Role of Glutathione Depletion in the Mechanism of Action of N-Methylformamide and N,N-Dimethylformamide in a Cultured Human Colon Carcinoma Cell Line

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ABSTRACT

The mechanism of the antitumor action of N-methylformamide (NMF), an agent currently undergoing clinical trials, and its congener, N,N-dimethylformamide (DMF), was examined in the DLD-1 Clone A human colon carcinoma cell line in vitro. The primary action of NMF and DMF on these cells is a depletion of cellular reduced glutathione levels which results in cytostasis. Evidence to support this hypothesis include (a) the extent of growth inhibition produced by NMF and DMF is directly proportional to the extent of depletion they effect on cellular reduced glutathione levels; (b) removal of NMF (170 mm) or DMF (103 mm) from the culture medium results in a parallel restoration of cell growth and cellular glutathione levels; (c) coaddition of the glutathione precursor, L-cysteine (0.5 mm), or certain precursors for this amino acid, significantly reverses NMF- and DMF-induced glutathione depletion and cytostasis; and (d) the specific glutathione-depleting agent, buthionine sulfoximine (7.5 mm) mimics the ability of NMF and DMF to induce cytostasis. In addition, NMF- and DMF-mediated reduced glutathione depletion accounts for the previously reported ability of these agents at high concentrations (>100 mm) to induce a more benign phenotype in DLD-1 Clone A human colon carcinoma cells. This is evidenced by the findings that (a) L-cysteine (0.5 mm) reverses NMF- and DMF-mediated (170 and 103 mm, respectively) increases in doubling time, decreases in saturation density, and decreases in clonogenicity; (b) buthionine sulfoximine (7.5 mm) mimics these actions of NMF and DMF; and (c) the tumorigenicity of DLD-1 Clone A cells in nude mice, which is completely eliminated by the in vitro treatment of these cells for passages with 170 mm NMF prior to inoculation, is fully restored if the cells are passaged in the presence of 170 mm NMF and 0.5 mm L-cysteine. Thus, NMF- and DMF-induced depletion of cellular reduced glutathione is responsible for not only the cytostatic effect of these agents on human colon carcinoma cells but also for their ability to induce more benign characteristics in these cells.

INTRODUCTION

NMF is a polar solvent that was shown in the early 1950s to be active against a wide spectrum of rat and murine tumors in vivo (1-4). Initial anticancer trials on NMF in humans were discontinued when evidence of hepatic toxicity emerged (5).

ABSTRACT

More recent studies have demonstrated that NMF and its congener DMF are active against a variety of experimental human tumors, including colon, lung, and mammary carcinomas, grown as xenografts either s.c. or in the subrenal capsule of nude mice (6, 7). These observations prompted the current reevaluation of NMF in the clinic (8).

To date, few studies have been done on the mechanism of the antitumor action of NMF or DMF. Early investigations demonstrated that NMF, which is an in vivo metabolite of DMF in humans (9) and in rodents (10), inhibits the incorporation of nucleotide precursors into the nucleic acids of cells responsive to this agent (11, 12). More recently, it has been shown that NMF and DMF are capable of inducing terminal differentiation in a variety of leukemic cell lines in vitro (13, 14). In certain cultured solid tumors, these agents can induce more differentiated characteristics (15-17). In the case of experimental human colon carcinomas, Dexter et al. (18), Dexter and Hager (19), and Hager et al. (20) have shown that high concentrations of DMF (>100 mm) in the growth medium induces a reversible expression of a more benign/differentiated phenotype in these cells, as evidenced by an increase in doubling time, a decrease in saturation density, a loss of clonogenicity in semisolid medium, a marked reduction of tumorigenicity in nude mice, and an increase in the expression of a mucoprotein antigen associated with normal colonic mucosa. NMF has also been shown to produce many of the same qualitative in vitro effects on solid tumor cell systems as DMF (17, 21). However, at present, it is not known whether NMF and/or DMF exert their antitumor action via the induction of differentiation or through some other mechanism.

Recently, this laboratory demonstrated that both NMF and DMF deplete the cellular total glutathione content of DLD-1 Clone A human colon carcinoma cells in vitro (21). Addition of L-cysteine, a precursor for glutathione, to NMF- or DMF-treated cells reverses the growth inhibitory and glutathione-depleting actions produced by either agent (21). These observations indicated that the mechanism of action of NMF and DMF on these cells, at least in vitro, is related to their effects on L-cysteine/glutathione metabolism. In the present study, it is demonstrated that a polar solvent-mediated depletion of reduced glutathione is responsible for the growth inhibitory effects of NMF and DMF on cultured human colon carcinoma cells, and also that this depletion can account for many of the broad biological actions of NMF and DMF, including their ability to induce a more benign phenotype in these cells.

MATERIALS AND METHODS

Materials. Reduced glutathione, oxidized glutathione, glutathione reductase, α-phthalaldehyde, NADPH, L-cysteine, L-glutamate, L-glycine,
**GLUTATHIONE IN THE MODE OF ACTION OF NMF AND DMF**

N-acetylcycteine, l-cystine, l-methionine, l-homocysteine thiolactone, (±)-cystathionine, β-mercaptoethylamine (cysteamine), and N,N-dimethylformamide were obtained from Sigma Chemical Co. (St. Louis, MO). N-Methylformamide was purchased from the Aldrich Chemical Co. (Milwaukee, WI). l-β-thionine-SR-sulfoximine was obtained from Bachem Inc. (Torrance, CA). All other chemicals were of standard reagent grade.

**Cell Culture.** The establishment of the human colon carcinoma cell lines designated DLD-1 (and its subpopulations, DLD-1 Clone A and DLD-1 Clone D), DLD-2, HCT-8, and HCT-15 has been described elsewhere (7, 18, 22, 23). These cells are routinely grown in RPMI Medium 1640 supplemented with 10% heat-inactivated fetal calf serum, buffers, and antibiotics (18) in a humidified incubator of 95% air, 5% CO2 at 37°C. In vitro growth studies with NMF and DMF as well as clonogenicity experiments in soft agar (Difco, Detroit, MI) were performed using previously reported methods (21).

Doubling time and saturation density experiments were carried out as described by Dexter et al. (18), with the following modifications. DLD-1 Clone A cells (2.5 x 105/dish) were plated in 35-mm Falcon tissue culture dishes in 2 ml of the above-described medium supplemented or not supplemented with 170 mM NMF, 103 mM DMF, or 7.5 mM BSO, and with or without 0.5 mM L-cysteine. Cells were harvested daily with trypsin-EDTA and counted with a hemocytometer. Doubling times were calculated from the logarithmic regions (days 2–4) of plotted growth curves. Saturation densities were determined from the plateau regions (days 4–6) of these curves.

**Tumorigenicity.** A-thymic nude mice bearing the nu/nu gene on a Swiss background were obtained from the Roger Williams Cancer Center Animal Care Facility (Providence, RI). Tumorigenicity studies were performed essentially as described by Dexter et al. (18). Male and female mice of 6–8 wk of age were randomized into groups of 8–15 animals and were given injections s.c. of DLD-1 Clone A cells (105 cells/mouse) that had been treated for 4 passages in RPMI 1640 medium (see above) and were given injections of DLD-1 Clone A cells (105 cells/mouse) that had been treated for 4 passages in RPMI 1640 medium (see above). These cells are routinely grown in RPMI Medium 1640 supplemented with 10% heat-inactivated fetal calf serum, buffers, and antibiotics (18) in a humidified incubator of 95% air, 5% CO2 at 37°C. In vitro growth studies with NMF and DMF as well as clonogenicity experiments in soft agar (Difco, Detroit, MI) were performed using previously reported methods (21).

**Measurement of Intracellular Glutathione.** The preparation of cell-free extracts for measurement of cellular total glutathione concentrations (i.e., the sum of reduced and oxidized glutathione) has been previously described (21); quantitation was done by the spectrophotometric assay of Halprin and Ohkawara (24) as modified by Hadley et al. (25). The cellular reduced glutathione content was assayed by the spectrophotometric method of Cohn and Lyle (26). Briefly, cells were harvested by trypsinization, washed twice in cold 0.9% saline, and resuspended in 5% TCA: 1.0 ml EDTA:0.1 N HCl (1:1:1, v/v) on ice (final cell concentration, 107 cells/ml). Cell lysates were centrifuged at 2500 x g for 20 min at 4°C. The deproteinized supernatant fluid (0.1 ml) was then added to assay mixtures (1.75 ml, final vol) containing 1.5 ml 500 mM sodium phosphate, 5 mM EDTA buffer (pH 8.0) and 0.15 ml o-phthalaldehyde (1 mg/ml methanol). After thorough mixing and incubation at room temperature for 15 min, the fluorescence at 420 nm resulting from activation at 350 nm was determined. Cellular oxidized glutathione content was assayed in the same manner, except that the reaction buffer was adjusted to pH 12.0. Using internal standards, the recovery of reduced and oxidized glutathione was found to be 99 and 105%, respectively. The sum of reduced and oxidized glutathione concentrations, as measured independently by the method of Cohn and Lyle (26), generated values within 7% of the total glutathione concentrations as measured by the method of Hadley et al. (25).

**RESULTS**

**Relationship between NMF- and DMF-induced Glutathione Depletion and Growth Inhibition.** If a depletion of glutathione is the primary mechanism by which NMF and DMF affect the inhibition of cell growth, then there should be a correlation between the ability of these compounds to inhibit growth and to deplete glutathione levels. As shown in Fig. 1, both NMF and DMF deplete reduced glutathione levels in DLD-1 Clone A cells in a dose-dependent manner (oxidized glutathione levels are not affected by either polar solvent). This effect is pronounced at concentrations that have been used to inhibit growth and induce a less malignant phenotype in these cells (170 and 103 mM for NMF and DMF, respectively) (18, 21); at these concentrations, reduced glutathione levels are decreased to less than 5% of control values. In both cases, there is an apparent correlation between the extent of reduced glutathione...
depletion and the degree to which growth is inhibited. For example, when DLD-1 Clone A cells are treated for 96 h with 100 mM NMF, reduced glutathione is depleted and their growth is inhibited by 68 and 47% relative to untreated controls, respectively. The concentration of DMF required to produce the same depletion of reduced glutathione in these cells, 72 mM, also inhibits DLD-1 Clone A cell growth to approximately the same extent as 100 mM NMF, i.e., by 51%. These findings are consistent with the hypothesis that a polar solvent-mediated depletion of reduced glutathione is responsible for the growth inhibition effected by NMF and DMF on these cells.

Since the inhibition of colon carcinoma cell growth mediated by 103 mM DMF has been shown to be reversed upon removal of the agent from the culture medium (18), one might predict that the effect of DMF (and NMF) on glutathione pools should also be reversed upon removal of these compounds from the medium if in fact a loss of glutathione is responsible for the observed growth inhibition. As shown in Fig. 2A, DLD-1 Clone A cell growth is inhibited in a time-dependent manner upon treatment with, in this case, 170 mM NMF. Accompanying this is a time-dependent depletion of cellular total glutathione, with maximal depletion (i.e., 88% relative to controls) occurring at 96 h (Fig. 2B). Total glutathione levels remain at this depressed level if NMF is maintained in the medium. However, if after 96 h the NMF-containing medium is replaced with drug-free medium, the total glutathione content is restored to control levels within 24 h. Correspondingly, the growth rate of NMF-treated DLD-1 Clone A cells is restored to that of their untreated counterparts (doubling time, 27 h) (Fig. 2A). Similar results are obtained upon treatment of the cells with 103 mM DMF (data not shown). Thus the recovery of growth parallels the restoration of glutathione levels, again supporting the postulate that NMF- (and DMF-) induced glutathione depletion can account for their growth inhibitory activity.

One corollary of this hypothesis is that a replenishment of cellular glutathione pools should reverse the growth inhibition effects by either polar solvent. Previous studies (21) demonstrated that the glutathione precursor, l-cysteine (0.5 mM), antagonizes both the NMF- and DMF-mediated inhibition of DLD-1 Clone A cell growth and decrease in cellular glutathione levels. In these studies, it was examined whether cells whose growth and glutathione content are suppressed by NMF (or DMF) treatment would recover upon the superaddition of l-cysteine. As depicted in Fig. 2B, when DLD-1 Clone A cells are treated for 96 h with 170 mM NMF and subsequently treated with medium containing 170 mM NMF plus 0.5 mM l-cysteine, their total glutathione content returns to that of untreated cells. As predicted, the growth rate of these cells also recovers to that of untreated controls (Fig. 2A). Thus the l-cysteine-mediated restoration of glutathione pools is paralleled by a restoration of the cellular growth rate, further supporting the concept that a depletion of glutathione results in an inhibition of DLD-1 Clone A cell growth.

Inasmuch as l-cysteine reverses both NMF-induced growth inhibition and glutathione depletion, a variety of l-cysteine precursors or derivatives were examined for their ability to abrogate these effects in the DLD-1 Clone A cell system. Of the l-cysteine precursors examined, l-cysteine (0.25 mM) produces the greatest reversal of these actions of NMF; i.e., growth and glutathione levels are restored 81 and 102% relative to untreated controls, respectively (Table 1). N-Acetylcysteine and reduced glutathione reverses of these actions of NMF; i.e., growth and glutathione levels are restored 81 and 102% relative to untreated controls, respectively (Table 1). N-Acetylcysteine and reduced glutathione itself also reverse these effects of NMF, although to a lesser extent. In contrast, intermediates along the transsulfuration pathway, namely l-methionine, l-homocysteine (administered in the form of l-homocysteine thiolactone), and (±)-cystathionine, fail to reverse the actions of NMF. Cysteamine, the sulfhydryl-containing decarboxylated derivative of l-cysteine which cannot

![Fig. 2. Time course of reversal of the effects of NMF on the growth (A) and total glutathione (total GSH) content (B) of DLD-1 Clone A cells. Cells were initially plated (for growth experiments, 1 x 105/60-mm dish; for total glutathione determination experiments, 1 x 103-1 x 104/100-mm dish) in RPMI 1640 Medium supplemented with 10% fetal calf serum and incubated at 37°C for 24 h. At this time, the medium was removed and the cells were washed twice with 0.9% saline. Medium containing either 170 mM NMF (C) or no drug (D) was then added. After a 96-h incubation, NMF-treated and untreated cells were washed twice with 0.9% saline and the medium was replaced with that containing no drug (C), 170 mM NMF alone (C), or 170 mM NMF plus 0.5 mM L-Cysteine (B). At the indicated time intervals, cells were harvested by trypsinization and counted by light microscopy with a hemacytometer. Cell-free extracts were prepared and assayed for total glutathione levels as described elsewhere (21). Values represent the mean ± SD (bars) of 4 samples obtained in 2 independent experiments.](image)

### Table 1

<table>
<thead>
<tr>
<th>Cell no. (x10^5) after 4 days' incubation</th>
<th>Total glutathione content (nmol/10^5 cells) after 4 days' incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-170 mM NMF</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
</tr>
<tr>
<td>Cystine (0.25 mM)</td>
<td>22.4 ± 1.2</td>
</tr>
<tr>
<td>N-Acetylcysteine (0.75 mM)</td>
<td>22.2 ± 0.5</td>
</tr>
<tr>
<td>Glutathione (reduced) (0.05 mM)</td>
<td>21.0 ± 1.7</td>
</tr>
<tr>
<td>Methionine (0.50 mM)</td>
<td>22.5 ± 0.9</td>
</tr>
<tr>
<td>Homocysteine thiolactone (0.25 mM)</td>
<td>22.7 ± 1.3</td>
</tr>
<tr>
<td>Cystathionine (0.50 mM)</td>
<td>21.5 ± 0.4</td>
</tr>
<tr>
<td>Cysteamine (0.25 mM)</td>
<td>23.4 ± 0.5</td>
</tr>
</tbody>
</table>

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be utilized as its precursor, also fails to restore the growth and glutathione content of NMF-treated cells. Taken together these data indicate that (a) cellular glutathione levels must be repleted with an appropriate L-cysteine precursor in order to reverse NMF-induced growth inhibition, and (b) based on the inability of cysteamine to restore the growth of NMF-treated cells, NMF does not act simply by altering the redox status of intracellular sulfhydryl groups.

It should be noted that all of the above results could be explained if NMF and DMF were to inhibit the cellular uptake of L-cystine from the medium. This would limit the amount of intracellular L-cysteine available for not only glutathione synthesis but protein synthesis as well. In such a case, the primary site of polar solvent action might be a limitation of protein synthesis, with glutathione depletion occurring secondarily. However, as shown in Fig. 3, 170 mM NMF does not inhibit the incorporation of [35S]cystine into either the acid-soluble (i.e., glutathione, cysteine, cysteine) or the acid-insoluble fraction (i.e., protein) of DLD-1 Clone A cells when examined over a 6-h period. During this time, total glutathione levels of NMF-treated cells are depleted by greater than 50% (see Fig. 2B). Incorporation of [35S]cystine into DLD-1 Clone A cells was also not inhibited in cells treated with 103 mM DMF (data not shown). These findings rule out the possibility that the growth inhibitory and glutathione-depleting effects of the polar solvents occur via an inhibition of cysteine utilization.

To further examine the relationship between glutathione and DLD-1 Clone A cell growth, the specific glutathione-depleting agent, buthionine sulfoximine was used. BSO is an irreversible inhibitor of γ-glutamylcysteine synthetase, the first step in the biosynthesis of glutathione (27), and has been shown to deplete glutathione in a variety of biological systems (28-30). As shown in Fig. 4A, BSO exerts a dose-dependent depletion of cellular reduced glutathione in DLD-1 Clone A cells. As in the case of NMF and DMF, a BSO-mediated depletion of reduced glutathione is paralleled by a dose-dependent inhibition of cell growth. BSO is approximately 25-fold more potent than NMF in that only 7.5 mM BSO is required to effect an inhibition of cell growth and depletion of glutathione levels similar to that produced by 170 mM NMF. Despite the differences in potency between NMF, DMF, and BSO, in all cases there is a striking correlation between the extent of depletion of reduced glutathione levels and the degree to which DLD-1 Clone A cell growth is inhibited (see Fig. 1 and Fig. 4A). This correlation becomes evident when cell growth is plotted as a function of intracellular glutathione content (Fig. 4B) using the data from Fig. 1 and Chart 4A. The finding that the specific glutathione-depleting agent BSO closely mimics the pattern of growth inhibition produced by NMF and DMF suggests that glutathione depletion is the primary action of these polar solvents. Nevertheless the effects of BSO are not identical to that of NMF or DMF. For example, 0.5 mM L-cysteine does not reverse the growth inhibitory and reduced glutathione-depleting effects of 7.5 mM BSO. This result is not unexpected because BSO is an irreversible inactivator of γ-glutamylcysteine synthetase (27). However, caution must be used in comparing BSO action with that of NMF or DMF, as different glutathione-depleting mechanisms may be involved.

To test whether the glutathione-depleting and growth-inhibitory effects of NMF are limited to the DLD-1 Clone A system, other human colon carcinoma lines were examined. In all the lines tested, including DLD-1 (the parent line of Clone A), Clone D (the sister clone of Clone A), DLD-2, HCT-15, and HCT-8, 170 mM NMF produces growth inhibition (up to a maximum of 80% in the case of HCT-15, relative to untreated controls) and a depletion of total glutathione pools (up to a maximum of 91% relative to controls in the case of HCT-15) (Table 2). In all instances, L-cysteine (0.5 mM) significantly antagonized the glutathione-depleting and growth inhibitory effects of NMF. In addition, the extent to which the growth of NMF-treated cells is restored by L-cysteine corresponds to the extent of L-cysteine-mediated restoration of total glutathione pools. Thus, these findings indicate that NMF inhibits growth via depletion of cellular glutathione levels in a wide spectrum of cultured human colon carcinoma cell lines.
Cytostatic Action of NMF and DMF. An additional issue is the nature of the observed growth inhibition effected by NMF and DMF, i.e., whether these polar solvents act via a cytotoxic or cytostatic mechanism. Earlier work on NMF (1) and DMF (18) suggested that these compounds were not cytotoxic. To address this question, the growth inhibition effected by NMF and DMF was compared to that effected by cis-dichlorodiamminemanganese(II) (cisplatin), a compound known to act via a cytotoxic mechanism (31). This was accomplished by examining DLD-1 Clone A cells after treatment with either NMF, DMF, or cisplatin for (a) viability by trypsin blue exclusion, and (b) clonogenicity by subculturing them in drug-free soft agar medium. As shown in Table 3, treatment of DLD-1 Clone A cells with either 170 mM NMF or 103 mM DMF for 96 h does not reduce their viability or their ability to clone in soft agar. In contrast, cells treated with cisplatin show decreases in viability and clonogenicity. These results indicate that NMF and DMF inhibit DLD-1 Clone A cell growth by a cytostatic rather than a cytotoxic mechanism. As might be expected, BSO also behaves as a cytostatic agent against this cell line (Table 3). Collectively, the above data suggest that glutathione depletion induces cytostasis, which is reversible upon restoration of glutathione pools.

Glutathione Depletion and Polar Solvent Induction of a More Benign Phenotype in DLD-1 Clone A Cells. Previously it has been demonstrated that treatment of cultured human colon carcinoma cells with DMF can induce the expression of a more benign/differentiated phenotype, as evidenced by an increase in doubling times, a decrease saturation density, abolished clonogenicity in soft agar, and markedly reduced tumorigenicity in nude mice (18, 19). If NMF- and DMF-mediated cellular glutathione depletion induces cytostasis, which is reversible upon restoration of glutathione pools.

Table 2

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Total glutathione content (nmol/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−170 mM NMF</td>
</tr>
<tr>
<td>DLD-1 Clone A</td>
<td>15.2 ± 0.5</td>
</tr>
<tr>
<td>DLD-1 Clone D</td>
<td>12.3 ± 0.6</td>
</tr>
<tr>
<td>DLD-2</td>
<td>9.9 ± 0.9</td>
</tr>
<tr>
<td>HCT-15</td>
<td>6.8 ± 0.7</td>
</tr>
<tr>
<td>HCT-8</td>
<td>5.3 ± 0.9</td>
</tr>
</tbody>
</table>

Table 3

<table>
<thead>
<tr>
<th>Exposure time (h)</th>
<th>% viability</th>
<th>% clonogenicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>96</td>
<td>39.7 ± 0.6</td>
</tr>
<tr>
<td>NMF (170 mM)</td>
<td>96</td>
<td>38.7 ± 1.1</td>
</tr>
<tr>
<td>DMF (103 mM)</td>
<td>96</td>
<td>37.3 ± 0.8</td>
</tr>
<tr>
<td>BSO (7.5 mM)</td>
<td>96</td>
<td>38.6 ± 0.4</td>
</tr>
<tr>
<td>Cisplatin (1.85 µM)</td>
<td>96</td>
<td>22.1 ± 1.7</td>
</tr>
<tr>
<td>Cisplatin (40 µM)</td>
<td>1</td>
<td>12.3 ± 0.6</td>
</tr>
</tbody>
</table>

Table 4

<table>
<thead>
<tr>
<th>Doubling time (h)</th>
<th>Saturation density (cells, x10^6/cm^2)</th>
<th>Clonogenicity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>−170 mM NMF</td>
<td>31</td>
<td>6.92 ± 0.43</td>
</tr>
<tr>
<td>L-cysteine (0.5 mM)</td>
<td>30</td>
<td>6.82 ± 0.31</td>
</tr>
<tr>
<td>NMF (170 mM)</td>
<td>64</td>
<td>2.80 ± 0.10</td>
</tr>
<tr>
<td>NMF (170 mM) + L-cysteine (0.5 mM)</td>
<td>37</td>
<td>5.28 ± 0.03</td>
</tr>
<tr>
<td>DMF (103 mM)</td>
<td>48</td>
<td>2.79 ± 0.13</td>
</tr>
<tr>
<td>DMF (103 mM) + L-cysteine (0.5 mM)</td>
<td>53</td>
<td>5.92 ± 0.21</td>
</tr>
<tr>
<td>BSO (7.5 mM)</td>
<td>59</td>
<td>2.52 ± 0.04</td>
</tr>
<tr>
<td>BSO (7.5 mM) + L-cysteine (0.5 mM)</td>
<td>65</td>
<td>2.85 ± 0.07</td>
</tr>
</tbody>
</table>

Table 4: Effect of L-cysteine on NMF-, DMF-, and BSO-induced alterations in DLD-1 Clone A cell culture characteristics

DLD-1 Clone A cells were plated (2.5 x 10^5/60-mm dish) in RPMI 1640 Medium supplemented with 10% fetal calf serum and incubated at 37°C for 24 h. At this time, the medium was replaced with that containing the indicated concentration of the appropriate agents. After the respective exposure times, the cells were harvested; some were assayed for viability by trypan blue exclusion and the remaining were cloned in drug-free semisolid medium by a previously described method (21). Values represent the mean ± SD of 4 samples.

Data from Ref. 21.
Table 5
Reversal by L-cysteine of the NMF-induced loss of DLD-1 Clone A cell tumorigenicity in nude mice

<table>
<thead>
<tr>
<th>In vitro treatment</th>
<th>% viability at time of inoculation</th>
<th>Intracellular reduced glutathione content at time of inoculation (nmol/10^6 cells)</th>
<th>Incidence of tumors 10 wk postinjection</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>87</td>
<td>12.0 ± 1.2</td>
<td>15/15</td>
</tr>
<tr>
<td>L-cysteine (0.5 mM)</td>
<td>85</td>
<td>12.7 ± 0.5</td>
<td>11/11</td>
</tr>
<tr>
<td>NMF (170 mM)</td>
<td>86</td>
<td>0.3 ± 0.3</td>
<td>0/10</td>
</tr>
<tr>
<td>NMF (170 mM) +</td>
<td>86</td>
<td>11.2 ± 1.0</td>
<td>8/8</td>
</tr>
<tr>
<td>L-cysteine (0.5 mM)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± SD (n = 4).

However, when DLD-1 Clone A cells are plated in soft agar medium containing 170 mM NMF (or 103 mM DMF) and 0.5 mM L-cysteine, their ability to clone in semisolid medium is fully restored to that of untreated controls (see Table 4 and Fig. 5). BSO-treated (7.5 mM) DLD-1 Clone A cells also are unable to form colonies in soft agar (Table 4). Coaddition of 0.5 mM L-cysteine fails to reverse this BSO-mediated action. These observations are in accord with the above-mentioned monolayer studies.

Finally, it was examined whether NMF can reduce the tumorigenicity of human colon carcinoma cells hosted in nude mice and whether L-cysteine could prevent this effect. As shown in Table 5, 15 of 15 mice that were given injections of untreated cells developed palpable tumors (>0.1 cm³) approximately 2 wk postinjection. These cells contained normal levels of reduced glutathione at the time of inoculation (12.0 nmol/10^6 cells). Similarly, 11 of 11 mice that were given injections of cells passaged in vitro 4 times in medium containing 0.5 mM L-cysteine alone developed tumors with 2 wk; the reduced glutathione content of these cells at the time of inoculation was comparable to that of controls. In contrast, no tumors developed throughout the length of the 10-wk experiment in the 10 mice inoculated with cells that had been treated for 4 passages with 170 mM NMF. The reduced glutathione content of these cells at the time of inoculation was less than 5% that of controls. However, when mice received cells that had been treated with 170 mM NMF and 0.5 mM L-cysteine, tumors were observed in 8 of 8 mice. The reduced glutathione content of these cells was near control values at the time of inoculation. Thus (a) four passages of treatment with 170 mM NMF completely eliminates the tumorigenicity of DLD-1 Clone A cells hosted in nude mice, and (b) preventing the NMF-mediated depletion of intracellular reduced glutathione by coaddition of L-cysteine permits the cells to express their tumorigenic potential. Taken together, these observations demonstrate that (a) NMF, like DMF, has the ability to induce a more benign phenotype in human colon carcinoma cells, and (b) NMF- and DMF-mediated glutathione depletion is involved in the induction of a more benign phenotype, at least in DLD-1 Clone A cells.

DISCUSSION
The results obtained in this study demonstrate that the primary mechanism by which NMF and DMF inhibit human colon carcinoma cell growth and induce a loss of the malignant phenotype in these cells involves a depletion of cellular reduced glutathione.
Finally, Cooksey et al. (37) found that low but detectable levels of 14C02 in the breath of mice finding has been challenged (35). In addition, Brindley et al. (36) hydrogenase activity, then S-hydroxymethylglutathione could ac to yield formaldehyde but have inadequate formaldehyde dehy.

The mechanism by which NMF and DMF, 2 relatively inert compounds, deplete cellular glutathione levels is not understood at the present time. Neither NMF nor DMF directly react with reduced glutathione. One possibility is that these compounds undergo oxidation to a more electrophilic metabolite, which is capable of conjugating with reduced glutathione. For example, these compounds could undergo a microsomal oxidase-dependent N-demethylation, generating formaldehyde. Formaldehyde is a highly electrophilic substance that reacts nonenzymatically with glutathione to yield S-hydroxymethylglutathione (32). In the liver, this glutathione conjugate can be catabolized via formaldehyde dehydrogenase to yield glutathione and formate (33), which can be further catabolized to CO2. According to one scheme, if human colon carcinoma cells can N-demethylate NMF and DMF to yield formaldehyde but have inadequate formaldehyde dehydrogenase activity, then S-hydroxymethylglutathione could accumulate and/or be exported from the cell (34), thereby depleting cellular glutathione levels. That DMF undergoes cytochrome P-450-dependent N-demethylation to yield NMF and formaldehyde has been described in isolated rat liver systems (10). NMF is found in the urine of industrial workers exposed to DMF, demonstrating that N-demethylation of DMF occurs in humans as well (9). There is some evidence that NMF also undergoes N-demethylation, resulting in the formation of formaldehyde. Barnes and Ranta (10) have shown that perfused rat livers will produce formaldehyde in the presence of NMF, although this finding has been challenged (35). In addition, Brindley et al. (36) found low but detectable levels of 14CO2 in the breath of mice treated with [methyl-14C]NMF, indicating the occurrence of oxidative N-demethylation. Finally, Cooksey et al. (37) found that N-hydroxymethylformamide, the putative intermediate that would be generated during the oxidative N-demethylation of NMF, readily dissociates to yield formaldehyde; in fact, the antitumor action of N-hydroxymethylformamide could be blocked by the formaldehyde trapping agent, semicarbazide (37). It is therefore possible that formaldehyde may be the metabolite of DMF and NMF that is responsible for the growth and glutathione-depleting action of these compounds in human colon carcinoma cells. The formation of electrophilic metabolites other than formaldehyde from NMF and DMF is also possible. As recently reviewed by Overton et al. (38) the oxidative metabolism of N-alkylamines or N-alkylamides (such as NMF or DMF) to N(1-hydroxyalkyl) amines (or amides) can lead to the formation of iminium ions

\[
\begin{align*}
\text{HC—N} & \rightarrow \text{HC—N} + \Phi \text{CH}_2
\end{align*}
\]

The 2 lines of evidence that support this postulate are that (a) coaddition of L-cysteine, the rate-limiting amino acid for glutathione synthesis, reverses all of the biological effects of NMF and DMF that were examined, and (b) the specific inhibitor of glutathione synthesis, BSO, duplicates the actions of NMF and DMF on DLD-1 Clone A cells when used at a concentration that effects a depletion of cellular reduced glutathione levels similar to that produced by biologically active concentrations of NMF or DMF.

An important question raised by the results of this study is how a decrease in glutathione levels results in an inhibition of colon carcinoma cell growth. It is well established that glutathione participates in a variety of important cellular processes, e.g., RNA synthesis, deoxyribonucleotide synthesis, and amino acid uptake (for reviews, see Refs. 39 and 40). Thus an inhibition of one or more of these pathways via a depletion of glutathione levels could account for the cytostatic effect of NMF and DMF. Currently we are examining which of these processes is critically affected by a polar solvent-mediated glutathione depletion.

One of the most intriguing properties of NMF and DMF is their ability to suppress the expression of the malignant phenotype in cells originating from solid tumors, in particular their clonogenicity in soft agar and tumorigenicity in nude mice. It is in fact by these criteria that a cell type is determined to be "malignant" (41, 42). Since, as demonstrated in this study, these malignant characteristics can be modulated by compounds that alter cellular glutathione content, such as NMF, DMF, BSO, and L-cysteine, it is tempting to propose that the expression of the malignant phenotype depends upon the reduced glutathione status of the cell, at least in the DLD-1 Clone A system. However, loss of these malignant characteristics may be more a result of the cytostasis caused by the depletion of reduced glutathione. In other words, any agent that could cause cytostasis by any a number of mechanisms (e.g., α-difluoromethylornithine, which induces cytostasis by depleting polyamine pools) (43) might also effect a loss in clonogenicity and tumorigenicity. This point remains to be studied. Indeed, a simple depletion of glutathione levels does not offer a complete explanation as to why treatment of DLD-1 Clone A cells for 4 passages in 170 mw NMF abolishes tumorigenicity.

To begin with, one would expect that cells whose glutathione levels were depleted by NMF treatment would replenish their glutathione pools and presumably regain their tumorigenicity once they had been injected, i.e., were no longer exposed to NMF. Moreover, treatment of DLD-1 Clone A cells for 96 h with 170 mw NMF, which depletes reduced glutathione levels to the same extent as treatment for 4 passages (3-4 wk) with 170 mw NMF (i.e., <5% of control values), does not alter tumorigenicity. It is possible that the cells respond to prolonged periods of low intracellular glutathione levels with adaptations that render them unable to carry out all the processes, such as angiogenesis, that enable them to establish a tumor. In any case, the ability to modulate the malignant behavior of DLD-1 Clone A cells by altering their glutathione levels will permit the study of some of the biochemical processes underlying the malignant phenotype.

Since NMF and DMF at the concentrations used in this study (i.e., >100 mw) can induce terminal differentiation in some human and murine leukemic systems (13, 14), it would be of interest to examine whether glutathione depletion is involved. It would also be of interest to determine if other differentiation-inducing agents, e.g., dimethyl sulfoxide and sodium butyrate, act in whole or in part by modulating cellular glutathione levels. A relationship
between glutathione and differentiation is not unprecedented: the differentiation of sheep reticulocytes has been shown to be accompanied by a marked decrease in cellular glutathione pools (44).

Is glutathione depletion the basis of the in vivo antitumor action of NMF and DMF? High concentrations of these formamide derivatives (>100 mm) are required to deplete glutathione and inhibit the growth of colon carcinoma cells in vitro, whereas the maximum achievable plasma level of NMF in rodents is in the range of 7 mm (36). In humans, the peak plasma levels at well-tolerated doses may only be in the range of 1.7 mm (45). It should be pointed out, however, that RPMI 1640 medium contains saturating (160 μM) concentrations of L-cysteine (the equivalent of 320 μM L-cysteine) as well as saturating levels of the other amino acids required for glutathione synthesis, glutamate and glycine (100 μM each). In a poorly vascularized tumor, however, the levels of these amino acids may be far below the saturating level for maximal glutathione synthesis, rendering the tumor cells more sensitive to the glutathione-depleting effects of NMF and DMF. In support of this, we have recently observed that the glutathione-depleting and growth-inhibitory actions of NMF is significantly potentiated in vitro if DLD-1 Clone A cells are plated in RPMI 1640 medium containing subsaturating levels of L-cysteine (<60 μM).4

A depletion of glutathione levels may be in part responsible for NMF-induced hepatotoxicity, which is the dose-limiting toxicity in experimental animals (46, 47) and in humans (5, 8). NMF causes a significant depletion of hepatic nonprotein thiol levels (by 50%) in mice receiving pharmacological dosages of this agent (400 mg/kg, single dose) (35). Similarly, isolated murine hepatocytes treated with relatively low concentrations of NMF (7 mm) undergo glutathione depletion and lipid peroxidation (48). Other mechanisms, such as an NMF-mediated alteration in hepatic mitochondrial Ca2+ homeostasis may also be involved (49). If glutathione depletion can account for the observed hepatotoxicity of NMF, it may be possible to replenish hepatic glutathione pools in patients receiving this agent by coadministering an appropriate L-cysteine precursor. Because the transsulfuration pathway, which converts methionine to L-cysteine, is highly active in hepatic tissue (50), whereas based on the data in Table 1, this pathway may be lacking in DLD-1 Clone A cells, it may be possible to rescue hepatocytes but not tumor tissue from the glutathione-depleting action of NMF by administering a transsulfuration intermediate such as cystathionine along with NMF. In this way, one might decrease the toxicity of NMF, allowing the use of higher doses of this agent in treatment regimens.

Since NMF (and DMF) effects a reversible cytostasis in vitro, one might question the use of this compound as a single agent in treating cancers in order to achieve long term survival. It is more appropriate to consider these formamide derivatives as biological modifiers which may potentiate the activity of other antitumor drugs or therapeutic modalities. NMF and DMF have recently been reported to sensitize human colon carcinoma cells to cisplatin (51), an agent known to react with intracellular nonprotein thiol pools (52), DMF (53) and NMF (54) also sensitize various human colon carcinoma lines to X-irradiation. Both of these effects may possibly be explained by the glutathione-depleting action of these polar solvents. In any case, it is hoped that these insights into the mode of action of NMF (and DMF) lead to a more rational as well as more effective use of this agent in the clinic.

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GLUTATHIONE IN THE MODE OF ACTION OF NMF AND DMF

Role of Glutathione Depletion in the Mechanism of Action of N-Methylformamide and N,N-Dimethylformamide in a Cultured Human Colon Carcinoma Cell Line

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