Evaluation of Difluoromethylornithine for the Chemoprevention of Barrett’s Esophagus and Mucosal Dysplasia

Frank A. Sinicrope1, Russell Broaddus2, Nina Joshi3, Eugene Gerner4, Elizabeth Half2, Ilan Kirsch3, Jan Lewin2, Bruce Morlan1, and Waun Ki Hong2

Abstract

Patients with Barrett’s esophagus (BE) and dysplasia are candidates for chemopreventive strategies to reduce cancer risk. We determined the effects of difluoromethylornithine (DMFO) on mucosal polyamines, gene expression, and histopathology in BE. Ten patients with BE and low-grade dysplasia participated in a single-arm study of DFMO (0.5 g/m²/d) given continuously for 6 months. Esophagoscopy with biopsies was conducted at baseline, 3, 6, and 12 months. Dysplasia was graded by a gastrointestinal pathologist. Audiology was assessed (at baseline and at 6 months). Mucosal polyamines were measured by high-performance liquid chromatography. Microarray-based gene expression was analyzed using a cDNA two-color chip. DFMO suppressed levels of the polyamines putrescine (P = 0.02) and spermidine (P = 0.02) and the spermidine/spermine ratio (P < 0.01) in dysplastic BE (6 months vs. baseline) that persisted at 6 months following drug cessation. Among the top 25 modulated genes, we found those regulating p53-mediated cell signaling (RPL11), cell-cycle regulation (cyclin E2), and cell adhesion and invasion (Plexin1). DFMO downregulated Krüppel-like factor 5 (KLF5), a transcription factor promoting cell proliferation, and suppressed RFC5 whose protein interacts with proliferating cell nuclear antigen. Histopathology showed regression of dysplasia (n = 1), stable disease (n = 8), and progression to high-grade dysplasia (n = 1). Polyamines were suppressed in the responder to a greater extent than in stable cases. DFMO was well tolerated, and one patient had subclinical, unilateral ototoxicity. DFMO suppressed mucosal polyamines and modulated genes that may be mechanistically related to its chemopreventive effect. Further study of DFMO for the chemoprevention of esophageal cancer in BE patients is warranted.

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Introduction

Barrett’s esophagus (BE) is a premalignant condition that is related to chronic gastroesophageal reflux disease and confers an increased risk of developing esophageal adenocarcinoma (1). It is estimated that BE increases the risk of developing esophageal adenocarcinoma by a factor of 30 to 40 times compared with patients without this condition. BE is a metaplastic change characterized by replacement of the normal squamous mucosa with gastric type columnar epithelium that is at risk for progression to low- and high-grade dysplasia (HGD) and cancer (1). The incidence of esophageal adenocarcinoma has been increasing rapidly in North America and Europe (2). Of the estimated that 16,400 new cases of esophageal cancer that will be diagnosed in the United States in 2009, approximately 60% will be adenocarcinomas (3). The majority of these adenocarcinomas are believed to arise either within Barrett’s epithelium or in the gastric cardiac mucosa of the distal esophagus. Although the prevalence of BE in the general population within the United States is unknown, extrapolating data from a population-based study from Sweden (4) would suggest that 1.5 to 2.0 million adults in the United States have this condition. Endoscopic surveillance of BE patients is the current standard of care that is aimed at detecting dysplasia and early malignancy. However, endoscopic surveillance is costly and labor-intensive and remains controversial owing to a lack of randomized trials supporting its efficacy in reducing cancer incidence (1). Furthermore, dysplasia and progression to cancer can occur in otherwise typical appearing Barrett’s epithelium and biopsy sampling error is also a limitation of current endoscopic surveillance. Accordingly, patients with BE and mucosal dysplasia are prime candidates for secondary prevention approaches, including chemoprevention, given their increased risk for developing esophageal adenocarcinoma.
Difluoromethylornithine (DMFO) is a chemopreventive agent that irreversibly inhibits ornithine decarboxylase (ODC) that has been shown to decrease tissue polyamine levels and tumorogenesis (5, 6). Polyamines are produced during cellular metabolism and act as signaling molecules for cell growth and differentiation (5). Abundant preclinical evidence supports DFMO as a potential chemopreventive agent for many types of cancer (7). Furthermore, DFMO has been shown to suppress polyamine levels in epithelial tissues, including human rectal mucosa (8). DFMO inhibits the growth of Barrett's epithelium–derived cell lines in culture (9) and was shown to reduce the incidence of carcinogen (N-nitrosomethylbenzylamine)-induced esophageal tumors in rodents (10, 11). ODC activity was shown to be increased in human Barrett’s mucosa compared with normal squamous esophageal or gastric mucosa (9), and ODC mRNA expression shows a progressive increase in BE-associated dysplasia and carcinoma (12). Together, these data suggest a role for polyamines in esophageal carcinogenesis and support the evaluation of DFMO as a chemopreventive agent in BE patients.

A recent randomized trial showed that the combination of DFMO and the nonsteroidal anti-inflammatory drug (NSAID) sulindac was associated with a 70% reduction in the recurrence of sporadic colorectal adenomas (13). This study lends further support for the evaluation of DFMO as an inhibitor of epithelial carcinogenesis. Early studies evaluating DFMO for cancer treatment found that this agent can cause ototoxicity in humans when given at high doses (6). However, more recent studies using lower doses have not detected clinically significant ototoxicity (14), yet the potential for subclinical ototoxicity remains an important issue for cancer prevention.

Given the critical need to develop chemopreventive strategies for patients at increased risk of developing esophageal cancer, we conducted a pilot study to determine the effect of DFMO on tissue polyamine levels and gene expression microarray in esophageal tissues from patients with BE and low-grade dysplasia (LGD). Such findings may identify potential drug targets and lead to further mechanistic insights related to DFMO. Tissue biomarkers were analyzed in biopsies obtained before, during, and after a 6-month period of continuous DFMO treatment. The effect of DFMO on the degree of dysplasia within Barrett’s epithelium was analyzed, as was its safety and tolerability that included pure tone audiometry and otoacoustic emission testing.

Materials and Methods

Study population

Patients with biopsy-proven BE and low-grade mucosal dysplasia (LGD) were eligible for study participation. Patients were identified at the time of routine surveillance endoscopy or by medical record review and contacted by mail regarding study participation. Patients were eligible if they had BE and LGD identified in more than 1 biopsy specimen, as determined by an expert gastrointestinal (GI) pathologist (R.F.B.) at independent pathology review. Eleven patients met study eligibility criteria; however, 1 was found to be a screen failure due to the absence of dysplasia within Barrett’s mucosa. Ten patients were subsequently enrolled, and all completed the baseline and end-of-study procedures, including endoscopic examinations. All patients were taking acid-suppressing medications at study entry and were allowed to continue their use for the duration of the study. Regular use of NSAIDs was not permitted, with the exception that patients would undergo a 3-week washout period and would refrain from their use during the study period. Corticosteroid use was not permitted. Data regarding alcohol and tobacco usage were not obtained. The study was approved by the Institutional Review Board at the University of Texas MD Anderson Cancer Center (Houston, TX).

Study design and procedures

The study objective was to determine the effect of DFMO on drug target modulation, that is, polyamine levels and gene expression profiles in Barrett’s mucosa. We also determined the histopathologic response rate and monitored for toxicity to obtain preliminary data to guide further study of DFMO in this patient population. DFMO was administered at a dose of 0.5 g/m²/d given once daily for 6 months. All patients underwent esophagogastroduodenoscopy (EGD) at baseline, 3 months, and 6 months with mucosal biopsies. Whenever possible, a 12-month follow-up EGD was done to assess for a delayed effect of the study medication and to evaluate the stability of any histologic changes observed.

The safety and tolerability of DFMO over the treatment period was determined. Safety evaluations included physical examinations and laboratory evaluations at baseline and at return visits at 3 and 6 months. Patients were allowed to continue any acid-suppressing medications that they were taking at study enrollment. Compliance with the study protocol, including in-person and telephone visits, study medication, and blood draws, was monitored throughout the duration of the study.

Tissue biopsies and histopathology

Endoscopic biopsies from Barrett’s appearing mucosa were conducted at 1-cm intervals and in 4 quadrants with jumbo biopsy forceps, using the “Seattle” protocol at all time points (15). Biopsies were directed by the endoscopist to be away from areas of obvious scarring related to prior biopsies. Biopsy specimens were divided and snap frozen in liquid nitrogen or placed in formalin for paraffin-embedding to generate tumor blocks. Paraffin blocks were later sectioned, slides were stained with hematoxylin and eosin, and submitted for histopathology review. At study completion, all biopsy specimens were rereviewed by an expert GI pathologist (R.F.B.), blinded to all clinical and biomarker data. At pathology, biopsies were classified as showing (i) no dysplasia; (ii) focal or extensive LGD; and (iii) HG or intramucosal carcinoma. Histology was compared between 6 months and baseline to determine potential modulation by DFMO.
**Audiometric evaluation**

Audiologic evaluation included pure tone audiograms and distortion product otoacoustic emission (DPOAE) testing that were performed both at baseline and at 6 months for ototoxic monitoring. The standard for ototoxic change is an audiometric decrease of 20 dB at any one-test frequency, a 10-dB decrease at any 2 adjacent test frequencies, or a loss of response at 3 consecutive test frequencies where responses were previously obtained (16). A 15-dB decrease was selected as the target threshold for audiometric change in this chemoprevention study to more conservatively monitor for ototoxicity. DPOAE results were based on a shift of 6 dB or more (17). DPOAEs are known to represent the contractile amplifier function of cochlear outer hair cell and may represent an earlier and more sensitive indicator of early ototoxicity (18).

**Polyamine analysis**

Polyamine contents of putrescine, histamine, spermidine, and spermine were assayed in snap-frozen esophageal tissue specimens obtained at each time point. Assays were conducted in the laboratory of Dr. E. Gerner (Arizona Cancer Center), as previously described (19). In brief, 3 individual biopsy specimens were minced in 300 mL of 9.2N perchloric acid (HClO₄), homogenized, and vortexed vigorously. The lysates were stored at 4°C overnight and were then rehomogenized and vortexed to precipitate cellular proteins, whereas free polyamines were soluble in 0.2N HClO₄. The acid-insoluble fraction was collected by centrifugation in a minifuge at 12,000 rpm for 10 minutes. Polyamines in the acid-soluble fraction were analyzed by high-performance liquid chromatography and were normalized to the protein content in the acid-insoluble fraction (19). Protein levels were assessed using the bicinchoninic acid method. Quality assurance procedures included regular measurements of standard polyamine preparations and use of internal standards in assessing polyamine amounts.

**Microarray-based gene expression profiling**

Total RNA was isolated from the snap-frozen biopsies, homogenized with a disposable generator and Micro-H Omni homogenizer in lysis buffer, RTL (Qiagen), and purified using Qiagen RNeasy Mini Kit columns according to the manufacturer’s instructions. mRNA was amplified per the modified Eberwine protocol (20). In brief, 5 μg of total RNA was used for first-strand cDNA synthesis using an oligo(dT)₄₃ primer containing 5'-T7 promoter primer using SuperScript II reverse transcriptase (Life Technologies). The second strand was synthesized with a mixture of RNase H, DNA polymerase, and DNA ligase. Purified cDNA was transcribed in vitro with T7 MEGAscript Kit (Ambion) according to the manufacturer’s instructions. Antisense RNA (aRNA) was purified using RNeasy mini spin columns (Qiagen). RNA quality was examined using the RNA 6000 Nano Assay on the Agilent 2100 Bioanalyzer.

**cDNA labeling and microarray hybridization**

Fluorescent-labeled cDNA was synthesized by reverse transcription of aRNA biopsies and human testis aRNA (prepared from total testis RNA as described for colon aRNA; Clontech, Inc.) with SuperScript II reverse transcriptase (Invitrogen) and random oligonucleotides primers in the presence of Cy3-dUTP or Cy5-dUTP (Amersham Pharmacia Biotech), respectively. For each hybridization experiment, 5 μg of biopsies and 6 μg of testis aRNA (reference material) were used to prepare a mixture of labeled cDNAs. Purified labeled biopsies and testis cDNA were dissolved in hybridization buffer containing 3.7× saline-sodium phosphate-EDTA, 2.5× Denhardt’s solution (Sigma), 0.5 μg/μL of poly(A)₄₀₋₆₀ (Amersham) and human Cot1 DNA (Invitrogen), 0.125 μg/μL yeast tRNA (Sigma), and 10% SDS in TE buffer.

Microarray containing cDNA clones (from Research Genetics, Inc.) spotted on lysine-coated glass slides were obtained from the Advanced Technology Center (National Cancer Institute; ref. 21). Microarrays contain 9,128 sequence-verified cDNAs, among which 7,102 represent name genes and 1,179 expressed sequence tag clusters, including 8,556 cDNAs with UniGene cluster ID (mapping to 7,777 unique UniGene clusters).

After hybridization at 65°C for 16 to 18 hours with 40 μL of hybridization mixture, slides were washed at room temperature in 2× SSC-0.1% SDS (5 minutes), 2× SSC (2 minutes), 1× SSC (1 minutes), 0.2× SSC (30 seconds) and dipped in 0.05% SSC. Slides were dried by centrifugation at 150 × g for 5 minutes at room temperature.

**Data acquisition and analysis**

Microarrays were scanned with an Axon 400 laser scanner and pretreatment image analysis was conducted using GenePix Pro 3.0 Software (Axon Instrument, Inc.). Images were also visually inspected, and questionable spots were flagged and excluded from the analysis. Data analysis was done with the BRB ArrayTools (version 3.2) software package developed by the Biometric Branch of the Division of Cancer Treatment and Diagnosis, National Cancer Institute, and the EMMES Corp. (22). Briefly, background intensities were subtracted and data were filtered for minimal spot intensities (100 units) in each of the 2 channels and for missing values (not more than in 20% of the array; ref. 23). Fluorescence intensity ratio data were transformed and normalized by subtracting the median log ratio from all log ratios on the array.

For analysis of the microarray data, we selected genes from the list of top 25 or top 100 genes modulated by DFMO. Objective criteria were used to select genes from these lists if their cellular function was known and was related to cellular transformation or processes associated with tumorigenesis based on a literature review.

**Statistical considerations**

The objective of this pilot study was to determine whether DFMO can suppress mucosal polyamines in Barrett’s mucosa and to explore gene modulation as potential biomarkers and...
drug targets. Polyamine levels both at baseline and at 6 months were compared using a linear mixed-effects model that adjusts for within-patient variability, with the patient as a random effect. Means, ranges, and the probability at baseline and 6-month levels were equivalent and are reported in Table 1. Polyamine data were also generated that show the change from baseline in the individual polyamine tissue levels, by patient, as shown in boxplots. Gene expression microarray data were normalized to control for nonlinear biases using the 2-channel fastlo procedure (24). Dotplots were generated from the microarray experiment show changes from baseline in gene expression in Barrett’s mucosa. Change was calculated by comparing end-of-study expression (6 months) with baseline expression. After transforming all data by taking logarithms, the genes were then ranked and their relative positions were determined using the log2 of the fold changes (x-axis) and the −log10 of the P values (y-axis) of tests of pre- and posttreatment values, adjusted by patient. The relative positions of the selected genes were grouped as being in the top 25 or top 100 modulated genes. Outcome groups were compared using a Wilcoxon signed-rank test to rank order the markers. Additional differential gene expression between pre- and posttreatment were computed using a linear mixed-effects model, with patient as a random effect.

### Results

#### Study population

The study population consisted of 10 patients with BE and low grade dysplasia (LGD) undergoing routine periodic endoscopic surveillance. Mean patient age was 58.3 years (range: 49–71), and 7 men and 3 women were included. All cases were independently reviewed by an expert GI pathologist to confirm the diagnosis of BE and LGD that was used to establish study eligibility. None of the patients had participated in prior chemoprevention studies, nor had received any endoscopic ablative therapies.

#### DFMO modulates polyamine levels in Barrett’s mucosa

DFMO taken continuously for 6 months was shown to significantly suppress mucosal polyamine levels in Barrett’s mucosa. Specifically, DFMO treatment significantly suppressed the levels of putrescine ($P = 0.008$) and spermidine ($P = 0.002$) and the spermidine/spermine ratio ($P < 0.001$) at 6 months compared with baseline, whereas no effect was observed on spermine or histamine levels (Table 1, Fig. 1). Table 1 shows the overall mean polyamine levels at the study time points, with a comparison of levels at baseline and at 6 months. The boxplots in Figure 1 display the change from baseline, by patient, in the individual polyamine levels at the various study time points. A reduction in the levels of putrescine and spermidine and the spermidine/spermine ratio was also detected in the 3-month biopsies, and suppression was maintained at 6 months after the cessation of DMFO (i.e., at 12 months) relative to baseline (Table 1, Fig. 1). A partial rebound in putrescine levels was seen at 12 months relative to 6-month time points. Neither spermine nor histamine levels were suppressed by DFMO treatment at any of the time points examined (Table 1). The relationship of polyamine expression to histopathologic response data for the intervention is described in the following text.

#### DFMO modulates gene expression in Barrett’s mucosa

The effect of DFMO treatment on microarray-based gene expression analysis was assessed in an effort to identify potential target genes of DFMO whose modulation of expression may be mechanistically related to the chemopreventive effects of this agent. We examined the top 25 genes modulated by the study agent, as shown in a dotplot as the change from baseline by subject (Fig. 2). The objective criteria used for the selection of these genes are explained in Materials and Methods. Genes believed to be of potential biological importance were identified and included those involved in cell-cycle or transcriptional regulation [CCNE2 (cyclin E2), TAF2, RPL11, PLXB1; Fig. 2, left]. Specifically, DFMO was shown to downregulate RPL11 whereas it upregulated cyclin E2, TAF2, and PLXB1. The RPL11 gene encodes a ribosomal protein that has been shown to regulate p53 activity through a direct binding with MDM2 (25). Knocking down the RPL11 gene in zebrafish was shown to activate the p53 pathway (26). TAF2 is a transcription factor that has been implicated in

### Table 1. DFMO modulates polyamine levels in Barrett’s mucosa

<table>
<thead>
<tr>
<th>Polyamine</th>
<th>Baseline (range)</th>
<th>3 mo (range)</th>
<th>6 mo (range)</th>
<th>12 mo (range)</th>
<th>P $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Putrescine</td>
<td>0.994 (0–3.26)</td>
<td>0.589 (0–3.14)</td>
<td>0.163 (0–0.67)</td>
<td>0.466 (0–1.22)</td>
<td>0.008</td>
</tr>
<tr>
<td>Spermidine</td>
<td>5.047 (1.79–8.56)</td>
<td>3.188 (1.13–6.18)</td>
<td>2.884 (0.87–6.93)</td>
<td>2.87 (0–4.77)</td>
<td>0.002</td>
</tr>
<tr>
<td>Spermine</td>
<td>6.660 (2.12–10.87)</td>
<td>7.134 (2.66–12.4)</td>
<td>6.378 (3.13–13.4)</td>
<td>5.576 (0–13.5)</td>
<td>0.809</td>
</tr>
<tr>
<td>Spermidine/spermine</td>
<td>0.732 (0.25–1.11)</td>
<td>0.458 (0.24–0.94)</td>
<td>0.457 (0.15–0.76)</td>
<td>0.497 (0.27–0.84)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Histamine</td>
<td>1.135 (0–2.24)</td>
<td>1.293 (0–3.12)</td>
<td>1.013 (0.13–2.99)</td>
<td>1.024 (0–3.49)</td>
<td>0.745</td>
</tr>
</tbody>
</table>

$^a$P values are for comparison of levels at 6 months with those at baseline.
core promoter selectivity of RNA polymerase II (27). DefA4 is a gene involved in neutrophil function (28) whose expression was increased by DFMO (Fig. 2). The cell-cycle regulator cyclin E2 and plexin B1 (PLXB1) were induced by DFMO. Accumulation of cyclin E can be found in Barrett’s epithelium and is upregulated in dysplasia and cancer (29, 30). Plexins have a role in the regulation of the actin cytoskeleton and can activate changes in extracellular matrix adhesion and motility that contribute to the invasive phenotype in cancer (31). They can also interact with and activate the receptor tyrosine kinases ErbB2 and c-Met (32). DFMO treatment was shown to upregulate the human homologue HLRC1 (MGC4293) known as the deoxyhypusine hydroxylase (DOHH) gene (Fig. 2). Hypusine is a polyamine-derived amino acid that is formed in the eukaryotic translation initiation factor 5A (eIF5A) by a posttranslational modification that involves 2 enzymatic steps (33). In the second step, DOHH converts the deoxyhypusine-containing intermediate to the hypusine-containing mature eIF5A (34). The eIF5A protein and deoxyhypusine/hypusine modification are essential for eukaryotic cell proliferation. Modulation of LOC134492, also known as Nuc-C-like protein 2 (NudCL2), by DFMO was also found (Fig. 2). NudCL2 is localized to the centrosome in interphase and spindle poles and kinetochores during mitosis (35). The data are also presented in volcano plots that show the relative ranking of the selected individual genes among the top 25 (Fig. 3A) and top 100 (Fig. 3B) genes. These plots show the distribution of the gene expression markers using the log2 of the fold change between pre- and posttreatment values for the x-axis and the −log10 of the P values for the y-axis. P values are used to rank order the genes. These values are shown on the y-axis to indicate statistical significance.

Figure 1. Boxplots display the change from baseline, by patient, in the individual polyamine levels in esophageal mucosal biopsies at the study time points. Patients received continuous DFMO treatment for 6 months, and off-study 12-month polyamine levels were also determined. The median value (line within box) and interquartile range are shown.
When expanding the microarray-based analysis to the top 100 or more genes, expression profiling revealed other genes modulated by DFMO that are of potential biological importance (KLF5, KAL1, RFC5, TIMM8a, TC10; Fig. 2, right). Of these, Krüppel-like factor 5 (KLF5) was downregulated whereas the others were upregulated. KLF5 is a transcription factor that inhibits cell proliferation, invasion, and induces proapoptotic BAX expression in esophageal cancer cells (36). DFMO treatment was associated with downregulation of KAL1 that has been shown to be suppressed in a metastasizing esophageal cancer cell line compared with nonmetastasizing parental cells (37). RFC5 was downregulated and is known to interact with proliferating cell nuclear antigen (PCNA) and is required for cell proliferation (38). CHEK1 is a p53-dependent survival gene and a DNA damage sensor (39), and TC10 is a Rho GTPase that regulates processes associated with cell growth (40). The TIMM8a gene, previously known as DDP1, is involved in the transport and sorting of proteins to the mitochondrial inner membrane (41). The small GTPase TC10 localizes predominantly to the plasma membrane and exhibits several of the cellular functions characterized for CDC42, Rac, and Rho and may play a pivotal role in actin cytoskeleton rearrangement, activation of gene expression, and oncogenic transformation (40). The relative ranking of selected genes among the top 100 genes is shown in a volcano plot (Fig. 3B). Taken together, the gene expression data suggest potential targets that may contribute to the chemopreventive effects reported for DFMO.

DFMO and histopathologic response

In patients treated with DFMO for 6 months, esophageal biopsies showed regression of LGD in 1 patient, progression to high grade in 1 patient, and stable disease in 8 patients compared with baseline (Table 2). Within the stable category, 2 patients went from having extensive to focal LGD, based on review of multiple biopsies. In these 2 cases, the change from extensive to focal LGD was detected at 3 months in one patient and following study completion at 12 months in the other patient. Of note, esophageal biopsies were obtained from 9 of 10 patients at 12 months,
representing 6 months postcessation of DFMO. In the patient who showed regression of LGD at 6 months, this finding was maintained at the 12-month time point (Table 2). The 12-month biopsies were otherwise unchanged histopathologically from the 6-month Barrett's biopsies. The patient who was found to have HGD at 6 months was recommended to undergo esophagectomy.

We also examined histopathologic response data, categorized as responder, improved, or stable (Table 2), in relation to polyamine modulation. Importantly, the patient classified as a responder with regression of LGD at 6 months showed the greatest suppression in mucosal levels of putrescine and spermidine and the spermidine/spermine ratio compared with patients with stable disease (Fig. 4). Regrettably, tissue for polyamine analysis was not available at the 6-month time point for the 1 patient with progression to HGD.

Toxicity
No clinically significant toxicities attributable to the study agent were observed during the course of the study. Two patients developed chest pain during the period of drug treatment, and both had a negative cardiac evaluation. In one of these patients, an episode of chest pain was relieved by nitroglycerin and the patient subsequently underwent cardiac catheterization, wherein no evidence of obstructive coronary artery disease was found. This same patient developed grade 2 fatigue and reported emotional stress related to domestic issues. Her symptoms resolved with lifestyle modification and medication for sleep. One participant developed grade 1 headaches, which relieved with acetaminophen use, and also reported emotional stress. Three patients experienced alterations in laboratory values that were grade 1 and not associated with symptoms. These included an elevation of serum lactate dehydrogenase levels in one patient and an isolated platelet count elevation in another without evidence of bleeding. Both abnormalities were found on the routine 6-month laboratory testing. One patient was noted to have a grade 2 reduction in the leukocyte count at the 3-month laboratory testing. DFMO was held and a repeat complete blood cell count 1 week later showed normalization of her leukocyte count, and the study drug was restarted at a reduced dose per protocol guideline.

Audiology
Three of the 10 patients treated with DFMO showed subclinical audiometric changes. One patient developed a unilateral 15-dB change at a single frequency at 3 months and ototoxic changes at 18 months characterized by a 10-dB decrease at 2 adjacent frequencies. The 2 remaining patients showed audiometric decline, without ototoxic change. Only one patient showed recovery of hearing to baseline at 8 months, based on pure tone audiometric data. We also utilized DPOAE testing as a more sensitive indicator of potential ototoxicity. Nine of 10 patients showed changes in DPOAEs within 6 months, and 1 patient whose testing was delayed showed changes at 7 months. Despite these changes in DPOAE, only 1 patient showed ototoxic effects according to both audiometric and DPOAE criteria. Eight patients were tested beyond 6 months and showed persistent changes on DPOAE; 3 of these patients had full recovery of DPOAEs at 12 or 13 months.

Discussion
We conducted a pilot study to determine the effect of DFMO on mucosal polyamine levels and microarray-based gene expression analysis in Barrett's mucosa and LGD. We also examined the effect of DFMO on the histopathology of Barrett's epithelium and monitored both toxicity and audiometry to guide further study of DFMO in this patient population. The relevance of this research is underscored by epidemiologic data showing a marked increase in the incidence of esophageal adenocarcinoma in the United States and developed countries (2). We found that a relatively low dose of DFMO (0.5 g/m²/d) suppressed mucosal polyamine content in dysplastic Barrett's epithelia. Specifically, significant reductions in the levels of putrescine and spermidine, but not spermine or histamine, were observed at 3 and 6 months compared with baseline biopsies consistent with the known mechanism of DFMO.

Table 2. DFMO and histopathologic response in BE

<table>
<thead>
<tr>
<th>Patients (N = 10)</th>
<th>Dysplasia grade</th>
<th>Response category</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>3 mo</td>
</tr>
<tr>
<td>6 Low grade</td>
<td>Low grade</td>
<td>Low grade</td>
</tr>
<tr>
<td>2 1, 2, low grade, extensive</td>
<td>1, low grade, focal</td>
<td>1, 2, low grade, focal</td>
</tr>
<tr>
<td>1 Low grade</td>
<td>Low grade</td>
<td>No dysplasia</td>
</tr>
<tr>
<td>1 Low grade</td>
<td>Low grade</td>
<td>High grade</td>
</tr>
</tbody>
</table>

*NA = not available.
as an inhibitor of polyamine biosynthesis (6, 42). Furthermore, the spermidine/spermine ratio was significantly suppressed at these same time points. Polyamine suppression occurred relatively early after treatment, and the suppressive effect of DFMO was maintained at 6 months following cessation of treatment. These data indicate that the DFMO dose used in our study is sufficient for drug target suppression. In a prior study, DFMO induced a similar reduction in the levels of putrescine and spermidine and the ratio of spermidine/spermine in human rectal mucosa (8, 43). We observed a partial rebound in putrescine levels at 12 months, but levels remained approximately 50% below those found at baseline. Putrescine is the most prevalent dietary polyamine (44) and is the preferred substrate for the polyamine transport system (45). A study in patients with prior colorectal adenomas found that putrescine levels rebounded in normal rectal mucosa after 12 months of continuous low-dose DMFO (500 mg/d), suggesting an adaptive response to prolonged ODC suppression with a compensatory cellular uptake of diet- or bacteria-derived putrescine (46).

We analyzed microarray-based gene expression data in Barrett’s esophageal tissues from patients treated with DFMO by comparing end-of-study versus baseline biopsies, with the objective of identifying genes that may represent targets of DFMO. Among the top 25 genes modulated by DFMO, we found those regulating p53-mediated cell signaling events (RPL11; ref. 25), cell-cycle regulation (cyclin E2; ref. 47), cell adhesion and invasion (Plexin1; ref. 31), and neutrophil function (DefA4; ref. 28) [Fig. 2]. DFMO was shown to downregulate RPL11 whose protein product can regulate p53 activity through a direct binding with...
However, areas of obvious scarring were avoided at biopsy could potentially impact subsequent histology at biopsy. Biopsies may induce a wound-healing response, which throughout the study. Another issue is whether repeated Of note, all patients were on acid-suppressing medications reserved for endoscopic surveillance of BE and HGD (51). This biopsy protocol is generally consistency of interpretation. Recognizing the potential for sampling error, we used the more rigorous biopsy technique logist reviewed pre- and postintervention biopsies for con-

MDM2 (25). Knocking down the RPL11 gene in zebrafish was shown to activate the p53 pathway (26), which may trigger apoptosis. In addition, DFMO was shown to down-regulate KLF5, a zinc-finger transcription factor that promotes cell proliferation, upregulates epithelial growth factor receptor, and is highly expressed in GI tract epithelia including the basal cells of the esophagus (48). DFMO was also found to downregulate RFC5 whose protein product interacts directly with PCNA (38). In contrast, DFMO treat-

We conducted an exploratory analysis of the relationship between DFMO treatment and esophageal histopathology. We found that continuous DFMO treatment for 6 months was associated with regression of LGD in 1 patient and this change was maintained in the 12-month off-study biopsies. Within the stable category, 2 patients went from having extensive to focal LGD over the 6-month treatment period. We also detected HGD in 1 patient at 6 months. Of the 9 of 10 patients with esophageal biopsies at 12 months (6 months off the study drug), no histopathologic changes were found compared with the 6-month end-of-study biopsies. Spontaneous regression in patients with BE and LGD has been described (50), and it remains unknown about whether this observation is related to histopathologic interpretation, sampling error, the effect of acid-suppressing medications, or true disease regression. An expert GI pathologist reviewed pre- and postintervention biopsies for consistency of interpretation. Recognizing the potential for sampling error, we used the more rigorous biopsy technique referred to as the “Seattle” protocol, which utilizes larger forceps and requires biopsies at 1-cm intervals throughout the Barrett’s segment (15). This biopsy protocol is generally reserved for endoscopic surveillance of BE and HGD (51). Of note, all patients were on acid-suppressing medications throughout the study. Another issue is whether repeated biopsies may induce a wound-healing response, which could potentially impact subsequent histology at biopsy. However, areas of obvious scarring were avoided at biopsy and Barrett’s epithelia was present on multiple biopsies from each level, which enabled an assessment for the presence or absence of dysplasia.

We also evaluated polyamine modulation in relation to histopathologic response data. Interestingly, DFMO suppressed polyamine levels in the responder (regression of LGD) to a greater extent than in patients with stable disease. Although this analysis is limited because of the small number of patients and that fact that most had stable findings at pathology, it nevertheless suggests a potential association between polyamine suppression and treatment response. In patients treated with DFMO and the NSAID sulindac for colon cancer chemoprevention, the data suggest that efficacy was greatest in subjects with low spermidine/spermine ratios at baseline, although polyamine modulation did not correlate with efficacy (46).

DFMO, at a dose of 0.5 g/m²/d, was well tolerated and toxicities were limited to fatigue, insomnia, or chest pain that were mild (grade 1 or 2) and whose attributions were regarded as unlikely to be related to the study drug. The 2 patients reporting chest pain had negative cardiac evaluations. Laboratory abnormalities were detected in 3 patients (grade 1 or 2), with grade 2 leukopenia resolving with drug cessation and not recurring with resumption and dose adjustment. Given the potential for ototoxicity from DFMO, pure tone audiograms were conducted and compared between end-of-study and baseline audiograms. One patient experienced subclinical, unilateral ototoxicity on pure tone audiograms that did not revert after 18 months of follow-up. In addition to pure tone audiometry, we also studied DPOAEs that are known to represent the contractile amplifier of the outer hair cells (18). Low or absent DPOAEs are associated with hearing loss on audiograms yet can also be associated with normal audiograms. It is unknown whether low DPOAEs in normal hearing ears are risk markers for subsequent early hearing loss when subjects are exposed to noise (18, 52). We sought to determine whether DPOAEs could indicate vulnerability or risk for developing DFMO-related ototoxicity. Our data suggest that DPOAE measurements may be an earlier and more sensitive indicator of DFMO-related abnormalities in pure tone audiometry, yet long-term testing needs to be conducted because protocol testing was completed at 6 months and additional testing was not consistent for all patients beyond the 6-month data collection point. A recent analysis of audiograms from participants in a phase II study of DFMO (500 mg/d) plus sulindac for sporadic colorectal adenomas has been reported (14). On the basis of 290 subjects, there were 14 of 151 subjects (9.3%) in the DFMO plus sulindac group and 4 of 139 subjects (2.9%) in the placebo group who experienced at least 15-dB hearing reduction from baseline in 2 or more consecutive frequencies across the entire range tested (P = 0.02). Follow-up air conduction audiograms done at least 6 months after end of treatment showed an adjusted mean difference in hearing thresholds of 1.08 dB (–0.81 to 2.96 dB; P = 0.26) between treatment arms. The authors concluded that there was no
significant difference in the proportion of subjects in the DFMO plus sulindac group who experienced clinically significant hearing loss compared with the placebo group. In conclusion, our data show that low-dose DFMO can inhibit polyamine levels in Barrett’s mucosa with dysplasia and can modulate genes of potential biological importance that may represent targets associated with its chemopreventive effects. Importantly, suppression of polyamines occurred after 3 and 6 months of treatment and was shown to persist 6 months following cessation of DFMO. The significance of histopathologic changes within Barrett’s mucosa of patients treated with DFMO will require further study in this patient population. Overall, DFMO was well tolerated and 1 participant experienced subclinical, unilateral ototoxicity on pure tone audiograms that did not revert, suggesting the need for audiometric evaluation in patients receiving DFMO. Abnormalities in otoacoustic emissions were found to correlate with audiometric findings and suggest their potential utility to identify ototoxic risk from DFMO, although further study is needed. Future study of the chemopreventive efficacy of DFMO in patients with BE and dysplasia is warranted.

Disclosure of Potential Conflicts of Interest

E.W. Gerner has an ownership interest in Cancer Prevention Pharmaceuticals, Tucson, Arizona.

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References

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Frank A. Sinicrope, Russell Broaddus, Nina Joshi, et al.


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