Unbiased metabolite profiling indicates that a diminished thymidine pools is the underlying mechanism of colon cancer chemoprevention by alpha-difluoromethylornithine (DFMO)

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ABBREVIATIONS: DFMO, α-difluoromethylornithine; ODC, ornithine decarboxylase; SAM, S-adenosylmethionine; CRC, colorectal cancer; dcSAM, decarboxylated S-adenosylmethionine; MTA, 5-methyl-thioadenosine; 5-FU, 5-fluorouracil; LC/MS, liquid chromatography/mass spectrometry; THF, tetrahydrofolate; MTHFR, methylenetetrahydrofolate reductase; TS, thymidylate synthase; MS, methionine synthase.
Abstract

The ornithine decarboxylase (ODC) inhibitor, α-difluoromethylornithine (DFMO), is a highly effective chemopreventative agent for colorectal cancer (CRC) thought to act via polyamine depletion. However, in DFMO treated patients, mucosal polyamine levels do not directly correlate with CRC risk. Untargeted metabolite profiling was used to broadly survey DFMO actions on colon cancer cell metabolism. Some studies revealed that DFMO treatment of ApcMin intestinal tumors and human CRC 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 (THF) from thymidylate synthase and thereby diminishing thymidine pools. In accord with this hypothesis, DFMO-treatment altered the folate cofactor balance and thymidine supplementation prevented DFMO-elicited 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 competing requirement of these pathways for a limited pool of tetrahydrofolate cofactor. This study identifies the first shared mechanism for CRC chemoprevention and chemotherapy, suggesting a common metabolic target for both pre-malignant and malignant colon cells.
Introduction

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

One of the most effective CRC chemoprevention regimens described to date is treatment with the ODC inhibitor DFMO, administered in combination with the non-steroidal anti-inflammatory agent sulindac sulfide. In a large 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 in advanced adenomas (3). The NCI Southwest Oncology Group is currently running a pivotal phase III FDA registration trial of DFMO/Sulindac for CRC chemoprevention, and trials of DFMO in combination with aspirin are also ongoing.

DFMO is an irreversible inhibitor of ODC activity, considered to be the basis for its chemotherapeutic benefit (4-7). Inhibition of ODC causes reduced levels of putrescine, the precursor of spermidine and spermine, polyamine biosynthetic pathway products that are known to promote cell proliferation (8, 9). In addition to the ODC product putrescine, polyamine synthesis requires the transfer of aminopropyl groups from decarboxylated S-adenosylmethionine (dcSAM) in two sequential enzymatic steps, yielding spermidine and then spermine as products (10, 11). Although DFMO is thought to inhibit proliferation of fast-growing colon adenoma and CRC cells by polyamine depletion, the chemopreventive mechanism of polyamine depletion is currently unknown. Moreover, in pre-clinical mouse studies DFMO treatment was proposed to induce cell cycle inhibitors p27^Kip1 and p21^{Cip1} through a mechanism that is not directly related to lowered polyamine levels (12). Importantly, in the Phase IIb/III DFMO/sulindac CRC chemoprevention trial, reduced mucosal polyamine levels did not correlate with CRC chemopreventative efficacy (13). Taken together, prior cancer chemoprevention studies highlight uncertainty regarding the molecular mechanism of colon cancer suppression by DFMO.
Untargeted metabolite profiling offers a powerful discovery approach for identification of diverse metabolic perturbations in cells and tissues. For example, metabolomic profiling has enabled the detection of hidden, but significant, drug actions (2, 14-18). Although DFMO is known to disrupt polyamine metabolism, how this relates to the cytostatic actions of DFMO and how ODC inhibition broadly impacts cell metabolism remains to be defined. Here, we used an untargeted metabolite profiling to study DFMO-treated cells and mice, leading to the unexpected finding that DFMO can elicit a marked reduction of thymidine metabolite pools due to apparent metabolic linkages between pathways for biosynthesis of polyamines, SAM and THF-dependent one-carbon transfer reactions. Further, 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-fluoruracil (5-FU), the most common chemotherapy drug used for CRC.
Results

Technical reproducibility of LC-MS-based metabolite profiling in the HT-29 colorectal cancer cell line.

Untargeted metabolite profiling was employed in 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 LC-MS platform (using aqueous normal phase chromatography and positive ion monitoring MS), a sample of HT-29 cell extract was analyzed 24 consecutive times, over a period of ~15 h. As shown in (Fig. S1A), an overlay of total ion chromatograms for these 24 repeat demonstrated high reproducibility. Considering 624 features that could be quantified in each of the 24 repeated measurements, the coefficient of variation did not exceed 15% (Fig. S1B). From these 624 features, extracted ion chromatograms are overlaid in (Fig.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 anti-tumor activity we performed LC/MS-based metabolite profiling on HT-29 cells, comparing 300 nM DFMO-treated vs. untreated cells. Using this approach, 1,350 molecular features in the range of 50 - 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 by 2-fold or greater after DFMO treatment (p < 0.05; Fig.1D).

Structural identification of differentially-expressed metabolites was performed by molecular formula generation and METLIN database searching, considering both LC retention times and 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 (Fig. 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 acetylspermidine (14-fold). Unexpectedly, we also observed significant decreases in the methionine cycle intermediates, S-adenosylmethionine (SAM; >5-fold), methionine (>5-fold), S-adenosylhomocysteine (SAH; >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 co-product is 5'-methylthioadenosine (MTA), which can subsequently be salvaged for regeneration of methionine, or further catabolized to adenine by MTA nucleosidase. As shown in (Fig. 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 4-fold 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) (19), an enzyme that irreversibly converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, switching the THF cofactor required for dTMP synthesis by thymidylate synthase (TS), to the obligate cofactor isoform used by methionine synthase (MS) for production of methionine, allowing for SAM regeneration.

**Thymidine supplementation reduces DFMO cytostasis.** Reduced availability and maintenance of thymidine metabolite pools causes rapidly growing CRC 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 anti-proliferative 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 ≈50% when administered alone to cells in culture, thymidine supplementation was found to reduce this DFMO-induced cell loss (Fig. 3A). In contrast, the anti-proliferative 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 anti-proliferative actions of the ODC inhibitor DFMO - it is instead 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 TS.

To assess the generality of thymidine supplementation as a protectant against DFMO-mediated cytostasis, studies were performed testing 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, D). However, at 300uM, 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, 300uM thymidine supplementation was found to reduce DFMO-elicited cytostasis (Fig. 3C, D).

**DFMO treatment is associated with 5-methylTHF accumulation.** In an irreversible NADPH-dependent reaction, MTHFR converts the essential TS cofactor 5,10-methyleneTHF to the MS cofactor, 5-methylTHF – this cofactor switch could explain attenuated thymidine synthesis, in favor of methionine production for SAM regeneration. Since 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 (Fig. S2), treatment of HT-29 cells with DFMO did indeed elicit an accumulation of the MS cofactor 5-methylTHF, identified by molecular formula assignment along with neutral loss of glutamate, a characteristic structural feature of folates ((26). Notably, whereas 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-methylene 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 CRC 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 cut-off limits for quantification and which could be detected with 100% frequency in all samples from least one treatment group (Fig. S3A). As for the *in vitro* studies of DFMO actions depicted in Fig. 1, PCA analysis of metabolites in tumors from DFMO-treated vs. untreated mice revealed distinct treatment group clusters, confirming distinct and reproducible drug effects on metabolism (Fig. S3B). Unsupervised hierarchical clustering analysis of all recognized features demonstrated a clear group separation at the first tree branch, consistent with coordinate changes in metabolic profiles of intestinal tumors from untreated vs. DFMO treated ApcMin mice (Fig. S3C). Volcano plot analysis identified 327 features that were >2-fold changed - either up or down - in association with DFMO treatment at p<0.05 (Fig. S3D). Taken together, untargeted assessment of metabolism in ApcMin mouse intestinal tumors indicated profound DFMO-elicited effects.

In Fig. 4, we summarize key DFMO-elicited 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. Further, levels of the nucleosides thymidine and cytidine, along with nucleotide precursors dTMP and dCMP, were significantly attenuated following 21 days of DFMO treatment. In contrast with the dramatic DFMO-elicited increase in adenosine in HT-29 cells, *in vivo* DFMO treatment was associated with a 3-fold 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 (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 CRC cells, where a pivotal role of SAM and thymidine metabolite pool depletion in DFMO-elicited suppression of CRC proliferation was found.

**Discussion**

DFMO is a promising compound for CRC chemoprevention. While it is well-established that DFMO inhibits ODC and attenuates cellular polyamine levels, its anti-proliferative mechanism of action is yet to be established. To broadly elucidate the metabolic consequences of DFMO treatment, we performed an untargeted metabolite profiling study of DFMO actions on both CRC cell lines *in vitro* and intestinal tumors from *ApcMin* mice. DFMO treatment was found to significantly deplete cellular polyamine levels and trigger ornithine accumulation *in vivo*, in accord with the recognized action of DFMO as a selective ODC inhibitor. Results described here further demonstrated that DFMO evokes broad changes in CRC 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 CRC.

As schematized in Fig.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 thymine-less 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 determinant of thymidine biosynthesis. Notably, two dcSAM-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 demonstrated a marked increase in SAM decarboxylase activity and accumulation of dcSAM in response to ODC inhibition by both pharmacological and genetic means (29, 31, 32) - including CRC treatment with DFMO (32). Although we found DFMO to elicit a paradoxical decrease in levels of the SAM decarboxylase product MTA (both in CRC cells in vitro and intestinal tumors in vivo), the observed >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 an essential substrate for polyamine biosynthesis. Indeed, it has been reported that 1-5% of SAM is directed to polyamine biosynthesis in healthy cells, with the remainder directed towards 1-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 non-productively 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 MTHFR activity (19, 25), an enzyme that catalyzes irreversible conversion of the TS cofactor 5,10-methyleneTHF, to the MS cofactor 5-methylTHF (see Fig.5). Accordingly, reduced levels of SAM, as we observed in DFMO-treated cells and mice, can elicit a de-
repression 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 addition of the polyamine putrescine to DFMO treated CRC cells restores the thymidine metabolite pools (Fig 6).

It is well established that via inhibition of TS, 5-FU and related anti-folate drugs deplete cellular thymidine, providing an important and well-characterized mechanism of CRC chemotherapy (20). Our data show that DFMO also acts through depletion of thymidine in rapidly proliferating CRC cells. In vivo 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. Since 5-FU mediated depletion of cellular thymidine is the cornerstone of anti-CRC chemotherapy, our study reveals the first shared mechanism for CRC 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 chemopreventative 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 where it 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 US 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 chemopreventative 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 demonstrate that DFMO-elicited anti-CRC 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 by DFMO in vivo. Development of enhanced analytical techniques will be needed to ascertain whether a “steal” of 5,10-methyleneTHF cofactor (i.e., conversion to 5-methylTHF for support of MS and SAM regeneration) provides the molecular basis for thymidine metabolite pool depletion by DFMO in mouse intestinal tumors, as inferred from CRC cell culture studies described herein.
Experimental Procedures

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 DCP clinical pharmacologists US 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 by http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM078932.pdf.

Cell Culture, DFMO Treatment and Thymidine Supplementation. Human colon cancer cell lines used for these investigations included HT-29, SW480, LoVo were obtained from 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’s 5A medium (Gibco), whereas SW480 and LoVo cells were maintained in Ham’s F12 medium (Gibco) in a humidified atmosphere 10% CO₂ in air. All culture media were supplemented with dialyzed 10% FBS (Gibco), 100U/mL penicillin and 100ug/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 hr 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 1cm sections, grown in 24-well tissue culture plates(Corning), maintained in DMEM/F12 containing nonessential amino acids, antibiotic-antimycotic, N2 supplement (Invitrogen), B27 supplement (Invitrogen), heparin (4 μg/mL; Sigma), epidermal growth factor (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 performed under an approved Weill Cornell IACUC protocol. *ApcMin* mice at 8 weeks of age given daily Intraperitoneal injections of 40 mg/kg DFMO, a generous gift from Marion Merrel Dow, administered in phosphate-buffered saline (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, Fisherscientic) using a *Tissuelyser* (Qiagen). Cells grown in 6-well plates were extracted in -70°C methanol:water, cell scrapers used to collect cells. Tissue and cell harvests were then centrifuged for 5 min at 5,000 rpm to pellet insoluble protein. Supernatant collected and pellets were twice re-extracted 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 (42).

**LC-MS and LC-MS/MS platforms for metabolite profiling.** The LC/MS setup was the same as described previously (42).

**Untargeted metabolomics data processing and statistical analysis** Raw data were processed by Agilent MassHunter Qualitative Analysis Software (version, B05), analyzed by MassProfiler Professional (MPP, version B2.02, Agilent Technology) as described (42). Briefly, MassHunter Qualitative Analysis untargeted molecular feature extraction (MFE) 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 biological 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 greater than 2 (p<0.05), compared to 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.

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References


35. Mudd SH, Poole JR. Labile methyl balances for normal humans on various dietary regimens. Metabolism 1975;24:721-35.


**Figure Legends**

**Figure 1. Untargeted LC/MS Metabolite Profiling of DFMO Treated HT-29 colorectal cancer cells.** Metabolite profiling identified differentially-expressed metabolites in DFMO-treated (300nM) compared to untreated HT-29 cells (P<0.05). **Panel A:** Plot of retention time vs. mass for 1,350 aligned features detected by untargeted molecular feature extraction in all 4 replicates from at least one treatment group. **Panel B:** Principal component analysis, showing that the DFMO-treated group clustered separately from the untreated HT-29 cell group along the principal component 1 (PC1) axis, which represents 67% of total variance. **Panel C:** Heat map representation of unsupervised hierarchical clustering analysis of data from Panel A. Relative expression of each metabolite is represented by a color code where red represents greatest abundance and blue represents lowest abundance, relative to mean values for the 1,350 denoted metabolites. **Panel D:** Volcano plot analysis indicates that 596 metabolites were altered in expression by 2-fold or greater after DFMO treatment (*p < 0.05).

**Figure 2. DFMO Affects Critical Metabolic Cycles in Human Colorectal Cancer Cells.** DFMO treated HT-29 cells (300nM) exhibited differential expression of key metabolites involved in polyamine synthesis, the methionine cycle trans-sulfuration pathway and nucleotide metabolism. Indicated values are ion intensities measured in HT-29 cell extracts from all samples in each group (DFMO-treated vs. untreated; n = 4), along with quantified differences in fold-change (FC) and statistical significance (P).

**Figure 3. Thymidine Supplementation Reduces DFMO Cytostasis** Comparison of HT-29 cells treated with DFMO (300µM) alone, or in combination with thymidine (panel A) or cytidine (panel B) over the concentration range of 30–300 µM, for a period of 7 days. Cell viability was determined based on trypan blue exclusion (Gibco) and cell counts were determined using a haemocytometer (counted in triplicate). Viability of two colorectal cancer cell lines, SW480 (panel C) and LoVo (panel D), assessed after 7-day treatment with the indicated concentrations of DFMO alone, DFMO in combination with thymidine (300 µM) or indicated concentrations of thymidine alone.
Cell viability was determined by MTT assay and indicated values represent means +/- SEM for triplicate determinations (*p<0.05).

**Figure 4. Metabolite Profiling of DFMO Treated ApcMin Mouse Tumors.** ApcMin 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 HPLC, 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 (see Fig. 3) – notably, this included markedly reduced thymidine levels. *denotes p<0.05 by unpaired t-test, comparing tumors from untreated and DFMO-treated ApcMin mice.

**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

**Figure 6. Putrescine Supplementation Modulates DFMO Mediated Thymidine Metabolite Pool depletion.** Colon cancer cell line HT29 cells were treated with DFMO(300nM) alone or in combination with putrescine(PUT) 100uM for 4 days (n=6). Metabolites were extracted and analyzed for thymidine pool metabolites, (A) thymidine triphosphate(TTP), (B) thymidine diphosphate. +/- SEM for Not significant(N.S)
Fig 2

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<td>Cystine</td>
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<tr>
<td>8.11E-04</td>
<td>2.85</td>
<td>Ornithine</td>
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</table>

Ion abundance (LOG10)
**Fig 3**

A. **HT29 Cells**  
**Thymidine Supplementation**

B. **HT29 Cells**  
**Cytidine Supplementation**

C. **Thymidine Concentration (mM)**

D. **Thymidine Concentration (mM)**

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For more information, please refer to the original publication [here](#).
Unbiased metabolite profiling indicates that a diminished thymidine pools is the underlying mechanism of colon cancer chemoprevention by alpha-difluoromethylornithine (DFMO)

Mavee Witherspoon, Qiuying Chen, Levy Kopelovich, et al.

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