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Inhibition of the Growth of Human Colon Cancer Xenografts by Polar Solvents[1]

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ABSTRACT

The effects of *N,N*-dimethylformamide (DMF) and *N*-methylformamide (NMF) on the growth of two human colon cancer cell lines xenografted in nude mice were assessed. Toxicological studies with mice heterozygous for the *nu/nu* gene showed that with both compounds the limiting organ toxicity was hepatic. The 10% lethal doses for DMF and NMF given i.p. daily for 21 days were 2219 and 374 mg/kg, respectively. Nude mice (10/group) received s.c. transplants of HCT-15 or DLD-2 human colon cancer cells. Mice were treated i.p. with the approximate 10% lethal doses of either DMF (daily for 21 days) or NMF (daily for 19 days) or with 0.9% NaCl solution when tumors became palpable. With the HCT-15 tumor, a growth inhibition of 65% was obtained using DMF compared to 0.9% NaCl solution-treated controls. Two independent experiments with DLD-2 demonstrated that DMF effected growth inhibitions of 45 and 67%. NMF treatment produced 48 and 75% growth inhibitions of HCT-15 and DLD-2 tumors, respectively. Weight loss of groups of treated mice in all experiments was between 2 and 14%, within the acceptable range for 10% lethal drug doses. Results indicate that some human cancer xenografts respond to the polar solvent DMF and to its metabolite NMF and that DMF may be acting at least in part by its metabolism to NMF. Furthermore, the data should alert clinical investigators to the possibility of hepatotoxicity when polar solvents are tested in Phase I clinical trials.

INTRODUCTION

Considerable work performed during the past 10 years has demonstrated that polar solvents can induce the differentiation of tumor cells. The early observations of Friend *et al.* (9) that DMSO[3] and DMF can induce the maturation of cultured cells from the MEL line to cells with erythrocyte-like properties (9, 19) have prompted numerous investigations with this system. Thus, many reports have appeared that document the alteration of growth properties and modulation of cellular markers upon exposure of cultured MEL cells to DMSO or DMF. Among these changes is an increase in the amount of hemoglobin (9, 19), heme (20), globin mRNA (18), and spectrin (8) and the appearance of an erythrocyte antigen (13). Growth property alterations include a loss of proliferative capacity (11) and a loss of clonogenicity in soft agar (17).

Work with polar solvent-induced differentiation of MEL cells has been extended to other culture systems. HL-60 human promyelocytic leukemia cells can be induced to differentiate to cells with properties of granulocytes upon treatment with DMSO or DMF (1). Tralka and Rabson (22) have shown that DMSO induces cilia formation in human lung cancer cells. Dexter (3) has demonstrated that DMF causes alterations in growth properties of a murine rhabdomyosarcoma cell line that are consistent with properties expected of a more benign cell type. Our laboratory has reported previously on the effects of DMF on cultured human colon carcinoma cells. Changes in growth characteristics of treated cells include morphological differentiation, increase in doubling time, decrease in saturation density, loss of clonogenicity in agar, and a marked reduction in tumorigenicity (4). Expression of tissue-specific or organ-specific markers was also modulated by DMF exposure. The treated colon cancer cells showed increased cell surface-associated carcinoembryonic antigen and tumor-specific colonic mucoprotein antigen (12). These results have been interpreted to indicate that human colon cancer cells can be used as a model to study differentiation induction in human solid tumors (6).

NMF, a polar solvent with chemical properties similar to those of DMF, has also been shown to be a good inducer of MEL cell and HL-60 cell differentiation (1, 21). We have reported in a recent preliminary communication that both DMF and NMF induce an increase in tyrosinase activity and in melanin content of cultured B16 melanoma cells (7). Investigations with these 2 polar solvents are also of special interest because DMF is metabolized to NMF in human subjects (16).

It is important to establish whether these studies with polar solvents and tumor cell lines have clinical relevance. The logical extension of studies with cultured cancer cells are *in vivo* experiments that would demonstrate whether responses could be obtained with polar solvent treatment of human tumors growing in nude mice. Accordingly, we have treated athymic nude mice bearing human colon carcinoma xenografts with either DMF or NMF. Confirmatory studies by the National Cancer Institute have provided a further impetus to submit an Investigational New Drug for Phase I studies with NMF.[4] Portions of our work have been presented in preliminary form (7).

MATERIALS AND METHODS

Cell Lines

Two human colon carcinoma cell lines established in our laboratory, HCT-15 and DLD-2, were used in this study. Details on the establishment and cultivation of these lines and on their properties have been published previously (2, 4). HCT-15 grows in culture with a doubling time of about 20 hr and produces tumors in nude mice that are

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[3] The abbreviations used are: DMSO, dimethyl sulfoxide; DMF, *N,N*-dimethylformamide; MEL, murine Friend erythroleukemia; NMF, *N*-methylformamide; LD_{10}, 10% lethal dose; LD_{50}, 50% lethal dose.

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histologically classified as well- to moderately differentiated adenocarcinomas. DLD-2 cells have a doubling time of about 50 hr in culture and in nude mice produce well-differentiated adenocarcinomas characterized by well-defined glandular structures and appreciable mucin and carcinoembryonic antigen production.

Toxicology Studies

Mice. These experiments used male and female outbred Swiss mice heterozygous for the *nu* gene (*nu*/+). The mice were bred and maintained at the Roger Williams Cancer Center Animal Care Facility. Animals were used when they were between 8 and 16 weeks of age and weighed 20 to 30 g (females) or 25 to 35 g (males). For each individual experiment, animals within a 2-g weight range were used. Male mice were housed individually and female mice were housed 10/cage. They had *ad libitum* access to feed and water.

Preparation of Agents and Injections. DMF was purchased from Sigma Chemical Company (St. Louis, Mo.) and was prepared as a 20% solution by dilution with 0.9% NaCl solution. NMF was obtained from Aldrich Chemical Company (Milwaukee, Wisc.) and was prepared as a 3% solution in 0.9% NaCl solution. Both the 20% DMF and the 3% NMF solutions were stored in the dark. Injections of DMF (20%) or NMF (3%) were given i.p. on a daily for 5- or daily for 21-day schedule. Needles were changed between each injection.

Histology and Chemistry Studies. All animals were weighed on Day 1, on Days 6 and 14 (both schedules), and on Day 21 (21-day schedule) using a Pelouze scale; weights were recorded to the nearest 0.5 g. Mice were observed daily for deaths for 60 days following the injections.

In addition, at least 3 animals/treatment group received the following studies: creatinine, blood urea nitrogen, serum glutamic-oxaloacetic transaminase, glucose, bilirubin, total WBC, and hematocrit determination. Blood for hematological analysis was obtained from the venous plexus of the eye while the mouse was anesthetized with ether. Hematocrits were performed using a Clay Adams MHct II centrifuge; WBC determinations were performed with a Becton Dickinson (Oxnard, Calif.) WBC Counting Kit (Unopette Test 5805).

Blood for biochemical analysis was obtained by enucleating the eye and exsanguinating the ether-anesthetized mice. Laboratory determinations were performed in the clinical chemistry laboratories of the Roger Williams General Hospital.

After cervical dislocation, mice received complete autopsy examinations; samples of tissues (brain, thymus, lung, heart, liver, spleen, esophagus, stomach, jejunum, sigmoid colon, muscle, kidney, and bone marrow) were preserved in 10% formalin:glutaraldehyde, stained with hematoxylin and eosin, and examined.

Determination of LD_{10} and LD_{50} Values. At least 6 dose levels, including a dose with no mortality and a dose with ≥50% mortality were used; these doses were geometrically spaced using a ratio of 1:3 (all groups contained a minimum of 13 mice). Data were analyzed using an SAS computer Probit Program and an ITEL AS-5 computer.

Treatment of Nude Mice Bearing Colon Cancers with Polar Solvents

Nude mice with the *nu/nu* gene on a Swiss background were bred and maintained at the Roger Williams Cancer Center Animal Care Facility. Female mice 6 to 8 weeks old were used in this study. In experiments with the HCT-15 line, mice were inoculated s.c. in the flank region with 1×10^7 cultured HCT-15 cells; tumors appeared with a 100% incidence in 4 to 7 days. Since DLD-2-cultured cells take 4 to 6 weeks to produce a tumor in nude mice, inoculation of hosts was done with mince from a DLD-2 tumor propagated *in vivo*. The tumor was excised and thoroughly minced, and 0.2 ml of the resulting suspension was injected s.c. into flanks of recipient mice. Using this method, DLD-2 carcinomas appeared within 1 week in all animals. Tumor-bearing mice were randomized into groups of 10 animals and were ear-tagged. When tumors reached a size of 5 x 5 mm, animals were weighed and tumor dimensions were recorded. Solutions of polar solvents in 0.9% NaCl solution were prepared as described above for the toxicology studies, and treated animals received i.p. either NMF (303 mg/kg daily for 19 days) or DMF (2000 mg/kg daily for 21 days). Mice in control groups received 0.2 ml of 0.9% NaCl solution i.p. daily. Weights and tumor dimensions were recorded twice weekly. After the final injection, mice were sacrificed, and tumors were excised and weighed individually. Response was calculated from the formula,

$$\% \text{ of growth inhibition} = \frac{(\text{Mean wt of control tumor}) - (\text{mean wt of treated tumor})}{\text{Mean wt control tumor}} \times 100\%$$

We used a daily for 21 days schedule because the effects of DMF on cultured human colon cancer cells are reversible (4, 12). Thus, the effectiveness of polar solvents *in vivo* could be quite schedule dependent, and we reasoned that a daily for 21 days schedule would be more efficacious than would be single, weekly, or daily for 5 days injections.

RESULTS

Toxicological Studies with Polar Solvents

DMF. DMF was injected on a daily for 21 days schedule. The following doses (mg/kg) were examined: 769, 1000, 1500, 2250, 2925, and 3802. LD_{10} and LD_{50} doses were 2219 and 3065 mg/kg, respectively. The 95% confidence limits on the LD_{10} dose were 1686 and 2539 mg/kg. Animals treated with doses >769 mg/kg on a daily for 21 days schedule exhibited altered behavior consisting of irritability and hyperactivity that was still noticeable 50 days after the completion of treatment. Gross autopsy examinations were normal. Histological examination showed that damage was confined to the liver, which showed centrilobular necrosis and areas of regeneration. Marrow cellularity was normal at the LD_{10} dose, as was histological examination of the brain.

NMF. The following doses (mg/kg) of NMF were studied on a daily for 21 days schedule: 156, 233, 350, 369, 500, 525, 584, and 788. LD_{10} was 374 mg/kg with 95% confidence limits of 285 and 422 mg/kg. LD_{50} was 512 mg/kg. Animals treated at doses >LD_{10} showed significant weight loss by Day 21. Irritability and hyperactivity were not seen. Total leukocyte counts were at the lower limits of normal; hematocrit was unchanged. Gross autopsy examination was normal. Histological examination revealed that the major changes were in the liver, which showed centrilobular necrosis and an increase in the number of mitotic cells present, indicating some regeneration. The bone marrow was of normal cellularity. In 2 of 6 animals, increased hemosiderin was present and 3 of 6 spleens also had hemosiderin-laden macrophages present. Mild focal peritonitis was seen in 3 of 6 mice.

To summarize, DMF and NMF manifested similar hepatotoxicity in mice. Gross autopsy examination was negative and histological damage was largely confined to the liver, which showed centrilobular necrosis and areas of regeneration. Serum glutamic-oxaloacetic transaminase values correlated with the observation of liver tissue damage from both drugs. Bone marrow cellularity was unaffected at LD_{10} doses. DMF-treated animals became irritable and hyperactive; this neurological side effect lasted for approximately 50 days after treatment ended. NMF-treated animals did not exhibit such altered behavior. The brain was histologically normal in DMF-treated mice despite the marked irritability exhibited by these animals.

Treatment of Xenografted Human Colon Cancers with Polar Solvents

Nude mice bearing the HCT-15 or DLD-2 human colon carcinomas were treated with 0.9% NaCl solution or with the approximate LD_{10} doses determined for mice heterozygous for the *nu* gene. Slight changes in doses (303 mg/kg for NMF; 2000 mg/kg for DMF) were made because nude mice tolerated DMF or NMF in a manner similar but not identical to that of their heterozygous littermates. Results are shown in Table 1. In all experiments with either agent, a tumor growth inhibition was observed. In our 2 initial studies with HCT-15 and DMF, tumors from control or treated animals were weighed as groups, and no statistical evaluation was made. In all other experiments, tumors were weighed individually and mean ± S.E. values were determined. Tumor weights were estimated from caliper measurements at various time points by the formula

$$\frac{l \times w^2}{2}$$

that closely approximates the volume of an ellipsoid and were plotted *versus* time. Linear regression analysis was performed from the graphs of (log) calculated tumor weight *versus* time. Slopes of the growth curves and their 95% confidence limits were determined using the appropriate *t* value with (N-2) d.f. (10). Statistical significance was assumed in those experiments containing no overlap of 95% confidence limits of slope and where the S.E. values did not overlap as well. Responses were statistically significant in the experiments with HCT-15 and DMF or with NMF where statistical analysis could be applied (Experiments 3 and 4) and in 2 of 3 of the experiments with DLD-2 and DMF (Experiments 6 and 7). The 75% inhibition of DLD-2 growth affected by NMF treatment was statistically significant and was the largest response obtained in this investigation.

Tumors in this study were approximately 50 to 75 mg when injections were started. We have made no attempt to correct our data for the weights of tumors at the time of treatment initiation; thus, our results underestimate the actual growth inhibition achieved (Chart 1). The temporal course of the growth inhibition and the relative extent of tumor growth in each group can be seen from Chart 1. The drug effect was quite pronounced during the last 10 days of treatment, when tumors in control mice grew rapidly.

There was some interexperimental variation in the response of the DLD-2 colon tumors to DMF. In 2 of the 3 experiments (Table 1, Experiments 6 and 7) where the results were statistically significant, some variability between responses was seen. This was more noticeable in Experiment 5. Most investigators are aware of the interanimal and interexperimental variability that is usually associated with *in vivo* tumor treatment work. Such differences in our study could be due to different extents of metabolism of DMF to NMF among the mice. They could also be caused by variable levels of natural killer cells (believed to be important in athymic mice) among animals within and between treatment groups.

The tumor growth inhibitions were achieved with weight losses acceptable for studies performed with LD_{10} doses (Table 1). Except for one experiment with DLD-2 and DMF where a 14% average weight loss occurred in treated animals, weight losses in all groups of animals treated with DMF or NMF were <10%. In several experiments, the loss was ≤5%. There were 13 deaths out of 160 animals treated in this study; 8 deaths occurred in control groups. A certain attrition can be expected

Table 1

Responses of human colon cancer xenografts to DMF or NMF

Age-matched s.c. tumor-bearing female nude mice were randomized (10 animals/group) and ear tagged. When carcinomas reached a size of 5 x 5 mm, treatment was initiated. Mice were given i.p. injections of 0.9% NaCl (controls), DMF (2000 mg/kg daily for 21 days), or NMF (303 mg/kg daily for 19 days). Tumor dimensions were measured twice weekly and all animals were weighed at these times. At the conclusion of the injection schedule, mice were sacrificed, and tumors were excised and weighed.

Treatment	Av. tumor wt (mg)	% of tumor growth inhibition	Av. % of polar solvent-induced change in host wt
HCT-15			
Experiment 1			
DMF	275	28	−9
Control	379		
Experiment 2			
DMF	210	36	No change
Control	330		
Experiment 3			
DMF	145 ± 42[a]	65	+2
Control	420 ± 64		
Experiment 4			
NMF	225 ± 36	43	−3
Control	396 ± 74		
DLD-2			
Experiment 5			
DMF	521 ± 132	33	−5
Control	778 ± 275		
Experiment 6			
DMF	222 ± 79	45	−14
Control	402 ± 104		
Experiment 7			
DMF	222 ± 77	67	−7
Control	663 ± 121		
Experiment 8			
NMF	308 ± 57	75	−1
Control	1222 ± 163		

[a] Mean ± S.E.

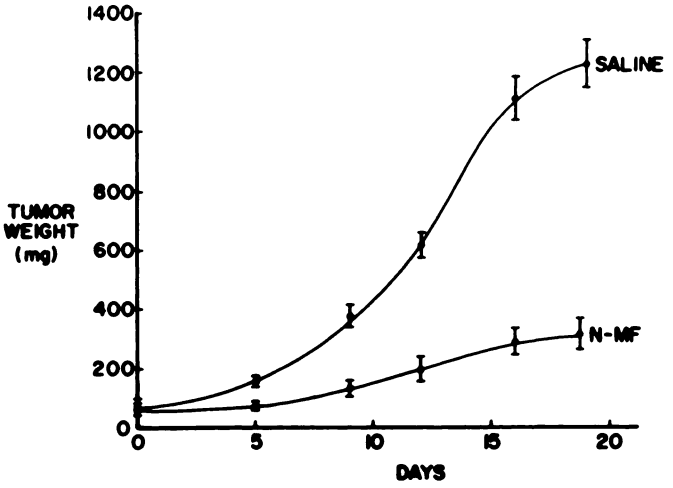

Chart 1. DLD-2 tumors were grown s.c. in nude mice. Mice were ear tagged, randomized (10/group), and treated i.p. daily for 19 days with 0.9% NaCl solution (*saline*) or NMF (303 mg/kg). Tumors were measured at intervals, and tumor weights (mg) were estimated from

$$\frac{l \times w^2}{2}$$

Mice were sacrificed after 19 days, and actual tumor weights were determined. *Bars*, ± S.E.

in experiments involving the administration of multiple injections to nude mice for a long period of time. It is noteworthy that NMF used in the studies with tumor-bearing mice produced very little toxicity as evidenced by virtually no weight loss and by the generally healthy appearance of treated animals. DMF, in contrast, seemed to be tolerated less than was NMF, and also produced neurological symptoms (hyperkinetic activity) in the treated mice. Our results suggest that a better therapeutic index may be achievable with NMF than with DMF.

DISCUSSION

This study demonstrates that the polar solvents DMF and NMF can effect growth inhibitions of 2 human colon carcinomas xenografted in nude mice. Earlier investigations in our laboratory had shown that DMF can alter growth properties of cultured human colon cancer cells and can modulate the expression of specific antigens associated with the differentiated state of human colon carcinoma (4, 12). Changes in both growth characteristics and expression of markers occur in the direction of a more normal, benign phenotype (6). The results obtained with cultured cells suggested the possible efficacy of polar solvents against human colon carcinoma. Support for this hypothesis was provided by other studies performed in our laboratories, which showed that DMF could sensitize cultured colon cancer cells to X-irradiation (14, 15). We have also reported that DMF modulates levels of key purine-metabolizing enzymes in these cells. Adenosine deaminase activity was reduced 11-fold in DMF-treated cells, suggesting that cells exposed to the polar solvent would be more sensitive to formycin or 8-azaadenosine, which are good substrates for adenosine deaminase (5). The *in vivo* responses reported here provide an important extension to the earlier tissue culture studies from our laboratory, and support consideration of polar solvents for Phase I clinical trials.

It is not known whether the responses observed in this study correlate with any differentiation changes occurring in the tumors of treated mice. This question will take time to resolve because of the lack of good markers specific for colon cell differentiation and because of the time-consuming and labor-intensive analyses that must be performed in order to demonstrate convincingly whether such correlations exist. For example, multiple sections of fixed tumor tissue from treated and control mice must be examined in a double-blind fashion using both the light and electron microscope to determine whether morphological (ultrastructural) changes have occurred with treatment and whether such changes are consistent with a maturational effect. Also, tumors would have to be assayed for changes in antigen expression or enzyme levels. This type of assessment on tumors from treated *versus* control mice represents a major undertaking; such studies have been initiated recently in our laboratory. Other mechanisms can also be presented to explain our results. The agents might simply be producing a toxic effect on the tumor with reduced toxicity to the host. Perhaps DMF or NMF affects the vasculature of the tumors, thus interfering with both the nutrition of the carcinomas and with their ability to eliminate waste products. Further studies with DMF and NMF in culture systems and in animal models will be necessary to elucidate the mechanism(s) whereby these agents induce changes in cancer cells. One must consider the possibility that polar solvents induce the differentiation of cultured cancer cells by a mechanism different from that responsible for the tumor growth inhibitions observed in this study.

DMF is known to be metabolized to NMF in humans (16). The extent of metabolism, as measured by NMF analysis in urine of workers exposed to DMF, appears to be small and is on the order of 0.5 to 2.0%. There are no good data available for plasma levels of NMF. The metabolism of DMF to NMF suggests that DMF might be a "double drug." It is active itself in cancer cell culture systems where demethylation should not occur (*N*-methylase is a liver enzyme). Furthermore, demethylation *in vivo* would produce the monomethyl derivative which is also active in culture (1, 21) and, as this study demonstrates, in tumor-bearing mice. Based on our LD_{10} values, NMF is about 5-fold more toxic than is DMF, and the ratio of doses used in our nude mice studies was about 6:1. Thus, the catabolism of a small percentage of injected DMF might result in NMF levels sufficient to produce an antitumor effect. These findings taken together suggest that the relatively innocuous DMF might be used clinically with acceptable toxicity and would produce biological activity through its own action and through catabolism to its active metabolite, NMF. The responses seen with NMF suggest that a favorable therapeutic index might also be achieved with this agent when given at the appropriate dose and schedule.

The following conclusions can be drawn from this investigation: (*a*) both DMF and its metabolite NMF effect responses against human colon carcinomas xenografted in nude mice; (*b*) NMF is more toxic than is DMF, but at LD_{10} appears to be better tolerated by nude mice than is DMF. Thus, a better therapeutic index might be achieved with NMF; (*c*) our toxicology data should alert clinical investigators that the dose-limiting organ toxicity to be expected in Phase I trials with either agent will be hepatic. Liver damage may be reversible, however, since regeneration was observed in our work. This preclinical study on the toxic and tumor growth-inhibiting properties of polar solvents should provide information useful in designing protocols for clinical trials with this promising new class of agents.

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