 mM) as cofactor and benzaldehyde (4 mM) as substrate.

^cGlutathione S-transferase; 1-Chloro-2,4-dinitrobenzene (1 mM) as substrate.

^dDT-diaphorase; NADH (0.16 mM) as cofactor and 2,6-dichlorophenol-indophenol (0.04 mM) as substrate.

Task # 7: Ascertain the ability of ligands for ARE to induce ALDH-3 activity and oxazaphosphorine-specific acquired resistance in our model system.

This task, as originally formulated, was completed in year-1 of the grant period. Results of that investigation were summarized in the year-1 report.

Briefly, phenolic antioxidants, already known to induce pan-GST and/or DT-D activities in various rodent and human organ/tissue/cells via AREs present in the 5'-upstream regions of these enzymes [reviewed in Talalay et al., 1987; Belinsky and Jaiswal, 1993], were found to rapidly, coordinately and reversibly induce ALDH-3, pan-GST, DT-D and UDP-GT in cultured breast and other tumor cells, the consequence of which was that such cells became less sensitive to certain anticancer drugs (multidrug resistance) and more sensitive to others (collateral sensitivity). These findings should be viewed as greatly expanding the number of recognized dietary, environmental and pharmaceutical agents that can potentially influence the sensitivity of breast and other tumor cells to cyclophosphamide, other oxazaphosphorines and still other antitumor agents since, in addition to phenolic antioxidants which themselves are abundantly present in a number of dietary materials, a number of other dietary, etc., agents are thought to effect enzyme induction via the signaling mechanism used by the phenolic antioxidants, Figure 12, *vide supra*.

These and other findings in our laboratory served as the basis for the second-generation investigation described herein under task # 1c.

Task # 7a: Ascertain whether limonene and/or any of several of its metabolites induce ALDH-3 levels in human breast adenocarcinoma MCF-7/0 cells.

d-Limonene, a monoterpene abundantly present in citrus fruits and various other foods common to most diets, has been shown to exhibit chemopreventive activity against many solid tumor types, particularly mammary tumors, induced by carcinogens in animal models [reviewed in Crowell and Gould, 1994; Gould, 1995]. Perillyl alcohol, a naturally occurring hydroxylated limonene analogue, is considerably more potent than limonene in that regard [reviewed in NCI, 1996]. The Chemoprevention Branch of the National Cancer Institute has therefore begun single-dose Phase I pharmacokinetic trials of perillyl alcohol in women at high risk for breast cancer.

Limonene is a demonstrated inducer of rodent hepatic enzymes that are known to catalyze the detoxification of certain carcinogens, e.g., pan-GST and UDP-GT [reviewed in Crowell and Gould, 1994]. It has been speculated that perillyl alcohol also has the potential to induce these enzymes since another hydroxylated monoterpene, viz., sobrerol, also induced hepatic pan-GST and UDP-GT in rodents [reviewed in Gould 1995]. Thus, the chemopreventive activity of limonene and perillyl alcohol against carcinogen-induced tumors is thought to be due to induction of these enzymes.

Perillic acid is a major circulating metabolite of limonene and perillyl alcohol [Crowell et al., 1994; Phillips et al., 1995; Poon et al., 1996]. Perillyl aldehyde is a probable intermediate in the biotransformation of limonene and perillyl alcohol to perillic acid [Regan and Bjeldanes, 1976].

Unknown is whether limonene, perillyl alcohol, perillyl aldehyde or perillic acid induce increased expression of pan-GST and UDP-GT in any human tissues, much less in extrahepatic human tissues such as breast. pan-GST and UDP-GT, as well as ALDH-3, belong to a battery of enzymes having in common XREs and AREs in the promoter regions of the genes that code for them, Figure 12, *vide supra*. Thus, cellular levels of these enzymes are coordinately elevated upon introduction of so-called XRE-B, e.g., 3-methylcholanthrene, and ARE-B, e.g., catechol, inducers, Figure 12 and Table 3, *vide supra*. Given the foregoing, the expectation was that the monoterpenes would induce the increased expression of ALDH-3 in MCF-7/0 cells.

The expectation was realized experimentally. Limonene, perillyl alcohol, perillyl aldehyde and perillic acid each induced ALDH-3 as well as pan-GST, in MCF-7/0 cells, Figure 13, but the amount of induction was less than dramatic. Perillyl aldehyde was easily the most potent inducer of these enzymes. Preliminary experiments showed that ALDH-3 catalyzed the oxidation of perillyl aldehyde, albeit much less effectively than ALDH-1 (data not shown).

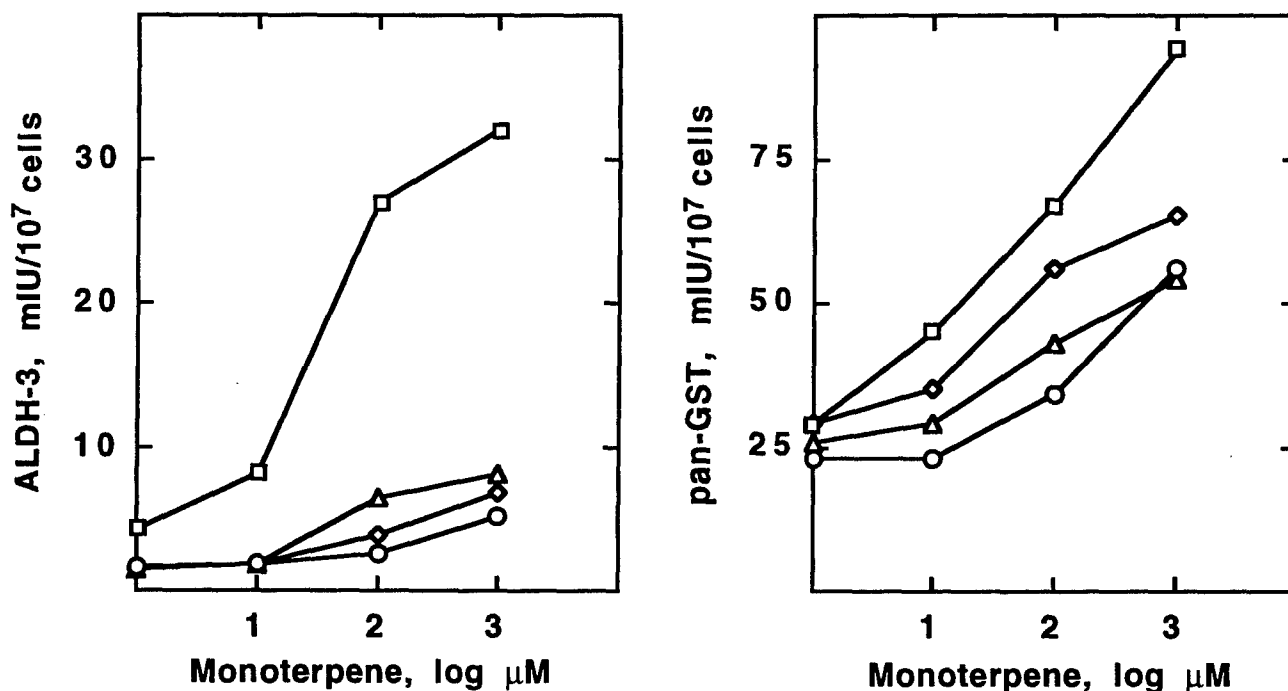


Figure 13. Induction of ALDH-3 and GST enzymes by monoterpenes in MCF-7/0 cells. Exponentially growing human breast adenocarcinoma MCF-7/0 cells were continuously exposed to of limonene (○), perillyl alcohol (Δ), perillyl aldehyde (◇), perillic acid (□) or vehicle for 5 days after which time they were harvested and NADP-linked ALDH-3 and pan-GST catalytic activities were quantified as described elsewhere [Sreerama and Sladek, 1994]. Substrates were 4 mM benzaldehyde and 1 mM 1-chloro-2,4-dinitrobenzene, respectively. ALDH-3 and GST catalytic activities in untreated cells were 1.8 and 22 mIU/10⁷ cells, respectively.

In addition to their chemopreventive activity, limonene and perillyl alcohol have been shown to inhibit the proliferation of cultured tumor cells and to exhibit a chemotherapeutic effect against mammary and other tumor types in animal models [reviewed in Gould 1995; NCI, 1996]. In our investigations, limonene, perillyl alcohol, perillyl aldehyde and perillic acid each inhibited the proliferation of MCF-7/0 cells; GI₉₀ values (concentrations of monoterpene required to effect a 90% inhibition of growth) were >1.0, 0.6, 0.85 and >1.0 mM, respectively (data not shown).

~~CONFIDENTIAL~~

Phase I clinical evaluation of limonene in the UK [reviewed in Gould, 1995] and of perillyl alcohol in the US [reviewed in NCI, 1996] has already been initiated in cancer patients with advanced solid tumors. The primary hope is that these agents will prove to be of value in the chemotherapy of breast cancer. Currently, cyclophosphamide constitutes the core of most treatment regimens used for advanced breast cancer. Thus, there is the potential for limonene or perillyl alcohol to be combined with cyclophosphamide in the treatment of advanced breast cancer. Our findings suggest that an unfavorable drug interaction (increased ALDH-3-catalyzed detoxification of cyclophosphamide) may occur in that event.

Task #7b: Generate a stably cyclophosphamide-resistant MCF-7/0 subline by exposing MCF-7/0 cells to gradually increasing concentrations of benzpyrene.

Heretofore, we were successful in effecting transient cellular resistance to oxazaphosphorines by transiently inducing ALDH-3 in MCF-7/0 cells with XRE-B inducers, e.g., polycyclic aromatic hydrocarbons such as 3-methylcholanthrene, and ARE-B inducers, e.g., phenolic antioxidants such as catechol, Figure 12 and Table 3, *vide supra*.

On the other hand, overexpression of ALDH-3 and resistance to oxazaphosphorines are stable in the MCF-7/OAP cell-line. In this model, only the ARE-B enzyme levels are elevated, Figure 12 and Table 3, *vide supra*.

Desired for future studies was a stably cyclophosphamide-resistant MCF-7/0 subline (model) in which XRE-B of drug-metabolizing enzymes was stably elevated. Benz(a)pyrene appeared to be a suitable mutagen/selecting agent for this purpose because 1) it is a known mutagen [Brookes et al., 1985], 2) it is toxic to MCF-7/0 cells [Sreerama and Sladek, 1993a], 3) it is a polycyclic aromatic hydrocarbon that has been shown to induce ALDH-3 in MCF-7/0 cells [Sreerama and Sladek, 1993a], 4) it is known to be an AhR-agonist and to induce CYP 1A1 [reviewed in Nebert et al., 1990] and 5) resistance to benz(a)pyrene on the part of MCF-7/0 cells has been effected by continuous exposure of them to it [Moore et al., 1994] Thus, MCF-7/0 cells were grown in the presence of gradually increasing concentrations of benz(a)pyrene over a period of four months and a benz (a)pyrene-resistant subline (MCF-7/0/BP) resulted. These cells were also resistant to mafosfamide ($LC_{90} > 2$ mM as compared to 60 μ M in the parent MCF-7/0 cell line). ALDH-3, pan-GST and DT-D levels were markedly elevated, but unexpectedly, that of CYP 1A1 was not, Table 6, i.e., our effort to generate a MCF-7/0 subline that stably expresses elevated levels of the XRE-B of enzymes was not rewarded. Increased expression of ALDH-3, pan-GST and DT-D was stable as indicated by unchanged levels of these enzymes even after the passage of the cells for over one year in the absence of benz(a)pyrene; predictably then, resistance to mafosfamide and benz(a)pyrene was fully stable as well.

Table 6. Cellular levels of various enzymes in human breast adenocarcinoma MCF-7/0 cells made resistant to benz(a)pyrene^a

Cells	Enzyme Activity, mIU/10 ⁷ cells			
	ALDH-3	pan-GST	DT-D	CYP 1A1
MCF-7/0	1.8	18	160	0.02
MCF-7/0/BP	704	749	567	0.02

^aMCF-7/0 cells were grown in the presence of gradually increasing concentrations of benzopyrene over a period of four months and the resulting subline (MCF-7/0/BP) was resistant to benz(a)pyrene (LC₉₀ >100 μM) as compared to the parent cell line (LC₉₀ = 0.3 μM). Enzyme activities were determined as described elsewhere [Sreerama and Sladek, 1994; Rekha and Sladek, 1997b]. Substrates were 4 mM benzaldehyde, 1 mM 1-chloro-2,4-dinitrobenzene, 0.04 mM 2,6-dichlorophenol-indophenol and 0.005 mM 7-ethoxyresorufin for NADP-linked ALDH-3, pan-GST, NADH-linked DT-D and NADPH-linked CYP 1A1 activities, respectively.

Task # 7c: Generate a stably cyclophosphamide-resistant MCF-7/0 subline by exposing MCF-7/0 cells once to a high concentration of mafosfamide for 30 minutes.

The stably oxazaphosphorine-resistant MCF-7/OAP cell line in which levels of ALDH-3 and the other ARE-B of enzymes are elevated, was obtained by continual exposure of MCF-7/0 cells to gradually increasing concentrations of 4-hydroperoxycyclophosphamide over several months [Frei et al., 1988]. Clinical drug resistance to chemotherapeutic agents is often acquired rapidly. Interestingly in that regard, rapid acquisition of resistance can be effected in cultured human leukemic cells by exposing them to a high concentration of an oxazaphosphorine for a short period of time [Andersson et al., 1994]. Specifically, in this study, cloned human chronic myelogenous leukemic KBM-7/b5 cells were exposed to a high concentration of 4-hydroperoxycyclophosphamide (200 μM) for 1 hr and several stably cyclophosphamide-resistant clones were obtained several weeks later. Similarly, resistance to 4-hydroperoxycyclophosphamide was acquired in human breast cancer MDA-MB-231 cells when they were exposed to 4-hydroperoxycyclophosphamide (25 μM) for 3 days [Graham et al., 1994], although resistance in this case was transient and depended on a 3-dimensional cell contact effect.

Encouraged by these reports, we set forth to see whether we could generate a cyclophosphamide-resistant subline of human breast adenocarcinoma MCF-7/0 cells by briefly exposing them to a high concentration of mafosfamide. MCF-7/0 cells (1 x 10⁶) were exposed to mafosfamide (1 mM) for 30 min at 37°C. after which they were cultured (monolayer) for 4-5 weeks at the end of which time 4 surviving colonies were recovered (surviving fraction of 4 x 10⁻⁶) and were further expanded as monolayer cultures. Three of the four were as sensitive to mafosfamide as were the parent MCF-7/0 cells (LC₉₀ = 65 μM); the fourth (MCF-7/0/MAF) was relatively insensitive (LC₉₀ > 2 mM). ALDH-3 levels in the three mafosfamide-sensitive clonal expansions did not differ from that in the parent MCF-7/0 cells, whereas the ALDH-3 level in the mafosfamide-resistant MCF-7/0/MAF cells was markedly elevated as were pan-GST and DT-D, but not CYP 1A1, levels, Table 7, i.e., only the ARE-B of enzymes, Figure 12, *vide supra*,

were elevated. Increased expression of ALDH-3, pan-GST and DT-D was stable as indicated by unchanged levels of these enzymes even after passage of these cells for over a year in the absence of mafosfamide; predictably then, resistance to mafosfamide was fully stable as well.

The implications of these findings with regard to high-dose clinical chemotherapy are self-evident.

Table 7. Cellular levels of various enzymes in human breast adenocarcinoma MCF-7/0 cells made resistant to mafosfamide with a single 30 minute high-dose exposure to mafosfamide^a

Cells	Enzyme Activity, mIU/10 ⁷ cells			
	ALDH-3	pan-GST	DT-D	CYP 1A1
MCF-7/0	1.8	18	160	0.02
MCF-7/0/MAF	373	521	763	0.02

^aEnzyme activities were determined as described elsewhere [Sreerama and Sladek, 1994; Rekha and Sladek, 1997]. Substrates were 4 mM benzaldehyde, 1 mM 1-chloro-2,4-dinitrobenzene, 0.04 mM 2,6-dichlorophenol-indophenol and 0.005 mM 7-ethoxyresorufin for NADP-linked ALDH-3, pan-GST, NADH-linked DT-D and NADPH-linked CYP 1A1 activities, respectively.

CONCLUSIONS

ALDH-3 and ALDH-1 catalyze the detoxification of oxazaphosphorines. In each case, detoxification is effected when the enzyme catalyzes the oxidation of aldophosphamide to carboxyphosphamide. Elevated levels of either of these enzymes in cultured tumor cell models account for the decrease in cellular sensitivity to the oxazaphosphorines exhibited by such models. Various lines of evidence indicate that variable levels of ALDH-1, and probably ALDH-3 as well, accounts, at least in some cases, for the variable response of breast tumors to cyclophosphamide and other oxazaphosphorines in the clinic. Cellular levels of glutathione may also be important in that regard.

A broad range of glutathione S-transferase, DT-diaphorase and cytochrome P450 1A1, as well as of ALDH-3, ALDH-1 and glutathione, levels was found in human breast tumor tissue samples. Glutathione S-transferases catalyze the detoxification of a number of chemotherapeutic agents, e.g., melphalan and chlorambucil. DT-diaphorase catalyzes the toxification of at least two chemotherapeutic agents, viz., mitomycin C and EO9. Cytochrome P450 1A1 catalyzes the toxification of ellipticine. Thus, the broad range of glutathione S-transferase, DT-diaphorase and cytochrome P450 1A1 levels found in surgically removed human breast tumor samples indicates that variable levels of these enzymes accounts, at least in some cases, for the variable response of breast tumors to these agents in the clinic.

As judged by *in vitro* experiments, at least three agents, viz., gossypol and two chlorpropamide derivatives, are of potential value *in vivo* with regard to relatively selectively inhibiting ALDH-3-catalyzed oxidative reactions, thereby sensitizing tumor cells, otherwise insensitive to cyclophosphamide and other oxazaphosphorines because they express large amounts of ALDH-3, to these agents. Moreover, human tumor cell ALDH-3 was found to be more sensitive to each of the chlorpropamide analogues than was human normal cell ALDH-3. Thus, it may be possible to develop a clinically useful selective inhibitor of tumor cell ALDH-3 thereby allowing the selective sensitization of tumor cells expressing large amounts of ALDH-3 to cyclophosphamide and other oxazaphosphorines.

Abundantly present dietary/environmental constituents coordinately induce ALDH-3, several glutathione S-transferases, DT-diaphorase, UDP-glucuronosyl transferase and other enzymes in our cultured human breast cancer models. As stated above, these enzymes catalyze the biotransformation (detoxification in most cases; toxification in some) of cancer chemotherapeutic agents. Induction is reversible. Thus, attention to the composition of the diet may be prudent, and even used advantageously, when using relevant cancer chemotherapeutic agents. However, whereas, in some samples, the levels of ALDH-3, glutathione S-transferase and DT-diaphorase were each elevated in the repository samples that we examined, the frequency of occurrence was no more than that expected by chance alone.

Salivary levels of ALDH-3, pan-glutathione S-transferase and DT-diaphorase were found to vary widely and to be induced by the consumption of various dietary constituents, viz., coffee and broccoli. Thus, assuming salivary enzyme levels mirror breast tumor tissue enzyme levels, there is the potential of measuring salivary levels of these enzymes, a noninvasive procedure, for the purpose of chemotherapeutic

prognostication. However, preliminary studies indicate that salivary ALDH-3 levels do not mirror breast tumor tissue levels of this enzyme.

Limonene and perillyl alcohol are currently in Phase 1 clinical trial. Preclinical experiments indicate that they may be of value in the treatment of breast cancer. We found that a putative metabolite of these monoterpenes, viz., perillyl aldehyde, induced ALDH-3 (and pan-GST) levels in cultured human breast adenocarcinoma MCF-7/0 cells albeit, relatively weakly. Cyclophosphamide is the mainstay of virtually every chemotherapeutic regimen currently used to treat advanced breast cancer. Should the monoterpenes prove to be of clinical utility, the likelihood that they would be given along with cyclophosphamide is quite high. An unwanted drug interaction may result.

As judged by the information that we have generated thus far, agents that induce ALDH-3, several glutathione S-transferases, DT-diaphorase, UDP-glucuronosyl transferase and other enzymes via Ah receptors and XREs (xenobiotic responsive elements) present in the 5'-upstream regions of the genes coding for these enzymes e.g., polycyclic aromatic hydrocarbons such as 3-methylcholanthrene, can only do so in estrogen-receptor positive cells, i.e., they cannot induce them in estrogen receptor-negative cells, whereas agents that induce ALDH-3, several glutathione S-transferases, DT-diaphorase, UDP-glucuronosyl transferase and other enzymes via AREs (antioxidant responsive elements) present in the 5'-upstream regions of the genes coding for these enzymes, e.g., antioxidants such as catechol, can do so in both estrogen receptor-positive and -negative cells. Hypermethylation of the gene coding for it appears to account for the failure of some cell lines to express estrogen receptors.

Stable overexpression of ALDH-3 protein in MCF-7/OAP cells was found to be the consequence of stable overexpression of ALDH-3 mRNA. Overexpression of the latter was not due to DNA amplification or demethylation of the relevant gene.

Transient overexpression of ALDH-3 protein in MCF-7/0/MC and MCF-7/0/CAT cells was found to be the consequence of transient overexpression of ALDH-3 mRNA. Overexpression of the latter was not due to demethylation of the relevant gene.

Stably cyclophosphamide-resistant breast adenocarcinoma MCF-7 cells can be generated with a single exposure of the parent cells to a very high concentration of mafosfamide for 30 minutes. The potential clinical relevance of this finding is self-evident.

Polycyclic aromatic hydrocarbons are capable of inducing both transient (low concentration of the hydrocarbon for a few days) and stable (continually increasing concentrations of the hydrocarbon over a period of several months) cellular resistance to mafosfamide (cyclophosphamide) in a cultured breast cancer cell (MCF-7/0) model.

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*Included in the appendix (reprints enclosed).

APPENDIX
(Reprints Enclosed)

- *Rekha, G. K., Devaraj, V. R., Sreerama, L., Lee, M. J. C., Nagasawa, H. T. and Sladek, N. E. Inhibition of human class 3 aldehyde dehydrogenase, and sensitization of tumor cells that express significant amounts of this enzyme to oxazaphosphorines, by chlorpropamide analogues. *Biochem. Pharmacol.*, 1997, In press.
- *Sreerama, L. and Sladek, N. E. Cellular levels of class 1 and class 3 aldehyde dehydrogenases and certain other drug-metabolizing enzymes in human breast malignancies. *Clin. Cancer Res.*, 1997, In press.

Cellular Levels of Class 1 and Class 3 Aldehyde Dehydrogenases and Certain Other Drug-metabolizing Enzymes in Human Breast Malignancies¹

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ABSTRACT

Molecular determinants of cellular sensitivity to cyclophosphamide, long the mainstay of chemotherapeutic regimens used to treat metastatic breast cancer, include class 1 and class 3 aldehyde dehydrogenases (ALDH-1 and ALDH-3, respectively), which catalyze the detoxification of this agent. Thus, interindividual variation in the activity of either of these enzymes in breast cancers could contribute to the wide variation in clinical responses that are obtained when such regimens are used to treat these malignancies. Consistent with this notion, ALDH-1 levels in primary and metastatic breast malignancies were found to range from 1–276 and 8–160 mIU/g tissue, respectively, and those of ALDH-3 from 1–242 and 6–97 mIU/g tissue, respectively. ALDH-1 and ALDH-3 levels in normal breast tissue predicted the levels of these enzymes in primary and metastatic breast malignancies present in the same individuals. Confirming and extending the observations of others, levels of glutathione, a molecular determinant of cellular sensitivity to various DNA cross-linking agents including cyclophosphamide, DT-diaphorase, glutathione S-transferases, and cytochrome P450 1A1, each of which is known to catalyze the detoxification/toxification of one or more anticancer agents (although not of cyclophosphamide), also varied widely in primary and metastatic breast malignancies. Given the wide range of ALDH-1, ALDH-3, and glutathione levels that were observed in malignant breast tissues, measurement of their levels in normal breast tissue and/or primary breast malignancies prior to the initiation of chemotherapy is likely to be of value in predicting the therapeutic potential, or lack thereof, of cyclophosphamide in the treatment of metastatic breast cancer, thus providing a rational

basis for the design of individualized therapeutic regimens when treating this disease.

INTRODUCTION

Cyclophosphamide is perhaps the most widely used chemotherapeutic drug in the conventional treatment, as well as the high-dose treatment (followed by autologous multipotent/pluripotent hematopoietic cell reinfusion to ameliorate the severe myelosuppression that accompanies it), of metastatic breast cancer (reviewed in Refs. 1–4). Unfortunately, its use, even in combination with other agents as is usual, rarely results in cures. Most often underlying the failure of cyclophosphamide to rid the patient of all malignant cells are the facts that drug-resistant mutant clones appear early in the natural history of tumor progression, *i.e.*, even before drug treatment (intrinsic resistance), and that new drug-resistant clones may develop quite rapidly after the initiation of therapy (acquired resistance).

Until resistant subpopulations become the dominant population, cyclophosphamide and related compounds are clinically effective in the treatment of metastatic breast cancer and, indeed, play a lead role in that regard, even when combined with other agents; therefore, an understanding of how resistance to these agents is effected would likely be of value because measures may then become apparent as to how to prevent and/or negate it. Molecular determinants of cellular sensitivity to cyclophosphamide and other oxazaphosphorines, *e.g.*, 4-hydroperoxycyclophosphamide, mafosfamide, and ifosfamide, include class 1 and class 3 ALDHs³ (ALDH-1 and ALDH-3, respectively). Specifically, cellular sensitivity to the oxazaphosphorines is inversely related to the cellular content of these enzymes because they each catalyze the detoxification of these agents (reviewed in Refs. 5 and 6; Refs. 7–18). Interindividual variation in ALDH-1 and/or ALDH-3 levels has been observed in colon, ovarian, and salivary gland malignancies (19–21). Not known is the extent of interindividual variation, if any, in the activity of either of these enzymes in breast malignancies.

Thus, the investigation reported herein sought to ascertain to what extent ALDH-1 and ALDH-3 levels varied in malignant (and normal) breast tissues, *i.e.*, whether clinical resistance to the oxazaphosphorines could be accounted for, at least in some cases, by relatively elevated levels of these enzymes. Surgically removed malignant (and normal) breast tissue samples were used for this purpose.

A second objective was to ascertain whether ALDH-1 and ALDH-3 levels in normal and malignant (primary and meta-

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³ The abbreviations used are: ALDH, aldehyde dehydrogenase; GST, glutathione S-transferase; DT-D, DT-diaphorase; CYP 1A1, cytochrome P450 1A1; GSH, glutathione; ER, estrogen receptor.

static) breast tissue samples taken from the same patients were quantitatively related. This was because, in the event that such a relationship did exist, determination of these enzyme levels in easily obtainable normal or malignant primary breast tissue samples would be of prognostic value with regard to the success, or lack thereof, that might be anticipated upon the subsequent use of an oxazaphosphorine to eliminate microscopic malignant metastatic nodules.

Xenobiotics that are abundantly present in the diet/environment, *e.g.*, 3-methylcholanthrene and catechol, rapidly, coordinately, and reversibly induce ALDH-3, DT-D, GSTs, UDP-glucuronosyl transferase and, in some cases, CYP 1A1 in cultured human breast cancer cell models. Consequently, reversible multienzyme-mediated multidrug resistance/collateral sensitivity to cyclophosphamide and certain other anticancer drugs is rapidly effected (22). Some of the latter are also already used, *e.g.*, mitoxantrone (reviewed in Ref. 23), or show promise, *e.g.*, E09 (24), in the treatment of metastatic breast cancer. Ingestion of various dietary substances, *e.g.*, coffee and broccoli, has been shown to result in the coordinated elevation of ALDH-3, DT-D, and the GSTs in human saliva (25). Stable (irreversible) intrinsic as well as acquired phenotypes of this sort have also been observed in cultured human cancer models (12, 13, 22). Not known is whether coordinated elevation of these enzymes ever occurs in normal and/or malignant breast tissue. Thus, in a first attempt to address this question, DT-D, pan-GST, GST α , GST μ , GST π , and CYP 1A1 levels in the malignant (and normal) breast tissue samples were also quantified.

The sulfhydryl, glutathione, appears to be yet another molecular determinant of cellular sensitivity to the oxazaphosphorines (reviewed in Ref. 5). Thus, its levels in malignant (and normal) breast tissues were determined as well.

MATERIALS AND METHODS

Normal ($n = 26$) and malignant ($n = 112$) female breast tissue samples obtained from 110 donors were procured from the Cooperative Human Tissue Network, Midwestern Division, Columbus, OH. Surgically removed normal and malignant breast tissue samples were snap-frozen in liquid nitrogen (within 6 h after removal), stored at -70°C (5 to 60 days), and shipped to us in dry ice. Patient characteristics, diagnosis, and cellular characteristics (Table 1) were provided by the pathology reports that accompanied the tissue specimens. Purified human GSTs α , μ , and π and affinity-purified polyclonal antibodies specific for each of these isozymes, *i.e.*, anti-GST α IgG, anti-GST μ IgG, and anti-GST π IgG, respectively (26), were generously provided by Dr. A. J. Townsend (Department of Biochemistry, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC). Microsomes, isolated from a cell line (h1A1 v2) transfected with human CYP 1A1 cDNA and constitutively expressing the enzyme (15 ng CYP 1A1/mg microsomal protein), were purchased from Gentest Corporation (Woburn, MA), as was polyclonal anti-CYP 1A1 IgG (obtained from goats immunized with rat CYP 1A1). Anti-goat IgG-alkaline phosphatase conjugate was purchased from Sigma Chemical Co. (St. Louis, MO). Enhanced protein binding 96-well ELISA plates were purchased from Corning, Inc. (New York, NY). All other chemicals, reagents, and supplies were

Table 1 Patient characteristics, diagnosis, and cellular characteristics^a

Parameter	%		
	Normal breast tissue	Malignant breast tissue	
		Primary	Metastatic
Patient age			
<45	27 (6/22)	16 (13/80)	27 (6/22)
45-60	27 (6/22)	43 (34/80)	32 (7/22)
>60	46 (10/22)	41 (33/80)	41 (9/22)
Smokers	50 (1/2)	50 (3/6)	
C		1 (1/90)	
IDC		92 (83/90)	82 (18/22)
ILA		4 (3/90)	14 (3/22)
Diagnosis			
LC			4 (1/22)
MA		1 (1/90)	
PC		1 (1/90)	
PDC		1 (1/90)	
ER ⁺	60 (6/10)	52 (17/33)	36 (4/11)
PR ⁺	50 (5/10)	42 (14/33)	18 (2/11)
Cellular status			
p53 ⁺	50 (2/4)	45 (5/11)	0 (0/2)
Bcl-2 ⁺		100 (1/1)	
c-erbB-2 ⁺		90 (9/10)	0 (0/3)

^a A total of 26 normal and 112 malignant (90 primary and 22 metastatic) breast tissue specimens obtained from 110 donors was evaluated, but the pathology reports that accompanied these specimens did not always include all of the information listed in this table. C, Carcinoma; IDC, infiltrating ductal carcinoma; ILA, infiltrating lobular adenocarcinoma; LC, lobular carcinoma; MA, mucinous adenocarcinoma; PC, papillary carcinoma; PDC, poorly differentiated carcinoma; PR, progesterone receptor; p53, tumor suppressor protein; Bcl-2, oncoprotein; c-erbB-2, oncoprotein. Known also is that 103 of the 110 donors had not been treated with a cancer chemotherapeutic agent prior to surgical removal of the tissue specimens. Unavailable was the information in that regard for the other seven donors [four normal and seven malignant (all metastatic) breast tissue specimens].

purchased from commercial sources or were prepared as described previously (7, 9, 25, 27).

Preparation of purified ALDH-1 and ALDH-3 from human stomach mucosa and chicken polyclonal antibodies specific for these enzymes, *i.e.*, anti-ALDH-1 IgY and anti-ALDH-3 IgY, respectively, was as described previously (7, 28).

Soluble (105,000 g supernatant) and particulate (105,000 g pellet) fractions of normal and malignant breast tissues were prepared as described previously for breast tissues (7). Soluble (105,000-g supernatant) fractions were used when tissue levels of ALDH-1, ALDH-3, DT-D, pan-GST, and GSTs α , μ and π were to be quantified. Lubrol (0.3%-solubilized particulate (105,000-g pellet) fractions were used when tissue levels of CYP 1A1 were to be quantified.

Direct quantification of ALDH-1, ALDH-3, DT-D, and pan-GST catalytic activities in soluble (105,000-g supernatant) fractions prepared from normal and malignant breast tissues was by spectrophotometric assay as described previously (7, 9, 28). Acetaldehyde and NAD, 4 mM each, were the substrate and cofactor, respectively, when ALDH-1 activity was quantified. Benzaldehyde and NADP, 4 mM each, were the substrate and cofactor, respectively, when ALDH-3 activity was quantified. Substrate, cofactor, and inhibitor were 2,6-dichlorophenol-endo-

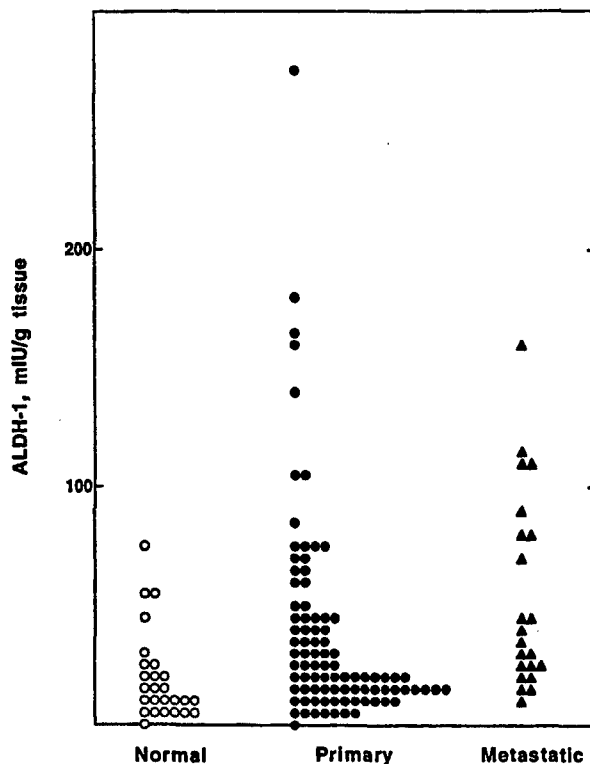


Fig. 1 ALDH-1 levels in human normal breast ($n = 26$) and primary ($n = 90$) and metastatic ($n = 22$) breast tumor tissue samples. ALDH-1 catalytic activity [NAD (4 mM)-linked oxidation of acetaldehyde (4 mM)] was quantified both directly by spectrophotometric assay and indirectly by an ELISA as described in "Materials and Methods." Only catalytic activity quantified indirectly is shown because the values obtained were independent of the method used to obtain them ($r^2 = 0.987$, $P \leq 0.0001$). Points are means, rounded off for clarity of presentation to zero if they were < 2.5 mIU/g, and to 5 mIU/g or the nearest multiple thereof if they were ≥ 2.5 mIU/g, of duplicate determinations made on single normal and malignant, or just malignant, tissue samples taken from each of 110 patients.

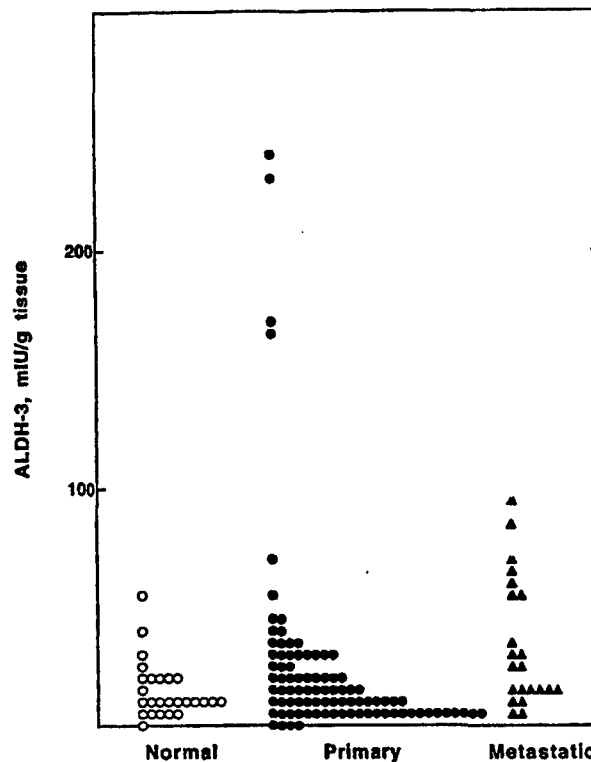


Fig. 2 ALDH-3 levels in human normal breast ($n = 26$) and primary ($n = 90$) and metastatic ($n = 22$) breast tumor tissue samples. ALDH-3 catalytic activity [NADP (4 mM)-linked oxidation of benzaldehyde (4 mM)] was quantified both directly by spectrophotometric assay and indirectly by an ELISA as described in "Materials and Methods." Only that quantified indirectly is shown because the values obtained were independent of the method used to obtain them ($r^2 = 0.976$, $P \leq 0.0001$). Points are means, rounded off for clarity of presentation to zero if they were < 2.5 mIU/g and to 5 mIU/g or the nearest multiple thereof if they were ≥ 2.5 mIU/g of duplicate determinations made on single normal and malignant, or just malignant, tissue samples taken from each of 110 patients.

phenol (40 μ M), NADH (160 μ M), and dicumarol (10 μ M), respectively, when DT-diaphorase activity was quantified. Co-substrates were 1-chloro-2,4-dinitrobenzene and GSH, 1 mM each, when pan-GST activity was quantified.

Spectrophotometric quantification of GSH levels in normal and malignant breast tissue was as described by Anderson (29). Spectrophotometric quantification of protein levels in soluble (105,000-g supernatant) and Lubrol-solubilized particulate (105,000-g pellet) fractions of normal and malignant breast tissues was as described previously (7).

Indirect quantification of ALDH-1, ALDH-3, and GSTs α , μ , and π catalytic activities in soluble (105,000-g supernatant) fractions was by ELISAs as described previously (22, 27). Dilution with blocking solution of primary antibodies was 1:1000 in the case of ALDH-1 and ALDH-3 and 1:2000 in the case of GSTs α , μ , and π . Normal and malignant breast tissue levels (catalytic activities/g of tissue) of ALDH-1, ALDH-3, and GSTs α , μ , and π were estimated from standard curves generated with purified enzymes; specific activities of the latter were 2,850, 60,500, 44,600, 24,100, and 56,800 mIU/mg protein,

respectively, when substrates and cofactors were as in the direct assays.

Quantification of CYP 1A1 levels in Lubrol-solubilized particulate (105,000-g pellet) fractions was by an ELISA as described immediately above, except that the: (a) primary antibody was anti-CYP 1A1 IgG diluted 1:1000 with blocking solution; and (b) secondary antibody was anti-goat IgG-alkaline phosphatase conjugate diluted 1:1000 with blocking solution. Normal and malignant breast tissue levels (pg/g tissue) of CYP 1A1 were estimated from standard curves generated with Lubrol-solubilized CYP 1A1-containing microsomes (15 ng of CYP 1A1/mg of microsomal protein).

The Macintosh-based STATView II (Brainpower, Inc., Calabas, CA) computer program was used to generate P s (one- and two-tailed, unpaired, Student's t -tests), linear regression lines, r^2 (regression coefficients), and P s thereof. A χ^2 test (2×2 table) was used to ascertain whether there were any statistically significant differences in frequencies of expression (positive or negative) of GST α , GST μ , and CYP 1A1 as a function of tissue type and of CYP 1A1 as a function of estrogen receptor

Table 2 ALDH-1, ALDH-3, DT-D, pan-GST, GSTs α , μ and π , CYP 1A1, and GSH levels in human normal and malignant (primary and metastatic) breast tissue samples: summary^a

Enzyme	Normal			Primary			Metastatic			All		
	n	Mean \pm SD	Range	n	Mean \pm SD	Range	n	Mean \pm SD	Range	n	Mean \pm SD	Range
ALDH-1	26	19 \pm 19	3-75	90	37 \pm 43	1-276	22	54 \pm 41	8-160	138	36 \pm 41	1-276
ALDH-3	26	14 \pm 12	2-54	90	24 \pm 41	1-242	22	34 \pm 27	6-97	138	24 \pm 36	1-242
DT-D	26	438 \pm 690	23-2700	90	1190 \pm 1520	10-6250	22	1110 \pm 810	84-2720	138	1030 \pm 1330	10-6250
pan-GST	26	920 \pm 1050	56-4550	90	2400 \pm 2030	123-8880	22	3330 \pm 2380	464-8130	138	2270 \pm 2070	56-8880
GST α	26	125 \pm 171	0-900	90	293 \pm 427	0-2500	22	501 \pm 605	0-1700	138	294 \pm 438	0-2500
GST μ	26	96 \pm 129	0-522	90	301 \pm 560	0-3400	22	295 \pm 654	0-3050	138	261 \pm 529	0-3400
GST π	26	694 \pm 829	100-3800	90	1730 \pm 1410	120-6200	22	2360 \pm 1800	400-6500	138	1630 \pm 1470	100-6500
CYP 1A1	21	1 \pm 5	0-24	80	36 \pm 92	0-570	19	52 \pm 120	0-485	120	33 \pm 90	0-570
GSH	25	264 \pm 681	2-3470	82	1130 \pm 1990	16-10400	18	2120 \pm 3360	16-11100	125	1100 \pm 2130	2-11100

^a Values are summaries of the data presented in Fig. 1, 2, and 5-11. Zero values obtained for GST α , GST μ , and CYP 1A1 are included in the calculation of mean values for these enzymes. Units are mIU/g tissue except in the cases of CYP 1A1 and GSH, where they are pg/g and nmol/g tissue, respectively. Statistically, mean enzyme and GSH levels in primary and metastatic breast tumor tissue are significantly higher ($P < 0.05$; one-tailed, unpaired, Student's *t*-test) than those in normal breast tissue in all cases except in that of metastatic breast tumor tissue GST μ ($P = 0.084$). Mean enzyme and GSH levels in metastatic breast tumor tissues are significantly higher ($P \leq 0.05$) than those in primary breast tumor tissue only in the cases of ALDH-1 and pan-GST.

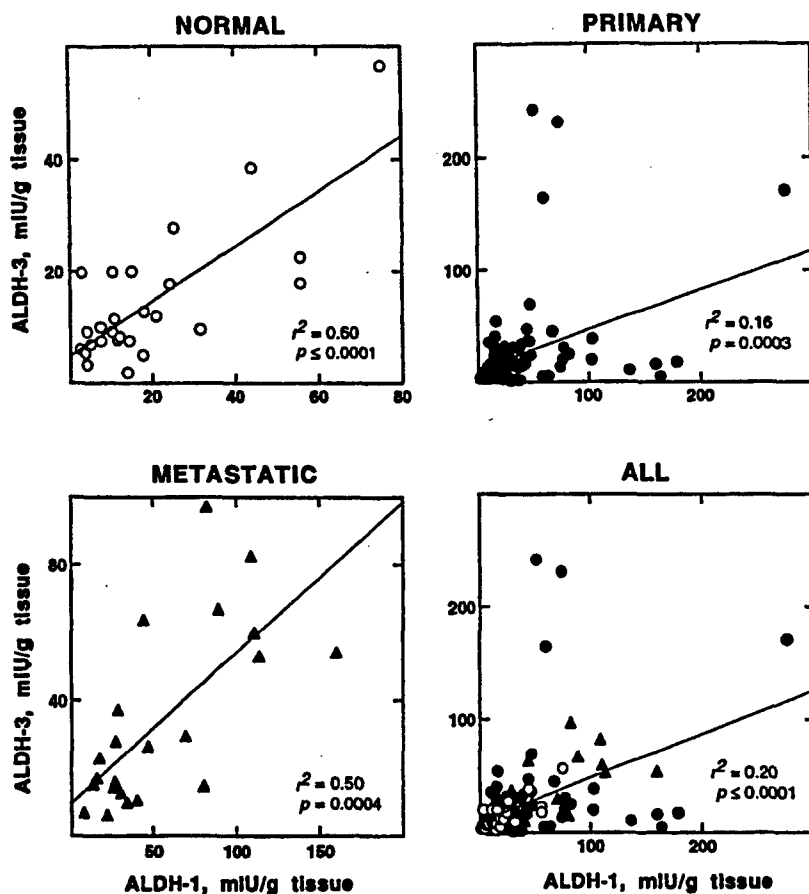


Fig. 3 ALDH-1 and ALDH-3 levels in normal ($n = 26$; \circ) and malignant [primary ($n = 90$; \bullet) and metastatic ($n = 22$; \blacktriangle)] human breast tissues. ALDH-1 [NAD (4 mM)-linked oxidation of acetaldehyde (4 mM)] and ALDH-3 [NADP (4 mM)-linked oxidation of benzaldehyde (4 mM)] catalytic activities were indirectly quantified by ELISAs as described in "Materials and Methods." Points are means of duplicate determinations made on single, normal and malignant, or just malignant, breast tissue samples taken from each of 110 patients.

status. This test was also used to ascertain whether the simultaneously elevated levels of ALDH-3, DT-D, GST (pan-GST and GST π), and CYP 1A1, and/or simultaneously elevated levels of ALDH-3, DT-D, and GST (pan-GST and GST π) that were observed were the consequence of a common or of independent event(s).

RESULTS

Shown in Figs. 1 and 2 are scatter plots of, respectively, ALDH-1 and ALDH-3 levels in normal and malignant (primary as well as metastatic) breast tissue samples. Mean values and SDs are presented in Table 2. Immediately apparent is that the

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T2

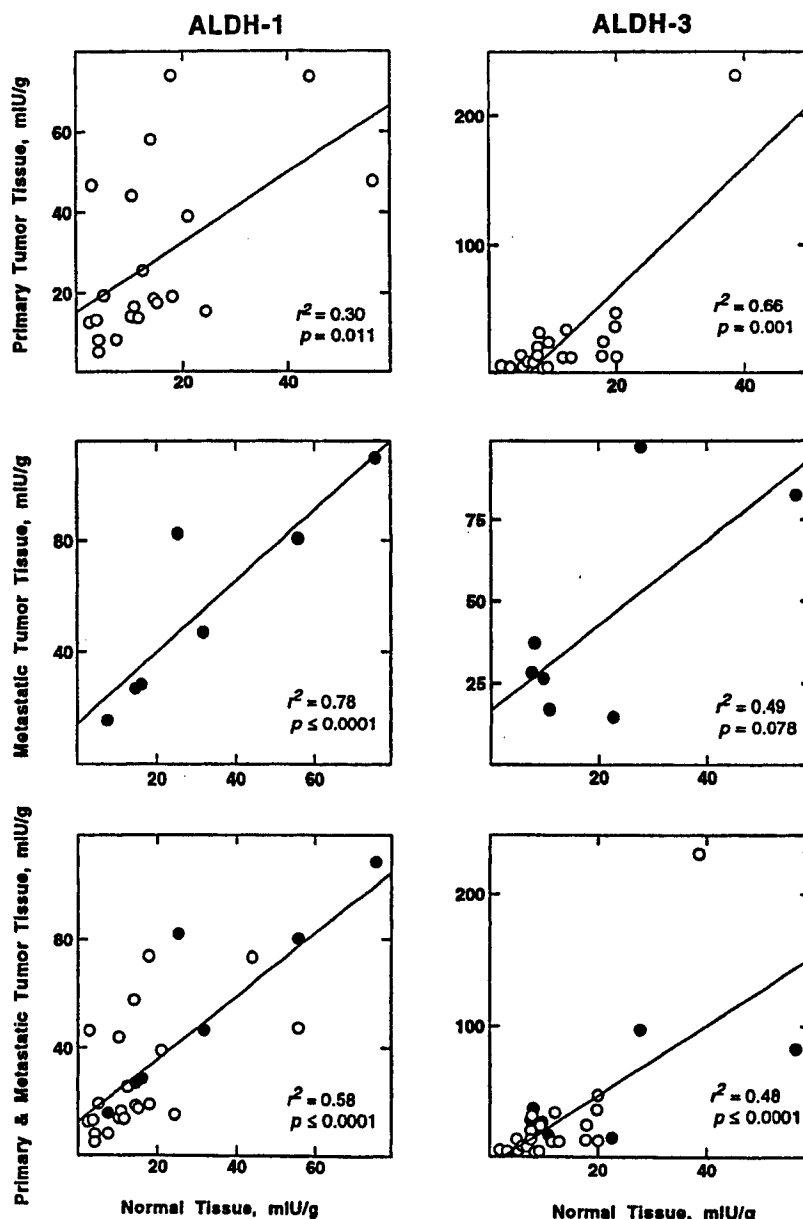


Fig. 4 ALDH-1 and ALDH-3 levels in paired human, normal, and primary tumor ($n = 21$; \circ), and normal and metastatic tumor ($n = 7$; \bullet) breast tissues. ALDH-1 [NAD (4 mM)-linked oxidation of acetaldehyde (4 mM)] and ALDH-3 [NADP (4 mM)-linked oxidation of benzaldehyde (4 mM)] catalytic activities were indirectly quantified by ELISAs as described in "Materials and Methods." Points are means of duplicate determinations made on single normal and malignant breast tissue samples taken from each of 26 patients.

level of each enzyme varies widely in all three tissues. For example, highest levels of ALDH-1 and ALDH-3 in primary breast malignancies were ~250-fold greater than the lowest levels of these enzymes in these tissues. Statistical analysis of this data revealed that mean levels of each enzyme in the malignant (primary or metastatic) breast tissue samples were significantly higher ($P \leq 0.05$) than those in the normal breast tissue samples. The mean ALDH-1 level in the metastatic breast tumor samples was significantly higher ($P = 0.029$) than that in the primary breast tumor samples. The mean ALDH-3 level in the metastatic breast tumor samples was not significantly higher than that in the primary breast tumor samples at a p level of 0.05 but was very nearly so because the p value was 0.051.

Unexpectedly, cellular levels of ALDH-1 and ALDH-3 appeared to be directly related in the normal, as well as in the

malignant (primary as well as metastatic), breast tissue samples (Fig. 3).

ALDH-1 and ALDH-3 levels in the normal breast tissue samples predicted the respective levels of these enzymes in paired primary, as well as metastatic, breast tumor tissue samples (Fig. 4). We did not have enough paired samples to ascertain whether cellular levels of ALDH-1 or ALDH-3 in primary breast malignancies predicted cellular levels of these enzymes in metastatic breast malignancies.

Confirming and extending the observations of others (Refs. 30 and 31; reviewed in Ref. 32), DT-D, pan-GST, GST α , GST μ , GST π , and CYP 1A1 levels also varied widely in normal and malignant (primary and metastatic) breast tissue (Figs. 5–10 and Table 2). As in the cases of ALDH-1 and ALDH-3, statistical analysis of the data revealed that mean

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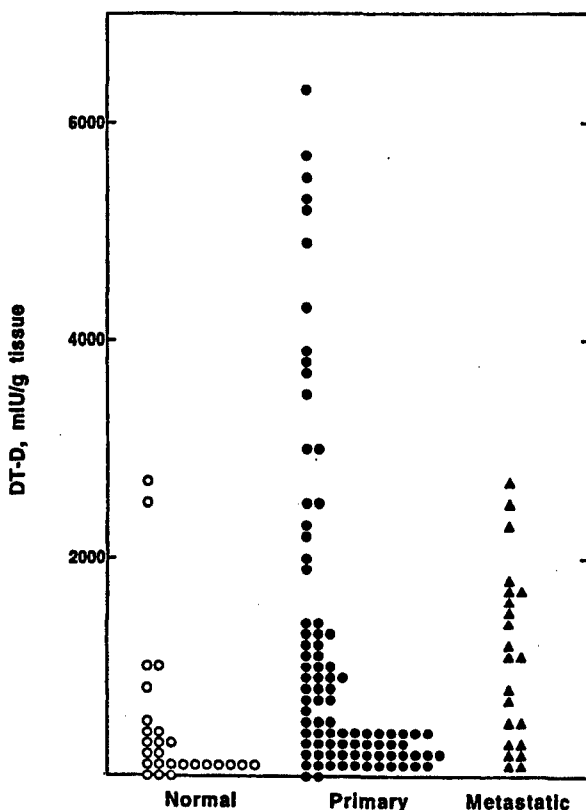


Fig. 5 DT-D levels in human normal breast ($n = 26$) and primary ($n = 90$) and metastatic ($n = 22$) breast tumor tissue samples. A spectrophotometric assay was used as described in "Materials and Methods" to directly quantify DT-D catalytic activity; substrate, cofactor, and inhibitor were 2,6-dichlorophenol-indophenol ($40 \mu\text{M}$), NADH ($160 \mu\text{M}$), and dicumarol ($10 \mu\text{M}$), respectively. Points are means, rounded off for clarity of presentation to zero if they were <50 mIU/g and to 100 mIU/g or the nearest multiple thereof if they were ≥ 50 mIU/g of duplicate determinations made on single normal and malignant, or just malignant, tissue samples taken from each of 110 patients.

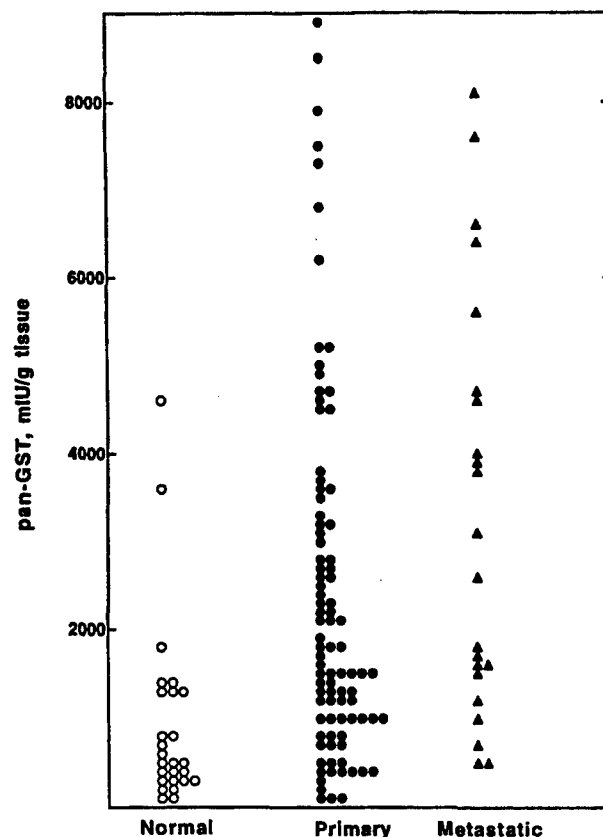


Fig. 6 Pan-GST levels in human normal breast ($n = 26$), and primary ($n = 90$) and metastatic ($n = 22$) breast tumor tissue samples. A spectrophotometric assay was used as described in "Materials and Methods" to directly quantify pan-GST catalytic activity; cosubstrates were 1-chloro-2,4-dinitrobenzene and GSH, 1 mM each. Points are means, rounded off for clarity of presentation to zero if they were <50 mIU/g and to 100 mIU/g or the nearest multiple thereof if they were ≥ 50 mIU/g of duplicate determinations made on single normal and malignant, or just malignant, tissue samples taken from each of 110 patients.

levels of each enzyme in the malignant (primary or metastatic) breast tissue samples were significantly higher ($P \leq 0.05$) than those in the normal breast tissue samples with one exception, metastatic breast tumor tissue $\text{GST}\mu$ ($P = 0.084$). The mean pan-GST level in the metastatic breast tumor samples was significantly higher ($P = 0.041$) than that in the primary breast tumor samples. Mean DT-D, $\text{GST}\alpha$, $\text{GST}\mu$, $\text{GST}\pi$, and CYP 1A1 levels in the metastatic breast tumor samples were not significantly higher at a P level of 0.05 than those in the primary breast tumor samples, although those of $\text{GST}\alpha$ ($P = 0.061$) and $\text{GST}\pi$ ($P = 0.058$) were very nearly so.

Statistically, lack of detectable $\text{GST}\alpha$ and $\text{GST}\mu$ (Figs. 7 and 8, respectively) was independent of tissue type ($P > 0.1$), as was the lack of detectable CYP 1A1 (Fig. 10; $P > 0.05$); however, a more frequent lack of detectable CYP 1A1 in normal breast tissue was very nearly statistically significant ($P = 0.0523$).

As expected, tissue levels of $\text{GST}\alpha$, $\text{GST}\mu$, $\text{GST}\pi$, and pan-GST were directly related to each other when all of the data ($n = 138$) was grouped ($P \leq 0.0001$ in each case; linear

regression analyses of data not shown). Similarly, there was a direct relationship between pan-GST and ALDH-1, ALDH-3, and DT-D levels ($P = 0.01, 0.02, \text{ and } 0.003$, respectively; linear regression analyses of data not shown). However, DT-D levels were not related to those of ALDH-1 or ALDH-3 ($P = 0.33$ and 0.42 , respectively), and CYP 1A1 levels were unrelated ($P > 0.1$) to those of the other enzymes (linear regression analyses of data not shown).

Evidence (levels that are each more than 1 SD above normal breast tissue mean levels) for the coordinated induction of ALDH-3, DT-D, pan-GST, and CYP 1A1 (induced gene expression effected by transactivation of a *cis*-acting DNA element, xenobiotic responsive element, present in the 5'-upstream regions of the genes coding for these enzymes; Refs. 13 and 33-41) was observed in only three samples, two primary and one metastatic breast tumors (Table 3). As judged by the same criteria, coordinated induction of ALDH-3, DT-D, and pan-GST, but not of CYP 1A1 (induced gene expression effected by transactivation of a *cis*-acting DNA element, antioxidant respon-

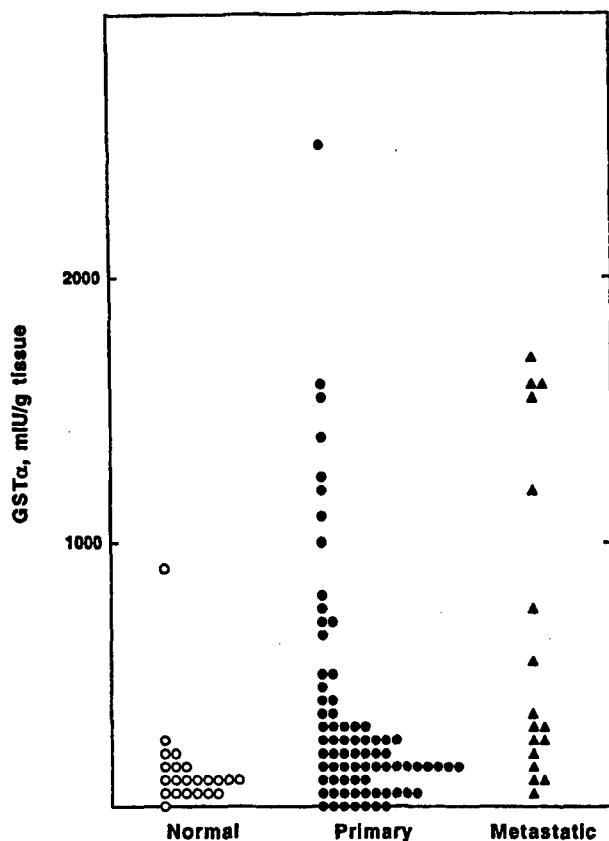


Fig. 7 GST α levels in human normal breast ($n = 26$) and primary ($n = 90$) and metastatic ($n = 22$) breast tumor tissue samples. GST α catalytic activity [conjugation of GSH to 1-chloro-2,4-dinitrobenzene (1 mM each)] was indirectly quantified by an ELISA as described in "Materials and Methods." Points are means, rounded off for clarity of presentation to zero if they were >0 and <25 mIU/g and to 50 mIU/g or the nearest multiple thereof if they were ≥ 25 mIU/g of duplicate determinations made on single normal and malignant, or just malignant, tissue samples taken from each of 110 patients. GST α was not detected in 4 of 26 (15%) normal breast tissue samples, 14 of 90 (16%) primary breast tumor samples, and 5 of 22 (23%) metastatic breast tumor samples. These zero values are not shown in this figure.

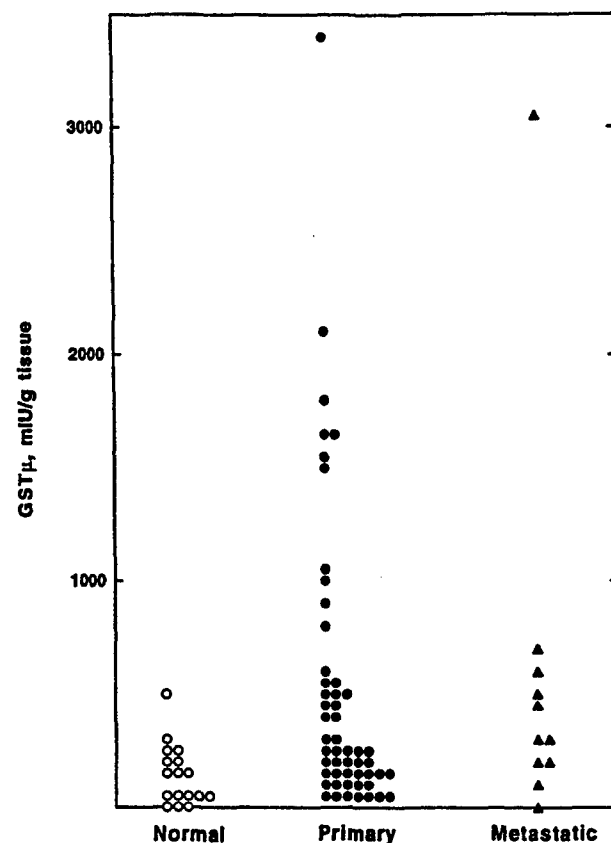


Fig. 8 GST μ levels in human normal breast ($n = 26$) and primary ($n = 90$) and metastatic ($n = 22$) breast tumor tissue samples. GST μ catalytic activity [conjugation of GSH to 1-chloro-2,4-dinitrobenzene (1 mM each)] was indirectly quantified by an ELISA as described in "Materials and Methods." Points are means, rounded off for clarity of presentation to zero if they were >0 and <25 mIU/g and to 50 mIU/g or the nearest multiple thereof if they were ≥ 25 mIU/g, of duplicate determinations made on single normal and malignant, or just malignant, tissue samples taken from each of 110 patients. GST μ was not detected in 9 of 26 (35%) normal breast tissue samples, 38 of 90 (42%) primary breast tumor samples, and 11 of 22 (50%) metastatic breast tumors samples. These zero values are not shown in this figure.

sive element, present in the 5'-upstream regions of the genes coding for these enzymes; Refs. 13 and 34-41) was observed in only seven additional samples, four primary and three metastatic breast tumors (Table 3). In one case, sample 6, a normal breast sample obtained from the same patient was available. ALDH-3 and DT-D levels in this sample were each more than 2 SDs, and pan-GST and GST π levels were nearly (~ 0.86) 1 SD, above corresponding normal breast tissue mean values; CYP 1A1 was not detected in this sample (data not shown).

DT-D, pan-GST, GST α , GST μ , GST π , and CYP 1A1 levels in normal breast tissue did not predict ($P > 0.1$) for corresponding enzyme levels in paired, primary, or metastatic breast tumor tissue (linear regression analyses of data not shown). However, detectable GST μ was always absent in malignant breast tissue samples when it was not found in normal breast tissue samples obtained from the same patient ($n = 11$), as would be expected if the absence of this enzyme was due to

a GST μ null genotype (reviewed in Ref. 41; data not shown). In contrast, GST α was detected in two of three malignant breast tissue samples obtained from patients from which normal breast tissue samples lacked detectable levels of this enzyme; moreover, in five cases, GST α was found in normal, but not malignant, breast tissue (data not shown). In the case of CYP 1A1, 15 of 19 malignant breast tissue samples tested negative when the paired normal breast tissue samples tested negative, and one malignant breast tissue sample tested negative when the paired normal breast tissue sample tested positive (data not shown). We did not have enough paired samples to ascertain whether cellular levels of these enzymes in primary breast malignancies predicted cellular levels of the corresponding enzyme in metastatic breast malignancies.

Again confirming and extending the observations of others (42, 43), GSH levels, also, varied widely in normal and malignant (primary and malignant) breast tissue (Fig. 11 and Table 2). As in the cases of the enzymes, statistical analysis of the data

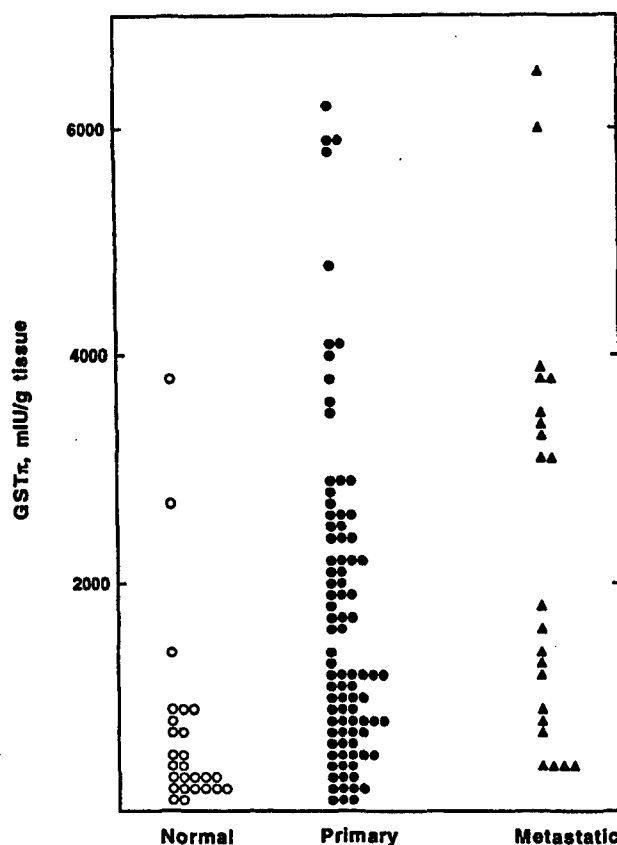


Fig. 9 GST π levels in human normal breast ($n = 26$) and primary ($n = 90$) and metastatic ($n = 22$) breast tumor tissue samples. GST π catalytic activity [conjugation of GSH to 1-chloro-2,4-dinitrobenzene (1 mM each)] was indirectly quantified by an ELISA as described in "Materials and Methods." Points are means, rounded off for clarity of presentation to zero if they were < 50 mIU/g and to 100 mIU/g or the nearest multiple thereof if they were ≥ 50 mIU/g of duplicate determinations made on single normal and malignant, or just malignant, tissue samples taken from each of 110 patients.

revealed that mean levels of GSH in the malignant (primary or metastatic) breast tissue samples were significantly higher ($P \leq 0.05$) than that in the normal breast tissue samples. The mean GSH level obtained for metastatic breast tumor samples was not significantly higher ($P > 0.1$) than that obtained for the primary breast tumor samples.

GSH levels were not related to any of the enzyme levels when all of the data ($n = 115-125$) were grouped ($P > 0.1$; linear regression analyses of data not shown).

GSH levels in normal breast tissue samples did not predict ($P > 0.1$) for the corresponding GSH levels in paired, primary, or metastatic breast tumor tissue samples (linear regression analyses of data not shown). We did not have enough paired samples to ascertain whether cellular levels of GSH in primary breast malignancies predicted cellular levels of GSH in metastatic breast malignancies.

Except for those of ALDH-1 and ALDH-3, enzyme and GSH levels in the four normal and seven metastatic specimens obtained from the seven donors for whom treatment histories (if any) prior to specimen removal were not known were not

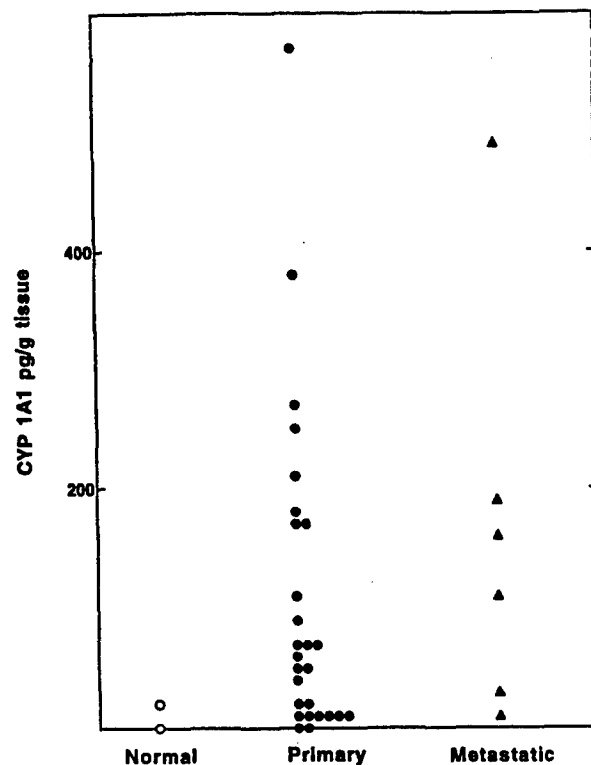


Fig. 10 CYP 1A1 levels in human normal breast ($n = 21$) and primary ($n = 80$) and metastatic ($n = 19$) breast tumor tissue samples. CYP 1A1 levels were quantified by an ELISA as described in "Materials and Methods." Points are means, rounded off for clarity of presentation to zero if they were > 0 and < 5 pg/g and to 10 pg/g or the nearest multiple thereof if they were ≥ 5 mIU/g of duplicate determinations made on single normal and malignant, or just malignant, tissue samples taken from each of 99 patients. CYP 1A1 was not detected in 19 of 21 (90%) normal breast tissue samples, 53 of 80 (66%) primary breast tumor samples, and 13 of 19 (68%) metastatic breast tumors samples. These zero values are not shown in this figure.

significantly different ($P > 0.1$) from those found in the 22 normal and 15 metastatic specimens, respectively, obtained from donors known not to have been treated with antitumor agents prior to specimen removal. Statistically, ALDH-1 and ALDH-3 levels were significantly greater ($P < 0.1$) in the four normal, as well as the seven metastatic, specimens obtained from the seven donors for whom treatment histories were not available.

Statistically, enzyme and GSH levels in normal and malignant (primary as well as metastatic) breast tissue samples were not always independent ($P \leq 0.05$) of patient age or of ER, progesterone receptor, or p53 status (Table 4). Perhaps meaningful, high levels of CYP 1A1 (> 50 pg/g tissue) may have occurred more frequently in ER⁺ than in ER⁻ tissue samples [4 of 26 (15%) versus 1 of 21 (5%), respectively; data not shown] as might be expected if xenobiotic induction of this enzyme cannot be effected in the absence of ER (reviewed in Refs. 13 and 44), although the putative more frequent appearance in ER⁺ tissue samples was not statistically significant ($P = 0.1073$).

Table 3 Malignant (primary and metastatic) breast tissue samples exhibiting markedly elevated levels of ALDH-3, DT-D, pan-GST, and GST π (each >1 SD above their respective normal breast tissue mean values)^a

Sample	Malignancy	ALDH-3	DT-D	pan-GST	GST π	CYP 1A1	
1	Primary	28	5520	2630	2100	0	
2		35	4310	7280	6200	177 ^b	
3		39	6250	4870	1900	5	
4		45	1410	3000	2900	206 ^b	
5 ^c		171	1890	4450	3600	5	
6 ^{c,d}		231	2950	5180	4150	0	
7		28	1730	5650	3850	ND ^e	
8		53	1510	6430	3800	0	
9		Metastatic	64	1600	4630	3900	485 ^b
10 ^{c,f}			97	2540	7600	6500	0

^a Included in this table are the 10 malignant samples, of 112, in which ALDH-3, DT-D, pan-GST, and GST π levels were each >1 SD above their respective normal breast tissue mean values (Table 2). ALDH-3, GST π , and CYP 1A1 levels were quantified by ELISAs, and those of DT-D and pan-GST were quantified by spectrophotometric assays, as described in "Materials and Methods." Values are means of duplicate determinations made on single malignant tissue samples taken from each of 10 patients. Units are mIU/g tissue except in the case of CYP 1A1, where they are pg/g. There were no normal breast tissue samples ($n = 26$) in which all four levels were >1 SD above their respective normal breast tissue mean values. There were no samples excluded from this listing because only pan-GST or only GST π failed to meet the criteria of levels >1 SD above their respective normal tissue mean values.

^b Value is >1 SD above the normal breast tissue mean value (Table 2).

^c Samples in which ALDH-3, DT-D, pan-GST, and GST π levels were each >2 SD above their respective normal breast tissue mean values (Table 2).

^d Samples in which ALDH-3, DT-D, pan-GST, and GST π levels were each >1 SD above their respective primary breast tumor tissue mean values (Table 2).

^e ND, not determined.

^f Samples in which ALDH-3, DT-D, pan-GST, and GST π levels were each >1 SD above their respective metastatic breast tumor tissue mean values (Table 2).

DISCUSSION

Given that: (a) metastatic breast cancer is usually treated with a combination of chemotherapeutic agents (reviewed in Refs. 1-4); (b) one of these agents is virtually invariably cyclophosphamide (reviewed in Refs. 1-4); (c) established molecular determinants of cellular sensitivity to cyclophosphamide and other oxazaphosphorines include ALDH-1 and ALDH-3 (reviewed in Refs. 5 and 6; Refs. 7-18); (d) ALDH-1 and ALDH-3 each catalyze the detoxification of cyclophosphamide and other oxazaphosphorines (reviewed in Refs. 5, 6, and 13; Ref. 16); and (e) ALDH-1 and ALDH-3 levels vary widely in primary and metastatic breast tumors as reported herein, it follows that the wide range of clinical responses to cyclophosphamide (oxazaphosphorine)-based combination chemotherapy of metastatic breast cancer must be, at least in part, due to the substantial variability of ALDH-1 and ALDH-3 levels in these malignancies. Furthermore, given that: (a) GSH is yet another established molecular determinant of cellular sensitivity to cyclophosphamide and other oxazaphosphorines (as well as to other DNA cross-linking agents, *e.g.*, melphalan, chlorambucil, and cisplatin; reviewed in Refs. 5, 45, and 46); (b) cellular sensitivity to cyclophosphamide and other oxazaphosphorines (as well as to other DNA cross-linking agents) decreases as cellular concentrations of GSH increase (reviewed in Refs. 5, 45, and 46); and (c) GSH levels vary widely in primary and metastatic breast tumors as reported herein and by others (42, 43), it follows that the wide range of clinical responses to cyclophosphamide (oxazaphosphorine)-based combination chemotherapy of metastatic breast cancer must be, at least in

part, due to the substantial variability of GSH levels in these malignancies.

Knowledge of ALDH-1 and ALDH-3 levels in metastatic breast tissue would be of value in the rational design of the conventional and high-dose cancer chemotherapeutic strategies that are ultimately used to treat breast cancer patients with metastatic disease. Thus, cyclophosphamide and other oxazaphosphorines may well be the drugs of choice when ALDH-1 and/or ALDH-3 levels are low, but they likely would not be when the level of one or both of these enzymes is high, as in metastatic breast tumors. Metastatic breast tumor samples of sufficient size or, indeed, any size, may only infrequently be obtainable for testing of this type, but that would not be a problem because ALDH-1 and ALDH-3 levels in normal breast tissues predict corresponding malignant metastatic, as well as primary, breast tissue levels of these enzymes. Whether cellular levels of ALDH-1 or ALDH-3 in primary breast malignancies predict cellular levels of these enzymes, respectively, in corresponding metastatic breast malignancies remain to be determined, but that is likely to be the case.

Cancer chemotherapeutic strategies could be beneficially individualized even further if cellular levels of DT-D, the GSTs, and CYP 1A1 in metastatic breast tumors were taken into account because these enzymes are known to catalyze the biotransformation of various anticancer agents, *e.g.*, mitomycin C and EO9; melphalan and chlorambucil; and ellipticine, respectively (reviewed in Refs. 45, 47, and 48), and the levels of these enzymes in metastatic breast tissue vary widely. For example, DT-D catalyzes the activation of EO9 (47), an agent that shows

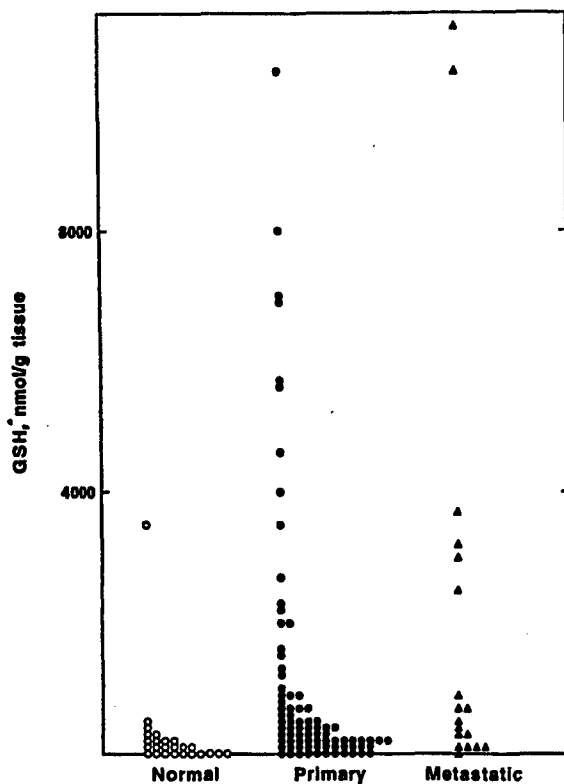


Fig. 11 GSH levels in human normal breast ($n = 25$) and primary ($n = 82$) and metastatic ($n = 18$) breast tumor tissue samples. GSH levels were quantified as described in "Materials and Methods." Points are means, rounded off for clarity of presentation to zero if they were < 50 mIU/g and to 100 mIU/g or the nearest multiple thereof if they were ≥ 50 mIU/g of duplicate determinations made on single normal and malignant, or just malignant, tissue samples taken from each of 100 patients.

preclinical promise in the treatment of breast cancer (24). Thus, EO9 may be of minimal value when DT-D levels are low but of substantial value when the levels of this enzyme are high. A potential problem is that DT-D, GST, and CYP 1A1 levels in normal breast tissue apparently do not predict the respective levels of these enzymes in malignant breast tissues. Whether DT-D, GST, and/or CYP 1A1 levels in primary breast malignancies predict the respective levels of these enzymes in metastatic breast malignancies remains to be determined.

UDP-glucuronosyl transferase is yet another enzyme known to catalyze the biotransformation of anticancer drugs, *e.g.*, detoxification of mitoxantrone (49). Variation in malignant breast tissue levels of this enzyme has been reported (50). Thus, knowledge of its levels in metastatic breast malignancies could also be of value when individualizing chemotherapeutic regimens to treat this disease.

Knowledge of GSH levels in metastatic breast tumor tissue could also be of value in the rational design of the conventional and high-dose cancer chemotherapeutic strategies that are ultimately used to treat breast cancer patients with metastatic disease because cellular sensitivity to various anticancer agents, *e.g.*, the oxazaphosphorines and melphalan, decreases as cellular levels of GSH increase (reviewed in Refs. 5 and 45), and the

level of GSH in metastatic breast tissue varies widely. Again, however, a potential problem is that GSH levels in normal breast tissue apparently do not predict the levels of GSH in malignant breast tissues. In this case, also, whether the levels of this determinant in primary breast malignancies predict the respective levels of it in metastatic breast malignancies remains to be determined.

Adriamycin is also generally included in chemotherapeutic regimens used to treat metastatic breast cancer (reviewed in Refs. 1-3), and paclitaxel (Taxol) shows promise in the treatment of this malignancy (51). Each is subject to transport out of cells by cell surface multidrug transporters, *i.e.*, P-glycoprotein 170 (P-170) and MRP (multidrug resistance-associated protein (reviewed in Refs. 52-55). Variations in malignant breast tissue levels of P-170 and MRP have been reported (56-58). Knowledge of transporter levels, in addition to GSH and relevant enzyme levels, in metastatic breast tumor tissue would provide the basis for even further beneficial individualization of the chemotherapeutic regimen.

Inhibitors of ALDH-1-mediated catalysis, ALDH-3-mediated catalysis, and GSH synthesis would be expected to sensitize, otherwise relatively insensitive, tumor cells to the oxazaphosphorines when high levels of one of these determinants is the basis for the relative insensitivity. Thus, such agents could be of therapeutic value.

Alcohol deterrents, *e.g.*, disulfiram and cyanamide, as well as certain other pharmacological agents, *e.g.*, certain cephalosporins, are known to inhibit ALDH-1-mediated catalysis (reviewed in Refs. 6 and 59). However, clinical use of these agents to sensitize tumor cells expressing large amounts of ALDH-1 to the oxazaphosphorines may not be strategically sound. This is because certain critical normal cells, *e.g.*, pluripotent and multipotent hematopoietic progenitor cells, appear to be insensitive to the oxazaphosphorines because they express relatively elevated levels of ALDH-1 or a very closely related enzyme, one that is also sensitive to these inhibitors (reviewed in Ref. 6; 60).

ALDH-3 is apparently not present in pluripotent and multipotent hematopoietic progenitor cells,⁴ although it may be present in certain other critical normal cells (8). Other than alternative substrates, *e.g.*, benzaldehyde, the only demonstrated inhibitor of ALDH-3-catalyzed oxazaphosphorine inactivation is gossypol (61). Predictably, nontoxic amounts of this agent markedly increased the sensitivity of cultured human breast adenocarcinoma cells that express large amounts of ALDH-3 to the oxazaphosphorines (61). Gossypol was found to be selectively toxic to tumors in several animal models (62-65), thus prompting clinical trials of this agent for the treatment of various cancers (63, 66, 67) including metastatic breast cancers (68). Thus, it can be envisaged that, in the case of cancer cells expressing large amounts of ALDH-3, gossypol could be of dual therapeutic value when combined with an oxazaphosphorine in the therapeutic regimen.

Inclusion of an inhibitor of ALDH-3 could especially be of value when autologous pluripotent and multipotent hematopoietic cells are used to repopulate bone marrow and other tissues

⁴ Unpublished observations.

Table 4 Enzyme levels in normal and malignant (primary and metastatic) breast tissues as function of patient age, ER status, progesterone receptor status, and p53 status: statistical analysis^a

Tissue and group	P (unpaired, two-tailed, Students's <i>t</i> test)						
	ALDH-1	ALDH-3	DT-D	pan-GST	GST α	GST μ	GST π
Normal							
<45 y vs 45-60 y	0.134	0.136	0.378	0.362	0.460	0.466	0.323
<45 y vs >60 y	0.973	0.370	0.320	0.713	0.967	0.054	0.941
45-60 y vs >60 y	0.023	≤ 0.0001	0.719	0.045	0.002	0.290	0.030
ER ⁺ vs ER ⁻	0.716	0.651	0.092	0.822	0.010	0.303	0.326
PR ⁺ vs PR ⁻	0.750	0.171	0.223	0.186	0.001	0.520	0.062
Primary							
<45 y vs 45-60 y	0.931	0.481	0.843	0.350	0.926	0.001	0.912
<45 y vs >60 y	0.250	0.688	0.008	0.962	0.196	0.071	0.616
45-60 y vs >60 y	0.185	0.346	0.004	0.327	0.152	0.056	0.706
ER ⁺ vs ER ⁻	0.799	0.635	0.281	0.188	0.590	0.136	0.096
PR ⁺ vs PR ⁻	0.885	0.777	0.335	0.665	0.886	0.022	0.283
p53 ⁺ vs p53 ⁻	0.120	0.005	0.509	0.571	0.706	0.500	0.097
Metastatic							
<45 y vs 45-60 y	0.204	0.407	0.136	0.538	0.918	0.214	0.980
<45 y vs >60 y	0.937	0.214	0.049	0.144	≤ 0.0001	0.055	0.324
45-60 y vs >60 y	0.019	0.031	0.549	0.016	≤ 0.0001	0.001	0.343
ER ⁺ vs ER ⁻	0.116	0.751	0.303	0.006	0.059	0.265	0.008
PR ⁺ vs PR ⁻	≤ 0.0001	0.002	0.632	0.037	0.027	0.930	0.057

^a Primary data is among that presented in Figs. 1, 2, and 5-9; *ns* are as listed in Table 1. CYP 1A1 and GSH levels were analyzed in a similar fashion, but the results of that analysis are not given in the table because $P > 0.1$ in all cases except GSH, <45 y vs 45-60 y ($P \leq 0.0001$). PR, progesterone receptor.

that have been depleted of these cells and their progeny as a consequence of very high-dose chemotherapy and/or radiation. This is because breast cancer cells frequently metastasize to the bone marrow, and they have been found in peripheral blood (69, 70); bone marrow and peripheral blood are the two most commonly used sources of multipotent/pluripotent hematopoietic cells. Analogues of cyclophosphamide, mafosfamide and 4-hydroperoxycyclophosphamide, are used to "purge" bone marrow and peripheral blood of these cells, thus allowing the use of such marrow and peripheral blood in autologous transplantation (reviewed in Ref. 2; Refs. 71 and 72). In some cases, however, purging is not complete, and tumor cells are reinfused into the patient with predictable consequences (72). Why purging is sometimes incomplete is not known. High levels of ALDH-3 are a possibility. It is in this scenario that an inhibitor of ALDH-3 could be especially useful.

Buthionine sulfoximine is a demonstrated inhibitor of GSH synthesis (73). Clinical trials designed to evaluate its efficacy in negating resistance to alkylating agents have been initiated (74, 75).

Uncertain is whether elevated levels of ALDH-3, DT-D, the GSTs, or elevated levels of these enzymes and of CYP 1A1 were ever the consequence of coordinated induction effected either by a relevant mutation (enzyme levels are stably elevated) or by the introduction of certain dietary or pharmacological agents (enzyme levels return to basal levels within days upon cessation of inducer intake). Malignant breast tissue levels of ALDH-3, DT-D, GST, and CYP 1A1 were each more than 1 SD above normal breast tissue mean levels in 3 of 99 samples (observed frequency of 0.030), and malignant breast tissue levels of ALDH-3, DT-D, and GST, but not those of CYP 1A1, were greater than 1 SD above normal breast tissue mean levels in 10 of 110 samples (observed frequency of 0.091). Expected

frequencies, based on the assumption that elevated expression (level greater than 1 SD above normal breast tissue mean level) of these enzymes is the consequence of independent events, were 0.011⁵ and 0.048,⁶ respectively. As judged by χ^2 analysis, observed frequencies did not differ significantly ($P = 0.09$ and 0.13, respectively) from expected frequencies. The reader is advised that the choice of 1 SD above normal breast tissue mean values as indication of coordinated induction was entirely arbitrary. The clinical ramifications of coordinated enzyme induction by pharmacological and/or dietary/environmental agents are potentially substantial, especially with regard to chemotherapeutic strategies. These have been detailed elsewhere (22, 25).

Unexpectedly, cellular levels of ALDH-1 and ALDH-3

⁵ ALDH-3, DT-D, GST (pan-GST and GST π), and CYP 1A1 levels were >1 SD above their respective normal breast tissue mean values in 30, 28, 43, and 29 of 99 samples, respectively. Therefore, the expected frequency = (0.303) (0.283) (0.434) (0.293) = 0.011.

⁶ ALDH-3, DT-D, and GST (pan-GST and GST π) levels were >1 SD above their respective normal breast tissue mean values in 32, 38, and 53 of 110 samples, respectively. Therefore, the expected frequency = (0.291) (0.345) (0.482) = 0.048. A denominator of 110, rather than 112, was used to calculate observed and expected frequencies because primary and metastatic tumor samples were obtained from the same patient in each of two instances; levels of any given enzyme, or groups of enzymes, in primary and metastatic tumor samples obtained from the same donor are likely to parallel each other. In none of the four samples were ALDH-3, DT-D, pan-GST, and GST π levels each >1 SD above their respective normal breast tissue mean values. Pan-GST and GST π levels were each >1 SD above their respective normal breast tissue mean values in each of one set of paired samples but not in either of the other paired samples. ALDH-3 and DT-D levels were not >1 SD above their respective normal breast tissue mean values in any of the paired samples.

appeared to be directly related in normal and malignant (primary as well as metastatic) breast tissue. This finding was unexpected because these enzymes are not known to have anything in common with regard to regulation of their expression. At this time, then, this observation can only be viewed as a curiosity.

In an effort to substantiate the contentions made herein with direct evidence, attempts are currently being made to collect information as to how the specimen donors were subsequently treated and, in those cases where anticancer agents were given, the clinical responses thereto. Clinical responses to anticancer drugs as a function of the enzyme and GSH levels reported herein will be the subject of a future publication if this effort proves sufficiently fruitful. In addition, an independent, also retrospective, study addressing this issue as it concerns cyclophosphamide and relevant aldehyde dehydrogenases is already in progress and will be the subject of a future communication.

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Inhibition of Human Class 3 Aldehyde Dehydrogenase, and Sensitization of Tumor Cells That Express Significant Amounts of This Enzyme to Oxazaphosphorines, by Chlorpropamide Analogues

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ABSTRACT. In some cases, acquired as well as constitutive tumor cell resistance to a group of otherwise clinically useful antineoplastic agents collectively referred to as oxazaphosphorines, e.g. cyclophosphamide and mafosfamide, can be accounted for by relatively elevated cellular levels of an enzyme, viz. cytosolic class 3 aldehyde dehydrogenase (ALDH-3), that catalyzes their detoxification. Ergo, inhibitors of ALDH-3 could be of clinical value since their inclusion in the therapeutic protocol would be expected to sensitize such cells to these agents. Identified in the present investigation were two chlorpropamide analogues showing promise in that regard, viz. (acetyloxy)[(4-chlorophenyl)sulfonyl]carbamic acid 1,1-dimethylethyl ester (NPI-2) and 4-chloro-*N*-methoxy-*N*-[(propylamino)carbonyl]benzenesulfonamide (API-2). Each inhibited NAD-linked benzaldehyde oxidation catalyzed by ALDH-3s purified from human breast adenocarcinoma MCF-7/0/CAT cells (IC_{50} values were 16 and 0.75 μ M, respectively) and human normal stomach mucosa (IC_{50} values were 202 and 5 μ M, respectively). The differential sensitivities of stomach mucosa ALDH-3 and breast tumor ALDH-3 to each of the two inhibitors can be viewed as further evidence that the latter is a subtle variant of the former. Human class 1 (ALDH-1) and class 2 (ALDH-2) aldehyde dehydrogenases were much less sensitive to NPI-2; IC_{50} values were >300 μ M in each case. API-2, however, did not exhibit a similar degree of specificity; IC_{50} values for ALDH-1 and ALDH-2 were 7.5 and 0.08 μ M, respectively. Each sensitized MCF-7/0/CAT cells to mafosfamide; the IC_{90} value decreased from >2 mM to 175 and 200 μ M, respectively. Thus, the therapeutic potential of combining NPI-2 or API-2 with oxazaphosphorines is established. *BIOCHEM PHARMACOL* 54:11:000–000, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. aldehyde dehydrogenase; cyclophosphamide; aldophosphamide; oxazaphosphorines; chlorpropamide analogues; breast cancer; drug resistance; gossypol.

ALDH-3§ is a demonstrated molecular determinant of cellular sensitivity to the cytotoxic action of certain widely used antineoplastic prodrugs collectively referred to as oxazaphosphorines, e.g. cyclophosphamide, ifosfamide, 4-hydroper-

oxycyclophosphamide, 4-hydroperoxyifosfamide and mafosfamide (cellular sensitivity to these drugs decreases as cellular levels of ALDH-3 increase) [1–10]. Thus, of therapeutic significance, relatively elevated levels of this enzyme can account for intrinsic, transient acquired, and stable acquired, resistance to the oxazaphosphorines on the part of malignant cells [2–4, 7, 9]. Resistance to the oxazaphosphorines mediated by ALDH-3 is due ostensibly to the enzyme-catalyzed oxidative detoxification of aldophosphamide, the pivotal metabolite of these prodrugs [2, 4, 7, 9, 10].

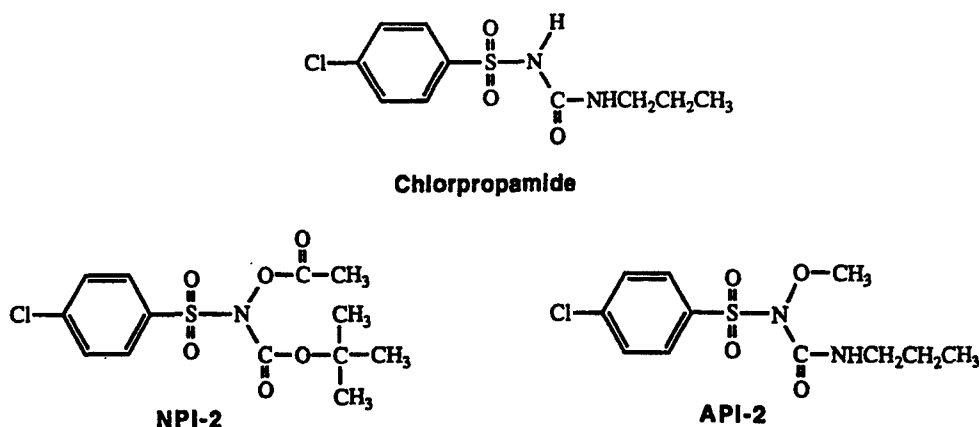
Inhibition of ALDH-3, therefore, would be expected to sensitize otherwise relatively insensitive tumor cells to the oxazaphosphorines when relatively high cellular levels of

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§ *Abbreviations:* ALDH-1, class 1 aldehyde dehydrogenase; ALDH-2, class 2 aldehyde dehydrogenase; ALDH-3, cytosolic class 3 aldehyde dehydrogenase; API-1, 4-chloro-*N*-ethyl-*N*-[(propylamino)carbonyl]benzenesulfonamide; API-2, 4-chloro-*N*-methoxy-*N*-[(propylamino)carbonyl]benzenesulfonamide; GAP, glyceraldehyde-3-phosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IC_{50} , concentration of agent required to effect 50% inhibition of enzyme catalysis; IC_{90} , concentration of drug required to effect 90% cell-kill; MCF-7/0/CAT cells, human breast adenocarcinoma MCF-7/0 cells cultured in the presence of 30 μ M catechol for 5 days to induce ALDH-3; mIU, milli-International Unit of enzyme activity (nmol NAD(P)H formed/min in the case of dehydrogenase activity, and nmol *p*-nitrophenol formed/min in the case of carboxylesterase or phosphatase activity); NPI-1, (benzoyloxy)[(4-chlorophenyl)sulfonyl]carbamic acid 1,1-dimethylethyl ester; NPI-2, (acetyloxy)[(4-chlorophenyl)sulfonyl]car-

bamic acid 1,1-dimethylethyl ester; NPI-3, *N*-acetyl-*N*-(acetyloxy)-4-chlorobenzenesulfonamide; rALDH-1, recombinant ALDH-1; rALDH-2, recombinant ALDH-2; tALDH-3 and nALDH-3, ALDH-3s purified from human tumor and normal cells/tissues, respectively; γ ALDH, yeast aldehyde dehydrogenase.

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Chlorpropamide: 1-(*p*-chlorobenzenesulfonyl)-3-*n*-propylurea

NPI-2: (acetyloxy)[(4-chlorophenyl)sulfonyl]carbamic acid 1,1-dimethylethyl ester

API-2: 4-chloro-*N*-methoxy-*N*-[(propylamino)carbonyl]benzenesulfonamide

FIG. 1. Structures of chlorpropamide and two analogues thereof.

ALDH-3 are the basis for the relative insensitivity. Thus, inhibitors of ALDH-3 could be of therapeutic value. However, known inhibitors of ALDH-1 and/or ALDH-2, e.g. disulfiram and chloral hydrate, do not, or only minimally, inhibit ALDH-3 [2, 4] and, predictably, do not sensitize tumor cells to the oxazaphosphorines when such cells are insensitive to these agents because of relatively high ALDH-3 levels [reviewed in Ref. 8]. Indeed, to date, only gossypol has been shown to inhibit ALDH-3 [11], although at least one chlorpropamide analogue showed promise in that regard [12], and inhibition of ALDH-3-catalyzed oxidative detoxification of aldophosphamide could be effected by alternative substrates, e.g. benzaldehyde and 4-(diethylamino)benzaldehyde [4, 7, 8].

Like the alcohol deterrents disulfiram and cyanamide, the oral hypoglycemic agent chlorpropamide is thought to be a pro-inhibitor of the aldehyde dehydrogenases, most notably ALDH-2, that catalyze the oxidation of ethanol-derived acetaldehyde [13]. Putative metabolites of cyanamide and chlorpropamide include nitroxyl (HNO) and *n*-propylisocyanate, respectively; they have been postulated to be the metabolites that inhibit hepatic aldehyde dehydrogenase-catalyzed reactions [14–16].

Based on these premises, a number of chlorpropamide analogues, viz. *N*¹-hydroxy-substituted ester, *N*¹-methoxy and *N*¹-alkyl derivatives, intended to be prodrugs with the potential of giving rise to HNO or *n*-propylisocyanate, have been designed and synthesized as potential alcohol deterrents [14–16].

Aldehyde dehydrogenases are bifunctional enzymes in that they catalyze the hydrolysis of esters in addition to catalyzing the oxidation of aldehydes [8, 17–19]. Whether catalysis of hydrolytic reactions by these enzymes is of physiological or pharmacological consequence is not known.

The ester analogues were therefore designed with the intent of exploiting the esterolytic activities exhibited by the aldehyde dehydrogenases, viz. to release HNO, a potent inhibitor of aldehyde dehydrogenase-catalyzed oxidations, upon ester hydrolysis catalyzed by these enzymes [16]. Indeed, some of the compounds that were synthesized have been shown to undergo hydrolytic cleavage catalyzed by γ ALDH and to inhibit γ ALDH-catalyzed acetaldehyde oxidation.

The *N*¹-methoxy and *N*¹-alkyl analogues, on the other hand, were designed to release *n*-propylisocyanate, a potent inhibitor of yeast and rodent hepatic aldehyde dehydrogenases, without the necessity of any enzyme participation [14, 15]. Some of the compounds that were synthesized have been shown to inhibit yeast and rodent hepatic mitochondrial aldehyde dehydrogenase-catalyzed oxidation, to decrease acetaldehyde clearance in rodents given ethanol, and to be devoid of a hypoglycemic effect.

Two of the ester analogues, viz. NPI-1 and NPI-3, and one *N*¹-ethyl analogue, viz. API-1, were studied more extensively [12]. They were found to inhibit ALDH-3 but they were not very potent in doing so, nor were they very selective, i.e. they inhibited ALDH-1 and/or ALDH-2 equally as well. Nonetheless, these findings encouraged us to ascertain the effects of some additional chlorpropamide analogues (Fig. 1) on the catalytic activities of these enzymes.

The ALDH-3 present in human tumor cells/tissues (τ ALDH-3), although otherwise seemingly identical to the ALDH-3 present in human normal tissues/fluids (n ALDH-3), differs from the latter in that it exhibits a much greater ability to catalyze the oxidative detoxification of the oxazaphosphorines [reviewed in Ref. 8]. Moreover, τ ALDH-3 is more sensitive to inhibition by NPI-1, NPI-3,

and API-1 than is nALDH-3 [12]. Hence, both tALDH-3 and nALDH-3 were included in our investigations.

Since aldehyde dehydrogenases catalyze hydrolytic as well as oxidative reactions, the effect of the chlorpropamide analogues on each of these reactions was determined though inhibition of the latter was our principal interest.

The ability of the chlorpropamide analogues to negate the influence of relatively high cellular levels of ALDH-3 on the cellular sensitivity of cultured tumor cells to oxazaphosphorines was also determined.

MATERIALS AND METHODS

Mafosfamide was provided by Dr. J. Pohl, Asta Medica AG. Phosphoramidate mustard · cyclohexylamine was supplied by the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute. *Escherichia coli* [BL21(DE3)pLysS] transfected with pET-19b vector, to which human ALDH-1 cDNA (cloned from human hepatoma Hep G2 cells [20]) was ligated, was provided by Dr. Jan Moreb, University of Florida. A vector, viz. pT7-7, to which human ALDH-2 cDNA (cloned from human liver [21]) was ligated, was provided by Dr. Henry Weiner, Purdue University. Transfection of human ALDH-2 cDNA ligated to the pT7-7 vector into *E. coli* [BL21(DE3)pLysS] was by Drs. P. A. Dockham and L. Sreerama of our laboratory as described by Sambrook *et al.* [22]. Generation and purification of human rALDH-1 and rALDH-2 were as described previously [12]. NPI-2 and API-2 were synthesized as described previously [15, 16]. Chromatographically purified γ ALDH, human erythrocyte GAPDH, and human placental alkaline phosphatase type XXIV were purchased from the Sigma Chemical Co. All other chemicals and reagents were obtained from the sources listed in previous publications [2, 4, 9, 12, 23].

Human normal stomach mucosa ALDH-3 (nALDH-3) and the ALDH-3 (tALDH-3) present in human breast adenocarcinoma MCF-7/0 cells cultured in the presence of 30 μ M catechol for 5 days to induce the enzyme (MCF-7/0/CAT cells) were purified as described previously [3, 9].

Primarily to remove dithiothreitol, all of the purified enzymes were transferred from the storage buffer [25 mM 2-(*N*-morpholino)ethanesulfonic acid buffer, pH 6.5, supplemented with 1 mM EDTA and 1 mM dithiothreitol] to 25 mM 2-(*N*-morpholino)ethanesulfonic acid buffer, pH 6.5, with the aid of a PD-10 (Sephadex G-25) column prior to their use.

NAD-linked oxidation of acetaldehyde catalyzed by rALDH-1 and rALDH-2 at 37° and pH 8.1, NAD(P)-linked oxidation of benzaldehyde catalyzed by nALDH-3 and tALDH-3 at 37° and pH 8.1, hydrolysis of *p*-nitrophenyl acetate catalyzed by each of these enzymes at 25° and pH 7.5, NAD-linked oxidation of GAP catalyzed by GAPDH at 37° and pH 7.6, and hydrolysis of *p*-nitrophenyl phosphate catalyzed by alkaline phosphatase at 25° and pH 9.8 were quantified spectrophotometrically as described previously [2, 23–25] except that, whenever previously it

had been included, glutathione was omitted from the reaction mixture when aldehyde dehydrogenase activity was quantified. Except where noted, preincubation of the putative inhibitor, viz. NPI-2 or API-2, or vehicle together with the complete reaction mixture except for the substrate was for 5 min. Preincubation temperatures and pH levels were the same as incubation temperatures and pH levels. All reactions were started by the addition of substrate. Stock solutions of NPI-2 and API-2 were prepared in dimethyl sulfoxide and were stored at –20°. The final concentration of dimethyl sulfoxide in the reaction mixture was always 5% (v/v); this concentration of dimethyl sulfoxide did not inhibit any of the enzyme-catalyzed reactions under investigation.

Enzyme · inhibitor complexes were subjected to gel permeation chromatography to determine whether inhibition effected by NPI-2 and API-2 was reversible. Briefly, the complete reaction mixture except for the substrate was first incubated at 37° for 5 min in a volume of 1 mL with vehicle or concentrations of inhibitor that effected >50% inhibition with one exception, viz. inhibition of rALDH-2 by NPI-2. Reaction mixtures were then chilled in an ice-bath for 2 min after which they were placed on a PD-10 (Sephadex G-25) column (2 × 5 cm; 2.5 mL void volume; 10 mL bed volume) that had been equilibrated with 25 mM 2-(*N*-morpholino)ethanesulfonic acid buffer, pH 6.5. The column was then eluted with 3.5 mL of equilibration buffer, and the resultant eluate was collected and saved for assay of enzyme activity. Gel permeation chromatography was at 4°. Preliminary experiments established that recoveries of free inhibitor and free enzyme protein were <2 and >98%, respectively. Enzyme activities were quantified before and after the gel permeation chromatography as described above.

Human breast adenocarcinoma MCF-7/0 and MCF-7/0/CAT cells were cultured (monolayer), harvested when still in exponential growth, resuspended in growth medium, and checked for viability (usually greater than 95% as judged by trypan blue exclusion) as described previously [2, 9]. Drug exposure and the colony-forming assay used to determine surviving fractions were also as described previously [2]. Briefly, freshly harvested cells were diluted with drug-exposure medium to a concentration of 1×10^5 cells/mL and then exposed to drug (mafosfamide or phosphoramidate mustard) or vehicle for 30 min at pH 7.4 and 37° after which they were harvested and cultured in drug-free growth medium for 15 days. Colonies (≥ 50 cells) were then visualized with methylene blue dye and counted. Stock solutions of mafosfamide and phosphoramidate mustard were prepared by dissolving them in water just before use. In some experiments, cells were preincubated with NPI-2, API-2, or vehicle for 5 min at 37° prior to the addition of mafosfamide or phosphoramidate mustard. Stock dimethyl sulfoxide solutions of NPI-2 or API-2 were diluted with drug-exposure medium just before use. The dimethyl sulfoxide concentration in the drug-exposure medium was 0.1% (v/v); this concentration of dimethyl sulfoxide did

not affect the rate of cell proliferation. At the concentrations used, viz. 100 and 50 μM , respectively, NPI-2 and API-2 effected only a small amount of cell-kill (<12%; LC_{50} values were >300 μM in each case), and this was taken into account when calculating the effect of including NPI-1 or API-2 in the drug-exposure medium on LC_{50} values for mafosfamide and phosphoramidate mustard.

Computer-assisted unweighted nonlinear regression analysis effected by the STATView statistical program (Brain Power Inc.) was used to generate the curves that best-fit plots of enzyme activities (% of control) as a function of inhibitor concentrations (four to eight) and, subsequently, to estimate the concentration of inhibitor that effected a 50% decrease in catalytic activity (IC_{50}). Double-reciprocal (Lineweaver-Burk) plots of initial catalytic rates as a function of substrate concentrations (at least three and usually five) were used to estimate the K_m and V_{max} values. Except in the case of rALDH-2-catalyzed oxidation of acetaldehyde, K_i values were determined by plotting the slopes of the lines generated by double-reciprocal (Lineweaver-Burk) plots as a function of inhibitor concentrations (three). In the case of rALDH-2-catalyzed oxidation of acetaldehyde, K_i values were determined by plotting the reciprocals of initial catalytic rates as a function of inhibitor concentrations (five) (Dixon plots) because K_m values were relatively small and, thus, K_i values were difficult to ascertain accurately from Lineweaver-Burk plots. In the case of double-reciprocal (Lineweaver-Burk) plots, computer-assisted Wilkinson weighted linear regression analysis [26] effected by the MacWilkins program (Microsoft) was used to generate the best-fit lines. Computer-assisted unweighted linear regression analysis effected by the STATView statistical program was used to generate best-fit lines for all other straight-line functions.

RESULTS

NPI-2 and API-2 were not substrates for the oxidative reactions catalyzed by any of the ALDHs studied.

Oxidative reactions catalyzed by rALDH-1, rALDH-2, the ALDH-3s, yALDH and GAPDH were inhibited by NPI-2 and API-2 (Fig. 2 and Table 1). API-2 was much more potent in that regard. Differential sensitivity to these inhibitors on the part of the human aldehyde dehydrogenases was observed. Thus, as judged by the concentrations of NPI-2 or API-2 required to effect 50% inhibition (IC_{50}), (1) tALDH-3 was, relative to rALDH-1, rALDH-2, nALDH-3, and GAPDH, far more sensitive to inhibition by NPI-2, and (2) tALDH-3 and, to an even greater extent, rALDH-2 were, relative to rALDH-1, nALDH-3, and GAPDH, far more sensitive to API-2. yALDH-catalyzed oxidation was relatively sensitive to inhibition by each agent.

Routinely, preincubation of NPI-2, API-2, or vehicle together with the complete reaction mixture except for substrate, viz. acetaldehyde or benzaldehyde, was for 5 min. Maximum inhibition of aldehyde dehydrogenase-catalyzed

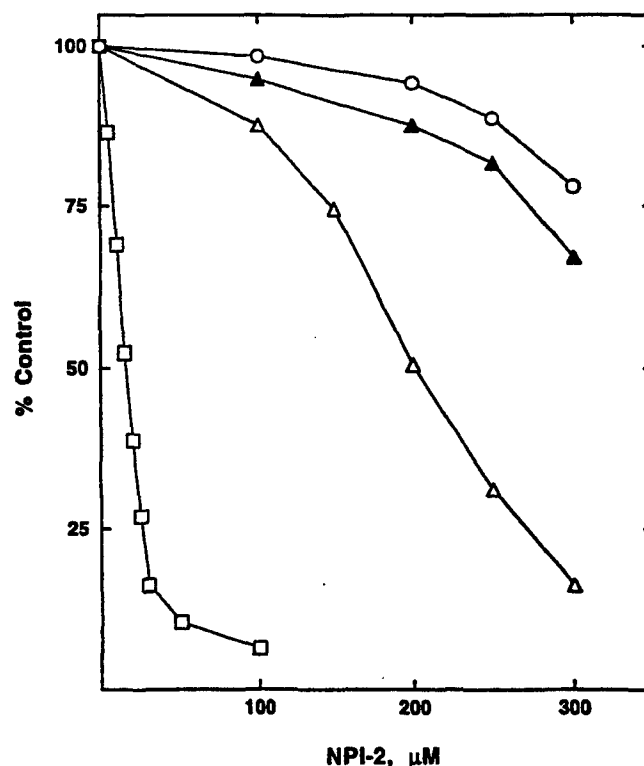


FIG. 2. Inhibition by NPI-2 of the NAD-linked oxidative reactions catalyzed by human aldehyde dehydrogenases: Concentration responses. The sensitivities of rALDH-1 (○), rALDH-2 (▲), nALDH-3 (△), and tALDH-3 (□) to inhibition by NPI-2 were determined as described in Materials and Methods and the footnote to Table 1. Data points are means of duplicate determinations. Control catalytic rates were 0.60, 2.4, 29, and 31 IU/mg for rALDH-1, rALDH-2, nALDH-3, and tALDH-3, respectively. Best-fit curves, and IC_{50} values estimated therefrom, were generated from the data presented in this figure, as described in Materials and Methods. The IC_{50} values thus obtained are given in Table 1.

oxidation by NPI-2 and API-2 (Fig. 3) was achieved within this time period in all cases except in that of rALDH-2 where a small amount of additional inhibition was observed between min 5 and 6 of preincubation in each case. Striking is the greater preincubation time that was required to achieve maximum inhibition of rALDH-2 by API-2 as compared with that required to achieve maximum inhibition of the other aldehyde dehydrogenases by this agent.

Inhibition could not be reversed by passing ALDH · NPI-2 complexes (enzyme · inhibitor complexes) through a PD-10 (Sephadex G-25) column (Fig. 4). It was somewhat reversed when the rALDH-1 · API-2 and rALDH-2 · API-2 complexes were passed through a PD-10 column. It was largely reversed when either of the ALDH-3 · API-2 complexes were passed through a PD-10 column.

Fifty percent inhibition of a hydrolytic reaction (hydrolysis of *p*-nitrophenyl acetate) catalyzed by the human ALDHs, and of one (hydrolysis of *p*-nitrophenyl phosphate) catalyzed by human placental alkaline phosphatase, was not achieved at the highest concentrations of NPI-2 tested and, except in the case of rALDH-2, was not

TABLE 1. Inhibition by chlorpropamide analogues of human aldehyde dehydrogenase-catalyzed oxidations and hydrolyses: IC_{50} values*

Enzyme	IC_{50} (μ M)					
	NAD-linked oxidation†		NADP-linked oxidation†		Hydrolysis‡	
	NPI-2	API-2	NPI-2	API-2	NPI-2	API-2
rALDH-1	>300	7.5			>200	>1000
rALDH-2	>300	0.08			>200	16
nALDH-3	202	5.0	267	0.90	>200	>1000
tALDH-3	16	0.75	39	0.62	>200	>1000
yALDH	15	0.15			50	2.7
GAPDH	111	38				
Alkaline phosphatase					>300	>1000

* Enzymes were incubated with vehicle or 4–8 different concentrations of one of the putative inhibitors for 5 min, substrate was added, and initial catalytic rates were quantified as described in Materials and Methods. Examples of plots of the primary data thus obtained are shown in Fig. 2. The IC_{50} values were estimated from such data, as described in Materials and Methods.

† Substrates and cofactors were: acetaldehyde and NAD (4 mM each) for rALDH-1, acetaldehyde (2 mM) and NAD (4 mM) for rALDH-2, benzaldehyde (4 mM) and NAD (1 mM) or NADP (4 mM) for the ALDH-3s, acetaldehyde (0.8 mM) and NAD (4 mM) for yALDH, and GAP and NAD (1 mM each) for GAPDH. Uninhibited catalytic rates (means of two determinations each made in duplicate) were 0.59, 2.1, 31, 53, 32, 52, 9.2, and 50 IU/mg protein for rALDH-1, rALDH-2, NAD-linked nALDH-3, NADP-linked nALDH-3, NAD-linked tALDH-3, NADP-linked tALDH-3, yALDH, and GAPDH, respectively.

‡ Substrates were *p*-nitrophenyl acetate (500 μ M) for the dehydrogenases and *p*-nitrophenyl phosphate (10 mM) for alkaline phosphatase. Uninhibited catalytic rates (means of two determinations each made in duplicate) were 147, 578, 8.7, 9.5, 433, and 15 IU/mg protein for rALDH-1, rALDH-2, nALDH-3, tALDH-3, yALDH, and alkaline phosphatase, respectively.

achieved at the highest concentration of API-2 tested either (Table 1). yALDH-catalyzed hydrolysis was relatively sensitive to inhibition by each agent.

rALDH-2- and yALDH-catalyzed oxidations and hydrolyses appear to be uniquely sensitive to inhibition by

N^1 -methoxy and N^1 -ethyl analogues of chlorpropamide, viz. API-2 (Table 1) and API-1 [12], respectively.

Kinetic constants, viz. K_m , V_{max} and K_i values, defining the catalysis of oxidative reactions by rALDH-1, rALDH-2, nALDH-3, tALDH-3, yALDH, and GAPDH, and inhibi-

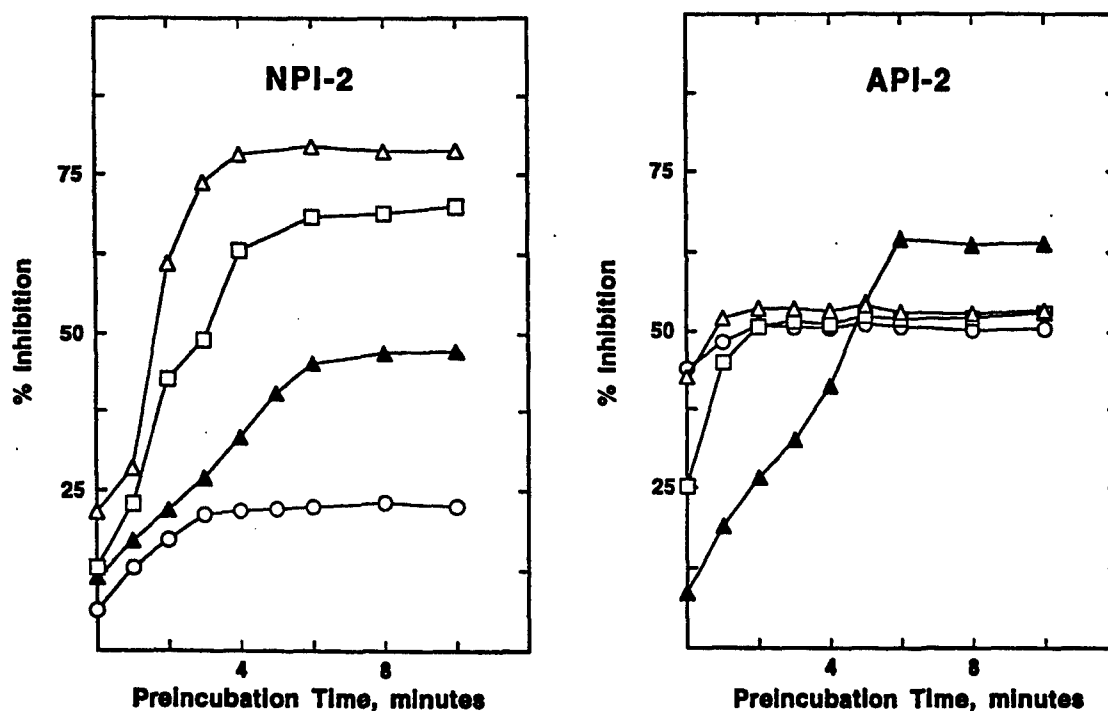


FIG. 3. Inhibition by NPI-2 and API-2 of the NAD-linked oxidative reactions catalyzed by human aldehyde dehydrogenases as a function of preincubation time. Sensitivities of rALDH-1 (O), rALDH-2 (\blacktriangle), nALDH-3 (\triangle), and tALDH-3 (\square) to inhibition by NPI-2 (300, 300, 300, and 30 μ M, respectively) and API-2 (7, 0.1, 4, and 0.75 μ M, respectively) were quantified. Experimental conditions were as described in Materials and Methods and a footnote to Table 1 except that preincubation of NPI-2, API-2, or vehicle together with the complete reaction mixture except for the substrate, viz. acetaldehyde or benzaldehyde, was for the length of time indicated in the figure. Data points are means of duplicate determinations. Mean control catalytic rates at the start, as well as at the end, of the preincubation period were 0.60, 2.5, 33, and 32 IU/mg for rALDH-1, rALDH-2, nALDH-3, and tALDH-3, respectively.

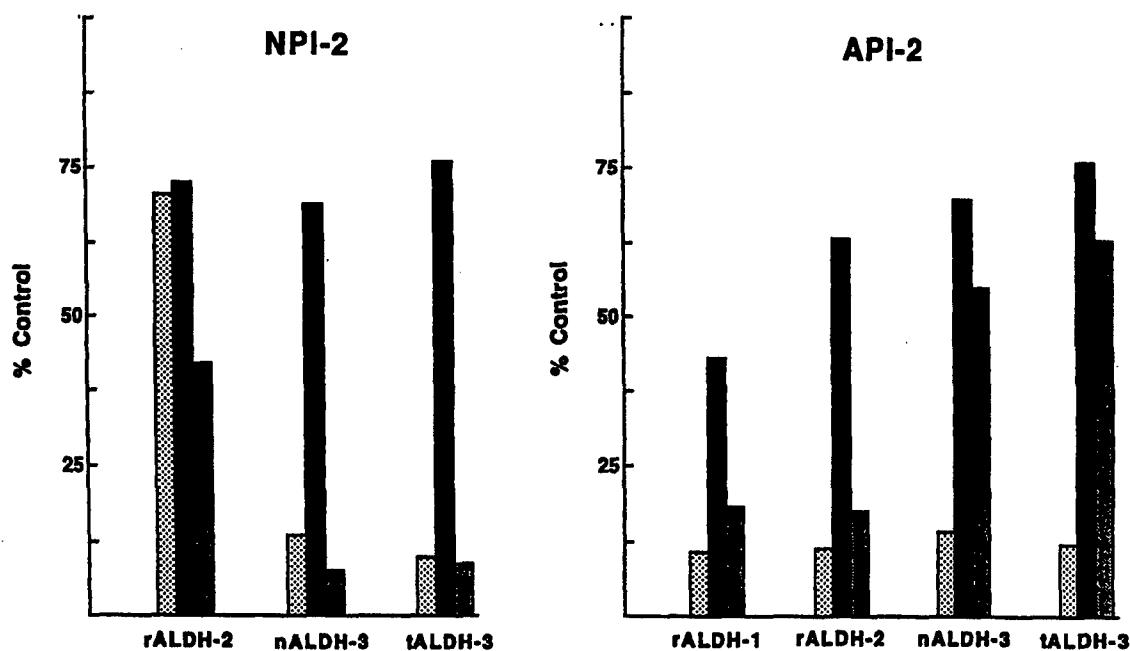


FIG. 4. NPI-2- and API-2-effected inhibition of NAD-linked aldehyde dehydrogenase-catalyzed oxidations: Reversibility studies. Purified enzyme was incubated with vehicle, NPI-2 (300 μ M in the cases of rALDH-2 and nALDH-3; 30 μ M in the case of tALDH-3), or API-2 (50, 1, 25, and 10 μ M in the cases of rALDH-1, rALDH-2, nALDH-3, and tALDH-3, respectively) for 5 min, and initial catalytic rates were quantified before and after passage through a PD-10 (Sephadex G-25) column as described in Materials and Methods. Substrates were acetaldehyde (4 and 2 mM; rALDH-1 and rALDH-2, respectively) or benzaldehyde (4 mM; nALDH-3 and tALDH-3). NAD concentrations were 4 (rALDH-1 and rALDH-2) and 1 (nALDH-3 and tALDH-3) mM. Values are means of duplicate determinations made on each of two samples. Mean control (no inhibitor; before passage through the column) rates were 0.60 (rALDH-1), 2.4 (rALDH-2), 36 (nALDH-3), and 32 (tALDH-3) IU/mg. Key: (■) no inhibitor; after passage through the column; (▨) and (▩) inhibitor; before and after the passage through the column, respectively.

tion thereof, as well as the type of inhibition, viz. competitive or noncompetitive, are given in Table 2. A representative of the plots from which these values were obtained is shown in Fig. 5. The K_m and V_{max} values obtained in the present investigation are in good agreement with those reported previously [2, 4, 9, 11, 12, 23, 25, 27].

As judged by K_i values determined with respect to the substrate, i.e. benzaldehyde, as well as those determined with respect to a cofactor, i.e. NAD or NADP, tALDH-3-catalyzed oxidation was inhibited to a greater extent by NPI-2 than was that catalyzed by nALDH-3 (Table 2). In the case of API-2, the rank order of enzyme sensitivity to the inhibitor was rALDH-2 > tALDH-3 > nALDH-3 > rALDH-1.

Inhibition of nALDH-3- and tALDH-3-catalyzed oxidation by NPI-2 was noncompetitive with respect to the substrate, i.e. benzaldehyde (Table 2 and data not shown). In contrast, inhibition of these enzymes, as well as of rALDH-2, by API-2 was competitive with respect to the substrate, i.e. benzaldehyde or acetaldehyde (Table 2, Fig. 5, and data not shown). Inhibition by NPI-2 and API-2 of human aldehyde dehydrogenase-catalyzed oxidations was always noncompetitive with respect to NAD (Table 2 and data not shown). In contrast, inhibition by NPI-2 and API-2 of ALDH-3-catalyzed oxidation was competitive with respect to NADP (Table 2 and data not shown).

K_i values defining the inhibition of γ ALDH- and rALDH-2-catalyzed hydrolysis by NPI-2 and API-2 are

given in Table 3. Inhibition of γ ALDH- and rALDH-2-catalyzed hydrolysis by these agents was of the mixed type (data not shown). Similar observations were made in the cases of NPI-1, NPI-3, and API-1 [12].

Addition of NPI-2 (100 μ M) or API-2 (50 μ M) to the drug-exposure medium prior to exposure to mafosfamide markedly increased the sensitivity of tumor cells that express large amounts of ALDH-3, viz. MCF-7/0/CAT, to the oxazaphosphorine (Fig. 6 and Table 4). As expected, identical treatment of tumor cells that express very small amounts of ALDH-3, viz. MCF-7/0, did not effect a statistically significant ($P > 0.1$; paired t -tests) increase in their sensitivity to mafosfamide. Also as expected because ALDH-3 does not catalyze the detoxification of phosphoramidate mustard, the ultimate cytotoxic metabolite of mafosfamide [28], addition of NPI-2 or API-2 to the drug-exposure medium prior to exposure to this agent essentially did not increase the sensitivity of MCF-7/0/CAT cells to it (Table 4).

DISCUSSION

NPI-2 and API-2 each showed some potential as useful inhibitors of tALDH-3, and, therefore, therapeutic potential when combined with an oxazaphosphorine in the treatment of certain cancers, since they were relatively potent and specific inhibitors of this enzyme. They join gossypol in that regard [11]. Attractive is that tALDH-3 is

TABLE 2. Inhibition by chlorpropamide analogues of human aldehyde dehydrogenase-catalyzed oxidations: K_i values*

Enzyme	Substrate and cofactor		K_m † (μM)	V_{max} † (IU/mg)	K_i (μM)			
	Variable (mM)	Fixed (mM)			NPI-2	API-2		
rALDH-1	Acetaldehyde (0.1–1.6)	NAD (4)	434	0.66	ND‡	6.0	(N)§	
	NAD (0.05–1)	Acetaldehyde (4)	33	0.63	ND	2.1	(N)	
rALDH-2	Acetaldehyde (0.16–2)	NAD (4)	3.6 [¶]	2.4	ND	0.03	(C)	
	NAD (0.125–4)	Acetaldehyde (2)	329	2.8	ND	0.08	(N)	
nALDH-3	Benzaldehyde (0.1–2)	NAD (1)	417	30	254	(N)	1.7	(C)
	NAD (0.05–1)	Benzaldehyde (4)	47	39	120	(N)	2.1	(N)
	Benzaldehyde (0.2–4)	NADP (4)	446	60	281	(N)	0.02	(C)
	NADP (1–4)	Benzaldehyde (4)	720	67	16	(C)	0.17	(C)
tALDH-3	Benzaldehyde (0.1–2)	NAD (1)	356	31	11	(N)	0.14	(C)
	NAD (0.075–1)	Benzaldehyde (4)	34	31	12	(N)	0.42	(N)
	Benzaldehyde (0.2–4)	NADP (4)	430	59	41	(N)	0.01	(C)
	NADP (0.5–4)	Benzaldehyde (4)	780	64	2.3	(C)	0.04	(C)
yALDH	Acetaldehyde (0.025–0.3)	NAD (4)	32	9.2	10	(N)	0.15	(N)
	NAD (0.5–4)	Acetaldehyde (0.8)	877	8.5	3.6	(C)	0.02	(C)
GAPDH	GAP (0.05–1)	NAD (1)	159	64	ND	31	(N)	

* Enzymes were preincubated with vehicle or various concentrations of the putative inhibitor for 5 min, substrate was added, and initial catalytic rates were quantified as described in Materials and Methods. A representative of the plots of the primary data from which the kinetic constants were obtained is given in Fig. 5.

† N = 2 except in the case of rALDH-1, rALDH-2, and GAPDH where N = 1.

‡ ND = not determined.

§ N: noncompetitive; C: competitive.

¶ Unlikely to be accurate because it is difficult to ascertain K_m values that are less than about 10 μM from the very flat Lineweaver–Burk plots that we generated. Thus, the K_m value was determined to be $<0.1 \mu\text{M}$ when a more appropriate experimental design and method of analysis, viz. integrated Michaelis analysis of a single enzyme–progress curve, was used [23].

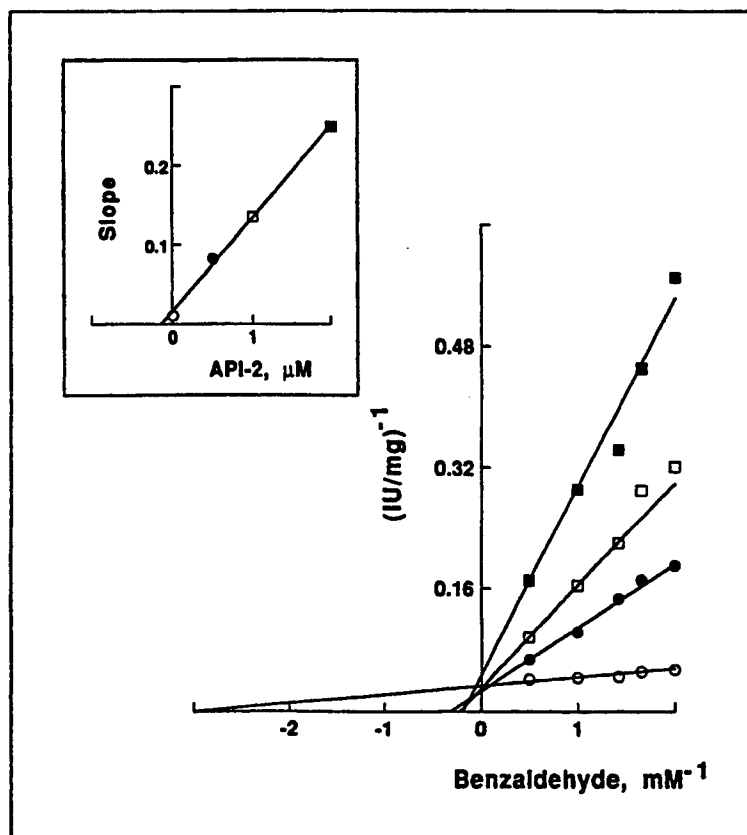


FIG. 5. Inhibition of NAD-linked tALDH-3-catalyzed oxidation of benzaldehyde by API-2: Lineweaver–Burk plot. tALDH-3 was incubated with 0 (○), 0.5 (●), 1.0 (□), or 2.0 (■) μM API-2 for 5 min, various concentrations of the substrate, benzaldehyde, were added, and initial catalytic rates were quantified as described in Materials and Methods. The NAD concentration was 1 mM. Data points are means of triplicate determinations. Inset: Slopes generated by the double-reciprocal (Lineweaver–Burk) plots were plotted as a function of API-2 concentrations for the purpose of determining the K_i value. K_m , V_{max} , and K_i values obtained in this experiment were 335 μM , 30 IU/mg, and 0.14 μM , respectively.

TABLE 3. Inhibition by chlorpropamide analogues of yeast and human recombinant class 2 aldehyde dehydrogenase-catalyzed hydrolyses*

Enzyme	p-Nitrophenyl acetate (μM)	K_m (μM)	V_{max} (IU/mg)	K_i (μM)	
				NPI-2	API-2
rALDH-2	50-400	30	584	ND†	11
yALDH	40-800	31	318	45	0.28

* Enzymes were preincubated with vehicle or various concentrations of the putative inhibitor for 5 min, substrate (*p*-nitrophenyl acetate) was added, initial catalytic rates were quantified, and K_i values were determined as described in Materials and Methods.

† ND = not determined; K_i values were not determined because inhibition was less than 20% at the highest concentration of inhibitor tested (Table 1).

differentially sensitive to inhibition effected by NPI-2 and that this inhibition is apparently irreversible. Differentially sensitive to inhibition by API-2 are tALDH-3 and rALDH-2, with the latter being the most sensitive. Thus, at first glance, although clearly of potential value as an alcohol deterrent given its greater specificity for rALDH-2, API-2 would not appear to have any future as a clinically useful inhibitor of tALDH-3 since it would inhibit ALDH-2 to an even greater extent. In fact, this is not the case because ALDH-2 is an enzyme that humans can apparently do without since 30-50% of Orientals lack a functional ALDH-2 and do not suffer any recognized ill-effects as a consequence thereof except for those following the ingestion of alcohol [29].

Whether these agents will inhibit tALDH-3 *in vivo* at doses that do not cause untoward effects remains to be tested. However, experiments with a limited number of animals showed that API-2, 1 mmol/kg, *i.p.*, did inhibit enzyme-catalyzed oxidation of acetaldehyde in rats, as judged by the markedly elevated plasma levels of acetaldehyde that were observed when the animals were treated with this agent prior to being given ethanol [15]. On the other hand, as judged by the same criteria, NPI-2, 1 mmol/kg, *i.p.*, failed to inhibit enzyme-catalyzed oxidation of acetaldehyde in rats [16]. Offered as the likely explanation was that NPI-2 was hydrolyzed prematurely by plasma esterases, thereby prematurely giving rise to the short-lived cytotoxic metabolite, *i.e.* before reaching the liver. However, the findings reported herein are consistent with another explanation. The aldehyde dehydrogenases that are thought to catalyze the bulk of acetaldehyde oxidation *in vivo*, *viz.* ALDH-2 and, to a lesser extent, ALDH-1, are simply not very sensitive to the inhibitory action of NPI-2. In contrast, the ALDH-3s, especially tALDH-3, are. Thus, the possibility that tolerated doses of NPI-2 will inhibit tALDH-3 *in vivo* remains viable.

NPI-2 is an ester analogue of N^1 -hydroxy-substituted chlorpropamide that, putatively, is without enzyme inhibitory activity *per se*, but that gives rise to nitroxyl upon ester hydrolysis catalyzed by the aldehyde dehydrogenases, which then irreversibly inhibits them. Inhibition of aldehyde dehydrogenase-catalyzed oxidations by NPI-2 was indeed

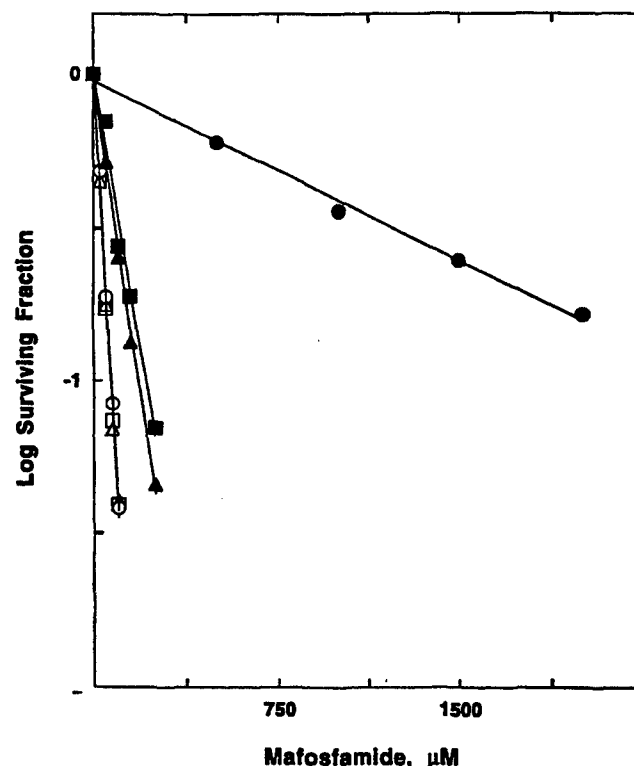


FIG. 6. Sensitivities of human breast adenocarcinoma MCF-7/0 and MCF-7/0/CAT cells to mafosfamide in the presence and absence of NPI-2 or API-2. Exponentially growing MCF-7/0 cells were cultured in the presence of vehicle (○, △, □; MCF-7/0) or 30 μM catechol (●, ▲, ■; MCF-7/0/CAT) for 5 days after which time they were each harvested and incubated with vehicle (○, ●), 100 μM NPI-2 (△, ▲), or 50 μM API-2 (□, ■) for 5 min at 37°. Mafosfamide was added and incubation was continued for an additional 30 min. The cells were then harvested and grown in drug-free growth medium for 15 days. The colony-forming assay described in Materials and Methods was used to determine surviving fractions. Data points are means of triplicate determinations. Cellular levels of aldehyde dehydrogenase activities (cofactor and substrate were 4 mM each of NADP and benzaldehyde, respectively) were 1.9 and 648 mIU/10⁷ cells in 105,000 g supernatant fractions obtained from Lubrol-treated whole homogenates of MCF-7/0 and MCF-7/0/CAT cells, respectively.

found to be apparently irreversible (Fig. 4). However, as judged by the relative rates at which yALDH, rALDH-1, rALDH-2, nALDH-3, and tALDH-3 catalyze the hydrolysis of *p*-nitrophenyl acetate (see footnote to Table 1), yALDH, rALDH-1, and rALDH-2 would be expected to catalyze the bioactivation of NPI-2 at a much faster rate. These enzymes are also much more sensitive to Piloty's acid than are nALDH-3 and tALDH-3 [12]. Piloty's acid spontaneously gives rise to HNO; the inhibitory action of Piloty's acid is thought to be effected by HNO, rather than by the parent compound [30]. Thus, the expectation was that yALDH-, rALDH-1-, and rALDH-2-catalyzed oxidation would be much more sensitive to inhibition by NPI-2 than would be that catalyzed by nALDH-3 and tALDH-3. As before when two structural analogues of NPI-2 were tested [12], this expectation was not realized. Why is not

TABLE 4. Sensitivity of human breast adenocarcinoma MCF-7/0 and MCF-7/0/CAT cells to mafosfamide and phosphoramidate mustard in the presence and absence of NPI-2 or API-2*

Cell line	ALDH-3 (mIU/10 ⁷ cells)	Inhibitor	LC ₉₀ (μM)	
			Mafosfamide	Phosphoramidate mustard
MCF-7/0	2	None	65	800
		NPI-2	60	ND†
		API-2	60	ND
MCF-7/0/CAT	665	None	>2000	1350
		NPI-2	175	1300
		API-2	200	1400

* Human breast adenocarcinoma MCF-7/0 cells were cultured in the presence of vehicle (MCF-7/0) or 30 μM catechol (MCF-7/0/CAT) for 5 days. At the end of this time, cells were harvested, washed, and resuspended in drug-exposure medium. Then the cells (1 × 10⁵ cells/mL) were incubated with NPI-2 (100 μM), API-2 (50 μM), or vehicle for 5 min at 37° after which time various concentrations of mafosfamide, phosphoramidate mustard, or vehicle were added, and incubation was continued as before for 30 min at 37°. The colony-forming assay described in Materials and Methods was used to determine surviving fractions. The LC₉₀ values were obtained from plots of log surviving fractions versus concentrations of drug (Fig. 6). Values are means of LC₉₀s obtained in two experiments. Cellular levels of ALDH-3 activity (NADP-linked enzyme-catalyzed oxidation of benzaldehyde; 4 mM each of cofactor and substrate) in 105,000 g supernatant fractions obtained from Lubrol-treated whole homogenates of tumor cells were determined as described in Materials and Methods.

† ND = not determined.

known. A detailed speculative discussion in that regard has been presented in a previous publication [12].

API-2 is a N¹-methoxy analogue of chlorpropamide that, putatively, is also without enzyme inhibitory activity *per se*, but that gives rise to *n*-propylisocyanate, a potent, presumably reversible, inhibitor of aldehyde dehydrogenases, without the necessity of any enzyme participation [15]. In harmony with this notion is our finding that inhibition of aldehyde dehydrogenase-catalyzed oxidation was partially reversible. Unexplained is the relatively greater preincubation time that was required to achieve maximum inhibition of tALDH-2 by API-2 as compared with that required to achieve maximum inhibition by API-2 of the other aldehyde dehydrogenases (Fig. 3).

Interestingly, but not totally unexpected, tALDH-3, as compared with nALDH-3, was significantly more sensitive to inhibition by each of the two chlorpropamide analogues. This observation further substantiates the notion that tALDH-3, putatively tumor-specific, is a subtle variant of nALDH-3 [8].

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