Cancer Chemother Pharmacol (1986) 17: 269–273

Cancer Chemotherapy and Pharmacology

N-methylformamide-mediated enhancement of in vitro tumor cell chemosensitivity*

Philip J. Tofilon, Charlotte M. Vines, and Luka Milas

Department of Experimental Radiotherapy, Box 66, The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston, 6723 Bertner Avenue, Houston, TX 77030, USA

Summary. The effects of the differentiation-inducing polar solvent *N*-methylformamide (NMF) on the in vitro response of murine hepatocarcinoma (HCa-1) cells to 1,3-bis(2-chloroethyl)-1-nitrosourea, *cis*-diamminedichloroplatinum (II), and melphalan were investigated using the sister chromatid exchange (SCE) and cell survival assays. When cells were exposed to 1.25% NMF, cell culture doubling time increased from 12 to 43 h and cell volume increased from 940 μm^3 to 1440 μm^3. Growth of HCa-1 cells in NMF for 96 h before drug treatment enhanced the SCEs induced by each of the three chemotherapeutic agents. For each drug, maximum enhancement occurred after 72 h of NMF pretreatment, and the enhancement was eliminated 48 h after NMF was removed. Pretreatment with 1.25% NMF for 96 h also enhanced the cell kill induced by each drug. NMF exposure modified primarily the low-dose shoulder region of each drug cell survival curve. The data indicate that NMF is an effective chemosensitizing agent for HCa-1 cells in vitro and suggest that NMF may provide clinical benefits when administered in combination with antineoplastic drugs.

Introduction

Though the polar solvent *N*-methylformamide (NMF) was earlier shown to have cytotoxic activity against certain murine tumors [2, 6], more recent interests have focused on its ability to induce cell differentiation [12]. Since a disorder in differentiation is considered a major component of cancer pathogenesis, it was suggested that NMF may provide a new and potentially significant form of cancer treatment [12]. Exposure of rodent and human leukemic cells grown in vitro to NMF was reported to result in terminal differentiation [3]. However, cells from most solid tumors thus far investigated do not terminally differentiate upon NMF exposure, but only proceed to form so-called better differentiated or less malignant phenotypes [12]. Cellular alterations reported to be associated the NMF-mediated induction of the putative better differentiated phenotype include reduction in clonogenicity, changes in cell morphology, increase in cell culture doubling time, decrease in tumorigenicity, and production of specialized cell products. While benefits may still be obtained from such changes in tumor cell characteristics, such as a decrease in tumor aggressiveness, the problem is that upon NMF withdrawal, the cells revert to their original form.

It has recently been found that differentiation-inducing polar solvents can augment the response of tumor cells to cytotoxic agents. Dimethylformamide, a differentiation-inducing polar solvent with activities similar to those of NMF, was shown to enhance radiation-induced cell kill in vitro when a clone isolated from a human colon carcinoma was used [7], and the administration of NMF for 19 consecutive days to nude mice was found to enhance the growth-inhibitory action of radiation on a human colon tumor xenograft [5]. In addition to radiosensitization, the in vitro exposure of a human colon carcinoma cell line to NMF was reported to enhance the activity of mitomycin C and *cis*-platinum [4]. Although NMF may have potential use as a chemosensitizer, a detailed investigation of its effects on the activity of chemotherapeutic agents has not been reported. We have recently found that the differentiation-inducing polar solvent dimethylsulfoxide (DMSO) enhances the drug response of a murine hepatocarcinoma (HCa-1) cell line [19]. However, DMSO has considerable toxicity and is not suitable for in vivo administration. Because NMF is relatively free of toxic side effects and can be used in a clinical setting [12], we have extended these studies to determine the effects of this differentiation-inducing polar solvent on the in vitro response of the HCa-1 cell line to three commonly used antineoplastic drugs: *cis*-platinum, BCNU, and melphalan.

Changes in cell drug response were evaluated according to clonogenic cell survival and the induction of sister chromatid exchanges (SCE). The SCE assay is a sensitive, rapid method for detecting agents that damage DNA [20], and we have frequently used it in chemotherapy studies with the rat 9L brain tumor [16] and murine hepatocarcinoma [18, 19] models. We found that SCE induction correlates with the in vitro cell kill induced by various chemotherapeutic agents [15, 16, 18]. This assay is useful also in detecting drug-resistant cells [17, 18], predicting the chemoresponse of in vivo primary tumors and metastases [18], and assessing the effect of agents that modify drug-induced cell kill [13, 14, 19]. Thus, the SCE assay appears to be a sensitive indicator of drug action. We now report that

* This investigation was supported in part by National Institutes of Health research grants CA-39465 and CA-06294. We thank Deborah Thomas Elum for her assistance in the preparation of this manuscript.

Offprint requests to: P. J. Tofilon

NMF pretreatment of hepatocarcinoma (HCa-1) cells increased the number of SCEs induced by BCNU, *cis*-platinum, and melphalan, and that this increase in drug-induced SCEs was accompanied by an NMF-mediated increase in drug-induced cell kill.

Materials and methods

Cell culture. The murine hepatocarcinoma cell line used in these studies was established from a spontaneously developed hepatocarcinoma (HCa-1) syngeneic to C_3Hf/Kam mice [18]. HCa-1 cells grow well in vitro, with a doubling time of about 12–14 h and a plating efficiency of 50%–60%. Cells were routinely cultured in Hsu's medium containing 20% fetal calf serum at 37 °C in a humidified 5% CO_2:95% air atmosphere. Before drug treatment, cells were seeded into 75-cm^2 flasks for at least 24 h to established log phase growth. Cell volume was determined electronically using a Coulter channelizer.

Drug treatment. HCa-1 cells were exposed to 1.25% NMF in normal growth medium for 96 h, unless otherwise stated. Stock solutions of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU, NSC # 409962-T), *cis*-platinum (*cis*-diamminedichloroplatinum, Sigma Chemical Co.), and melphalan (Sigma Chemical Co.) were made immediately before use. BCNU was dissolved in ethanol; melphalan dissolved in solution A (8.0 g NaCl, 0.4 g glucose, and 0.35 g $NaHCO_3$ per water) containing 3% 1 *N* HCl (v/v); and *cis*-platinum dissolved in warm solution A.

SCE assay. After treatment for 1 h with graded concentrations of BCNU, *cis*-platinum, or melphalan, cells were rinsed and 15 ml fresh, NMF-free medium containing 10 μ*M* bromodeoxyuridine (BrdUrd) was added to each culture. Cells were allowed to replicate in the presence of BrdUrd for two cell cycles, which resulted in the incorporation of BrdUrd into two DNA strands of one chromatid and only one strand of its sister chromatid. At 2–3 h before harvesting, cultures were treated with Colcemid (0.04 μg/ml). Mitotic cells were shaken from the flasks and treated with 0.075 *M* KCl. Cells were fixed and washed in freshly prepared methanol: glacial acetic acid (3:1 v/v). Sister chromatids were differentially stained using the method of Perry and Wolff [10].

Cell survival assay. After treatment for 1 h with graded concentrations of BCNU, *cis*-platinum, or melphalan, cells were trypsinized, counted, diluted, and plated into dishes containing NMF-free medium and 5×10^4 heavily irradiated (40 Gy) HCa-1 feeder cells. After 7 days of incubation, colonies in all groups were approximately the same size and were fixed, stained, and counted. Plating efficiency was determined from colony counts and surviving fractions were calculated.

Results

The presence of NMF in the growth medium altered the morphology and growth rate of HCa-1 cells. The volume of cells exposed to 1.25% NMF increased from a control value of 940 μm^3 to 1440 μm^3, and cell doubling time increased from 12 to about 43 h. These changes were similar to those reported for other types of cells exposed to NMF [12].

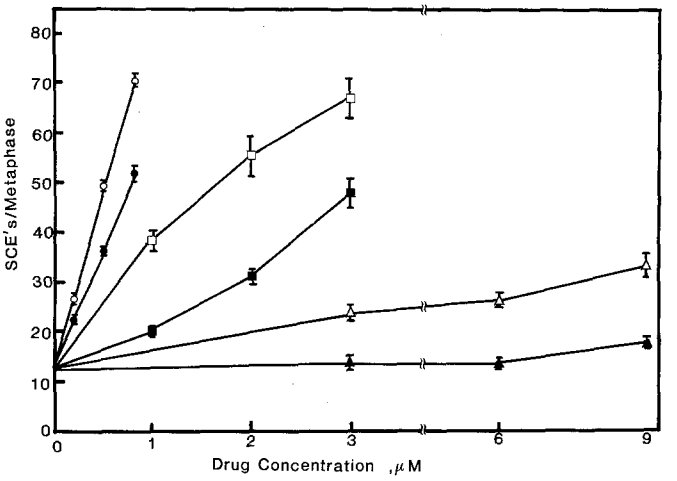

Fig. 1. Effect of growing cells in the presence of NMF on SCEs induced with BCNU (△), *cis*-platinum (□), and melphalan (○). *Open symbols* represent values for cells grown in NMF and *closed symbols*, those for cells grown in NMF-free medium. HCa-1 cells were grown in medium containing 1.25% NMF for 96 h and then treated for 1 h with graded concentrations of each drug. After treatment cells had been rinsed, NMF-free medium containing 10 μ*M* BrdUrd was added. Cells were collected about 30 h, later, and SCE analysis was performed as described in *Materials and methods.* SCEs induced by NMF alone are subtracted from NMF-plus-drug results. Values represent the means ± of SEM of 20 well-spread metaphase cells

As reported earlier [18], HCa-1 cells exhibit different sensitivities to SCE induction by melphalan, *cis*-platinum, and BCNU. The results presented in Fig. 1 confirmed that observation, showing that HCa-1 cells are most sensitive to melphalan, less sensitive to *cis*-platinum, and essentially resistant to BCNU. Figure 1 also shows that the growth of HCa-1 cells in 1.25% NMF for 96 h before drug treatment enhanced the SCEs induced by each of the three chemotherapeutic agents. An enhancement factor was determined for each drug by calculating the ratio of the slopes of the SCE dose responses for cells grown in NMF-containing and NMF-free medium. With respect to SCE induction, the NMF enhancement factors for melphalan and *cis*-platinum were approximately 1.5 and for BCNU approximately 3.4. In these experiments, NMF was present during the 1-h drug treatment period; after drug treatment, cells were allowed to replicate for two cycles in NMF-free medium containing BrdUrd. SCEs induced as a result of NMF exposure alone ranged from 1.9 to 3.7.

As predicted by the SCE assay [15], HCa-1 cells were also most sensitive to the cell killing effects of melphalan, less sensitive to *cis*-platinum, and essentially resistant to BCNU. Figure 2 shows that the presence of 1.25% NMF enhanced the cytotoxicity induced by each of the three chemotherapeutic agents. NMF exposure modified primarily the shoulder region of the melphalan and *cis*-platinum cell survival curves. This NMF effect on the shoulder region was reflected by the change in the dose enhancement factor (DEF) as the surviving fraction decreased. At a surviving fraction of 0.5 the DEFs for melphalan and *cis*-platinum were 2.4 and 2.1, respecitvely, whereas at a sur-

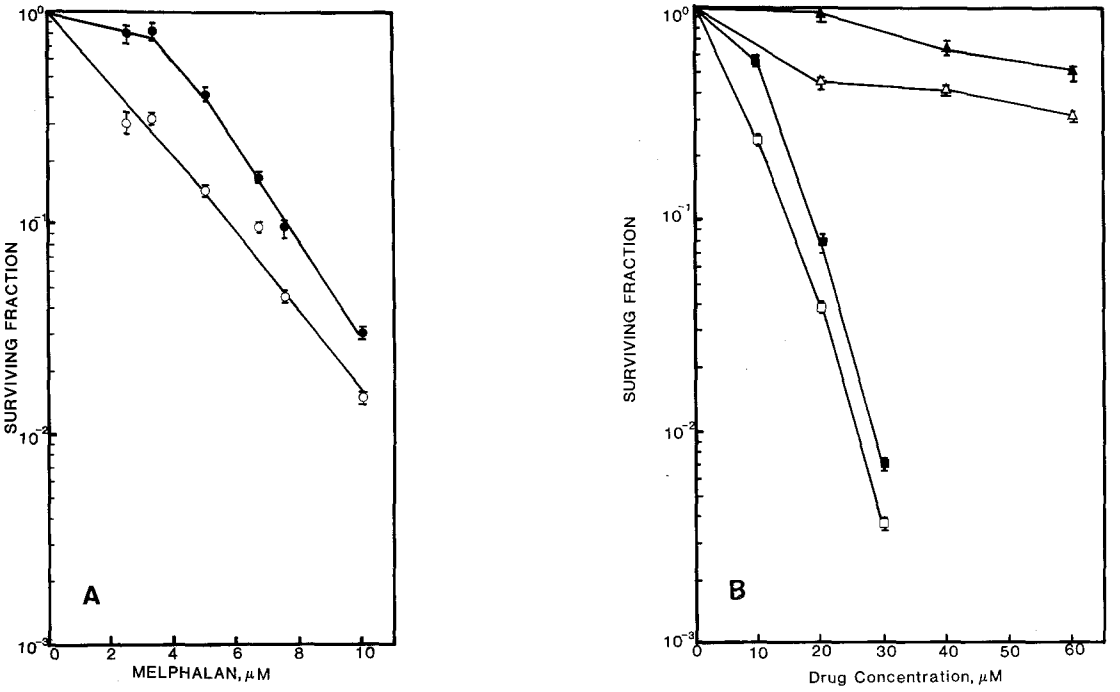

Fig. 2A, B. Effect of growing cells in the presence of NMF on cell survival after treatment with **A** melphalan (O), **B** BCNU (△) and *cis*-platinum (□) (*open* and *closed symbols* as in Fig. 1). HCa-1 cells were grown in the presence of 1.25% NMF for 96 h and then treated for 1 h with graded concentrations of each drug. After treatment, cells were trypsinized and plated into NMF-free medium. Values represented the means ± SEM for 8–12 petri dishes, and curves were fitted by eye. Values were normalized to account for the difference in plating efficiencies between cells maintained in 1.25% NMF and those maintained in NMF-free medium

vival of 0.1 the DEFs for both melphalan and *cis*-platinum decreased to 1.3. Although it is difficult to assign a shoulder region to the BCNU cell survival curve, the largest NMF-mediated enhancement in cell kill was also found in the low-dose region. At a survival of 0.5, the DEF for BCNU was 3.5. As in the SCE experiments, NMF was present during drug treatment; after treatment cells were plated into NMF-free medium. Plating efficiency of cells grown in 1.25% NMF was 0.46, as against 0.53 for control cells, indicating a slight amount of NMF-induced cytotoxicity.

To determine the NMF exposure period required to enhance the actions of BCNU, *cis*-platinum, and melphalan, cells were exposed to 1.25% NMF for 0, 48, or 72 h before drug treatment and the SCE assay performed (Table 1). For each drug investigated, 48 h of NMF exposure before treatment had essentially no effect on drug-induced SCEs, whereas 72 h in NMF significantly increased the number of SCEs induced by each drug. No further increase in drug-induced SCEs was detected for times longer than 72 h in NMF. Since previous studies had noted the effects of differentiation-inducing polar solvents on various cell growth characteristics to be reversible [12], we investigated the reversibility of the NMF-mediated enhancement of cell chemosensitivity. Cells were grown in 1.25% NMF for 72 h; NMF was then removed and replaced with NMF-free medium 24 h or 48 h before drug treatment. As the results in Table 1 show, the increase in drug-induced

Table 1. Effects of NMF pretreatment protocols on drug-induced SCEs[a]

			NMF (1.25%) pretreatment time		
	No NMF	48 h	72 h	72 h and removed 24 h before drug treatment	72 h and removed 48 h before drug treatment
Melphalan (0.5 μ*M*)	29.6 ± 1.5	32.8 ± 1.7	40.1 ± 2.1[b]	38.6 ± 1.3[b]	30.9 ± 2.2
cis-Platinum (2 μ*M*)	36.7 ± 1.9	35.1 ± 2.5	57.8 ± 2.4[b]	55.4 ± 3.8[b]	37.8 ± 1.7
BCNU (6 μ*M*)	16.2 ± 1.4	17.9 ± 1.0	28.9 ± 3.6[b]	25.5 ± 2.3[b]	19.3 ± 1.0

[a] See text for details of NMF pretreatment. After drug treatment cells were rinsed, and fresh NMF-free medium containing 10 μ*M* BrdUrd was added. Cells were collected about 30 h later and SCE analysis was performed as described in *Materials and methods*. Values represent the means ± SEM for 20 well-spread metaphases

[b] Significantly greater than drug treatment alone ($P < 0.01$) as determined by two-tailed *t*-test

SCEs mediated by NMF declined only slightly at 24 h and was essentially eliminated within 48 h of NMF removal. Thus, like other cellular changes, the NMF-mediated enhancement of chemosensitivity is reversible.

Discussion

The effects of NMF on HCa-1 cells in vitro included an increase in cell doubling time and a change in cell morphology, findings that agree with previous investigations of the effects of NMF or other polar solvents on in vitro cell lines obtained from solid tumors [12]. Although these changes in cell characteristics may suggest a progression to a better differentiated phenotype, it is not possible to draw firm conclusions from our observations with respect to the state of cell differentiation of the HCa-1 cells used in this study. However, our data do indicate that in vitro exposure of these cells to NMF enhances cell sensitivity to three commonly used chemotherapeutic agents. The enhancement occurred for drugs that were both effective and ineffective against HCa-1. The increase in drug activity was detected at the chromosome level based on SCE induction and was verified by the cell survival assay. As previously mentioned, agents that modify drug-induced cell kill in the in vitro 9L system also modify drug-induced SCEs [13, 14]. A similar correlation was also shown for the modification of hepatocarcinoma drug response by DMSO [19]. Our present studies extended the relationship between SCEs and cell kill to NMF, another response-modifying agent. Thus, these data further support the use of the SCE assay as an indicator of cell sensitivity to certain antineoplastic drugs.

Because NMF enhances the activity of three different types of antineoplastic drugs, elucidation of the mechanism or mechanisms involved in NMF-mediated chemosensitization may, in addition to aiding in the clinical application of NMF, contribute to the general understanding of cytotoxic drug action. Since NMF was present during the time of drug treatment in our studies, it is possible that NMF acts directly on the drug, facilitating its uptake into the cell. This seems unlikely, however, because the enhancement requires NMF to be present for more than 48 h before drug treatment. This suggests that the effects of NMF are on the cell and not on the cytotoxic drugs themselves.

The cell membrane is one of the potential cellular sites at which NMF may act to modify drug action. NMF treatment results in an increase in cell volume, and DMSO, which is also a differentiation-inducing polar solvent, has been shown to alter the fluidity of cell membranes [9]. NMF may therefore modify the cellular membrane in such a way as to increase the uptake of these cytotoxic drugs. If this is the mechanism responsible of NMF-mediated enhancement of drug activity, any proposed membrane change must result in increased cellular uptake of three drugs that penetrate cells by three different processes; melphalan and *cis*-platinum are actively transported, each by a different mechanism [1], whereas BCNU is extremely lipid-soluble and passively diffuses into cells [8]. We did not measure drug uptake, and thus cannot at this time evaluate the role of the cell membrane in the NMF-mediated enhancement of drug action.

The three antineoplastic drugs used in this study exert their cytotoxic effects through an initial alkylation of nuclear DNA with the subsequent formation of DNA – DNA and/or DNA – protein cross-links. Therefore, a potential mechanism for enhancement may include an NMF-mediated alteration in drug – DNA interactions. The effects of NMF on DNA have not to our knowledge been investigated. However, DMSO, which also enhances the response of HCa-1 cells to *cis*-platinum, BCNU, and melphalan [13], has been shown to induce DNA damage in a dose-dependent manner, primarily in the form of single-strand breaks [11]. In our experiments, the growth of cells in NMF-containing medium resulted in a slight increase in SCEs, suggesting that NMF also induces DNA damage, which may be similar to that induced by DMSO. The induction of DNA strand breaks has been hypothesized to induce the unwinding of complimentary DNA strands and/or a change in general chromatin structure [11]. It was previously postulated that a change in DNA tertiary structure might be responsible for the modification of *cis*-platinum activity by polyamine depletion [14] and of BCNU activity by polyamine depletion [14] and low doses of X-irradiation [13]. Thus, it is possible that an alteration in the structure or conformation of DNA resulting from NMF exposure affects the drug – DNA interactions and consequently influences the effectiveness of these cytotoxic agents. Further experiments are required to evaluate the effects, if any, of NMF on the interactions between these chemotherapeutic agents and DNA.

In addition to its potential alteration of initial drug-induced damage, NMF may also modify the repair of damage. Leith et al. [7] found that DMF, of which NMF is a metabolite, affected the low-dose shoulder region of the X-ray cell survival curve of a human colon carcinoma cell line. Since a decrease in the size of the shoulder is indicative of an inhibition of the repair of radiation-induced sublethal damage, they suggested that DMF might interfere with cellular repair capbilities. Our data showed that NMF modifies the low-dose shoulder regions of the melphalan, *cis*-platinum, and BCNU cell survival curves. The decrease in the size of these shoulders may thus suggest that NMF interferes with the cell's ability to repair drug-induced damage. However, the interpretation of cell survival curves generated after drug treatment are not as well characterized as for those obtained after ionizing radiation. A change in the shoulder region of the drug cell survival curve may also be indicative of an alteration in transport into the cell at low drug doses or perhaps a change in the interaction between the drug and its critical target. Since nuclear DNA is the critical target of the three chemotherapeutic agents used in this study, it will be necessary to use DNA-level assays to clearly define the effects, if any, of NMF on the repair of drug-induced damage.

Animal studies showed that NMF toxicity is limited primarily to the liver [12]. The absence of NMF hematopoietic toxicity combined with its chemosensitizing effects reported here and its radiosensitizing ability reported by others [5, 7] suggest that NMF may be an appropirate agent for use in combination protocols with other standard modalities. Obviously, before the optimal clinical application of NMF as a response modifier can be developed, the selectivity of the chemosensitizing actions of NMF for tumor cells over normal cells must be demonstrated in experimental in vivo tumor systems. Experiments are currently in progress to evaluate the effects of combinations of NMF and cytoxic agents on normal tissue, and the

mechanisms involved in the observed NMF-mediated chemosensitization are also being investigated. Studies of this nature should aid in the successful assimilation of NMF into cancer treatment strategies.

References

1. Byfield JE, Calabro-Jones PM (1981) Carrier-dependent and carrier-independent transport of anti-cancer alkylating agents. Nature 294: 281
2. Clarke CA, Philips FS, Sternberg SS, Barclay RK, Stock CC (1953) Effects of *N*-methylformamide and related compounds in sarcoma 180. Proc Soc Exp Biol Med 84: 203
3. Collins SJ, Ruscetti FW, Gallager RE, Gallo RC (1978) Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar solvents. Proc Natl Acad USA 75: 2458
4. Dexter DL, DeFusco, DJ, McCarthy K, Calabresi P (1983) Polar solvents increase the sensitivity of cultured human colon cancer cells to *cis*-platinum and mitomycin C. Proc Am Assoc Cancer Res 26: 267
5. Dexter DL, Lee ES, Bliven SF, Glicksman AS, Leith JT (1984) Enhancement by *N*-methylformamide of the effect of ionizing radiation on a human colon tumor xenografted in nude mice. Cancer Res 44: 4942
6. Gescher A, Gibson NW, Hickman JA, Langdon SP, Ross D, Atassi G (1982) *N*-Methylformamide: antitumor activity and metabolism in mice. Br J Cancer 45: 843
7. Leith JT, Gaskins LA, Dexter DC, Calabresi P, Glicksman AS (1982) Alteration of the survival response of two human colon carcinoma subpopulations to X-irradiation by *N,N*-dimethylformamide. Cancer Res 42: 30
8. Levin VA, Kabra PM (1974) Effectiveness of the nitrosoureas as a function of lipid solubility in the chemotherapy of experimental rat brain tumors. Cancer Chemother Rep 58: 787
9. Lyman GH, Preisler HD, Papahodjopoules D (1976) Membrane action of DMSO and other chemical inducers of Friend leukaemic cell differentiation. Nature 262: 260
10. Perry P, Wolff S (1974) New Giemsa method for differential staining of sister chromatids. Nature 251: 156
11. Schwer W, Friend C (1978) Breakage of DNA and alterations in folded genomes by inducers of differentiation of Friend erythroleukemic cells. Cancer Res 38: 841
12. Spremulli EN, Dexter DL (1984) Polar solvent – a novel class of antineoplastic agents. J Clin Oncol 2: 227
13. Tofilon PJ, Deen DF (1984) BCNU-induced sister chromatid exchanges are increased by X irradiation. Radiat Res 97: 171
14. Tofilon PJ, Oredsson SM, Deen DF, Marton LJ (1982) Polyamine depletion influences drug-induced chromosomal damage. Science 217: 1044
15. Tofilon PJ, Williams ME, Barcellos MH, Deen DF (1983a) Comparison of the sister chromatid exchange and cell survival assays as a measure of tumor cell sensitivity in vitro to *cis*-diamminedichlorplatinum (II). Cancer Res 43: 3511
16. Tofilon PJ, Williams ME, Deen DF (1983b) Nitrosourea-induced sister chromatid exchanges and correlation to cell survival in 9L rat brain tumor cells. Cancer Res 43: 473
17. Tofilon PJ, Gerosa MA, Rosenblum ML, Bodell WJ, Deen DF (1984) Prediction of the relative in vitro sensitivity of 9L rat brain tumor cells to nitrosoureas by the sister chromatid exchange assay. Life Sci 35: 1611
18. Tofilon PJ, Basic I, Milas L (1985) Prediction of in vivo tumor response to chemotherapeutic agents by the in vitro sister chromatid exchange assay. Cancer Res 45: 2025
19. Tofilon PJ, Vines CM, Milas L (1985) Enhancement of in vitro chemotherapeutic activity by dimehtylsulfoxide. Clinical and Experimental Metastases 3: 141
20. Wolff S (1981) Measurement of sister chromatid exchanges in mammalian cells: In: Friedberg EC, Hanawalt PC (eds) DNA repair: a laboratory manual of research procedures, vol 1 pt B. . Dekker, New York, p 577

Received November 21, 1985/Accepted January 21, 1986