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Mechanism of cytotoxic activity of 5′-deoxy-5-fluorouridine

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Summary. *The purpose of these studies was to characterize the effect of the new fluoropyrimidine nucleoside 5′-deoxy-5-fluorouridine (5′dFUrd) on macromolecular processes in correlation with its cytotoxicity in Ehrlich ascites tumor cells. Following a 2-h exposure, 5′-dFUrd exhibited an LD_{50} (as determined by clonogenicity) of 48 μM. In cells supplemented with 10 μM dThd, the LD_{50} for 5′-dFUrd increased to 660 μM. DNA synthesis was markedly and rapidly suppressed by all cytotoxic concentrations of 5′-dFUrd. There was no apparent direct measurable effect of 5′-dFUrd on either RNA or protein synthesis, although both were suppressed 24 h after the drug exposure. Thymidylate synthetase activity was completely inhibited by all cytotoxic concentrations of 5′-dFUrd. FUra incorporation into RNA was also measured and appeared to correlate with the dThd-nonreversible toxicity of 5′-dFUrd. These studies indicate that the mechanism of 5′-dFUrd cytotoxicity is directly analogous to that reported for 5-fluorouracil. The inhibition of thymidylate synthetase leading to an inhibition of DNA synthesis was the most potent cytotoxic mechanism (i.e., dThd-reversible) for 5′-dFUrd, and was found to be highly time-dependent. Higher concentrations of 5′-dFUrd resulted in dThd-nonreversible toxicity, which appeared to be related to the incorporation of FUra into RNA.*

Introduction

5′-dFUrd, a new fluoropyrimidine nucleoside, is of current interest because of its demonstrated [1, 5] superior therapeutic antineoplastic activity in rodents compared with 5-fluorouracil (FUra), 5-fluorouridine (FUrd), 5-fluoro-2′-deoxyuridine (FdUrd), or N_1 (furanidyl)-5-fluorouracil (ftorafur). Previous studies suggested that 5′-dFUrd may have a cytotoxic mechanism similar to other fluoropyrimidines since it was found to be cleaved to FUra in sensitive tumor cells. Additionally, no other novel potentially toxic metabolites of 5′-dFUrd have been detected. Both an incorporation of FUra into RNA and an inhibition of thymidylate synthetase activity, the two reported cytotoxic mechanisms for FUra, result in cells exposed to 5′-dFUrd [1, 2]. While these events potentially should contribute to any resulting cytotoxicity from 5′-dFUrd, it is possible that other effects on macromolecular processes may result from this nucleoside analog. There has also been a recent report suggesting that 5′-dFUrd may have an alternative route of activation compared with FUra [7]. The purpose of the present study was to further clarify and establish the mechanisms of cytotoxicity from 5′-dFUrd.

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The abbreviations used in this paper are: 5′-dFUrd, 5′-deoxy-5-fluorouridine; FUra, 5-fluorouracil; FUTP, 5-fluorouridine-5′-monophosphate; FdUMP, 5-fluoro-2′-deoxyuridine-5′-monophosphate; UTP, uridine-5′-triphosphate; dGua, 2′-deoxyguanosine; dThd, thymidine; E.A., Ehrlich ascites

Materials and methods

Chemicals. 5′-dFUrd was supplied by Hoffmann-LaRoche, Inc., (Nutley, NJ) and was purified by reversed-phase liquid chromatography [1] prior to use. [^{3}H]-5′-dFUrd (16 mCi/μmol), [^{3}H]-cytosine (19 mCi/μmol), and [^{3}H]-leucine (61 mCi/μmol) were purchased from Moravek Biochemicals (City of Industry, CA, USA). [^{3}H]-Deoxyguanosine (18 mCi/μmol) was purchased from Amersham/Searle Corp. (Arlington Heights, IL, USA). Thymidine, deoxyuridine, uridine, and all other biochemicals, unless otherwise designated, were purchased from Sigma Chemical Co., (St Louis, MO, USA).

Cells and media. Ehrlich ascites (E.A.) tumor cells were maintained as a suspension spinner culture at 37° C in an atmosphere of 5% CO_2: 95% air. Cells were grown in a minimal essential medium with spinner salts supplemented with dialyzed fetal calf serum (100 ml/l), 1-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin (all obtained from Grand Island Biological Co., Grand Island, NY, USA). A Model ZBI Coulter Counter (Coulter Electronics, Inc., Hialeah, FA, USA) was used for cell quantitation, and viability was assessed by trypan-blue exclusion and soft agar cloning.

Drug toxicity studies. Exponentially growing cells were exposed to various concentrations of fluoropyrimidine for variable time periods. At the end of this time the cells were centrifuged (300 *g*) and the supernatant (containing any remaining drug) discarded. In an attempt to circumvent DNA-directed toxicity secondary to inhibition of thymidylate synthetase activity, 10 μ*M* dThd was added following the removal of 5′-dFUrd. Drug toxicity was assessed using a soft-agar cloning methodology described previously [6]. Viability was defined as the ability of a cell to produce progeny, which are visible as distinct individual clones or cell colonies. Percent viability (or clonal growth) was determined by the ratio of clonal growth of the treated cells compared to the clonal growth of untreated cells × 100.

Measurement of DNA, RNA, and protein synthesis and thymidylate synthetase activity. At designated times following the start of incubation, 5-ml cell aliquots were removed from the spinner flasks and placed in 15-ml centrifuge tubes which contained 5 μCi of [^{3}H]-cytidine, [^{3}H]-deoxyguanosine, or [^{3}H]-leucine. The tubes were capped and placed in a 37° C shaking water bath for 1 h, after which they were centrifuged and the supernatant discarded. The cell pellet was disrupted with 0.2 *N* perchloric acid, the acid-soluble fraction discarded, and the acid-insoluble RNA, DNA and protein fractions isolated as described previously [4]. The RNA fraction extracted from the cells exposed to [^{3}H]-cytidine was divided into two parts, with one part measured for radioactivity by liquid scintillation counting and the other part assayed for RNA content by the orcinol reaction [12]. The DNA fraction from cells exposed to [^{3}H]-deoxyguanosine was treated similarly, but with quantitation of DNA by the diphenylamine reaction [12]. Protein from cells incubated with [^{3}H]-leucine was isolated, with measurement of radioactivity and quantitation of protein by a modified Lowry assay [10]. Incorporation of [^{3}H]-cytidine into RNA was used as a measure of RNA synthesis, incorporation of [^{3}H]-deoxyguanosine into DNA as a measure of DNA synthesis, and incorporation of [^{3}H]-leucine into protein as a measure of protein synthesis. The results are expressed as: % Synthesis (or activitiy) = (dpm/μg RNA, DNA or protein in control cells) × 100. Thymidylate synthetase activity was measured by a modified tritium-release assay that we have described in detail in other publications [2, 3].

Determination of FUra incorporation into RNA. [^{3}H]-5′-dFUrd (10 mCi/mmol) was substituted for the unlabeled 5′-dFUrd under the incubation conditions described above. At various times during the incubation period aliquots were removed, added to 15-ml centrifuge tubes containing iced saline, and immediately centrifuged. The cell pellet was isolated, washed twice with iced saline, and the RNA extracted and analyzed as described above. The results are expressed as pmol FUra per μg RNA.

Results

Cytotoxic activity of 5′-dFUrd

Figure 1 illustrates the clonal growth of E.A. cells following a 2-h exposure to 1, 10, 100, or 1,000 μ*M* 5′-dFUrd. Cells were cloned both with and without the presence of 10 μ*M* dThd, as indicated. 5′-dFUrd exhibited a LD_{50} of 48 μ*M* under these conditions without dThd supplementation, and had a dThd-nonreversible LD_{50} of 660 μ*M*. (The present studies were completed with 5′-dFUrd purified prior to use by HPLC. We have found that 5′-dFUrd used without this purification is more toxic, and the increased cytotoxicity is totally dThd-nonreversible.)

Effect of 5′-dFUrd on DNA, RNA and protein synthesis and thymidylate synthetase activity

Thymidylate synthetase activity and DNA synthesis in cells from 2 to 96 h following a 2-h exposure to 5′-dFUrd is illustrated in Fig. 2A (thymidylate synthetase activity) and B (DNA synthesis). Thymidylate synthetase activity was completely inhibited by the 10, 100, and 1,000 μ*M* concentrations of 5′-dFUrd following the completion of the 2-h exposure

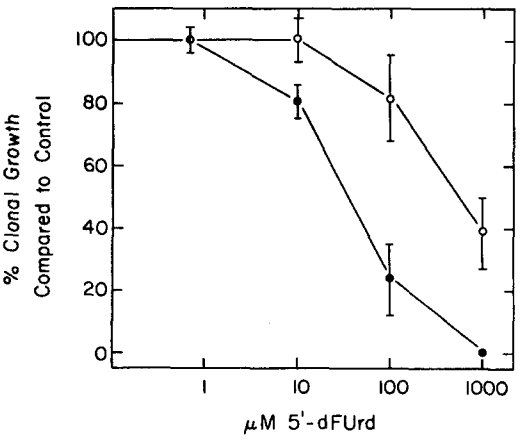

Fig. 1. Clonal growth of Ehrlich ascites tumor cells exposed to 5′-dFUrd. Cells were incubated for 2 h with different concentrations of 5′-dFUrd and then cloned in soft agar either with (○) or without (●) 10 μ*M* dThd. *Bars* ± SD ($n = 4$)

period, although the inhibition was not maintained in cells treated with 10 μ*M* 5′-dFUrd, where a rapid and full recovery of thymidylate synthesis began by 12–24 h. Some recovery of thymidylate synthetase activity was also observed by 48 h in cells treated with 100 μ*M* 5′-dFUrd, although enzyme activity was only 28% of control values at 96 h. Only a 75% initial suppression of thymidylate synthetase activity, which was followed by full recovery, resulted in cells exposed to 1 μ*M* 5′-dFUrd. DNA synthesis was similarly assessed, and the results are illustrated in Fig. 2B. DNA synthesis was 62%–72% suppressed by the end of a 2-h exposure with 10, 100, or 1,000 μ*M* 5′-dFUrd. DNA synthesis was further suppressed to 2%–3% of control with over 1,000 μ*M* 5′-dFUrd. At 100 μ*M* 5′-dFUrd, DNA synthesis dropped to 5%, exhibiting recovery to 18% at 96 h. In cells treated with 10 μ*M* 5′-dFUrd, DNA synthesis was reduced to 15% of control but then rapidly recovered. RNA synthesis and protein synthesis were also assessed in these cells. None of the concentrations of 5′-dFUrd used appeared to directly alter either RNA or protein synthesis, as a 100% rate of synthesis was detected for both RNA and protein at the immediate completion of the 2-h drug exposure. There was, however, measurable suppression (30%–50%) of both protein and RNA synthesis at 24 h in cells treated with 100 μ*M* and 1,000 μ*M* 5′-dFUrd.

Effect of exposure time on 5′-dFUrd cytotoxicity

Figure 3A illustrates the clonal growth of E.A. cells following 2, 6, 12, or 24 h exposure to 10 μ*M* 5′-dFUrd cloned both with and without dThd supplementation. Cytotoxicity at this concentration of 5′-dFUrd was highly time-dependent, and the inhibition of clonal growth that resulted with the longer incubation periods was almost entirely reversible with dThd. Figure 3B depicts thymidylate synthetase activity in these cells following 2, 6, 12, or 24 h exposure to 5′-dFUrd. As we observed in Fig. 2A, the 2-h exposure resulted in only a temporary inhibition of enzyme activity. However, with increasing lengths of exposure, the recovery of thymidylate synthetase activity was increasingly delayed: the 24-h exposure maintained a complete inhibition of enzyme activity for 96 h.

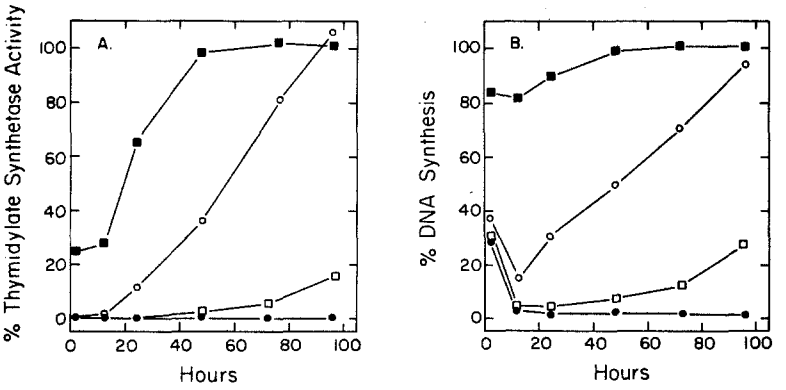

Fig. 2A and B. Effect of 5′-dFUrd on thymidylate synthetase activity (**A**) and DNA synthesis (**B**). Ehrlich ascites tumor cells were exposed for 2 h to 1 μ*M* (■), 10 μ*M* (○), 100 μ*M* (□), or 1,000 μ*M* (●) 5′-dFUrd and then resuspended in drug-free medium. At times from 2 to 96 h cells were assayed for thymidylate synthetase activity and DNA synthesis as described under *Materials and methods*. These results are representative of three separate experiments

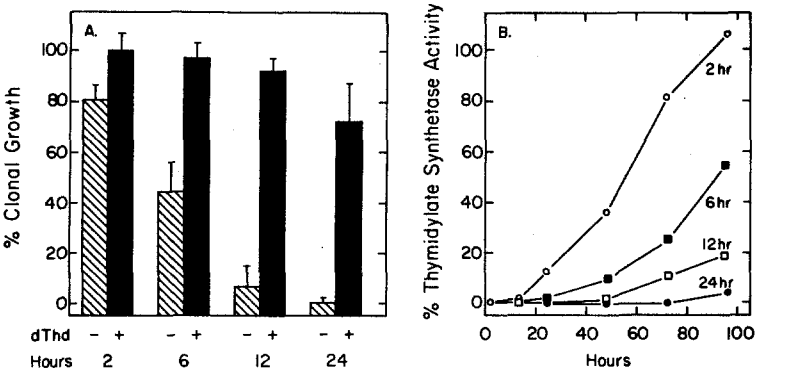

Fig. 3A and B. Cells exposed for 2–24 h to 10 μ*M* 5′-dFUrd. Ehrlich ascites tumor cells were exposed to 10 μ*M* 5′-dFUrd and either cloned with (+) or without (−) is the addition of 10 μ*M* TdR (**A**), or assayed for thymidylate synthetase activity (**B**). Incubation times: 2 h (○), 6 h (■), 12 h (□), or 24 h (●). *Bars* ± SD ($n = 3$)

Incorporation of FUra into RNA

The exposure of cells to [^{3}H]-5′-dFUrd for 2 h resulted in a concentration-dependent level of FUra incorporation into RNA. The respective levels of incorporation are listed as follows: 10 μ*M* 5′-dFUrd = 0.26 pmol FUra/μg RNA; 100 μ*M* 5′-dFUrd = 1.1 pmol FUra/μg RNA, and 1,000 μ*M* 5′-dFUrd = 5.2 pmol FUra/μg RNA.

Discussion

These studies have examined the mechanism of cytotoxicity for 5′-dFUrd in E.A. tumor cells. Toxicity from FUra and other fluoropyrimidines has been previously characterized as two-fold: (1) dThd-reversible, resulting from the inhibition of thymidylate synthetase by FdUMP, which leads to a depletion of cellular thymidylate and the subsequent inhibition of DNA synthesis; and (2) dThd-nonreversible, which has been speculated to results from the incorporation of FUra into RNA [8, 9, 13]. FUra incorporation into RNA can lead to a disruption of RNA function and maturation [8, 13] without directly altering synthesis and may be a mechanism of cytotoxicity. Supplementation of cells with dThd can bypass the FdUMP block of thymidylate synthetase, restore thymidylate pools, and circumvent this mode of fluoropyrimidine toxicity, without altering the so-called RNA-directed mode. The results of the present studies demonstrate that all cytotoxic concentrations of 5′-dFUrd produce a complete inhibition of thymidylate synthetase activity and cause a rapid and marked inhibition of DNA synthesis. DNA synthesis was the only macromolecular process that appeared to be acutely affected by 5′-dFUrd. Although both RNA and protein synthesis were suppressed 48 h following drug exposure, this was probably secondary to the rapid and sustained inhibition of DNA synthesis produced by these concentrations of 5′-dFUrd. A 2-h exposure to 10 μ*M* 5′-dFUrd also resulted in an apparent complete inhibition of thymidylate synthetase activity, although it produced only minimal toxicity. This could be incorrectly interpreted to suggest that the inhibition of thymidylate synthetase would not be a mediating factor in 5′-dFUrd cytotoxicity. However, it is important to note that the thymidylate synthetase inhibition produced by 10 μ*M* 5′-dFUrd, although initially complete, was not maintained over time, and enzyme activity recovered completely. Cells exposed for 2 h to 10 μ*M* 5′-dFUrd did exhibit a temporary cytostatic effect (data not shown), but had only a minimal loss of clonogenicity. However, with the longer exposure periods of cells to 10 μ*M* 5′-dFUrd, as well as with higher drug concentrations, a maintained inhibition of thymidylate synthetase was the most potent mediating event in the cytotoxicity of 5′-dFUrd, and was also found to be highly time-dependent. This distinguished this mode of toxicity from dThd-nonreversible toxicity, which appeared to be primarily concentration-dependent, and contributed to toxicity only at concentrations exceeding 100 μ*M*. With increasing drug concentrations, increasing dThd-nonreversible toxicity resulted and the degree of dThd-nonreversible toxicity directly correlated with the level of FUra incorporation into RNA, although the synthesis of RNA was not inhibited. These concepts were illustrated with 10 μ*M* 5′-dFUrd, which exhibited an 80% increase in cytotoxic activity from a 2-h to a 24-h exposure. This increased toxicity was almost entirely reversible with dThd, as the dThd-nonreversible toxicity only slightly increased, a fraction of which may have been due simply to the 24-h thymidylate depletion. These results are all analogous to what has been reported for FUra and FUrd [8–10, 13], thereby further establishing that 5′dFUrd cytotoxicity is probably dependent upon its cleavage to FUra.

In conclusion, it appears that 5′-dFUrd cytotoxicity is related primarily to an inhibition of thymidylate synthetase. However, it is important to note that at higher concentrations a

dThd-nonreversible mechanism contributes to cytotoxicity, which may be the result of an incorporation of FUra into RNA and subsequent alteration of RNA function. As a result, 5′-dFUrd should be toxic to cells resistant to thymidylate synthetase inhibition, although higher concentrations of 5′-dFUrd would be required. Although the results presented are only from one cell line, we have completed identical studies in the sarcoma-180 murine tumor line, and partial studies in several other murine and human tumor lines, all of which gave similar results. From this, we feel the conclusions made here should generally be true for 5′-dFUrd in most mammalian cell lines.

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