Unbiased Metabolite Profiling Indicates That a Diminished Thymidine Pool Is the Underlying Mechanism of Colon Cancer Chemoprevention by Alpha-Difluoromethylornithine

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INTRODUCTION

Colorectal cancer is the second-leading cause of cancer-related death in the United States. Colorectal cancer is a model for a cancer that progresses through multiple distinct stages in its evolution. Overall, the colorectal cancer multistep progression process may last more than 15 years (1, 2). The long duration of this multistep process makes chemoprevention an attractive approach to reducing the incidence of colorectal cancer.

One of the most effective colorectal cancer chemoprevention regimens described to date is treatment with the ornithine decarboxylase inhibitor α-difluoromethylornithine (DFMO), administered in combination with the nonsteroidal anti-inflammatory agent sulindac sulfide. In a large U.S. Food and Drug Administration (FDA) phase IIb/III trial, patients treated with DFMO/sulindac for 3 years exhibited a 70% reduction in the incidence of adenomas and a 92% reduction of polyamine depletion. However, in DFMO-treated patients, mucosal polyamine levels do not directly correlate with colorectal cancer risk. Untargeted metabolite profiling was used to broadly survey DFMO actions on colon cancer cell metabolism. We found that DFMO treatment of Apcmin intestinal tumors and human colorectal cancer cells is associated with reduced levels of folate-dependent metabolites, including S-adenosylmethionine (SAM), thymidine pools, and related pathway intermediates. We hypothesized that unrestrained SAM consumption/regeneration constitutes a futile DFMO-triggered cascade that can steal tetrahydrofolate from thymidylate synthase and thereby diminish thymidine pools. In accord with this hypothesis, DFMO treatment altered the folate cofactor balance and thymidine supplementation prevented DFMO-elicted cytostasis without restoring polyamine levels. These findings suggest that thymidine metabolite pool insufficiency is a fundamental mechanism of DFMO cytostatic activity.

SIGNIFICANCE: A previously unappreciated metabolic linkage between polyamine and thymidine biosynthesis is revealed, based on the competing requirement of these pathways for a limited pool of tetrahydrofolate cofactor. This study identifies the first shared mechanism for colorectal cancer chemoprevention and chemotherapy, suggesting a common metabolic target for both premalignant and malignant colon cells. Cancer Discov; 3(9): 1072–81. © 2013 AACR.
marked reduction of thymidine metabolite pools due to apparent metabolic linkages between pathways for biosynthesis of polyamines, S-adenosylmethionine (SAM), and tetrahydrofolate (THF)-dependent one-carbon transfer reactions. Furthermore, our finding that DFMO-mediated cytostasis of colon cancer epithelial cells can be prevented by thymidine supplementation identifies diminished thymidine pools as the predominant basis for DFMO-mediated colon cancer chemoprevention. This mechanism of DFMO action bears a striking, but unanticipated, similarity with 5-fluorouracil (5-FU), the most common chemotherapy drug used for colorectal cancer.

RESULTS
Technical Reproducibility of LC/MS-Based Metabolite Profiling in the HT-29 Colorectal Cancer Cell Line

Untargeted metabolite profiling was used in an attempt to discover molecules that are differentially expressed in colon cancer cells and tumors in response to DFMO treatment. The efficacy of this approach relies on highly reproducible metabolite quantification to enable confident detection of bona fide DFMO-evoked changes. To quantify the reproducibility of our liquid chromatography/mass spectrometry (LC/MS) platform (using aqueous normal-phase chromatography and positive-ion monitoring mass spectrometry), a sample of HT-29 cell extract was analyzed 24 consecutive times over a period of approximately 15 hours. As shown in Supplementary Figure S1A, an overlay of total ion chromatograms for these 24 repeats showed high reproducibility. Considering 624 features that could be quantified in each of the 24 repeated measurements, the coefficient of variation did not exceed 15% (Supplementary Fig. S1B). From these 624 features, extracted ion chromatograms are overlaid in Supplementary Figure S1C for a group of exemplary molecules that were subsequently found to be of importance in the present study of DFMO actions. Given the high technical reproducibility of these repeated analyses, we conclude that DFMO-induced changes in HT-29 colon cancer cell metabolite levels exceeding 15% can be reliably identified.

Untargeted Profiling of DFMO Actions in HT-29 Colorectal Cells

To define metabolic effects of DFMO that potentially contribute to antitumor activity, we conducted LC/MS-based metabolite profiling on HT-29 cells, comparing 300 nmol/L DFMO-treated cells versus untreated cells. Using this approach, 1,350 molecular features in the range of 50 to 1,000 Da m/z were quantified in all samples from either DFMO-treated or untreated HT-29 cell groups (n = 4/group; Fig. 1A). Principal component analysis (PCA) clearly differentiated between DFMO-treated and untreated groups (Fig. 1B), and unsupervised hierarchical cluster analysis revealed a clear and reproducible pattern of within-group metabolite expression similarities and between-group differences (Fig. 1C). Of 1,350 features quantified in all samples from at least one treatment group, volcano plot analysis indicated that 596 were altered in expression twofold or more after DFMO treatment (P < 0.05; Fig. 1D).

Structural identification of differentially expressed metabolites was conducted by molecular formula generation and METLIN database searching, considering both LC retention times and tandem mass spectrometry (MS/MS) fragmentation of reference standards. The most significant effects of DFMO treatment on structurally identified metabolite expression levels in HT-29 cells are summarized in Figure 2. As expected, DFMO was found to effectively inhibit ODC, documented by an increase in cellular levels of the ODC substrate ornithine (2.9-fold) and a reciprocal decrease in levels of the downstream ODC products spermidine (>3.0-fold), spermine (1.8-fold), and acetylpyridoxamine (14-fold). Unexpectedly, we also observed significant decreases in the methionine cycle intermediates S-adenosylmethionine (SAM) (>5-fold), methionine (>5-fold), S-adenosylhomocysteine (>1.6-fold), and homocysteine (3-fold). Notably, SAM is an essential precursor for polyamine biosynthesis, and in addition to the ODC product putrescine, two SAM-derived propylamine molecules are needed for the sequential production of spermidine, then spermine, in that order. After loss of propylamine, the SAM-derived coproduct is 5'-methylthioadenosine (MTA), which can be subsequently salvaged for regeneration of methionine or further catabolized to adenine by MTA nucleosidase. As shown in Figure 2, DFMO treatment of HT-29 cells was associated with a decrease in MTA (>3-fold) and its product adenine (>6-fold), but a >100-fold increase in adenosine and deoxyadenosine levels. Along with this dramatic increase in adenosine, DFMO elicited fourfold decreases in levels of the pyrimidines uridine and cytidine as well as a near-complete loss of cellular thymidine in this experiment. Notably, production of the obligate biosynthetic precursor to thymidine, dTMP, is controlled by cellular levels of SAM. Indeed, SAM is an allosteric inhibitor of methylene tetrahydrofolate reductase (MTHFR; ref. 19), an enzyme that irreversibly converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which can be subsequently salvaged for regeneration of methionine or further catabolized to adenine by MTA nucleosidase. As shown in Figure 2, DFMO treatment of HT-29 cells was associated with a decrease in MTA (>3-fold) and its product adenine (>6-fold), but a >100-fold increase in adenosine and deoxyadenosine levels. Along with this dramatic increase in adenosine, DFMO elicited fourfold decreases in levels of the pyrimidines uridine and cytidine as well as a near-complete loss of cellular thymidine in this experiment. Notably, production of the obligate biosynthetic precursor to thymidine, dTMP, is controlled by cellular levels of SAM. Indeed, SAM is an allosteric inhibitor of methylene tetrahydrofolate reductase (MTHFR; ref. 19), an enzyme that irreversibly converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, switching the THF cofactor required for dTMP synthesis by thymidylate synthase to the obligate cofactor isofom used by methionine synthase for production of methionine, allowing for SAM regeneration.

Thymidine Supplementation Reduces DFMO Cytostasis

Reduced availability and maintenance of thymidine metabolite pools causes rapidly growing colorectal cancer cells to undergo apoptosis due to uridine misincorporation into DNA, leading to DNA double-strand breaks and apoptosis (20). To test the possibility that reduced thymidine metabolite pool levels contribute to the antiproliferative effect of DFMO in HT-29 cells, we investigated whether thymidine supplementation would oppose DFMO-induced cytostasis. Using a DFMO treatment regimen that reduced cell numbers by approximately 50% when administered alone to cells in culture, thymidine supplementation was found to reduce this DFMO-induced cell loss (Fig. 3A). In contrast, the antiproliferative effect of DFMO was unaffected by supplementation of cells with an identical concentration of cytidine, a pyrimidine that was depleted by DFMO treatment along with thymidine (Fig. 3B). Although thymidine supplementation reduced DFMO-induced cytostasis in HT-29 cells, it is notable that thymidine supplementation failed to restore polyamine levels (data not shown). This finding establishes that it is not polyamine depletion per se that mediates the antiproliferative actions of the ODC inhibitor DFMO, it is the associated
depletion of thymidine metabolite pools that is responsible. Through a related biochemical mechanism, this mode of thymine-less cell death parallels that of 5-FU, which depletes thymidine by direct inhibition of thymidylate synthase.

To assess the generality of thymidine supplementation as a protectant against DFMO-mediated cytostasis, studies were conducted that tested DFMO effects on additional colon cancer cell lines, SW480 (Fig. 3C) and LoVo (Fig. 3D). In the literature, it has been reported that high levels of exogenous thymidine can be toxic to cells (21–24). Consistent with previous data, treatment of SW480 or LoVo cells with millimolar-range thymidine reduces cell viability (Fig. 3C and D). However, at 300 μmol/L, thymidine does not dramatically impact SW480 or LoVo cell viability. Therefore, we used this concentration of thymidine to test whether it can ameliorate the impact of DFMO treatment on cell viability. In each case, 300 μmol/L thymidine supplementation was found to reduce DFMO-elicited cytostasis (Fig. 3C and D).

**DFMO Treatment Is Associated with 5-MethylTHF Accumulation**

In an irreversible NADPH-dependent reaction, MTHFR converts the essential thymidylate synthase cofactor 5,10-methyleneTHF to the methionine synthase cofactor 5-methylTHF—this cofactor switch could explain attenuated thymidine synthesis in favor of methionine production for SAM regeneration. As MTHFR is known to be activated by lowering SAM levels (19, 25), we wondered whether DFMO-elicited SAM depletion leads to 5-methylTHF accumulation as a potential mechanism for depleting cellular thymidine levels. As shown in Supplementary Figure S2, treatment of HT-29 cells with DFMO did indeed elicit an accumulation of the methionine synthase cofactor 5-methylTHF, identified by molecular formula assignment along with neutral loss of glutamate, a characteristic structural feature of folates (26). Notably, although 5-methylTHF could be detected in HT-29 cell cultures treated with DFMO, it was essentially undetectable in untreated HT-29 cells, in accord with a relatively low native abundance. Notably, 5,10-methyleneTHF was not detected in extracts from either untreated or DFMO-treated HT-29 cells, perhaps due to relative instability.

**Metabolomic Profiling of DFMO-Treated ApcMin Intestinal Tumors**

ApcMin mice are a well-established model of intestinal tumorigenesis and commonly used in studies of intestinal cancer chemoprevention (27, 28). To assess whether in vitro
actions of DFMO on colorectal cancer cells in culture extend to the in vivo setting, we compared metabolic profiles of intestinal tumors excised from ApcMin mice that were either untreated or treated for 21 days with DFMO (40 mg/kg daily). Tumor metabolite profiling results confidently identified 1,165 distinct metabolites that exceeded cutoff limits for quantification and which could be detected with 100% frequency in each sample from least one treatment group (Supplementary Fig. S3A). As for the in vitro studies of DFMO actions depicted in Figure 1, PCA analysis of metabolites in tumors from DFMO-treated versus untreated mice revealed distinct treatment group clusters, confirming distinct and reproducible drug effects on metabolism (Supplementary Fig. S3B). Unsupervised hierarchical clustering analysis of all recognized features showed a clear group separation at the first tree branch, consistent with coordinate changes in metabolic profiles of intestinal tumors from untreated versus DFMO-treated ApcMin mice (Supplementary Fig. S3C). Volcano plot analysis identified 327 features that were changed more than twofold, either up or down, in association with DFMO treatment at P < 0.05 (Supplementary Fig. S3D). Taken together, untargeted assessment of metabolism in ApcMin mouse intestinal tumors indicated profound DFMO-elicted effects.

In Figure 4, we summarize key DFMO-elicted changes in metabolite expression in ApcMin intestinal tumors. Results confirm the anticipated robust accumulation of DFMO in intestinal tumors from DFMO-treated mice, but absence from tumors in untreated mice. As expected from the in vitro findings with HT-29 cells, and knowledge that DFMO is a selective inhibitor of ODC, treatment with DFMO was associated with significant accumulation of the ODC substrate ornithine (2-fold) and decreased levels of polyamine pathway products, spermine and spermidine (>3-fold). Also in accord with in vitro findings, in vivo DFMO treatment significantly diminished intestinal tumor levels of SAM and its methionine cycle intermediate, homocysteine. Furthermore, levels of the nucleosides thymidine and cytidine, along with the nucleotide precursors dTMP and dCMP, were significantly attenuated following 21 days of DFMO treatment. In contrast with the dramatic DFMO-elicted increase in adenosine in HT-29 cells, in vivo DFMO treatment was associated with a threefold decrease in tumor adenosine levels.

Finally, we also show that DFMO treatment reduced SAM and methionine cycle metabolites in normal human colon epithelial cell cultures (Supplementary Fig. S4). Overall, our observed in vivo actions of DFMO on intestinal tumor cells and in vitro studies using normal human colon epithelial cells were concordant with findings in colorectal cancer cells, where a pivotal role of SAM and thymidine metabolite pool depletion in DFMO-elicted suppression of colorectal cancer proliferation was found.

DISCUSSION

DFMO is a promising compound for colorectal cancer chemoprevention. Although it is well-established that DFMO inhibits ODC and attenuates cellular polyamine levels, its
To broadly elucidate the metabolic consequences of DFMO, we conducted an untargeted metabolite profiling study of DFMO actions on both colorectal cancer cell lines in vitro and intestinal tumors from ApcMin mice. DFMO treatment was found to significantly deplete cellular polyamine levels and to trigger ornithine accumulation in vivo, in accord with the recognized action of DFMO as a selective ODC inhibitor. Results described here further showed that DFMO evokes broad changes in colorectal cancer cell and tumor metabolism, including SAM- and THF-associated one-carbon transfer networks, associated with a dramatic depletion of one-carbon–dependent thymidine metabolite pools in different contexts. Consistent with a model in which the anti-proliferative effect of DFMO is a consequence of reduced thymidine pools, we found that supplementation of cells with thymidine reduced DFMO-induced cytostasis, despite no restoration of polyamine levels (i.e., neither spermine nor spermidine). These studies establish thymidine metabolite pool reduction as the key mechanistic basis for DFMO-mediated cytostatic activity against colorectal cancer.

As schematized in Figure 5, SAM is known to play a central role in multiple biochemical pathways. Our study provides the first evidence for a functional linkage between inhibition of polyamine synthesis and thymineless death of colon cancer cells. This link is reconciled by a role for SAM as both a polyamine precursor (in conjunction with ODC-derived putrescine) and a determinant of thymidine biosynthesis. Notably, two deSAM-derived polyamine groups are required for polyamine synthesis, one adding to putrescine, forming spermidine, and the second adding to spermidine, yielding the pathway end-product spermine (29). DFMO treatment markedly reduced SAM levels in the HT-29 colon cancer cell line, intestinal tumors from ApcMin mice, and normal human colon epithelial cells—this was concomitant with reduced levels of methionine cycle and one-carbon–dependent thymidine metabolite pool biosynthesis pathway intermediates. Although the mechanism of SAM depletion by DFMO awaits precise molecular definition, it is notable that SAM decarboxylase has a short half-life due to 26S proteasomal degradation, and polyamines negatively regulate the translation of SAM decarboxylase (30)—accordingly, depletion of polyamines by DFMO would predictably enhance SAM decarboxylase protein activity and thereby promote SAM consumption. Indeed, prior studies have consistently shown a marked increase in SAM decarboxylase activity and accumulation of deSAM in response to ODC inhibition by both pharmacologic and genetic means (29, 31, 32), including colorectal cancer treatment with DFMO (32). Although we found DFMO to elicit a paradoxical decrease in levels of the SAM decarboxylase product...
**Figure 4.** Metabolite profiling of DFMO-treated Apc<sup>Min</sup> mouse tumors. Apc<sup>Min</sup> mice were given DFMO by intraperitoneal injection (40 mg/kg daily) or vehicle (saline) for 21 days and intestinal tumors were microdissected and collected (3 tumors/mouse, n = 4 mice/group). Metabolites were extracted from tumors for analysis by high-performance liquid chromatography, as described in Methods. Along with accumulation of DFMO itself and expected decreases in polyamines, DFMO-treated tissues showed altered expression of methionine cycle and nucleotide/nucleoside levels, similar to *in vitro* findings (Fig. 3). Notably, this included markedly reduced thymidine levels. *, P < 0.05 by unpaired t test, comparing tumors from untreated and DFMO-treated Apc<sup>Min</sup> mice. N.D., not detected.

MTA (both in colorectal cancer cells *in vitro* and in intestinal tumors *in vivo*), the observed more than 100-fold increase in adenosine and deoxyadenosine levels in HT-29 cells may be ascribed to rapid MTA catabolism to adenine and then adenosine/deoxyadenosine. In contrast, DFMO did not increase adenosine levels in intestinal tumors *in vivo*, likely due to further catabolic and/or anabolic reactions, perhaps involving systemic redistribution of adenosine from tumor cells.

SAM is most often considered for its substrate role in one-carbon transfer reactions; however, as noted above, SAM is also

**Figure 5.** Schematic description of the metabolic network proposed to link DFMO-mediated inhibition of polyamine synthesis to observed perturbations in the methionine cycle, trans-sulfuration pathway, purine synthesis/salvage, and de novo thymidine production. SAH, S-adenosylhomocysteine.
an essential substrate for polyamine biosynthesis. Indeed, it has been reported that 1% to 5% of SAM is directed to polyamine biosynthesis in healthy cells, with the remainder directed toward one-carbon transfer reactions, including synthesis of methionine and thymidine for protein and DNA synthesis, respectively (33–36). Thus, decreased SAM levels may effectively limit the availability of SAM for use by metabolic pathways, including regulation of THF cofactor availability (37–39). Indeed, when polyamine levels are insufficient, such as occurs when ODC is inhibited by DFMO, a futile cycle can result wherein SAM is nonproductively consumed for polyamine biosynthesis, at the expense of other SAM-dependent activities (40). One recognized activity of SAM that does not depend on methyl-group transfer is as a repressor of activity of MTHFR (19, 25), an enzyme that catalyzes irreversible conversion of the thymidylate synthase cofactor 5,10-methyleneTHF to the methionine synthase cofactor 5-methylTHF (Fig. 5). Accordingly, reduced levels of SAM, as we observed in DFMO-treated cells and mice, can elicit a derepression of MTHFR activity and thereby divert THF cofactor for support of methionine and SAM regeneration, away from support and maintenance of thymidine metabolite pools. Consistent with this proposed model, we show that the addition of the polyamine putrescine to DFMO-treated colorectal cancer cells restores the thymidine metabolite pools (Fig. 6).

It is well established that, via inhibition of thymidylate synthase, 5-FU and related antifolate drugs deplete cellular thymidine, providing an important and well-characterized mechanism of colorectal cancer chemotherapy (20). Our data show that DFMO also acts through depletion of thymidine in rapidly proliferating colorectal cancer cells. In vitro depletion of cellular thymidine levels by DFMO is clearly less dramatic than that caused by 5-FU. However, 5-FU is given at high doses for short periods of time, typically by intravenous bolus. In contrast, DFMO for chemoprevention is orally ingested daily at relatively low doses for multiple years and rarely causes significant side effects. Our data are consistent with a model whereby DFMO chemoprevention therapy inhibits proliferation of rapidly dividing colon epithelial cells by long-duration summation of the effects of moderately reduced thymidine levels. As 5-FU–mediated depletion of cellular thymidine is the cornerstone of anti–colorectal cancer chemotherapy, our study reveals the first shared mechanism for colorectal cancer chemoprevention and therapy. This is conceptually similar to the shared mechanism of estrogen receptor signaling blockade for both chemoprevention and chemotherapy of breast cancer. We anticipate that metabolomic studies of other chemotherapy and chemoprevention drugs may similarly reveal shared chemoprevention/chemotherapy mechanisms that can provide new insights for designing novel metabolism-based approaches that enhance drug efficacy and safety. Our work also highlights the potential for repurposing of “old” chemoprevention and chemotherapy drugs for new cancer indications.

SAM itself is a nutraceutical supplement, sold over the counter in the United States and other countries, and is readily available to individuals who take DFMO. Also, folate is a common constituent of multivitamins and its supplementation in grain-containing food products is mandated by U.S. law (41). Overall, our data support meticulous monitoring of dietary folate and SAM intake for participants in ongoing and future DFMO clinical trials. We anticipate that correlative studies of DFMO trial participants may well show that reduced intestinal mucosal SAM, 5,10-methyleneTHF, and thymidine levels are more closely associated with chemopreventive efficacy than polyamine levels. For trials that do not meet their primary endpoint, analysis of trial participant intestinal mucosal SAM, folate cofactors, and thymidine levels may help identify responders that otherwise could not easily be identified. For DFMO trials that have not yet initiated enrollment, our data suggest a mechanistic rationale for using dietary SAM intake as an exclusion criterion for trial participation.

Taken together, our findings show that DFMO-elicited anti–colorectal cancer activity arises from impaired de novo thymidine synthesis, owing to a network of interconnected metabolic pathways that we identified by untargeted metabolite profiling to be linked by SAM and THF cofactor dependencies. These suggested in vitro DFMO-induced pathway perturbations were found to extend to intestinal tumors of mice, including depletion of thymidine metabolite pools that we hypothesize to be a key molecular underpinning of colorectal cancer chemoprevention.

**Figure 6.** Putrescine supplementation modulates DFMO-mediated thymidine metabolite pool depletion. Colon cancer cell line HT-29 cells were treated with DFMO (300 nmol/L) alone or in combination with putrescine (PUT) 100 μmol/L for 4 days (n = 6). Metabolites were extracted and analyzed for thymidine pool metabolites, thymidine triphosphate (TTP; **A**), thymidine diphosphate (TDP; **B**). ± SEM for not significant (N.S.).
by DFMO in vivo. Development of enhanced analytical techniques will be needed to ascertain whether stealing 5,10-methylenetetrahydrofolate cofactor (i.e., conversion to 5-methyltetrahydrofolate) provides the molecular basis for thymidine metabolite pool depletion by DFMO in mouse intestinal tumors, as inferred from colorectal cancer cell culture studies described herein.

**METHODS**

**Calculating DFMO Human Dose Equivalence**

For *in vivo/*in vitro studies, relevant Human Dose Equivalence (HDE) of DFMO doses were calculated using the recommended NCI Division of Cancer Prevention clinical pharmacologists’ FDA Center for Drug Evaluation and Research “Guidance for Industry: Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers.” Equations used for calculating starting HDE were previously described at http://www.fda.gov/downloads/Drugs/GuidanceCompliance RegulatoryInformation/Guidances/UCM078932.pdf.

**Cell Culture, DFMO Treatment, and Thymidine Supplementation**

Human colon cancer cell lines used for these investigations included HT-29, SW680, and LoVo, and were obtained from the American Type Culture Collection (ATCC) in 2012. For HT-29, experiments were repeated in new cells purchased from ATCC in 2013. HT-29 cells were maintained in McCoy 5A medium (Gibco), whereas SW680 and LoVo cells were maintained in Ham F12 medium (Gibco) in a humidified atmosphere of 10% CO2 in air. All culture media were supplemented with dialyzed 10% FBS (Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). Human colon cancer cells were harvested from 4-day-old cultures grown to near confluence in 6-well tissue culture plates (Corning). After 24-hour incubation post-plating, cells were treated with DFMO at the indicated concentrations in cell culture media. For supplementation studies, thymidine or cytidine (Sigma) was added to the culture medium at the indicated concentrations concurrently with DFMO. Cell viability was quantified by MTT assay (Sigma) or by Trypan blue exclusion.

**Normal Human Colon Studies**

Normal colon discarded tissues from colon cancer surgical resections were used under a protocol approved by the Weill Cornell Institutional Review Board. Tissues were extensively washed (PBS), dissected into 1-cm sections, grown in 24-well tissue culture plates (Corning), maintained in Dulbecco’s Modified Eagle Medium/F12-containing nonessential amino acids, antibiotic–antimycotic, N2 supplement (Invitrogen), B27 supplement (Invitrogen), heparin (4 μg/mL; Sigma), EGF (40 ng/mL), and basic fibroblast growth factor (20 ng/mL) at 37°C and 5% CO2. DFMO was added to the culture medium at the indicated concentrations. Normal colonic epithelium was mechanically removed after treatment and used for metabolomic analysis.

**Mouse Studies**

Mice were housed under temperature control conditions and fed standard diet (Harlan LM-485). All studies were carried out under an approved Weill Cornell Institutional Animal Care and Use Committee protocol. ApoE−/− mice at 8 weeks of age were given intraperitoneal injections of 40 mg/kg DFMO, a generous gift from Marion Merrell Dow (Cincinnati, OH), administered daily in PBS solution. Control mice were given PBS injections without DFMO. On the desired day after DFMO treatment, mice were sacrificed by CO2 asphyxiation and intestinal tumors and were excised and subjected to metabolite extraction for untargeted metabolite profiling.

**Metabolite Extraction for Untargeted Analysis**

Intestinal tumor isolates were lysed in 80%−70°C methanol/water (LC/MS grade methanol, Fisher Scientific) using a TissueLyser (Qiagen). Cells grown in 6-well plates were extracted in −70°C methanol/water and cell scrapers were used to collect cells. Tissue and cell harvests were then centrifuged for 5 minutes at 5,000 rpm to pellet insoluble protein. Supernatant was collected and pellets were twice reextracted with fresh 80% methanol each time. The three supernatants were pooled, dried in a speed-vac (Savant), and stored at −80°C until reconstituted in LC mobile phase buffer A on the day of analysis by LC/MS (17). For *in vitro* studies, HT-29 cells were treated with 300 nmol/L DFMO for 4 days before metabolite extraction.

**LC/MS and LC/MS–MS Platforms for Metabolite Profiling**

The LC/MS setup was the same as described previously (17).

**Untargeted Metabolomics Data Processing and Statistical Analysis**

Raw data were processed by Agilent MassHunter Qualitative Analysis Software (version, B05) and analyzed by MassProfiler Professional (MPP, version B2.02, Agilent Technology) as described (17). Briefly, MassHunter Qualitative Analysis untargeted molecular feature extraction generates features (compounds/metabolites) based on the elution profile of identical mass and retention times, within a defined mass accuracy (5 ppm). Aligned molecular features detected in all biologic replicates of at least one group were directly applied for statistical analysis across treatment groups by MPP. The Bonferroni Family-wise error-rate correction was applied for multiple testing correction of *P* values (corrected *P* < 0.05).

**Differentially Expressed Metabolite Identification**

Differential metabolites with fold changes more than 2 (*P* < 0.05), compared with untreated control, were searched against an in-lab annotated METLIN Personal Metabolite Database (Agilent Technologies), based on accurate monoisotopic neutral masses (<5 ppm). A molecular formula generator (MFG) algorithm in MPP was used to generate and score empirical molecular formulae based on a weighted consideration of monoisotopic mass accuracy, isotope abundance ratios, and spacing between isotope peaks. Notably, MFG imposes additional constraints on the list of candidate molecular formulas detected by a METLIN database search. A putative compound ID was tentatively assigned when METLIN and MFG concurred for a given candidate. Tentatively assigned compounds were further verified by a match of LC retention time and/or MS/MS fragmentation pattern of pure molecular standards.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** M. Witherspoon, S.S. Gross

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