[CANCER RESEARCH 41, 2783-2785, July 1981]
0008-5472/81/0041-0000$02.00

Sensitization of 9L Rat Brain Gliosarcoma Cells to 1,3-bis(2-chloroethyl)-1-nitrosourea by α-Difluoromethylornithine, an Ornithine Decarboxylase Inhibitor[1]

David T. Hung, Dennis F. Deen, Jerome Seidenfeld,[2] and Laurence J. Marton[3]

Brain Tumor Research Center, Department of Neurological Surgery [D. T. H., D. F. D., J. S., L. J. M.], Department of Radiation Oncology [D. F. D.], and Department of Laboratory Medicine [L. J. M.], School of Medicine, University of California, San Francisco, California 94143

ABSTRACT

α-Difluoromethylornithine, a known inhibitor of polyamine biosynthesis, significantly enhanced the cytotoxic effect of 1,3-bis(2-chloroethyl)-1-nitrosourea, a cell cycle-nonspecific agent, in 9L rat brain gliosarcoma cells *in vitro*. Administered as a single agent, α-difluoromethylornithine was not cytotoxic to 9L cells and, compared to untreated control cells, caused no perturbation of cell cycle kinetics. α-Difluoromethylornithine-induced depletion of intracellular polyamine levels appears to have caused the observed sensitization of 9L cells to 1,3-bis(2-chloroethyl)-1-nitrosourea. Restoration of intracellular polyamine levels by the addition of exogenous putrescine to treated 9L cells reversed this phenomenon.

INTRODUCTION

The use of chemical adjuncts to enhance the effectiveness of chemotherapeutic agents has been rather limited, and many chemosensitizing agents are toxic in doses that optimize tumor cell kill (6, 7, 21). We have found, however, that DFMO,[4] an inhibitor of polyamine biosynthesis, is an effective and nontoxic chemosensitizer.

The polyamines PU, SD, and SP are polycationic aliphatic amines present in all mammalian systems and are related to normal and neoplastic tissue growth (29). ODC catalyzes the initial and rate-limiting step in polyamine biosynthesis, and rapid cell proliferation is accompanied by a dramatic increase in ODC activity that leads to an increase in intracellular polyamine concentrations (17). The depletion of intracellular polyamine levels generally leads to the cessation of cell growth (17). Among the known inhibitors of polyamine biosynthesis are α-methyl ornithine, methylglyoxal-bis(guanylhydrazone), and DFMO. We report here the chemosensitivity that results from depleting the polyamine content of rat 9L gliosarcoma cells with DFMO.

MATERIALS AND METHODS

Cell Culture. 9L rat brain gliosarcoma cells were seeded into 75-sq cm tissue culture flasks (Falcon Plastics, Oxnard, Calif.) and grown in 14.5 ml of Earle's minimum essential medium supplemented with nonessential amino acids, 10% fetal calf serum, and gentamicin (50 μg/ml). Cells were incubated for 24 hr (all incubations were at 37° in a humidified 5% CO_2-95% air atmosphere) to establish early log phase growth. Flasks to receive 10 mM DFMO treatment were initially seeded with 5 × 10^5 9L cells, whereas control flasks were seeded with 2.5 × 10^5 9L cells in order to minimize the difference in cell density between the 2 flasks at the onset of BCNU treatment.

Drug Treatment. After the initial incubation, cells were treated with either 0.5 ml of 300 mM DFMO in HBSS (to achieve a final DFMO concentration of 10 mM) or 0.5 ml of HBSS alone. After incubation for 48 hr, both DFMO-treated and control cells were treated with BCNU as follows. Stock solutions of ethanolic BCNU were prepared at concentrations of 1000, 800, 600, 400, 200, or 0 μg/ml. To each flask containing 9L cells (total liquid volume, 15 ml), 150 μl of a stock solution were added to achieve a final BCNU concentration of either 10, 8, 6, 4, 2, or 0 μg/ml. After a 1-hr incubation, cells were rinsed to remove BCNU and DFMO, trypsinized, and plated to determine colony-forming efficiency.

In the PU reversal experiments, the above protocol was followed except that, in both DFMO-treated and control experiments, flasks were seeded with 2.5 × 10^5 9L cells and, after the 48-hr incubation, treated with either DFMO or HBSS. PU-reversed cells received 0.5 ml of 31 mM PU (Calbiochem-Behring, La Jolla, Calif.) to achieve a final PU concentration of 1 mM, while control cells received an additional 0.5 ml of HBSS. After incubation for an additional 24 hr, both PU-treated and HBSS-treated cells were treated with BCNU for 1 hr as described above, rinsed to remove drug, trypsinized, and plated to determine colony-forming efficiency.

Colony-forming Efficiency Assay. This assay has been described (10).

Polyamine Analysis. This assay has been described (25).

RESULTS

The cell kill in cultures treated with DFMO before BCNU treatment averaged approximately 0.5 log more than controls (Chart 1). At 10, 1, and 0.1% survival levels, DER's were 1.2 to 1.3. PU and SD levels in DFMO-pretreated cells were less than 5% of control levels, while SP levels were essentially unchanged (24).

Cells receiving 1 mM PU after DFMO pretreatment plated at efficiencies averaging approximately 0.5 log more than cells receiving DFMO pretreatment only (Chart 2). PU, SD, and SP levels in DFMO-pretreated cells treated with PU were >200, 80, and 125%, respectively, of polyamine levels in control cells

[1] Supported by National Cancer Institute Grants CA-15515 and CA-13525 and a gift from the Phi Beta Psi Sorority.

[2] Present address: The Ben May Laboratory for Cancer Research, University of Chicago, Chicago, Ill.

[3] To whom requests for reprints should be addressed.

[4] The abbreviations used are: DFMO, α-difluoromethylornithine; PU, putrescine; SD, spermidine; SP, spermine; ODC, ornithine decarboxylase; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; HBBS, Hanks' balanced salt solution; DER, dose enhancement ratio.

Received January 12, 1981; accepted April 15, 1981.

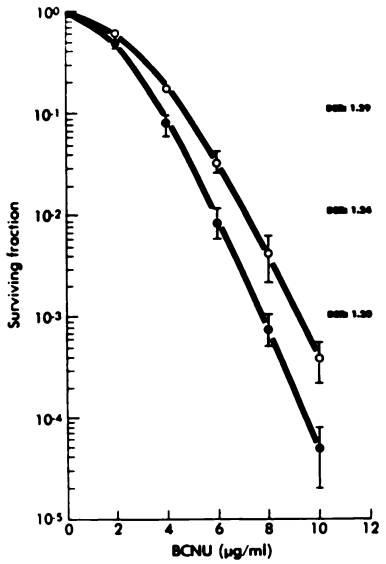

Chart 1. ○, cells pretreated with HBSS for 48 hr and then treated with BCNU. ●, cells pretreated with 10 mM DFMO for 48 hr and then treated with BCNU. *Points*, means of 3 separate experiments; *bars*, S.E.

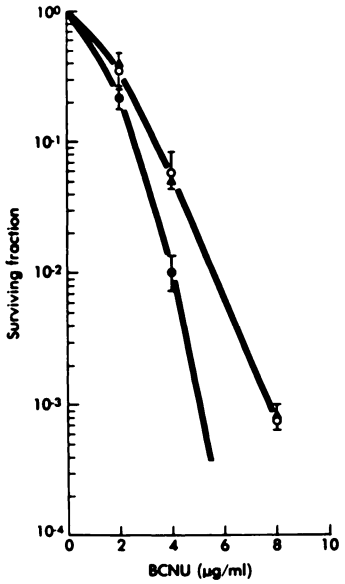

Chart 2. ○, cells receiving HBSS for 48 hr, 1 mM PU for 24 hr, and then treated with BCNU. ●, cells receiving 10 mM DFMO for 48 hr, HBSS for 24 hr, and then treated with BCNU. ▲, cells receiving 10 mM DFMO for 48 hr, 1 mM PU for 24 hr, and then treated with BCNU. *Points*, means of 8 Petri dishes; *bars*, S.D.

receiving only HBSS. Cells treated with only PU and HBSS plated at efficiencies identical to cells treated with DFMO and PU, indicating that PU alone is neither toxic nor stimulatory to 9L cells. PU, SD, and SP levels in cells pretreated with PU only were >200, 40, and 105% of control levels, respectively.

DISCUSSION

DFMO is an effective, highly selective, enzyme-activated, irreversible inhibitor of ODC (17, 19, 24). Within 48 hr of treatment, 10 mM DFMO depletes PU and SD levels in 9L cells to less than 5% of control levels and inhibits cell proliferation (24). However, addition of 1 mM PU to cell cultures restores the polyamine content of the cell, which immediately reinitiates cell growth and division (24). DFMO concentrations of up to 25 mM are nontoxic to 9L cells (23) and cause no perturbation of normal 9L cell cycle distribution, as measured by flow cytometry. Cell cycle synchronization induced by DFMO was apparently responsible for enhanced cell kill observed in previous studies (22, 26).

Our data indicate that DFMO sensitizes 9L cells to BCNU. However, DFMO sensitization does not appear to be the result of an interaction of BCNU and DFMO; cells treated simultaneously with DFMO and BCNU for 1 hr have the same plating efficiency as cells treated only with BCNU for 1 hr. We feel that low intracellular polyamine levels are responsible for the observed sensitization, because restoration of intracellular polyamine levels reverses the phenomenon.

Preliminary *in vivo* data corroborate the phenomenon of DFMO-sensitization observed *in vitro*. C57BL/6 mice bearing an intracerebral glioma 26 tumor that were given 3% DFMO in drinking water *ad libitum* for up to 21 days had the same median life span as untreated tumor-bearing mice. However, DFMO administered in drinking water for 4 days before treatment with a single i.p. dose of either 20 or 30 mg of BCNU per kg increased the median life span of glioma 26 tumor-bearing mice by approximately 20% over control mice that received BCNU alone ($p = 0.01 < 0.05$ by the Gehan modification of the Wilcoxon rank sum analysis) without increasing nonfatal toxicity.

Although the mechanism by which DFMO sensitizes 9L cells to BCNU is not known, the well-documented effects of polyamines on the structure and stability of DNA obtained from cell lysates suggest that DFMO sensitization is due to the alterations in DNA structure caused by intracellular polyamine deficiency. Polyamines protect DNA in solution from enzymatic degradation (1), from denaturation by X-rays (4) and heat (27), from photochemical formation of psoralen monadducts and interstrand cross-links (2), and from *N*-methyl-*N*-nitrosourea methylation of the N^7 and O^6 positions of guanine residues and of the N^3 position of adenine residues (20). Polyamines also aid in the packaging of DNA in T-7 phage heads (12) and lead to the condensation and compaction of DNA in solution (5, 13). Considerable X-ray crystallographic evidence indicates that the primary and secondary amine groups of SD and SP can bind ionically to adjacent phosphate groups on one strand of DNA while the 4-carbon chain stretches across the minor groove of the double helix, forming a cross-bridge between phosphate groups on opposite strands (8, 9, 16, 27). The noncovalent cross-bridging and consequent charge neutralization by polyamines may stabilize DNA structure. We have conducted preliminary studies utilizing viscoelastometry (3, 14, 15, 18) that indicate that the DNA obtained from 9L cells depleted of polyamines by 10 mM DFMO pretreatment *in vitro* is considerably more susceptible to shear during the cell lysis procedure than the DNA obtained from untreated cells.[5] This is consistent with a destabilization of the DNA caused by polyamine depletion.

By inhibiting polyamine biosynthesis, DFMO may destabilize the DNA in 9L cells, which would render the DNA more susceptible to nonspecific alkylation by the reactive alkylating

[5] D. T. Hung, K. L. W. Wun, R. H. Shafer, and L. J. Marton, unpublished data.

moiety of BCNU (28). If this is indeed the case, DFMO should sensitize cells to alkylating agents in general.

Doses of DFMO that inhibit cellular proliferation have been essentially nontoxic in virtually all *in vitro* and *in vivo* systems studied. Preliminary data on long-term administration of large doses of DFMO to rodents show that only mild toxicity (hematological and gastrointestinal) occurred in some instances, which was readily reversed when the drug was withdrawn. Our data with mice bearing glioma 26 indicate that only short pretreatment times with DFMO are necessary to enhance the cytotoxic effect of BCNU.

The DER's of 1.2 to 1.3 produced by DFMO sensitization of 9L cells to BCNU are significant. For most tumors, the probability of cure increases very rapidly with dose, and only minute increments in either dose or dose effectiveness are necessary to produce substantial increases in tumor cell kill. Cure rates can be augmented from 10% to 90% by DER's of 1.13 to 1.16 (11). Therefore, the DER's produced by DFMO could significantly alter the outcome of chemotherapy with alkylating agents.

Finally, the capacity of DFMO to arrest cell growth without toxic effects may prevent or retard tumor regrowth in the interval between courses of conventional chemotherapy. Given in drinking water, 3% DFMO is at least as effective in preventing the proliferation of the EMT6 mammary tumor in mice as 100 mg cyclophosphamide per kg i.p. Both therapies limit the tumor weight to less than 10% of the tumor weight in control-treated mice 36 days after inoculation (19). In this study, a combination of DFMO and cyclophosphamide given simultaneously produced a slightly increased effect over either agent alone, but DFMO pretreatment was not attempted. Another recent study investigated the interaction of polyamine synthesis inhibitors with other chemotherapeutic agents (26). However, the observed interactions were apparently caused by cell cycle perturbations, a phenomenon not found in our study.

Clearly, the polyamine synthesis inhibitors are a class of drug that deserve intensive investigation, not only for their potential as molecular biological probes but also for their potential use as chemotherapeutic agents.

ACKNOWLEDGMENTS

We thank Warren P. Lubich for expert technical assistance, Beverly J. Hunter for manuscript preparation, and Neil Buckley for excellent editorial assistance. DFMO was the generous gift of the Merrell Research Center, Cincinnati, Ohio.

REFERENCES

1. Bachrach, U., and Eilon, G. The effect of spermine and oxidized spermine on the enzymatic degradation of DNA. Biochim. Biophys. Acta, *179:* 494–496, 1969.
2. Ben-Hur, E. The effect of polyamines on the photochemical reactivity of DNA. Isr. J. Chem., *13:* 221–228, 1975.
3. Bowen, B. C., and Zimm, B. H. Molecular weight of T2 Nu DNA from viscoelasticity. Biophys. Chem., *7:* 235–252, 1978.
4. Brown, P. E. Some effects of binding agents on the x-irradiation of DNA. Radiat. Res., *34:* 24–35, 1968.
5. Chattoraj, D. K., Gosule, J. A., and Schellman, J. A. DNA condensation with polyamines. II. Electron microscopic studies. J. Mol. Biol., *121:* 327–337, 1978.
6. Cohen, M. H. Enhancement of the antitumor effect of 1,3-bis(2-chloroethyl)-1-nitrosourea by various psychotropic drugs in combination with caffeine. J. Pharmacol. Exp. Ther., *194:* 475–479, 1975.
7. Cohen, M. H., and Carbone, P. P. Enhancement of the antitumor effects of 1,3-bis(2-chloroethyl)-1-nitrosourea and cyclophosphamide by vitamin A. J. Natl. Cancer Inst., *48:* 921–926, 1972.
8. Damiani, A., Giglio, E., Puliti, R., and Ripamonti, A. X-ray study of spermidine trihydrochloride. J. Mol. Biol., *11:* 441–442, 1965.
9. Damiani, A., Liquori, A. M., Puliti, R., and Ripamonti, A. X-ray study of spermine tetrahydrochloride. J. Mol. Biol., *11:* 438–440, 1965.
10. Deen, D. F., Bartle, P. M., and Williams, M. E. Response of cultured 9L cells to spirohydantoin mustard and x rays. Int. J. Radiat. Oncol. Biol. Phys., *5:* 1663–1667, 1979.
11. Elkind, M. M. Fundamental questions in the combined use of radiation and chemicals in the treatment of cancer. Int. J. Radiat. Oncol. Biol. Phys., *5:* 1711–1720, 1979.
12. Gosule, L. C., and Schellman, J. A. Compact form of DNA induced by spermine. Nature (Lond.), *259:* 333–335, 1976.
13. Gosule, L. C., Schellman, J. A. DNA condensation with polyamines. I. Spectroscopic studies. J. Mol. Biol., *121:* 311–326, 1978.
14. Klotz, L. C., and Zimm, B. H. Size determination by viscoelastic measurements: results on bacteriophages, *Bacillus subtilis* and *Escherichia coli*. J. Mol. Biol., *72:* 779–800, 1972.
15. Klotz, L. C., and Zimm, B. H. Retardation time measurements on replicating *Bacillus subtilis* chromosomes: effect of EDTA concentration. Macromolecules, *5:* 471–481, 1972.
16. Liquori, A. M., Constantino, L., Crescenzi, V., Elia, V., Giglio, E., Puliti, R., DeSantis Savino, M., and Vitagliano, V. Complexes between DNA and polyamines: a molecular model. J. Mol. Biol., *24:* 113–122, 1967.
17. Mamont, P. S., Duchesne, M., Joder-Ohlenbusch, A., and Grove, J. Effects of ornithine decarboxylase inhibitors on cultured cells. N. Seiler, M. J. Jung, and J. Koch-Weser (eds), Enzyme-activated irreversible inhibitors, pp. 43–53. Amsterdam: Elsevier/North-Holland Biomedical Press, 1978.
18. Muller, W. A., and Klotz, L. C. Retardation times measurements on replicating *Bacillus subtilis* chromosomes: effect of EDTA concentration. Biochim. Biophys. Acta, *378:* 171–185, 1975.
19. Prakash, N. J., Schecter, P. J., Mamont, P. S., Grove, J., Koch-Weser, J., and Sjoerdsma, A. Inhibition of EMT6 tumor growth by interference with polyamine biosynthesis; effects of α-difluoromethyl ornithine, an irreversible inhibitor of ornithine decarboxylase. Life Sci., *26:* 181–194, 1980.
20. Rajalakshmi, S., Rao, P. M., and Sarma, D. S. R. Modulation of carcinogen chromatine-DNA interaction by polyamines. Biochem. Biophys. Res. Commun., *81:* 936–940, 1978.
21. Roizin-Towle, L. E., and Hall, J. Studies with bleomycin and misonidazole on aerated and hypoxic cells. Br. J. Cancer, *37:* 254–260, 1978.
22. Rupniak, H. T., and Paul, D. Selective killing of transformed cells by exploitation of their defective cell cycle control by polyamines. Cancer Res., *40:* 293–297, 1980.
23. Seidenfeld, J., Deen, D. F., and Marton, L. J. Depletion of intracellular polyamine content does not alter the survival of 9L rat brain tumour cells after x-irradiation. Int. J. Radiat. Biol., *38:* 223–230, 1980.
24. Seidenfeld, J., Gray, J. W., and Marton, L. J. Depletion of polyamine content by treatment with DL-α-difluoromethyl ornithine inhibits 9L rat brain tumor cell proliferation and the G_1 to S transition. Exp. Cell Res., *131:* 209–216, 1981.
25. Seidenfeld, J., and Marton, L. J. Effects of DL-α-methylornithine on proliferation and polyamine content of 9L rat brain tumor cells. Cancer Res., *40:* 1961–1966, 1980.
26. Sunkara, P. S., Fowler, S. K., Nishioka, K., and Rao, P. N. Inhibition of polyamine biosynthesis by α-difluoromethyl ornithine potentiates the cytotoxic effects of arabinosyl cytosine in HeLa cells. Biochem. Biophys. Res. Commun., *95:* 423–430, 1980.
27. Tsuboi, M. On the melting temperature of nucleic acid in solution. Bull. Chem. Soc. Jpn., *37:* 1514–1522, 1964.
28. Weinkam, R. J., and Lin, H. S. Reactions of 1,3-bis (2-chloroethyl)-1-nitrosourea and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea in aqueous solution. J. Med. Chem., *22:* 1193–1198, 1980.
29. Williams-Ashman, H. G., and Canellakis, Z. N. Polyamines in mammalian biology and medicine. Perspect. Biol. Med., *22:* 421–453, 1979.