**Advances in Brief**

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

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**Abstract**

Molecular determinants of cellular sensitivity to cyclophosphamide, long the mainstay of chemotherapeutic regimens used to treat metastatic breast cancer, include class 1 and class 3 aldehyde dehydrogenases (ALDH-1 and ALDH-3, respectively), which catalyze the detoxification of this agent. Thus, interindividual variation in the activity of either of these enzymes in breast cancers could contribute to the wide variation in clinical responses that are obtained when such regimens are used to treat these malignancies. Consistent with this notion, ALDH-1 levels in primary and metastatic breast malignancies were found to range from 1–276 and 8–160 mIU/g tissue, respectively, and those of ALDH-3 range from 1–242 and 6–97 mIU/g tissue, respectively. ALDH-1 and ALDH-3 levels in normal breast tissue predicted the levels of these enzymes in primary and metastatic breast malignancies present in the same individuals. Confirming and extending the observations of others, levels of glutathione, a molecular determinant of cellular sensitivity to various DNA cross-linking agents including cyclophosphamide, and of DT-diaphorase, glutathione S-transferases, and cytochrome P450 IA1, each of which is known to catalyze the detoxification/toxification of one or more anticancer agents (although not of cyclophosphamide), also varied widely in primary and metastatic breast malignancies. Given the wide range of ALDH-1, ALDH-3, and glutathione levels that were observed in malignant breast tissues, measurement of their levels in normal breast tissue and/or primary breast malignancies prior to the initiation of chemotherapy is likely to be of value in predicting the therapeutic potential, or lack thereof, of cyclophosphamide in the treatment of metastatic breast cancer, thus providing a rational basis for the design of individualized therapeutic regimens when treating this disease.

**Introduction**

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

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

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

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

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3 The abbreviations used are: ALDH, aldehyde dehydrogenase; GST, glutathione S-transferase; DT-D, DT-diaphorase; CYP 1A1, cytochrome P450 1A1; GSH, glutathione; ER, estrogen receptor.
static) breast tissue samples taken from the same patients were quantitatively related. This was because, in the event that such a relationship did exist, determination of these enzyme levels in easily obtainable normal or malignant primary breast tissue samples would be of prognostic value with regard to the success, or lack thereof, that might be anticipated upon the subsequent use of an oxazaphosphorine to eliminate microscopic malignant metastatic nodules.

Xenobiotics that are abundantly present in the diet/environment, e.g., 3-methylcholanthrene and catechol, rapidly, coordinately, and reversibly induce ALDH-3, DT-D, and GSTs in human saliva (25). The sulphydryl, glutathione, appears to be yet another molecular determinant of cellular sensitivity to the oxazaphosphorines (reviewed in Ref. 5). Stable (irreversible) intrinsic as well as acquired phenotypes of this sort have also been observed in cultured human cancer models (12, 13, 22). Not known is whether coordinated elevation of these enzymes ever occurs in normal and/or malignant breast tissue. Thus, in a first attempt to address this question, DT-D, pan-GST, GST, GSTα, GSTπ, and CYP 1A1 levels in the malignant (and normal) breast tissue samples were also quantified.

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

### Materials and Methods

Normal (n=26) and malignant (n=112) female breast tissue samples obtained from 110 donors were procured from the Cooperative Human Tissue Network, Midwestern Division, Columbus, OH. Surgically removed normal and malignant breast tissue samples were snap-frozen in liquid nitrogen (within 6 h after removal), stored at -70°C (5 to 60 days), and shipped to us in dry ice. Patient characteristics, diagnoses, and cellular characteristics (Table 1) were provided by the pathology reports that accompanied the tissue specimens. Purified human GSTs α, μ, and π and affinity-purified polyclonal antibodies specific for each of these isoforms, i.e., anti-GSTα IgG, anti-GSTμ IgG, and anti-GSTπ IgG, respectively (26), were generously provided by Dr. A. J. Townsend (Department of Biochemistry, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, NC). Microsomes, isolated from a cell line (h1A1 v2) transfected with human CYP 1A1 cDNA and constitutively expressing the enzyme (15 ng CYP 1A1/mg microsomal protein), were purchased from Gentest Corporation (Woburn, MA), as was polyclonal anti-CYP 1A1 IgG (obtained from goats immunized with rat CYP 1A1). Anti-goat IgG-alkaline phosphatase conjugate was purchased from Sigma Chemical Co. (St. Louis, MO). Enhanced protein binding 96-well ELISA plates were purchased from Corning, Inc. (New York, NY). All other chemicals, reagents, and supplies were purchased from commercial sources or were prepared as described previously (7, 9, 25, 27).

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

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

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

### Table 1: Patient characteristics, diagnoses, and cellular characteristics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal breast tissue</th>
<th>Malignant breast tissue</th>
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<tbody>
<tr>
<td>Patient age</td>
<td></td>
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<tr>
<td>&lt;45</td>
<td>27 (6/22)</td>
<td>16 (13/80)</td>
</tr>
<tr>
<td>45-60</td>
<td>27 (6/22)</td>
<td>43 (34/80)</td>
</tr>
<tr>
<td>&gt;60</td>
<td>46 (10/22)</td>
<td>41 (33/80)</td>
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* A total of 26 normal and 112 malignant (90 primary and 22 metastatic) breast tissue specimens obtained from 110 donors were evaluated, but the pathology reports that accompanied these specimens did not always include all of the information listed in this table. C, carcinoma; IDC, infiltrating ductal carcinoma; ILA, infiltrating lobular adenocarcinoma; LC, lobular carcinoma; MA, mucinous adenocarcinoma; PC, papillary carcinoma; PDC, poorly differentiated carcinoma; PR, progesterone receptor; p53, tumor suppressor protein; Bcl-2, oncoprotein; c-erbB-2, oncoprotein. Known also is that 103 of the 110 donors had not been treated with a cancer chemotherapeutic agent prior to surgical removal of the tissue specimens. Unavailable was the information in that regard for the other seven donors [four normal and seven malignant (all metastatic) breast tissue specimens].
Benzaldehyde and NADP, 4 mM each, were the substrate and cofactor, respectively, when ALDH-1 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. Co-substrates were 1-chloro-2,4-dinitrobenzene and GSH, 1 mM each, when pan-GST activity was quantified.

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

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

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

The Macintosh-based STATView II (Brainpower, Inc., Calabas, CA) computer program was used to generate Ps (one- and two-tailed, unpaired, Student’s t-tests), and linear regression lines, r² (regression coefficients), and Ps thereof.
Table 2  ALDH-1, ALDH-3, DT-D, pan-GST, GSTs α, μ, and π, CYP 1A1, and GSH levels in human normal and malignant (primary and metastatic) breast tissue samples: summarya

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Normal</th>
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<td>Mean ± SD</td>
<td>Range</td>
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<td>Range</td>
<td>n</td>
<td>Mean ± SD</td>
<td>Range</td>
<td>n</td>
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<td>Mean ± SD</td>
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<td>ALDH-1</td>
<td>26</td>
<td>19 ± 19</td>
<td>3–75</td>
<td>90</td>
<td>37 ± 43</td>
<td>1–276</td>
<td>22</td>
<td>54 ± 41</td>
<td>8–160</td>
<td>138</td>
<td>36 ± 41</td>
<td>1–276</td>
<td>22</td>
<td>34 ± 27</td>
<td>6–97</td>
<td>138</td>
<td>24 ± 36</td>
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<td>ALDH-3</td>
<td>26</td>
<td>14 ± 12</td>
<td>2–54</td>
<td>90</td>
<td>24 ± 41</td>
<td>1–242</td>
<td>22</td>
<td>34 ± 27</td>
<td>6–97</td>
<td>138</td>
<td>24 ± 36</td>
<td>1–242</td>
<td>22</td>
<td>34 ± 27</td>
<td>6–97</td>
<td>138</td>
<td>24 ± 36</td>
</tr>
<tr>
<td>GSTα</td>
<td>26</td>
<td>125 ± 171</td>
<td>0–900</td>
<td>90</td>
<td>293 ± 427</td>
<td>0–2500</td>
<td>22</td>
<td>501 ± 605</td>
<td>0–1700</td>
<td>138</td>
<td>294 ± 438</td>
<td>0–2500</td>
<td>22</td>
<td>501 ± 605</td>
<td>0–1700</td>
<td>138</td>
<td>294 ± 438</td>
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<tr>
<td>GSTμ</td>
<td>26</td>
<td>96 ± 129</td>
<td>0–522</td>
<td>90</td>
<td>301 ± 560</td>
<td>0–3400</td>
<td>22</td>
<td>295 ± 654</td>
<td>0–3050</td>
<td>138</td>
<td>261 ± 529</td>
<td>0–3400</td>
<td>22</td>
<td>295 ± 654</td>
<td>0–3050</td>
<td>138</td>
<td>261 ± 529</td>
</tr>
<tr>
<td>GSTπ</td>
<td>26</td>
<td>694 ± 829</td>
<td>100–3800</td>
<td>90</td>
<td>1730 ± 1410</td>
<td>120–6200</td>
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<td>400–6500</td>
<td>138</td>
<td>1630 ± 1470</td>
<td>100–6500</td>
<td>22</td>
<td>2360 ± 1800</td>
<td>400–6500</td>
<td>138</td>
<td>1630 ± 1470</td>
</tr>
<tr>
<td>CYP 1A1</td>
<td>21</td>
<td>1 ± 5</td>
<td>0–24</td>
<td>80</td>
<td>36 ± 92</td>
<td>0–570</td>
<td>19</td>
<td>52 ± 120</td>
<td>0–485</td>
<td>120</td>
<td>33 ± 90</td>
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<td>120</td>
<td>33 ± 90</td>
<td>0–570</td>
<td>120</td>
<td>33 ± 90</td>
</tr>
</tbody>
</table>

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

A χ² test (2 × 2 table) was used to ascertain whether there were any statistically significant differences in frequencies of expression (positive or negative) of GSTα, GSTμ, and CYP 1A1 as a function of tissue type and of CYP 1A1 as a function of estrogen receptor status. This test was also used to ascertain whether the observed simultaneously elevated levels of ALDH-3, DT-D, GST (pan-GST and GSTπ), and CYP 1A1, and/or simultaneously elevated levels of ALDH-3, DT-D, and GST (pan-GST and GSTπ), were the consequence of a common or of independent event(s).

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

Shown in Figs. 1 and 2 are scatter plots of, respectively, ALDH-1 and ALDH-3 levels in normal and malignant (primary as well as metastatic) breast tissue samples. Mean values and SDs are presented in Table 2. Immediately apparent is that the level of each enzyme varies widely in all three tissues. For example, highest levels of ALDH-1 and ALDH-3 in primary breast malignancies were ~250-fold greater than the lowest levels of these enzymes in these tissues. Statistical analysis of this data revealed that mean levels of each enzyme in the malignant (primary or metastatic) breast tissue samples were significantly higher (P ≤ 0.05) than those in the normal breast tissue samples. The mean ALDH-1 level in the metastatic breast tumor samples was not significantly higher than that in the primary breast tumor samples at a P value of 0.05 but was very nearly so as the P value was 0.051.

Unexpectedly, cellular levels of ALDH-1 and ALDH-3 appeared to be directly related in the normal, as well as in the malignant (primary as well as metastatic), breast tissue samples (Fig. 3).

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

Confirming and extending the observations of others (Refs.
Levels in Human Breast Malignancies

Duplicate determinations made on single normal and malignant, or just malignant, tissue samples taken from each of 110 patients. A spectrophotometric assay was used as described in "Materials and Methods" to directly quantify DT-D catalytic activity; substrate, cofactor, and inhibitor were 2,6-dichlorophenol-indophenol (40 μM), NADH (160 μM), and dicumarol (10 μM), respectively. Points are means (rounded off for clarity of presentation to zero if they were <50 mIU/g, and to 100 mIU/g or the nearest multiple thereof if they were ≥50 mIU/g) of duplicate determinations made on single normal and malignant, or just malignant, tissue samples taken from each of 110 patients.

![Figure 5](image1.png)

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

![Figure 6](image2.png)

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

30 and 31; reviewed in Ref. 32), DT-D, pan-GST, GSTα, GSTμ, GSTπ, and CYP 1A1 levels also varied widely in normal and malignant (primary and metastatic) breast tissue (Figs. 5–10 and Table 2). As in the cases of ALDH-1 and ALDH-3, statistical analysis of the data revealed that mean levels of each enzyme in the malignant (primary or metastatic) breast tissue samples were significantly higher (P ≤ 0.05) than those in the normal breast tissue samples with one exception, metastatic breast tumor tissue GSTμ (P = 0.084). The mean pan-GST level in the metastatic breast tumor samples was significantly higher (P = 0.041) than that in the primary breast tumor samples. Mean DT-D, GSTα, GSTμ, GSTπ, and CYP 1A1 levels in the metastatic breast tumor samples were not significantly different at a P level of 0.05 than those in the primary breast tumor samples, although those of GSTα (P = 0.061) and GSTπ (P = 0.058) were very nearly so.

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

As expected, tissue levels of GSTα, GSTμ, GSTπ, and pan-GST were directly related to each other when all of the data (n = 138) was grouped (P ≤ 0.0001 in each case; linear regression analyses of data not shown). Similarly, there was a direct relationship between pan-GST and ALDH-1, ALDH-3, and DT-D levels (P = 0.01, 0.02, and 0.003, respectively; linear regression analyses of data not shown). However, DT-D levels were not related to those of ALDH-1 or ALDH-3 (P = 0.33 and 0.42, respectively), and CYP 1A1 levels were unrelated (P > 0.1) to those of the other enzymes (linear regression analyses of data not shown).

Evidence (levels that are each more than 1 SD above normal breast tissue mean levels) for the coordinated induction of ALDH-3, DT-D, pan-GST, and CYP 1A1 (induced gene expression effected by transactivation of a cis-acting DNA element, xenobiotic responsive element, present in the 5'–upstream regions of the genes coding for these enzymes; Refs. 13 and...
These zero values are not shown in this figure.

taken from each of 110 patients. GSTα was not detected in 4 of 26 tumor samples,
to zero if they were >0 and <25 mIU/g, and to 50 mIU/g or the nearest multiple thereof if they were ≥25 mIU/g) of duplicate determinations made on single normal and malignant, or just malignant, tissue samples taken from each of 110 patients. GSTα was not detected in 4 of 26 (15%) normal breast tissue samples, 14 of 90 (16%) primary breast tumor samples, and 5 of 22 (23%) metastatic breast tumor samples. These zero values are not shown in this figure.

33–41) was observed in only three samples, two primary and one metastatic breast tumors (Table 3). As judged by the same criteria, coordinated induction of ALDH-3, DT-D, and pan-GST, but not of CYP 1A1 (induced gene expression effected by transactivation of a cis-acting DNA element, antioxidant responsive element, present in the 5'-upstream regions of the genes coding for these enzymes; Refs. 13 and 34–41) was observed in only seven additional samples, four primary and three metastatic breast tumors (Table 3). In one case, sample 6, a normal breast sample obtained from the same patient was available. ALDH-3 and DT-D levels in this sample were each more than 2 SDs, and pan-GST and GSTπ levels were nearly (~0.86) 1 SD, above corresponding normal breast tissue mean values; CYP 1A1 was not detected in this sample (data not shown).

DT-D, pan-GST, GSTα, GSTμ, GSTπ, and CYP 1A1 levels in normal breast tissue did not predict (P > 0.1) for corresponding enzyme levels in paired, primary, or metastatic breast tumor tissue (linear regression analyses of data not shown). However, detectable GSTμ was always absent in malignant breast tissue samples when it was not found in normal breast tissue samples obtained from the same patient (n = 11), as would be expected if the absence of this enzyme was due to a GSTμ null genotype (reviewed in Ref. 41; data not shown). In contrast, GSTα was detected in two of three malignant breast tissue samples obtained from patients from which normal breast tissue samples lacked detectable levels of this enzyme; moreover, in five cases, GSTα was found in normal, but not malignant, breast tissue (data not shown). In the case of CYP 1A1, 15 of 19 malignant breast tissue samples tested negative when the paired normal breast tissue samples tested negative, and one malignant breast tissue sample tested negative when the paired normal breast tissue sample tested positive (data not shown). We did not have enough paired samples to ascertain whether cellular levels of these enzymes in primary breast malignancies pre-
dicted cellular levels of the corresponding enzyme in metastatic breast malignancies.

Again confirming and extending the observations of others (42, 43), GSH levels, also, varied widely in normal and malignant (primary and metastatic) breast tissue samples (Fig. 11 and Table 2). As in the cases of the enzymes, statistical analysis of the data revealed that mean levels of GSH in the malignant (primary or metastatic) breast tissue samples were significantly higher ($P \leq 0.05$) than that in the normal breast tissue samples. The mean GSH level obtained for metastatic breast tumor samples was not significantly higher ($P > 0.1$) than that obtained for the primary breast tumor samples.

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

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

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

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

Discussion

Given that: (a) metastatic breast cancer is usually treated with a combination of chemotherapeutic agents (reviewed in Refs. 1–4); (b) one of these agents is virtually invariably cyclophosphamide (reviewed in Refs. 1–4); (c) established molecular determinants of cellular sensitivity to cyclophosphamide and other oxazaphosphorines include ALDH-1 and ALDH-3 (reviewed in Refs. 5 and 6; Refs. 7–18); (d) ALDH-1 and ALDH-3 each catalyze the detoxification of cyclophosphamide and other oxazaphosphorines (reviewed in Refs. 5, 6, and 13; Ref. 16); and (e) ALDH-1 and ALDH-3 levels vary widely in primary and metastatic breast tumors as reported herein, it follows that the wide range of clinical responses to cyclophosphamide (oxazaphosphorine)-based combination chemotherapy of metastatic breast cancer must be, at least in part, due to the substantial variability of GSH levels in these malignancies.

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

Cancer chemotherapeutic strategies could be beneficially individualized even further if cellular levels of DT-D, the GSTs, and CYP 1A1 in metastatic breast tumors were also taken into account because these enzymes are known to catalyze the biotransformation of various anticancer agents, e.g., mitomycin C and EOP; melphalan and chlorambucil; and ellipticine, respectively (reviewed in Refs. 45, 47, and 48), and the levels of these enzymes in metastatic breast tissue vary widely. Perhaps most relevant with regard to the chemotherapeutic treatment of breast

<table>
<thead>
<tr>
<th>Sample</th>
<th>Malignancy</th>
<th>ALDH-3</th>
<th>DT-D</th>
<th>pan-GST</th>
<th>GST(\pi)</th>
<th>CYP 1A1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Primary</td>
<td>28</td>
<td>5520</td>
<td>2630</td>
<td>2100</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Primary</td>
<td>35</td>
<td>4310</td>
<td>7280</td>
<td>6200</td>
<td>177(a)</td>
</tr>
<tr>
<td>3</td>
<td>Primary</td>
<td>39</td>
<td>6250</td>
<td>4870</td>
<td>1900</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>Primary</td>
<td>45</td>
<td>1410</td>
<td>3000</td>
<td>2900</td>
<td>206(b)</td>
</tr>
<tr>
<td>5(c)</td>
<td>Primary</td>
<td>171</td>
<td>1890</td>
<td>4450</td>
<td>3600</td>
<td>5</td>
</tr>
<tr>
<td>6(d)</td>
<td>Primary</td>
<td>231</td>
<td>2950</td>
<td>5180</td>
<td>4150</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>Metastatic</td>
<td>28</td>
<td>1730</td>
<td>5650</td>
<td>3850</td>
<td>ND(e)</td>
</tr>
<tr>
<td>8</td>
<td>Metastatic</td>
<td>53</td>
<td>1510</td>
<td>6430</td>
<td>3800</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>Metastatic</td>
<td>64</td>
<td>1600</td>
<td>4630</td>
<td>3900</td>
<td>485(f)</td>
</tr>
<tr>
<td>10(f)</td>
<td>Metastatic</td>
<td>97</td>
<td>2540</td>
<td>7600</td>
<td>6500</td>
<td>0</td>
</tr>
</tbody>
</table>

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

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

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

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

\(e\) ND, not determined.

\(f\) Samples in which ALDH-3, DT-D, pan-GST, and GST\(\pi\) levels were each \(>1\) SD above their respective metastatic breast tumor tissue mean values (Table 2).
cancer is that DT-D catalyzes the activation of EO9 (47), an agent that shows preclinical promise in the treatment of this malignancy (24). Thus, EO9 may be of minimal value when DT-D levels are low but of substantial value when the levels of this enzyme are high. A potential problem is that DT-D, GST, and CYP 1A1 levels in normal breast tissue apparently do not predict the levels of these enzymes in malignant breast tissues. Whether DT-D, GST, and/or CYP 1A1 levels in primary breast malignancies predict the respective levels of these enzymes in metastatic breast malignancies remains to be determined.

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

Knowledge of GSH levels in metastatic breast tumor tissue could also be of value in the rational design of the conventional and high-dose cancer chemotherapeutic strategies that are ultimately used to treat breast cancer patients with metastatic disease because cellular sensitivity to various anticancer agents, e.g., the oxazaphosphorines and melphalan, decreases as cellular levels of GSH increase (reviewed in Refs. 5 and 45), and the level of GSH in metastatic breast tissue varies widely. Again, however, a potential problem is that GSH levels in normal breast tissue apparently do not predict the levels of GSH in malignant breast tissues. In this case, also, whether the levels of this determinant in primary breast malignancies predict the respective levels of it in metastatic breast malignancies remains to be determined.

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

Inhibitors of ALDH-1-mediated catalysis, ALDH-3-mediated catalysis, and GSH synthesis would provide the basis for even further beneficial individualization of the chemotherapeutic regimen. Inclusion of an inhibitor of ALDH-1 could especially be of therapeutic value.

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

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

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

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

Uncertain is whether elevated levels of ALDH-3, DT-D, and the GSTs, and/or elevated levels of these enzymes and of CYP 1A1 were ever the consequence of coordinated induction effected either by a relevant mutation (enzyme levels are stably elevated) or by the introduction of certain dietary or pharmacological agents that transiently induce the expression of these enzymes (enzyme levels return to basal levels within days upon cessation of inducer intake). Malignant breast tissue levels of ALDH-3, DT-D, GST, and CYP 1A1 were each more than 1 SD above normal breast tissue mean levels in 3 of 99 samples (observed frequency of 0.030), and malignant breast tissue levels of ALDH-3, DT-D, and GST, but not those of CYP 1A1, were greater than 1 SD above normal breast tissue mean levels in 10 of 110 samples (observed frequency of 0.091). Expected frequencies, based on the assumption that elevated expression (level greater than 1 SD above normal breast tissue mean level) of these enzymes is the consequence of independent events, were 0.011 and 0.048, respectively. As judged by \( \chi^2 \) analysis, observed frequencies did not differ significantly (\( P = 0.09 \) and 0.13, respectively) from expected frequencies. The reader is advised that the choice of 1 SD above normal breast tissue mean values as indication of coordinated induction was entirely arbitrary. The clinical ramifications of coordinated enzyme induction by pharmacological and/or dietary/environmental agents

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

<table>
<thead>
<tr>
<th>Tissue and group</th>
<th>ALDH-1</th>
<th>ALDH-3</th>
<th>DT-D</th>
<th>pan-GST</th>
<th>GST( \alpha )</th>
<th>GST( \mu )</th>
<th>GST( \tau )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;45 y vs 45–60 y</td>
<td>0.134</td>
<td>0.136</td>
<td>0.378</td>
<td>0.362</td>
<td>0.460</td>
<td>0.466</td>
<td>0.323</td>
</tr>
<tr>
<td>&lt;45 y vs &gt;60 y</td>
<td>0.973</td>
<td>0.370</td>
<td>0.320</td>
<td>0.713</td>
<td>0.967</td>
<td>0.054</td>
<td>0.941</td>
</tr>
<tr>
<td>45–60 y vs &gt;60 y</td>
<td>0.023</td>
<td>≤0.0001</td>
<td>0.719</td>
<td>0.045</td>
<td>0.002</td>
<td>0.290</td>
<td>0.030</td>
</tr>
<tr>
<td>ER( ^+ ) vs ER( ^- )</td>
<td>0.716</td>
<td>0.651</td>
<td>0.092</td>
<td>0.822</td>
<td>0.010</td>
<td>0.303</td>
<td>0.326</td>
</tr>
<tr>
<td>PR( ^+ ) vs PR( ^- )</td>
<td>0.750</td>
<td>0.171</td>
<td>0.223</td>
<td>0.186</td>
<td>0.001</td>
<td>0.520</td>
<td>0.062</td>
</tr>
<tr>
<td>Primary</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;45 y vs 45–60 y</td>
<td>0.931</td>
<td>0.481</td>
<td>0.843</td>
<td>0.350</td>
<td>0.926</td>
<td>0.001</td>
<td>0.912</td>
</tr>
<tr>
<td>&lt;45 y vs &gt;60 y</td>
<td>0.250</td>
<td>0.688</td>
<td>0.008</td>
<td>0.962</td>
<td>0.196</td>
<td>0.071</td>
<td>0.616</td>
</tr>
<tr>
<td>45–60 y vs &gt;60 y</td>
<td>0.185</td>
<td>0.346</td>
<td>0.004</td>
<td>0.327</td>
<td>0.152</td>
<td>0.056</td>
<td>0.706</td>
</tr>
<tr>
<td>ER( ^+ ) vs ER( ^- )</td>
<td>0.799</td>
<td>0.635</td>
<td>0.281</td>
<td>0.188</td>
<td>0.590</td>
<td>0.136</td>
<td>0.096</td>
</tr>
<tr>
<td>PR( ^+ ) vs PR( ^- )</td>
<td>0.885</td>
<td>0.777</td>
<td>0.335</td>
<td>0.665</td>
<td>0.886</td>
<td>0.022</td>
<td>0.283</td>
</tr>
<tr>
<td>p53( ^+ ) vs p53( ^- )</td>
<td>0.120</td>
<td>0.005</td>
<td>0.509</td>
<td>0.571</td>
<td>0.706</td>
<td>0.500</td>
<td>0.097</td>
</tr>
<tr>
<td>Metastatic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;45 y vs 45–60 y</td>
<td>0.204</td>
<td>0.407</td>
<td>0.136</td>
<td>0.538</td>
<td>0.918</td>
<td>0.214</td>
<td>0.980</td>
</tr>
<tr>
<td>&lt;45 y vs &gt;60 y</td>
<td>0.937</td>
<td>0.214</td>
<td>0.049</td>
<td>0.144</td>
<td>≤0.0001</td>
<td>0.055</td>
<td>0.324</td>
</tr>
<tr>
<td>45–60 y vs &gt;60 y</td>
<td>0.019</td>
<td>0.031</td>
<td>0.549</td>
<td>0.016</td>
<td>≤0.0001</td>
<td>0.001</td>
<td>0.343</td>
</tr>
<tr>
<td>ER( ^+ ) vs ER( ^- )</td>
<td>0.116</td>
<td>0.751</td>
<td>0.303</td>
<td>0.006</td>
<td>0.059</td>
<td>0.265</td>
<td>0.008</td>
</tr>
<tr>
<td>PR( ^+ ) vs PR( ^- )</td>
<td>≤0.0001</td>
<td>0.002</td>
<td>0.632</td>
<td>0.037</td>
<td>0.027</td>
<td>0.930</td>
<td>0.057</td>
</tr>
</tbody>
</table>

\( P \) (unpaired, two-tailed, Student's \( t \) test)

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

5 ALDH-3, DT-D, GST (pan-GST and GST\( \tau \)), and CYP 1A1 levels were >1 SD above their respective normal breast tissue mean values in 30, 28, 43, and 29 of 99 samples, respectively. Therefore, the expected frequency = (0.303) (0.283) (0.434) (0.293) = 0.091. Expressed frequencies, based on the assumption that elevated expression (level greater than 1 SD above normal breast tissue mean level) of these enzymes is the consequence of independent events, were 0.011 and 0.048, respectively. As judged by \( \chi^2 \) analysis, observed frequencies did not differ significantly (\( P = 0.09 \) and 0.13, respectively) from expected frequencies. The reader is advised that the choice of 1 SD above normal breast tissue mean values as indication of coordinated induction was entirely arbitrary. The clinical ramifications of coordinated enzyme induction by pharmacological and/or dietary/environmental agents

6 ALDH-3, DT-D, and GST (pan-GST and GST\( \tau \)) levels were >1 SD above their respective normal breast tissue mean values in 32, 38, and 53 of 110 samples, respectively. Therefore, the expected frequency = (0.303) (0.283) (0.434) (0.293) = 0.091. Expressed frequencies, based on the assumption that elevated expression (level greater than 1 SD above normal breast tissue mean level) of these enzymes is the consequence of independent events, were 0.011 and 0.048, respectively. As judged by \( \chi^2 \) analysis, observed frequencies did not differ significantly (\( P = 0.09 \) and 0.13, respectively) from expected frequencies. The reader is advised that the choice of 1 SD above normal breast tissue mean values as indication of coordinated induction was entirely arbitrary. The clinical ramifications of coordinated enzyme induction by pharmacological and/or dietary/environmental agents
are potentially substantial, especially with regard to chemotherapeutic strategies. These have been detailed elsewhere (22, 25).

Unexpectedly, cellular levels of ALDH-1 and ALDH-3 appeared to be directly related in normal and malignant (primary as well as metastatic) breast tissue. This finding was unexpected because these enzymes are not known to have anything in common with regard to regulation of their expression. At this time, then, this observation can only be viewed as a curiosity.

In an effort to substantiate the contentsions made herein with direct evidence, attempts are currently being made to collect information as to how the specimen donors were subsequently treated and, in those cases where anticancer agents were given, the clinical responses thereto. Clinical responses to anticancer drugs as a function of cancer drugs as a function of time, then, this observation can only be viewed as a curiosity.

In an effort to substantiate the contentsions made herein with direct evidence, attempts are currently being made to collect information as to how the specimen donors were subsequently treated and, in those cases where anticancer agents were given, the clinical responses thereto. Clinical responses to anticancer drugs as a function of time, then, this observation can only be viewed as a curiosity.

Acknowledgments

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Cellular levels of class 1 and class 3 aldehyde dehydrogenases and certain other drug-metabolizing enzymes in human breast malignancies.

L Sreerama and N E Sladek


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