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BIOLOGIC EFFECT OF 5-FLUORO-2′-DEOXYURIDINE INCORPORATION IN L1210 DEOXYRIBONUCLEIC ACID*

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Abstract—Recent studies have demonstrated that 5-fluoro-2′-deoxyuridine (FdUrd) misincorporates in eukaryotic DNA. We have extended these findings and studied the relationship between DNA incorporation of FdUrd and cell lethality. This required an approach that would distinguish the DNA and RNA effects of this agent. The specific effect of (FdUrd)DNA formation on L1210 clonogenic survival was, therefore, monitored using deoxyuridine (dUrd) to inhibit the metabolism and subsequent incorporation of FdUrd into RNA. The results demonstrated that misincorporation of FdUrd in DNA resulted in lethal cellular events. These observations suggest a new mechanism of action for this cytotoxic and mutagenic agent.

Several mechanisms of action have been proposed for the fluorinated pyrimidines, 5-fluorouracil (5-FUra) and 5-fluoro-2′-deoxyuridine (FdUrd). These drugs are converted to 5-fluorodeoxyuridine-monophosphate (FdUMP) which binds irreversibly to thymidylate synthetase and inhibits DNA synthesis [1, 2]. The fluorinated pyrimidines also incorporate in RNA and disrupt RNA processing [3–7]. There is a highly significant relationship between incorporation of FUra in RNA and loss of clonogenic survival [8, 9]. This relationship is maintained even in the presence of excess thymidine which bypasses the effect of FdUMP on DNA synthesis [8]. These findings suggest that the formation of (FUra)RNA is the major mechanism of cytotoxic action in some cell lines.

More recently, we and others have also demonstrated the incorporation of FUra residues in eukaryotic DNA [10–13]. The FUra residues are excised from DNA [14, 15], and methotrexate has been shown to enhance this excision process [14]. The excision of FUra from eukaryotic DNA may contribute to the cytoxicity and mutagenicity of the fluorinated pyrimidines [16]. This effect on DNA, however, has not been distinguished from the effect of these agents on inhibition of thymidylate synthetase and on disruption on RNA processing.

In the present report, we extend our previous studies on the incorporation of FdUrd in L1210 DNA. We have employed high concentrations of dUrd to limit degradation of FdUrd and to prevent reutilization of FUra for incorporation into RNA. This approach distinguishes the DNA and RNA effects of this agent. The results demonstrate that FdUrd incorporation in DNA results in lethal cellular events as determined by loss of L1210 clonogenic survival.

MATERIALS AND METHODS

Cell culture. The L1210 cells were grown as a suspension culture in Eagle's minimum essential medium for suspension (S-MEM) with 10% heat-inactivated dialyzed fetal calf serum, 1% L-glutamine, 100 μg penicillin/ml, 100 units streptomycin/ml and 0.05 mM 2-mercaptoethanol at 37° in a 5% CO_2 atmosphere.

Incorporation of [^{3}H]*FdUrd in L1210 nucleic acids.* L1210 cells at a concentration of 1×10^6 cells/ml were incubated in the S-MEM medium with 10^{-6} to 10^{-2} M dUrd (Sigma Chemical Co., St. Louis, MO) for 1 hr prior to adding 10^{-6} M [^{3}H]FdUrd (20 Ci/mmole; Moravek Biochemicals, Inc., Brea, CA) and/or 10 μCi/ml of $H_3{}^{32}PO_4$ (carrier-free, New England Nuclear Corp., Boston, MA) for an additional 6 hr. The cells were then washed twice with 5 ml of phosphate-buffered saline (PBS), resuspended in 1 ml PBS, and placed at −20° overnight. The cells were thawed and digested by addition of 1.5 mg of nuclease-free pronase (Calbiochem-Behring Corp., La Jolla, CA) in 2 ml of 0.01 M Tris (pH 7.4), 0.01 M EDTA, and 0.5% sodium dodecyl sulfate. After 3 hr at 37°, the nucleic acids were precipitated with 0.3 ml of 4 M NaCl and 2 vol. of absolute ethanol. The nucleic acids were then dissolved in 1 ml of 0.2 M Tris, 0.2 M EDTA (pH 7.4) and analyzed by cesium sulfate gradient centrifugation as previously described [8].

Clonogenic survival of L1210 cells. L1210 cells in logarithmic growth phase were washed twice in phosphate-buffered saline and incubated at 1×10^6 cells/ml for 1 hr at 37° in medium containing various concentrations of dUrd (10^{-8} to 10^{-2} M) prior to

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adding 10^{-6} M FdUrd for an additional 6 hr. The effects of incubating cells with 10^{-2} M dUrd for 1 hr prior to adding FdUrd (10^{-8} to 10^{-4} M) for an additional 6 hr were also monitored in a separate experiment. After drug exposure, the cells were collected in drug-free medium, counted in a model Z Coulter Counter, and plated in 1.5% methylcellulose (S-MEM) containing 20% fetal calf serum. Viability was determined after 7 days by scoring colonies greater then 50 cells. The percent viability was determined by the ratio of colonies formed by treated cells as compared to untreated cells multiplied by 100. Cloning efficiency of the untreated L1210 cells in this system ranged between 55 and 65%.

RESULTS

We have monitored the incorporation of [^{3}H]FdUrd into L1210 nucleic acids using cesium sulfate density centrifugation which separates RNA (banding at a density of 1.65 g/ml) and DNA (banding at a density of 1.45 g/ml). Simultaneous labeling with ^{32}P was employed to measure newly synthesized RNA and DNA. A representative profile of 10^{-6} M [^{3}H]FdUrd and ^{32}P incorporation in L1210 nucleic acids is illustrated in Fig. 1A. Incubation with increasing concentrations (10^{-6} to 10^{-2} M) of dUrd resulted in a progressive decline in (FUra)RNA formation (Fig. 1, B–F). The synthesis of RNA as determined by ^{32}P labeling demonstrated that the absence of detectable (FUra)RNA formation was not due to inhibition of RNA synthesis. The effect of dUrd on incorporation of [^{3}H]FdUrd in L1210 DNA is also illustrated in Fig. 1, A–F. Increasing concentrations of dUrd resulted in an enhancement of DNA synthesis, while the absolute amount of FdUrd incorporated into DNA remained similar to that obtained when using [^{3}H]FdUrd alone.

The biologic significance of FdUrd incorporated into DNA was monitored by clonogenic survival of L1210 cells following exposure to 10^{-6} M FdUrd and various concentrations of dUrd (Fig. 2). Clonogenic survival increased with the addition of 10^{-4} to 10^{-2} M dUrd. However, survival of L1210 cells remained at nearly 80% even following incubation with 10^{-6} M FdUrd and 10^{-2} M dUrd, which resulted in inhibition of (FUra)RNA formation.

Figure 3 also illustrates that 10^{-2} M dUrd significantly reduced the cytotoxic effects of FdUrd obtained at concentrations ranging from 10^{-8} to 10^{-6} M. In contrast, dUrd had little, if any, effect on the clonogenic survival of L1210 cells exposed to 10^{-5} or 10^{-4} M FdUrd. These findings are consistent

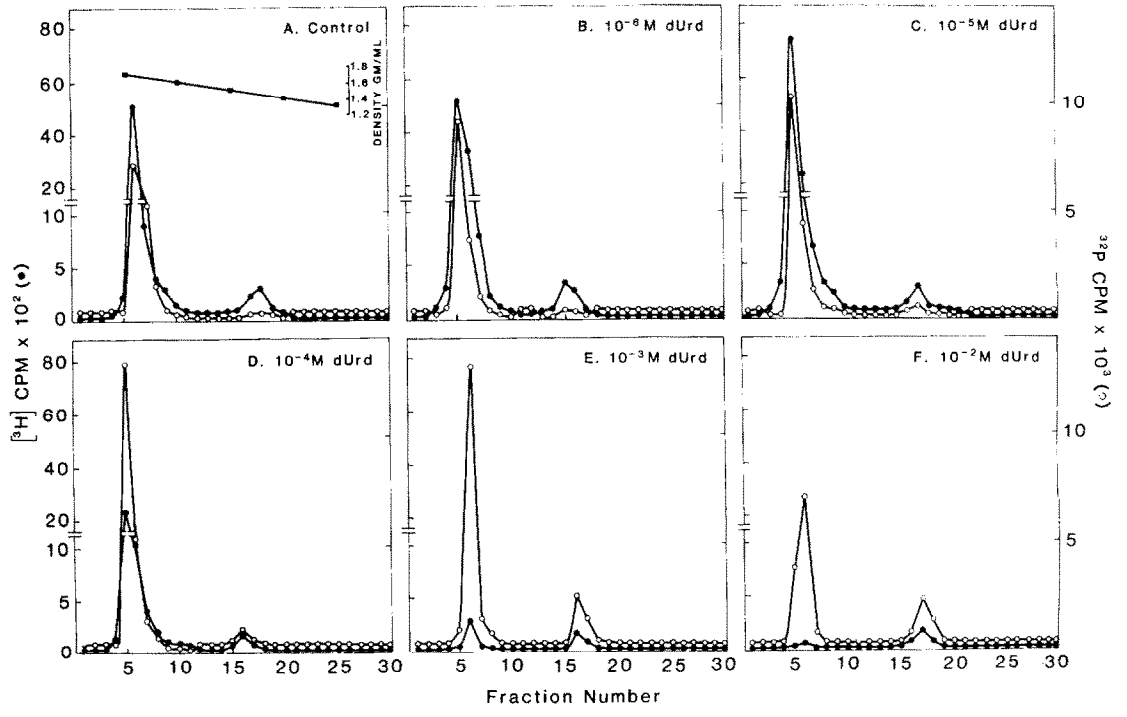

Fig. 1. Effect of dUrd on incorporation of tritium in nucleic acids of L1210 cells treated with [^{3}H]FdUrd. L1210 cells in logarithmic growth phase were incubated with 10^{-6} M [^{3}H] FdUrd and 10 μCi/ml ^{32}P for 6 hr. The total cellular nucleic acids from 10^6 cells were purified and analyzed by cesium sulfate gradient centrifugation (A). Cells were also treated with 10^{-6} to 10^{-2} M dUrd (B–F) for 1 hr prior to the addition of 10^{-6} M [^{3}H]FdUrd and ^{32}P for an additional 6 hr. The tritium radioactivity banding in the RNA region (between densities 1.62 and 1.68 g/ml) and the DNA region (between densities 1.42 and 1.48 g/ml) was then determined as a measure of FUra (pmoles) incorporation: (A) RNA: 0.468, DNA: 0.039; (B) RNA: 0.654, DNA: 0.052; (C) RNA: 0.754, DNA: 0.042; (D) RNA: 0.275, DNA: 0.024; (E) RNA: 0.026, DNA: 0.023; and (F) RNA: 0.006, DNA: 0.031. The detectable tritium radioactivity specifically represents FUra incorporation as determined by high pressure liquid chromatographic analysis [8] of these RNA and DNA fractions.

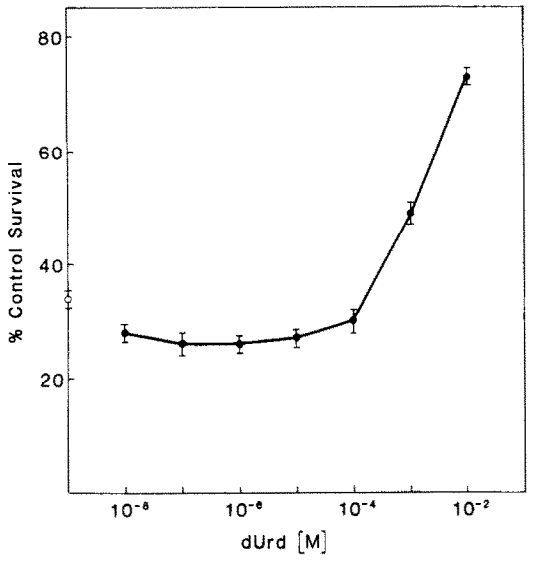

Fig. 2. Clonogenic survival of L1210 cells following exposure to FdUrd and dUrd. L1210 cells in logarithmic growth phase were exposed to 10^{-6} M FdUrd for 6 hr (○) or to dUrd (10^{-8} to 10^{-2} M) for 1 hr prior to the addition of 10^{-6} M FdUrd for an additional 6 hr (●). After the appropriate drug exposure, cells were washed and resuspended in drug-free medium without serum, prior to plating in 1.5% methylcellulose containing 20% fetal calf serum. Viability was determined after 7 days by scoring colonies greater than 50 cells. Percentage of viability was determined by the ratio of colonies formed by treated cells as compared to untreated cells × 100. Results are expressed as the means ± S.D. for three separate determinations.

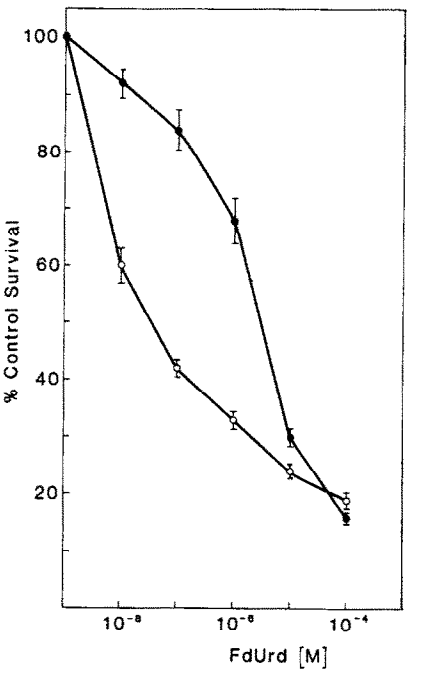

Fig. 3. Clonogenic survival of L1210 cells following exposure to 10^{-2} M dUrd and various concentrations of FdUrd. L1210 cells in logarithmic growth phase were exposed to various concentrations of FdUrd alone for 6 hr (○) or to 10^{-2} M dUrd for 1 hr prior to the addition of various concentrations of FdUrd for an additional 6 hr (●). Clonogenic survival was assayed according to the legend to Fig. 2.

with the effect of dUrd on the formation of (FUra)RNA following exposure to these concentrations of FdUrd.

In the absence of detectable (FUra)RNA formation, this cytotoxic effect observed with 10^{-6} M FdUrd could have been due to either incorporation of FdUrd in DNA or to inhibition of DNA synthesis. Figure 4 illustrates the effects of 10^{-3} and 10^{-2} M dUrd on RNA and DNA syntheses as measured by ^{32}P incorporation. Incubation of L1210 cells with dUrd (10^{-3} or 10^{-2} M) for 7 hr did not result in cytotoxicity and yet inhibition of DNA synthesis was similar to that obtained with 10^{-6} M FdUrd.

DISCUSSION

It has been demonstrated recently that FdUrd incorporates in eukaryotic DNA, including that

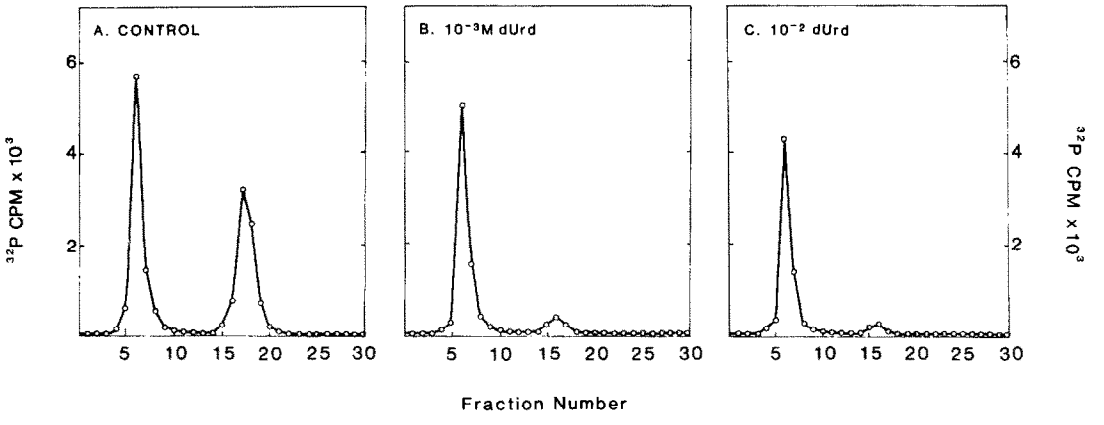

Fig. 4. Effect of dUrd on nucleic acid synthesis in L1210 cells. L1210 cells in logarithmic growth phase were incubated with 10 μCi/ml ^{32}P alone (A) and with 10^{-3} M dUrd (B) or 10^{-2} M dUrd (C) for 7 hr. Nucleic acids were purified and analyzed by cesium sulfate gradient centrifugation.

obtained with L1210 cells [8–13]. The biologic significance of this finding, however, has remained unclear, although the excision of the FUra residues by uracil-DNA glycosylase could result in DNA strand breaks or mutagenesis [14, 15, 17]. The present study was undertaken to determine whether FdUrd incorporation in DNA results in lethal cellular events.

Our previous studies demonstrated a highly significant relationship between incorporation of FUra in total cellular RNA and loss of clonogenic survival [8]. Identical results were obtained using thymidine to bypass inhibition of thymidylate synthesis [8]. This finding suggested that formation of (FUra)RNA and not inhibition of DNA synthesis is the major mechanism of cytotoxic action. In the present studies, we have employed deoxyuridine to inhibit formation of (FUra)RNA in FdUrd-treated cells. The deoxyuridine probably competed with FdUrd for pyrimidine nucleoside phosphorylase [17] and thereby significantly reduced the metabolism of FdUrd to FUra for incorporation of this residue into RNA. In contrast, this effect was not observed with either uridine or uracil.

The inhibition of (FUra)RNA formation resulted in a decrease in cytotoxicity and further supports the finding that a major mechanism of action of the fluorinated pyrimidines is through incorporation into RNA. However, losses in clonogenic survival exceeding 20% occur even in the absence of (FUra)RNA formation. This result suggests that cytotoxicity without (FUra)RNA formation is due to either inhibition of DNA synthesis or incorporation of FdUrd residues in DNA. Experiments with dUrd demonstrate that this agent can be employed to inhibit DNA synthesis at a level similar to that obtained by treating cells with 10^{-6} M FdUrd but without producing cytotoxic effects. These observations indicate that, under these experimental conditions, slowing of DNA synthesis itself is not sufficient to cause cell death and that the formation of (FdUrd)DNA may be responsible for the cytotoxic DNA effects with FdUrd. It will now be of interest to determine whether the misincorporation of FUra in DNA or the excision of these residues is responsible for cell lethality.

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