Biochemical Pharmacology, Vol. 36, No. 10, pp. 1673–1677, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

POTENTIATION OF ARA-C INDUCED CYTOTOXICITY BY HYDROXYUREA IN LoVo COLON CARCINOMA CELLS*

NORMAN J. ROBICHAUD and ROBERT J. FRAM†
Departments of Medicine and Pharmacology, University of Massachusetts Medical School, Worcester, MA 01605, U.S.A.

(*Received* 21 *July* 1986; *accepted* 20 *October* 1986)

Abstract—The present study was undertaken to determine whether cytotoxicity by 1-β-D-arabinofuranosylcytosine (ara-C) in LoVo colon carcinoma cells, which are resistant to high concentrations of ara-C, would be enhanced by concurrent exposure to hydroxyurea (HU). Since mechanisms underlying the effects of HU on ara-C induced cytotoxicity are unclear, we also evaluated the effect of HU on the incorporation of ara-C into DNA, as well as potential consequences of misincorporation. Our results demonstrate that HU synergistically enhances cytotoxicity by ara-C in these cells. This effect was not present when HU was combined with aphidicolin, an agent that resembles ara-C in competing with dCTP for binding to polymerase alpha but that is not incorporated into DNA. Further, cells exposed to HU and ara-C incorporated up to 5-fold as much ara-C into DNA as cells solely treated with ara-C. While the extent of inhibition of DNA synthesis was comparable with cells exposed to HU and aphidicolin as those treated with HU and ara-C, recovery of DNA synthesis was delayed more significantly by the latter combination. These findings suggest that HU synergistically potentiates ara-C induced cytotoxicity by enhancing incorporation of ara-C in LoVo cell DNA.

The lethal effects of 1-β-D-arabinofuranosylcytosine (ara-C) are correlated significantly with the extent of ara-C incorporated into DNA during DNA synthesis [1–5]. Misincorporated ara-C acts as a chain terminator during *in vivo* and *in vitro* DNA synthesis [3–6].

Several approaches have been employed to enhance the incorporation of ara-C into DNA in order to increase cytotoxicity by this agent. These include the use of inhibitors of ribonucleotide reductase and of *de novo* pyrimidine synthesis to increase the formation of ara-C nucleotide pools [7, 8].

A prior study demonstrated that hydroxyurea (HU) synergistically enhances cytotoxicity by ara-C in a human B cell line, in blasts from patients with acute leukemia, as well as in murine L1210 leukemia [9–10]. HU reversibly inhibits eukaryotic ribonucleotide reductase by binding to its B2 protein [11–13]. While declines in dATP pools have been documented in eukaryotic cells, HU either does not affect or increases pools of the other nucleotides [14–16]. Thus, the effect of HU, unlike that of thymidine, in enhancing ara-C induced cytotoxicity is complex and may not be related to declines in dCTP pools [9, 17]. Further, HU while increasing ara-CTP pools decreases the incorporation of ara-C into acid insoluble macromolecules of cells in which the combination causes synergistic cytotoxic effects [9]. In contrast, others have demonstrated that HU increases incorporation of ara-C into acid insoluble macromolecules, but they did not analyze the effect of HU on ara-C induced cytotoxicity and inhibition of DNA synthesis [8].

The present study was undertaken to determine whether cytotoxicity by ara-C in LoVo colon carcinoma cells, which are resistant to high concentrations of ara-C, was enhanced by concurrent exposure to HU [18, 19]. Since mechanisms underlying the effects of HU on ara-C induced cytotoxicity are unclear, we also wished to evaluate the effect of HU on the incorporation of ara-C into DNA as well as potential consequences of misincorporation.

MATERIALS AND METHODS

Cell culture. LoVo colon cancer cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were routinely tested for mycoplasma and maintained by serial passage in Ham's F-12 medium with 15% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Reagents. Cytosine arabinoside and hydroxyurea were obtained from Sigma (St. Louis, MO). Aphidicolin was provided by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. Drugs were prepared in medium without serum just prior to use except for aphidicolin which was dissolved in dimethyl sulfoxide.

Cytotoxicity. Flasks (25 cm^2) (Corning Glassworks, Corning, NY) were seeded with 10^6 cells in 10 ml medium, and drug exposure was carried out on day 3. Cytotoxicity was analyzed by exposing non-confluent cells to drug for 3 hr at 37°. Cells were washed twice, trypsinized, and plated on 100 mm

* This investigation was supported by PHS Grant R32 CA38613-02 awarded by the National Cancer Institute, DHHS (R. J. F.)

† Address reprint requests to: Dr. R. J. Fram, Division of Oncology, University of Massachusetts Medical School, 55 Lake Ave. North, Worcester, MA 01605.

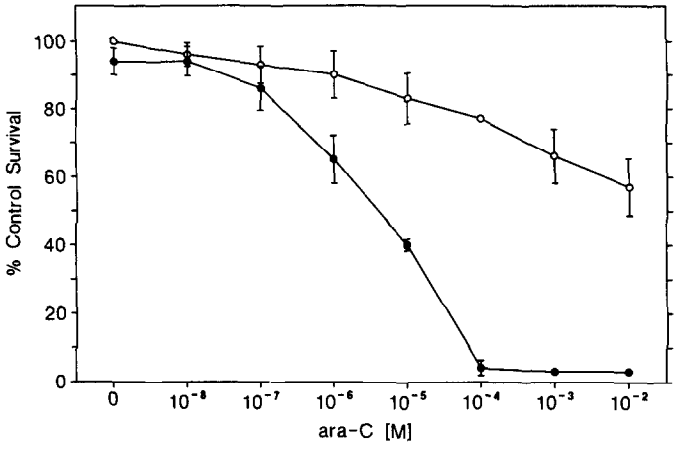

Fig. 1. Cytotoxicity by ara-C and hydroxyurea. LoVo colon carcinoma cells were incubated with no ara-C or 10^{-8}–10^{-2} M ara-C either without HU (○) or with HU at 2×10^{-3} M (●) for 3 hr at 37°. Cells were washed twice, trypsinized, and plated. Percent (%) clonogenic survival was analyzed as described in the text. Of 1000 cells plated, 276 ± 4.65 (SD) colonies were noted in untreated controls. For the other figures, the control value varied slightly. Values represent the mean and standard deviations of triplicate determinations from a representative experiment.

petri dishes (Corning Glassworks). Colonies with at least 50 cells were counted after incubation for 14 days at 37°, 5% CO_2. Cytotoxicity was determined by counting six control plates and three plates for each drug treatment. Percent control survival was determined by the ratio of colonies formed by treated cells as compared to untreated cells multiplied by 100. Cloning efficiency of the untreated LoVo cells was 24% (mean of ten experiments). Experiments were done at least twice. Representative experiments are shown.

Incorporation of ara-C into DNA. LoVo cells in logarithmic growth phase were exposed to 10^{-7}–10^{-5} M [^{3}H]ara-C (Amersham International, Amersham, U.K.) in the presence or absence of hydroxyurea for 3 hr at 37°. Cells were washed twice with cold phosphate-buffered saline (PBS) and cells were trypsinized. Cells (10^6) in 2 ml PBS were digested with 50 μg/ml proteinase K and 2 ml of 0.01 M Tris, 0.01 M EDTA, 0.5% sodium dodecyl sulfate (SDS), pH 7.4, for 12 hr at 37°. The solution was extracted with phenol, and the aqueous phase was treated with 50 μg/ml ribonuclease (Sigma) for 90 min at 37°. A second phenol extraction was performed, and the DNA was precipitated with 0.1 vol. of 4 M NaCl and 2 vol. of absolute ethanol overnight at 4°. Specimens were centrifuged at 2800 rpm for 30 min at 4°. Aliquots were assessed spectrophotometrically at 280 and 260 nm for purity. Samples were then filtered on Whatman GF/A filters (Whatman, Maidstone, U.K.), and radioactivity was analyzed after the addition of 5 ml hydroflour. Results are expressed as pmoles [^{3}H]ara-C incorporated per 10^6 cells.

Inhibition of DNA synthesis. LoVo cells in logarithmic growth phase were incubated with no drug, 10^{-6}–10^{-3} M aphidicolin in the presence or absence of 2×10^{-3} M HU or 10^{-6}–10^{-3} M ara-C with or without 2×10^{-3} M HU. ^{32}P (5 μCi/ml) was added, and cells were incubated for 3 hr at 37°. DNA was isolated as previously described, and samples were evaluated for radioactivity. Results are expressed as counts per min/10^6 cells.

Recovery of DNA synthesis. Cells were treated with no drug; 10^{-4} M aphidicolin or 10^{-4} M ara-C alone, or in combination with 2×10^{-3} M HU; or 2×10^{-3} M HU alone. Cells were incubated for 3 hr, washed twice, and then exposed to 5 μCi/ml ^{32}P for 3 and 12 hr at 37°. DNA was isolated as previously described, and radioactivity was analyzed. ^{32}P incorporation into DNA/10^6 cells was calculated for each treatment group. The experiment was performed twice, and a representative experiment is shown. Values represent the percent of control DNA synthesis and are the mean of duplicate determinations ± standard deviations.

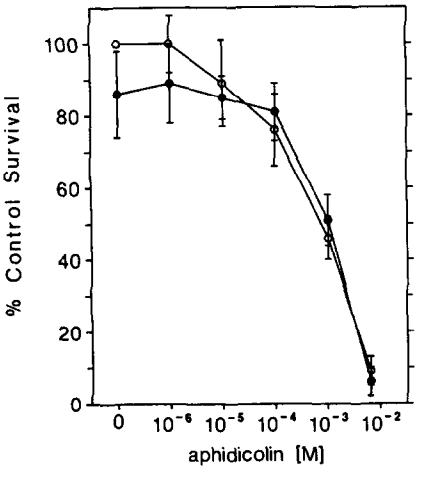

Fig. 2. Cytotoxicity by aphidicolin and hydroxyurea. LoVo colon carcinoma cells were exposed to no drug or 10^{-6}–10^{-2} M aphidicolin either without HU (○) or with HU at 2×10^{-3} M (●) for 3 hr at 37°. Cells were washed twice, trypsinized, and plated. Percent (%) clonogenic survival was evaluated as described in the text. Values represent the mean and standard deviations of triplicate determinations from a representative experiment.

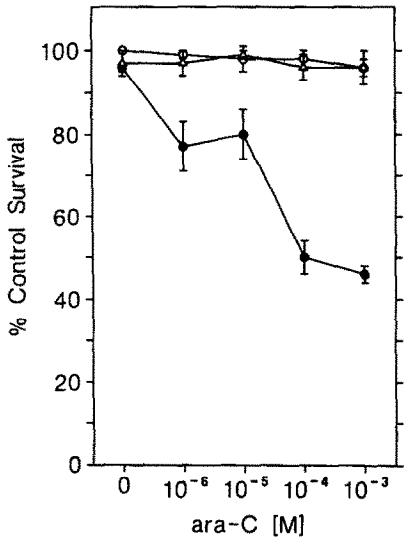

Fig. 3. Effect of deoxycytidine on cytotoxicity by ara-C and HU. LoVo colon carcinoma cells were incubated with no drug or 10^{-6}–10^{-3} M ara-C (○), 2×10^{-3} M HU with either no ara-C or 10^{-6}–10^{-3} M ara-C (●), or 10^{-5} M dCyd and 2×10^{-3} M HU with either no ara-C or 10^{-6}–10^{-3} M ara-C (△). Incubations with drug were for 3 hr at 37°. Cells were washed twice, trypsinized, and plated. Percent (%) clonogenic survival was analyzed as described in the text. Values represent the mean and standard deviations of triplicate determinations from a representative experiment.

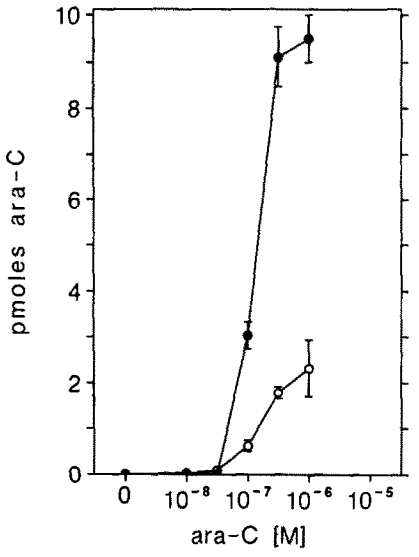

Fig. 4. Effect of hydroxyurea on the incorporation of [^{3}H]ara-C into LoVo cell DNA. Cells were exposed to [^{3}H]ara-C at 10^{-8}–10^{-6} M with 2×10^{-3} M HU (●) or without HU (○). Cells were washed twice and trypsinized, and DNA was isolated. Results represent the mean of duplicate determinations ± standard deviation. See Materials and Methods for details.

RESULTS

The effect of HU on ara-C induced cytotoxicity is shown in Fig. 1. HU markedly potentiated ara-C induced cytotoxicity. In this representative experiment, the combination of 2×10^{-3} M HU with 10^{-4} M ara-C increased cytotoxicity 19-fold compared to treatment with 10^{-4} M ara-C alone.

The interaction of HU with ara-C was analyzed further by evaluating the effect of HU on aphidicolin induced cytotoxicity. Aphidicolin resembles ara-C in competing with dCTP for binding to polymerase alpha, but differs from ara-C in not being misincorporated into DNA [20]. Figure 2 demonstrates that, in contrast to HU and ara-C, HU in combination with aphidicolin caused less than additive cytotoxic effects.

Differing cytotoxic effects by HU in combination with ara-C and aphidicolin suggest that misincorporation of ara-C may underlie these results. To evaluate this hypothesis, cytotoxicity was analyzed after the addition of 10^{-5} M deoxycytidine to cells treated with either ara-C or ara-C and HU. We reasoned that incorporation of ara-C into DNA should be slowed in the presence of increased dCTP pools and the effect of HU on ara-C induced cytotoxicity abrogated. The representative experiment shown in Fig. 3 confirms that 2×10^{-3} M HU caused more than additive cytotoxic effects in combination with ara-C and further that this potentiation of cellular lethality did not occur after treatment with 10^{-5} M deoxycytidine.

The effect of HU on the incorporation of [^{3}H]ara-C into LoVo carcinoma cell DNA is shown in Fig. 4. Cells were exposed to 10^{-8}–10^{-6} M [^{3}H]ara-C in the presence or absence of 2×10^{-3} M HU for 3 hr at 37°. The results demonstrate that HU caused up to a 5-fold increase in the incorporation of ara-C into the DNA of these cells. Further, the most significant increments in incorporation of ara-C into DNA occurred in the range of ara-C concentrations where cytotoxicity was potentiated most markedly by HU.

Since both HU and ara-C inhibit DNA synthesis, the effects of these agents in combination were

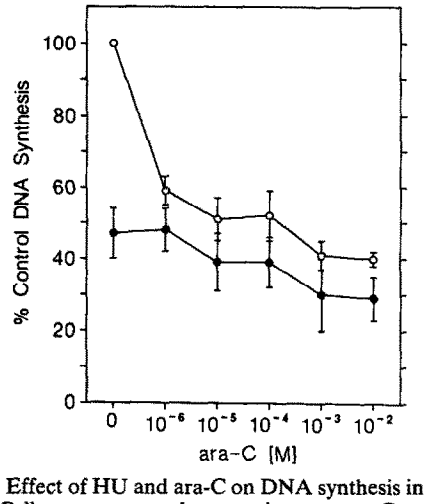

Fig. 5. Effect of HU and ara-C on DNA synthesis in LoVo cells. Cells were exposed to no drug or ara-C at 10^{-6}–10^{-2} M with 2×10^{-3} M HU (●) or without HU (○). Cells were incubated for 3 hr at 37° with drug and 5 μCi/ml ^{32}P, washed and trypsinized; DNA was isolated and evaluated for incorporation of radioactivity as described in the text. Values represent ^{32}P incorporation into DNA/10^6 cells and are the mean and standard deviations of duplicate determinations from a representative experiment.

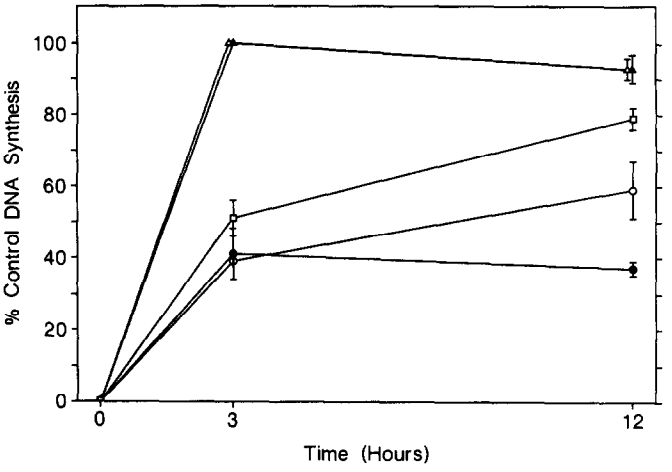

Fig. 6. Recovery of DNA synthesis after exposure of cells to HU in combination with ara-C or aphidicolin. LoVo cells were exposed to no drug; 10^{-4} M aphidicolin (△); 10^{-4} M aphidicolin and 2×10^{-3} M HU (▲); 10^{-4} M ara-C (○); 10^{-4} M ara-C and 2×10^{-3} M HU (●); or 2×10^{-3} M HU (□). Cells were incubated for 3 hr, washed twice, and then incubated after drug removal with 5 μCi/ml ^{32}P for 3 and 12 hr at 37°. DNA was isolated, and incorporation of ^{32}P into DNA was measured as described in the text. Values are the percent control DNA synthesis as measured by ^{32}P incorporation into DNA/10^6 cells. The mean of duplicate determinations ± standard deviations are shown from a representative experiment.

analyzed on both DNA synthesis as well as the recovery of DNA synthesis after removal of drug. Cells were exposed to either 10^{-6}–10^{-2} M ara-C alone or 2×10^{-3} M HU in combination with 10^{-6}–10^{-2} M ara-C for 3 hr, and DNA synthesis was analyzed. Figure 5 demonstrates that a less than additive effect on the inhibition of DNA synthesis occurred when HU was combined with ara-C. In fact, DNA synthesis inhibition by ara-C and HU was not significantly different from that caused by HU alone in the range of ara-C concentrations in which HU markedly potentiates ara-C induced cytotoxicity. Effects of HU on the inhibition of DNA synthesis by aphidicolin also were less than additive (data not shown).

While the incorporation of ara-C into DNA may not alter the extent of inhibition of DNA synthesis, incorporated ara-C residues could slow the recovery of DNA synthesis by causing DNA chain termination. To evaluate this possibility, cells were exposed to 10^{-4} M ara-C and 10^{-4} M aphidicolin in the presence and absence of 2×10^{-3} M HU for 3 hr and washed twice; then the incorporation of ^{32}P into DNA was measured over a 12-hr interval. Figure 6 demonstrates that the combination of ara-C and HU markedly inhibited the recovery of DNA synthesis up to 12 hr after drug exposure as did, albeit to a lesser extent, 10^{-4} M ara-C alone. In contrast to ara-C and HU, exposure to aphidicolin and HU did not inhibit the recovery of DNA synthesis after drug removal.

DISCUSSION

Our results confirm prior studies demonstrating more than additive cytotoxic effects when ara-C is combined with HU. Further, in contrast to the previously cited reports, our data demonstrate that enhanced incorporation of ara-C into DNA in the presence of HU likely underlies the effects of HU on ara-C induced cytotoxicity [9]. Thus, more than additive cytotoxic effects were not seen when HU was combined with aphidicolin, an agent that resembles ara-C in competing with dCTP for binding to polymerase alpha but that is not incorporated into DNA [20]. In addition, the incorporation of [^{3}H]ara-C into DNA was enhanced up to 5-fold in the presence of HU. Lastly, the effects of HU were reversed in the presence of 10^{-5} M dCyd. The latter result might be expected if increased pools of dCyd competed with ara-C for phosphorylation and incorporation into DNA.

A probable effect of enhanced incorporation of ara-C into DNA caused by HU is that, while the extent of DNA synthesis is not altered during incubation with both agents, recovery of DNA synthesis is delayed markedly. The latter did not occur after treatment with aphidicolin and HU, also suggesting that recovery of DNA synthesis is inhibited as a result of incorporation of ara-C into DNA. Ara-C incorporated into DNA may, because it acts as a chain terminator during DNA synthesis, have irreversible effects on the recovery of DNA synthesis after removal of drugs from the medium. A prior study, for example, has shown that 10^{-4} M ara-C results in the irreversible inhibition of DNA synthesis in L1210 murine leukemia cells [5]. Our findings demonstrate in LoVo colon cells, that while recovery of DNA synthesis was inhibited by 10^{-4} M ara-C, this effect was even more marked in the presence of HU.

The mechanisms underlying enhanced incorporation of ara-C into DNA after exposure to HU are not known. An effect of HU in lowering dCTP pools has not been documented in prior reports and, in fact, dCTP pools either do not change or increase after HU treatment [14–16]. An effect by HU on the localization of deoxyribonucleotide pools within the

cell nucleus, however, may occur, leading to decreases in dCTP pools that would prove relevant in determining the extent of incorporation of ara-C DNA [15]. Another possible mechanism by which hydroxyurea may enhance incorporation of ara-CTP into DNA is by increasing phosphorylation of ara-C perhaps by effects on kinase activity. As yet, no evidence has been presented to show that HU directly affects enzymes participating in the phosphorylation of ara-C, although ara-CTP pools do increase in the presence of HU [9]. Future work will be directed at clarifying these potential effects of HU.

REFERENCES

1. D. W. Kufe, P. P. Major, E. M. Egan and G. P. Beardsley, *J. biol. Chem.* **255**, 8997 (1980).
2. P. P. Major, E. M. Egan, G. P. Beardsley, M. D. Minden and D. W. Kufe, *Proc. natn. Acad. Sci. U.S.A.* **78**, 3235 (1981).
3. D. W. Kufe, D. Spriggs, E. Egan and D. Monroe, *Blood* **64**, 54 (1984).
4. D. W. Kufe, D. Monroe, D. Herrick, E. Egan and D. Spriggs, *Molec. Pharmac.* **26**, 128 (1984).
5. P. P. Major, E. M. Egan, D. J. Herrick and D. W. Kufe, *Biochem. Pharmac.* **31**, 2937 (1982).
6. G. P. Beardsley and T. Mikita, *Proc. Am. Ass. Cancer Res.* **27**, 305 (1986).
7. R. Fram, P. Major, E. Egan, P. Beardsley, D. Rosenthal and D. Kufe, *Cancer Chemother. Pharmac.* **11**, 43 (1983).
8. P. Plagemann, R. Marz and R. Wohlhueter, *Cancer Res.* **38**, 978 (1978).
9. J. A. Streifel and S. B. Howell, *Proc. natn. Acad. Sci. U.S.A.* **78**, 5132 (1981).
10. F. M. Schabel, Jr., W. R. Laster, Jr. and M. W. Trader, *Proc. Am. Ass. Cancer Res.* **12**, 67 (1971).
11. I. W. Krakoff, N. C. Brown and P. Reichard, *Cancer Res.* **28**, 1559 (1968).
12. Y. Engstrom, S. Eriksson, L. Thelander and M. Akerman, *Biochemistry* **18**, 2941 (1979).
13. J. F. Whitfield and T. Youdale, *Pharmac. Ther.* **29**, 407 (1985).
14. L. Skoog and B. Nordenskjold, *Eur. J. Biochem.* **19**, 81 (1971).
15. R. L. P. Adams, S. Berryman and A. Thomson, *Biochim. biophys. Acta* **240**, 455 (1971).
16. R. A. Walters, R. A. Tobey and R. L. Ratliff, *Biochim. biophys. Acta* **319**, 336 (1973).
17. S. E. Grant, C. Lehman and E. Cadwan, *Cancer Res.* **40**, 1525 (1980).
18. S. P. Bergerat, B. Drewinko, P. Corry, G. Barlogie and D. H. Ho, *Cancer Res.* **41**, 25 (1981).
19. R. J. Fram and N. Robichaud, *Proc. Am. Ass. Cancer Res.* **27**, 103 (1986).
20. J. A. Huberman, *Cell* **23**, 647 (1981).