5-Fluoro-2'-deoxyuridine Incorporation in L1210 DNA*

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We have employed cesium sulfate density gradient centrifugation to separate RNA and DNA of L1210 cells labeled with [3H]fluorodeoxyuridine. We have analyzed nucleotide and nucleoside digests of purified DNA from the [3H]fluorodeoxyuridine-labeled cells and demonstrate by reverse phase and anion exchange high pressure liquid chromatography the presence of tritium radioactivity co-migrating with fluorodeoxyuridine 5'-monophosphate or fluorodeoxyuridine. These observations demonstrate the internucleotide incorporation of fluorodeoxyuridine in DNA and suggest a new mechanism of action for this cytotoxic and mutagenic agent.

Fluorouracil and fluorodeoxyuridine are effective agents in the treatment of certain human epithelial tumors (1-3). Several mechanisms of action have been proposed for these agents, among which are 1) incorporation of FURA in RNA with disruption of RNA synthesis and function and 2) conversion to FdUMP with irreversible binding to thymidylate synthetase and inhibition of DNA synthesis by limiting production of dTMP (3-8). Either mechanism could be responsible for the cytotoxic effects of these agents. FURA incorporation in RNA has been correlated with antitumor activity, while the misincorporation of FdUrd in eukaryotic DNA has not been demonstrated previously (9).

The enzymes deoxyuridine-triphosphate nucleotidohydrolase and uracil-DNA-glycosylase are responsible for preventing the incorporation of uracil nucleotides in DNA (10-12). The nucleotide hydrolase degrades intracellular dUTP, and uracil DNA-glycosylase removes uracil residues incorporated in the DNA strand. These enzymes can also utilize the fluorinated derivatives of uracil as substrates, and this has been suggested as an explanation for the failure to detect FdUrd in cellular DNA (10, 11). Although fluorodeoxyuridine-triphosphate and dUTP are hydrolyzed at similar rates, the exclusion of FURA from DNA by uracil DNA-glycosylase is much less efficient than that of uracil (11). This finding, as well as the detection of FURA in bacterial DNA (13) and of uracil in DNA of human lymphoid cells treated with methotrexate (14), suggests that FdUrd residues could be identified in mammalian DNA.

We have studied the incorporation of FdUrd in DNA of L1210 murine leukemia cells. The results demonstrate that FdUrd incorporates in internucleotide linkage in DNA.

MATERIALS AND METHODS

Cell Culture—The L1210 cells were grown as a suspension culture in minimum essential medium for suspension supplemented with 10% heat-inactivated dialyzed fetal calf serum, 1% l-glutamine, 100 μg of penicillin/ml, 100 units of streptomycin/ml, 0.06 mM 2-mercaptopethanol at 37 °C in a 5% CO₂ atmosphere.

Incorporation of [3H]FdUrd in L1210 Nucleic Acids—L1210 cells at a concentration of 10⁶/ml were incubated in complete medium with 10⁻⁷, 10⁻⁴, or 10⁻³ M [3H]FdUrd (19 Ci/mmol, Moravek Biochemicals, City of Industry, CA) and 10 μCi/ml of H₂¹⁶PO₄ carrier-free, New England Nuclear) for varying time periods (4 to 12 h). The cells were then washed twice with 5 ml of phosphate-buffered saline, resuspended at 10⁶ cells/ml in phosphate-buffered saline, and digested by the addition of 2.5 mg of proteinase-K (Boehringer Mannheim) 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 purified by phenol extraction and then precipitated by the addition of 1/10 volume of 4 M NaCl and 2 volumes of absolute ethanol. The nucleic acids were analyzed by cesium sulfate gradient centrifugation using a Varian Model 5020 gradient liquid chromatograph.

Digestion of [3H]FdUrd-labeled Nucleic Acids—L1210 cells were labeled with [3H]FdUrd for 6 h as described for the incorporation studies. The total nucleic acids were purified as described above and separated by centrifugation on cesium sulfate gradients. The purified DNA was digested to 5'-nucleotides using DNase I and snake venom phosphodiesterase. The digestion of [3H]FdUrd-labeled DNA to 3'-nucleotides was performed by the sequential action of micrococcal nuclease and spleen phosphodiesterase. Further digestion of the 5'- or 3'-nucleotides to nucleosides was accomplished with bacterial alkaline phosphatase. (All enzymes were purchased from Worthington Biochemicals.) After precipitating the remaining macromolecular species with perchloric acid, nucleotides or nucleosides were analyzed by high pressure liquid chromatography using a Varian Model 5020 gradient liquid chromatograph under reverse phase and anion exchange conditions.

The reverse phase isocratic chromatography was performed on a Varian Micropak MCH-10 column using 0.01 M KH₂PO₄ (pH 5.25) buffer at a flow rate of 1 ml/min. Anion exchange analysis was performed using a Varian Micropak AX-10 column with a gradient elution. Buffer A was 0.01 M KH₂PO₄ (pH 2.85)/acetonitrile (20/80), and buffer B was 0.01 M KH₂PO₄ (pH 2.85). The elution program utilizing buffer B was as follows: 1) 0% for 10 min; 2) 0-100% for 10 min, and 3) 100% for 40 min. Each elution was performed after the addition of appropriate markers. The 5-fluoro-2'-deoxycytidine marker was kindly provided by Dr. J. Fox, Sloan-Kettering Institute for Cancer Research, Rye, NY. Fractions were collected during the elution and assayed for tritium counts.

RESULTS

The amount of [3H]FdUrd incorporation into L1210 nucleic acids was monitored by cesium sulfate gradient centrifugation which separates RNA (banding between density 1.62 to 1.68 g/ml) and DNA (banding between density 1.42 and 1.48 g/ml). Fig. 1 shows the incorporation of [3H]FdUrd and ³²P in L1210 nucleic acids at various concentrations of FdUrd (10⁻⁷ to 10⁻⁵ M) during an incubation period of 6 h. There is significant incorporation of tritium radioactivity in both the DNA and RNA regions of the gradient. The amount of tritium incorporated in DNA and RNA is dependent on drug concentration. The labeling with ³²P serves as a measure of newly incorporated ³²P.
synthesized RNA and DNA and demonstrates that RNA synthesis is not affected by increasing the concentration of FdUrd, while DNA synthesis is inhibited at each drug level. The incorporation of tritium radioactivity into RNA and DNA also increases with time of exposure, as illustrated in Fig. 2.

It is important to demonstrate that the tritium radioactivity detectable in the DNA region of the Cs2SO4 gradient represents [3H]FdUrd. The labeled DNA was digested to nucleosides using DNase I, snake venom phosphodiesterase, and BAP for analysis by high pressure liquid chromatography. The profile seen in Fig. 3A illustrates that the tritium radioactivity co-migrates with FdUrd and not with other metabolites such as fluorouridine or deoxyuridine. Although not shown in this profile, the radioactivity co-migrating with FdUrd does not co-migrate with any of the pyrimidine and purine deoxyribonucleosides or ribonucleosides. Furthermore, the radioactivity does not co-migrate with 5-fluoro-2'-deoxycytidine (Fig. 3B). Similar results are obtained when the DNA fraction is first treated with either NaOH (0.3 M for 6 h at 37 °C) or RNase A (0.1 mg/ml, with 10 mM EDTA for 2 h at 37 °C) and then precipitated with perchloric acid prior to the enzyme digest. These observations confirm that the FdUrd residues are incorporated into DNA and not RNA. Cells were exposed to equimolar (10⁻⁶ M) concentrations of FdUrd and dThd. The amount of each nucleoside incorporated in DNA was determined and the ratio of FdUrd to dThd was 1:900.

The [3H]FdUrd-labeled DNA was also digested to 5'- and 3'-nucleotides to determine whether the FdUrd residues are in internucleotide linkage. As illustrated in Fig. 4, digestion of the labeled DNA with DNase I and snake venom phosphodiesterase results in a peak of tritium radioactivity co-migrating with 5'-FdUMP. A similar digest of the same labeled DNA with the addition of BAP results in co-migration of the tritium radioactivity with FdUrd. A similar analysis has been performed using micrococcal nuclease and spleen phosphodiesterase. This approach results in the co-migration of tritium counts adjacent to the 5'-FdUMP marker. The unavailability of a 3'-FdUMP marker prevents a more precise definition of

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**Fig. 1.** Dose dependence of [3H]FUra incorporation in nucleic acids of L1210 cells treated with [3H]FdUrd. L1210 cells in logarithmic growth phase were incubated with 10⁻⁷, 10⁻⁶, or 10⁻⁵ M [3H]FdUrd and 10 μCi/ml of 32P for 6 h. The total cellular nucleic acids from 10⁶ cells were purified and analyzed by cesium sulfate gradient centrifugation. The amount of [3H]FUra incorporation in the DNA region of the gradients was: A, 0.06 pmol; B, 0.16 pmol; and C, 0.95 pmol.

**Fig. 2.** Time dependence of [3H]FUra incorporation in DNA and RNA of L1210 cells treated with [3H]FdUrd. L1210 cells in logarithmic growth phase were incubated with 10⁻⁷ M [3H]FdUrd and 10 μCi/ml of 32P. The purified total cellular nucleic acids from 10⁶ cells were analyzed by cesium sulfate gradient centrifugation. Amounts of [3H]FUra incorporated in the DNA region of the gradient was 0.12 pmol at 4 h, 0.19 pmol at 8 h, and 0.21 pmol at 12 h.
On cesium sulfate gradients. The DNA was then digested with DNase I, snake venom phosphodiesterase, and bacterial alkaline phosphatase. The nucleosides were purified and analyzed by reverse phase (A, flow rate: 1 ml/min; 1.0-ml fractions) and anion exchange (B, flow rate: 1 ml/min; 0.5-ml fractions) high pressure liquid chromatography.

This suggested that FUra could incorporate into DNA and cytotoxicity will be explored in subsequent studies. The relationship between FdUrd incorporation in DNA and cytotoxicity will be explored in subsequent studies. It will also now be of interest to determine the rate of excision of FdUrd residues from DNA in different cell lines. The comparison of tumor and normal cell repair of FdUrd misincorporation might provide a basis for the selectivity of this agent against malignant cells.

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REFERENCES

1. Heidelberger, C., Chaudhur, N. K., Danneberg, P., Mooren, D., Griesbach, L., Duschinsky, R., Schnitzer, R. J., Pleven, E., and Scheiner, J. (1975) Nature 179, 663-666

2. Heidelberger, C., Griesbach, L., Monigal, B., Mooren, D., Cruz, O., Schnitzer, R. J., and Grunberg, E. (1968) Cancer Res. 18, 305-317

3. Heidelberger, C. (1975) in Antineoplastic and Immunosuppressive Agents (Sartorelli, A. C., and Johns, D. G., eds) pp. 193-231, Springer-Verlag, New York

4. Reyes, P., and Heidelberger, C. (1965) Mol. Pharmacol. 1, 14-30

5. Hartmann, K., and Heidelberger, C. (1961) J. Biol. Chem. 236, 3006-3013
6. Glazer, R., and Peale, A. (1979) Mol. Pharmacol. 16, 270-277
7. Glazer, R., and Hartmann, K. (1980) Mol. Pharmacol. 17, 245-249
8. Glazer, R., and Legraverend, M. (1980) Mol. Pharmacol. 17, 279-282
9. Chaudhuri, N. K., Montag, B., and Heidelberger, C. (1968) Cancer Res. 18, 318-328
10. Ingraham, H., Tseng, B., and Goulian, M. (1980) Cancer Res. 40, 998-1001
11. Caradonna, S., and Cheng, Y. (1980) Mol. Pharmacol. 18, 513-520
12. Caradonna, S., and Cheng, Y. (1980) J. Biol. Chem. 255, 2293-2300
13. Warner, H., and Rockstroh, P. (1980) J. Bacteriol. 141, 680-686
14. Goulian, M., Bleile, B., and Tseng, B. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 1956-1960
15. Kufe, D. W., Major, P. P., Egan, E. M., and Beardsley, G. P. (1980) J. Biol. Chem. 255, 8997-9000
16. Tseng, B., and Goulian, M. (1977) Cell 12, 483-489
17. Grafstrom, R., Tseng, B., and Goulian, M. (1978) Cell 15, 131-140
18. Kufe, D. W., and Egan, E. M. (1981) Biochem. Pharmacol. 30, 129-133