Eur J Cancer Clin Oncol, Vol. 20, No. 3, pp. 417–420, 1984.
Printed in Great Britain.

0277-5379/84$3.00+0.00

Potentiation of 1,3-Bis(2-chloroethyl)-1-nitrosourea Cytotoxicity in 9L Rat Brain Tumor Cells by Methylglyoxal-bis(guanylhydrazone), an Inhibitor of *S*-Adenosyl-L-methionine Decarboxylase*

DAVID T. HUNG,† STINA M. OREDSSON,† ANTHONY E. PEGG,‡ DENNIS F. DEEN†§ and LAURENCE J. MARTON†‖ ¶

†*Brain Tumour Research Center of the Department of Neurological Surgery,* §*Department of Radiation Oncology and* ‖*Department of Laboratory Medicine, School of Medicine, University of California, San Francisco, CA* 94143, *U.S.A. and* ‡*Department of Physiology and Specialized Cancer Center, The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, PA* 17033, *U.S.A.*

Abstract—*Methylglyoxal-bis(guanylhydrazone) (MGBG), a potent inhibitor of the spermidine and spermine biosynthetic enzyme* S-*adenosyl-*L-*methionine decarboxylase, enhanced the cytotoxicity of* 1,3-*bis-*(2-*chlorethyl*)-1-*nitrosourea in* 9*L rat brain tumor cells* in vitro, *as measured by a colony-forming efficiency assay, by an amount that was approximately the same as the potentiation caused by the ornithine decarboxylase inhibitor α-difluoromethylornithine. Dose enhancement ratios at* 10, 1 *and* 0.1% *survival levels were approximately* 1.3 *for both inhibitors.* 9*L cells that were treated for* 48 hr *with* 40 μM *MGBG had putrescine, spermidine and spermine levels that were* 112, 41 *and* 21%, *respectively, of polyamine levels in control cells. MGBG treatment does not increase intracellular levels of decarboxylated* S-*adenosyl-*L-*methionine (AdoMet) as α-difluoromethylornithine treatment does. Elevated levels of decarboxylated AdoMet could modify intracellular methylation reactions and could affect the cytotoxicity of a chloroethylnitrosourea. Despite the fact that MGBG treatment caused a slight increase in intracellular levels of AdoMet, it is unlikely that this elevation will increase the amount of intracellular methylation. Thus it appears that effects caused by the decrease in polyamine levels are responsible for the potentiation of chloroethylnitrosourea cytotoxicity against* 9*L cells.*

Accepted 17 August 1983.

*Supported by American Cancer Society Grant RD-137, NIH Program Project Grant CA-13525, NIH Grant CA-18138, The Aaron Silvera Fund and travel grants (to S.M.O) from the Swedish Natural Science Research Council (R-RA 4685-100) and the Swedish Medical Research Council (B81-04R-6065-504106065).

¶To whom requests for reprints should be addressed at: Department of Laboratory Medicine, University of California, San Francisco, CA 94143, U.S.A.

Abbreviations: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; MeCCNU, 1-(2-chloroethyl)-3-*trans*-4-methylcyclohexyl-1-nitrosourea; DFMO, α-difluoromethylornithine; CENU, chloroethylnitrosourea; AdoMet, *S*-adenosyl-L-methionine; MGBG, methylglyoxal-bis(guanylhydrazone); DER, dose enhancement ratio; Pu, putrescine; Sd, spermidine; Sp, spermine; HBSS, Hanks' balanced salt solution; NBCS, newborn calf serum.

INTRODUCTION

WE HAVE recently reported that the *in vitro* cytotoxicity of the alkylating anticancer agents BCNU [1] and MeCCNU (Oredsson *et al.*, submitted for publication) against 9L rat brain tumor cells is significantly enhanced by depletion of intracellular polyamine levels caused by treatment with DFMO, an enzyme-activated, irreversible inhibitor of ornithine decarboxylase. DERs for both CENUs were approximately 1.3. Potentiation caused by treatment with DFMO can be readily prevented for either CENU by addition of exogenous Pu before treatment with the CENU.

Stabilization of the structure of DNA caused by polyamines is well-documented [2–5]. We

postulated that polyamine-related destabilization of the double helix of DNA might render DNA bases more susceptible to alkylation and subsequent interstrand cross-linking, the mechanisms through which alkylating agents are presumed to kill cells [1]. We found that DFMO pretreatment does not increase the number of monoadducts formed between the reactive alkylating moiety of MeCCNU and DNA bases (Oredsson *et al.*, submitted for publication). However, using alkaline elution techniques we have recently shown that cross-linking of 9L DNA by BCNU is potentiated by DFMO pretreatment of cells and that the potentiation of cross-linking is prevented by the addition of Pu to DFMO pretreated cells before BCNU treatment (Tofilon *et al.*, submitted for publication). Thus it appears that DFMO potentiates the cytotoxicity of these alkylating agents by increasing DNA interstrand cross-links. Nevertheless, factors other than DNA alkylation and cross-link formation may also affect or influence the synergism between DFMO and either BCNU or MeCCNU.

A substantial increase in the intracellular concentration of decarboxylated AdoMet caused by DFMO has been documented in human fibroblasts [6], hepatoma cells [7] and 9L tumor cells (Oredsson *et al.*, submitted for publication), approximately 50-fold for the latter. Increased levels of decarboxylated AdoMet could modify intracellular methylation reactions and could contribute to or produce the synergism observed between DFMO and BCNU (or MeCCNU) [6].

To investigate this possibility we studied the effect of MGBG-induced polyamine depletion on BCNU cytotoxicity in 9L cells *in vitro*. MGBG is an effective agent in numerous neoplastic cell lines [8], and is a potent inhibitor of Pu-activated AdoMet decarboxylase, the rate-limiting enzyme in the biosynthesis of Sd and Sp [9]. Intracellular levels of Sd and Sp are markedly reduced in 9L cells pretreated with 40 μM MGBG for 48 hr [10]; intracellular levels of decarboxylated AdoMet are decreased. These data are consistent with those of Hibasami *et al.* [11] for liver cells. Our data suggest that potentiation of BCNU cytotoxicity is independent of formation of decarboxylated AdoMet and is related to the reduction of intracellular polyamine levels.

MATERIALS AND METHODS

Drugs

DFMO was the generous gift of the Merrell-Dow Research Center (Cincinnati, OH). MGBG was purchased from Aldrich (Milwaukee, WI), and Sd and aminoguanidine were purchased from Calbiochem-Behring (La Jolla, CA). BCNU was generously provided by Dr Robert R. Engle of the National Cancer Institute.

Cell culture

9L cells were seeded into 75-cm^2 tissue culture flasks (Falcon Plastics, Oxnard, CA) and grown in 14.5 ml of Earle's minimum essential medium supplemented with nonessential amino acids, 10% NBCS and gentamicin (50 μg/ml). Cells were incubated for 24 hr to establish early log phase growth (all incubations were at 37°C in a humidified 5% CO^2–95% air atmosphere). To minimize differences in cell density between the flasks at the time of BCNU treatment, flasks to receive 40 μM MGBG treatment were initially seeded with 5×10^5 9L cells and control flasks were seeded with 2.5×10^5 9L cells.

Drug treatment

After the initial incubation, cells were treated with either 0.5 ml of 1200 μM MGBG in HBSS (to achieve a final MGBG concentration of 40 μM) or 0.5 ml of HBSS alone (control). After incubation for 48 hr, both MGBG-treated and control cells were treated with BCNU. 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 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 MGBG, trypsinized and plated to determine colony-forming efficiency [12].

Polyamine replenishment experiments

In these experiments the protocol described above was used except that, in both MGBG treatment and control experiments, flasks were seeded with 2.5×10^5 9L cells and, after the 48-hr incubation, treated with either Sd plus aminoguanidine or HBSS plus aminoguanidine. Cells were treated with 0.5 ml of 32 mM aminoguanidine (final concentration of 1 mM) and 0.5 ml of 3.2 mM Sd (final concentration of 0.1 mM). Control cells were treated with 0.5 ml of 32 mM aminoguanidine and an additional 0.5 ml of HBSS. After incubation for an additional 24-hr, both Sd/aminoguanidine-treated and HBSS/aminoguanidine-treated cells were treated with BCNU for 1 hr as described above, rinsed to remove drug, trypsinized and plated to determine colony-forming efficiency [12].

Polyamine assay

Cells for polyamine analysis were pelleted at 2000 rpm for 10 min at 4°C and stored at -20°C before being assayed [13].

AdoMet and decarboxylated AdoMet content

Cells were pelleted at 1000 rpm for 5 min at 4°C and stored at −70°C until assayed for AdoMet and decarboxylated AdoMet content [14].

RESULTS

Pretreatment of 9L cells with 40 μM MGBG significantly increased the cytotoxicity of BCNU (Fig. 1). The DERs at 10, 1 and 0.1% survival levels were 1.3. The potentiation phenomenon was only slightly prevented by the addition of 0.1 mM Sd to the culture medium 24 hr before BCNU treatment (data not shown). Pu, Sd and Sp levels in MGBG-pretreated cells were 112, 41 and 21%, respectively, of polyamine levels in control cells. Addition of 0.1 mM Sd produced Pu, Sd and Sp levels that were 72, 113 and 25%, respectively, of levels in control cells. Survival curves generated using either 48 or 72 hr MGBG pretreatment were virtually identical.

Cells treated with 1 mM aminoguanidine and 0.1 mM Sd for 24 hr plated at efficiencies identical to untreated control cells, indicating that aminoguanidine and Sd are neither toxic nor stimulatory to 9L cells at the concentrations used. However, higher concentrations or longer treatment times were toxic.

9L cells treated with 40 μM MGBG for either 24, 48 or 96 hr had colony-forming efficiencies that were approximately 80% of control cells. Figure 1 was plotted to show potentiation effects only.

AdoMet and decarboxylated AdoMet levels in 9L cells treated with 40 μM MGBG for 48 hr were 230 and 53%, respectively, of levels in control cells.

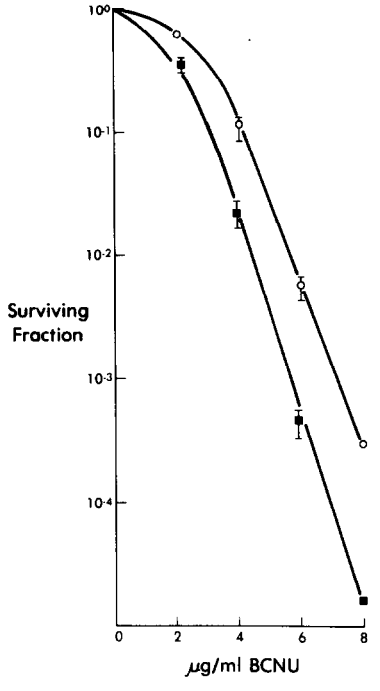

Fig. 1. Effects of MGBG-induced polyamine depletion on the cytotoxicity of BCNU against 9L cells in vitro. O, *Cells pretreated with HBBS for* 48 hr *and then treated with BCNU for* 1 hr. ■, *Cells pretreated with* 40 μM *MGBG and then treated with BCNU for* 1 hr. *Points are the means of* 8–12 *Petri dishes, and the bars represent standard deviations. If error bars are not shown, the error is within the symbol.*

DISCUSSION

The fact that MGBG potentiates the cytotoxicity of BCNU against 9L cells in monolayer culture at a level equivalent to the potentiation cause by DFMO, and the fact that the only similarity between MGBG and DFMO is that they are polyamine biosynthesis inhibitors that, moreover, inhibit different steps in the polyamine biosynthetic pathway, indicates that polyamine depletion is probably responsible for the potentiation. Potentiation could not be prevented significantly by the addition of small amounts of exogenous Sd. Because oxidases in NBCS catalyze the production of toxic metabolites of Sd and Sp, sufficient Sd to reverse polyamine deficiency completely could not be administered to 9L cells, even in the presence of the oxidase inhibitor aminoguanidine (1 mM).

Because MGBG depletes intracellular Sd and Sp levels without increasing levels of decarboxylated AdoMet, it can be argued that both agents potentiate cell kill by depleting intracellular polyamine levels. While it is possible that DFMO and MGBG potentiate BCNU cytotoxicity by different mechanisms, and that the increase in AdoMet levels caused by MGBG treatment is responsible for the potentiation, because higher intracellular levels of AdoMet do not increase the amount of intracellular methylation unless *S*-adenosylhomocysteine levels are elevated [15], and because the DERs for DFMO and MGBG are identical, this seems unlikely. In addition, although DFMO increases the level of decarboxylated AdoMet in 9L cells by approximately 50-fold, MGBG increases AdoMet levels by only 2.3-fold.

We have shown that G_1 and G_2/M phase 9L cells are more sensitive to BCNU than S phase cells [16]. However, although treatment with 40 μM MGBG for either 48 or 72 hr causes a G_1 block in 9L cells, flow cytometric analysis has shown that the increase in the percentage of G_1 phase cells is matched quite closely by a decrease in G_2/M phase cells; the percentage of S phase cells remained fairly constant. Therefore the potentiation observed here cannot be attributed to cell cycle perturbations.

Acknowledgements—We thank Corie Tofilon for typing the manuscript in draft and Neil Buckley for editing it.

REFERENCES

1. HUNG DT, DEEN DF, SEIDENFELD J, MARTON LJ. Sensitization of 9L rat brain gliosarcoma cells to 1,3-bis(2-chloroethyl)-1-nitrosourea by α-difluoromethylornithine, an ornithine decarboxylase inhibitor. *Cancer Res* 1981, **41**, 2783–2785.
2. BLOOMFIELD VA, WILSON RW. Interaction of polyamines with polynucleotides. In: MORRIS DR, MARTON LJ, eds. *Polyamines in Biology and Medicine*. New York, Marcel Dekker, 1981, 183–206.
3. BROWN PE. Some effects of binding agents on the x-irradiation of DNA. *Radiat Res* 1968, **34**, 24–35.
4. LIQUORI AM, CONSTANTINO V, CRESCENZI V *et al.* Complexes between DNA and polyamines: a molecular model. *J Mol Biol* 1967, **24**, 113–122.
5. TSUBOI M. On the melting temperature of nucleic acid in solution. *Bull Chem Soc Jpn* 1966, **37**, 1514–1522.
6. PEGG AE, PÖSÖ H, SHUTTLEWORTH K, BENNETT RA. Effect of inhibition of polyamine synthesis on the content of decarboxylated *S*-adenosylmethionine. *Biochem J* 1982, **202**, 519–526.
7. MAMONT PS, DANZIN C, WAGNER J, SAIT M, JODER-OHLENBUSCH A-M, CLAVERIE N. Accumulation of decarboxylated *S*-adenosyl-L-methionine in mammalian cells as a consequence of the inhibition of putrescine biosynthesis. *Eur J Biochem* 1982, **123**, 499–520.
8. HEBY O, JÄNNE J. Polyamine antimetabolites: biochemistry, specificity, and biological effects of inhibitors of polyamine synthesis. In: MORRIS DR, MARTON LJ, eds. *Polyamines in Biology and Medicine*. New York, Marcel Dekker, 1981, 243–310.
9. WILLIAMS-ASHMAN HG, SCHENONE A. Methylglyoxal bis(guanylhydrazone) as a potent inhibitor of mammalian and yeast *S*-adenosylmethionine decarboxylases. *Biochem Biophys Res Commun* 1972, **46**, 288–295.
10. HEBY O, MARTON LJ, WILSON CB, GRAY J. Effect of methylglyoxal bis-(guanlhydrazone), an inhibitor of spermidine and spermine synthesis, on cell cycle traverse. *Eur J Cancer* 1977, **13**, 1009–1017.
11. HIBASAMI H, HOFFMAN JL, PEGG AE. Decarboxylated *S*-adenosylmethionine in mammalian cells. *J Biol Chem* 1980, **255**, 6675–6678.
12. DEEN DF, BARTLE PM, WILLIAMS ME. Response of cultured 9L cells to spirohydantoin mustard and x rays. *Int J Radiat Oncol Biol Phys* 1979, **5**, 1663–1667.
13. SEIDENFELD J, MARTON LJ. Effects of DL-α-methylornithine on proliferation and polyamine content of 9L rat brain tumor cells. *Cancer Res* 1980, **40**, 1961–1966.
14. PEGG AE, PÖSÖ H, SHUTTLEWORTH K, BENNETT RA. Effect of inhibition of polyamine synthesis on the content of decarboxylated *S*-adenosylmethionine, *Biochem J* 1982, **202**, 519–526.
15. DUERRE JA. Regulation of *S*-adenosylmethionine and *S*-adenosylmethionine levels in isolated rat liver. In: USDIN E, BORCHADT RT, CREVELING CR, eds. *Biochemistry of S-adenosylmethionine and Related Compounds*. Macmillan, London, 1982, 595–602.
16. BJERKVIG R, OREDSSON SM, MARTON LJ, LINDEN M, DEEN DF. The cell cycle age response of 9L cells to 1,3-bis(2-chloroethyl)-1-nitrosourea and modification by α-difluoromethylornithine, *Cancer Res* 1983, **43**, 1497–1500.