Effects of α-Difluoromethylornithine on Markers of Proliferation, Invasion, and Apoptosis in Breast Cancer

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Abstract. *Aim: To examine changes in biomarkers expressed in breast tumors in response to patient treatment with the polyamine synthesis inhibitor α-difluoromethylornithine (DFMO). Patients and Methods: The expression of Ki-67 (MIB-1), matrix metalloproteinases (MMP) 2 and 9, urokinase-type plasminogen activator (uPA) were examined by immunohistochemistry in breast tissue specimens (controls: n=15, 13 evaluable, and DFMO group; n=27, 21 evaluable). Apoptosis was evaluated by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL). Results: Significant increases in apoptosis, MMP-9 and uPA (tumor) were observed in 7 patients ≥50 years who received DFMO for ≥14 days relative to patients <50 years and/or who received <14 days of treatment (n=11). No other measured characteristics, including tumor estrogen and progesterone receptor status, hormone replacement therapy history, histopathological characteristics or tumor grade were correlated with these biomarker changes. Conclusion: Unexpected correlation of proapoptotic DFMO activity in postmenopausal women with breast cancer warrants further study.*

The incidence of breast cancer has been increasing steadily over the past several decades. In the past 15 years, the mortality due to breast cancer has declined somewhat due to advances in early detection and the development of more effective treatments. The National Surgical Adjuvant Breast and Bowel Project (NSABP) P1 and P2 trials have evaluated the effects of tamoxifen and raloxifene on the incidence of invasive breast cancer in high-risk women (1, 2). Compared to placebo, tamoxifen treatment resulted in a 69% decrease in the occurrence of invasive estrogen receptor (ER)-positive breast cancer but it did not significantly alter the occurrence of ER-negative cancer. In another study that compared tamoxifen and raloxifene in ER-negative breast cancer, no benefit was found for treatment with α-difluoromethylornithine (DFMO) (3). Given the enormous toll exacted by breast cancer, it is vitally important to explore potential therapies that could be effective in reducing the incidence of ER-negative disease.

Polyamines are formed by the enzymatic decarboxylation of the amino acids ornithine and arginine and are involved in cellular proliferation, differentiation, migration and apoptosis. Ornithine decarboxylase (ODC) is the rate-limiting enzyme in the polyamine synthesis pathway (4). ODC is essential for normal cellular functions including normal growth and tissue repair. Overexpression of ODC, with accumulation of intracellular polyamines, is thought to play an important role in breast cancer proliferation and progression to a hormone-independent, aggressive phenotype. DFMO is an orally available irreversible inhibitor of ODC that has a relatively favorable systemic toxicity profile (5). Inhibition of ODC-mediated polyamine synthesis by DFMO has been shown to thwart proliferation of both ER-positive and ER-negative breast tumors in both *in vitro* and *in vivo* studies (6-8).

Low daily doses of DFMO have been evaluated in phase I studies. A daily dose of 0.5 g/m^2 was the lowest dose that was able to achieve at least 50% inhibition of ODC activity in one phase I trial (9). A randomized phase II chemoprevention trial was conducted with low-dose DFMO (0.5 g/m^2) in 119 women at high risk of developing breast cancer (10). A modest reduction in urine polyamines was observed but the biological breast cancer risk markers, including p53, proliferating cell nuclear antigen (PCNA), and epidermal growth factor receptor (EGFR), were not modulated, suggesting that this dose of DFMO may be sub-optimal in modulating proliferation of breast intraepithelial neoplasia.

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High-dose DFMO has also been evaluated in cancer patients (11). A DFMO dose of 4.8 g/m^2, three times a day for 14 days followed by a 2-week drug break was chosen for the treatment of 22 patients with metastatic breast cancer. Urinary polyamine levels were suppressed by the treatment and remained low during the 2-week break. There was no objective tumor response in the 18 evaluable patients. An alternate DFMO dose regimen with 3.6 g/m^2 administered three times a day for 14 days followed by a 1-week drug holiday was evaluated in 98 patients with recurrent glioma (12). Ototoxicity was the major toxicity, with 14% of patients developing grade 3 hearing loss. Antitumor activity was seen in patients with anaplastic gliomas as well in patients with glioblastoma multiforme (13). Based on these experiences indicating some degree of antitumor activity, both lower and higher doses of DFMO were evaluated in this pre-operative study in early breast cancer.

We have assessed the effects of DFMO on markers of breast cancer proliferation, invasion and apoptosis in the tumors of patients diagnosed with ductal carcinoma *in situ* (DCIS) and/or early-stage invasive breast cancer. Tumor proliferation was evaluated by Ki-67/MIB-1 expression. Metastatic potential was evaluated by measuring expression of matrix metalloproteinase (MMP) 2 and 9, and of urokinase-type plasminogen activator (uPA) fibroblast and tumor (uPA(F), uPA(T)). Apoptosis induction was measured by terminal deoxyribonucleotide transferase-mediated dUTP nick-end labeling (TUNEL) assay.

Patients and Methods

Patients. Forty-three patients newly diagnosed with DCIS and/or invasive breast cancer agreed to a study of biomarkers in their diagnostic core biopsies and in the tissue removed at definitive surgical excision. The interval between the initial biopsy and definitive excision was approximately 10 to 14 days. Twenty-seven patients received oral DFMO between the initial core biopsy and the tumor excision. Sixteen patients declined DFMO treatment but agreed to have their tissues analyzed as untreated controls. The treatment arm patients received either 0.5 g/m^2 daily (n=11) or 3 g/m^2 daily (n=16) in two sequential cohorts in order to study whether the possible antiproliferative and proapoptotic effects of DFMO are dose dependent. A dose of 0.5 g/m^2/d was chosen by the NCI Chemoprevention Branch as the optimal daily dose for phase II chemoprevention trials (10). The dose of 3.0 g/m^2/d, which has been shown to be safe in advanced cancer studies, was chosen to allow evaluation of any superior benefit relative to the lower dose. Patient demographic data are presented in Table I. The investigations were performed after approval by a local Institutional Review Board (IRB) and in accordance with an assurance filed with and approved by the U.S. Department of Health and Human Services. All patients signed an IRB-approved informed consent. The study (#DFMO232) was supported by Ilex Oncology, San Antonio, TX, USA.

Biomarker analysis. Expression level of the proliferation marker Ki-67/MIB-1 and four biomarkers of tumor invasiveness: MMP-2 and MMP-9, uPA(F) and uPA(T), were evaluated in paraffin sections from preoperative biopsy tissue and tumor resected by surgery by immunostaining with human-specific antibodies; apoptosis was determined by TUNEL assay.

Table I. *Patient demographics*.

Patient characteristic	DFMO group	Control group
Number of patients	27	15
Median age (years)	51	56
On HRT at diagnosis	11	4
Infiltrating ductal	26	13
Infiltrating lobular	6	4
DCIS only	4	1
ER$^+$	16	12
PR$^+$	19	12

HRT, Hormone replacement therapy; DCIS, ductal carcinoma *in situ*; ER, estrogen receptor; PR, progesterone receptor.

Ki-67/MIB-1 determination. Anti-human Ki-67 (clone MIB-1) monoclonal murine antibody was obtained from DAKO Cytomation (Carpinteria, CA, USA) and used at dilutions of 1:100 and 1:50. Preparation of the samples was carried out as described elsewhere (14). The percentage of MIB-1-positive cells was counted on the entire section in one slide per patient (15).

MMP-2 determination. MMP-2 monoclonal murine antibody (Lab Vision Corp., Neo Markers, Fremont, CA, USA) was used at 1:100 dilution with normal placenta as a control. The expression of MMP-2 was determined using the scoring system of Allred *et al*. (15) whereby immunostaining signals are assigned a proportion score (PS, range 0-5) that represents the estimated proportion of positive tumor cells on the entire slide and an intensity score (IS) that estimates the average staining intensity of positive tumor cells (range 0-3). The PS and IS are added to obtain a total score (TS, range 0-8) (15). The numbers of cells on each slide (one slide per patient) and their TS were determined.

MMP-9 determination. Anti-MMP-9 polyclonal antibody (R&D Systems, Minneapolis, MN, USA) was used at 1:20 dilution. Normal human placenta was used as a control. The expression of MMP-9 was determined using the scoring method of Allred *et al*. (15).

Urokinase plasminogen activator determination. Anti-uPA monoclonal antibody (American Diagnostica, Stamford, CT, USA) was used at a dilution of 1:20, with a normal kidney specimen used as a control. Blocking, incubations with antibodies and diaminobenzidine (DAB) staining were carried out in a humidity chamber, and samples were scored as above (14).

In situ apoptosis determination. Apoptosis was determined *in situ* by TUNEL following the standard procedure. TUNEL indices were calculated by counting 500 tumor cells.

Statistical analysis. Biomarkers in tumor biopsy and tumor excision specimens were compared in the control group and in the DFMO groups and identified subgroups using a one-tailed Student's *t*-test assuming heteroskedastic variance with *p*-values less than or equal to 0.05 considered significant.

Table II. *Histological results in the control group*.

ID	Control group	Ki-67%	MMP-2	MMP-9	uPA(F)	uPA(T)	TUNEL%
C1	Pre-control	1	4	7	7	7	0
	Post-control	11	5	7	7	7	3
C2	Pre-control	2	5	7	6	7	6
	Post-control	3	5	7	6	7	2
C3	Pre-control	24	4	5	7	7	12
	Post-control	3	3	5	5	7	8
C4	Pre-control	5	0	6	4	6	2
	Post-control	33	0	7	7	7	7
C5	Pre-control	25	2	5	6	7	7
	Post-control	20	0	7	7	7	15
C6	Pre-control	nd	4	7	nd	nd	nd
	Post-control	11	5	6	5	6	5
C7	Pre-control	25	2	6	3	4	9
	Post-control	23	4	6	4	6	12
C8	Pre-control	5	0	6	4	6	3
	Post-control	nd	nd	nd	nd	nd	nd
C9	Pre-control	4	0	5	3	4	2
	Post-control	9	0	6	4	5	2
C10	Pre-control	4	6	6	7	7	1
	Post-control	nd	nd	nd	nd	nd	nd
C11	Pre-control	3	3	5	5	5	2
	Post-control	1	0	5	7	7	0
C12	Pre-control	7	0	7	6	7	3
	Post-control	8	2	7	6	7	1
C13	Pre-control	52	3	4	7	7	10
	Post-control	5	0	6	7	7	1
C14	Pre-control	31	0	6	7	7	17
	Post-control	31	0	5	7	7	4
C15	Pre-control	0	2	7	6	7	1
	Post-control	3	2	6	6	7	0

nd: Not determined.

Results

Because of the limitations imposed by the relatively small size of the diagnostic core biopsy specimens, biomarker expression determination was not possible in all patients. Data were first sorted to remove patients with incomplete sets of measurements. As a result, evaluable data from 22 preoperative and 21 postoperative measurements from the DFMO groups and 14 preoperative and 12 postoperative tissue specimens from the control group were collected. The data for the control and DFMO groups are presented in Tables II and III, respectively.

In the control group, there were no significant differences in tumor cell proliferation and invasiveness as measured by Ki-67, MMP-2 and MMP-9 expression levels. No significant differences in apoptosis were seen as measured by TUNEL between tumor core biopsies and the corresponding surgically removed tumors. The one-tailed *p*-values were 0.254, 0.300, 0.257, 0.286, 0.141, and 0.198 for Ki-67, MMP-2, MMP-9, uPA(F), uPA(T) and TUNEL, respectively.

Analysis of biomarker expression in both DFMO dose groups together revealed no significant differences in the tissue biomarkers pre- and post-DFMO except for TUNEL. The one-tailed *p*-values were 0.306, 0.360, 0.447, 0.152, and 0.409 for Ki-67, MMP-2, MMP-9, uPA(F), and uPA(T), respectively. There was a trend towards increased apoptosis (TUNEL) after DFMO exposure (p=0.056).

Analysis of the DFMO patients by dose revealed no statistically significant differences between pre- and post-treatment biomarker expression in pair-wise comparison of the 0.5 g/m^2/d and the 3.0 g/m^2/d groups except that the 3.0 g/m^2/d group had greater decreases in MMP-2 levels post-treatment (p=0.052) relative to the lower dose group (Table IV).

Comparison of the biomarker expression levels in the post-DFMO resected tissues of patients who received 0.5 g/m^2/d of DFMO to the pre-treatment values of all patients (DFMO and control) yielded *p*-values of 0.452, 0.073 (decrease with DFMO), 0.158, 0.105, 0.260 and 0.093 for Ki-67, MMP-2, MMP-9, uPA(F), uPA(T) and TUNEL, respectively.

Comparison of the biomarker expression levels evident in the post-DFMO resected tissues of patients who received 3.0 g/m^2/d of DFMO to the pre-treatment values of all patients (DFMO and control) yielded *p*-values of 0.403, 0.053 (decrease with DFMO), 0.086, 0.115, 0.200 and 0.144 for Ki-67, MMP-2, MMP-9, uPA(F), uPA(T) and TUNEL, respectively.

Comparison of the markers in biopsies of the control group specimens to the pre-treatment drug treatment group biopsy specimens revealed a borderline significant decrease in uPA(F) (p=0.051) and significantly increased TUNEL (p=0.040) values for the control group. This observation is possibly due to the relatively limited volume of the biopsy specimens and the attendant risk of non-representative sampling giving rise to erroneous determinations.

During the initial evaluation of the data we noticed the tendency for patients with more DFMO treatment days to show greater changes in biomarker expression. In addition, we observed that patients older than 50 years tended to have more significant changes in biomarker levels. Thus, bearing in mind the limited statistical power of the smaller sample set sizes we analyzed the data as described above.

Segregating those patients that completed more than 14 days of treatment (n=16) and comparing their biomarker expression levels to those in all of the pre-treatment core biopsies in the DFMO group yielded p-values of 0.213, 0.342, 0.409, 0.129, 0.395 and 0.032 (increase) for Ki-67, MMP-2, MMP-9, uPA(F), uPA(T) and TUNEL, respectively. Comparison of the >14-day treatment group post treatment biomarker values to those of all study patients (DFMO and control groups) pre-treatment core biopsy biomarker levels yielded p-values of 0.150, 0.423, 0.384, 0.301, 0.477 and 0.078 for MIB-1, MMP-2, MMP-9, uPA(F), uPA(T) and TUNEL, respectively.

Segregating those patients older than 50 years (n=10) and comparing their biomarker expression levels pre- and post-treatment yielded p-values of 0.388, 0.318, 0.060 (increase), 0.073 (decrease), 0.057 (increase) and 0.107 for Ki-67, MMP-2, MMP-9, uPA(F), uPA(T) and TUNEL respectively. Comparing of biomarker values for the group aged >50 years to those of all study patients (drug and control groups), pretreatment (biopsy) biomarker levels yielded p-values of 0.481, 0.322, 0.049 (increase), 0.114, 0.014 (increase), 0.085 for Ki-67, MMP-2, MMP-9, uPA(F), uPA(T) and TUNEL, respectively. Comparison of the biomarker levels of those aged >50 years to the levels measured in the sample set consisting of all pre-dose drug treated (biopsy), pre-dose (biopsy) control and post-dose control (surgical) patient specimens yielded p-values of 0.491, 0.363, 0.055 (increase), 0.348, 0.002 (increase) and 0.138 for Ki-67, MMP-2, MMP-9, uPA(F), uPA(T) and TUNEL, respectively. No statistically significant differences were observed for analogous comparisons within the group of patients aged less than 50 years.

To investigate the possibility of synergy of patient age and treatment duration, we evaluated biomarker levels in the double stratification group of patients older than 50 years who were on treatment for more than 14 days (n=7). Comparing the pre-treatment to post-treatment-values in this subset yielded p-values of 0.496, 0.232, 0.101, 0.212, 0.053 (increase), and 0.074 (increase) for Ki-67, MMP-2, MMP-9, uPA(F), uPA(T) and TUNEL, respectively. Comparison of the levels for this subset to levels measured in all pre treatment (biopsy) specimens yielded p-values of 0.353, 0.263, 0.129, 0.269, 0.006 (increase) and 0.089 for Ki-67, MMP-2, MMP-9, uPA(F), uPA(T) and TUNEL, respectively.

Finally, we evaluated the effects of tumor histologic subtype on biomarker outcome measurements. The patients were separated into groups based on diagnostic subtype including invasive ductal carcinoma (IDCA), invasive lobular carcinoma (ILCA) and DCIS. Insufficient data were available to extend the analyses to other classes, for example node-positive *vs*. node-negative patients. The only statistically significant difference was found for Ki-67 (p=0.038; decrease) levels in the comparison of non-IDCA (ILCA and DCIS) patients pre-dose *vs*. post-dose in the treatment group (n=12). A trend towards increased (p=0.072) apoptosis (TUNEL) was observed in the comparison of pre- *vs*. post-dose IDCA patients. Tables IV and V give a complete listing of p-values derived from the statistical analyses of the patients grouped by dosage, tumor type, treatment days and age. In addition, we investigated the relation of pre-diagnosis hormone replacement therapy (HRT) status to treatment related biomarker status. We were unable to discern any statistically significant change in the measured values for any of the biomarkers evaluated when comparing groups of patients segregated by pre-diagnosis HRT status (data not shown).

Discussion

In this study, we investigated the effects of DFMO treatment on tumor proliferation, invasiveness and apoptosis in breast cancer patients' diagnostic core biopsies prior to surgery and on their resected tissues at definitive surgery. The size of the study group and the sizes of the important subgroups therein limit the statistical strength of the observations but, taken together, the results demonstrate that DFMO has significant effects on several biomarkers associated with apoptosis and invasiveness. We observed that patients who took DFMO for 14 or more days showed a statistically significant increase in tumor cell apoptosis compared to pre-treatment values. In addition, we observed a trend towards increased apoptosis in specimens from patients who were 50 years of age or older over those from patients less than 50 years old. Data from specimens from patients in the older group who took 14 or more days of treatment (n=7) were compared to the pre-treatment biomarker values in the entire DFMO treatment group and this showed increased apoptosis along with significantly increased levels of uPA(T) (p=0.014). In addition, these patients' tumors showed borderline significant increases in MMP-9 expression relative to pre-treatment levels (p=0.054). This observation is consistent with those reported by Stabellini *et al*. who showed that DFMO treatment tended to increase expression of MMP-1 and MMP-2 in cultured

Table III. *Immunohistochemical results in DFMO group treated with A: 0.5g/m^2/d and B: 3g/m^2/d.*

A

ID	Treatment	Ki-67%	MMP-2	MMP-9	uPA(F)	uPA(T)	TUNEL%
D1	Pre-DFMO	7	3	6	7	6.5	0
	Post-DFMO	2	2	6	6	7	2
D3	Pre-DFMO	35	3	6	7	7	2
	Post-DFMO	23	2	5	7	7	4
D10	Pre-DFMO	11	8	5	7	7	1
	Post-DFMO	0	2	7	6	7	1
D14	Pre-DFMO	11	0	6	7	7	2
	Post-DFMO	21	0	7	6	8	26
D17	Pre-DFMO	19	0	5	7	7	3
	Post-DFMO	38	4	6	7	7	8
D18	Pre-DFMO	20	4	7	7	7	4
	Post-DFMO	22	4	6	7	7	7
D25	Pre-DFMO	16	4	7	7	7	8
	Post-DFMO	12	5	6	6	7	37
D26	Pre-DFMO	22	0	6	6	6	3
	Post-DFMO	0	3	5	7	7	4
D27	Pre-DFMO	nd	0	6	4	6	2
	Post-DFMO	1	0	0	0	0	3

B

ID	Treatment	Ki-67%	MMP-2	MMP-9	uPA(F)	uPA(T)	TUNEL%
D2	Pre-DFMO	nd	nd	nd	nd	nd	nd
	Post-DFMO	0.5	0	6	7	7	2
D4	Pre-DFMO	4	0	7	8	7	3
	Post-DFMO	5	0	7	7	7	1
D5	Pre-DFMO	5	2	7	7	7	0
	Post-DFMO	5	0	7	5	7	2
D6	Pre-DFMO	16	0	6	7	7	11
	Post-DFMO	11	0	7	7	7	3
D7	Pre-DFMO	2	0	7	nd	nd	nd
	Post-DFMO	1	4	6	7	7	1
D8	Pre-DFMO	nd	nd	nd	nd	nd	nd
	Post-DFMO	7	0	6	7	7	1
D9	Pre-DFMO	6	6	7	7	4	1
	Post-DFMO	8	4	5	7	7	3
D11	Pre-DFMO	5	4	6	6	7	3
	Post-DFMO	0	3	5	6	7	20
D12	Pre-DFMO	7	0	3	6	7	0
	Post-DFMO	5	0	7	6	7	0
D13	Pre-DFMO	5	4	6	6	7	3
	Post-DFMO	0	3	5	6	7	20
D15	Pre-DFMO	33	2	6	7	7	2
	Post-DFMO	45	4	6	6	7	2
D16	Pre-DFMO	nd	nd	nd	nd	nd	nd
	Post-DFMO	49	6	6	7	7	9
D19	Pre-DFMO	0	0	5	6	6	1
	Post-DFMO	0	0	5	7	7	1
D20	Pre-DFMO	24	2	5	5	7	1
	Post-DFMO	26	2	6	7	6	2
D21	Pre-DFMO	5	0	7	7	7	0
	Post-DFMO	0	3	6	7	7	2
D22	Pre-DFMO	0	0	4	5	5	14
	Post-DFMO	14	3	6	6	7	10
D24	Pre-DFMO	19	0	7	7	7	1
	Post-DFMO	19	0	7	7	7	4

nd: Not determined.

Table IV. *Intergroup p-values.*

Comparison	Ki-67%	MMP-2	MMP-9	uPA(F)	uPA(T)	TUNEL%
Drug group pre *vs.* post	0.306	0.360	0.447	0.152	0.409	0.056
Pre control *vs.* pre drug	0.349	0.267	0.426	0.050	0.124	0.040
Control group pre *vs.* post	0.254	0.300	0.257	0.286	0.141	0.198
0.5 g dose post *vs.* pre drug controls	0.452	0.073	0.158	0.105	0.260	0.093
3.0 g dose post *vs.* pre drug controls	0.289	0.419	0.181	0.435	0.122	0.286
3.0 g dose post *vs.* 0.5 g dose post	0.403	0.053	0.086	0.115	0.200	0.144
All >14 d *vs.* pre drug controls	0.213	0.342	0.409	0.129	0.395	0.033
Pre non IDCA *vs.* post non IDCA	0.038	0.290	0.403	0.153	0.317	0.139
Pre IDCA *vs.* post IDCA patients	0.395	0.111	0.488	0.446	0.227	0.072
<50 pts. pre drug *vs.* post drug	0.150	0.492	0.102	0.321	0.219	0.159
>50 pts. pre drug vs post drug	0.388	0.318	0.060	0.073	0.057	0.107
All pre drug controls *vs.* >50 pts. post drug	0.481	0.322	0.049	0.114	0.014	0.085
All controls pre and post *vs.* >50 pts. post drug	0.491	0.363	0.055	0.487	0.002	0.138
>14 d, >50 years old pre *vs.* post-treatment	0.496	0.232	0.101	0.212	0.053	0.074

Table V. *Detailed evaluation of age-based differentiation.*

Pts >50 years old	Ki-67%	MMP-2	MMP-9	uPA(F)	uPA(T)	TUNEL%
Post drug *vs.* pre drug treated pts. only	0.388	0.319	0.060	0.073	0.056	0.107
Post drug *vs.* all pre treatment pts.	0.481	0.322	0.049	0.114	0.014	0.085
Post drug *vs.* all pre treatment and control pts.	0.491	0.363	0.055	0.487	0.002	0.137
Post >14 days on drug *vs.* pre drug treated pts. only	0.496	0.232	0.101	0.212	0.053	0.074
Post >14 days on drug *vs.* all pre treatment pts.	0.353	0.263	0.129	0.269	0.006	0.089
Post >14 days on drug *vs.* all pre treatment and control pts.	0.393	0.240	0.165	0.209	0.007	0.090
Pts <50 years old	**Ki-67%**	**MMP-2**	**MMP-9**	**uPA(F)**	**uPA(T)**	**TUNEL%**
Post drug *vs.* pre drug treated pts. only	0.150	0.492	0.102	0.321	0.219	0.159
Post drug *vs.* all pre treatment pts.	0.174	0.448	0.177	0.250	0.237	0.370
Post drug *vs.* all pre treatment and control pts.	0.126	0.453	0.162	0.406	0.313	0.452
Post >14 d on drug *vs.* pre drug treated pts. only	0.257	0.365	0.115	0.333	0.048	0.127
Post >14 d on drug *vs.* all pre treatment pts.	0.317	0.307	0.494	0.464	0.019	0.134
Post >14 d on drug *vs.* all pre treatment and control pts.	0.253	0.366	0.468	0.136	0.003	0.257

gingival fibroblasts through induction of molecular markers of hypoxic stress (16). A number of investigators have observed that oxygen deprivation induces the expression of MMPs in various lines of cultured cancer cells (17-19). In addition, evaluation of tissue derived from resected colon tumors has shown that treatment with DFMO induces expression of MMPs and other mediators of metastasis and angiogenesis (20). The pharmacologic effects of DFMO in tumors thus operate at cross purposes, *i.e.* DFMO inhibits ODC and polyamine synthesis-mediated gene transcription, but it also induces a tumor hypoxia survival program. Given the modest clinical antitumor activity of DFMO, it is unlikely that it will be generally effective as cytotoxic monotherapy, but the proapoptotic effects of DFMO might be enhanced by combining it with agents that target and inhibit the hypoxia-inducible factor (HIF) mechanistic complex. For example, agents that inhibit fatty acid synthetase such as cerulin (21) and C75 (22) used in combination with DFMO may unbalance hypoxia-inducible factor-mediated homeostasis in cancer cells and initiate apoptosis.

Although not of statistical significance in this study, enrolling patients in this study who were on hormone replacement therapy at the time of diagnosis can confound interpretation of the biomarker data. The effects of hormone replacement therapy cessation in decreasing expression of Ki-67 and proliferating cell nuclear antigen were recently reported, arguing for a careful selection of subjects for preoperative biomarker studies if proliferation is used as an endpoint and if cessation of hormone replacement therapy occurs between the diagnostic biopsy and definitive surgery (23, 24).

Our results, although statistically limited, support the contention that DFMO induces apoptosis in some patients

with early breast cancer and that the resistance to DFMO seen in other patients may be due to induction of tumor survival mechanisms associated with hypoxia tolerance. Of note is our observation that DFMO does not have antiproliferative effects in DCIS or invasive breast cancer. The borderline significant *p*-values observed for many of the intergroup comparisons argue for continued clinical and laboratory investigation of DFMO and investigation of potential proapoptotic synergies that could be exploited.

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