A Randomized, Placebo-controlled Trial of Low-Dose α-Difluoromethylornithine in Individuals at Risk for Colorectal Cancer[1]

Richard R. Love,[2] Russell Jacoby, Michael A. Newton, Kendra D. Tutsch, Kris Simon, Marcia Pomplun, and Ajit K. Verma

Departments of Medicine [R. R. L., R. J., K. D. T., K. S., M. P.], Biostatistics and Medical Informatics [M. A. N.], and Human Oncology [A. K. V.], University of Wisconsin School of Medicine, Madison, Wisconsin

Abstract

DFMO is an irreversible inhibitor of ornithine decarboxylase (ODC), the key enzyme in mammalian polyamine biosynthesis. The goal of this study was to determine the effects of DFMO 0.5 g/m^2/day as a single oral dose on polyamine and ODC levels in rectal, rectosigmoidal, and cecal colonic mucosae of individuals at risk for colon cancer because of a personal history of adenomatous polyps of the colon or a family history of colon cancer in at least one first-degree relative. A second goal was to determine toxicity of this treatment given over 1 year. Forty-five randomized subjects had a flexible sigmoidoscopy with no preparation and a colonoscopy after lavage preparation at baseline, a sigmoidoscopy with no preparation after 3 months, and both procedures (as at baseline) after 12 months, with mucosal biopsies taken from the rectosigmoid area (sigmoidoscopy) or rectal and cecal areas (colonoscopy) for evaluations of ODC and polyamine levels. Significantly decreased levels of putrescine and spermidine were found in rectosigmoid colonic mucosae of DFMO-treated (n = 24) compared with placebo (n = 21) subjects at 3 months (P = 0.03 and 0.04) and 12 months (P = 0.005, P = 0.004). Similar trends, none reaching statistical significance, were found for individual polyamine levels in rectal and cecal mucosae. No significant differences in ODC levels were detected marginally. There was evidence of global suppression of ODC and polyamine levels in the treatment group (P = 0.035). Three DFMO recipients (12.5%) developed clinically noticeable and audiologically demonstrated hearing loss, which was reversible and attributed to DFMO after 3 months (two subjects) and 12 months (one subject). The tissue polyamine changes demonstrated in this study are consistent with findings in other studies in colon and other tissues. The ototoxicity findings here suggest that investigation of other DFMO schedules, such as ones with a drug "holiday," will be a necessary step before Phase III chemoprevention studies can be pursued.

Introduction

Given the multiyear preclinical history of malignancies, in the evaluation stages of a potential preventive intervention, it is very useful to have an intermediate end point, a biological "marker," changes in which might be expected to be linked to a change in risk for the eventual development of cancer. Additionally, optimally the change in the marker should be specifically demonstrated in the target tissue of interest.

A spectrum of laboratory and clinical data have suggested that the enzyme ODC[3] as an appropriate target for control of preclinical cancers (1, 2). ODC, which decarboxylates ornithine to putrescine, is the first and controlling enzyme in the biosynthetic pathway for mammalian polyamines (1). The specific roles of polyamines in cell growth, differentiation, and neoplastic development and the potential usefulness of measurements of ODC activity and polyamine contents as biomarkers for cancer risk in humans are all not well defined. Levels of ODC and polyamines can be assessed in small samples of skin, colon, and other tissues. ODC activity can be inhibited by the suicide inhibitor DFMO (2). In animal studies, DFMO can significantly suppress the appearance of several cancers (3–5). In humans, in patients with advanced tumors, only in cancers of the brain and cervix has DFMO been shown to be promising as a therapeutic intervention (6, 7). At clinical doses defined by Phase I studies, gastrointestinal and reversible ototoxicity have been the major side effects of DFMO (8).

In these contexts, recent investigations have focused on defining nontoxic doses of DFMO that demonstrably affect target tissue ODC and polyamine levels (9, 10). In 1993, the present authors reported that at a dose of 0.5 g/m^2/day, five patients developed no ototoxicity after 10–12 months and demonstrated consistent evidence of suppression of 12-*O*-tetradecanoylphorbol-13-acetate-induced ODC activity in skin biopsy specimens (9). To extend observations at this low dose of DFMO, we have now studied polyamines and ODC in colon mucosal biopsies in a randomized control trial of this treatment given for 1 year.

Materials and Methods

Subjects. Under a protocol approved by the University of Wisconsin Committee for the Protection of Human Subjects (UWCPHS 92-480-269), healthy adults >18 years of age were

Received 12/22/97; revised 8/3/98; accepted 8/10/98.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] Supported by NIH Grant CA 59352.
[2] To whom requests for reprints should be addressed, at 7C MSC, 1300 University Avenue, Madison, WI 53706.

[3] The abbreviations used are: ODC, ornithine decarboxylase; DFMO, α-difluoromethylornithine.

recruited who had demonstrated presence of colonic adenomatous polyps on colonoscopic examination and/or had a family history of colorectal cancer in at least one first-degree relative. Patients with a hearing deficit acknowledged by the individual, known other colorectal disease, or women of childbearing potential were ineligible. Enrolled subjects were asked not to take aspirin or nonsteroidal anti-inflammatory drugs during any 7 days prior to study investigations. All subjects provided written informed consent according to institutional and federal guidelines.

Drug Formulation. DFMO was supplied in liquid form with 200 mg/ml of active drug as was an identical tasting placebo. Subjects self-administered a measured dose once daily in the evening.

Drug Administration. DFMO was given to randomly assigned subjects at a dose of 0.5 g/m^2 BSA at baseline. There was no pretreatment run-in period. There were no dose changes during the study. Any subject developing any Eastern Cooperative Oncology Group toxicity greater than grade 1 had his/her medication stopped.

Subject Monitoring. Prior to study entry and every 3 months, a complete blood count, medical assessment, toxicity check, and physical examination were performed. A liver function and renal function chemistry panel was done prior to study entry. An audiogram was done before the study, then if any audiotoxicity developed, or at off study.

DFMO Assessment. At 3, 6, 9, and 12 months, 13–16 h after previous DFMO dose, blood samples were taken from subjects for plasma, which was immediately frozen. DFMO was assayed as reported previously (9).

Study Design and ODC and Polyamine Marker Assessments. At the baseline evaluation, subjects underwent flexible sigmoidoscopy without any enema, lavage, or other preparation. Pinch biopsies of rectosigmoid mucosae were taken at 15 cm from the anal verge. After a lavage preparation, full colonoscopy was done ~16 h later, and biopsies were taken at 5 cm from the anal verge ("rectal") and in the cecal area. At 3 months, flexible sigmoidoscopy was again done with rectosigmoid biopsies as at baseline; at 12 months, the baseline evaluation sequence with both flexible sigmoidoscopy and colonoscopy was followed.

Thus, the rectosigmoid mucosal biopsies were always taken after no colonic preparation, and the 3- and 12-month specimens were obtained ~13–16 h after the previous DFMO/placebo administration. In contrast, the colonic mucosal biopsies of the cecum and rectum were always taken 12 h after lavage preparation, and the 12-month specimens were obtained ~12 h after the previous DFMO/placebo administration.

Biopsy tissue samples for measurement of ODC activity were placed in minimal essential medium on ice and immediately assayed according to our method reported previously (9).

Biopsy tissue samples for polyamine assay were immediately placed into 2% perchloric acid and homogenized in a Polytron. The acid extracts were frozen at −70°C until assay. The polyamines putrescine, spermidine, and spermine were analyzed in the acid extract by the high-performance liquid chromatography method of Kabra *et al.* (11). Briefly, after addition of the internal standard (1,7-diaminoheptane), the polyamines in the acid extracts were derivatized with dansyl chloride, and the derivatized samples were purified using Bond-Elut C_{18} SPE columns (Varian, Walnut Creek, CA). The derivatized polyamines were separated on a Waters 8 × 10 Novapak C_{18} cartridge (Waters, Milford, MA) using a gradient of 48 to 100% acetonitrile over 30 min against 10 mM sodium acetate buffer with fluorescent detection (excitation, 340 nm; emission, 515 nm). Polyamines were quantitated by comparison of chromatographic peak areas of each polyamine compared with that of the internal standard. The standard curve for putrescine was linear from 0.325 to 10 nmol/ml of extract; the standard curve for spermidine was linear from 1.56 to 50 nmol/ml of extract; and the standard curve for spermine was linear from 1.56 to 50 nmol/ml ($r^2 > 0.999$ in each case). The coefficient of variation in duplicate determinations was <2% for low and high standards of each polyamine. The interday coefficient of variation was <5% for high standards of each polyamine ($n = 4$; putrescine, 10 nmol/ml; spermidine and spermine, 50 nmol/ml). The interday coefficient of variation for low standards of each was <7% for each polyamine ($n = 4$; putrescine, 0.62 nmol/ml; spermidine and spermine, 3.12 nmol/ml). Sensitivity was optimized by minimizing the volume of perchloric acid used to homogenize the tissue samples as well as the volume of methanol used to elute the derivatized polyamines from the SPE columns. The resulting polyamine solutions were within the range of the standard curve and well above the limit of detection of 6 pmol of injected polyamine. These assay ranges were found to be sufficiently sensitive to measure polyamines in 5–10 mg of colon biopsy specimens. The resulting polyamine levels in each biopsy specimen were normalized to the DNA content of each individual sample. Duplicate tissue samples showed a mean variability of 20% when normalized to DNA. This agrees with the variability in individual tissue polyamine measurements reported by Higuchi and Wang (12).

Statistical Considerations. Two-sample, unpaired, two-sided *t* tests were used to assess differences in ODC and three polyamine measures between the treatment and control groups, and comparisons were done separately on baseline data on changes from baseline to 3 months for rectosigmoid biopsy data and on changes from baseline to 12 months for all responses. Replicate measures were averaged prior to analysis. Data on these measures were transformed to their natural logarithms to stabilize sample variance.

A global assessment of treatment effect was performed to account for the multivariate nature of the data. A linear model was fit with treatment group, tissue source, and specific polyamine or enzyme as predictors of log concentration. Interactions among these major factors were tested. Significance levels were calculated by randomization test in which subjects were experimental units to account for potential dependencies among the response measures within subjects. A Monte Carlo sample size of 10,000 was used. This assessment investigated changes between baseline and 12 months only.

Results

Forty-five subjects were entered into the study; 24 received DFMO, and 21 received placebo. Subject characteristics are shown in Table 1. All subjects were non-Hispanic Caucasians, except one black woman. Five additional subjects were canceled before any procedures or treatment was given (but after baseline/flexible sigmoidoscopy and colonoscopy procedures were accomplished). Mean steady-state plasma DFMO concentrations (*Cp*) of 13.6 ± 4.5 μM were found in 22 DFMO recipients still taking DFMO at the 3-month evaluation. *Cp* values were insignificantly different in subjects evaluated at 6, 9, and 12 months.

Toxicity. All of the 21 placebo subjects completed the 12 months of the study. Five of the 24 subjects receiving DFMO (21%) did not complete the 12 months of the study as planned.

Table 1 Demographic Characteristics of Study Subjects (N = 45)

Characteristic	Category	*n*	%
Sex	Male	23	51
	Female	22	49
Ages (yr)	31–50	15	33
	51–60	14	31
	61–80	16	36
Family history of colorectal cancer	Yes	18	40
	No	27	60
Personal history of adenomatous polyps	Yes	33	73
	No	12	27

Table 2 Baseline tissue polyamine levels (nmol/mg DNA natural logarithmic values)

	Rectosigmoid No preparation	Rectum lavage preparation	Cecum lavage preparation
Putrescine			
DFMO	2.22	2.40	2.32
Placebo	2.39	2.62	2.27
Spermidine			
DFMO	3.64	3.40	3.57
Placebo	3.52	3.48	3.55
Spermine			
DFMO	5.04	4.75	4.98
Placebo	4.86	4.71	4.79

Two withdrew for unspecified reasons apparently unrelated to any toxicity at 3 months. Two developed both tinnitus and clinical hearing loss, which they reported at the 3-month evaluation. Each of these had audiograms showing 15 decibels or greater hearing loss in both ears at two or more frequencies. Medication was stopped, and both had reduction of the hearing loss and tinnitus over 2 months. The fifth subject reported unilateral hearing loss at 6 months and was found to have an ear canal tumor on the affected side. One additional subject was not aware of any hearing loss and reported no tinnitus at his 12-month evaluation but was nevertheless found to have 15-decibel losses at three frequencies in one ear, a 20-decibel loss in the same ear at an additional frequency, and 10- or 5-decibel losses at three frequencies in the opposite ear. These resolved with time and were considered likely to be due to DFMO. All hearing losses tended to be at low frequencies. In summary, 3 of 24 DFMO recipients (12.5%) developed reversible audiotoxicity ascribed to the drug; two of these developed after total doses of 45 g/m^2. No other toxicities developed that were irreversible or of greater than grade 1 in either placebo or DFMO recipients.

ODC and Polyamine Effects. In contrast to the similar tissue levels at baseline (Table 2), putrescine and spermidine levels in sigmoid colon mucosae, obtained by flexible sigmoidoscopy, were significantly lower at both 3- and 12-month assessments in DFMO as compared with placebo recipients (Fig. 1). Differences in levels of spermine and ODC at these time points were not of statistical significance in marginal tests.

At 3 months, the changes in the spermidine:spermine ratio were significantly different in the two groups ($P = 0.05$; mean change for DFMO group −0.26; for placebo, 0.07). At 12 months, the changes in this ratio were not significantly different ($P = 0.17$; mean change for DFMO group, −0.66; for placebo, −0.36). ODC activity levels were 4.36 and 4.45 nmol/mg DNA in the DFMO and placebo groups, respectively, at baseline;

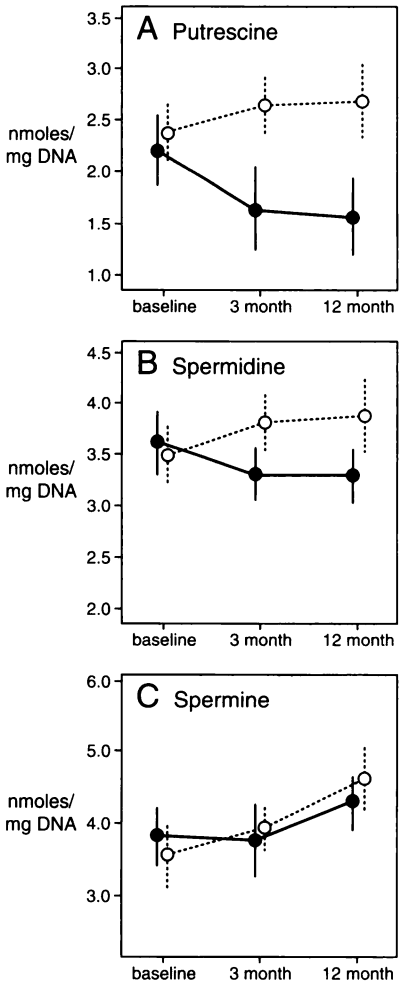

Fig. 1. *Solid lines*, the DFMO recipient group ($n = 24$); *dotted lines*, the placebo group ($n = 21$). The dots show mean levels; *bars*, ± 2 SE. The tissue levels in the two randomized groups did not differ significantly for either putrescine or spermidine levels at baseline. At 3 and at 12 months, mean putrescine levels were significantly lower in the DFMO group ($P = 0.03$ and $P = 0.005$). At 3 and 12 months, mean spermidine levels were significantly lower in the DFMO group ($P = 0.04$ and $P = 0.004$). The differences in levels at 3 and 12 months for both putrescine and spermidine were not significant. For spermine, the changes in the two groups between baseline and 3 months was not significant ($P = 0.40$); between baseline and 12 months, the difference in change in the two groups was marginally significant ($P = 0.08$).

mean changes of −0.50 and −0.17 at 3 months and −0.60 and −0.36 at 12 months were not statistically different between the two groups.

In rectal colonic mucosae obtained at colonoscopy, similar comparisons at 12 months showed a suggestion of decreased levels of putrescine in the DFMO group ($P = 0.07$) but no statistically significant differences in spermidine, spermine, or ODC levels when paired time point evaluations were studied. Similarly, with colonoscopically obtained cecal colonic mucosae, although there were trends for slightly lower values for levels of all three polyamines and ODC after 12 months, none of the differences were statistically significant.

A global analysis of treatment effect demonstrated a significant decrease in the activity levels of treated subjects for all tissue sites and all measures ($P = 0.035$), with no significant interactions. On the logarithm scale, the expected concentration

measurement was 0.22 unit lower in the treatment group. Thus, raw concentrations in the DFMO patients were ~80% of the control values. Four subjects were found to have adenomas at their 12-month evaluations (three on DFMO and one on placebo).

Discussion

In this double-blind placebo-controlled trial, findings showed modest reductions in sigmoid colonic mucosae polyamines (putrescine and spermidine) 13–16 h after the last dose of oral DFMO 0.5 g/m^2. We could not demonstrate effects of this dose of DFMO in rectal or cecal mucosae, perhaps because these tissues were exposed to a lavage solution 12 h before the biopsies. We have demonstrated previously an effect of lavage preparation on colonic mucosal ODC levels (13). Other explanations for this finding of no effect in rectal and cecal tissues are possible: for example, the time of day samples were taken was regularly earlier (a.m.) than for the rectosigmoid samples (14).

These findings are consistent with our previous findings of suppression of 12-*O*-tetradecanoylphorbol-13-acetate-induced ODC in skin biopsies at this dose of DFMO (9), with findings of Boyle *et al.* (10) of reduced polyamine concentrations in rectal mucosae at a dose of 3 g/m^2/day of DFMO, and with findings of decreased polyamines in prostate tissue at the 0.5 g/m^2 DFMO dose.[4] Whether any of these intermediate biomarker changes would result in long-term reductions in cancer development in these tissues with long-term therapy are uncertain but worthy of evaluation.

Unfortunately, in contrast to our initial experience with the 0.5 g/m^2 DFMO dose, in this study, three subjects developed reversible audiotoxicity attributed to the DFMO. Analyses of the relationship of DFMO to audiotoxicity suggests that this side effect is related to total daily dose (15, 16). The data presented here with a dose of 0.5 g/m^2/day suggest that we are working at the border of ability to demonstrate a regular biomarker tissue effect in colon mucosae, whereas other data suggest that with slightly lower doses, polyamine effects can be demonstrated and no ototoxicity is seen (17). Because the mechanisms of this toxicity are unknown (it is atypical in being reversible and characterized by low frequency hearing loss), it cannot be predicted whether a threshold for ototoxicity exists. In a brain tumor study by Levin *et al.* (6), an intermittent treatment schedule with much higher doses of DFMO than were used in this study, was associated with a frequency of ototoxicity similar to that observed here. Our interpretation of this situation is that an intermittent drug schedule is more likely to be associated with absence of ototoxicity, if doses in the range of 0.25–0.5 g/m^2/day are to be used.

[4] E. Messing, unpublished data.

References

1. Pegg, A. E. Polyamine metabolism and its importance in neoplastic growth and a target for chemotherapy. Cancer Res., *48:* 759–774, 1988.

2. Verma, A. K. The enzyme-activated irreversible inhibitor of ornithine decarboxylase, DL-α-difluoromethyl ornithine: a chemopreventive agent. Prev. Med., *18:* 646–652, 1989.

3. Fozard, J. R., and Prakash, N. J. Effects of D,L-α-difluoromethylornithine, an irreversible inhibitor of ornithine decarboxylase, on the rat mammary tumor induced by 7,12-dimethylbenz[*a*]anthracene. Arch. Pharmacol., *320:* 72–77, 1982.

4. Tutton, P. J. M., and Barkla, D. H. Comparison of the effect of an ornithine decarboxylase inhibitor on the intestinal epithelium and on intestinal tumors. Cancer Res., *46:* 6091–6094, 1986.

5. Homma, Y., Kakizoe, T., Samma, S., and Oyasu, R. Inhibition of *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine-induced rat urinary bladder carcinogenesis by α-difluoromethylornithine. Cancer Res., *47:* 6176–6179, 1987.

6. Levin, V. A., Prados, M. D., Yung, W. K. A., Gleason, M. J., Ictech, S., and Malec, M. Treatment of recurrent gliomas with eflornithine. J. Natl. Cancer Inst., *84:* 1432–1437, 1992.

7. Nishioka, K., Melgarejo, A. B., Lyon, R. R., and Mitchell, M. F. Polyamines as biomarkers of cervical intraepithelial neoplasia. J. Cell Biochem. Suppl., *23:* 87–95, 1995.

8. Abeloff, M. D., Slavik, M., Luk, G. D., Greffin, C. A., Hermann, J., Blanc, O., Sjoerdsma, A., and Baylin, S. B. Phase I trial and pharmacokinetic studies of α-difluoromethylornithine–an inhibitor of polyamine biosynthesis. J. Clin. Oncol., *2:* 124–130, 1984.

9. Love, R. R., Carbone, P. P., Verma, A. K., Gilmore, D., Carey, P., Tutsch, K. D., Pomplun, M., and Wilding, G. Randomized phase I chemoprevention dose-seeking study of α-difluoromethylornithine. J. Natl. Cancer Inst., *85:* 732–737, 1993.

10. Boyle, J. O., Meyskens, F. L., Jr., Garewal, H. S., and Gerner, E. W. Polyamine contents in rectal and buccal mucosae in humans treated with oral difluoromethylornithine. Cancer Epidemiol. Biomark. Prev., *1:* 131–136, 1992.

11. Kabra, P. M., Lee, H. K., Lubich, W. P., and Marton, L. J. Solid-phase extraction and determination of dansyl derivatives of unconjugated and acetylated polyamines by reversed-phase liquid chromatography: improved separation systems for polyamines in cerebral spinal fluid, urine and tissue. J. Chromatogr., *380:* 19–32, 1986.

12. Higuchi, C. M., and Wang W. Comodulation of cellular polyamines and proliferation: biomarker application to colorectal mucosa. J. Cell. Biochem., *57:* 256–261, 1995.

13. Love, R. R., Verma, A. K., Surawicz, T. S., and Morrissey, J. F. Colon ornithine decarboxylase activity following standard endoscopy preparation regimens. J. Surg. Oncol., *42:* 150–153, 1989.

14. Fujimoto, K., Granger, D. N., Johnson, L. R., Price, V. H., and Tso, P. Diurnal rhythm of L-ornithine decarboxylase activity in small intestine of fasted rat. Gastroenterology, *100* (Suppl.)*:* A523, 1991.

15. Pasic, T. R., Heisey, D., and Love, R. R. α-Difluoromethylornithine ototoxicity: chemoprevention clinical trial results. Arch. Otolaryngol. Head Neck Surg., *123:* 1281–1286, 1997.

16. Croghan, M. K., Aickin, M. G., and Meyskens, F. L. Dose-related α-difluoromethylornithine ototoxicity. Am. J. Clin. Oncol., *14:* 331–335, 1991.

17. Meyskens, F. L., Jr., Emerson, S. S., Pelot, D., Meshkinpour, H., Shassetz, L. R., Einspahr, J., and Alberts, D. S. Dose de-escalation chemoprevention trial of α-difluoromethylornithine in patients with colon polyps. J. Natl. Cancer Inst., *86:* 1122–1130, 1994.