Minireview

Development of Difluoromethylornithine (DFMO) as a Chemoprevention Agent

Frank L. Meyskens, Jr. and Eugene W. Gerner

Abstract

D,L-\(\alpha\)-difluoromethylornithine (DFMO) was synthesized over 20 years ago. It was hoped that this enzyme-activated, irreversible inhibitor of ornithine decarboxylase, the first enzyme in polyamine synthesis, would be effective as a chemotherapy for hyperproliferative diseases, including cancer and/or infectious processes. DFMO was generally found to exert cytostatic effects on mammalian cells and tissues, and its effectiveness as a therapeutic agent has been modest. DFMO was also found to cause treatment-limiting (but reversible) ototoxicity at high doses. This side effect, along with its minimal therapeutic activity, contributed to the loss of interest by many clinicians in further developing DFMO as a cancer therapeutic agent. However, DFMO was subsequently shown to inhibit carcino genesis-induced cancer development in a number of rodent models, and interest in developing this compound as a preventive agent has increased. The rationale for the inhibition of ornithine decarboxylase as a cancer chemopreventive agent has been strengthened in recent years because this enzyme has been shown to be transactivated by the \(c\)-myc oncogene in certain cell/tissue types and to cooperate with the \(ras\) oncogene in malignant transformation of epithelial tissues. Recent clinical cancer chemoprevention trials, using dose de-escalation designs, indicate that DFMO can be given over long periods of time at low doses that suppress polyamine contents in gastrointestinal and other epithelial tissues but cause no detectable hearing loss or other side effects. Current clinical chemoprevention trials are investigating the efficacy of DFMO to suppress surrogate end point biomarkers (e.g., colon polyp recurrence) of carcinogenesis in patient populations at elevated risk for the development of specific epithelial cancers, including colon, esophageal, breast, cutaneous, and prostate malignancies.

Early Rationale for the Development of Inhibitors of Polyamine Metabolism

Studies on the diamine putrescine and its polyamine products spermidine and spermine date to the 17th century with the observation by Leeuwenhoek of spermine phosphate crystals in human semen (1). The strong association between high levels of the polyamines and rapid proliferation in prokaryotes and eukaryotes was recognized more than 25 years ago (2–4). These investigations led scientists at the Merrell Research Institute in Strasbourg to synthesize specific inhibitors of ODC (5), the first enzyme in mammalian polyamine synthesis, and of other enzymes involved in polyamine metabolism (6–7). It was hoped that the inhibition of polyamine metabolism would be a successful strategy for chemotherapy for cancer and/or other hyperproliferative diseases or infectious diseases such as protozoal parasiticism (8).

Subsequent studies by the Merrell group and others, using specific ODC inhibitors (9–14) or genetic approaches (15, 16) to manipulate levels of endogenous polyamines, confirmed that amines derived from ornithine are essential for mammalian cell viability, and high levels are necessary for optimal mammalian cell growth. Corroborative results, demonstrating the importance of the polyamines for viability and growth, were also obtained in nonmammalian systems. Mutant strains of Escherichia coli and Saccharomyces cerevisiae, incapable of synthesizing the diamine putrescine, the first amine in the polyamine pathway, do not grow (17, 18). Null mutants of S. cerevisiae, which makes putrescine but not the triamine spermidine because of the deletion of the gene encoding the S-adenosylmethionine decarboxylase, also do not grow (19). E. coli apparently lacks this spermidine requirement for growth (20).

Misconceptions Regarding DFMO Effects on Cells and Tissues

Because polyamines are ubiquitous and, apparently, essential molecules in cells, it was reasonable to presume that the inhibition of polyamine synthesis might be toxic. In some protozoal parasites, this hypothesis is true (21). The mechanism of cell death induced by DFMO in Trypanosoma brucei involves the limitation of the production of an essential antioxidant, trypanothione (22). The parasites die because of their inability to eliminate endogenous reactive oxygen species. The suppression of polyamine synthesis does not, however, generally cause cell death in mammalian cells (23–25). Although DFMO has been reported to kill some human tumor cells, concentrations required...
for cytotoxicity are greatly in excess of those required to suppress ODC activity (26). DFMO is usually cytostatic, causing a reduction in the rate of cell proliferation in the absence of cell death.

Several recent exceptions to this generalization have been described. Treatment of human colon cancer derived CaCo-2 cells, (constitutively expressing an activated Ki-raf oncogene) with DFMO suppressed colony formation. However, DFMO suppressed the growth, but not the colony formation, of non-transfected CaCo-2 cells. DFMO also caused regression of epidermal papillomas induced by low doses of the chemical carcinogen 7,12-dimethylbenz[a]anthracene in transgenic mice overexpressing ODC (27). It has been shown that the activation of the oncogene c-myc influences cell proliferation and apoptosis by separable pathways (some involving ODC expression), presumably by modulating the production of cell survival and cell death factors (28). Mutations in the ras oncogene are prevalent in the transgenic skin carcinogenesis model (29). Thus, a plausible mechanism for selective cytotoxicity of DFMO in cells overexpressing an activated ras is that polyamines are required for either the formation of cell survival factors or the inhibition of cell death factors in cells expressing an activated ras oncogene. The suppression of polyamine pools would lead to a loss of viability.

In the few models in which DFMO seems to induce cell death [e.g., in Lawson et al. and Peralta Soler (27)], the mechanism of death is not apoptosis. In fact, DFMO has been shown to suppress apoptosis in several cell culture models (28, 30, 31). In these models, apoptosis induction requires overexpression of ODC.

**Rationale for DFMO as an Inhibitor of Carcinogenesis**

Polyamine contents are often elevated in rodent and human neoplastic cells/tissues, compared with relevant normal cells/tissues (32). A well-documented example of this relationship involves colonic polyps and cancers, compared with adjacent normal colonic mucosa (33–35). The mechanism of this elevation likely involves activation of signaling pathways influencing processes affecting intracellular polyamine pools. For example, nearly 70% of human colon cancers are associated with the activation of the c-myc oncogene (36). ODC is one target gene for the transcriptional transactivating activity of c-myc (37, 38). We have recently found that the loss of function of the APC tumor suppressor gene in the min mouse model of gastrointestinal cancer (39) causes steady-state levels of ODC RNA to increase 6–10-fold in both small and large intestinal tissue. The increased ODC RNA expression is associated with an increase in especially small intestinal polyamine contents. DFMO suppresses both the increased polyamine contents and tumorigenicity in the small intestines of min mice. These data suggest a signaling pathway between the tumor suppressor gene APC and ODC. Others have recently shown that APC acts to suppress expression of c-myc (40). Because ODC is one of the transcriptional activation targets of c-myc, it is likely that the elevation of ODC RNA in the min mouse, a model in which the loss of functional APC is associated with gastrointestinal tumorigenesis, is mediated by activation of c-myc.

Intracellular polyamine pool sizes are determined by a number of factors in addition to ODC, as depicted in Fig. 1. The identities of other oncogenes and tumor suppressor genes influencing the expression of ODC and/or other proteins affecting polyamine contents, as described in Fig. 1, remain to be elucidated for specific tissues.

**Effects of DFMO on Cell and Tissue Polyamine Contents**

Treatment of mammalian cell cultures, rodents, or humans with DFMO generally causes a suppression of putrescine and spermidine contents in cells/tissues in which intracellular pools depend on ODC activity (see Fig. 1), without affecting spermine pool sizes (41, 42). A notable exception to this finding is in human prostate tissue, in which spermine is the major polyamine. The administration of DFMO to men who are scheduled for surgical interventions to treat some form of prostate hyperplasia or neoplasia causes a suppression in all prostate polyamine pools, including the spermine pool. Supplying cells or

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animals with sufficient amounts of exogenous polyamines to restore normal intracellular pools can reverse most of the effects of DFMO (8, 10, 41).

Several groups reported that polyamine metabolism was an integral component of the mechanism of carcinogenesis, especially in epithelial tissues. Inhibitors of ODC were found to suppress tumor formation in experimental models of bladder, breast, colon, and skin carcinogenesis (32, 43–45). Inactivation of the FAD-dependent polyamine oxidase (PAO), the second enzyme in polyamine catabolism, impeded colon carcinogenesis in the dimethylhydrazine-treated rat model (46).

The mechanism of cancer prevention by DFMO probably involves more than simple inhibition of cell proliferation. Studies in animals suggested that DFMO acts late in models of chemical carcinogenesis, affecting the transition of noninvasive tumors to invasive cancers (47). Consequently, several groups have demonstrated that the expression of genes affecting tumor invasion, including the matrix metalloproteinases, are dependent on polyamines and inhibited by DFMO in several cell types (48–50).

Validation of SEBs for DFMO Effect

To assess DFMO effects in humans, we sought to validate specific markers of effects of this drug in specific tissues. ODC is the target for DFMO, and consequently should be an appropriate SEB for DFMO effect. However, ODC is a highly regulated and labile protein. Consequently, measurement of its activity in uninduced tissues is difficult. Because polyamines are stable molecules, we reasoned that the measurement of ODC products may be a meaningful measure of DFMO effect in some cases, and we have used high performance liquid chromatography (HPLC) techniques to measure tissue polyamine contents, in addition to ODC enzyme activity. We evaluated our ability to measure ODC activity and polyamine contents in colonic and rectal mucosa under a number of conditions relevant to our methods of obtaining colorectal mucosal biopsies (51). These conditions included bowel preparation procedures, size of the biopsy, number of biopsies evaluated in a single measure, and biopsy location in the bowel. Our results indicated that the bowel preparation method did not influence our measurements. Polyamine contents, and especially the spermidine/spermine ratio, were less variable than the measurement of ODC enzyme activity. Spermidine/spermine ratios were least variable, because this parameter did not depend on a second measurement (e.g., tissue protein or DNA content) for normalization. Consequently, we routinely measure polyamine contents as primary SEBs of DFMO effects in human colonic tissues (e.g., see Ref. 42).

Clinical Studies of DFMO in Malignant and Precancerous Conditions

Early clinical cancer therapeutic trials with DFMO were disappointing, and at high doses (greater than 3 g/m²/day), several side effects occurred, including diarrhea, abdominal pain, and emesis, as well as moderate anemia, leukopenia, and thrombocytopenia (52–57). Some responses were noted in Phase I toxicity and uncontrolled Phase II efficacy studies, but controlled studies failed to establish DFMO as a useful agent in specific disease sites. In addition, DFMO treatment was associated with treatment-limiting ototoxicity (58), which curtailed its utility as a cancer therapeutic agent. Recently DFMO combined with BCNU has been shown to have considerable effect on glioblastomas (59), and a reexploration of the drug in combination may be worthwhile.

We and several other groups have been actively involved in the development of DFMO as a chemoprevention agent with a systematic emphasis on the skin (60, 61), cervix (62–64), and colon (42, 65, 66). The side effects of DFMO at intermediate (1–3 g/m²/day) doses are few and limited to mild gastrointestinal upset and reversible hearing changes. At the doses (less than 0.50 g/m²/day) of DFMO being proposed for long-term chemoprevention trials, no systematic side effects (including hearing loss), have been seen (discussed below). A comparison of the side effects seen with DFMO at low, intermediate, and high doses is shown in Table 1.

Two major issues have been of prime importance in consideration of the development of DFMO as a chemoprevention agent: (a) its ability to lower polyamine levels in the tissue of interest; and (b) its effect on hearing; and the key elements of the major chemoprevention trials with DFMO are summarized in Table 2. We have performed a series of studies that have demonstrated that DFMO lowers polyamines in rectal mucosa (42, 65, 66) and does so in a dose-response manner without a rebound increase of polyamine levels after discontinuation of the drug (42). Additionally, at a dose below 0.40 g/m²/day, side effects and hearing changes did not occur more frequently than

### Table 1 Side effects of DFMO (dose, g/m²/day)

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<thead>
<tr>
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<th>High (&gt;3)</th>
<th>Intermediate (1–3)</th>
<th>Low (&lt;1)</th>
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<tbody>
<tr>
<td>Diarrhea</td>
<td>Frequent, severe</td>
<td>Occasional, mild</td>
<td>Uncommon</td>
</tr>
<tr>
<td>Abdominal pain/bloating</td>
<td>Frequent, severe</td>
<td>Occasional, mild</td>
<td>Uncommon</td>
</tr>
<tr>
<td>Nausea/Vomiting</td>
<td>Frequent, moderate</td>
<td>Uncommon</td>
<td>Rare</td>
</tr>
<tr>
<td>Hematological</td>
<td>Modest</td>
<td>Not seen</td>
<td>Not seen</td>
</tr>
<tr>
<td>Reversible hearing loss</td>
<td>Common, cumulative dose-related</td>
<td>Occasional, dose-related</td>
<td>Uncommon, may be absent at &lt;0.5 g/m²/day</td>
</tr>
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* Unpublished data.
DFMO as a Chemoprevention Agent

In the placebo group, even after 1 year of therapy. Because the overall effect of DFMO on rectal mucosal levels of polyamines (putrescine levels, spermidine:spermine ratio) was equivalent at daily doses of 0.20 or 0.40 g/m²/day, these studies suggested that a dose of DFMO of 0.20 g/m²/day would be effective in lowering colon mucosal polyamines without producing side effects, including audiometric decreases in hearing threshold (see Ref. 42). In a smaller study, Love et al. has reported on the effect of 0.50 g/m²/day of DFMO on rectal mucosal polyamines (67). Compared with the placebo group, polyamines were decreased after 3 and 12 months of therapy. A recent case-control study of patients with colon cancer (68) indicates that increases in mucosal polyamine measurements were significantly associated with risk (odds ratio, 4.8). This study provides additional evidence for polyamines as biomarkers for identifying high-risk individuals and/or as intermediate end points in colon prevention trials.

In a complex Phase I study of oral DFMO for 1 month at multiple doses, an effect of the drug on TPA-induced ODC activity in the skin biopsies was demonstrated (60). On the basis of limited data, the investigators concluded that a dose of 0.50 g/m²/day produced this biochemical response and that no side effects were demonstrated. In a two-step Phase I study of DFMO, piroxicam, or the combination in 31 subjects the combination of DFMO alone at 0.50 g/m²/day significantly reduced cutaneous TPA-induced ODC levels (61). In this study, no objective changes in hearing were demonstrated (61). Mitchell et al. (64) have also reported the results from a 1 month dose de-escalation Phase I trial of oral DFMO in grade 3 cervical intraepithelial neoplasia. A dose of 1.0 g/m²/day produced a significant decrease in the spermidine:spermine ratio in the cervix tissue. Surprisingly, 15 patients experienced a complete or partial histological response that was not dose-dependent. Lower doses and longer-term randomized trials will be necessary to determine whether lower doses also produce these effects because the effect on tissue polyamines takes time and, except for one agent [topical trans-retinoic acid (69)], evaluation of chemoprevention agents in the Phase III setting has not borne out promising Phase II results in cervical intraepithelial neoplasia (70–73). We have also measured the effect of 1 month of oral DFMO on polyamines in the prostate in patients undergoing a definitive surgical procedure, and we have demonstrated a marked lowering of polyamines (6). (R. Love of Wisconsin has also obtained similar results.)

Although DFMO has been highly effective as a chemoprevention agent in combination in preclinical models, to date only one clinical study has been reported using DFMO in combination (61). Using a complicated but rational two-step approach, the effect of 6 months of oral daily DFMO and piroxicam alone and in combination on TPA-induced ornithine ODC in skin biopsies and urinary 11-dehydrothromboxane B₂ was measured, and an effect on these biomarkers demonstrated. On the basis of these responses and a favorable clinical profile, doses of DFMO of 0.50 g/m²/day and piroxicam 10 mg every other day was recommended for Phase IIa and IIb trials.

In therapeutic trials, hearing loss was frequent and considerable, although reversible (58). However, the doses being used in chemoprevention trials are considerably lower. There are three reports that have examined the issue of hearing loss from DFMO in detail (42, 74, 75). Our original meta-analysis of patients receiving DFMO for therapeutic reasons suggested that hearing loss rarely occurred below a total cumulative dose of about 150 g and that above this dose, the hearing loss was cumulative but reversible (74). However, these patients were receiving doses of DFMO above 1 g/m² daily, and, therefore, the direct relevance of this finding to hearing changes at the lower doses used in chemoprevention trials is problematic. Pasic et al. (75) has done an analysis of hearing changes in 66 patients entered into their Phase I and II trials. The oral doses of DFMO ranged between 0.5 and 5.0 g/m² daily. A complex analysis was performed, and the conclusions were made that small predictable shifts in auditory thresholds occurred, which

<table>
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<th>Table 2 Chemoprevention trials of DFMO</th>
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<tbody>
<tr>
<td>Phase</td>
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<tr>
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</tr>
<tr>
<td>Pilot</td>
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<td>IIA (1 month, de-escalation)</td>
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<td>I (1 month, de-escalation)</td>
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<td>II (12 months, randomized)</td>
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7 R. Love, personal communication.
increased as the daily dose of DFMO increased, but that the changes were not related to cumulative dosage. However, an analysis of the mean thresholds at the beginning and end of the study for all of the subjects receiving a dose of 0.50 g/m²/day of DFMO indicated that there was no discernible shift of audiometric threshold at any frequency measured. The most relevant study addressing the issue of hearing loss by DFMO is our 1-year placebo-controlled randomized trial of DFMO (42). The doses of DFMO were low but effective in lowering tissue polyamines. Pretreatment and serial audiometry were performed. There was no evidence for a dose-related effect of DFMO on hearing at the three doses tested, 0.075, 0.20, and 0.40 g/m²/day. Subsequent detailed analyses of the data indicate that there is no evidence to suggest that hearing loss at any frequency at the lowest and intermediate dose occurred. At the highest dose tested (0.40 g/m²/day), there may be a 3-dB decrease (which was clinically unimportant) at the two lowest of the eight frequencies tested.

Overall, we conclude that the effect of DFMO on hearing at doses relevant for usage as a chemoprevention agent is not significant. In a study of different doses (0.125–1.0 g/m²/day) of DFMO given to patients with superficial bladder cancers, Loprinzi et al. (76) have found that little to no side effects were demonstrated. We have also found that the effect of DFMO on nonaudiological side effects at doses below 0.40 g/m²/day is not greater than placebo (41), thereby providing considerable strength for its usage at low doses as a chemoprevention agent. Recently, two detailed studies of aging and hearing have been published (77, 78), which will help considerably in the long-term evaluation of subtle hearing changes in response to DFMO and other potentially ototoxic drugs; a set of guidelines for hearing changes and chemoprevention drug development is currently being developed by the National Cancer Institute.

Current Lessons and Future Development

Several important lessons have emerged from the development of DFMO that have relevance to the development of chemoprevention agents in general, particularly those which are currently used for other indications.

These key issues include:

(1) The relevance of in vitro and preclinical models to identify appropriate SEBs for the intervention, and to predict consequences for the intervention, in humans (e.g., inhibit proliferation, induce apoptosis, inhibit invasion).

(2) Side effects that occur at high therapeutic doses of the drug may not be present or relevant at lower doses.

(3) A dose de-escalation design is a powerful method by which to determine the lowest dose of an agent that can consistently modulate the relevant biochemical markers without side effects.

(4) A dose of DFMO 0.20–40 g/m² daily is probably the best estimate of the proper dose for subsequent colon cancer chemoprevention trials. DFMO doses required to suppress polyamine contents in other tissues need to be verified for each tissue under study.

(5) Although DFMO is a potent inhibitor of epithelial carcinogenesis, it does not totally suppress tumorigenesis in animal models. Consequently, combinations of DFMO with other agents, such as the NSAIDs should be considered. Our group is conducting both preclinical and clinical investigations combining DFMO with the NSAID sulindac at this time.

At the clinical level, interest in the exploration of DFMO as a chemoprevention agent has recently increased markedly. Currently, we are aware of the following clinical trials using DFMO as a chemoprevention agent: breast (C. Fabian, University of Kansas), Barrett’s esophagus (D. Brenner, University of Michigan), cervix (M. Follen Mitchell, M. D. Anderson Cancer Center, Houston, TX), and prostate (A. Simoneau, University of California-Irvine). Additionally, DFMO is being studied in combination with piroxicam in a Phase II nonmelanoma skin cancer trial (P. Carbone, University of Wisconsin) and with sulindac in a Phase IIb colon cancer prevention study (F. Mey- skens, University of California-Irvine, and E. Gerner, University of Arizona).

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