MOLECULAR PHARMACOLOGY, 25:322-326

Lethal Effects of 1-β-D-Arabinofuranosylcytosine Incorporation into Deoxyribonucleic Acid during Ultraviolet Repair

DONALD W. KUFE, RALPH WEICHSELBAUM, E. MICHAEL EGAN, WILLIAM DAHLBERG, AND ROBERT J. FRAM

Departments of Medicine and Radiation Therapy, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115

Received September 19, 1983; Accepted October 27, 1983

SUMMARY

We have previously demonstrated that 1-β-D-arabinofuranosylcytosine (ara-C) incorporates into leukemic cell DNA and that the extent of this incorporation correlates significantly with loss of clonogenic survival. These studies have now been extended by monitoring the incorporation of ara-C in DNA undergoing repair of UV-induced damage. The present studies have been performed using human foreskin diploid fibroblasts that undergo density-dependent growth arrest. The results demonstrate that ara-C incorporates specifically into DNA undergoing repair of damage by UV light. The extent of ara-C incorporation in AG1522 DNA during UV repair correlates significantly ($p < 0.0006$) with cell lethality. These findings confirm that incorporation of ara-C into DNA undergoing either semiconservative or unscheduled DNA synthesis results in lethal cellular events. The present findings may provide the experimental basis for the development of new therapeutic approaches using ara-C in combination with agents that damage DNA.

INTRODUCTION

Ara-C[1] is the most effective agent in the treatment of myelogenous leukemia (1, 2). However, the precise biochemical mechanism of action of ara-C and the basis for this selectivity against leukemic cells remains unclear.

We have demonstrated that ara-C incorporates into DNA and not RNA of murine and human leukemic cells (3, 4). Furthermore, there is a highly significant relationship between formation of (ara-C)DNA and loss of clonogenic survival. The extent of ara-C incorporation in DNA correlates with inhibition of DNA synthesis, and the ara-C residue behaves as a relative chain terminator (5). These findings are consistent with conformational and hydrogen bonding differences of the arabinose sugar moiety altering reactivity at the 3′-terminus and slowing chain elongation (6, 7). The disruption of DNA replication by ara-C also results in DNA fragmentation (8) and reinitiation of synthesis in previously replicated segments (9, 10).

Ara-C is a potent inhibitor of the repair of DNA damage of the type induced by UV light, certain alkylating agents, and to some extent x-ray (11–19). However, the precise mechanism by which ara-C inhibits repair has not been defined at the molecular level. Ara-C has been shown to inhibit repair of UV-induced single-strand DNA breaks in fibroblasts (12). While this study demonstrated that ara-C was incorporated into DNA undergoing repair after UV irradiation, the incorporated ara-C residues did not substantially inhibit the repair of UV-induced single-strand breaks after a deoxycytidine pulse. Furthermore, no attempt was made to correlate with cytotoxicity the presence of ara-C residues incorporated into DNA undergoing repair. In view of our recent observations on the effect of ara-C incorporation into DNA on the inhibition of replicative synthesis, we have reassessed the effect of ara-C incorporation into DNA undergoing repair after UV irradiation.

The results of the present study demonstrate that ara-C incorporates in DNA during UV excision repair of human diploid fibroblasts. Furthermore, there is a highly significant relationship ($p < 0.0006$) between the extent of ara-C incorporation during UV repair and loss of clonogenic survival. These findings are consistent with the hypothesis that the incorporation of ara-C into DNA undergoing either unscheduled or semiconservative synthesis is an initial event leading to cell lethality.

MATERIALS AND METHODS

Cell culture. The AG1522 human diploid fibroblast cells (obtained from Institute for Medical Research, Camden, N. J.) were grown in minimal essential medium supplemented with 10% heat-inactivated

This investigation was supported by United States Public Health Service Grant CA-29431 awarded by the National Cancer Institute, Department of Health and Human Services, an American Cancer Society Faculty Research Award (to D. W. K.), and American Cancer Society Grant PRTF-17 (to R. J. F.).

[1] The abbreviations used are: ara-C, 1-β-D-arabinofuranosylcytosine, BrdUrd, bromodeoxyuridine; FdUrd, fluorodeoxyuridine.

0026-895X/84/020322-05$02.00/0

fetal calf serum (GIBCO, Grand Island, N. Y.), glucose (900 mg/liter), sodium pyruvate (0.66 mg/ml), streptomycin (100 units/ml), and penicillin (100 μg/ml). Cells were plated in 6-cm culture dishes and grown to confluency. The culture medium was renewed daily for 3 days and experiments were performed thereafter.

Clonogenic survival of human diploid fibroblasts. The growth-arrested AG1522 cells were incubated with varying concentrations of ara-C (10^{-7} to 10^{-4} M) for 3, 6, 12, and 24 hr alone or after exposure to 5 J/m² UV irradiation. After treatment, the cells were collected by mild trypsinization in drug-free medium without serum and then washed twice. Appropriate numbers of cells were then seeded in culture dishes (100 × 20 mm) with complete medium. Viability was determined after 7–14 days by scoring colonies containing more than 20 cells. Percentage colony formation was determined by the ratio of colonies formed by ara-C-treated cells to untreated cells. The dose of UV light selected gave a mean surviving fraction of 0.63 (result of four separate determinations). The UV and ara-C data points were normalized by this value.

Incorporation of [³H]ara-C in AG1522 DNA. Growth-arrested AG1522 cells were incubated for 2 hr in the presence of 10^{-5} M BrdUrd and 10^{-6} M FdUrd followed by treatment with 5 or 20 J/m² UV irradiation. The cells were then incubated for 3, 6, 12, or 24 hr in the presence of 2×10^{-3} M hydroxyurea, 10^{-6} M FdUrd, 10^{-5} M BrdUrd, and 10^{-7} M [³H]ara-C (Radiochemical Centre, Amersham, England; specific activity 15.5 Ci/mmole). The cells were trypsinized and DNA was extracted as previously described (3) after lysis with 2.5 mg of pronase B in 2 ml of 0.01 M Tris (pH 7.4), 0.01 M EDTA, and 0.5% sodium dodecyl sulfate. The nucleic acids were precipitated with 4 M NaCl and ethanol. The DNA was then isolated on CsCl gradients as described elsewhere (20), except that 0.01 M Tris/1 mM EDTA (pH 8) was used as the buffer. The purified DNA was also digested to nucleotides for analysis by high-pressure liquid chromatography as previously described (3).

Determination of percentage of labeled nuclei. The relative proliferative rate of growth-arrested AG1522 cells was determined using autoradiographic techniques. The increase in percentage of labeled nuclei with time was measured by incubating cells in drug-free medium containing [³H]thymidine (0.5 μCi/ml; specific activity 50 Ci/mmole; New England Nuclear Corporation, Boston Mass.). The cells were fixed at 3, 6, 12, or 24 hr of incubation and prepared for autoradiography. Data points were determined by scoring at least 300 nuclei under a light microscope.

RESULTS

Our previous studies monitoring incorporation of ara-C into DNA were performed with cells in logarithmic growth phase and did not distinguish between incorporation into DNA undergoing semiconservative or repair synthesis. The monitoring of ara-C incorporation into DNA undergoing repair requires the use of cells that undergo density-dependent growth arrest to avoid significant incorporation of ara-C in DNA undergoing semiconservative synthesis. The following studies were thus performed using human foreskin diploid fibroblast (AG1522) that undergo density-dependent growth arrest.

The extent of [³H]ara-C incorporation into AG1522 DNA was determined by CsCl gradient centrifugation, which separates DNA undergoing repair and replicative synthesis. Figure 1 illustrates the incorporation of [³H] ara-C in AG1522 DNA during UV excision repair. There was little detectable incorporation of ara-C in the growth-arrested AG1522 cells not treated with UV irradiation. In contrast, significant amounts of ara-C incorporated into DNA following treatment of growth-arrested cells with 5 or 20 J/m² UV light. The pattern of incorporation (Fig. 1) indicated that over 90% of the detectable radioactivity is present in DNA undergoing repair. Furthermore, the extent of ara-C incorporation during DNA repair was dependent upon drug concentration (Fig. 2) and time of exposure.

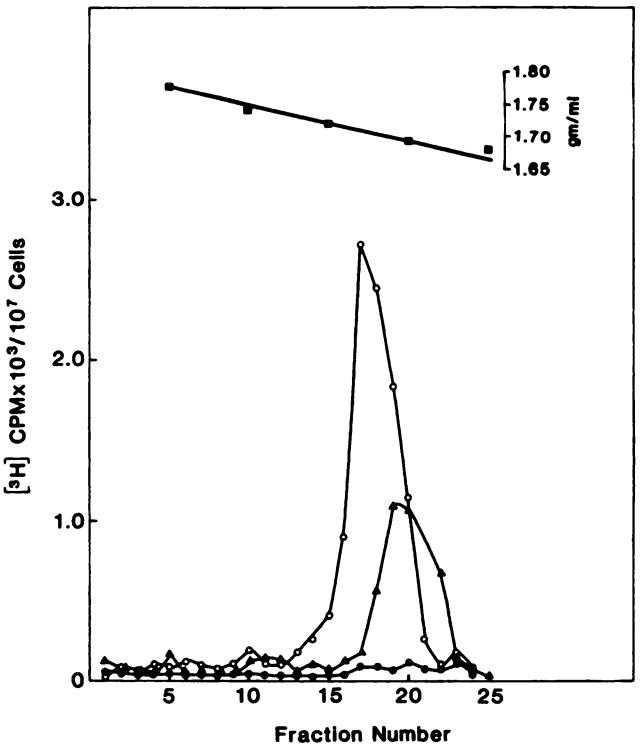

FIG. 1. *Incorporation of [³H]ara-C into DNA following UV treatment*

Growth-arrested AG1522 cells were treated with 10^{-5} M BrdUrd and 10^{-6} M FdUrd for 2 hr and then irradiated with 0, 5, or 20 J/m² UV. The cells were then incubated with 2×10^{-3} M hydroxyurea, 10^{-6} M FdUrd, 10^{-5} M BrdUrd, and 10^{-7} M [³H]ara-C for 24 hr. The DNA was then purified and analyzed by CsCl gradient centrifugation (20). Cells treated with 20 J/m² UV and ara-C (○), 5 J/m² UV and ara-C (▲), or ara-C alone (●). Under these conditions, the DNA undergoing repair bands at a density of 1.700 g/ml, and the DNA undergoing semiconservative synthesis bands at 1.751 g/ml.

It is relevant to demonstrate that the tritium radioactivity detectable in DNA undergoing repair synthesis actually represents [³H]ara-C. The labeled DNA was thus digested to nucleotides for analysis by high-pressure liquid chromatography with the addition of appropriate markers. The profile (Fig. 3) illustrates that the tritium radioactivity co-migrated with both ara-C and 3′-ara-CMP, and was not detectable in other fractions of the high-pressure liquid chromatogram. These findings indicate that the ara-C residues were positioned in both internucleotide linkage and at the chain terminus.

The relationship of ara-C incorporation into AG1522 DNA undergoing DNA repair to drug-induced cytotoxicity was studied by comparing the amount of ara-C incorporated with loss of clonogenic survival. The effect of ara-C on clonogenic survival of untreated and UV-

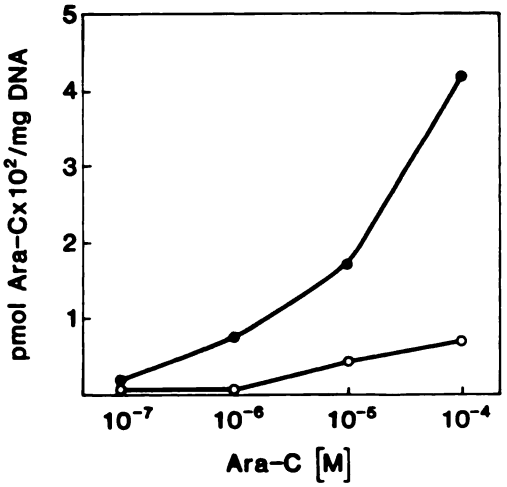

FIG. 2. *Incorporation of varying concentrations of [³H]ara-C into DNA undergoing UV repair synthesis*

Growth-arrested AG1522 cells were treated with UV irradiation (5 J/m²) and varying concentrations (10^{-7} to 10^{-4} M) [³H]ara-C for 24 hr. The DNA fraction was then purified and analyzed for tritium incorporation. ●, Cells treated with UV and [³H]ara-C; ○, cells treated with [³H]ara-C alone.

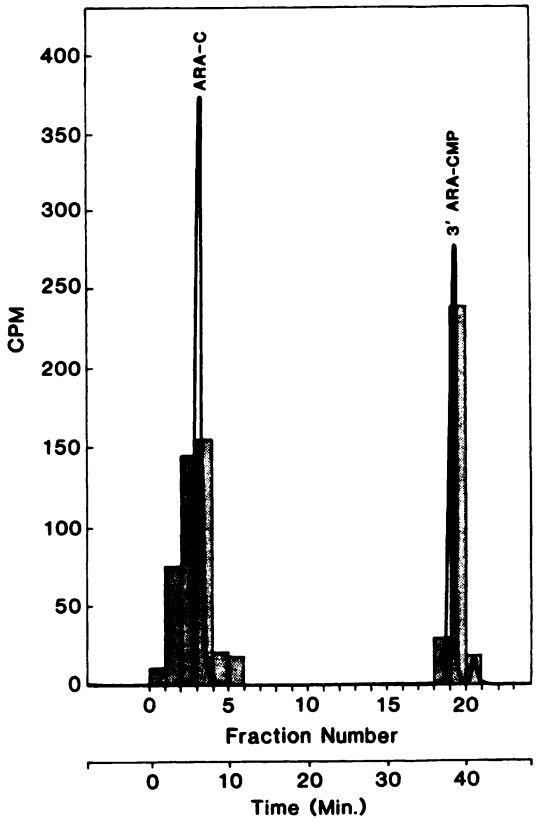

FIG. 3. *Nucleotide chromatogram of digested [³H]ara-C-labeled AG1522 DNA*

Growth-arrested AG1522 cells were treated with UV irradiation (20 J/m²) and 10^{-7} M [³H]ara-C for 24 hr. The DNA was purified and digested to nucleotides prior to analysis by high-pressure liquid chromatography (3). Similar results were obtained with DNA obtained from cells treated with 5 J/m² UV irradiation and ara-C.

treated AG1522 cells was determined by exposure to concentrations of 10^{-7} M to 10^{-4} M for 3, 6, 12, and 24 hr. The results are illustrated in Fig. 4. While there was little difference between untreated and UV-treated cells following exposure to ara-C for 3 hr (Fig. 4A), a progressive enhancement of cell lethality occurred with increasing time of incubation. The loss of clonogenic survival for both untreated and UV-treated cells was also dependent upon drug concentration.

In order to ensure further that the effect of ara-C on clonogenic survival was the result of drug incorporation during DNA repair synthesis, we monitored the percentage of labeled nuclei using autoradiography. These results (Fig. 5) indicate that less than 10% of the growth-arrested AG1522 cells underwent replicative DNA synthesis during the 24-hr exposure. Thus, the enhancement of cell lethality at 24 hr (Fig. 4D) resulted from the effect of ara-C on unscheduled DNA synthesis and confirms our findings of [³H]ara-C incorporation into DNA undergoing repair (Fig. 1).

The relationship between incorporation of ara-C into DNA undergoing UV repair synthesis and loss of clonogenic survival was determined by measuring the amount of [³H]ara-C incorporated into DNA under the same conditions used for studies monitoring clonogenic survival. Incorporation studies were performed at [³H]ara-C concentrations ranging from 10^{-7} to 10^{-4} M during incubation periods of 3, 6, 12, and 24 hr. Clonogenic survival at each ara-C concentration was obtained from the cloning data shown in Fig. 4. The extent of ara-C incorporation into DNA undergoing repair synthesis correlated significantly, by probit analysis (21), with loss of clonogenic survival. The probit analysis of this relationship as determined by a computer-assisted program is illustrated in Fig. 6.

DISCUSSION

Although the incorporation of ara-C during the repair of UV-damaged DNA has been demonstrated by others (12), the biological significance of this finding was not investigated further. In fact, it was suggested that ara-C incorporation into DNA does not play a significant role in the inhibition of repair synthesis. The results of the present study also demonstrate the incorporation of ara-C into DNA undergoing UV excision repair. These findings have been extended by our demonstration of a highly significant relationship between incorporation of ara-C into DNA undergoing repair and loss of clonogenic survival. Our data suggest that the incorporation of ara-C into DNA undergoing unscheduled DNA synthesis, as we have previously shown for replicative synthesis (3, 4), is at least one mechanism responsible for producing lethal cellular events.

The repair of UV damage is thought to occur by excision and resynthesis steps (22). These observations have also been derived from experiments using xeroderma pigmentosa cells defective in excision repair. Other observations have suggested that the repair of UV-like damage is accomplished by a complex containing an exonuclease and a polymerase function (16, 23). The effect of ara-C on UV excision and resynthesis repair could thus be secondary to any of the following events: (a) inhibition of the exonuclease function, (b) inhibition of repair synthesis after excision, or (c) incorporation of ara-C into the repair patch.

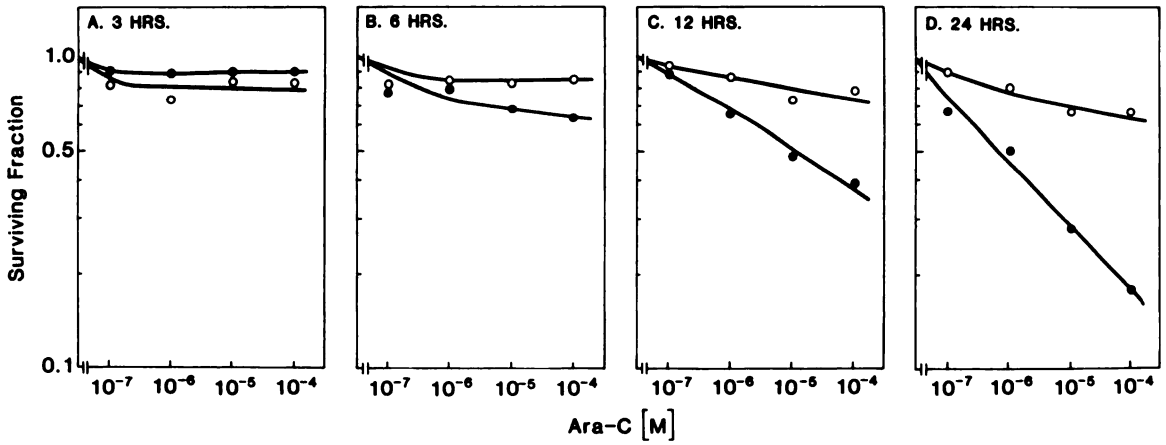

FIG. 4. *Clonogenic survival of AG1522 cells exposed to UV irradiation and varying concentrations of ara-C*

Growth-arrested AG1522 cells were treated with 5 J/m^2 UV irradiation and then incubated with varying concentrations (10^{-7} to 10^{-4} M) of ara-C for 3, 6, 12, or 24 hr. After drug exposure, the cells were washed, trypsinized, and resuspended in drug-free medium. Clonogenic survival was determined after 7–14 days by scoring colonies containing more than 20 cells. ●, Cells treated with UV and ara-C; ○, cells treated with ara-C alone.

The available evidence suggests that the inhibition of UV excision repair by ara-C occurs at the resynthesis step. The accumulation of DNA strand breaks induced by UV damage has been demonstrated in the presence of ara-C (22). This accumulation occurs to a lesser extent in excision-deficient fibroblasts from patients with xeroderma pigmentosa (22). In contrast, inhibition of the resynthesis step could occur as a result of ara-C incorporation into the repair patch. We have previously demonstrated that the extent of ara-C incorporation into DNA correlates with inhibition of semiconservative DNA synthesis and that the ara-C residue behaves as a relative chain terminator (5). Thus, an ara-C residue incorporated into the repair patch could also act as a relative chain terminator and force the polymerase to stutter at this site. Furthermore, the inhibition of the resynthesis step by ara-C could sequester the polymerase at a repair site and make it unavailable to perform excision functions at other damaged DNA sites.

The present observations thus provide further insights into the mechanism of action of ara-C. It will now be of interest to determine whether ara-C incorporates into DNA undergoing repair of damage caused by alkylating agents or anthracyclines. This finding may provide the experimental basis for the development of therapeutic protocols which would employ a chemotherapeutic agent that damages DNA to be followed by ara-C during a period required for repair.

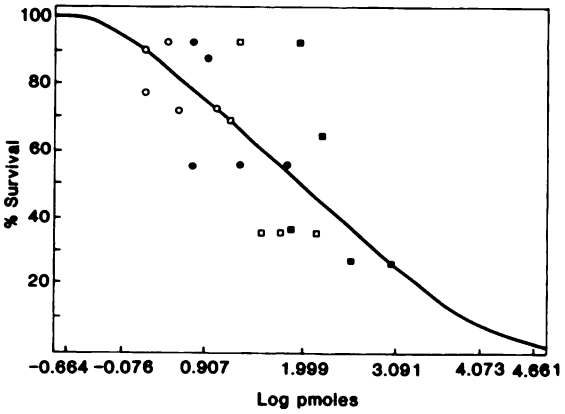

FIG. 6. *Relationship between AG1522 clonogenic survival and incorporation of [^{3}H]ara-C into DNA*

Clonogenic survival and incorporation of [^{3}H]ara-C (picomoles per milligram of DNA) were compared at ara-C concentrations of 10^{-7} M (○), 10^{-6} M (□), 10^{-5} M (●), and 10^{-4} M (■) during periods of 3, 6, 12, and 24 hr.

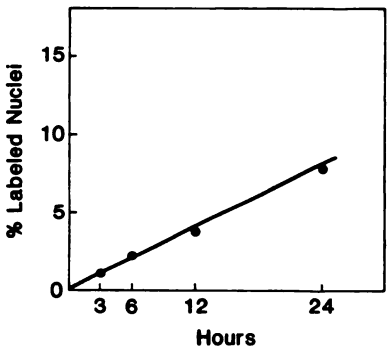

FIG. 5. *Percentage of labeled nuclei of growth-arrested AG1522 cells*

Percentage of labeled nuclei was determined at 3, 6, 12, and 24 hr for growth-arrested AG1522 cells.

REFERENCES

1. Frei, E., J. Bickers, J. Hewlett, M. Lane, W. Leary, and R. Tally. Dose schedule and antitumor studies of arabinosyl cytosine. *Cancer Res.* **29**:1325–1332 (1969).
2. Bodey, G., E. Freireich, R. Monto, and J. Hewlett. Cytosine arabinoside therapy for acute leukemia in adults. *Cancer Chemother. Rep.* **53**:59–66 (1969).
3. Kufe, D. W., P. P. Major, E. M. Egan, and P. Beardsley. Correlation of cytotoxicity with incorporation of ara-C into DNA. *J. Biol. Chem.* **255**:8997–9000 (1980).
4. Major, P., E. M. Egan, G. P. Beardsley, M. D. Minden, and D. W. Kufe. Lethality of human myeloblasts correlates with the incorporation of ara-C into DNA. *Proc. Natl. Acad. Sci. U. S. A.* **78**:3235–3239 (1981).
5. Major, P., E. Egan, D. Herrick, and D. Kufe. The effect of ara-C incorporation on deoxyribonucleic acid synthesis in cells. *Biochem. Pharmacol.* **31**:2937–2940 (1982).
6. Cohen, S., and W. Plunkett. The utilization of nucleotides by animal cells. *Ann. N. Y. Acad. Sci.* **255**:269–286 (1975).
7. Burgoyne, L. Effect of cytosine arabinoside triphosphate on deoxyribonucleic acid synthesis in permealysed cells from Ehrlich ascites tumour. *Biochem. Pharmacol.* **23**:1619–1627 (1974).

8. Fram, R., and D. Kufe. DNA strand breaks caused by inhibitors of DNA synthesis: 1-β-D-arabinofuranosylcytosine and aphidicolin. *Cancer Res.* **42**:4050–4053 (1982).
9. Woodcock, D., R. Fox, and I. Cooper. Evidence for a new mechanism of cytotoxicity of 1-β-D-arabinofuranosyl cytosine. *Cancer Res.* **39**:1416–1424 (1979).
10. Woodcock, D., and I. Cooper. Evidence for double replication of chromosomal DNA segments as a general consequence of DNA replication inhibition. *Cancer Res.* **41**:2483–2490 (1981).
11. Hiss, E., and R. Preston. The effect of cytosine arabinoside on the frequency of single-strand breaks in DNA of mammalian cells following irradiation or chemical treatment. *Biochim. Biophys. Acta* **478**:1–8 (1977).
12. Dunn, W., and J. Regan. Inhibition of DNA excision repair in human cells by arabinofuranosyl cytosine: effect on normal and xeroderma pigmentosa cells. *Mol. Pharmacol.* **15**:367–374 (1979).
13. Erixon, K., and G. Ahnstrom. Single-strand breaks in DNA during repair of UV-induced damage in normal human and xeroderma pigmentosum cells as determined by alkaline DNA unwinding and hydroxylapatite chromatography. *Mutat. Res.* **59**:257–271 (1979).
14. Preston, R. The use of inhibitors of DNA repair in the study of the mechanisms of induction of chromosome aberrations. *Cytogenet. Cell Genet.* **33**:20–26 (1982).
15. Preston, R. The effect of cytosine arabinoside on the frequency of x-ray-induced chromosome aberrations in normal human leukocytes. *Mutat. Res.* **69**:71–79 (1980).
16. Snyder, R. D., W. Carrier, and J. Regan. Application of arabinofuranosyl cytosine in the kinetic analysis and quantitation of DNA repair in human cells after ultraviolet irradiation. *Biophys. J.* **35**:339–350 (1981).
17. Collins, A., S. Squires, and R. Johnson. Inhibitors of repair DNA synthesis. *Nucleic Acids Res.* **10**:1203–1213 (1982).
18. Miller, M., and D. Chinault. Evidence that DNA polymerases α and β participate differentially in DNA repair synthesis induced by different agents. *J. Biol. Chem.* **257**:46–49 (1982).
19. Miller, M., and D. Chinault. The roles of DNA polymerases α, β, and γ in DNA repair synthesis induced in hamster and human cells by different DNA damaging agents. *J. Biol. Chem.* **257**:10204–10209 (1982).
20. Smith, C. A., P. K. Cooper, and P. C. Hanawalt. Measurement of repair replication by equilibrium sedimentation, in *A Laboratory Manual of Research Procedures* (E. Friedburg and P. Hanawalt, eds.). Marcel Dekker, Inc., New York, 289–305 (1981).
21. Cox, D. *Analysis of Binary Data.* Barnes and Noble, New York, 76–79 (1969).
22. Regan, J., and R. Setlow. Two forms of repair in the DNA of human cells damaged by chemical carcinogens and mutagens. *Cancer Res.* **34**:3318–3325 (1974).
23. Hanawalt, P., P. Cooper, A. Ganesan, and C. Smith. DNA repair in bacteria and mammalian cells. *Annu. Rev. Biochem.* **48**:783–836 (1979).

Send reprint requests to: Dr. Donald W. Kufe, Department of Medicine, Dana-Farber Cancer Institute, Harvard Medical School, 44 Binney Street, Boston, Mass. 02115.