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13. ABSTRACT *(Maximum 200)*

Cyclophosphamide is perhaps the most widely used drug in the treatment of breast cancer. Unfortunately, its use is rarely curative because cyclophosphamide-resistant tumor cell populations rapidly emerge. Our investigations sought to identify at least one mechanism by which cyclophosphamide-resistance is effected, thereby providing the rationale for strategies that would reverse or even prevent it. At a minimum, the expectation was that our studies would identify a prognostic determinant that could be used to identify patients for whom cyclophosphamide therapy woud be futile, thus sparing them that therapy and the accompanying untoward effects.

Two suspects were identified in preclinical models, viz., the enzymes ALDH3A1 and ALDH1A1. Each was found to catalyze the inactivation of cyclophosphamide. Therefore, the more there was of either of these enzymes in cancer cells, the better able they were to defend themselves against this drug. Amounts of these enzymes in clinical breast cancer samples were found to vary over a 300-fold range. Moreover, ALDH3A1 levels could be increased more than 200-fold in cultured breast cancer cells by exposing them to any of several chemicals that are commonly present in various foods and beverages, e.g., broccoli and coffee. Three agents, each potentially useful in overcoming ALDH3A1-mediated cyclophosphamide-resistance, were identified.

Retrospective studies established that ALDH1A1 levels in clinical breast cancers were of sufficient magnitude to affect the clinical outcome of cyclophosphamide-based chemotherapy, whereas those of ALDH3A1 were not.

14. SUBJECT TERMS	15. NUMBER OF PAGES
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M. E. Sladek 10.30.98

PI - Signature Date

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ABBREVIATIONS

ALDH1A1:	human cytosolic aldehyde dehydrogenase; formerly referred to as a class 1 aldehyde dehydrogenase, specifically, ALDH-1
rALDH1A1:	recombinant ALDH1A1; formerly referred to as rALDH-1
ALDH2A1:	human mitochondrial aldehyde dehydrogenase; formerly referred to as a class 2 aldehyde dehydrogenase, specifically, ALDH-2
rALDH2A1:	recombinant ALDH2A1; formerly referred to as rALDH-2
ALDH3A1:	human cytosolic aldehyde dehydrogenase; formerly referred to as a class 3 aldehyde dehydrogenase, specifically, ALDH-3
nALDH3A1:	"normal" ALDH3A1, viz., ALDH3A1 purified from human normal stomach mucosa
tALDH3A1:	"tumor" ALDH3A1, viz., ALDH3A1 purified from human breast adenocarcinoma MCF-7/0/CAT cells
yALDH:	yeast aldehyde dehydrogenase
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
CYP1A1:	cytochrome P4501A1
G6PDH:	glucose-6-phosphate dehydrogenase
GAPDH:	glyceraldehyde-3-phosphate dehydrogenase
MCF-7/0 cells:	cultured human breast adenocarcinoma cells
MCF-7/0/CAT cells:	MCF-7/0 cells cultured in the presence of 30 μM catechol for 5 days to transiently induce ALDH3A1
MCF-7/0/MC cells:	MCF-7/0 cells cultured in the presence of 3 μM 3-methylcholanthrene for 5 days to transiently induce ALDH3A1
MCF-7/0/IC cells:	MCF-7/0 cells cultured in the presence of 80 μM indole-3-carbinol for 5 days to transiently induce ALDH3A1.
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 ALDH3A1
MCF-7/OAP/CAT cells:	MCF-7/OAP cells cultured in the presence of 30 μM catechol for 5 days to transiently induce ALDH3A1
MCF-7/OAP/MC cells:	MCF-7/OAP cells cultured in the presence of 3 μM 3-methylcholanthrene for 5 days to transiently induce ALDH3A1
MCF-7/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 ALDH3A1
MCF-7/MAF cells:	MCF-7/0 cells surviving exposure to 1 mM mafosfamide for 30 minutes and stably overexpressing ALDH3A1
MCF-10A/0 cells:	cultured human normal breast epithelial cells
MCF-10A/0/CAT cells:	MCF-10A/0 cells cultured in the presence of 30 μM catechol for 5 days to transiently induce ALDH3A1
MCF-10A/0/MC cells:	MCF-10A/0 cells cultured in the presence of 3 μM 3-methylcholanthrene for 5 days to transiently induce ALDH3A1
MCF-10A/0/IC cells:	MCF-10A/0 cells cultured in the presence of 80 μM indole-3-carbinol for 5 days to transiently induce ALDH3A1
Colon C cells:	cultured human colon carcinoma cells

ABBREVIATIONS cont'd

GSH: glutathione

ARE: antioxidant responsive element
ARE-B: battery of enzymes that have in common an ARE in the 5'-upstream regions of the genes that code 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 code for them

AhR: aromatic hydrocarbon receptor
AhR^+: AhR-positive
ARNT: Aromatic hydrocarbon receptor nuclear translocator
$ARNT^+$: ARNT-positive
ER: estrogen receptor
ER^+: ER-positive
ER^-: ER-negative

TCDD: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin
PAH: polycylic aromatic hydrocarbon
IC: indole-3-carbinol

API-1: 4-chloro-N-ethyl-N-[(propylamino)carbonyl]benzenesulfonamide
API-2: 4-chloro-N-methoxy-N-[(propylamino)carbonyl]benzenesulfonamide

NPI-1: (benzoyloxy)[(4-chlorophenyl)sulfonyl]carbamic acid 1,1-dimethylethyl ester
NPI-2: (acetyloxy)[(4-chlorophenyl)sulfonyl]carbamic acid 1,1-dimethylethyl ester
NPI-3: N-acetyl-N-(acetyloxy)-4-chlorobenzenesulfonamide (designated JAE 34 37/20 in the grant application)
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

EPP: ethylphenyl(2-formylethyl)phosphinate

CAD 567C91: 3-(3,4-dimethoxyphenyl)-2-(3-pyridyl)acrylonitrile
CAD 690C88: α-cyano-4-hydroxy-3,5-diisopropylcinnamamide
CAD 707C91: 2-cyano-3-(3,4-dihydroxyphenyl)-N-(3-phenylpropyl)acrylamide
CAD 764C89: α-cyano-3-ethoxy-4-hydroxy-5-phenylthiomethylcinnamamide

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 to be of value because measures may then become apparent as to how to reverse and/or prevent it. It is this understanding which was the overall objective of our first-generation investigations.

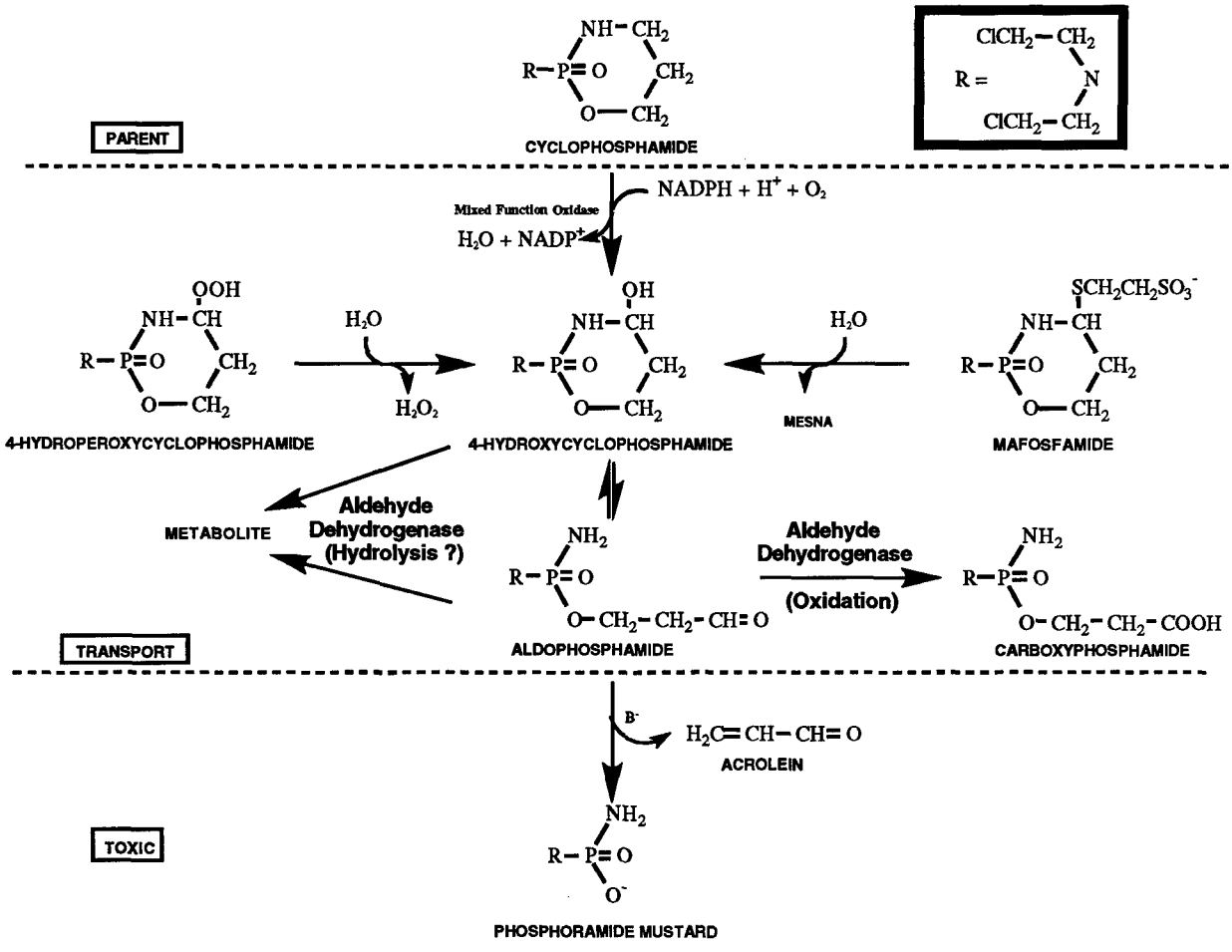


Figure 1. Salient features of oxazaphosphorine metabolism. The prodrugs, cyclophosphamide, mafosfamide and 4-hydroperoxycyclophosphamide, each give rise to 4-hydroxycyclophosphamide which exists in equilibrium with its ring-opened tautomer, aldophosphamide. 4-Hydroxycyclophosphamide and aldophosphamide are, themselves, also without cytotoxic activity. However, aldophosphamide gives rise to acrolein and phosphoramide 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., ALDH1A1, ALDH2A1 and ALDH3A1, 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, Figure 2. Whether aldehyde dehydrogenases catalyze the hydrolysis of either of these intermediates to an irreversibly inactive metabolite was not known.

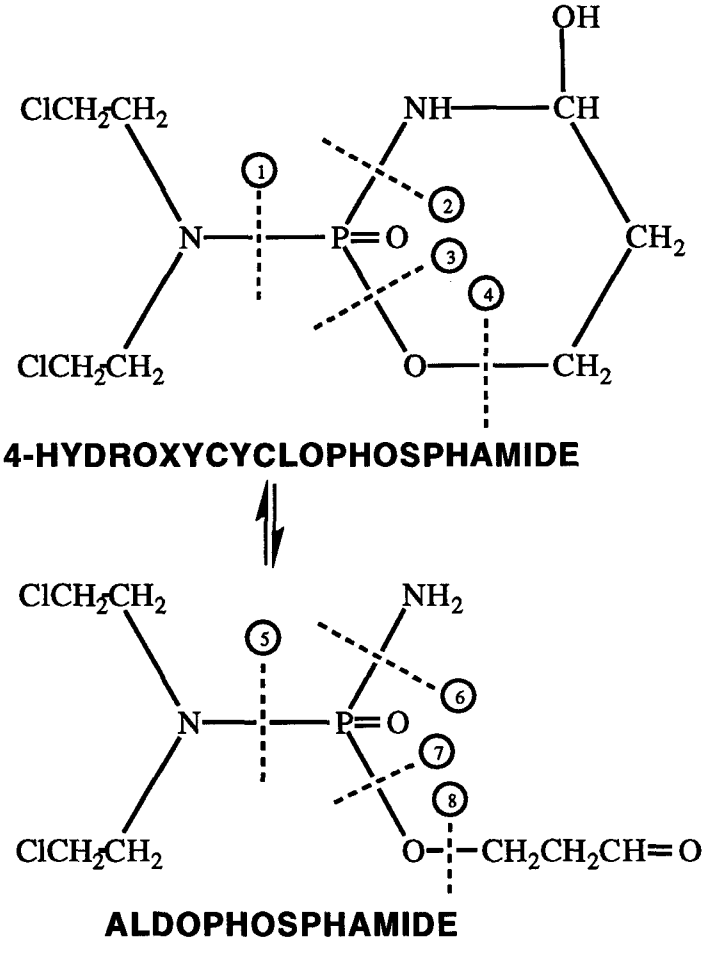


Figure 2. Aldehyde dehydrogenase-catalyzed hydrolysis of 4-hydroxycyclophosphamide and aldophosphamide: possibilities. Hydrolysis of 4-hydroxycyclophosphamide at (1) and aldophosphamide at (5) would give rise to bis-(2-chloroethyl)-amine. Thus, hydrolysis at (1) or (5) cannot account for oxazaphosphorine detoxification, because bis-(2-chloroethyl)-amine is more cytotoxic than are the prodrugs, e.g., mafosfamide and 4-hydroperoxycyclophosphamide, from whence it would originate [Sreerama and Sladek, 1993a; Sladek, 1994]. Hydrolysis of aldophosphamide at (7) and (8) would give rise to phosphoramide mustard. Thus, hydrolysis at (7) and (8) cannot account for the oxazaphosphorine detoxification, because phosphoramide mustard is more cytotoxic than are the prodrugs, e.g., mafosfamide, 4-hydroperoxycyclophosphamide, from whence it would originate [Sreerama and Sladek, 1993a, 1994; Sladek, 1994].

Using cultured human breast adenocarcinoma MCF-7/0 cells and two oxazaphosphorine-resistant models 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, viz., 3 μM 3-methylcholanthrene, for 5 days [Sreerama and Sladek, 1993b, 1994]), we had demonstrated that ALDH3A1 is a 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:

- clinical breast cancer cellular resistance to cyclophosphamide and other oxazaphosphorines is the consequence of elevated ALDH3A1 levels
- ALDH3A1 mediates cellular resistance to oxazaphosphorines by catalyzing the oxidative and/or hydrolytic detoxification of these agents
- inhibitors of the detoxifying reaction can be identified and utilized to reverse the resistance
- hypomethylation of ALDH3A1 genomic DNA accounts for oxazaphosphorine- and activated Ah receptor-induced ALDH3A1 overexpression
- activated Ah receptor-induced ALDH3A1 overexpression can only occur in cells that are estrogen receptor-positive
- agents known to induce xenobiotic-metabolizing enzymes via the antioxidant responsive element (ARE) will also induce ALDH3A1 overexpression, since the ARE consensus sequence is present in the 5'-flanking region of the ALDH3A1 gene

Testing of these hypotheses was divided into **seven tasks** (statement of work):

1. quantify cellular ALDH3A1 levels (and those of ALDH1A1 because, it, too, was a demostrated molecular determinant of cellular sensitivity to the oxazaphosphorines in preclinical models) in surgically removed human breast tumor samples

2. ascertain the ability of ALDH3A1 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

3. synthesize and identify agents that inhibit the ALDH3A1-catalyzed oxidative and/or hydrolytic detoxification of cyclophosphamide (aldophosphamide)

4. evaluate identified inhibitors of the relevant ALDH3A1 activity with respect to their ability to sensitize our oxazaphosphorine-resistant models to the oxazaphosphorines

5. identify the molecular basis for the apparent overexpression of ALDH3A1 in our model systems

6. ascertain the ability of Ah receptor ligands to induce ALDH3A1 overexpression and oxazaphosphorine-specific acquired resistance in estrogen receptor-positive and -negative breast cancer cell lines that lack and express Ah receptors

7. ascertain the ability of ligands for ARE to induce ALDH3A1 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 included 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 directed towards completing the seven (original) tasks and conducted in months 1 through 48 are summarized in the text that follows. They will support the following:

Original Tasks	Months	Progress
1	1 - 48	Nearly completed
2	1 - 24	Completed
3	1 - 24	Completed
4	6 - 30	Nearly completed
5	24 - 48	Nearly completed
6	12 - 42	Nearly completed
7	36 - 48	Completed

Additionally, several added 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 **added tasks**):

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

1b. Ascertain whether simultaneous elevation of ALDH3A1, glutathione S-transferase and DT-diaphorase, or of these three enzymes and cytochrome P4501A1, 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.

1c. Ascertain whether breast tumor ALDH3A1 levels parallel salivary ALDH3A1 levels.

7a. Ascertain whether limonene and/or any of several of its metabolites induce ALDH3A1 levels in human breast adenocarcinoma MCF-7/0 cells.

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

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 48 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	Completed
7a	Completed
7b	Completed
7c	Completed

Parts of the investigations described herein were conducted during the time period between submission of this grant proposal and its initiation on October 1, 1994; funding during that interval was by grants awarded by the National Institutes of Health and Bristol-Myers Squibb Co.

BODY

Task # 1: Quantify cellular ALDH3A1 levels in surgically removed human breast tumor samples.

Cellular levels of ALDH3A1 in human normal breast (n = 29), benign breast tumor (n = 6), and primary (n = 119) and metastatic (n = 37) breast tumor, tissue samples obtained from the Cooperative Human Tissue Network, Midwestern Division were quantified with the aid of an ELISA. In addition, cellular levels of ALDH3A1 in 171 surgically removed primary (122) and metastatic (49) human breast tumor samples collected at the University of Minnesota were semiquantified with the aid of immunocytochemical staining methodology. ALDH1A1 is another known determinant of cellular sensitivity to the oxazaphosphorines [reviewed in Sladek, 1993]. Thus, ALDH1A1 levels in breast tumor tissues, together with those of ALDH3A1, are more likely to correlate with clinical outcome than are ALDH3A1 levels alone. Hence, (semi)quantification of ALDH1A1 levels was added to the original task.

ALDH3A1 levels (determined by an ELISA) in human normal breast, benign breast tumor, and primary and metastatic breast tumor, tissue samples obtained from the Cooperative Human Tissue Network, Midwestern Division are shown in Figure 3. A summary of this data also appears in Table 2, *vide infra.* Noteworthy, ALDH3A1 levels varied widely (356-fold).

ALDH1A1 levels (determined by an ELISA) in human normal breast, benign breast tumor, and primary and metastatic breast tumor, tissue samples obtained from the Cooperative Human Tissue Network, Midwestern Division are shown in Figure 4. A summary of this data also appears in Table 2, *vide infra.* Noteworthy, ALDH3A1 levels varied widely (276-fold).

ALDH3A1 and ALDH1A1 levels in primary breast tumor tissues were found to predict the corresponding malignant metastatic breast tissue levels of these enzymes, Figure 5. Moreover, ALDH3A1 and ALDH1A1 levels in normal breast tissues may predict the corresponding malignant metastatic, as well as primary, breast tissue levels of these enzymes, Figure 5. Metastatic breast tumor samples of sufficient size, or indeed, any size, may only infrequently be obtainable for testing of this type, thus, the potential significance of these relationships.

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. When they do, clinical responses to cyclophosphamide as a function of ALDH3A1 and ALDH1A1 levels will be evaluated.

Immunocytochemical staining methodology to visualize and semiquantify any ALDH3A1 and ALDH1A1 present in breast tumor tissue 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 ALDH3A1 and/or ALDH1A1 activities. Specifically, repository frozen tissue sections were first incubated with polyclonal antibodies specific for ALDH3A1 or ALDH1A1 and then with a biotin-linked secondary antibody, after which they were incubated with an avidin-biotin complex conjugated to horse radish peroxidase and then with diaminobenzidine and H_2O_2 for color development. This method proved to be highly sensitive and reproducible when used on the frozen

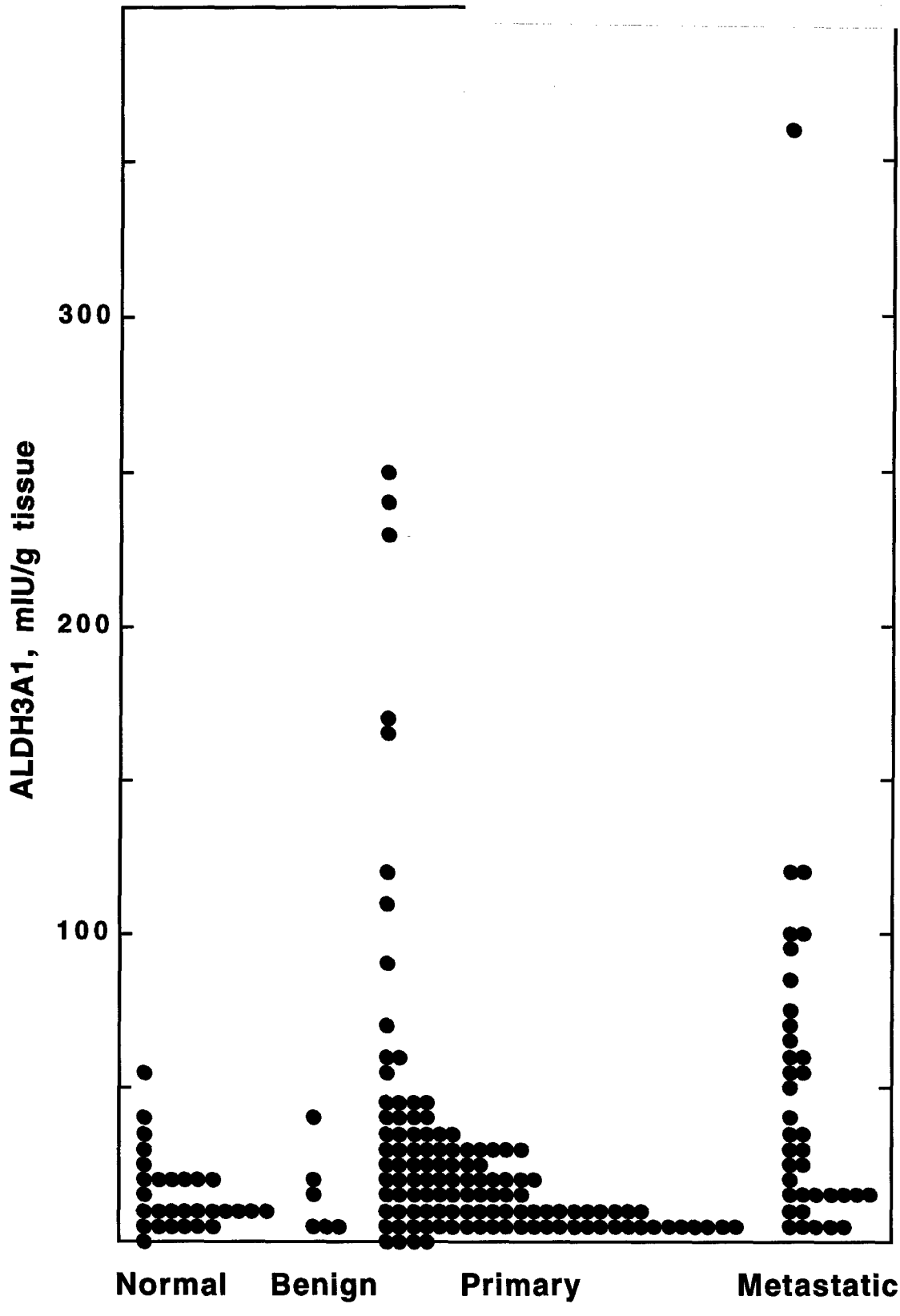


Figure 3. ALDH3A1 levels in human normal breast (n = 29), benign breast tumor (n = 6), and primary (n = 119) and metastatic (n = 37) breast tumor, tissue samples. ALDH3A1 catalytic activity (NADP [4 mM] -linked oxidation of benzaldehyde [4 mM]) was quantified indirectly by an ELISA as described previously [Sreerama and Sladek, 1997]. 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 breast, and/or single benign or malignant breast tumor, tissue samples taken from each of 148 patients.

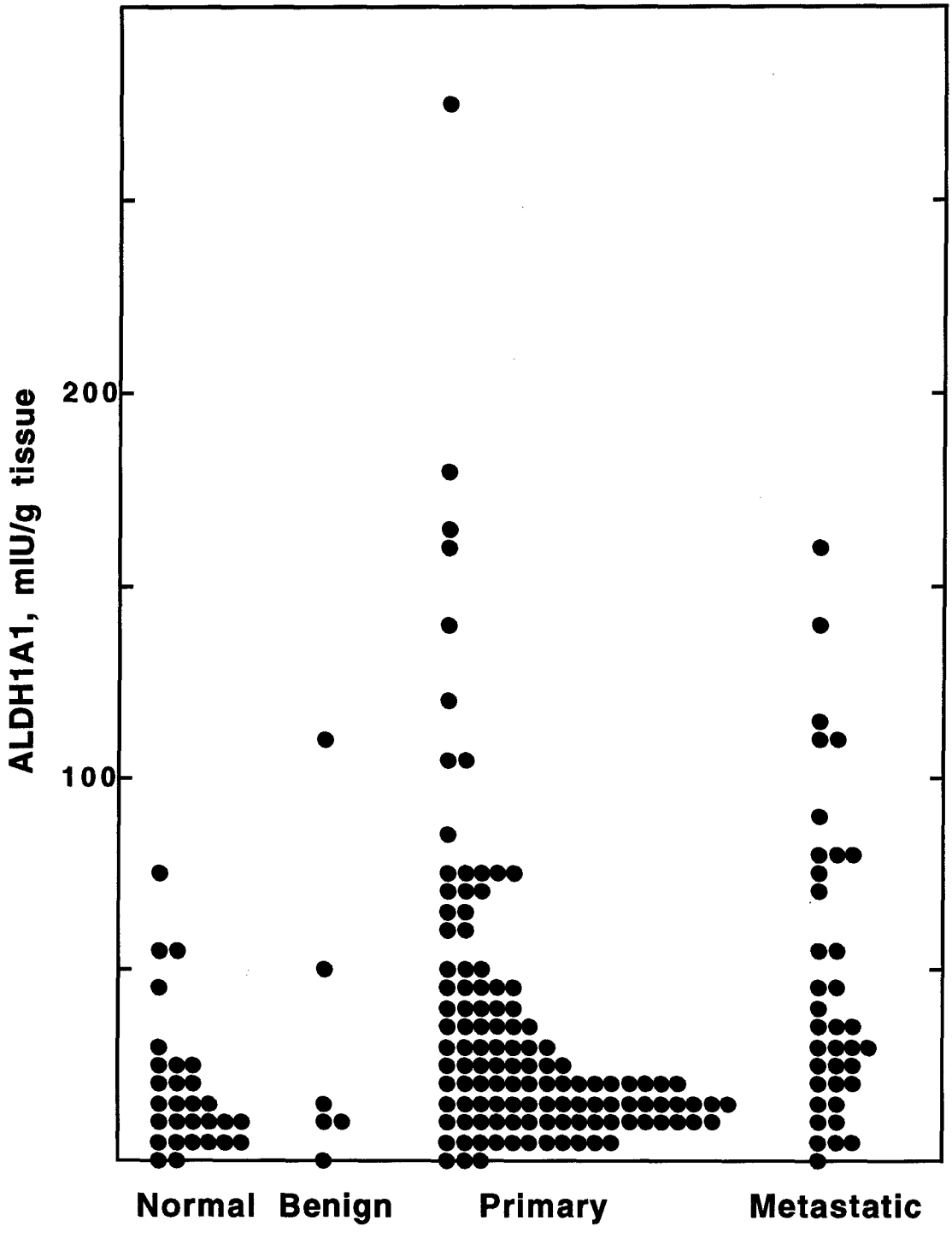


Figure 4. ALDH1A1 levels in human normal breast (n = 29), benign breast tumor (n = 6), and primary (n = 119) and metastatic (n = 37) breast tumor, tissue samples. ALDH1A1 catalytic activity (NAD [4 mM] -linked oxidation of acetaldehyde [4 mM]) was quantified indirectly by an ELISA as described previously [Sreerama and Sladek, 1997]. 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 breast, and/or single benign or malignant breast tumor, tissue samples taken from each of 148 patients.

breast tumor tissue sections which normally contain significantly lower amounts of aldehyde dehydrogenase as compared to that contained by human liver and stomach mucosa. The optimized procedure was used to semiquantify the levels of ALDH3A1 and ALDH1A1 present in our repository of breast tumor (122 primary and

49 metastatic) tissue samples. Representative photomicrographs are shown, and scoring of enzyme levels is illustrated, in Figure 6.

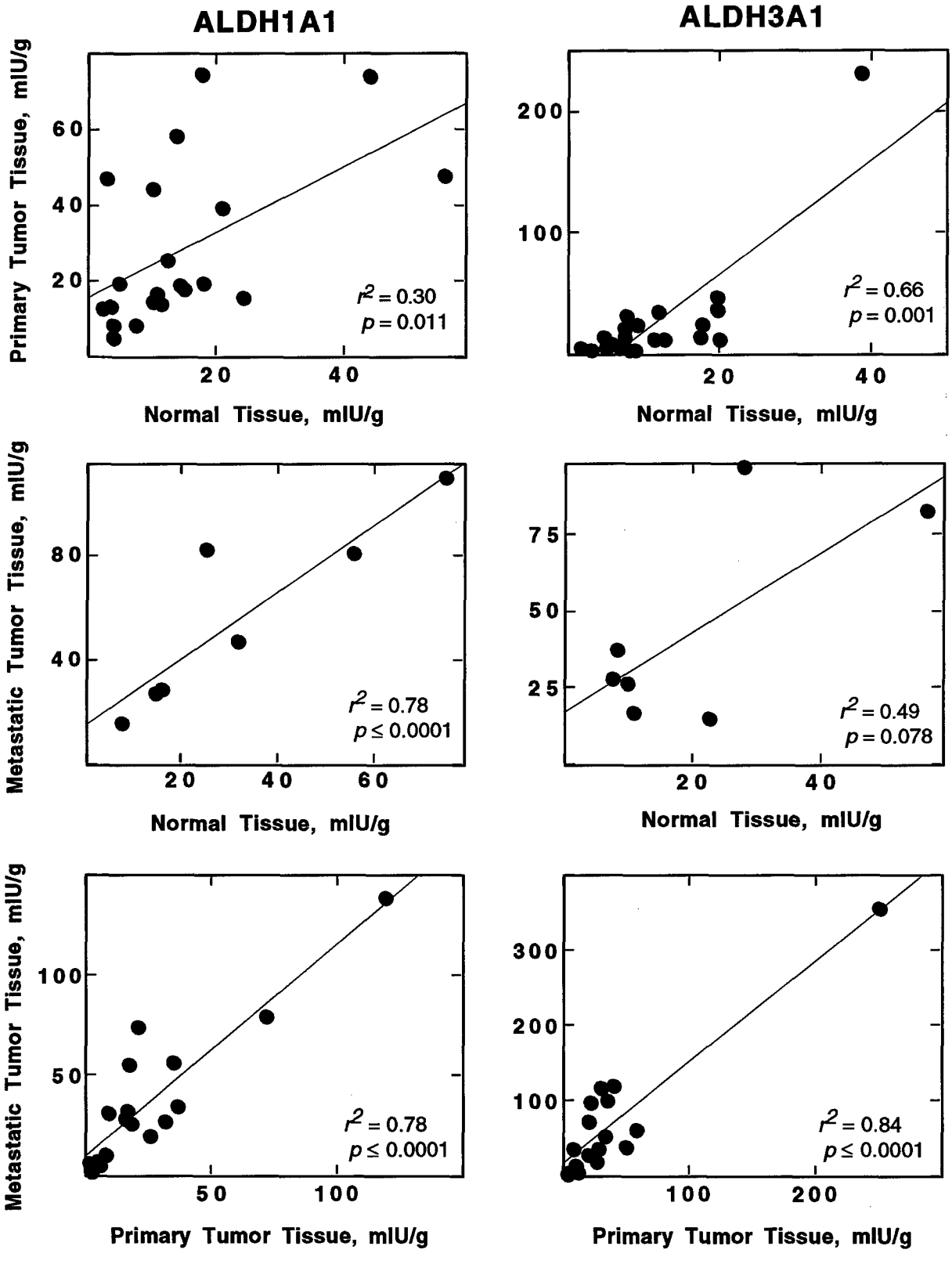


Figure 5. ALDH3A1 and ALDH1A1 levels in paired human normal breast and primary malignant breast (n = 21), normal breast and metastatic malignant breast (n = 7), and primary and metastatic malignant breast (n = 17), tissue samples. ALDH3A1 (NADP [4 mM] -linked oxidation of benzaldehyde [4 mM]) and ALDH1A1 (NAD [4 mM] -linked oxidation of acetaldehyde [4 mM]) catalytic activities were quantified by ELISAs as described previously [Sreerama and Sladek, 1997]. Points are means of duplicate determinations made on single normal breast, and/or primary and/or metastatic malignant breast, tissue samples taken from each of 41 patients.

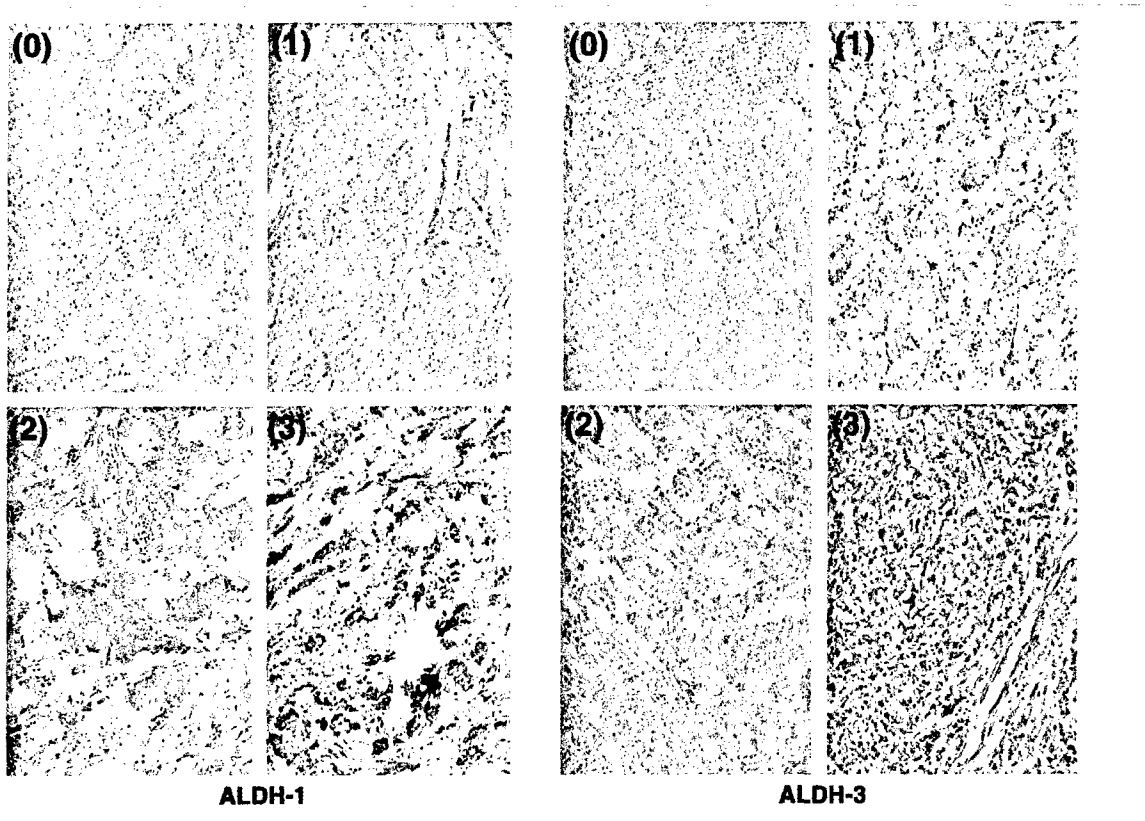


Figure 6. Immunocytochemical visualization/quantification of aldehyde dehydrogenase in breast malignancies. Human breast cancer tissue samples had been stored in liquid nitrogen from the time of biopsy; in most cases, associated medical charts were available for review. Immunocytochemical staining was of formalin-fixed 4 μm sections. Blocking was with, successively, hydrogen peroxide, goat serum, avidin, biotin and BSA. Primary antibodies were chicken anti-ALDH3A1 IgY and anti-ALDH1A1 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.

Albeit the sample size was small, the good relationship obtained between immunocytochemical- and ELISA-determined aldehyde dehydrogenase levels further validated the former as meaningfully and consistently semiquantifying cellular levels of the two aldehyde dehydrogenases, Figure 7.

Cellular levels of ALDH3A1 and ALDH1A1 in 122 primary and 49 metastatic human breast tumor samples were semiquantified with the aid of the immunocytochemical staining methodology described above. Results of these investigations are summarized in Table 1.

As in the preceding studies, ALDH3A1 and ALDH1A1 levels varied substantially in both primary and metastatic breast tumor tissue. Average ALDH3A1 and ALDH1A1 values were 0.84 and 1.40, respectively, in the primary breast cancer samples. They were 1.31 and 1.53, respectively, in the metastatic breast cancer samples. Also as in the preceding studies, the mean ALDH3A1 level in the metastatic breast tumor samples was significantly higher ($P = 0.0006$) than that in the primary breast tumor samples. As in the preceding study, too, the mean ALDH1A1 level in the metastatic breast tumor samples was not significantly higher ($P = 0.18$) than that in the primary breast tumor samples.

Whereas in an earlier study [Sreerama and Sladek, 1997] cellular levels of ALDH3A1 and ALDH1A1 in malignant breast tissue appeared to be directly related, no correlation between ALDH3A1 and ALDH1A1 levels was observed in the present investigation, nor was any correlation found between either enzyme level and the patient's age, smoking history, or estrogen or progesterone receptor status (data not shown).

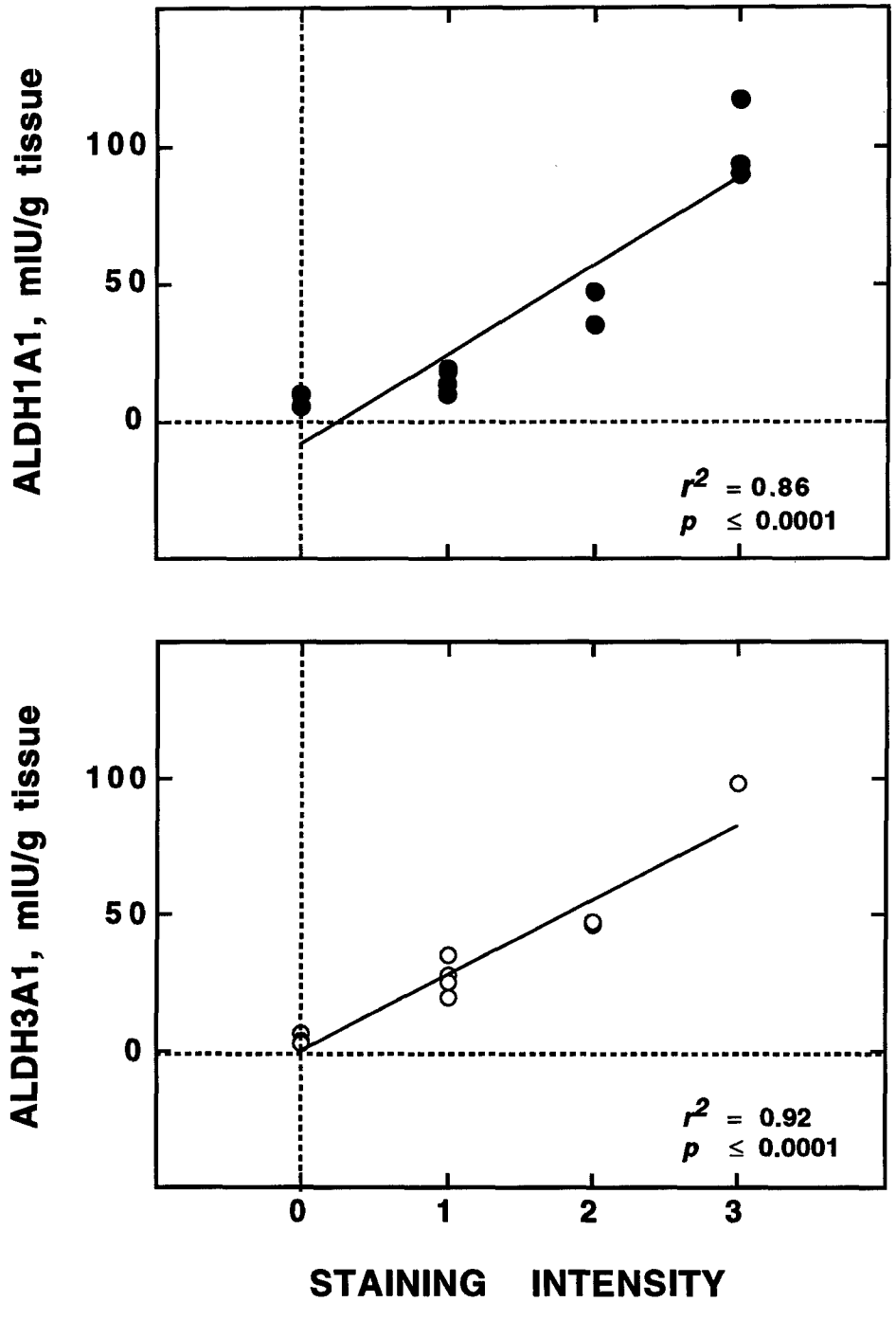


Figure 7. Relationship between aldehyde dehydrogenase levels (semi)quantified immunocytochemically and by an ELISA. ALDH3A1 (O) and ALDH1A1 (●) levels in metastatic malignant breast tissue samples (n = 11) were (semi)quantified by an ELISA [Sreerama and Sladek, 1997] as well as by the immunocytochemical assay illustrated in Figure 6. Immunocytochemical assay: the staining intensities were scored on a scale of 0 (no staining) to 3 (intense staining). ELISA: catalytic activities of ALDH3A1 and ALDH1A1 were estimated from standard curves generated with purified proteins; specific activities of purified ALDH3A1 and ALDH1A1 were 60,500 and 2,850 mIU/mg protein, respectively. Computer-assisted unweighted regression analysis was carried using the STATView (Brainpower, Inc., Calabas, CA) statistical program to generate straight-line functions.

Table 1. Immunocytochemical semiquantification of ALDH3A1 and ALDH1A1 levels in human primary and metastatic breast tumor samples.

Enzyme	Staining Intensity	% of Total	
		Primary (n = 122)	Metastatic (n = 49)
ALDH3A1	0	40	12
	1	38	49
	2	20	35
	3	2	4
ALDH1A1	0	17	8
	1	34	41
	2	41	41
	3	8	10

[a]Staining intensities were rated on a scale of 0 (no staining) to 3 (intense staining) as illustrated in Figure 6. Mean ± SD values for ALDH3A1 were 0.84 ± 0.82 (primary) and 1.31 ± 0.74 (metastatic), and were significantly different ($P = 0.0006$) from each other. Mean ± SD values for ALDH1A1 were 1.40 ± 0.87 (primary) and 1.53 ± 0.79 (metastatic), and were not significantly different ($P = 0.18$) from each other.

Most of the primary tumor samples were obtained from patients who had not been, nor were they going to be at the time that samples were taken, treated with chemotherapeutic agents. On the other hand, almost all of the metastatic tumors were obtained from patients who had been, and/or were going to be, treated with chemotherapeutic agents, most commonly, cyclophosphamide, doxorubicin, methotrexate, 5-fluorouracil and/or vincristine. Usually, these agents were given in one of several combinations.

Given that ALDH3A1 and ALDH1A1 are operational molecular determinants of cellular sensitivity to cyclophosphamide clinically, the expectation was that cellular levels of these enzymes would be higher in tumor cell populations that had survived exposure to cyclophosphamide as compared to their levels in tumor cell populations that had never been exposed to this agent. This is because cells capable of defending themselves against the otherwise cytocidal action of cyclophosphamide by virtue of their relatively greater content of ALDH3A1 and/or ALDH1A1 would be selected for survival when exposed to cyclophosphamide, but not when exposed to other chemotherapeutic agents for which these enzymes are not molecular determinants of cellular sensitivity.

Distributions of ALDH3A1 and ALDH1A1 staining intensities in breast tumor tissues obtained from patients that, earlier, had, and had not, been treated with cyclophosphamide-based chemotherapeutic regimens are shown in Figure 8.

Average ALDH3A1 and ALDH1A1 levels were slightly higher (approximately + 0.2 and + 0.4 units, respectively) in metastatic tumor cells that survived exposure to cyclophosphamide alone or to cyclophosphamide and methotrexate, 5-fluorouracil and/or vincristine than were those in metastatic tumor cells that had not been exposed to these drugs, Figure 8. The difference in ALDH3A1 levels was not statistically significant; that in ALDH1A1 levels was.

ALDH3A1 staining intensities were high (score: 2 or 3) in 39% of the samples obtained from patients that had been treated with cyclophosphamide as well as in 39% of those obtained from patients that had not been treated with cyclophosphamide (breakdown of data not shown). ALDH1A1 staining intensities were high (score: 2 or 3) in 61% of the samples obtained from patients that had been treated with cyclophosphamide; they were high in only 42% of the control samples, i.e., those obtained from patients that had not been treated with cyclophosphamide (breakdown of data not shown). This difference was statistically significant ($P = 0.05$).

Further, in that fraction of the sample population where ALDH1A1 staining intensities were low (score: 0 or 1), ALDH3A1 staining intensities were high (score 2 or 3) in 11% and 13% of the samples obtained from patients from patients that had, and had not (controls), been treated with cyclophosphamide, respectively (breakdown of data not shown). In contrast, in that fraction of the sample population where ALDH3A1 staining intensities were low (score: 0 or 1), ALDH1A1 staining intensities were high (score: 2 or 3) in 43% and 19% of the samples obtained from patients that had, and had not (controls), been treated with cyclophosphamide, respectively (breakdown of data not shown). This difference was statistically significant ($P = 0.0042$).

Given that ALDH3A1 and ALDH1A1 are operational molecular determinants of cellular sensitivity to cyclophosphamide clinically, the expectation is that low tumor cell levels of these enzymes would predict for a more favorable therapeutic response to this agent as compared to that obtained when tumor cell levels of these enzymes are high.

A favorable response (tumor size decreased; PR/CR) was observed in 18 of the 26 (69%) patients treated with a therapeutic regimen that included cyclophosphamide, Figure 9.

As compared to the average ALDH3A1 level present in metastatic tumors that, upon subsequent treatment, "did respond" (PR/CR) to chemotherapeutic regimens that included cyclophosphamide, the average ALDH3A1 level in metastatic tumors that, upon subsequent treatment, "did not respond" (PD/SD) to these chemotherapeutic regimens was higher (+ 0.3 units). However, this difference was not statistically significant, Figure 9. Moreover, in a control study, the average ALDH3A1 level was also higher (+ 0.3 units) in metastatic tumors that, upon subsequent treatment, "did not respond" to therapeutic strategies that did not include the use of cyclophosphamide as compared to the average ALDH3A1 level in metastatic tumors that, upon subsequent treatment, "did respond" to these strategies, Figure 9.

In the case of ALDH3A1, frequencies of false positives (cyclophosphamide was not effective (PD/SD) when a low level (score: 0 or 1) of ALDH3A1 predicted it would be) and false negatives (cyclophosphamide was effective (PR/CR) when a high level (score: 2 or 3) of ALDH3A1 predicted it would not be) were 0.25 and 0.60, respectively, Figure 10. False positive and false negative frequencies were 0.33 and 1.00, respectively, when

staining intensities "0/1/2" and "3" were viewed as predictive of a favorable response (PR/CR) and a lack thereof (PD/SD), respectively, to cyclophosphamide-based therapy, and 0.00 and 0.64, respectively, when staining intensities "0" and "1/2/3" were viewed as predictive of a favorable response (PR/CR) and a lack thereof (PD/SD), respectively, to cyclophosphamide-based therapy (breakdown of data not shown). False positive and false negative frequencies were 0.00 and 1.00, respectively, when ALDH3A1 levels (0/1 *vs* 2/3) were viewed as the predictive indicator and ALDH1A1 staining intensities were "0/1," i.e., the predictive value of ALDH3A1 levels did not improve in the absence of the potentially confounding presence of putatively pharmacologically-meaningful ALDH1A1 levels (breakdown of data not shown).

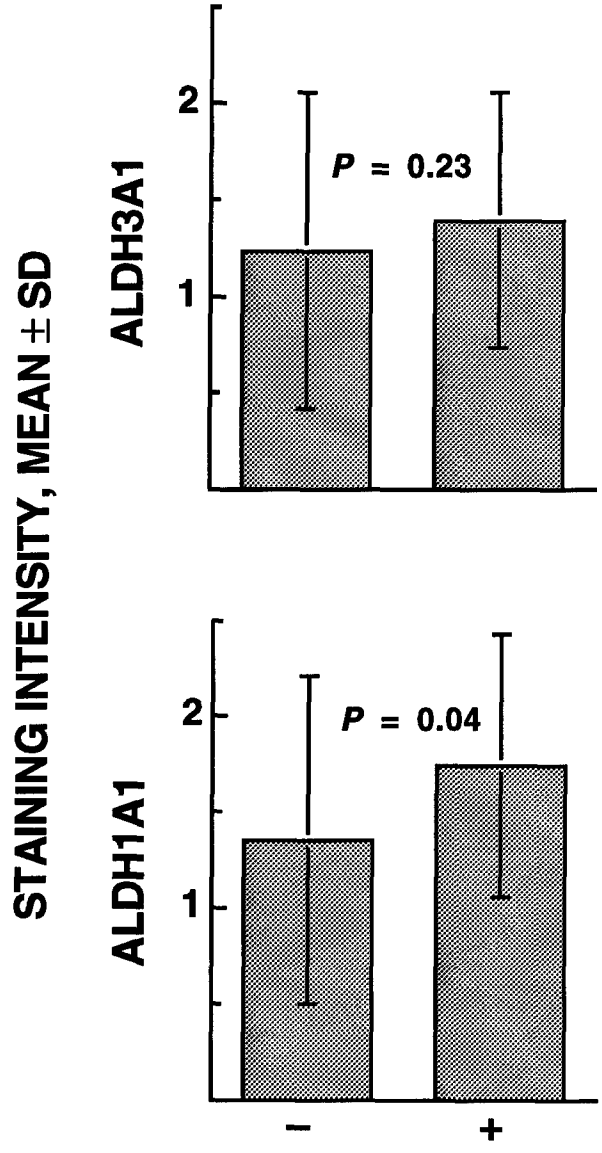


Figure 8. Aldehyde dehydrogenase levels in human metastatic breast tumors obtained from patients that, earlier, had not (–; n = 26) and had (+; n = 23) been treated with cyclophosphamide-based chemotherapeutic regimens. Staining intensities were rated on a 0 to 3 scale as illustrated in Figure 6: no 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.

Looking at the data in another way, ALDH3A1 staining intensities were high (score: 2 or 3) in 50% of the samples obtained from patients exhibiting PD/SD after subsequent administration of cyclophosphamide; they were high in 33% of the samples obtained from patients exhibiting a PR/CR after subsequent administration of cyclophosphamide, Figure 11. This difference was not statistically significant ($P = 0.0833$). Moreover, nearly identical distributions (44% *vs* 31%, respectively; $P = 0.0844$) were observed in control samples, i.e., those obtained from patients that were subsequently subjected to therapeutic strategies that did not include the use of cyclophosphamide, Figure 11.

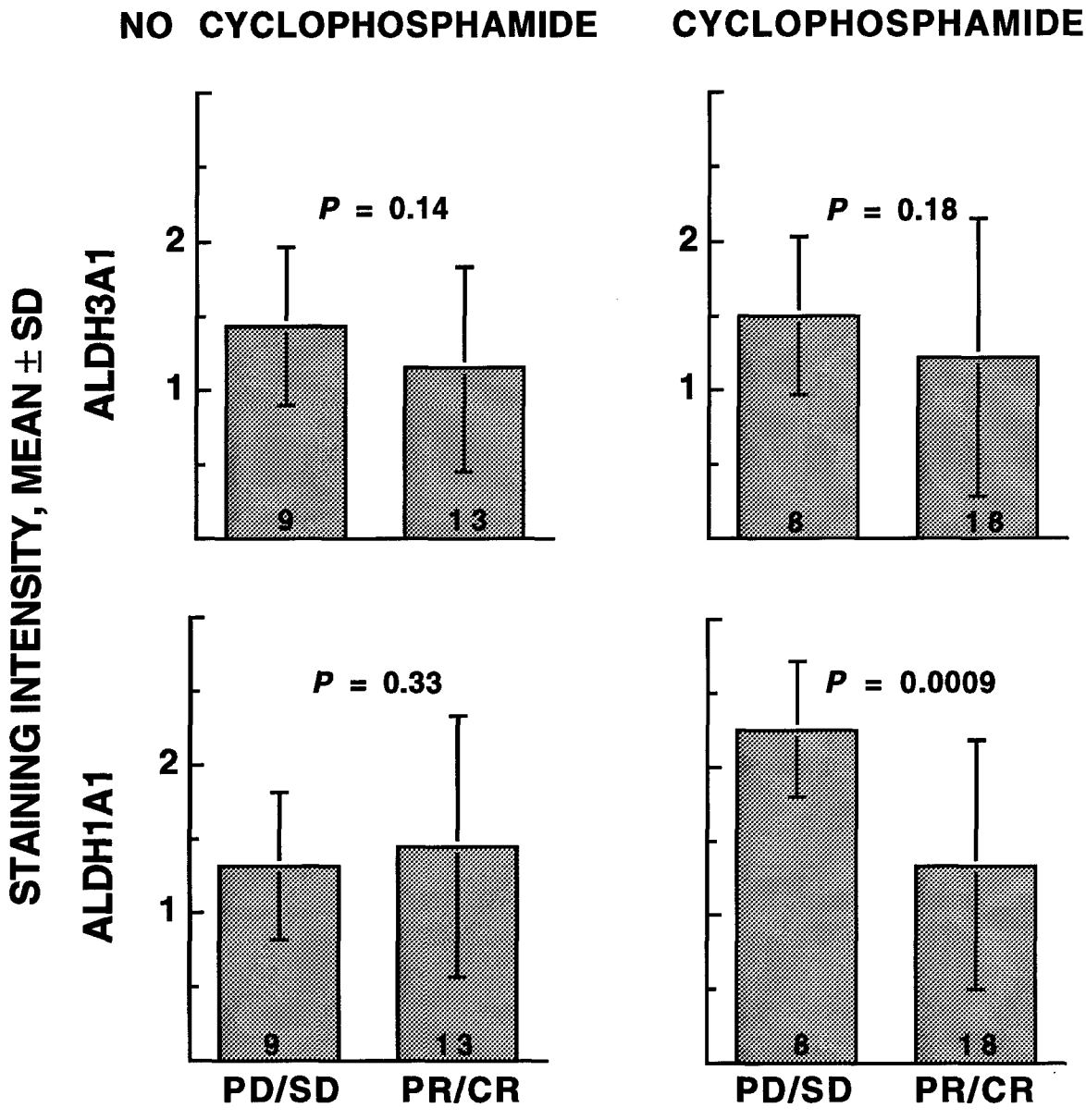


Figure 9. Mean aldehyde dehydrogenase levels in human metastatic breast tumors as a function of subsequent cyclophosphamide-based treatment outcome. Immunocytochemical staining of formalin-fixed breast tumor tissue sections for ALDH3A1 and ALDH1A1 was as described in the legend to Figure 6. Staining intensities were scored on a scale of 0 (no staining) to 3 (intense staining) as illustrated in Figure 6. PD, progressive disease; SD, stable disease; PR, partial response; CR, complete response. Data given under "no cyclophosphamide" is that for patients that did not subsequently receive cyclophosphamide, i.e., patients serving as controls. Numbers inside bars are number of samples (patients) assigned to each group.

As compared to the average ALDH1A1 level in metastatic tumors that, upon subsequent treatment, "did respond" (PR/CR) to therapeutic regimens that included cyclophosphamide, the average ALDH1A1 level in metastatic tumors that, upon subsequent treatment, "did not respond" (tumor size did not decrease or even increased; SD/PD) to these therapeutic regimens was higher (+ 0.9 units), Figure 9. This difference was, statistically, highly significant. In a control study, average ALDH1A1 levels were not statistically different in metastatic tumors that, upon subsequent treatment, "did, and did not, respond" to therapeutic strategies that did not include the use of cyclophosphamide, Figure 9.

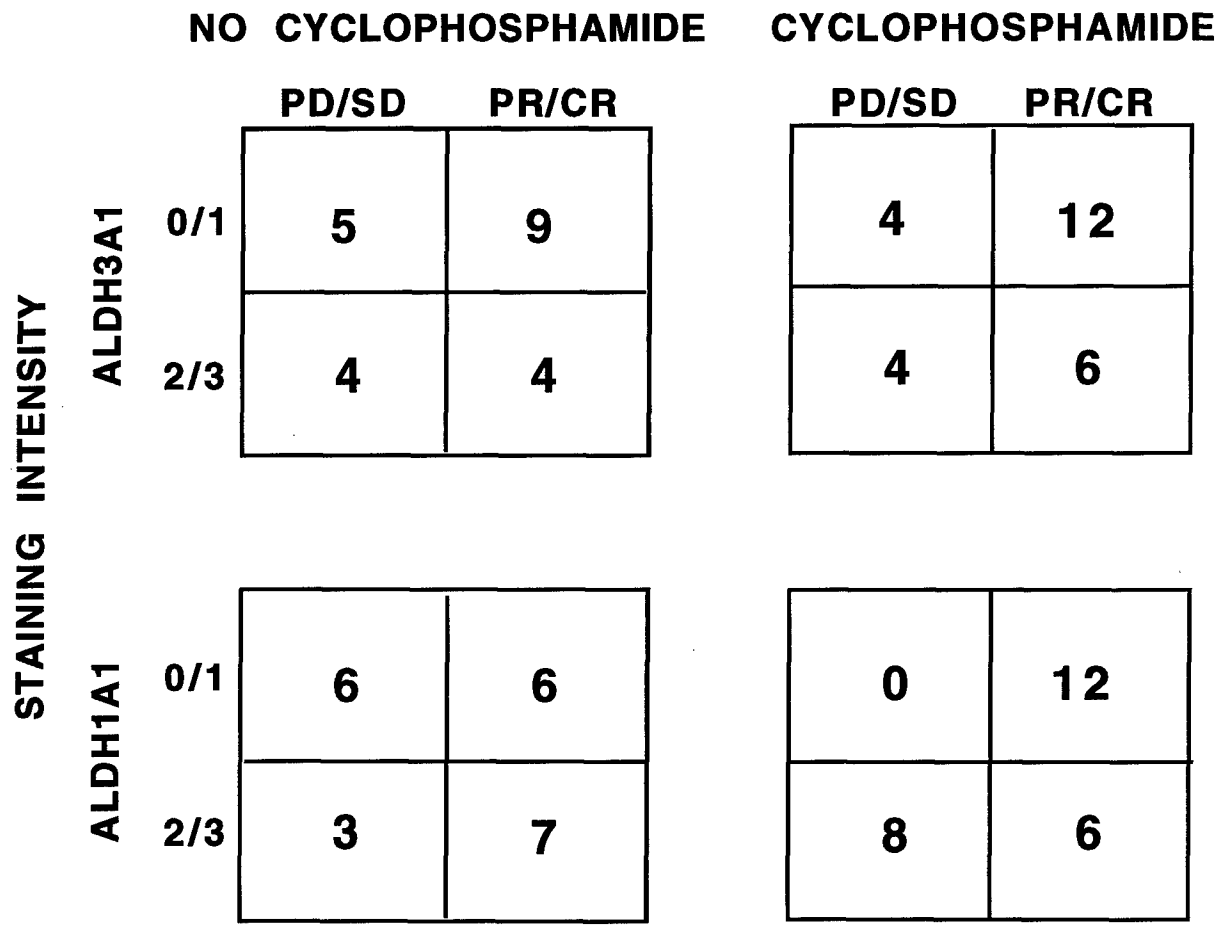


Figure 10. Predictive relationship between subsequent cyclophosphamide-based treatment outcome and aldehyde dehydrogenase levels in human metastatic breast tumors. Original data is the same as that presented in Figure 9. Numbers in the four quadrants of each of the four boxes are samples that fall into each category. False positives (cyclophosphamide was not effective (PD/SD) when aldehyde dehydrogenase levels predicted it would be) and false negatives (cyclophosphamide was effective (PR/CR) when aldehyde dehydrogenase levels predicted it would not be) are those in the upper left corner quadrants and lower right corner quadrants, respectively, of each box.

In the case of ALDH1A1, frequencies of false positives (cyclophosphamide was not effective (PD/SD) when a low level (score: 0 or 1) of ALDH1A1 predicted it would be) and false negatives (cyclophosphamide was effective (PR/CR) when a high level (score: 2 or 3) of ALDH1A1 predicted it would not be) were 0.00 and 0.43, respectively, Figure 10. False positive and false negative frequencies were 0.27 and 0.50, respectively, when staining intensities "0/1/2" and "3" were viewed as predictive of a favorable response (PR/CR) and a lack thereof (PD/SD), respectively, to cyclophosphamide-based therapy, and 0.00 and 0.67, respectively, when staining intensities "0" and "1/2/3" were viewed as predictive of a favorable response (PR/CR) and a lack thereof (PD/SD), respectively, to cyclophosphamide-based therapy (breakdown of data not shown). False positive and false negative frequencies were 0.00 and 0.33, respectively, when ALDH1A1 levels (0/1 *vs* 2/3) were viewed as the

predictive indicator and ALDH3A1 staining intensities were "0/1," i.e., the predictive value of ALDH1A1 levels was only minimally improved in the absence of the potentially confounding presence of putatively pharmacologically-meaningful ALDH3A1 levels (breakdown of data not shown).

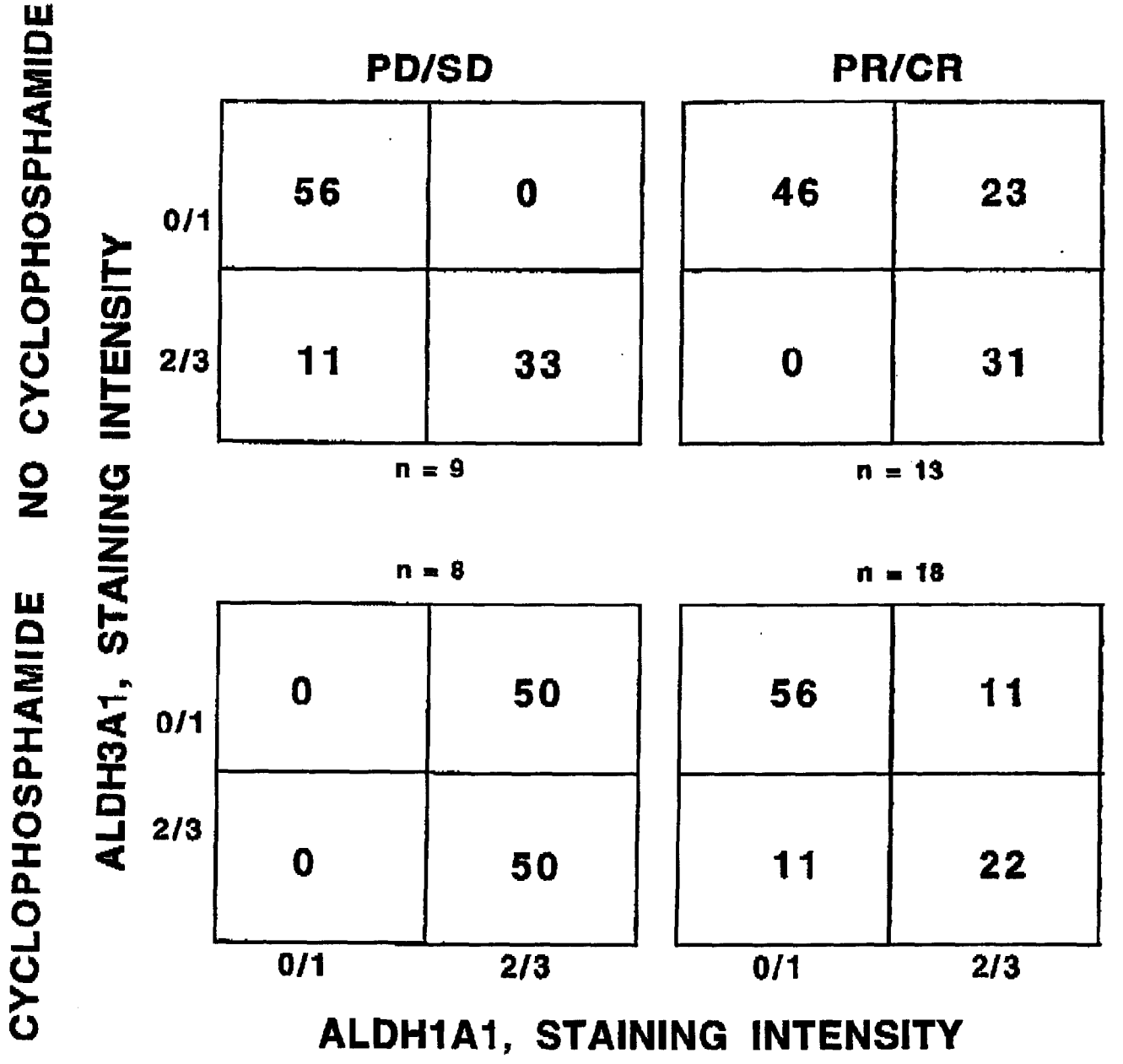


Figure 11. Aldehyde dehydrogenase levels in human metastatic breast tumors obtained from patients for whom subsequent cyclophosphamide-based chemotherapy was, and was not, effective: summary of distribution. Original data is the same as that presented in Figure 9. Numbers in boxes are per cents of totals.

Again looking at the data in another way, ALDH1A1 staining intensities were high (2/3) in all (100%) of the samples obtained from patients exhibiting PD/SD after subsequent administration of cyclophosphamide; they were high in only 33% of the samples obtained from patients exhibiting a PR/CR after subsequent administration of cyclophosphamide, Figure 11. This difference was statistically significant ($P \leq 0.0001$). It was not observed in control samples, i.e., those obtained from patients that were subsequently subjected to therapeutic strategies that

did not include the use of cyclophosphamide. Thus, ALDH1A1 staining intensities were high (2/3) in 33% and 54% of the samples obtained from patients exhibiting PD/SD and a PR/CR, respectively, after subsequent subjection to therapeutic strategies that did not include the use of cyclophosphamide, Figure 11.

Further, in that fraction of the sample population where ALDH3A1 staining intensities were low (score: 0 or 1), ALDH1A1 staining intensities were high (score: 2 or 3) in 100% and 17% of the samples obtained from patients exhibiting PD/SD and a PR/CR, respectively, after subsequent administration of cyclophosphamide, Figure 11. This difference, too, was statistically significant ($P \leq 0.001$). Again this difference was not observed in samples obtained from patients that were not subsequently treated with cyclophosphamide, i.e., controls. Thus, in that fraction of the sample population where ALDH3A1 staining intensities were low (score: 0 or 1), ALDH1A1 staining intensities were high (score: 2 or 3) in 0% and 33% of the samples obtained from patients exhibiting PD/SD and a PR/CR, respectively, after subsequent subjection to therapeutic strategies that did not include the use of cyclophosphamide, Figure 11.

The false negative (cyclophosphamide was effective (PR/CR) when aldehyde dehydrogenase levels predicted it would not be) frequency was 0.50, when ALDH1A1 and ALDH3A1 levels were each high (score: 2 or 3), Figure 11.

Only seven of the primary breast tumor samples, for which the corresponding medical records were available, were obtained from patients who were surgically resected and subsequently treated with a cyclophosphamide-based chemotherapeutic regimen to prevent recurrence. Four of the seven were disease-free for at least two years. Mean ALDH1A1 and ALDH3A1 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.

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

Knowledge of glutathione (GSH) levels in breast tumor tissue could also be of value in the rational use of cyclophosphamide in breast cancer patients because cellular sensitivity to cyclophosphamide decreases as cellular levels of GSH increase [reviewed in Sladek, 1993, and in 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 [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 12.

GSH levels in normal breast tissue samples did not predict for the corresponding GSH levels in paired, primary, or metastatic, breast tumor tissue samples, Figure 13. However, cellular levels of GSH in primary breast malignancies did predict cellular levels of this tripeptide in paired metastatic breast malignancies, albeit the value for r^2 was less than impressive, Figure 13.

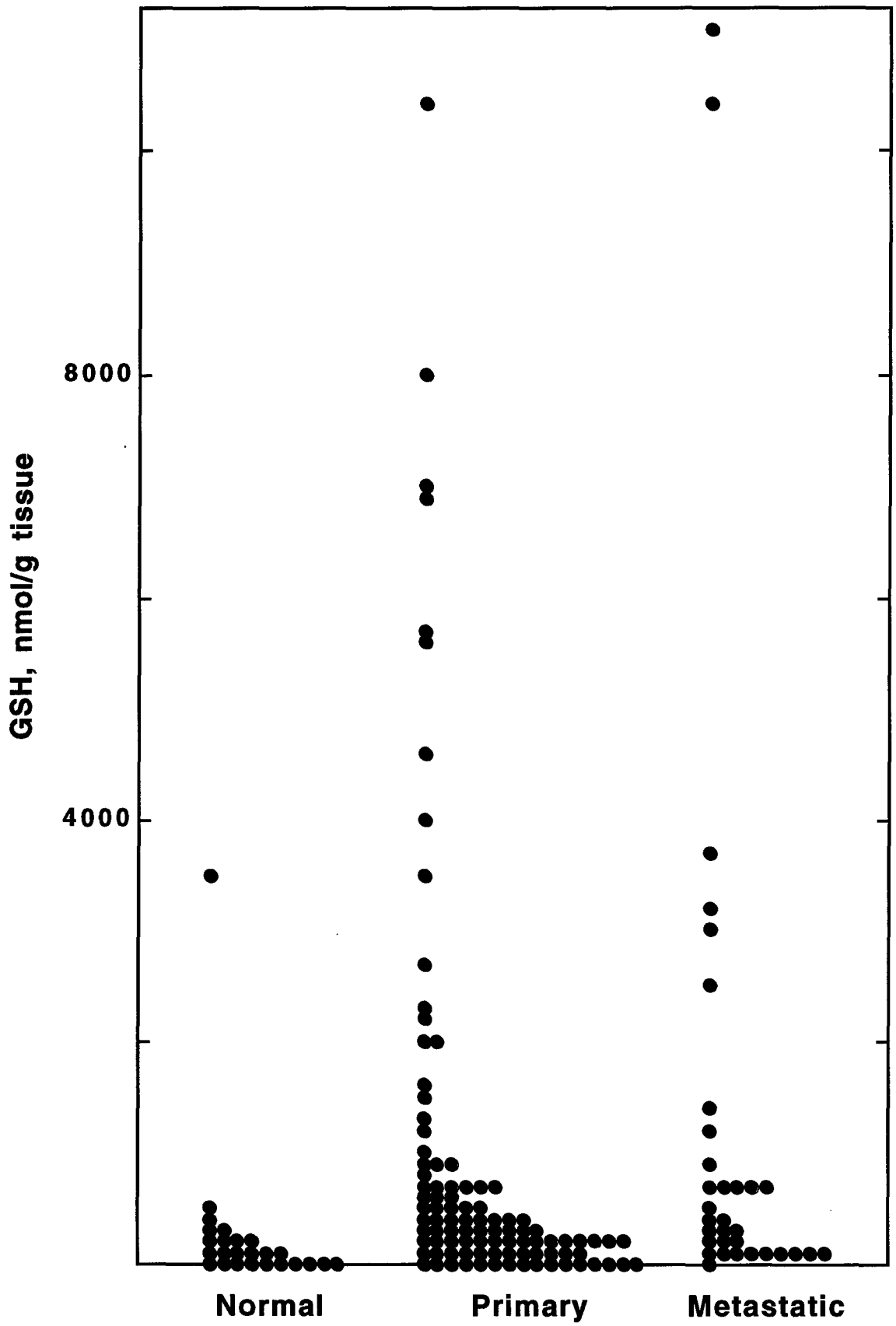


Figure 12. GSH levels in human normal breast (n = 25), and primary (n = 97) and metastatic (n = 33) breast tumor, tissue samples. GSH levels were quantified as described previously [Sreerama and Sladek, 1997]. Points are means, rounded off for clarity of presentation to zero if they were < 50 mIU/g, and to 100 mIU/g or the nearest multiple thereof if they were ≥ 50 mIU/g, of duplicate determinations made on single normal breast, and/or single malignant breast tumor, tissue samples taken from each of 115 patients.

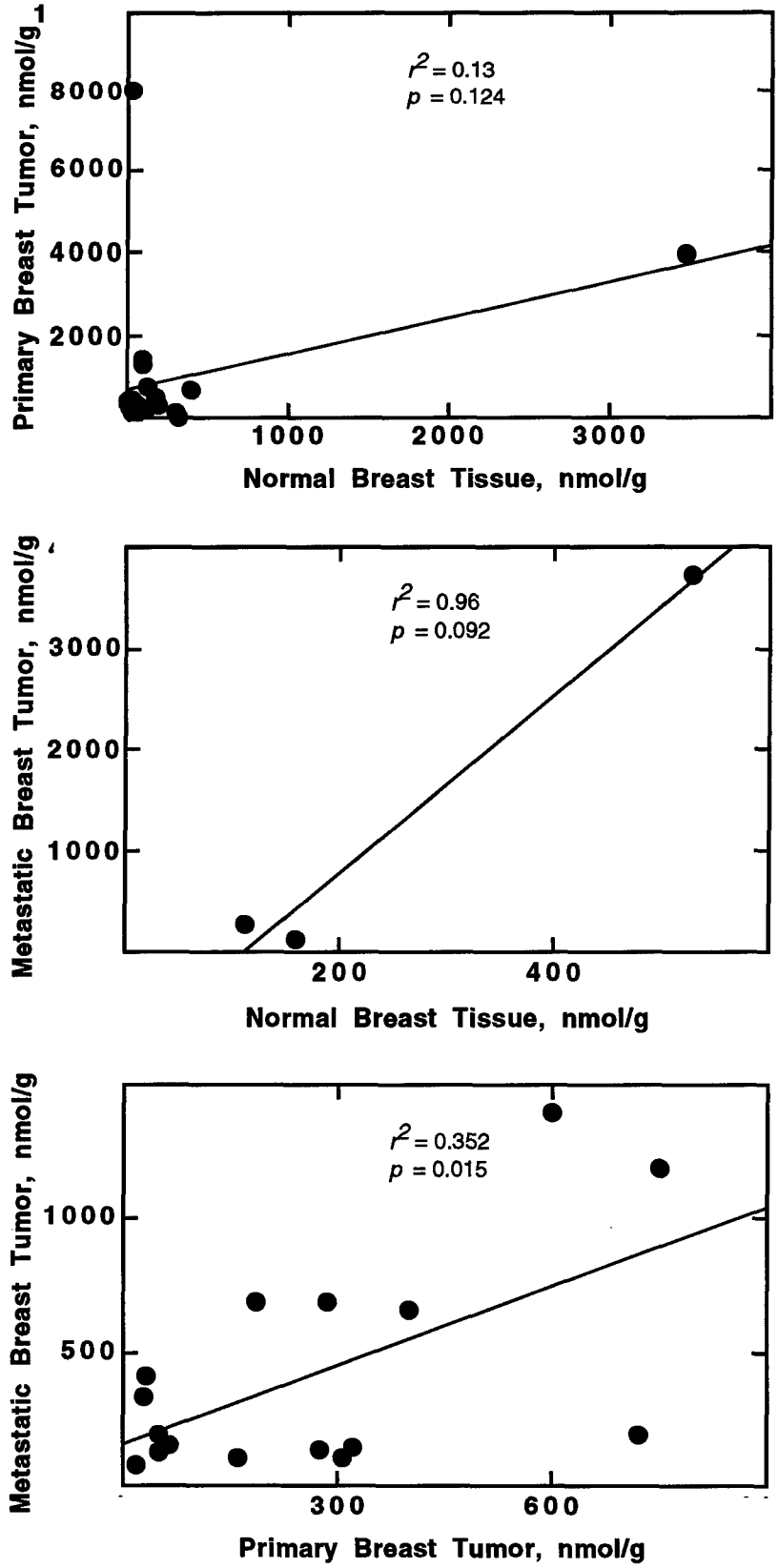


Figure 13. GSH levels in paired human normal breast and primary malignant breast (n = 21), normal breast and metastatic malignant breast (n = 3), and primary and metastatic malignant breast (n = 16), tissue samples. GSH levels were quantified by a spectrophotometric assay as described previously [Sreerama and Sladek, 1997]. Points are means of duplicate determinations made on single normal breast, and/or primary and/or metastatic malignant breast, tissue samples taken from each of 39 patients.

Table 2. ALDH3A1, ALDH1A1, DT-D, panGST, GSTs α, μ and π, CYP IA1 and GSH levels in human normal breast, and benign and malignant breast tumor, tissue samples: summary[a]

Enzyme	Normal			Benign			Primary			Metastatic		
	n	Mean ± SD	Range	n	Mean ± SD	Range	n	Mean ± SD	Range	n	Mean ± SD	Range
ALDH3A1	29	15 ± 12	2 - 56	6	15 ± 13	6 - 39	119	29 ± 43	1 - 251	37	50 ± 62	3 - 356
ALDH1A1	29	19 ± 18	2 - 75	6	33 ± 41	2 - 108	119	35 ± 41	1 - 276	37	48 ± 40	2 - 160
DT-D	29	410 ± 658	23 - 2700	6	214 ± 155	68 - 415	119	1000 ± 1370	6 - 6250	37	1060 ± 940	84 - 4280
panGST	29	1070 ± 1240	56 - 4920	6	3300 ± 3750	658 - 10300	119	2110 ± 1880	23 - 8880	37	3060 ± 2390	460 - 8220
GSTα[b]	29	150 ± 218	0 - 900	6	117 ± 132	0 - 360	119	266 ± 404	0 - 2500	37	434 ± 593	0 - 1950
GSTμ[c]	29	99 ± 127	0 - 522	6	108 ± 126	0 - 260	119	275 ± 516	0 - 3400	37	319 ± 598	0 - 3050
GSTπ	29	806 ± 989	100 - 3950	6	2850 ± 3620	375 - 9900	119	1550 ± 1320	39 - 6200	37	2200 ± 1730	230 - 6500
CYP1A1[d]	24	1 ± 5	0 - 24	6	11 ± 16	0 - 41	109	39 ± 113	0 - 640	34	33 ± 91	0 - 485
GSH	25	264 ± 681	2 - 3470	-	-	-	97	995 ± 1860	16 - 10400	33	1350 ± 2610	16 - 11100

[a]Preparation of soluble (105,000 g supernatant) and Lubrol-solubilized particulate (105,000 g pellet) fractions from human normal breast, and benign and malignant breast tumor, tissue samples, and spectrophotometric quantification of DT-diaphorase and panGST levels were as described by Sreerama and Sladek [1997]. 1-Chloro-2,4-dinitrobenzene and glutathione, 1 mM each, were the substrate and cofactor respectively, when panGST activity was quantified. Substrate, cofactor and inhibitor were 2,6-dichlorophenol-indophenol (40 μM), NADH (160 μM) and dicumarol (10 μM), respectively, when DT-diaphorase activity was quantified. Indirect quantification of ALDH3A1, ALDH1A1, and GSTs α, μ and π levels in soluble (105,000 g supernatant) fractions, and CYP1A1 in Lubrol-solubilized particulate (105,000 g pellet) fractions, was by ELISAs as described by Sreerama and Sladek [1997]. Spectrophotometric quantification of GSH levels in whole tissue homogenates was also as described by Sreerama and Sladek [1997]. The values are means of duplicate determinations made on single normal breast, and/or single benign or malignant breast tumor, tissue samples taken from each of 148 patients. The values are expressed as mIU/g tissue in each case except for CYP1A1 and GSH, in which case they are pg/g, and nmol/g, tissue, respectively.

[b]GSTα values were zero in 5 of 29 (17%) normal breast tissue samples, 2 of 6 (33%) benign breast tumor samples, 20 of 119 (17%) primary breast tumor samples, and 9 of 37 (24%) metastatic breast tumor samples. Zero values were included in the calculation of mean values.

[c]GSTμ values were zero in 10 of 29 (34%) normal breast tissue samples, 3 of 6 (50%) benign breast tumor samples, 51 of 119 (43%) primary breast tumor samples, and 16 of 37 (43%) metastatic breast tumor samples. Zero values were included in the calculation of mean values.

[d]CYP1A1 values were zero in 21 of 24 (88%) normal breast tissue samples, 3 of 6 (50%) benign breast tumor samples, 71 of 109 (65%) primary breast tumor samples, and 16 of 34 (47%) metastatic breast tumor samples. Zero values were included in the calculation of mean values.

As in the cases of ALDH3A1 and ALDH1A1, 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.

We also quantified pan-glutathione S-transferase (pan-GST), glutathione S-transferases α, μ and π (GSTs α, μ and π), DT-diaphorase (DT-D), and cytochrome P4501A1, levels in the normal and malignant (primary and 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]. While not known to be molecular determinants of cellular sensitivity to the oxazaphosphorines, these enzymes are demonstrated molecular determinants of cellular sensitivity to a host of other anticancer agents [Rekha and Sladek, 1997a]. Our findings are summarized in Table 2. The wide range of activity that was observed in the case of each enzyme may be of pharmacological significance clinically.

Whereas ALDH3A1 and ALDH1A1 levels in normal breast tissue samples did predict for the corresponding ALDH3A1 and ALDH1A1 levels in paired, primary, or metastatic, breast tumor tissue samples, Figure 5 and Table 3, DT-D, GST and CYP1A1 levels in normal breast tissue samples did not predict for the corresponding DT-D, GST and CYP1A1 levels in paired, primary, or metastatic, breast tumor tissue samples, Table 3. However, cellular levels in primary breast malignancies of each of the five enzymes did predict their cellular levels in paired metastatic breast malignancies, Table 3.

Table 3. Enzyme and GSH levels in paired human normal breast and primary malignant breast, paired normal breast and metastatic malignant breast, and paired primary malignant breast and metastatic malignant breast, tissue samples: statistical analysis[a]

Enzyme	Normal and Malignant Breast Tissue Pairs								
	Normal *vs* Primary			Normal *vs* Metastatic			Primary *vs* Metastatic		
	n	r^2	*p*	n	r^2	*p*	n	r^2	*p*
ALDH3A1	21	0.66	0.001	7	0.49	0.078	17	0.84	<0.0001
ALDH1A1	21	0.30	0.011	7	0.78	<0.0001	17	0.78	<0.0001
DT-D	21	0.01	0.614	7	0.02	0.790	17	0.40	0.006
pan-GST	21	0.004	0.799	7	0.27	0.233	17	0.55	0.001
GSTα	21	0.04	0.399	7	0.21	0.301	17	0.52	0.001
GSTμ	21	0.02	0.537	7	0.74	0.013	17	0.46	0.003
GSTπ	21	0.01	0.650	7	0.18	0.339	17	0.48	0.002
CYP1A1	17	0.01	0.779	4	-	-	16	0.19	0.096
GSH	21	0.13	0.124	3	0.96	0.092	16	0.35	0.015

[a]Primary data is amongst that presented in Figures 3, 4, and 12, and in Table 2.

Task # 1b: Ascertain whether simultaneous elevation of ALDH3A1, glutathione S-transferase and DT-diaphorase, or of these three enzymes and cytochrome P4501A1, 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 ALDH3A1, DT-D, GSTs, UDP-glucuronosyl transferase (UDP-GT) and, in some cases, cytochrome P4501A1 (CYP1A1) 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 ALDH3A1, 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 20, *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 20, *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, the ALDH3A1, DT-D, pan-GST, GST α, μ and π, and CYP1A1 levels determined to be present in the normal and malignant (primary and metastatic) breast tissue samples procured from the Cooperative Human Tissue Network, Midwestern Division, Table 2, were 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 ALDH3A1, DT-D, pan-GST and CYP1A1 (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 encoding these enzymes [Figure 20, *vide infra*]), was observed in only five samples, viz., two primary, and three metastatic, breast tumors, Table 4, an observed frequency of 0.039 (5/128). As judged by the same criteria, coordinated induction of ALDH3A1, DT-D, and pan-GST, but not of CYP1A1 (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 20, *vide infra*]), was observed in only eight additional samples, viz., five primary, and three metastatic, breast tumors, Table 4, an observed frequency of 0.088 (13/148). In one case, sample 7, a normal breast sample obtained from the same patient was available. ALDH3A1 and DT-D levels in this sample were each more than two standard deviations, and pan-GST and GST π levels were nearly (~0.79) one standard deviation, above corresponding normal breast tissue mean values; CYP1A1 was not detected in this sample (data not shown).

Table 4. Malignant (primary and metastatic) breast tissue samples exhibiting markedly elevated levels of ALDH3A1, DT-D, pan-GST and GST π (each > 1 SD above their respective normal breast tissue mean values)[a]

Sample	Malignancy	ALDH3A1	DT-D	pan-GST	GST π	CYP1A1
1		28	5520	2630	2100	0
2		30	1090	2320	1900	0
3		35	4310	7280	6200	177[b]
4	Primary	39	6250	4870	1900	5
5		45	1410	3000	2900	206[b]
6[c]		171	1890	4450	3600	5
7[c, d]		231	2950	5180	4150	0
8		28	1730	5650	3850	ND[e]
9		53	1510	6430	3800	0
10	Metastatic	64	1600	4630	3900	485[b]
11[c]		97	2540	7600	6500	0
12[c]		100	2650	8220	5400	25[b]
13		118	1560	5240	3640	15[b]

[a]Included in this table are the 13 malignant samples, out of 191, in which ALDH3A1, DT-D, pan-GST and GST π levels were each > 1 SD above their respective normal breast tissue mean values. ALDH3A1, GST π and CYP1A1 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 13 patients. Units are mIU/g tissue except in the case of CYP1A1 where they are pg/g. There were no normal breast tissue samples (n = 29) 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.

[c]Samples in which ALDH3A1, DT-D, pan-GST and GST π levels were each > 2 SD above their respective normal breast tissue mean values.

[d]Samples in which ALDH3A1, DT-D, pan-GST and GST π levels were each > 1 SD above their respective primary breast tumor tissue mean values.

[e]Not determined

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 0.012 (ALDH3A1, DT-D, pan-GST and CYP1A1 levels were > 1 SD above their respective normal breast tissue mean values in 51, 34, 48 and 40 of 128 samples, respectively) and 0.044 (ALDH3A1, DT-D and pan-GST levels were > 1 SD above their respective normal breast tissue mean values in 56, 44 and 59 of 148 samples,

respectively). As judged by chi-squared analysis, observed frequencies did not differ significantly ($P = 0.089$ and 0.087, 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., 1995a,b; Rekha and Sladek, 1997a].

Task # 1c: Ascertain whether breast tumor ALDH3A1 levels parallel salivary ALDH3A1 levels.

Given that 1) ALDH3A1 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 ALDH3A1 levels are elevated following the ingestion of broccoli or other dietary materials, e.g., coffee, known to contain agents that induce ALDH3A1 [Sreerama et al., 1995b], and 4) cellular levels of ALDH3A1 vary widely in normal and malignant breast tissues [*vide supra*; Sreerama and Sladek, 1997], it follows that salivary levels of ALDH3A1 may reflect normal and tumor tissue, e.g., breast, levels of this enzyme, and, thus, that salivary levels of ALDH3A1 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 was initiated. Unfortunately, breast (normal, benign and primary malignant) tissue ALDH3A1 levels do not parallel salivary ALDH3A1 levels, Figure 14.

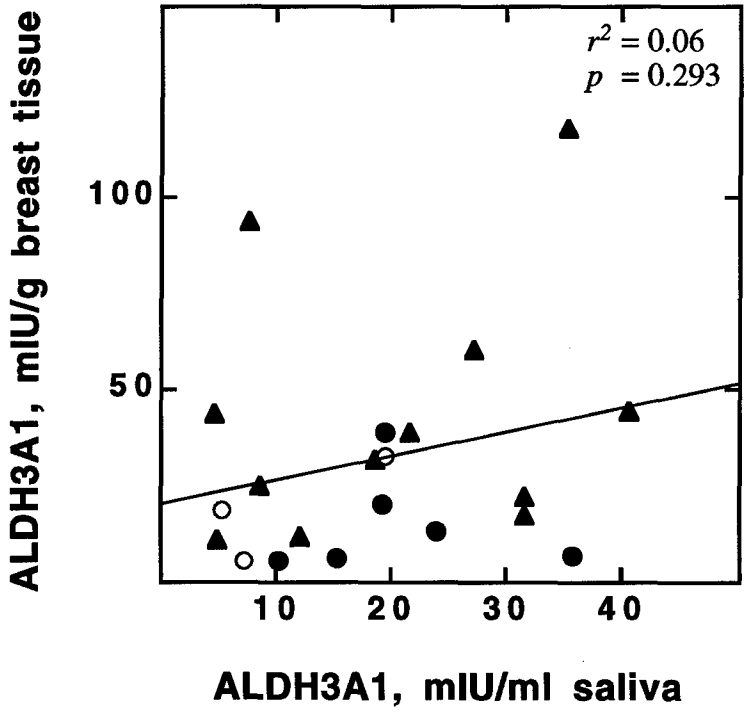


Figure 14. ALDH3A1 levels in presurgery saliva samples (n = 21), and in subsequently surgically removed normal (n = 3; ○), benign (n = 6; ●) and malignant primary (n = 12; ▲)] breast tissue samples. Saliva and surgically removed breast tissue samples (normal, benign and malignant primary) were obtained through the Tissue Procurement Facility at University of Minnesota Cancer Center, Minneapolis. Processing of saliva and breast tissue samples, and quantification of ALDH3A1 activity (NADP [4 mM] -linked oxidation of benzaldehyde [4 mM]) by ELISA, were as described previously [Sreerama et al., 1995b; 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.

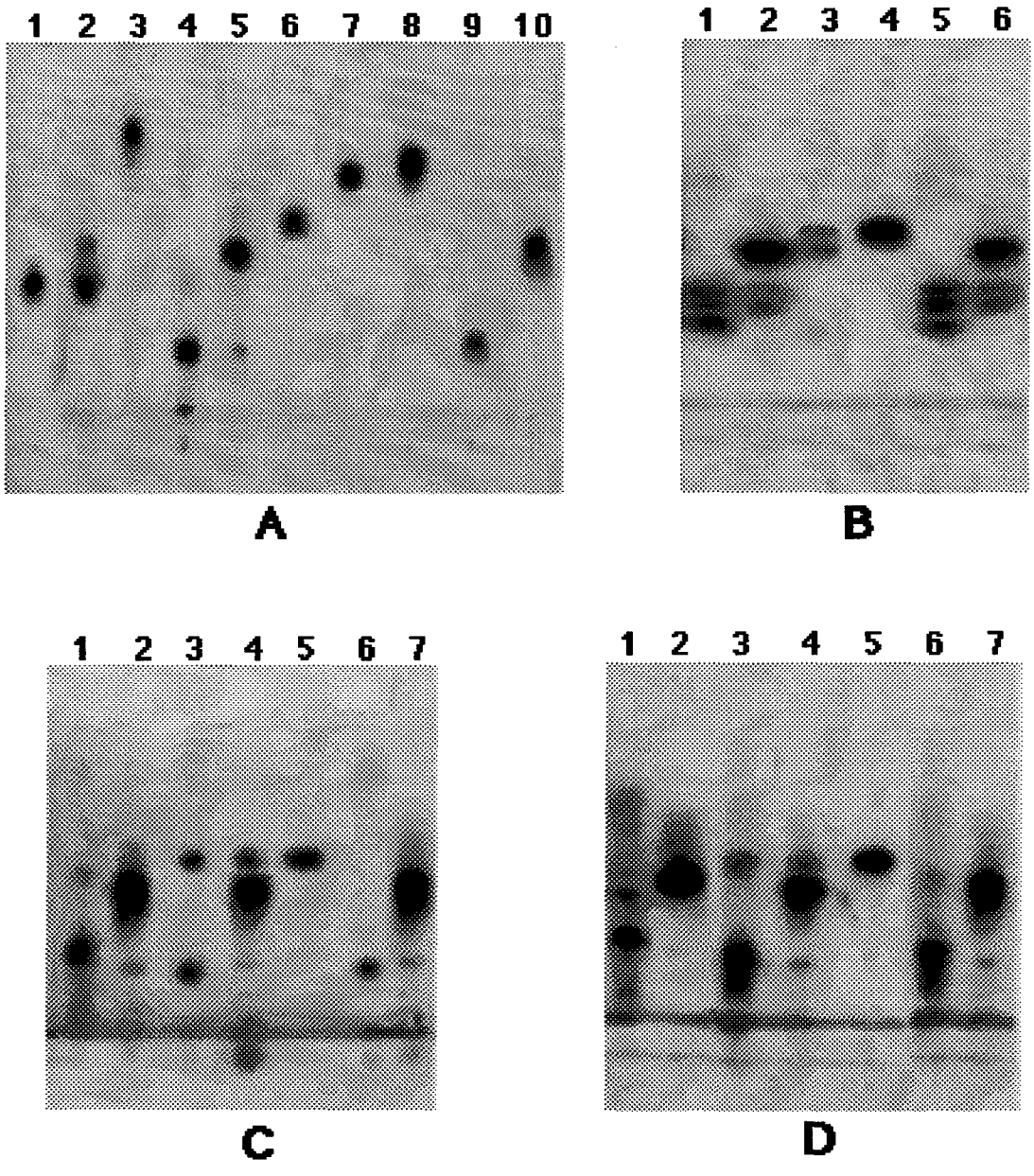


Figure 15. Oxazaphosphorine metabolism catalyzed by purified tALDH3A1 and rALDH1A1, and intact MCF-7/0/CAT cells: thin-layer chromatographic analysis. Purification of tALDH3A1, and preparation of cytosolic fractions, from MCF-7/0/CAT cells was as before [Sreerama and Sladek, 1993a, 1994]. Generation and purification of human recombinant ALDH1A1 (rALDH1A1) was as described by Devaraj et al. [1997]. Incubation of purified tALDH3A1 or rALDH1A1 with 4-hydroxycyclophosphamide/aldophosphamide was for 15 min, and exposure of 3 x 10^7 MCF-7/0/CAT cells to mafosfamide was for 30 min, as described previously [Dockham et al, 1992; Sreerama and Sladek, 1993a; Sreerama et al., 1995a]. A thin-layer chromatography/NBP/computer-assisted scanning/image analysis assay was used to separate and then quantify 4-hydroperoxycyclophosphamide, mafosfamide or 4-hydroxycyclophosphamide/aldophosphamide and their respective metabolites. Semicarbazide was added, when called for, to the incubation mixture at the end of the initial 15 min incubation period with purified enzymes, to a suspension of sonicated (4°C, 10 sec) MCF-7/0/CAT cells that had been exposed to 1 mM mafosfamide for 30 min and then separated from the culture medium by centrifugation (9,000 g, 4°C, 2 min) through a silicone oil/mineral oil (4:1) layer, and to the cell-free aqueous layer (incubation media) remaining on top of the oil layer after centrifugation, and incubation was continued for 5 min to trap 4-hydroxycyclophosphamide/aldophosphamide(hydrate) as the semicarbazone. Sonicated MCF-7/0/CAT cells were then centrifuged at 105,000 g and 4°C for 60 min to obtain 105,000 g soluble fractions. Aliquots of this and the other preparations were

then separated on HPTLC high performance LHP-K silica gel plates (Whatman Inc., Clifton, NJ) that had been pre-eluted twice with methanol and dried at 140°C each time for 30 min. TLC was carried out in glass TLC tanks. The mobile phase was butanol:water (20:3). Following separation, the plates were dried with cold air, sprayed with a 5% solution of 4-(p-nitrobenzyl)pyridine (NBP) in acetone/0.2M sodium acetate buffer pH 4.6 (8:2 v/v), baked in a hot air oven at 140°C for 10 min, cooled by blowing cold air over them, and dipped in a 3% methanolic KOH solution to visualize (brilliant violet-blue spots) the compounds of interest [Sladek, 1973]. Because the color is stable for only a few minutes, the developed, still moist, plate was immediately transferred into a polyethylene bag which was then sealed, and scanned 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). Image analysis and quantification of 4-hydroperoxycyclophosphamide, mafosfamide and their metabolites on the scanned image was performed with the aid of Image 1.6 software (NIH, Bethesda, MD) in a Power Macintosh with reference to authentic standards. Plate **A**: Authentic cyclophosphamide analogues and metabolites. Lane 1, mafosfamide; Lane 2, mafosfamide incubated with semicarbazide; Lane 3, 4-hydroperoxycyclophosphamide; Lane 4, 4-hydroxycyclophosphamide/aldophosphamide; Lane 5, 4-hydroxycyclophosphamide/aldophosphamide incubated with semicarbazide; Lane 6, carboxyphosphamide; Lane 7, 4-ketocyclophosphamide; Lane 8, alcophosphamide; Lane 9; phosphoramide mustard; and Lane 10, nornitrogen mustard. Plate **B**: MCF-7/0/CAT cells incubated with mafosfamide for 30 min. Lane 1, cell-free aqueous layer; Lane 2 cell-free aqueous layer incubated with semicarbazide; Lane 3, MCF-7/0/CAT cell-lysate incubated with semicarbazide; Lane 4, authentic carboxyphosphamide; Lane 5, complete incubation medium except for MCF-7/0/CAT cells (blank reaction); and lane 6, complete incubation medium except for MCF-7/0/CAT cells incubated with semicarbazide. Plate **C**: rALDH1A1 incubated with 4-hydroxycyclophosphamide/aldophosphamide for 15 min. Lane 1, 4-hydroxycyclophosphamide/aldophosphamide; Lane 2, 4-hydroxycyclophosphamide/aldophosphamide incubated with semicarbazide; Lane 3, rALDH1A1 incubated with 4-hydroxycyclophosphamide/aldophosphamide; Lane 4, rALDH1A1 incubated with 4-hydroxycyclophosphamide/aldophosphamide and then with semicarbazide; Lane 5, authentic carboxyphosphamide; Lane 6, complete reaction mixture except for rALDH1A1 (blank reaction); and Lane 7, complete reaction mixture except for rALDH1A1 incubated with semicarbazide. Plate **D**: tALDH3A1 incubated with 4-hydroxycyclophosphamide/aldophosphamide for 15 min. Lane 1, 4-hydroxycyclophosphamide/aldophosphamide; Lane 2, 4-hydroxycyclophosphamide/aldophosphamide incubated with semicarbazide; Lane 3, tALDH3A1 incubated with 4-hydroxycyclophosphamide/aldophosphamide; Lane 4, tALDH3A1 incubated with 4-hydroxycyclophosphamide/aldophosphamide and then with semicarbazide; Lane 5, authentic carboxyphosphamide; Lane 6, complete reaction mixture except for tALDH3A1 (blank reaction); and Lane 7, complete reaction mixture except for tALDH3A1 incubated with semicarbazide.

We have previously 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 μmol/min/10^9 cells, respectively [Sreerama and Sladek, 1993a, 1994]. Subsequently, we found MCF-7/0 cells to exhibit oxazaphosphorine-specific resistance when purified nALDH3A1 or tALDH3A1 was electroporated into them [Sreerama and Sladek, 1995]. In the present investigation, we ascertained that, as judged by the formation of carboxyphosphamide itself, purified tALDH3A1, as well as cytosolic fractions prepared from MCF-7/0/CAT cells (MCF-7/0 cells grown in the presence of a phenolic antioxidant, viz., catechol (CAT), for five days to transiently induce ALDH3A1 and the associated oxazaphosphorine-specific resistance [Sreerama et al., 1995a]), catalyze the oxidation of aldophosphamide to carboxyphosphamide, Figure 15. 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, ALDH3A1-mediated oxazaphosphorine-specific resistance results solely from ALDH3A1-catalyzed oxidation of aldophosphamide to carboxyphosphamide, the latter being without cytotoxic activity.

Task # 3: Synthesize and identify agents that inhibit the ALDH3A1-catalyzed oxidative and/or hydrolytic detoxification of cyclophosphamide (aldophosphamide).

Tested was whether ethylphenyl(2-formylethyl)phosphinate (EPP), any of four cinnamic acid analogues, any of eight chlorpropamide analogues, and/or gossypol would inhibit ALDH3A1-catalyzed oxidation of benzaldehyde.

EPP is structurally similar to aldophosphamide. It was synthesized for us by Dr. C.-H. Kwon, St. John's University, Jamaica, NY.

Others [Poole et al., 1993] have found that a wide variety of cinnamic acid derivatives inhibited "rat low Km mitochondrial aldehyde dehydrogenase" (ALDH2A1) but not lactate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase or a "rat high Km mitochondrial aldehyde dehydrogenase." Inhibition was competitive with respect to the cofactor, viz., NAD. The sensitivity of class 3 aldehyde dehydrogenases to these agents was not tested. We were able to procure four of these agents, viz., CAD 567C91, CAD 690C88, CAD 707C91 and CAD 764C89, from Dr. A. T. Hudson of The Wellcome Foundation, Beckenham, Kent, England and did so.

Eight chlorpropamide analogues, of which six are N^1-hydroxy-substituted esters (putatively, nitroxyl proinhibitors; NPIs), viz., NPI-1 - NPI-6, and the other two are N^1-methoxy and N^1-alkyl derivatives (putatively, *n*-propylisocyanate proinhibitors; APIs), viz., API-1 and API-2, Table 5, were synthesized in Dr. H. T. Nagasawa's laboratory [Lee et al., 1992a,b] and evaluated with regard to their ability to inhibit aldehyde dehydrogenase-catalyzed oxidation. The NPIs were designed with the intent that they generate nitroxyl (HNO) as a consequence of ester bond hydrolysis. ALDH3A1, like other human aldehyde dehydrogenases, is a bifunctional enzyme, i.e., it catalyzes ester bond hydrolysis as well as the oxidation of aldehydes to acids. Hydrolysis of the NPIs catalyzed by yeast aldehyde dehydrogenase (yALDH) thus giving rise to HNO, and inhibition of yALDH-catalyzed oxidation of acetaldehyde by the newly generated HNO, have been demonstrated [Lee et al., 1992a]. The expectation was that ALDH3A1 would also catalyze the hydrolysis of NPIs thereby generating HNO. Preliminary experiments established that HNO inhibits ALDH3A1-catalyzed oxidation of benzaldehyde. In contrast, the APIs were designed with the intent that they generate *n*-propylisocyanate without benefit of enzymatic involvement; this metabolite, too, inhibits yeast, as well as rodent class 2, aldehyde dehydrogenase-catalyzed oxidation of acetaldehyde [Lee et al., 1992a,b].

Messiha [1991a,b] has reported that gossypol, a polyphenolic aldehyde, Table 5, inhibits "hepatic aldehyde dehydrogenase activity" when given to mice. Again, the sensitivity of class 3 aldehyde dehydrogenases to this agent was not tested, thus, we did so.

The ALDH3A1 present in human tumor cells/tissues (cultured breast carcinoma MCF-7, Zr-75 and T-47D cells, colon carcinoma C cells, and salivary gland Warthin tumors and mucoepidermoid carcinomas), although otherwise seemingly identical to the ALDH3A1 present in human normal tissues/fluids (cultured normal breast MCF-10A cells, stomach mucosa, saliva, and parotid gland), differs from the latter in that it exhibits a much greater ability to catalyze the oxidative detoxification of the oxazaphosphorines, and may be tumor-specific [Sladek et al., 1995; Sreerama and Sladek, 1995; Sreerama and Sladek, 1996; Sreerama and Sladek, unpublished observations]. Hence, the inhibitory potency of the compounds enumerated above was tested against normal stomach mucosa ALDH3A1 (nALDH3A1) as well as against breast adenocarcinoma MCF-7 cell ALDH3A1 (tALDH3A1).

Human ALDH1A1, known to also catalyze the irreversible oxidation (detoxification) of aldophosphamide [Dockham et al., 1992], and human ALDH2A1 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.

Purified aldehyde dehydrogenases and spectrophotometric assays were used in these investigations.

E. coli [BL21(DE3)pLysS] transfected with pET-19b vector to which human ALDH1A1 cDNA was ligated, was provided by Dr. Jan Moreb, University of Florida, Gainsville, FL. Recombinant human ALDH1A1 (rALDH1A1) was overexpressed in E. coli by growing them in the presence of isopropylthio-β-D-galactoside (IPTG) and the overexpressed rALDH1A1 was purified by Ni-Sepharose CL 6B affinity column chromatography according to the manufacturers protocol [Novagen, Inc., Madison, WI]. A vector, viz., pT7-7, to which human ALDH2A1 cDNA was ligated, was provided by Dr. Henry Weiner, Purdue University, Lafayette, IN. The pT7-7 vector was transfected into E. coli [BL21(DE3)] and recombinant human ALDH2A1 (rALDH2A1) was overexpressed by growing them in the presence of IPTG. The overexpressed rALDH2A1 was purified by ion-exchange chromatography on DEAE-Sephacel followed by affinity chromatography on 5'-AMP-Sepharose CL 6B [Dockham et al., 1992]. nALDH3A1 and tALDH3A1 were purified from human stomach mucosa and MCF-7/CAT cells (MCF-7/0 cells cultured in the presence of 30 μM catechol for 5 days; contain >100-fold more tALDH3A1 activity as compared to that contained by MCF-7/0 cells cultured in the presence of vehicle alone), respectively, as described previously [Sreerama and Sladek, 1993a; Sreerama et al., 1995a].

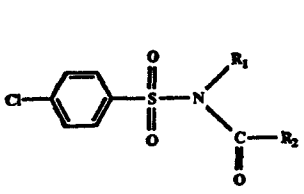
EPP did not inhibit any of the human aldehyde dehydrogenases tested; however, it was found to be a relatively good substrate (Km = 32 μM) for ALDH1A1.

None of the cinnamic acid derivatives proved to be a very potent inhibitor of the human ALDH3A1s, nor were either of these enzymes differentially more sensitive to the inhibitory action of these agents (data presented in the year-2 report).

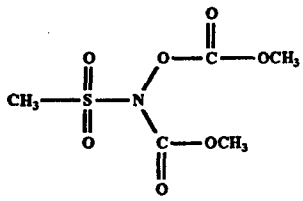
As judged by IC_{50} values, NPIs -1 to -6 and APIs -1 and -2 exhibited differential inhibitory potencies towards the human aldehyde dehydrogenases, Table 5. Inhibitory constants (Ki) values are given in Table 6. These experiments revealed that, of the eight chlorpropamide analogues investigated, NPI-2 and API-2 showed the most promise as selective *in vivo* inhibitors of ALDH3A1. Although NPI-2 was only moderately potent with regard to inhibiting tALDH3A1 (IC_{50} = 16 μM), tALDH3A1 was differentially much more sensitive to inhibition effected by NPI-2 and the inhibition appeared to be irreversible [Rekha et al., 1998]. Inhibition of tALDH3A1 by API-2 was effected at a very low concentration (IC_{50} = 0.75 μM), but appeared to be easily reversible [Rekha et al., 1998] and was not entirely specific for tALDH3A1 since rALDH2A1 was approximately ten times more sensitive to this agent. Thus, at first glance, API-2 would not appear to have any future as a clinically useful inhibitor of tALDH3A1 since it would inhibit ALDH2A1 to an even greater extent. In fact, this is not the case because ALDH2A1 is an enzyme that humans can apparently do without since 30-50% of Orientals lack a functional ALDH2A1 and do not suffer any recognized ill-effects as a consequence thereof except for those following the ingestion of alcohol [Goedde and Agarwal, 1990].

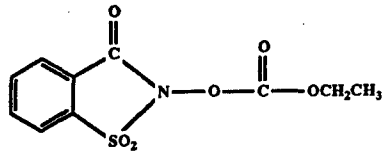
The differential inhibitory potency of NPIs and APIs towards nALDH3A1 and tALDH3A1 further supports the notion that tALDH3A1 is different from the nALDH3A1.

Table 5. Inhibition by chlorpropamide analogues and gossypol of human aldehyde dehydrogenase-catalyzed oxidation: IC_{50} values*

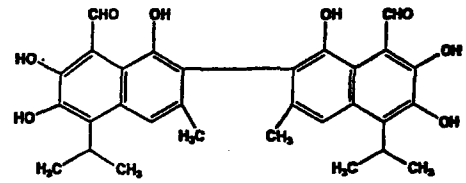


NPI-5





NPI-6



Gossypol

Code	Inhibitor R_1	Inhibitor R_2	IC_{50}, μM rALDH1A1	rALDH2A1	nALDH3A1	tALDH3A1
Chlorpropamide	—H	—$NHCH_2CH_2CH_3$	>2000	>2000	>2000	>1000
API-1	—CH_2CH_3	—$NHCH_2CH_2CH_3$	150	23	223	93
API-2	—OCH_3	—$NHCH_2CH_2CH_3$	7.5	0.08	5	0.75
NPI-1	—O—C(=O)—C$_6$H$_5$	—O—C—$(CH_3)_3$	49	47	121	45
NPI-2	—O—C(=O)—CH_3	—O—C—$(CH_3)_3$	>300	>300	202	16
NPI-3	—O—C(=O)—CH_3	—CH_3	217	900	775	305
NPI-4	—O—C(=O)—OCH_2CH_3	—OCH_2CH_3	1.0	4.0	2.0	1.9
NPI-5			0.7	ND	3.2	2.3
NPI-6			5.0	ND	>400	>400
Gossypol			75	45	7.5	6.6

*Enzymes were incubated with vehicle or 4 - 8 different concentrations of one of the putative inhibitors for 5 (chlorpropamide, API-1, API-2, NPI-1, NPI-2, NPI-3 and gossypol) or 20 (NPI-4, NPI-5 and NPI-6) min at 37°C and pH 8.1, substrate was added, and initial catalytic rates were quantified spectrophotometrically. Substrate was acetaldehyde (4 mM and 2 mM, respectively) for rALDH1A1 and rALDH2A1, and benzaldehyde (4 mM) for ALDH3A1s. Cofactor was NAD (4 mM for rALDH1A1 and rALDH2A1; 1 mM for ALDH3A1s). Uninhibited catalytic rates (mean; n = 2) were 0.59, 2.1, 31, 32 IU/mg protein for rALDH1A1, rALDH2A1, nALDH3A1 and tALDH3A1, respectively. Computer-assisted unweighted nonlinear regression analysis effected by the STATview statistical program 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 (IC_{50}). Data for chlorpropamide, API-1, API-2, NPI-1, NPI-2, NPI-3 and gossypol have been published [Devarai et al., 1997; Rekha and Sladek, 1997b; Rekha et al., 1998].

ND: Not Determined.

Table 6. Inhibition by chlorpropamide analogues and gossypol of human aldehyde dehydrogenase-catalyzed oxidation: Ki values*

Enzyme	Substrate and Cofactor		Km† (μM)	Vmax† (IU/mg)	Ki (μM)					
	Variable	Fixed (mM)			NPI-2		API-2		Gossypol‖	
rALDH1A1	Acetaldehyde	NAD (4)	490	0.66	ND‡		6.0	(N)§	69	
	NAD	Acetaldehyde (4)	46	0.63	ND		2.1	(N)	5.3	
rALDH2A1	Acetaldehyde	NAD (4)	4.6**	2.9	ND		0.03	(C)	37	
	NAD	Acetaldehyde (2)	328	3.2	ND		0.08	(N)	7.0	
nALDH3A1	Benzaldehyde	NAD (1)	416	29	254	(N)	1.7	(C)	10	
	NAD	Benzaldehyde (4)	42	34	120	(N)	2.1	(N)	0.32	
	Benzaldehyde	NADP (4)	427	61	281	(N)	0.02	(C)	5.8	
	NADP	Benzaldehyde (4)	735	69	16	(C)	0.17	(C)	0.56	
tALDH3A1	Benzaldehyde	NAD (1)	354	32	11	(N)	0.14	(C)	4.3	
	NAD	Benzaldehyde (4)	38	31	12	(N)	0.42	(N)	0.10	
	Benzaldehyde	NADP (4)	393	62	41	(N)	0.01	(C)	4.4	
	NADP	Benzaldehyde (4)	775	67	2.3	(C)	0.04	(C)	0.19	
yALDH	Acetaldehyde	NAD (4)	32	9.2	10	(N)	0.15	(N)	ND	
	NAD	Acetaldehyde (0.8)	877	8.5	3.6	(C)	0.02	(C)	ND	
GAPDH	GAP	NAD (1)	159	64	ND		31	(N)	ND	

* 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 elsewhere [Devaraj et al., 1997; Rekha and Sladek, 1997b; Rekha et al., 1998].

† n = 1 in the case of GAPDH, n = 2 in the case of rALDH1A1, rALDH2A1 and yALDH, and n = 3 in the case of nALDH3A1 and tALDH3A1.

‡ ND: Not Determined.

§ N: Noncompetitive; C: Competitive.

‖ Inhibition was always noncompetitive with respect to the aldehyde and always competitive with respect to the cofactor.

** Unlikely to be accurate because it is difficult to ascertain Km values that are less than about 10 μM from the very flat Lineweaver-Burk plots that we generated. Thus, the Km value was determined to be <0.1 μM when a more appropriate experimental design and method of analysis, viz., integrated Michaelis analysis of a single enzyme-progress curve, was used [Dockham et al., 1992].

The effect of NPI-2 and API-2 on aldehyde dehydrogenase-catalyzed hydrolysis was also determined [Rekha et al., 1998]. In the case of NPI-2, 50% inhibition not achieved at the highest concentration, 200 uM. tested. In

the case of API-2, 50% inhibition was not achieved at the highest concentration, 1,000 uM, tested, with one exception - the IC_{50} was 16 μM in the case of rALDH2A1.

As judged by IC_{50} values, gossypol also proved to be a good inhibitor of the human ALDH3A1s, Table 5. Moreover, the ALDH3A1s were differentially more sensitive to the inhibitory action of this agent. The inhibitory action of gossypol was competitive with respect to the cofactor, viz., NAD or NADP, Table 6, and was virtually instantaneous and irreversible [data not presented].

The effect of gossypol on aldehyde dehydrogenase-catalyzed hydrolysis was also determined. In no case was 50% inhibition achieved at the highest concentration, 200 uM, of gossypol tested.

Thus, gossypol exhibited all of the properties that we were looking for, viz., a potent and relatively specific, irreversible inhibitor of the ALDH3A1s, and it was, therefore, submitted to further testing (see task # 4). For much the same reasons, *vide supra*, also submitted to further testing were two analogues of chlorpropamide, viz., NPI-2 and API-2 (see task # 4).

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.

The ability of two chlorpropamide analogues, viz., NPI-2 and API-2, and gossypol to negate the influence of relatively high cellular levels of ALDH3A1 on the cellular sensitivity of tumor cells to an oxazaphosphorine, viz., mafosfamide, was determined for the reasons given under task # 3.

Addition of NPI-2, API-2 or gossypol to the drug-exposure medium prior to exposure to mafosfamide markedly increased the sensitivity of tumor cells that constitutively express large amounts of ALDH3A1, viz., cultured human colon C carcinoma cells, or that are induced to do so, viz., cultured human breast adenocarcinoma MCF-7/0/CAT cells, to the oxazaphosphorine, Table 7. NPI-2, API-2 and gossypol, at the concentrations used in these experiments, were minimally (<10% cell-kill) toxic to the cultured cells, and, in fact, these concentrations were well below those that effected a 90% cell-kill, Table 8. As expected, identical treatment of tumor cells that express very small amounts of ALDH3A1, viz., MCF-7/0, only very minimally increased their sensitivity to mafosfamide. Also as expected because ALDH3A1 does not catalyze the detoxification of phosphoramide mustard, the ultimate cytotoxic metabolite of mafosfamide [Sladek, 1994], addition of NPI-2, API-2 or gossypol 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 7.

The findings reported herein establish the therapeutic potential of combining NPI-2, API-2 or gossypol with an oxazaphosphorine in the treatment of certain cancers, viz., those that express pharmacologically significant amounts of ALDH3A1 and are otherwise sensitive to the oxazaphosphorines. Moreover, given the antitumor activity that gossypol itself exhibits, Table 8 and *vide infra*, it can be envisaged that in the case of some of these cancers, viz., those that are also gossypol-sensitive, gossypol could be of dual therapeutic value when combined with an oxazaphosphorine in the therapeutic protocol. Uncertain is whether these agents will inhibit ALDH3A1 *in vivo* at doses that do not cause untoward effects since this possibility remains essentially untested.

Table 7. Sensitivity of human breast adenocarcinoma MCF-7/0 and MCF-7/0/CAT cells and human colon C carcinoma cells to mafosfamide and phosphoramide mustard in the presence and absence of inhibitors of ALDH3A1[a]

Cells	ALDH3A1 (mIU/10^7 cells)	Inhibitor	LC_{90}, μM	
			Mafosfamide	Phosphoramide Mustard
MCF-7/0	2	None	65	800
		NPI-2	60	*b*
		API-2	60	*b*
		Gossypol	65	*b*
MCF-7/0/CAT	665	None	>2000	1350
		NPI-2	175	1300
		API-2	200	1400
		Gossypol	200	1100
Colon C	412	None	335	*b*
		Gossypol	80	*b*

[a]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. The MCF-7/0 and MCF-7/0/CAT, as well as human colon C carcinoma, cells (1 x 10^5 cells/ml) were then incubated with NPI-2 (100 μM), API-2 (50 μM), gossypol (75 μM) or vehicle for 5 min at 37°C after which time various concentrations of mafosfamide, phosphoramide mustard or vehicle were added and incubation was continued as before for 30 min at 37°C. The colony-forming assay described previously [Sreerama and Sladek, 1993a] was used to determine surviving fractions. LC_{90} values (concentrations of drug required to effect 90% cell-kills) were obtained from plots of log surviving fractions versus concentrations of drug. Values are means of LC_{90}s obtained in two experiments. Cellular levels of ALDH3A1 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 previously [Sreerama and Sladek, 1993a].

[b]Not determined.

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, Table 5. In contrast, the class 3 aldehyde dehydrogenases, especially tALDH3A1, are, Table 5. Thus, the possibility that tolerated doses of NPI-2 will inhibit tALDH3A1 *in vivo* remains viable. However, as judged by LC_{90} values, NPI-2-mediated restoration of MCF-7/0/CAT sensitivity to mafosfamide was minimal at less than 100 μM NPI-2; specifically,

LC_{90} values were 175, 1,600, >2,000 and >2,000 μM when 100, 75, 50 and 0 μM NPI-2, respectively, were evaluated (Table 7 and data not presented). A NPI-2 concentration of 100 μM may be difficult to achieve *in vivo* without also inducing various untoward effects.

Table 8. Sensitivity of human breast adenocarcinoma MCF-7/0 and MCF-7/0/CAT, human normal breast epithelial MCF-10A, and human colon C carcinoma, cells to NPI-2, API-2 and gossypol[a]

Cell Line	LC_{90}, μM		
	NPI-2	API-2	Gossypol
MCF-7/0	>300	>300	155
MCF-7/0/CAT	>300	>300	160
MCF-10A	>300	>300	145
Colon C	*b*	*b*	138

[a]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. The MCF-7/0 and MCF-7/0/CAT, as well as human normal breast epithelial MCF-10A and human colon C carcinoma, cells (1×10^5 cells/ml) were then incubated with vehicle or various concentrations of NPI-2, API-2 or gossypol for 35 min at 37°C. The colony-forming assay described previously [Sreerama and Sladek, 1993a] was used to determine surviving fractions. LC_{90} values (concentrations of drug required to effect 90% cell-kills) were obtained from plots of log surviving fractions versus concentrations of drug.

[b]Not determined.

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]. However, the aldehyde dehydrogenase that is thought to catalyze the bulk of acetaldehyde oxidation *in vivo*, viz., ALDH2A1, is about 10 times more sensitive to inhibition by API-2 than is tALDH1A1, Table 5. Encouragingly, as judged by LC_{90} values, API-2-mediated restoration of MCF-7/0/CAT sensitivity to mafosfamide was evident even at 10 μM API-2; specifically, LC_{90} values were 200, 635, 1,110 and >2,000 μM when 50, 25, 10 and 0 μM API-2, respectively, were evaluated (Table 7 and data not presented).

Gossypol has been shown to effect male contraception and *in vitro* tumor cell-kill [Tuszynski and Cossu, 1984; Joseph et al., 1986; Band et al., 1989 Wu, 1989; Wu et al., 1989; Benz et al., 1990; Ford et al., 1991; Hu et al., 1993; Coyle et al., 1994; Gilbert et al., 1995] More importantly, it was demonstrated in several animal models that tumor cell-kill could be achieved *in vivo* with doses of gossypol that were not injurious to the host

animal [Tso, 1984; Wu et al., 1989; Chang et al., 1993; Naik et al., 1995]. These observations prompted phase 1, and, subsequently, phase 2, clinical trials of gossypol for the treatment of certain cancers, viz., metastatic carcinomas of the ovary [Wu, 1989], various advanced cancers [Stein et al., 1992], metastatic adrenocortical carcinomas [Flack et al., 1993], recurrent malignant gliomas [Bushunow et al., 1998] and metastatic breast cancers [Seidman, 1996; Seidman et al., 1998]. Thus, much is known about the pharmacokinetic behavior and toxicity of gossypol in humans [Wu, 1989]. Whether doses of gossypol sufficient to inhibit tALDH3A1 *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 tALDH3A1 *in vitro* (IC_{50} = ~7 μM, Table 5) is much less than that needed to inhibit tumor cell proliferation (LC_{90} = ~150 μM) in our *in vitro* models, Table 8. However, as judged by LC_{90} values, gossypol-mediated restoration of MCF-7/0/CAT sensitivity to mafosfamide was minimal at less than 50 μM gossypol; specifically, LC_{90} values were 200, 1,260, >2,000 and >2,000 μM when 75, 50, 25 and 0 μM gossypol, respectively, were evaluated (Table 7 and data not presented). Predictably, given that inhibition of ALDH3A1 by gossypol is essentially instantaneous and irreversible, *vide supra*, preincubation with gossypol for periods greater than 5 minutes did not further sensitize MCF-7/0/CAT cells to mafosfamide (data not presented). A gossypol concentration of 50 μM may be difficult to achieve *in vivo* without also inducing various untoward effects. Plasma C_{max} values were only about 1.8 μM 6-8 hours after the administration of gossypol, 20 mg, *per os* to healthy male subjects [Wu et al., 1986]. The maximum tolerated daily dose of gossypol is about 40 mg [Seidman et al., 1998].

Racemic gossypol was used in all of our investigations. Significant differences in the potency of racemic gossypol and its enantiomers with respect to antitumor properties and inhibition of enzyme catalysis have been reported [Yu, 1987; Band et al., 1989; Benz et al., 1990; Blackstaffe et al., 1997].

Task # 5: Identify the molecular basis for the apparent overexpression of class 3 aldehyde dehydrogenases (ALDH3A1s) in our model systems.

RT-PCR was utilized to quantify cellular levels of ALDH3A1 mRNA in the first of a series of experiments intended to ascertain whether the elevated levels of ALDH3A1 that we observed in MCF-7/OAP, MCF-7/0/MC and MCF-7/0/CAT cells [task # 7 - Table 12; Sreerama and Sladek, 1993a, 1994; Sreerama et al., 1995a; Sladek et al., 1995] were the consequence of transcriptional activation, as we suspected, or of some other mechanism.

ALDH3A1 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 ALDH3A1 mRNA level in MCF-7/0 cells, Figure 16. The latter was in agreement with a previous observation, viz., as judged by Northern blot analysis, ALDH3A1 mRNA levels were elevated in MCF-7/0/CAT cells [task # 7 - Figure 21; Sreerama et al., 1995a].

As judged by ALDH3A1 mRNA half-lives, Figure 17, the relatively elevated ALDH3A1 mRNA levels that we observed in MCF-7/OAP, MCF-7/0/MC and MCF-7/0/CAT cells were not the consequence of ALDH3A1 mRNA stabilization. An additional experiment, Figure 18, revealed that, in each case, the relatively elevated ALDH3A1 mRNA levels were the consequence of an increase in the rate of ALDH3A1 mRNA synthesis.

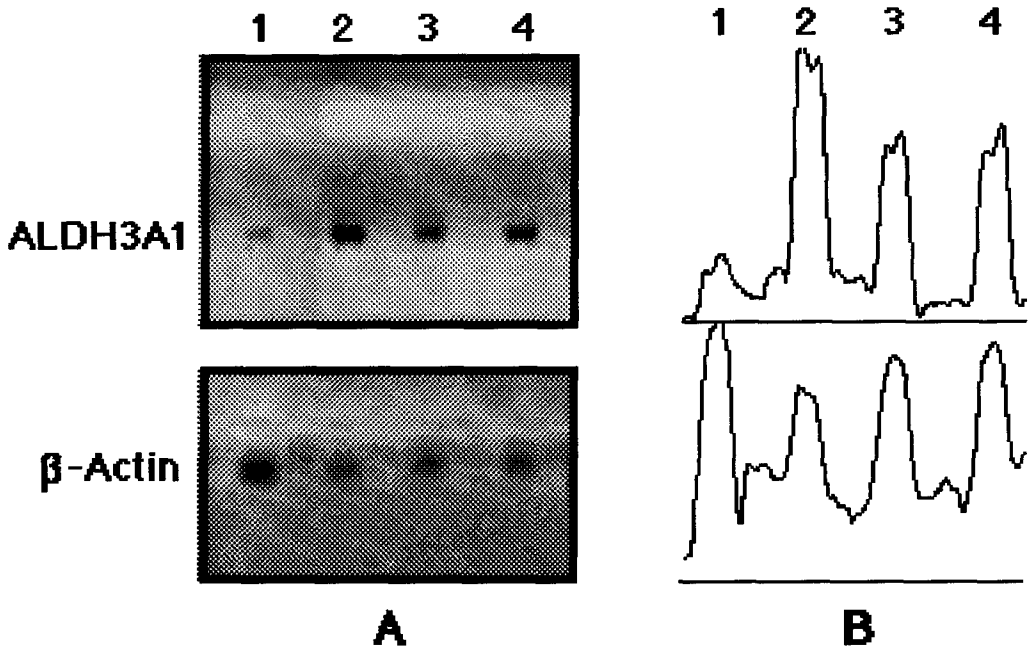


Figure 16. ALDH3A1 mRNA levels in MCF-7/0 (lane 1), MCF-7/0/CAT (lane 2), MCF-7/0/MC (lane 3) and MCF-7/OAP (lane 4) cells as judged by RT-PCR. Isolation of total RNA from MCF-7/0, MCF-7/0/CAT, MCF-7/0/MC and MCF-7/OAP cells was with the aid of a RNA isolation kit (Gentra Systems, Inc., Minneapolis, MN). Two oligonucleotide primers specific to human stomach mucosa ALDH3A1 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 ALDH3A1 and human β-actin cDNA sequences, respectively [Nakajima-Iijima et al., 1985; Hsu et al., 1992]. Thus designed ALDH3A1 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'-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3' (3'-end; corresponds to bases 1876-1905 of the antisense strand). 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 μg of the isolated total cellular RNA with 300 ng of oligo(dT) primer in 40 μl 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 μl of freshly-prepared RT mixture [5 μl of a buffer solution (500 mM Tris-HCl, 50 mM dithiothreitol, 50 mM $MgCl_2$, 500 mM KCl and 0.5 mg/ml BSA), 1 μl of RNase block (ribonuclease inhibitor; 40 IU/μl), 3 μl of deoxyribonucleotide (dNTP) mixture (100 mM each of dATP, dGTP, dCTP and dTTP) and 1 μl of Moloney murine leukemia virus reverse transcriptase (50 IU/μl)], and incubating at 37°C for 60 min. The reaction was stopped by heat inactivation (90°C for 2 min). An aliquot (1-5 μl) of thus synthesized cDNA was mixed with 5 μl of a buffer solution [200 mM Tris-HCl, 100 mM KCl, 20 mM $MgSO_4$, 100 mM $(NH_4)_2SO_4$ and 0.1% (w/v) Triton X-100], 1 μl of dNTP mixture (100 mM each), 1 μl of 5'-end primer (10 μM), 1 μl of 3'-end primer (10 μM), and sufficient sterile water to give a final volume of 49 μl in a 500 μl 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 μl of Vent DNA polymerase (2 IU/μl). 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 *ALDH3A1* DNA copies, thus demonstrating that increased ALDH3A1 mRNA levels in these cells was not due to gene amplification, Figure 19. As expected, given that methylcholanthrene- and catechol-induced elevations of ALDH3A1 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 *ALDH3A1*

DNA copies, Figure 19. Elevated levels of ALDH3A1 mRNA in MCF-7/OAP, MCF-7/0/MC and MCF-7/0/CAT cells were not the consequence of hypomethylation of a relevant *ALDH3A1* DNA regulatory element because neither the ALDH3A1 mRNA level nor the ALDH3A1 catalytic activity was increased when MCF-7/0 cells were cultured in the presence of 1, 5 or 10 μM 5'-azacytidine, a known hypomethylating agent (task # 6, Table 10).

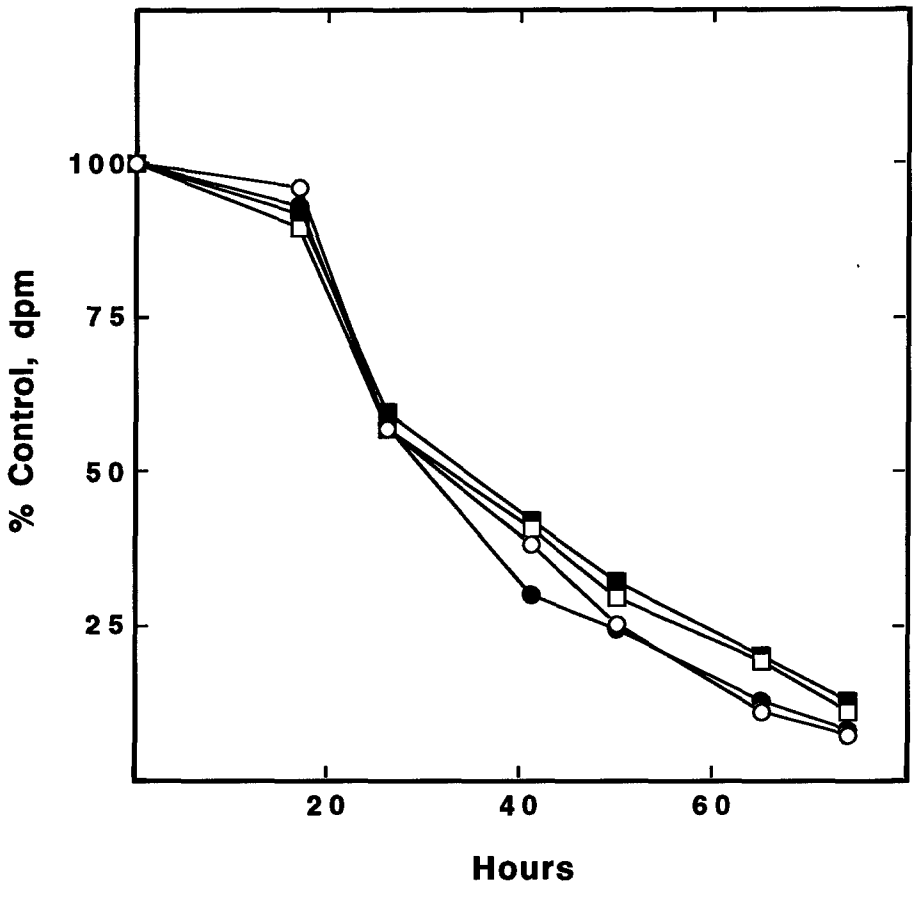


Figure 17. ALDH3A1 mRNA half-lives. MCF-7/0 (○), MCF-7/OAP (●), MCF-7/0/MC (□) and MCF-7/0/CAT (■) cells suspended in growth medium (1 x 10^7 cells/ml) were first pulse-labeled with the aid of ^{32}P-UTP (200 μCi/ml) for 3 hrs after which time labeling was terminated by the addition of an aqueous solution of glucosamine, uridine and cytidine (5 mM each). Cells were then pelleted by low speed centrifugation (500 g for 10 min), resuspended in PBS, washed twice by low speed centrifugation and cultured in growth medium. Subsequently, cells were withdrawn from culture at the times indicated, lysed and subjected to a RNAse protection assay with the aid of a "Direct Protect" RNase protection assay kit and the protocol provided by the manufacturer (Ambion, Inc., Austin TX). Briefly, cell lysates were first incubated overnight with *in vitro*-transcribed antisense ALDH3A1 transcripts to hybridize sense to antisense ALDH3A1 transcripts. Following RNAse digestion to remove unhybridized RNA, ALDH3A1 mRNA hybrids were precipitated with ethanol and pelleted by centrifugation (14,000 g for 10 min). Radioactivity in the RNA precipitate was determined by liquid scintillation. Values are mean of duplicate determinations made in a single experiment. Control values were 18,800, 100,800, 156,800 and 154,000 dpm/10^6 cells for MCF-7/0, MCF-7/OAP, MCF-7/0/MC and MCF-7/0/CAT cells, respectively. The half-lives of ALDH3A1 mRNA in MCF-7/0, MCF-7/OAP, MCF-7/0/MC and MCF-7/0/CAT cells were 32, 31, 33 and 34 hrs, respectively.

Given the foregoing, elevated levels of ALDH3A1 in MCF-7/OAP, MCF-7/0/MC and MCF-7/0/CAT cells appear to be the consequence of transcriptional activation mediated by relevant trans-acting factors.

Whereas the transiently elevated levels of ALDH3A1 in MCF-7/0/MC and MCF-7/0/CAT cells are almost certainly the consequence of transient upregulation of the rate-limiting event in the XRE and ARE signaling

pathways, respectively, Figure 20, the stably elevated levels of ALDH3A1 in MCF-7/OAP cells are probably the consequence of stable upregulation of the rate-limiting event in the ARE signaling pathway. This notion is based on the facts (Figure 20 and task # 7, Table 12) that 1) ALDH3A1, pan-GST, DT-D and UDP-GT levels, but not those of CYP1A1, 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) ALDH3A1, pan-GST, DT-D and UDP-GT levels, but not those of CYP1A1, are coordinately elevated in MCF-7/OAP cells.

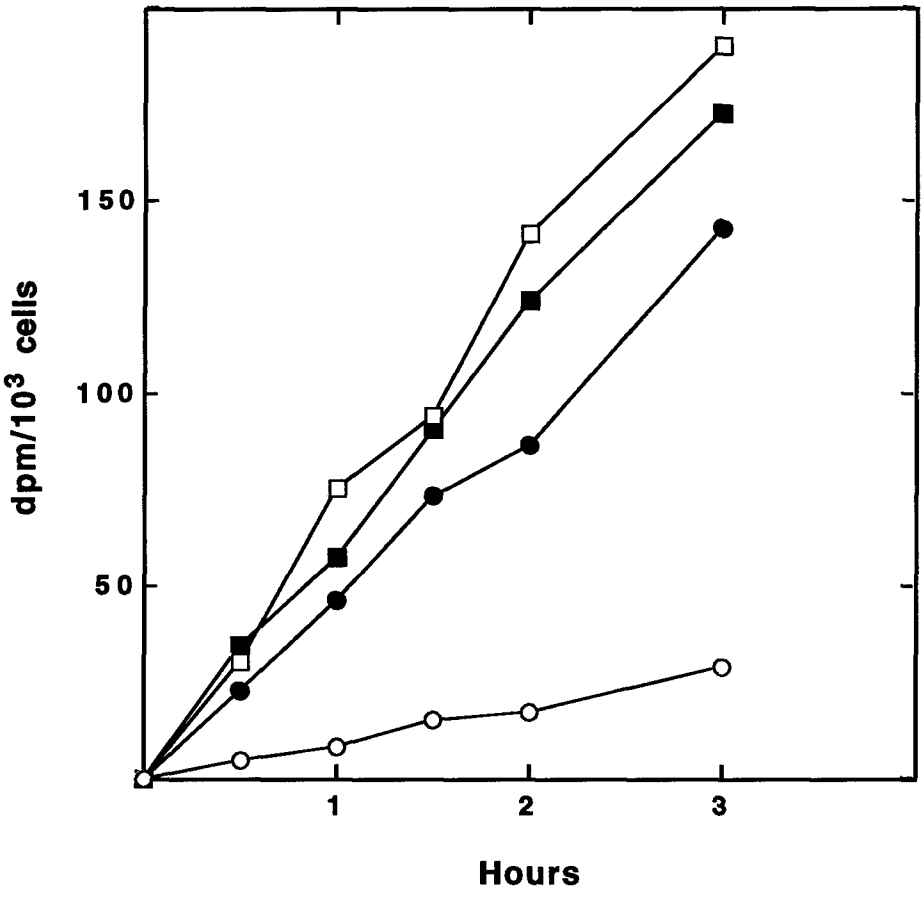


Figure 18. ALDH3A1 mRNA synthesis. MCF-7/0 (○), MCF-7/OAP (●), MCF-7/0/MC (□) and MCF-7/0/CAT (■) cells suspended in growth medium (1 x 10^7 cells/ml) were pulse-labeled with the aid of ^{32}P-UTP (200 μCi/ml) for up to 3 hrs. Aliquots of cells were withdrawn at the times indicated and an aqueous solution of glucosamine, uridine and cytidine (5 mM each) was added to terminate labeling. Cells were then washed and subjected to a RNase protection assay as described in the legend to Figure 17. Values are means of duplicate determinations made in a single experiment. As compared to MCF-7/0 cells, incorporation of ^{32}P-UTP into ALDH3A1 mRNA was 6-, 8- and 7-times faster in MCF-7/OAP, MCF-7/0/MC and MCF-7/0/CAT cells, respectively.

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 ALDH3A1 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 ALDH3A1 and oxazaphosphorine-specific resistance in both ER$^+$ and ER$^-$ cells [Sladek et al., 1995; Sreerama et al., 1995a and unpublished observations].

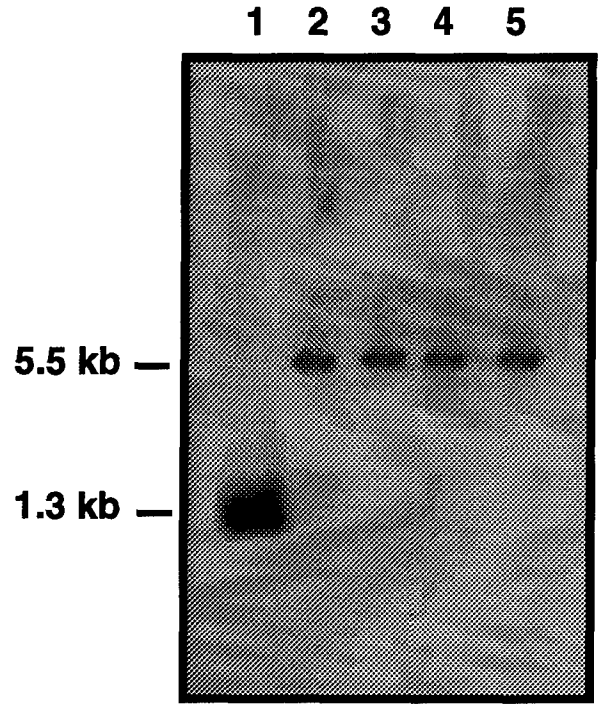


Figure 19. Southern blot analysis of genomic DNA isolated from MCF-7/0 (lane 2), MCF-7/0/CAT (lane 3), MCF-7/0/MC (lane 4) and MCF-7/OAP (lane 5) cells. Southern blot analysis of genomic DNA was essentially as described by Hsu et al. [1992]. Briefly, isolation of genomic DNA from MCF-7/0, MCF-7/0/CAT, 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 μg in each case) was first digested with EcoRI (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 human stomach mucosa ALDH3A1 cDNA (successfully cloned in our laboratory). 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 ALDH3A1 cDNA (lane 1) was inserted at the EcoRI site was used as the reference.

Updating the above, we have now confirmed/established that each of the nine human breast (adeno)carcinoma cell lines that we carry are AhR$^+$ and ARNT$^+$, that the ARNT is of the wild-type in each of these cell lines, that the MCF-7/0, MCF-7/OAP, T-47D, ZR-75-1 and MDA-MB-468 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 9. Finally, we have shown that polycyclic aromatic hydrocarbons, e.g., 3-methylcholanthrene, as well as phenolic antioxidants, e.g., catechol, induce ALDH3A1 in ER$^+$ cell lines, but that only the latter induce ALDH3A1 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 ALDH3A1 gene, induce ALDH3A1 overexpression in ER$^+$ breast cancer cells, but not in ER$^-$ breast cancer cells, whereas, induction of ALDH3A1 effected by agents that cause the activation of AREs present in the 5'-upstream region of the ALDH3A1 gene is estrogen receptor-independent.

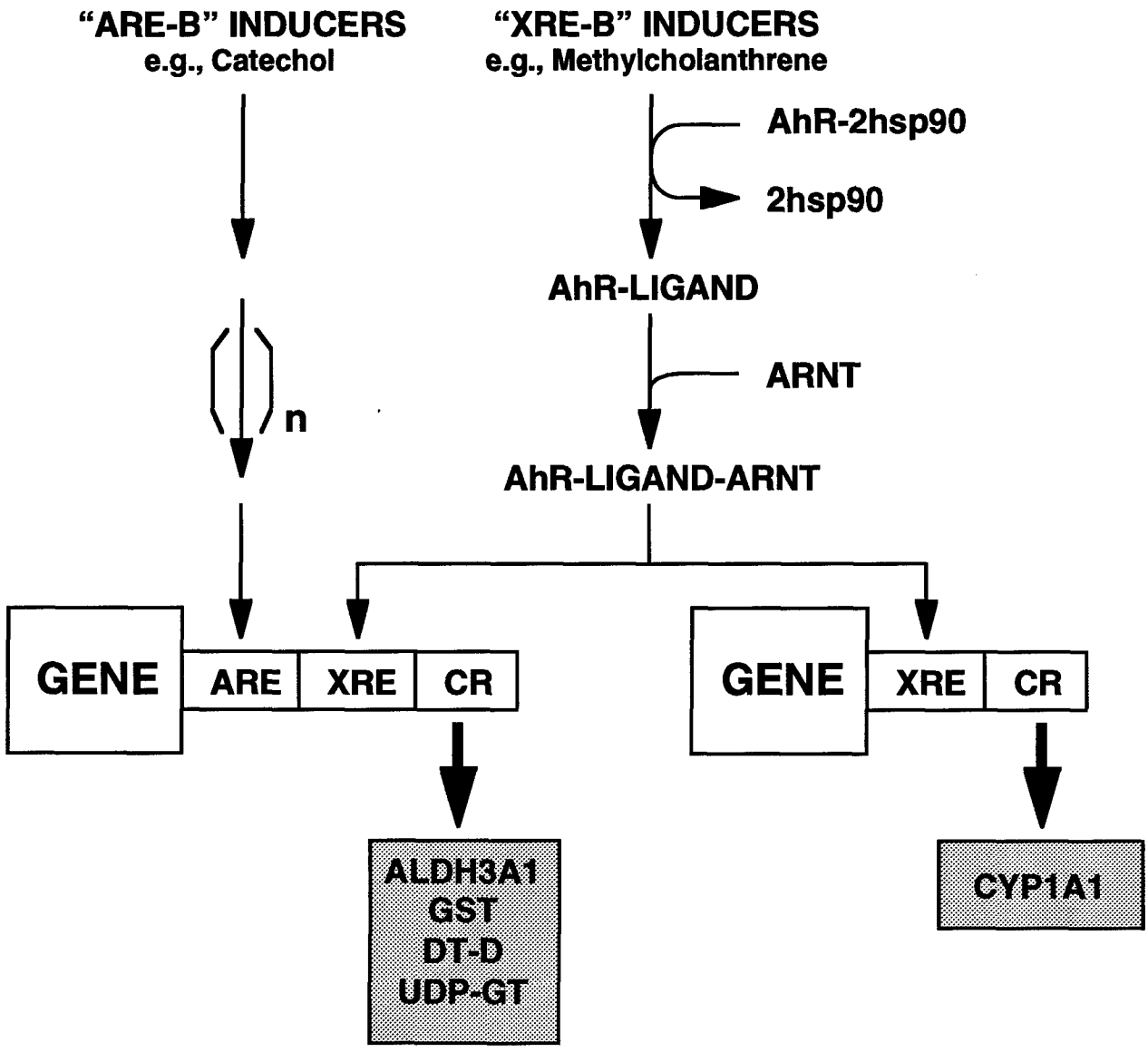


Figure 20. Induction of ALDH3A1 and other drug metabolizing enzymes: signaling pathways. ARE-B inducers: agents, e.g., phenolic antioxidants such as catechol, that induce the transcription of a battery (B) of genes having in common an antioxidant responsive element (ARE) in their promoter (5'-upstream) 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 are 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 are largely known and are 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; CYP1A1, cytochrome P4501A1.

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 CYP1A1 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.

Table 9. Estrogen receptor status and the ability of 3-methylcholanthrene and catechol to induce ALDH3A1 and resistance to mafosfamide in human breast (adeno)carcinoma cell lines and a human non-malignant breast epithelial cell line[a]

Cell Line	ARNT[b]	Vimentin[c]	ER[d]	AhR[e]	ALDH3A1, mIU/10^7 cells			LC_{90}, μM Mafosfamide		
					Inducing agent			Inducing agent		
					None	MC	CAT	None	MC	CAT
MCF-7/0	wt	–	(+) 0.160	15	1.2	310	668	60	>2000	>2000
MCF-7/OAP	wt	*f*	(+) 0.168	16	254	3534	1593	>2000	>2000	>2000
T-47D	wt	–	(+) 0.260	19	1.8	169	982	32	600	>2000
ZR-75-1	wt	–	(+) 0.105	19	3.8	150	18	35	550	100
MDA-MB-468[g]	wt	–	(+) 0.052	*f*	3.8	28	105	25	60	155
MDA-MB-231	wt[h]	+	(–) 0.009	23	1.0	1.2	846	50	50	>2000
SK-BR-3	wt	–	(–) 0.006	15	1.4	1.2	23	48	47	110
HS-578-T	wt	+	(–) 0.015	15	3.2	4.3	22	85	90	225
MDA-MB-435	wt[h]	+	(–) 0.010	10	2.1	2.6	27	60	60	140
MCF-10A[i]	wt	*f*	(–) 0.018	*f*	2.4	223	195	260	475	525

[a]Cells were grown (monolayer) in the presence of vehicle, 3 μM methylcholanthrene (MC) or 30 μM catechol (CAT) for 5 days and harvested. ALDH3A1 activity (NADP-linked enzyme-catalyzed oxidation of benzaldehyde by Lubrol-treated whole homogenates) was quantified spectrophotometrically as described previously [Sreerama and Sladek, 1993a]. A colony-forming assay was used to quantify cellular sensitivity to mafosfamide [Sreerama and Sladek, 1993a].

[b]Aromatic hydrocarbon receptor nuclear translocator; wt = wild type. RT-PCR analysis of ARNT mRNA of each of the cell lines was essentially as described by Wilson et al. [1997].

[c]From Sommers et al. [1989]. Vimentin is an intermediate filament protein.

[d]Absorbance values of *p*-nitrophenol formed per 50 μg protein obtained from whole cell homogenates. Lower limit of detection above background is 0.01. Semiquantification of estrogen receptor levels was by an enzyme-linked immunosorbent assay (ELISA) as described by Hornbeck et al. [1991].

[e]Radioactivity counts (dpm/1000) from nuclear extracts of 5 x 10^6 cells incubated *ex vivo* with ^{3}H-labeled benzpyrene for 2 hr; dpms are the sum of those in fractions that contributed to the binding peak of interest. Semiquantification of Ah receptor levels was essentially as described by Harris et al. [1989] except that ^{3}H-labeled benzpyrene, instead of ^{3}H-labeled 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), was used as the receptor ligand.

[f]Not determined.

[g]CYP1A1 is induced in this cell line by TCDD according to published reports [Wang et. al., 1997]; no ALDH1A1 present (unpublished observations).

[h]Both wild and variant types of ARNT are present according to Wilson et al. [1997].

[i]Human non-malignant breast epithelial cell line; no ALDH1A1 present (unpublished observations).

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 ALDH3A1 and other relevant enzymes, upon treatment with the DNA demethylating agent 5'-azacytidine. Our hypothesis proved to be correct, Table 10. 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.

Table 10. ALDH3A1, 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		
		ALDH3A1[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

[a]MDA-MB-231 cells (1 x 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].

[b]Class-3 aldehyde dehydrogenase; NADP (4 mM) as cofactor and benzaldehyde (4 mM) as substrate.

[c]Glutathione S-transferase; 1-Chloro-2,4-dinitrobenzene (1 mM) as substrate.

[d]DT-diaphorase; NADH (0.16 mM) as cofactor and 2,6-dichlorophenol-indophenol (0.04 mM) as substrate.

Challenging the notion that an estrogen receptor must be present if Ah-receptor agonists are to induce ALDH3A1 and other XRE-B enzymes is our finding that 3-methylcholanthrene markedly induced ALDH3A1 activity in a human non-malignant breast epithelial ER$^-$ cell line, viz., MCF-10A, Table 9. Perhaps of relevance, MCF-10A cells exhibit the characteristics of normal breast epithelial cells including lack of tumorigenicity in nude or SCID mice [reviewed in Russo et al., 1993], and they express an ALDH3A1 that, functionally, is identical to that expressed in all other human normal tissues/fluid thus far examined, but that, functionally, is subtly different from that found in all human malignant cells/tissues thus far examined [reviewed in Sladek et al., 1995; Sreerama

and Sladek, 1995; unpublished observations]. The latter is unlikely to be relevant since 3-methylcholanthrene also induced other members of the XRE-B of enzymes in MCF-10A cells including CYP1A1 [data not presented]. Induction of ALDH3A1 activity by 3-methylcholanthrene in MCF-10A cells may be via a 4 S polycyclic aromatic hydrocarbon-binding protein [Bhat and Bresnick, 1997], identified as glycine N-methyltransferase, rather than via the Ah receptor, thus obviating the need for the estrogen receptor for 3-methylcholanthrene-effected induction in this cell line, the implication being that the 4 S polycyclic aromatic binding protein is not present in the malignant ER^- cell lines listed in Table 9.

Task # 7: Ascertain the ability of ligands for ARE to induce ALDH3A1 activity and oxazaphosphorine-specific acquired resistance in our model system.

MCF-7/0 cells were cultured in the presence of agents, viz., phenolic antioxidants (30 μM each for 5 days), known to induce glutathione S-transferase and/or DT-diaphorase via AREs present in the 5'-upstream regions of the genes that encode these enzymes. Cellular levels of ALDH3A1 activity were then quantified. Each of the phenolic antioxidants tested induced ALDH3A1 activity in MCF-7/0 cells, but the degree of induction varied, Table 11. Immunoblot analysis (specific polyclonal antibodies raised against stomach mucosa ALDH3A1 were used) confirmed the induction of ALDH3A1 protein in catechol-treated MCF-7/0 cells (data not presented). Northern blot (an oligonucleotide probe specific for the mRNA sequence that codes for the 9 N-terminal end amino acids of stomach mucosa ALDH3A1 was used) and RT-PCR analyses confirmed the induction of ALDH3A1 mRNA in catechol-treated MCF-7/0 cells, Figures 21 and 16 (task # 5), respectively. ALDH3A1 induction by catechol was concentration dependent; maximum induction was achieved at a concentration of 60 μM, [Sreerama et al., 1995a]. In addition to MCF-7/0 cells, phenolic antioxidants, e.g., catechol (30 μM for 5 days), induced ALDH3A1 in other human breast tumor cells, viz., T-47D, ZR-75-1, MDA-MB-231 and SK-BR-3, as well as in human colon carcinoma cells, viz., HCT 116b and colon C [Sreerama et al., 1995a].

Table 11. Class 3 aldehyde dehydrogenase activity in untreated and phenolic antioxidant-treated human breast adenocarcinoma MCF-7/0 cells[a]

Phenolic Antioxidant	ALDH3A1 Activity, mIU/10^7 cells
Control	1.7
Catechol	768
Hydroquinone	438
3,5-Di-tert-butyl-4-hydroxyanisole	163
Ethoxyquin	132
t-Butylhydroquinone	107
2,6-Di-tert-butyl-4-hydroxytoluene	46
Vitamin E	9.0

[a]Exponentially growing MCF-7/0 cells (1×10^5) were cultured in the presence of vehicle (control) or a phenolic antioxidant, 30 μM, for 5 days. They were then harvested and Lubrol-treated whole homogenates (2.5×10^4 - 5×10^6 cells) were prepared and assayed for aldehyde dehydrogenase activity as described in Sreerama and Sladek [1993a, 1994]. Each value is the mean of four determinations.

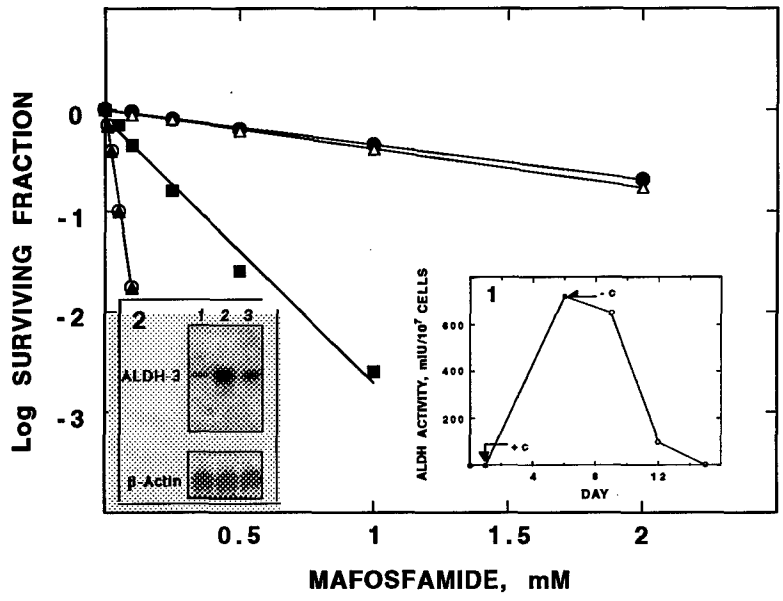


Figure 21. Effect of adding and then removing catechol from the culture medium on the sensitivity of MCF-7/0 cells to mafosfamide. Exponentially growing MCF-7/0 cells were cultured in the presence of 30 μM catechol (C) for 5 days. At the end of this time, cells were harvested, washed, resuspended in catechol-free growth medium, and cultured for an additional 9 days. Sensitivity to mafosfamide was determined as described in Sreerama and Sladek [1993a, 1994] on days 0 (○), 6(●), 9 (△), 12 (■), and 15 (▲). Each point is the mean of measurements on triplicate cultures. LC_{90} values (concentrations of drug required to kill 90% of cells) obtained from these plots were 55 (○), >2,000 (●), >2,000 (△), 370 (■), and 55 (▲) μM. *Inset 1*: Aldehyde dehydrogenase activity at corresponding time points. NADP (4 mM)-linked aldehyde dehydrogenase-catalyzed oxidation of benzaldehyde (4 mM) was quantified in Lubrol-treated whole homogenates, as described in Sreerama et al [1995a], at the times indicated. Reaction mixtures (1 ml) contained whole homogenates prepared from 1.5 x 10^5 to 1 x 10^7 cells. Each value is the mean of duplicate determinations. *Inset 2*: Northern blot analysis of poly(A)$^+$-enriched RNA isolated from MCF-7/0 cells (*Lane 1*), MCF-7/0 cells treated with catechol (30 μM for 5 days) (*Lane 2*), and MCF-7/0 cells treated with catechol (30 μM for 5 days) and then cultured in the absence of catechol for 3 days (*Lane 3*). Isolation of total RNA, poly(A)$^+$-enriched RNA, electrophoresis of poly(A)$^+$-enriched RNA, transfer of poly(A)$^+$-enriched RNA onto a Zeta-Probe nylon membrane, and probing of the blotted membrane with ^{32}P-labeled oligonucleotide specific for human stomach mucosa ALDH3A1 and with full-length β-actin cDNA were described in Sreerama et al [1995a]. Placed on the gels were 10 μg of poly(A)$^+$-enriched RNA in each case.

Polycyclic aromatic hydrocarbons, e.g., 3-methylcholanthrene, coordinately induced ALDH3A1, glutathione S-transferase, DT-diaphorase, UDP-glucuronosyl transferase and cytochrome P4501A1 in MCF-7/0 cells, Table 12 [reviewed in Sladek et al., 1995], whereas phenolic antioxidants, e.g., catechol, coordinately induced all of the above enzymes except cytochrome P4501A1, Table 12. Coordinate induction by the polycyclic aromatic hydrocarbons is effected via the Ah receptor and xenobiotic responsive elements present in the 5'-upstream region of each of the genes encoding ALDH3A1, glutathione S-transferase, DT-diaphorase, UDP-glucuronosyl transferase and cytochrome P4501A1, whereas, coordinated induction by phenolic antioxidants is effected via an ARE present in the 5'-upstream region of each of the genes encoding ALDH3A1, glutathione S-transferase, DT-diaphorase and UDP-glucuronosyl transferase, Figure 20 [reviewed in Sladek et al., 1995]. An ARE is not present in the 5'-upstream region of cytochrome P4501A1 [reviewed in Sladek et al., 1995].

Table 12. Enzyme activities in catechol-, methylcholanthrene- and indole-3-carbinol-treated human breast adenocarcinoma and human normal breast epithelial cells[a]

Cells	Enzyme Activity, mIU/10^7 cells[b]										GSH[c]	LC_{90}[d] (μM)
	ALDH3A1	GST				DT-D	UDP-GT	CYP1A1	G6PDH	GAPDH		
		pan-	α	μ	π							
MCF-7/0	2	25	9	13	11	82	0.04	0.03	2,650	3,980	185	60
MCF-7/0/CAT	768	250	29	121	62	6,395	0.07	0.03	6,090	11,530	182	>2,000
MCF-7/0/MC	310	150	20	113	34	495	0.78	0.48	6,880	13,250	190	>2,000
MCF-7/0/IC	45	82	*e*	*e*	*e*	1,228	*e*	0.22[f]	4,688	6,552	176	175
MCF-7/OAP[g]	254	157	27	81	32	340	0.16	0.03	155	1,630	199	>2,000
MCF-7/OAP/CAT	1,593	227	36	135	51	12,400	0.23	0.03	350	2,165	185	>2,000
MCF-7/OAP/MC	3,534	192	38	96	36	15,300	1.30	0.67	183	1,886	180	>2,000
MCF-10A/0	2	461	74	261	126	163	*e*	<0.04[f]	220	4,050	192	260
MCF-10A/0/CAT	195	1,052	152	669	260	2,284	*e*	<0.04[f]	440	4,850	192	525
MCF-10A/0/MC	223	807	82	662	78	644	*e*	0.45[f]	390	6,120	176	475
MCF-10A/0/IC	34	860	*e*	*e*	*e*	815	*e*	0.24[f]	588	6,880	189	320

[a]Human breast adenocarcinoma MCF-7/0 or MCF-7/OAP, and human normal breast epithelial MCF-10A/0, cells were cultured in the presence of vehicle (MCF-7/0, MCF-7/OAP, MCF-10A/0), 30 μM catechol (MCF-7/0/CAT, MCF-7/OAP/CAT, MCF-10A/0/CAT), 3 μM methylcholanthrene (MCF-7/0/MC, MCF-7/OAP/MC, MCF-10A/0/MC) or 80 μM indole-3-carbinol [MCF-7/0/IC, MCF-10A/0/IC] for 5 days.

[b]Class 3 aldehyde dehydrogenase (ALDH3A1), pan-glutathione S-transferase (pan-GST), DT-diaphorase (DT-D), UDP-glucuronosyl transferase (UDP-GT), cytochrome P4501A1 (CYP1A1), glucose-6-phosphate dehydrogenase (G6PDH) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) catalytic activities were quantified as described previously [Anonymous, 1967; Lambeir et al., 1991; Sreerama and Sladek, 1993a; Sreerama and Sladek, 1994; Rekha and Sladek, 1997a]. Substrates were 4 mM benzaldehyde, 1 mM 1-chloro-2,4-dinitrobenzene, 0.04 mM 2,6-dichlorophenol-indophenol, 0.05 mM α-naphthol, 0.005 mM 7-ethoxyresorufin, 0.6 mM glucose-6-phosphate and 1 mM glyceraldehyde-3-phosphate, respectively. Cofactors were 4 mM NADP, 1 mM GSH, 0.16 mM NADH, 2 mM UDP-glucuronic acid, 0.25 mM NADPH, 0.2 mM NADP and 1 mM NAD, respectively. Also quantified were glutathione *S*-transferase α, μ and π levels. ELISAs were used for this purpose as described previously [Rekha and Sladek, 1997a].

[c]GSH levels (nmol/10^7 cells) were quantified as described previously [Sreerama and Sladek, 1994].

[d]Freshly harvested cells (1 x 10^5 cells/ml) were exposed to various concentrations of mafosfamide for 30 min at 37°C. They were then harvested and grown in drug-free growth medium for 10-20 days. A colony-forming assay was used to determine cellular sensitivity to mafosfamide as described previously [Sreerama and Sladek, 1993a].

[e]Not determined.

[f]CYP1A1 catalytic activities were not determined. However, as determined by ELISA [Sreerama and Sladek, 1997], there were 2.38, 4.88 and 2.56 pg of CYP1A1 protein/mg microsomal protein in MCF-7/0/IC, MCF-10A/0/MC and MCF-10A/0/IC cells, respectively, whereas there was <0.4 pg of CYP1A1 protein/mg microsomal protein in MCF-7/0, MCF-10A/0 and MCF-10A/0/CAT cells. In MCF-7/0/MC cells the amount of CYP1A1 protein was 5.20 pg/mg microsomal protein. Estimates of CYP1A1 catalytic activity, based on CYP1A1 catalytic activity and protein determined to be present in MCF-7/0/MC cells, viz., 0.48 mIU/10^7 cells and 5.20 pg/mg microsomal protein, respectively, are presented in the Table for MCF-7/0/IC, MCF-10A/0, MCF-10A/0/CAT, MCF-10A/0/MC and MCF-10A/0/IC cells.

[g]MCF-7/0 cells that had been cultured in the presence of gradually increasing concentrations of 4-hydroperoxycyclophosphamide for several months to generate a stable oxazaphosphorine-resistant subline (MCF-7/OAP) [Frei et al., 1988].

MCF-7/0 cells cultured in the presence of catechol (30 μM for 5 days) were less sensitive to mafosfamide than were untreated MCF-7/0 cells, Figure 21 and Table 12. Sensitivity to phosphoramide mustard was not decreased [Sreerama et al., 1995a]. The relative insensitivity to mafosfamide exhibited by these cells was transient since, following the removal of catechol from the culture medium, sensitivity to mafosfamide, as well as ALDH3A1 activity and the cellular content of ALDH3A1 mRNA, returned to basal levels within 10 days, Figure 21.

The ALDH3A1 induced by catechol was purified, Figure 22, and physically and kinetically characterized [Sreerama et al., 1995a]; it was found to be identical to the ALDH3A1 present in MCF-7/0/MC cells [Sreerama and Sladek, 1994].

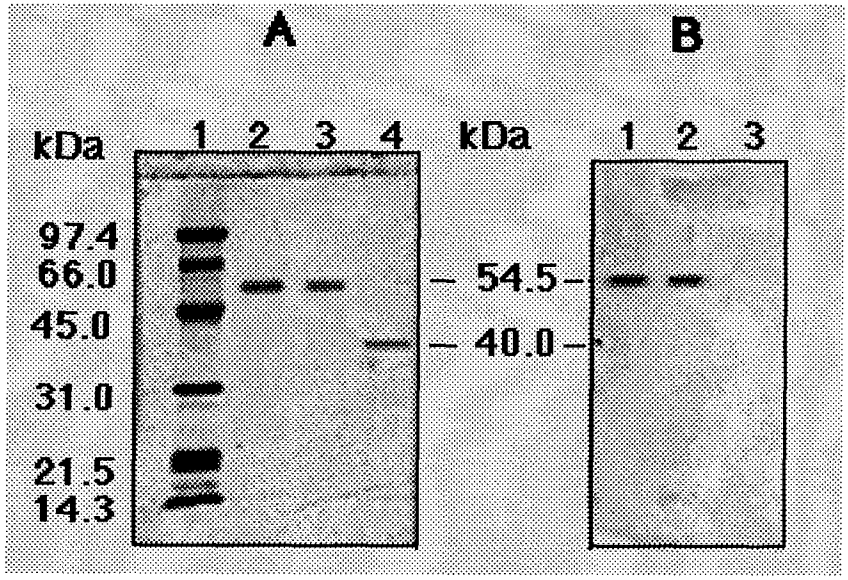


Figure 22. Class 3 aldehyde dehydrogenase purified from catechol-treated MCF-7/0 cells: subunit molecular weight and recognition of the denatured enzyme by anti-stomach mucosa ALDH3A1 IgY. Induction of ALDH3A1 by catechol, and the subsequent purification of this enzyme, were as described in Sreerama et al [1995a]. Panel **A**: SDS-PAGE of molecular weight markers (*Lane 1*) and 5 μg each of purified stomach mucosa ALDH3A1 (*Lane 2*), catechol-induced enzyme (*Lane 3*), and MCF-7/OAP ALDH3A1 (*Lane 4*) was as described in Sreerama and Sladek [1993a]. Molecular weight markers were lysozyme (14.3 kDa), trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), BSA monomer (66 kDa) and phosphorylase B (97.4 kDa). Proteins in each lane were visualized by staining with Coomassie Brilliant Blue R-250. A plot of log M_r *versus* mobility was used to estimate the subunit molecular weight of the catechol-induced enzyme [Sreerama and Sladek, 1993a]. Panel **B**: Purified stomach mucosa ALDH3A1 (*Lane 1*), purified catechol-induced enzyme (*Lane 2*), and purified MCF-7/OAP ALDH3A1 (*Lane 3*) were submitted to SDS-PAGE and electrotransferred onto a Immobilon-PVDF transfer membrane; attempted visualization of the denatured enzymes with anti-stomach mucosa ALDH3A1 IgY was as described in Sreerama and Sladek [1993a]. Placed on the gel were 5 μg of each purified enzyme.

The ALDH3A1 isolated from MCF-7/0/CAT cells, like the ALDH3A1 isolated from other tumor sources [reviewed in Sladek et al., 1995], was found to be a subtle, putatively tumor specific, variant of the ALDH3A1 isolated from normal cells/secretions, e.g., stomach mucosa, parotid gland and whole saliva [reviewed in Sladek et al., 1995]. Tumor cell ALDH3A1, relative to normal cell ALDH3A1, was more able to catalyze the oxidation of aldophosphamide [reviewed in Sladek et al., 1995] and, thus, the detoxification of oxazaphosphorines [Sreerama and Sladek, 1995]. It was also more sensitive to inhibition by chlorpropamide analogs, Table 5 (task # 3). Physical and all other catalytic properties appeared to be identical

As in MCF-7/0/CAT cells, coordinately elevated levels of ALDH3A1, glutathione S-transferases, DT-diaphorase and UDP-glucuronosyl transferase, but not of cytochrome P4501A1, were also found in MCF-7/OAP cells, Table 12. As a consequence of increased enzyme levels (relative to those in MCF-7/0 cells), the sensitivity of MCF-7/0/MC, MCF-7/0/CAT and MCF-7/OAP cells to certain anticancer drugs was (relative to the sensitivity of MCF-7/0 cells to these same drugs) markedly altered. Thus, the rate at which some drugs were bioinactivated, e.g., mafosfamide, melphalan and mitoxantrone, was increased and sensitivity to these drugs was decreased (multidrug resistance), and the rate at which some other drugs were bioactivated, e.g., ellipticine and the indoloquinone EO9, was also increased and sensitivity to these drugs was increased (collateral sensitivity) [Rekha and Sladek, 1997a]. Xenobiotic-induced multienzyme-mediated multidrug resistance/collateral sensitivity thus demonstrated is mechanistically different from, and pertains to a largely different group of anticancer agents than does, the multidrug resistance effected by multidrug resistance-associated protein (MRP) or the P-glycoprotein encoded by the *mdr* 1 gene. Indeed, multidrug resistance mediated by enzymes simultaneously induced by a xenobiotic is a newly recognized mechanism by which multidrug resistance can be effected.

In light of the foregoing, we initiated an investigation in which glutathione S-transferase, DT-diaphorase and cytochrome P4501A1, as well as ALDH3A1 (and ALDH1A1 and GSH), levels in normal and malignant breast tissue samples procured from the Cooperative Human Tissue Network, Midwestern Division, Columbus, Ohio, were quantified. Results are presented under task # 1a (Table 2) and task # 1b (Table 4).

As mentioned under task # 6, human MCF-10A cells exhibit the characteristics of normal breast epithelial cells including lack of tumorigenicity in nude or SCID mice [reviewed in Russo et al., 1993], and they express an ALDH3A1 that, functionally, is identical to that expressed in all other human normal tissues/fluid thus far examined, viz., stomach mucosa, parotid gland and whole saliva, but that, functionally, is subtly different from that found in all human malignant cells/tissues thus far examined, e.g., MCF-7/0 and several other breast tumor cell lines [reviewed in Sladek et al., 1995; Sreerama and Sladek, 1995; unpublished observations]. As in MCF-7 cells, 3-methylcholanthrene, coordinately induced ALDH3A1, glutathione S-transferase, DT-diaphorase and cytochrome P4501A1 activities in MCF-10A/0 cells, Table 12, whereas catechol coordinately induced all of the above enzyme activities except that of cytochrome P4501A1, Table 12. Also as in the case of the malignant human breast adenocarcinoma MCF-7/0 cell line, 3-methylcholanthrene- and catechol-treated human non-malignant breast epithelial MCF-10A cells were, relative to untreated MCF-10A/0 cells, less sensitive to mafosfamide, but the decrease in sensitivity induced by these two agents was only about 2-fold in the case of MCF-10A cells, whereas it was more than 33-fold in the case of MCF-7/0 cells, Table 12. This discrepancy cannot be fully explained by the degree of ALDH3A1 induction, 100-fold (MCF-10A) *vs* 150- to 384-fold (MCF-7/0), Table 12. Probably also contributing is that MCF-10A/0 cells express an ALDH3A1 that is about 10-fold less efficient in catalyzing the oxidation (detoxification) of aldophosphamide than is the ALDH3A1 expressed by MCF-7/0 cells (reviewed in Sladek et al., 1995; unpublished observations).

Indole-3-carbinol is a demonstrated Ah receptor agonist, Figure 20, and its chemopreventive potential (putatively, effected by virtue of its ability to induce drug-detoxifying enzymes) is currently undergoing clinical trial [reviewed in Greenwald,, 1993; NCI, 1996a]. Potentially, it could alter the sensitivity of malignant cells to a number of anticancer agents, e.g., cyclophosphamide, as well. As expected, indole-3-carbinol induced

ALDH3A1, glutathione S-transferase, DT-diaphorase, UDP-glucuronosyl transferase and cytochrome P4501A1 in the malignant MCF-7/0 cell line, Table 12. It also induced the levels of these enzymes in the non-malignant MCF-10A cell line, Table 12. Each of the indole-3-carbinol-treated cell lines exhibited a relatively decreased sensitivity to mafosfamide, Table 12. Thus, the potential for drug interactions of this type in the clinic is established.

Agents that coordinately induce ALDH3A1, glutathione S-transferases, DT-diaphorase, UDP-glucuronosyl transferase and cytochrome P4501A1 are also abundantly present in the human diet, e.g., beverages such as coffee and vegetables such as broccoli [summarized in Sreerama et al., 1995b]. We have demonstrated that consumption of large amounts of coffee or broccoli by human subjects results in the coordinated elevation of ALDH3A1, glutathione S-transferases and DT-diaphorase levels in saliva [Sreerama et al., 1995b]. Thus, assuming that the xenobiotic-induced coordinated elevation of salivary enzymes mirrors the coordinated elevation of these enzymes in various cancer cells, dietary and other environmental factors could greatly influence the therapeutic efficacy of anticancer drugs that are bioinactivated or bioactivated by these enzymes [Sreerama et al., 1995b; Rekha and Sladek 1997a]. However, salivary levels of ALDH3A1 did not reflect the levels of this enzyme in malignant breast tissue, Figure 14 (task # 1c).

Task # 7a: Ascertain whether limonene and/or any of several of its metabolites induce ALDH3A1 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, 1996b]. 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 monoterpenoid, 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 ALDH3A1, belong to a battery of enzymes having in common XREs and AREs in the promoter regions of the genes that encode, Figure 20, *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 20 and Table 12, *vide supra*. Given the foregoing, the expectation was that the monoterpenes would induce the increased expression of ALDH3A1 in MCF-7/0 cells.

The expectation was realized experimentally. Limonene, perillyl alcohol, perillyl aldehyde and perillic acid each induced ALDH3A1 as well as pan-GST, in MCF-7/0 cells, Figure 23, but the amount of induction was less than dramatic. Perillyl aldehyde was easily the most potent inducer of these enzymes. Preliminary experiments showed that ALDH3A1 catalyzed the oxidation of perillyl aldehyde, albeit much less effectively than did ALDH1A1 (data not shown).

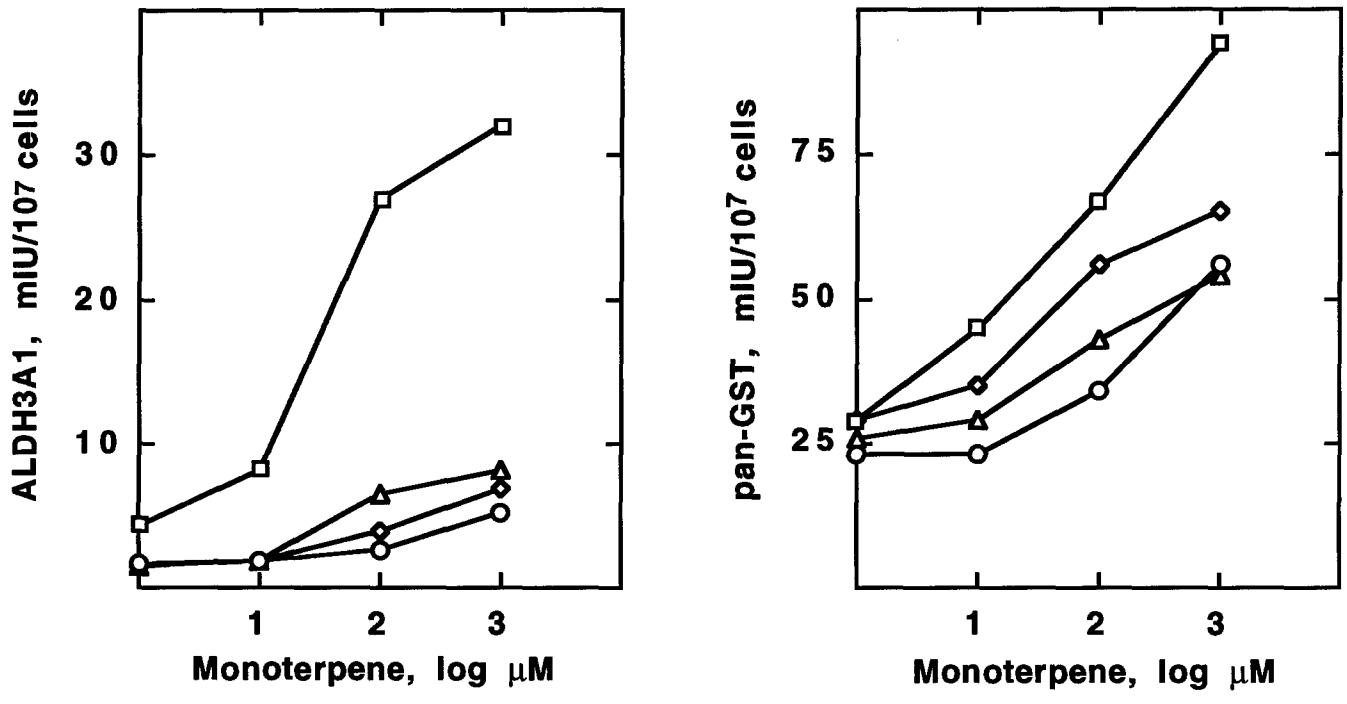


Figure 23. Induction of ALDH3A1 and GST enzymes by monoterpenes in MCF-7/0 cells. Exponentially growing human breast adenocarcinoma MCF-7/0 cells were continuously exposed to of limonene (○), perillyl alcohol (△), perillyl aldehyde (◇), perillic acid (□) or vehicle for 5 days after which time they were harvested and NADP-linked ALDH3A1 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. ALDH3A1 and pan-GST catalytic activities in untreated cells were 1.8 and 22 mIU/10^7 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, 1996b]. In our investigations, limonene, perillyl alcohol, perillyl aldehyde and perillic acid each inhibited the proliferation of MCF-7/0 cells; GI_{90} 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).

Phase I clinical evaluation of limonene in the UK [reviewed in Gould, 1995] and of perillyl alcohol in the US [reviewed in NCI, 1996b] 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 ALDH3A1-catalyzed detoxification of cyclophosphamide) may occur in that event.

Tasks # 7b & 7c: Generate a stably cyclophosphamide-resistant MCF-7/0 subline by exposing MCF-7/0 cells to gradually increasing concentrations of benzpyrene for several months, or once to a high concentration of mafosfamide for 30 minutes.

Heretofore, we were successful in effecting transient cellular resistance to oxazaphosphorines by transiently inducing ALDH3A1 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 20 and Table 12, *vide supra.*

On the other hand, overexpression of ALDH3A1 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 20 and Table 12, *vide supra.*

Desired for future studies was a stably cyclophosphamide-resistant MCF-7/0 subline (model) in which the 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 ALDH3A1 in MCF-7/0 cells [Sreerama and Sladek, 1993a], 4) it is known to be an AhR-agonist and to induce CYP1A1 [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/BP) resulted. These cells were also resistant to other polycyclic aromatic hydrocarbons, viz., dimethylbenzanthracene and 3-methylcholanthrene, as well as to mafosfamide, Table 13. ALDH3A1, pan-GST and DT-D levels were markedly elevated, but unexpectedly, that of CYP1A1 was not, Table 14, 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 ALDH3A1, 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.

The stably oxazaphosphorine-resistant MCF-7/OAP cell line in which levels of ALDH3A1 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., 1996]. Specifically, in that 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.

Table 13. Sensitivity of MCF-7/BP and MCF-7/MAF cells to mafosfamide and to several environmental carcinogens

Cells	LC_{90} (μM)			
	Mafosfamide[a]	BP[b]	DMBA[b]	MC[b]
MCF-7/0	60	1.00	0.95	1.00
MCF-7/BP[c]	>2,000	>50	>50	>50
MCF-7/MAF[d]	>2,000	>50	>50	>50

[a]Freshly harvested cells (1 x 10^5 cells/ml) were exposed to various concentrations of mafosfamide for 30 min at 37°C. They were then harvested and grown in drug-free growth medium for 10-20 days. A colony-forming assay was used to determine cellular sensitivity to mafosfamide as described previously [Sreerama and Sladek, 1993a].

[b]Freshly harvested cells were incubated (2 x 10^5 cells/plate) in a CO_2 incubator for 24 hr at 37°C and various concentrations (4-6) of benzpyrene (BP), 7,12-dimethylbenzanthracene (DMBA) or 3-methylcholanthrene (MC) were added and the incubation was continued for an additional 24 hr, after which time they were harvested and grown in drug-free growth medium for 15-20 days. A colony-forming assay was used to determine cellular sensitivity to BP, DMBA and MC as described previously [Sreerama and Sladek, 1993a].

[c]Human breast adenocarcinoma MCF-7/0 cells that had been grown in the presence of gradually increasing concentrations of benzpyrene for four months to generate a stably resistant subline (MCF-7/BP).

[d]MCF-7/0 cells that had been exposed once to mafosfamide (1 mM) for 30 min at 37°C and that had then been cultured (monolayer) for 4-5 weeks resulting in 4 surviving colonies (surviving fraction of 4 x 10^{-6}) which had then been further expanded as monolayer cultures, among which three of the four were as sensitive to mafosfamide as were the parent MCF-7/0 cells (LC_{90} = 65 μM) but the fourth (MCF-7/MAF) was relatively and stably insensitive (LC_{90} > 2 mM).

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 10^6) 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 (LC_{90} = 65 μM); the fourth (MCF-7/MAF) was relatively insensitive, Table 13. Unexplainably, MCF-7/MAF cells were also resistant to the cytotoxic action of several polycyclic aromatic hydrocarbons, viz., benzpyrene, dimethylbenzanthracene and 3-methylcholanthrene, Table 13. ALDH3A1 levels in the three mafosfamide-sensitive clonal expansions did not differ from that in the parent MCF-7/0 cells, whereas the ALDH3A1 level in the mafosfamide-resistant MCF-7/MAF cells was markedly elevated as were pan-GST and DT-D, but not CYP1A1, levels, Table 14, i.e., only the ARE-B of enzymes, Figure 20, *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 14. Enzyme activities in stably benzpyrene- and mafosfamide-resistant human breast adenocarcinoma cells.

Cells	Enzyme Activity, mIU/10^7 cells[a]									GSH[b] (nmol/10^7 cells)
	ALDH3A1	GST				DT-D	CYP1A1	G6PDH	GAPDH	
		pan-	α	μ	π					
MCF-7/0	2	25	9	13	11	82	0.03	2,650	3,980	185
MCF-7/BP[c]	704	749	80	361	260	567	0.02	520	1,850	171
MCF-7/MAF[d]	373	521	36	280	170	763	0.02	530	3,770	168

[a]Class 3 aldehyde dehydrogenase (ALDH3A1), pan-glutathione S-transferase (pan-GST), DT-diaphorase (DT-D), cytochrome P4501A1 (CYP1A1), glucose-6-phosphate dehydrogenase (G6PDH) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) catalytic activities were quantified as described previously [Anonymous, 1967; Lambeir et al., 1991; Sreerama and Sladek, 1993a; Sreerama and Sladek, 1994; Rekha and Sladek, 1997a]. Substrates were 4 mM benzaldehyde, 1 mM 1-chloro-2,4-dinitrobenzene, 0.04 mM 2,6-dichlorophenol-indophenol, 0.005 mM 7-ethoxyresorufin, 0.6 mM glucose-6-phosphate and 1 mM glyceraldehyde-3-phosphate, respectively. Cofactors were 4 mM NADP, 1 mM GSH, 0.16 mM NADH, 0.25 mM NADPH, 0.2 mM NADP and 1 mM NAD, respectively. Also quantified were glutathione *S*-transferase α, μ and π levels. ELISAs were used for this purpose as described previously [Rekha and Sladek, 1997a].

[b]Glutathione (GSH) levels were determined as described previously [Sreerama and Sladek, 1994].

[c]Human breast adenocarcinoma MCF-7/0 cells that had been grown in the presence of gradually increasing concentrations of benzpyrene for four months resulting in a stable benzpyrene-resistant (LC_{90} > 50 μM) subline (MCF-7/BP).

[d]MCF-7/0 cells that had been exposed once to mafosfamide (1 mM) for 30 min at 37°C and that had then been cultured (monolayer) for 4-5 weeks resulting in 4 surviving colonies (surviving fraction of 4 x 10^{-6}) which had then been further expanded as monolayer cultures, among which three of the four were as sensitive to mafosfamide as were the parent MCF-7/0 cells (LC_{90} = 65 μM), but the fourth (MCF-7/MAF) was relatively and stably insensitive (LC_{90} > 2 mM).

Perhaps of significance, glucose-6-phosphate dehydrogenase activities were markedly lower in both the MCF-7/BP and MCF-7 sublines when compared to that in the wild-type MCF-7/0 cell line, Table 14. Whether low glucose-6-phosphate dehydrogenase activities in any way accounts for the benzpyrene-resistance exhibited by these two sublines is unknown. Glucose-6-phosphate dehydrogenase activity is also relatively low in the MCF-7/OAP subline, Table 12; it, too, is benzpyrene resistant (data not presented).

In the first of a series of experiments intended to ascertain whether the elevated levels of ALDH3A1 that we observed in the MCF-7/BP and MCF-7/MAF sublines were the consequence of stable, upregulated transcriptional activation, as we suspected, or of some other mechanism, RT-PCR was utilized to quantify cellular levels of

ALDH3A1 mRNA in the two sublines. As expected, ALDH3A1 mRNA levels were found to be elevated (~5-fold) in each case, Figure 24.

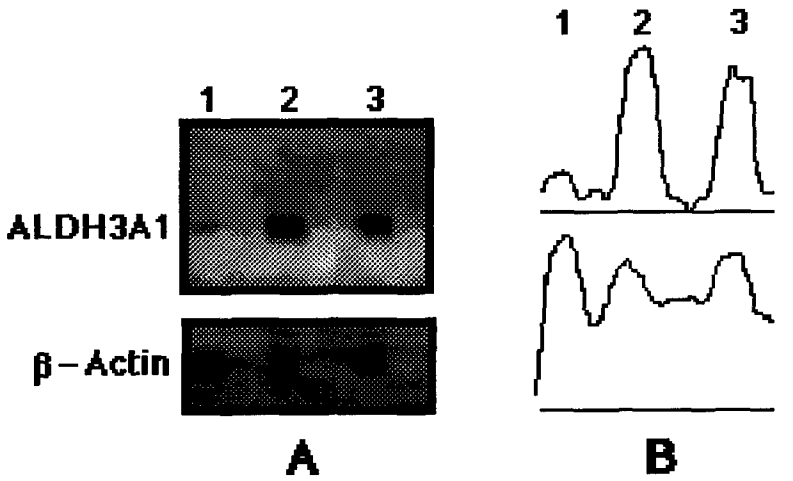


Figure 24. ALDH3A1 mRNA levels in MCF-7/0 (lane 1), MCF-7/BP (lane 2) and MCF-7/MAF (lane 3) cells as judged by RT-PCR. Isolation of total RNA from MCF-7/0, MCF-7/BP and MCF-7/MAF cells, synthesis of first-strand cDNA catalyzed by reverse transcriptase, and the subsequent PCR catalyzed by Vent DNA polymerase were as described in the legend to Figure 16. 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).

As judged by ALDH3A1 mRNA half-lives, Figure 25, the relatively elevated ALDH3A1 mRNA levels that we observed in the MCF-7/BP and MCF-7/MAF sublines were not the consequence of ALDH3A1 mRNA stabilization. An additional experiment, Figure 26, revealed that the relatively elevated ALDH3A1 mRNA levels were the consequence of increases in the rates of ALDH3A1 mRNA synthesis.

Southern blot analysis of genomic DNA isolated from MCF-7/BP and MCF-7/MAF cells showed no increases in genomic *ALDH3A1* DNA copies, thus demonstrating that increased ALDH3A1 mRNA levels in these cells were not due to gene amplification, Figure 27. The elevated levels of ALDH3A1 mRNA in MCF-7/BP and MCF-7/MAF cells were not the consequence of hypomethylation of a relevant *ALDH3A1* DNA regulatory element because neither the ALDH3A1 mRNA level nor the ALDH3A1 catalytic activity was increased when MCF-7/0 cells were cultured in the presence of 1, 5 or 10 μM 5'-azacytidine, a known hypomethylating agent (task # 6, Table 10).

Given the foregoing, the elevated levels of ALDH3A1 in the MCF-7/BP and MCF-7/MAF sublines appears to be, as in the case of the MCF-7/OAP subline (task # 5), the consequence of stable upregulated transcriptional activation mediated by relevant trans-acting factors, specifically, stable upregulation of the rate-limiting event in the ARE signaling pathway. This notion is based on the facts that 1) ALDH3A1, pan-GST and DT-D levels, but not those of CYP1A1, are coordinately elevated when induced expression is effected via the ARE signaling pathway, 2) cellular levels of all four enzymes are coordinately elevated when induced expression is effected via the XRE signaling pathway, and 3) ALDH3A1, pan-GST and DT-D levels, but not those of CYP1A1, are coordinately elevated in MCF-7/BP and MCF-7/MAF cells, Figure 20 and Table 14.

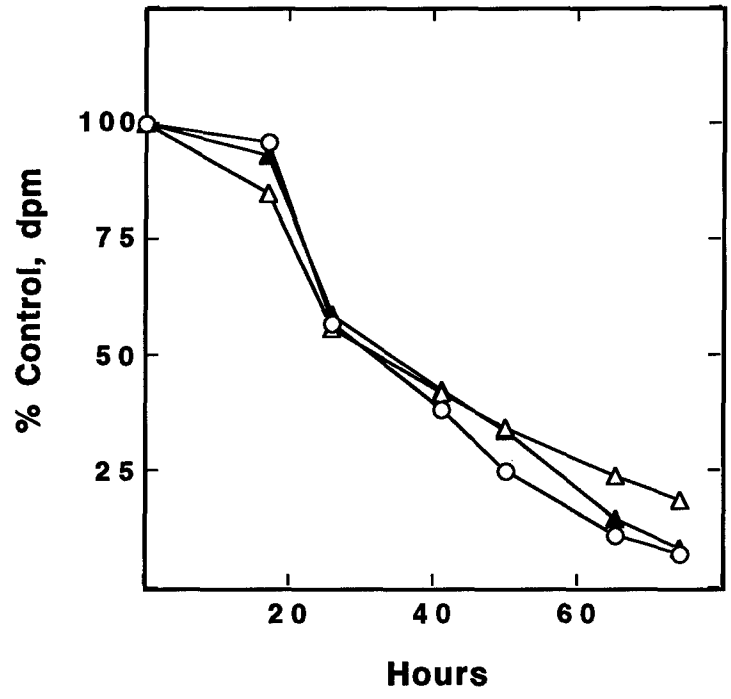


Figure 25. ALDH3A1 mRNA half-lives. MCF-7/0 (○), MCF-7/BP (△) and MCF-7/MAF (▲) cells suspended in growth medium (1×10^7 cells/ml) were first pulse-labeled with the aid of ^{32}P-UTP (200 μCi/ml) for 3 hrs after which time labeling was terminated by the addition of an aqueous solution of glucosamine, uridine and cytidine (5 mM each). Cells were then pelleted by low-speed centrifugation (500 g for 10 min), resuspended in PBS, washed twice by low-speed centrifugation and cultured in growth medium. Subsequently, cells were withdrawn from culture at the times indicated, lysed and subjected to a RNase protection assay as described in the legend to Figure 17. Values are mean of duplicate determinations made in a single experiment. Control values were 18,800, 137,800 and 104,400 dpm/10^6 cells for MCF-7/0, MCF-7/BP and MCF-7/MAF cells, respectively. The half-lives of ALDH3A1 mRNA in MCF-7/0, MCF-7/BP and MCF-7/MAF cells were 32, 32 and 34 hrs, respectively.

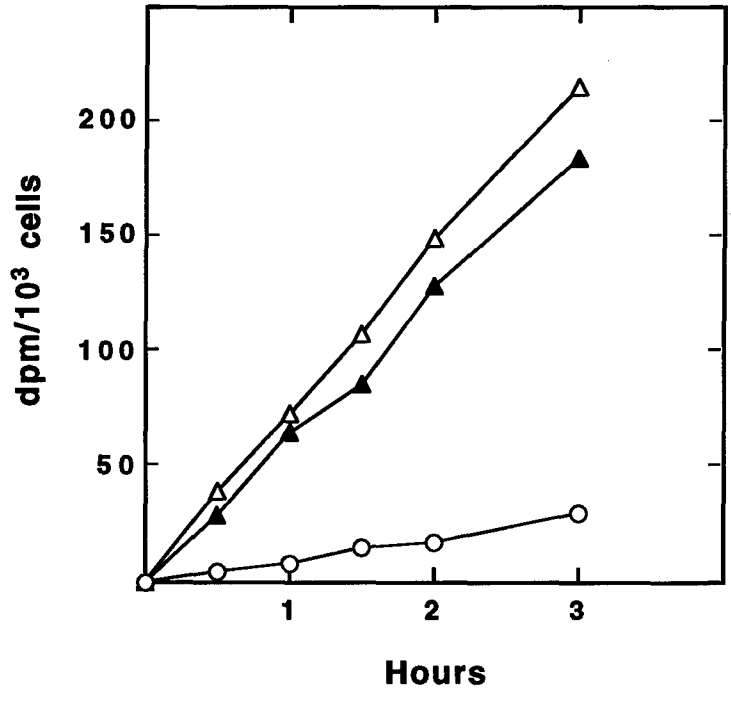


Figure 26. ALDH3A1 mRNA synthesis. MCF-7/0 (○), MCF-7/BP (△) and MCF-7MAF (▲) cells suspended in growth medium (1×10^7 cells/ml) were pulse-labeled with the aid of ^{32}P-UTP (200 μCi/ml) for up to 3 hrs. Aliquots of cells were withdrawn at the times indicated and an aqueous solution of glucosamine, uridine and cytidine (5 mM each) was added to terminate labeling. Cells were then washed and subjected to a RNase protection assay as described in the legend to Figure 17. Values are means of duplicate determinations made in a single experiment. As compared to MCF-7/0 cells, incorporation of ^{32}P-UTP into ALDH3A1 mRNA was 9- and 7-times faster in MCF-7/BP and MCF-7/MAF cells, respectively.

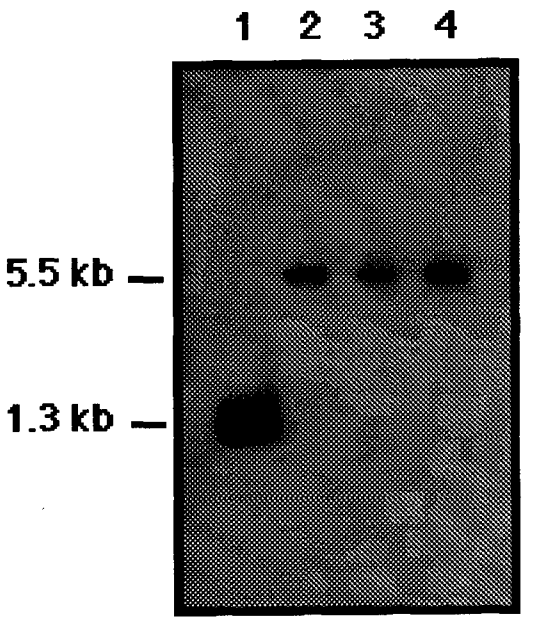


Figure 27. Southern blot analysis of genomic DNA isolated from MCF-7/0 (lane 2), MCF-7/BP (lane 3), and MCF-7/MAF (lane 4) cells. Southern blot analysis of genomic DNA was as described in the legend to Figure 19. A plasmid, viz., pCR 3.1, (50 ng) into which the full-length stomach mucosa ALDH-3 cDNA (lane 1) was inserted at the EcoRI site was used as a positive control.

CONCLUSIONS

Known at the start of this investigation was that, in preclinical models, ALDH3A1, ALDH1A1 and glutathione were operative molecular determinants of cellular sensitivity to cyclophosphamide and other oxazaphosphorines, e.g., ifosfamide, 4-hydroperoxycyclophosphamide and mafosfamide, that is, cellular sensitivity to these agents decreased as cellular levels of either of these cellular constituents increased. Known, also, was that ALDH1A1 effected the detoxification of these prodrugs by catalyzing the oxidation of a pivotal intermediate aldehyde to the corresponding acid, the latter being without cytotoxic activity and incapable of giving rise to a cytotoxic metabolite. Established/concluded in the present investigation was that:

- ALDH3A1 catalyzes the oxidative detoxification of the oxazaphosphorines as does ALDH1A1.

- Human breast tumor ALDH3A1, ALDH1A1 and glutathione levels vary widely (>250-fold).

- Exposure to low concentrations of abundantly present pharmacological/dietary/environmental constituents, e.g., 3-methylcholanthrene, indole-3-carbinol, perillyl alcohol or catechol, for a few days markedly induces ALDH3A1 levels (in some cases, >200-fold) in preclinical breast cancer models.

  - Induction of ALDH3A1 levels (thus, insensitivity to the oxazaphosphorines) by short-term (a few days) exposure to low concentrations of these agents is transient.

  - Transient induction of ALDH3A1 levels by polycyclic aromatic hydrocarbons such as 3-methylcholanthrene:

    - occurs only in estrogen receptor-positive cells, i.e., it does not occur in estrogen receptor-negative cells.

    - is the consequence of transient overexpression of ALDH3A1 mRNA which, in turn, is not due to demethylation of the gene encoding it.

    - is via Ah receptors and XREs (xenobiotic responsive elements) present in the 5'-upstream regions of the gene encoding this enzyme.

  - Transient induction of ALDH3A1 levels by phenolic antioxidants such as catechol:

    - is estrogen receptor independent, i.e., it occurs in both estrogen receptor-positive and -negative cells.

    - is the consequence of transient overexpression of ALDH3A1 mRNA which, in turn, is not due to demethylation of the gene encoding it.

    - is via AREs (xenobiotic responsive elements) present in the 5'-upstream regions of the gene encoding this enzyme.

- Cultured human breast adenocarcinoma MCF-7/0 sublines, stably overexpressing ALDH3A1 and relatively insensitive to oxazaphosphorines, can be generated by:
  - continuous exposure to gradually increasing concentrations of 4-hydroxycyclophosphamide over a period of several months (MCF-7/OAP).
    - Stable overexpression of ALDH3A1 protein was the consequence of stable overexpression of ALDH3A1 mRNA.
    - Overexpression of ALDH3A1 mRNA was not due to DNA amplification or demethylation of the relevant gene.
  - continuous exposure to gradually increasing concentrations of benz(a)pyrene over a period of several months (MCF-7/BP).
    - Stable overexpression of ALDH3A1 protein was the consequence of stable overexpression of ALDH3A1 mRNA.
    - Overexpression of ALDH3A1 mRNA was not due to DNA amplification or demethylation of the relevant gene.
  - exposure to 1 mM mafosfamide for 30 minutes (MCF-7/MAF).
    - Stable overexpression of ALDH3A1 protein was the consequence of stable overexpression of ALDH3A1 mRNA.
    - Overexpression of ALDH3A1 mRNA was not due to DNA amplification or demethylation of the relevant gene.
- Transient induction of salivary ALDH3A1 levels could be effected by short-term (a few days) ingestion of coffee or broccoli.
  - Salivary ALDH3A1 levels did not predict breast tumor ALDH3A1 levels.
- As judged by *in vitro* and *ex vivo* experiments, at least three agents, viz., gossypol and two chlorpropamide derivatives, are of potential value *in vivo* with regard to relatively selectively inhibiting ALDH3A1-catalyzed oxidative reactions, thereby sensitizing tumor cells, otherwise insensitive to the oxazaphosphorines because they express large amounts of ALDH3A1, to these agents.
  - Human tumor cell ALDH3A1 was found to be more sensitive to each of the chlorpropamide analogues than was human normal cell ALDH3A1. Thus, it may be possible to develop a clinically useful selective inhibitor of tumor cell ALDH3A1, thereby allowing the selective sensitization of tumor cells expressing large amounts of ALDH3A1 to cyclophosphamide and other oxazaphosphorines.

- Retrospective studies indicated that human breast tumor:
  - **ALDH3A1 levels are not predictive of the clinical outcome of cyclophosphamide-based chemotherapy.**
    - Failure to observe the expected inverse relationship between clinical responses to cyclophosphamide-based chemotherapeutic regimens and ALDH3A1 levels was probably because that even the highest breast tumor tissue ALDH3A1 level thus far reported appears to be below the threshold level at which ALDH3A1-catalyzed detoxification of the oxazaphosphorines becomes pharmacologically meaningful.
    - ALDH3A1 levels in certain malignancies, e.g., those of the alimentary tract and lung, may be pharmacologically meaningful.
  - **ALDH1A1 levels are predictive of the clinical outcome of cyclophosphamide-based chemotherapy.**
    - Therapeutic outcome of cyclophosphamide-based chemotherapy was correctly predicted by cellular ALDH1A1 levels in 77% of cases.
    - Frequencies of false positives (cyclophosphamide-based chemotherapy was not effective when a low level of ALDH1A1 predicted it would be) and false negatives (cyclophosphamide-based chemotherapy was effective when a high level of ALDH1A1 predicted it would not be) were 0.00 and 0.43, respectively.
- **Clinical outcome of cyclophosphamide-based chemotherapy of breast cancer as a function of glutathione has yet to be retrospectively evaluated.**

- Human breast tumor glutathione S-transferase (72-fold), DT-diaphorase (625-fold) and cytochrome P4501A1 (>570-fold) levels also vary widely.
  - Along with those of ALDH3A1, glutathione S-transferase, DT-diaphorase and cytochrome P4501A1 levels are coordinately and transiently induced (via the XRE signaling pathway) by short-term (a few days) exposure of human breast adenocarcinoma MCF-7 cells to low concentrations of 3-methylcholanthrene.
  - Along with those of ALDH3A1, glutathione S-transferase and DT-diaphorase levels, but not those of cytochrome P4501A1, are:
    - coordinately and transiently induced (via the ARE signaling pathway) by short-term (a few days) exposure of human breast adenocarcinoma MCF-7 cells to low concentrations of catechol.
    - coordinately and stably elevated (via stable upregulation of the ARE signaling pathway) by:
      - continuous exposure to gradually increasing concentrations of 4-hydroxycyclophosphamide over a period of several months (MCF-7/OAP).
      - continuous exposure to gradually increasing concentrations of benz(a)pyrene over a period of several months (MCF-7/BP).
      - exposure to 1 mM mafosfamide for 30 minutes (MCF-7/MAF).
- 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 P4501A1 catalyzes the toxification of ellipticine. Thus, the broad range of glutathione S-transferase, DT-diaphorase and cytochrome P4501A1 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. Further, attention to the composition of the diet may be prudent, and even used advantageously, when using relevant cancer chemotherapeutic agents.
  - Along with those of ALDH3A1, salivary glutathione S-transferase and DT-diaphorase levels were markedly elevated in human subjects ingesting coffee or broccoli.
  - Whereas, in some samples, the levels of ALDH3A1, glutathione S-transferase and DT-diaphorase were each elevated in the repository breast tumor samples that we examined, the frequency of occurrence was no more than that expected by chance alone, thus indicating that coordinated induction of these enzymes in breast tumors by pharmacological/dietary/environmental agents may not occur *in vivo*.

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PERSONNEL FUNDED BY AWARD

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Shoeman, Donald	Research Associate
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Kollander, Rahn	Assistant Scientist

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Rayman, Amber	Student Lab Worker
Khieu, Kelly	Student Lab Worker
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7/19/2000

DEPARTMENT OF THE ARMY
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MCMR-RMI-S (70-1y) 6 Jul 00

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FOR THE COMMANDER:

Phyllis Rinehart

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Deputy Chief of Staff for
Information Management