DNA Image Cytometric Measurement as a Surrogate End Point Biomarker in a Phase I Trial of α-Difluoromethylornithine for Cervical Intraepithelial Neoplasia


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Abstract

Cervical intraepithelial neoplasia grade 3 (CIN 3) is considered a high-risk precursor of invasive cervical cancer. α-Difluoromethylornithine (DFMO) is a promising antiproliferative chemopreventive agent. The purpose of this study was to evaluate image cytometric measurement of nuclear DNA (ICM-DNA) as a surrogate end point biomarker (SEB) in a Phase I trial of DFMO for CIN. Thirty patients with CIN 3 were treated with DFMO at five doses, ranging from 0.0625 to 1.0 g/m²/day, for 1 month. Half of the patients had histological responses. Twenty-five pre- and posttreatment cervical biopsies from 11 responders and 14 nonresponders were available for this analysis. ICM-DNA was performed on 4-μm sections cut from formalin-fixed tissue blocks and stained with thionin-SO₂ Feulgen reaction. ICM-DNAs for each case were expressed as normalized measurements (against the nuclear modal absorbance of lymphocytes) of the absorbance of each cell of interest and were presented in bar histograms. The mean normalized summed absorbance (ΣODₙ) was obtained as a mean histogram of the cell population of interest. Nineteen (76%) of 25 patients had a significant decrease in ΣODₙ after DFMO treatment. Posttreatment values were significantly lower than pretreatment values in a paired analysis, and responders had significantly lower values than nonresponders. Analyses of different ICM-DNA references, including percentile values of ΣODₙ, distribution, DNA malignancy grade, and 5c exceeding rate, showed a decrease of mean ΣODₙ during DFMO treatment. In addition, the summed posttreatment ΣODₙ histograms also showed progressively shorter right shoulders compared with pretreatment histograms in both responders and nonresponders. We concluded that the modulation of ΣODₙ reflected the chemoprevention effect of DFMO even before morphological changes appeared, and thus, ICM-DNA may be useful as a SEB in chemoprevention trials of DFMO.

Introduction

Cervical cancer remains an important health problem; it is the second most common cancer in women worldwide (1). CIN 3, which includes severe dysplasia and carcinoma in situ, is a high-risk precursor of invasive cervical cancer and is thus suitable for the study of chemoprevention of cancer (2, 3). One chemopreventive agent that is promising in the cervix is DFMO, a potent antiproliferative agent that was used in a Phase I chemoprevention trial in CIN 3 (3). DFMO will soon be tested in a Phase II placebo-controlled trial in CIN.

DFMO, a specific inhibitor of ornithine decarboxylase, inhibits polyamine synthesis. Despite extensive research efforts, the precise function of polyamines in cellular physiology is not yet well understood. Basic polyamines are capable of noncovalent interactions with nucleic acids and proteins (4–6), and these interactions may stabilize DNA structure and protect DNA from nuclease digestion (5, 7), alter sequence-specific DNA-protein interactions, change the regulatory process in the chromatin of nuclei (8, 9), and affect the attachment of DNA to the nuclear matrix, possibly affecting replication and transcription (5, 10, 11). Because DFMO inhibits ornithine decarboxylase activity and polyamine synthesis, it can inhibit or delay DNA synthesis both in vitro and in vivo (12, 13). This process has been associated with decreased cell proliferation (12, 14), loss of the mitogenic response to growth factor stimulation (15), and reduction of proto-oncogene (c-fos and c-myc) expression (16).

Here, we investigated two hypotheses. The first was that DFMO decreases DNA synthesis or total DNA content in CIN...
3 lesions. The second involved a potential SEB for evaluating the effects of DFMO.

The end point of interest in chemoprevention studies is cancer incidence reduction (17). However, this end point requires years of follow-up, and such studies are expensive. SEBs are intermediate markers of carcinogenesis that allow trials to be of shorter duration, to require fewer subjects, to be lower in cost, to use small tissue samples, and to aid in learning more about the carcinogenic process (17).

For several decades, flow cytometric measurement of nuclear DNA and ICM-DNA have been used for research and diagnostic purposes as biomarkers of proliferation and neoplastic transformation (18–24). For example, an increase in nuclear DNA during the progression of CIN lesions has been shown (21, 22). ICM-DNA has also been used for monitoring dose effectiveness in some chemotherapeutic studies (23, 24).

Recently, ICM-DNA has been proposed for purposes of SEB development because it uses an intact tissue sample, and thus, important information about tissue architecture and the degree of neoplastic progression is retained (17). Despite relative limitations of ICM-DNA in tissue sections (25, 26), well-standardized analysis (27) of nuclear summed absorbance can still reflect the relative total amount of cell DNA. Thus, our second hypothesis was that DFMO decreases DNA synthesis and/or total DNA content in CIN 3 lesions and that ICM-DNA measures that reflect DFMO’s effect on DNA content may serve as SEBs of the effects of chemoprevention. To test our hypotheses, we used ICM-DNA to examine pre- and posttreatment biopsies from a group of CIN 3 patients during a chemoprevention trial of DFMO.

Materials and Methods

Patient Groups and Tissue Sampling. Thirty patients with CIN 3 involving over one-third of the surface area of the cervix (i.e., areas ~1.0–1.5 cm in diameter) were treated in a Phase 1 chemoprevention trial. Six patients were assigned to each of five dose levels of DFMO, ranging from 0.0625 to 1.0 g/m2 day, for 1 month. Pretreatment colposcopically directed biopsy samples (measuring 1 × 2 × 2 mm) were compared with posttreatment colposcopically directed cone biopsy samples (measuring 3 cm in diameter at the cone base and 2 cm in height). Fifteen (50%) of these patients had histological responses (previously classified as complete responses (a posttreatment diagnosis of metaplasia or reactive and granulation tissue) and 10 were evaluated as partial responses [CIN lesions of lower grade (i.e., 1 or 2) than before treatment]. Samples from 25 of the 30 patients were available for ICM-DNA analysis because of biopsy size and the need for adequate field size.

Preparation of Materials. The tissue blocks were randomly ordered, and the same microtome was used to cut all of the specimens at the same time. Serial 4-μm-thick tissue sections were stained with H&E for light microscopic evaluation, and other sections were stained for the Feulgen reaction using the protocol of Xillix Technologies (Vancouver, British Columbia, Canada; Ref. 28). All slides were stained at the same time, under the same conditions. To increase the accuracy of this study, paired pre- and posttreatment samples from each patient were cut and treated under the same conditions, at the same time. Briefly, following deparaffinization, the samples were rehydrated and postfixed in Boehm-Sprenger fixative. After 45 min of acid hydrolysis (5 N HCl; 23°C), the sections were stained with thionin-SO2, washed, dehydrated, cleared in xylene, and mounted for image analysis. The pre- and posttreatment H&E-stained slides were reviewed to identify the pathological areas of interest, including dysplastic and reactive areas in the epithelium, by two pathologists (A. M. and J. V. B.) and used as templates to map Feulgen-stained slides.

Image Analysis. At The University of Texas M. D. Anderson Cancer Center (Houston, TX), the CytoSavant computer-assisted image analysis system (Oncometrics Imaging Co., Vancouver, British Columbia, Canada) was used for ICM-DNA analysis (29). This system has a scientific-grade charge-coupled device, which is able to give a small pixel size, 100% fill factor, high quantum efficiency, low readout noise, wide dynamic range, good linearity, and geometric stability (Xillix Technologies). The charge-coupled device transducer is positioned in the primary image plane of a Nikon 2000.75 Plan Apo objective. This arrangement results in the acquisition of a chromatically and geometrically correct image with square pixels. On average, each nucleus is covered by over 500 pixels. The device uses algorithms for the automated detection of the nuclear boundaries along the highest local gradient between the nuclear stain and unstained cytoplasmic background, which defines the nuclear boundary in a precise, objective, and reproducible way.

Cell Selection. Lymphocytes were used as internal standard controls to normalize each slide and to correct for staining variations. From each tissue section, nuclear images of 50 ± 15 (mean ± SD) lymphocytes and 130 ± 40 epithelial nuclei from previously mapped CIN 3 areas were collected by a pathologist (J. V. B.) in a semi-interactive procedure and stored in the computer memory. The collection of nuclei in interactive mode helped to control selection bias (only nonoverlapping nuclei with easily detected boundaries and without “capping” were chosen). Additional control of cell quality was performed by examination of a computer-stored cell gallery, which allowed a magnified display of the stored image of each nucleus. In this way, the scored cells could be reviewed, and fragmented cells were eliminated from the analysis. The number of selected nuclei depended on how much of the pathological area of interest was on the analyzed slide. The coefficient of variation of ΣOD of lymphocytes was 5%.

Epithelial cells (about 100 per sample) from normal-appearing regions that were adjacent to CIN lesions were also analyzed in available samples to determine the mean ΣOD of normal epithelial cells in relation to the modal ΣOD of lymphocytes. A correction factor of 1.15 was used to account for the down-shift in the ΣOD distribution due to the difference in chromatin compaction in lymphocytes and epithelial cells. After the correction factor was applied, the modal ΣOD of lymphocytes was summed as a normalized unit of measurement (which corresponded to the unit “2c,” which has been used in previous studies) and linearly extended to establish the normalized ΣOD scale.

Interpretation of DNA Histograms. ICM-DNAs for each case were expressed as normalized measurements (against the nuclear modal absorbance of lymphocytes) of the ΣOD of each cell of interest and were presented in bar histograms. The following data were obtained and used as ICM-DNA references: (a) ΣODn for each case, obtained as a mean ΣOD, histogram of the cell population of interest (30); (b) DNA-MG, based on the “2c deviation index” and presented as a continuous scale ranging from 0.01 to 3.0 (31); (c) scER, defined as the percentage of cells having a “DNA content” of more than 5c (32); and (d) percentile value of ΣOD, distribution, defined as rates of ΣODn at the 5th, 25th, 50th, 75th, and 95th percentile points of the ΣODn histogram.
Statistical Analysis. A nonparametric (Wilcoxon) test of significance was performed to assess the differences between \( \Sigma OD_n \) in pre- and post-DFMO treatment CIN 3 samples. The SPSS statistical software was used for analysis.

The modulation of \( \Sigma OD_n \) during DFMO treatment was analyzed also using a mixed effect linear model. Specifically, we assumed

\[
y_{ik} = \beta_0 + \beta_1 x_i + \beta_2 x_i + \beta_3 x_i + \mu_j + \gamma_i + \epsilon_{ik},
\]

where: (a) \( y_{ik} \) is the logarithm of the \( \Sigma OD_n \) for patient \( i \), group \( j \), and replication \( k \); (b) group 1 is composed of pretreatment samples from nonresponders; group 2 is composed of pretreatment samples from responders; group 3 is composed of posttreatment samples from nonresponders; and group 4 is composed of posttreatment samples from responders; (c) \( x_i \) is 0 for pretreatment data and 1 for posttreatment data; (d) \( x_i \) is 0 for data from nonresponders and 1 for responders; (e) \( \mu \), which represents the random effect for patient \( i \), is normally distributed with mean 0 and variance \( \sigma^2_{\mu} \); (f) \( \gamma_i \), which represents the random effect of group \( j \) within patient \( i \), is normally distributed with mean 0 and variance \( \sigma^2_{\gamma} \); and (g) \( \epsilon_{ik} \) is normally distributed with mean 0 and variance \( \sigma^2 \).

Thus, the patient is considered a random factor, response/nonresponse is a between-patient factor with both fixed and random components, and pretreatment/posttreatment is a within-patient factor with both fixed and random components. The design is balanced with unequal replicates. The variance of case means around the group mean is permitted to differ among the four groups. The model was fit using the lme function of S-PLUS, version 3.4. All significant tests were performed using the likelihood ratio test.

Results

Data for patient ages, doses of DFMO, \( \Sigma OD_n \) of pre- and posttreatment samples, and histological response are presented in Table 1. The mean age was the same (29 years) in responders and nonresponders. Nineteen (76%) of the 25 patients studied showed a decrease in \( \Sigma OD_n \) after DFMO treatment. Three patients had no \( \Sigma OD_n \) change, and three showed an increase of \( \Sigma OD_n \). Although no significant dose-dependent response in \( \Sigma OD_n \) was detected as a fraction of dose, perhaps due to the small number of cases, all patients who had increases of \( \Sigma OD_n \) following treatment had received low (0.0625–0.125 g/m²/day) doses of DFMO.

According to histological diagnosis, there were 11 responders and 14 nonresponders among the 25 patients who had evaluable pre- and posttreatment samples available for the study. Only 1 of 11 responders had no \( \Sigma OD_n \) change after DFMO treatment (all other responders had decreases in \( \Sigma OD_n \)). 5 of 14 nonresponders had either no change or an increase in posttreatment \( \Sigma OD_n \).

The group mean values of \( \Sigma OD_n \) according to histological response and treatment category are shown in Table 2. There was a trend for lower pretreatment \( \Sigma OD_n \) in responders than in nonresponders; however, this trend was not significant. Although both the responders and nonresponders showed an overall significant decrease in \( \Sigma OD_n \) after DFMO treatment, the responders’ values dropped more than the nonresponders’ values, which resulted in a significant difference between \( \Sigma OD_n \) values for responders and nonresponders in posttreatment samples (1.25 versus 1.5, respectively).

The values obtained from DNA-MG analysis are presented in Table 3. DNA-MG, which is based on a 2c deviation index distribution, is defined as the sum of the squares of the differences between the \( \Sigma OD_n \) of single cells and the 2c value, divided by the number of measured cells (31, 32). In our study, 2c was evaluated as the modal \( \Sigma OD \) of lymphocytes multiplied by a correction factor. Mean DNA-MG in this study was significantly lower in posttreatment than in pretreatment samples for both responders \((P < 0.01)\) and nonresponders \((P < 0.04)\).

Values for 5cER, the number of cells with \( \Sigma OD_n \) exceeding 5c, were also lower in posttreatment samples than in pretreatment samples (Table 4). There was a posttreatment decrease in both responders and nonresponders.
DNA Image Cytometry in a Cervix Chemoprevention Trial

Table 4 5cER by DFMO treatment and histological response category

<table>
<thead>
<tr>
<th>Histological response</th>
<th>No. of patients</th>
<th>No. of patients with 5cER Cells</th>
<th>Mean 5cER (%)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pretreatment</td>
<td>Posttreatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonresponders</td>
<td>11</td>
<td>9</td>
<td>2</td>
<td>5.5</td>
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<tr>
<td>Responders</td>
<td>14</td>
<td>13</td>
<td>9</td>
<td>9.4</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
<td>22</td>
<td>11</td>
<td>8.3</td>
</tr>
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</table>

Table 5 Percentile values of ΣODₙ distribution by DFMO treatment and histological response category

<table>
<thead>
<tr>
<th>Treatment and histological response</th>
<th>Percentile of ΣODₙ</th>
<th>Pretreatment</th>
<th>Posttreatment</th>
<th>Pretreatment</th>
<th>Posttreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5th</td>
<td>25th</td>
<td>50th</td>
<td>75th</td>
</tr>
<tr>
<td>Responders</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pretreatment</td>
<td>0.96</td>
<td>1.25</td>
<td>1.49</td>
<td>2.01</td>
<td>2.92</td>
</tr>
<tr>
<td>Posttreatment</td>
<td>0.80</td>
<td>1.03</td>
<td>1.21</td>
<td>1.40</td>
<td>1.82</td>
</tr>
<tr>
<td>Nonresponders</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pretreatment</td>
<td>0.97</td>
<td>1.29</td>
<td>1.62</td>
<td>2.16</td>
<td>3.04</td>
</tr>
<tr>
<td>Posttreatment</td>
<td>0.80</td>
<td>1.13</td>
<td>1.37</td>
<td>1.76</td>
<td>2.64</td>
</tr>
<tr>
<td>Post/Pretreatment ratio</td>
<td>0.83</td>
<td>0.88</td>
<td>0.85</td>
<td>0.81</td>
<td>0.87</td>
</tr>
<tr>
<td>Responders</td>
<td>0.83</td>
<td>0.82</td>
<td>0.81</td>
<td>0.70</td>
<td>0.62</td>
</tr>
</tbody>
</table>

Table 6 Mean area, ΣODₙ, and chromatin structure values of lymphocytes in pre- and posttreatment samples

<table>
<thead>
<tr>
<th>Area</th>
<th>ΣODₙ</th>
<th>Entropy</th>
<th>Energy</th>
<th>Correlation</th>
<th>Contrast</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreatment</td>
<td>199</td>
<td>91.7</td>
<td>3.2096</td>
<td>0.073</td>
<td>220.6</td>
</tr>
<tr>
<td>Posttreatment</td>
<td>205</td>
<td>92</td>
<td>3.2088</td>
<td>0.073</td>
<td>217.7</td>
</tr>
</tbody>
</table>

σ₁ = σ₂ = σ₃ = 0.1816, σ₄ = 0.0298, σ₅ = 0.0035, and σ = 0.2987. Thus, posttreatment values were lower than pretreatment values, and responders had lower values than did nonresponders. These results of the mixed modal analysis support the hypothesis that DFMO significantly affected the ΣODₙ.

Fig. 1 shows the individual ΣODₙ histograms for biopsy specimens from three patients: a nonresponder, a partial responder, and a complete responder. These histograms demonstrate a shift in the DNA index for the patients who responded and no shift for the patients who did not respond.

For each patient, two histograms were constructed, one for pretreatment ΣODₙ and one for posttreatment ΣODₙ. The histograms represented the proportion of cells falling in a bin, based on rank percentile of optical densities. The individual histograms were then averaged, giving them equal weight, to get the composite histogram. A composite histogram of each group was then created by averaging the groups’ individual histograms, thus giving equal weight to each case.

As shown in Fig. 2, the pretreatment biopsies from responders and nonresponders showed similar ΣODₙ distributions; however, there was a tendency toward a more normalized pattern in the responders. Following treatment, there was a left shift in distribution for both responders and nonresponders.

Discussion

CIN 3 is considered a high-risk precursor to invasive cervical cancer (2, 3). Both genomic instability and proliferative dysregulation play important roles in the progression of CIN 3 lesions and could result in increased mean DNA content (17). Cells with high DNA content have been found to have a particular proliferative behavior and could play an important role in tumor evolution (33) and in the progression of CIN 3 to invasive cervical carcinoma. It has been shown that “high-risk” CIN 3 lesions, those lesions residing beside invasive cancers, have a higher percentage of cells with high DNA content than do CIN 3 lesions that are not associated with cancer (22). Therefore, the opposite process, decreasing the number of cells with high DNA content in CIN 3, may reflect an abolition of “malignant” behavior of CIN 3 and a reversal of precancerous lesions.

The “slicing problem” makes the precise determination of DNA content and modal stem-line ploidy difficult on tissue sections. The method of correcting DNA ploidy measurements in tissue sections (25, 26) can be used to address this problem only for special cell populations with “spherical nuclei and uniform DNA concentration throughout the nucleus.”
Because the CIN 3 cell populations in our study were quite heterogenic, we did not use this method. However, we believe that the slicing problem did not affect our data, because we measured \( \Sigma OD_n \) in pairs: pre- and posttreatment samples from each patient. Under these conditions, paired comparison was achieved in genetically similar (especially for nonresponders) cell populations before and after DFMO treatment. Uniform conditions of slide preparation and measurement of pre- and posttreatment samples were used to ensure consistency. The histograms in Fig. 1 illustrate the OD changes for different histological responses: A, a nonresponsive patient; B, a partially responsive patient; C, a complete responsive patient.
posttreatment lymphocytes were used as controls to demonstrate that no factors other than DFMO affected $\Sigma$OD$_{n}$. Under these circumstances, we believe that $\Sigma$OD$_{n}$ changes reflect a modulation of DNA content during DFMO treatment.

Our data show that all patients with CIN 3 lesions had high levels of $\Sigma$OD$_{n}$, and nonresponders showed a trend to have more high pretreatment $\Sigma$OD$_{n}$ than responders. These data mean that both responders and, especially, nonresponders had in pretreatment samples high numbers of cells with high DNA content that contribute to the malignant behavior of CIN 3 (22).

After 1 month of DFMO administration, reduction of $\Sigma$OD$_{n}$ was achieved in 10 of 11 responders and in 9 of 14 nonresponders. If a posttreatment $\Sigma$OD$_{n}$ decrease in responders can be associated with a change in the histological grade of CIN lesions (21, 22), the reduction of $\Sigma$OD$_{n}$ in nonresponders suggests that DFMO could decrease “DNA amount” in CIN 3 lesions even before the appearance of morphological changes. Whether this change is due to a decrease in proliferation or elimination of particular clones is unknown. We believe that these results suggest antiproliferative activity of DFMO because, as was shown in an animal model, DFMO can inhibit or delay DNA synthesis and decrease proliferation (12–14). These data agree with our findings that DFMO can decrease the level of cell proliferation, which has been detected by proliferating cell nuclear antigen and MPM-2 modulation (34, 35).

To study the antiproliferative activity of DFMO, we analyzed the $\Sigma$OD$_{n}$ histograms with different descriptors. The visual left shift of posttreatment $\Sigma$OD$_{n}$ histograms, which was quantitatively detected by analysis of percentile values of $\Sigma$OD$_{n}$ distributions and a decrease of DNA-MG and ScER, shows a depletion in cell number and in the right shoulder of the histogram. These changes probably correspond to depletion in cells in the S-phase and $G_2$-M of the cell cycle. These data suggest that DFMO has an antiproliferative and cytostatic effect. The evidence that DFMO did not affect $\Sigma$OD in lymphocytes also confirms the cytostatic, rather than cytotoxic, effect of DFMO; it selectively affects DNA content in the proliferative cell population (CIN 3) but not the quiescent one (lymphocytes).

To be sure that this result was not dependent on the quality of the image analysis system or the operator, additional measurements of $\Sigma$OD$_{n}$ in the same samples were performed in the Cancer Imaging Department of the British Columbia Cancer Agency (Vancouver, British Columbia, Canada) on their CytoSavant system by another operator. This independent study

Fig. 2. The summed pre- and posttreatment $\Sigma$OD$_{n}$ histograms for responders (A and B) and nonresponders (C and D).
also detected a significantly lower $\Sigma OD_n$ after DFMO treatment than that in pretreatment samples (data not shown).

In conclusion, our study suggests that: (a) the $\Sigma OD_n$ in CIN 3 tissues significantly decreased during the DFMO chemoprevention trial, and this decrease was due to a depletion of cells with high DNA content; and (b) the modulation of $\Sigma OD_n$ reflected the chemoprevention effect of DFMO before the appearance of morphological changes, providing a rationale for the possible use of ICM-DNA as a SEB in chemoprevention trials with DFMO. Additional reasons for using ICM-DNA as a SEB are its relative simplicity, low cost of reagents, ability to use small tissue samples, objectivity, and reproducibility.

Acknowledgments

We are grateful for helpful comments from Branko Palic, Ph.D., Calum MacAuley, Ph.D., and James Bacus, Ph.D. Editorial assistance from Sunita Patterson and manuscript preparation by Pat Williams are appreciated.

References


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