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EVIDENCE THAT 5'-DEOXY-5-FLUOROURIDINE MAY NOT BE ACTIVATED BY THE SAME MECHANISM AS 5-FLUOROURACIL

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5'-Deoxy-5-fluorouridine (5'dFUrd; Roche 21-9738) has been reported to be a more effective antitumor agent with less toxicity than 5-fluorouracil (FUra), 5-fluoro-2'-deoxyuridine and 5-fluorouridine [1,2]. It has been suggested that 5'-dFUrd is a prodrug from which FUra is generated by cellular nucleoside phosphorylase [1,2]. FUra was found to accumulate more selectively in tumor tissue when 5'-dFUrd was administered than when FUra was given to the animals [3]. Further, 5'-dFUrd was found to be less immunosuppressive than FUra [4].

FUra has been shown to be converted to FUTP with subsequent incorporation into RNA [5,6] and to 5-fluoro-2'-deoxyuridine 5'-monophosphate (FdUMP), a potent inhibitor of thymidylate synthetase [7]. The incorporation of FUra into RNA has been shown to interfere with the maturation of ribosomal RNA [7]. The cytotoxicity of FUra was reversed by uridine, suggesting that the incorporation of FUra into RNA was one of the cytotoxic mechanisms [8,9].

It was observed that a Novikoff hepatoma cell line (N1-S1/FdUrd) which lacked thymidine kinase and therefore was resistant to 5-fluoro-2'-deoxyuridine [10] was also resistant to FUra [8]. The basis for the resistance to FUra in the N1-S1/FdUrd cells was found to be the lack of formation of FUra ribonucleotides [8]. Further studies showed that the resistance to FUra in the N1-S1/FdUrd cell line could be reversed by the presence of inosine in the culture medium [9,11].

In the presence of inosine, FUra was converted to FUra ribonucleotides, and there was increased incorporation into the RNA of these cells. This combination inhibited ribosomal RNA maturation and inhibited tumor cell growth in culture. There was no evidence that FUra plus inosine led to the formation of FdUMP [9].

This cell line, N1-S1/FdUrd, offered an excellent test system in which to compare the effects of FUra and 5'-dFUrd on several biochemical parameters and on tumor cell growth. In this report we present data to show that, in this mutant Novikoff hepatoma cell line (N1-S1/FdUrd), FUra and 5'-dFUrd are not activated in the same manner since inosine does not potentiate the effects of 5'-dFUrd.

The Novikoff hepatoma cell line (N1-S1/FdUrd) was obtained from Dr. Van R. Potter, McArdle Laboratory for Cancer Research, Madison, WI [10]. The cells were grown in Medium S-69 supplemented with calf serum and Pluronic F-68 as previously described [8]. The incorporation of [^{14}C]FUra and [^{3}H]5'-dFUrd, in the absence and presence of inosine, into the acid-soluble and RNA fractions was determined as previously described [12]. The acid-soluble fractions were analyzed by HPLC to determine the formation of nucleotide derivatives [11]. The 5'-deoxy-5-fluorouridine and the [6-^{3}H]5'-dFUrd were gifts from Hoffmann-LaRoche, Inc., Nutley, NJ, and Dr. Youcef Rustum of Roswell Park Memorial Institute, Buffalo, NY, respectively. [^{14}C]FUra was purchased from Moravek Biochemical, City of Industry, CA.

As seen in Table 1, inosine (1 mM) increased the incorporation of FUra into the acid-soluble fraction (nucleotide fraction) 1.7-fold while increasing the incorporation of FUra into RNA 4.5-fold. Under the same conditions, inosine had essentially no effect on the uptake of 5'-dFUrd into the acid-soluble fraction and incorporation of FUra from [6-^{3}H]-5'-dFUrd into the RNA. When the nucleotide pools were analyzed for distribution of mono-, di- and triphosphate derivatives of FUra, it was found that inosine markedly stimulated the formation of FUTP from FUra as previously reported [9].

Table 1. Effects of inosine on incorporation of [^{14}C]fluorouracil and [^{3}H]5'-deoxy-5-fluorouridine into the acid-soluble and RNA fractions of N1-S1/FdUrd cells*

Treatment	Acid-soluble	RNA
	(cmp/mg RNA x 10^{-3})	
[^{14}C]FUra	87.2	28.9
[^{14}C]FUra + inosine, 1 mM	151.1	130.1
[^{3}H]dFUrd	43.6	4.7
[^{3}H]dFUrd + inosine, 1 mM	49.6	3.4

*N1-S1/FdUrd cells (20 x 10^6 cells) were incubated in the presence of [^{14}C]FUra (2.5 μCi, 56 mCi/mmole) or [^{3}H]dFUrd (2.5 μCi, 750 mCi/mmole) in the presence or absence of inosine (1 mM) for 2 hr. The cells were harvested by centrifugation, washed and treated by the Schmidt-Thannhauser procedure [12] to separate the acid-soluble and RNA fractions. The flasks were set up in triplicate.

As seen in Fig. 1, in the presence of inosine in the culture medium FUra was converted to FUTP. Approximately 53% of the FUra in the acid-soluble fraction was found as FUTP when inosine was present. In the absence of inosine very little FUTP was generated. With [6-^{3}H]5'-dFUrd as the precursor, there was very little generation of FUTP in the acid-soluble fractions of the cells incubated with [6-^{3}H]5'-dFUrd alone (Fig. 2, panel A) or with [6-^{3}H]5'-dFUrd plus inosine (Fig. 2, panel B). The distribution of FUra metabolites in the presence of inosine was essentially identical to the pattern seen for 5'-dFUrd alone. Greater than 90% of the radioactivity in the acid-soluble fractions eluted from the column in the peak fraction in which FUra, FUrd and 5'-dFUrd would elute under the conditions used.

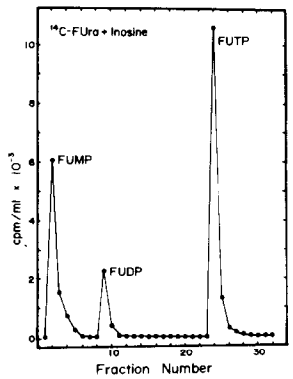

Fig. 1. Formation of fluorouracil nucleotides in N1-S1/FdUrd hepatoma cells from [^{14}C]-fluorouracil. The acid-soluble fractions, prepared in the experiment described in Table 1, were neutralized, lyophilized and dissolved in a small volume of H_2O (200 μl). The samples were analyzed by HPLC on a Partisil SAX column as previously described [11]. This figure shows the FUra metabolite profile obtained from cells incubated in the presence of [^{14}C]FUra and inosine (1 mM). Under the conditions used with the HPLC, FUra, FUrd and FUMP elute in the same peak.

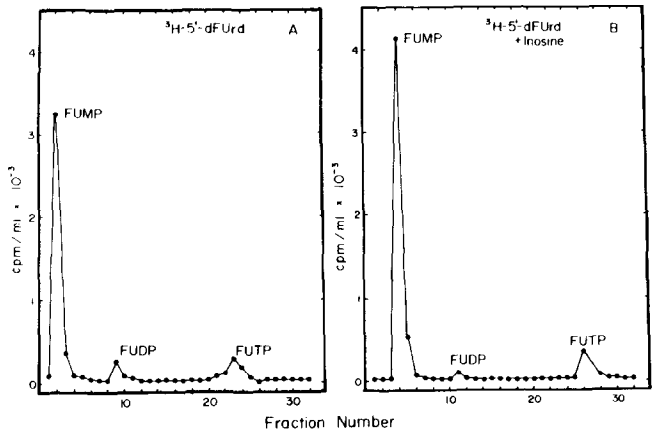

Fig. 2. Formation of fluorouracil nucleotides in N1-S1/FdUrd hepatoma cells from [^{3}H]-5'-deoxy-5-fluorouridine. The acid-soluble fractions were prepared and analyzed as described in Fig. 1. Panel A shows the FUra metabolite profile in the N1-S1/FdUrd cells incubated in the presence of [^{3}H]dFUrd alone. Panel B shows the FUra metabolite profile obtained from cells incubated in the presence of [^{3}H]dFUrd and inosine (1 mM).

The levels of pyrimidine nucleoside phosphorylase, uracil phosphoribosyl transferase, PRPP and ribose-1-phosphate were compared in both the wild type N1-S1 and mutant N1-S1/FdUrd Novikoff hepatoma cell lines and were found to be the same (data not shown). In both the N1-S1 and N1-S1/FdUrd Novikoff hepatoma cell lines, FUra was a better substrate than Ura in the uracil phosphoribosyl transferase reaction. These data indicate that, at least in these FUra-resistant cells, the metabolic activation of these 5-fluoropyrimidines is not limited by decreased enzymes or ribose donors.

[^{14}C]Fluorouridine was taken up to the same extent by both the sensitive (N1-S1) and resistant (N1-S1/FdUrd) Novikoff hepatoma cell lines. The incorporation of [^{14}C]-fluorouracil into the RNA was also the same. Inosine did not stimulate the incorporation of [^{14}C]FUrd into the RNA in either of the cell lines. These data are summarized in Table 2. In the N1-S1/FdUrd cell line greater than 70% of the radioactivity from [^{14}C]FUrd was found as FUTP in the nucleotide pool when analyzed by HPLC. Inosine did not alter the distribution of FUra metabolites in the acid-soluble pool when FUrd was the labeled precursor. These data indicate that uridine kinase is not a limiting factor in the activation of the fluoroprymidines in these cells.

Table 2. Effect of inosine on incorporation of [^{14}C]fluorouridine into the acid-soluble and RNA fractions of Novikoff hepatoma cells*

Cell Line	Acid-soluble (cmp/mg RNA x 10^{-3})	RNA
N1-S1	1020	478
+ inosine	865	480
N1-S1/FdUrd	634	573
+ inosine	506	566

*Novikoff hepatoma cells (30 x 10^6 cells) were incubated in the presence of [^{14}C]-FUrd (2.5 μCi, 52 mCi/mmole) in the presence or absence of inosine (1 mM) for 2 hr. The cells were treated as described in Table 1. The flasks were set up in triplicate.

The N1-S1/FdUrd cells grew normally in the presence of FUra (0.01 mM) (Fig. 3, panel A). The growth of the wild type N1-S1 Novikoff hepatoma cells was completely inhibited by this concentration of FUra. However, in the presence of the combination of FUra (0.01 mM) plus inosine (1 mM), there was essentially complete inhibition of tumor cell growth. Inosine alone, at this concentration, had no effect on the growth of the

Nl-Sl/FdUrd cells. On the other hand, 5'-dFUrd even at the higher concentration (0.1 mM) in combination with inosine (1 mM) did not alter the cell growth pattern observed with 0.1 mM 5'-dFUrd (Fig 3, panel B). The growth of the cells was only partially inhibited by 5'-dFUrd at a concentration of 1 mM.

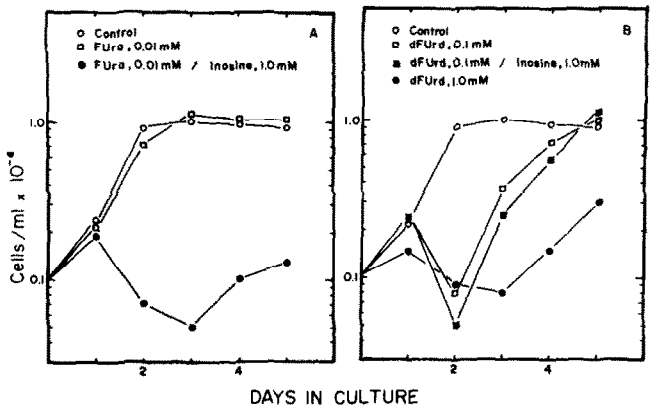

Fig. 3. Effects of inosine and fluorouracil or 5'-deoxy-5-fluorouridine on the growth of Nl-Sl/FdUrd Novikoff hepatoma cells. Nl-Sl/FdUrd cells were grown in culture in the presence of FUra and inosine (panel A) or in the presence of 5'-dFUrd and inosine (panel B). All cultures were set up in triplicate.

The incorporation of FUra into the RNA of the sensitive Novikoff hepatoma cell line (Nl-Sl) was much greater than the incorporation of FUra into the RNA of the Nl-Sl/FdUrd cell line. However, when inosine was added to the culture medium, the level of FUra incorporation increased to that seen in the sensitive cell line [9]. This could be correlated with the effect of inosine on the increased formation of FUTP in the nucleotide pool [9]. On the other hand, there was no difference in the level of incorporation of FUrd into the acid-soluble or RNA fractions between the Nl-Sl and Nl-Sl/FdUrd cell lines. Further, the uptake and incorporation of the FUrd into RNA was not stimulated by inosine in contrast to the observed effects of inosine on FUra uptake and incorporation into RNA. These data would suggest that the phosphorylation of FUrd is not a limiting factor in the metabolism of the fluoroprymidines.

These data suggest that the metabolism of FUra and 5'-dFUrd to the active metabolites (FUTP and FdUMP) does not proceed through identical pathways. It may be that the mechanism by which 5'-dFUrd is activated is more prevalent in tumor cells leading to increased tumor cell kill and reduced host toxicity which has been observed [1-4].

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