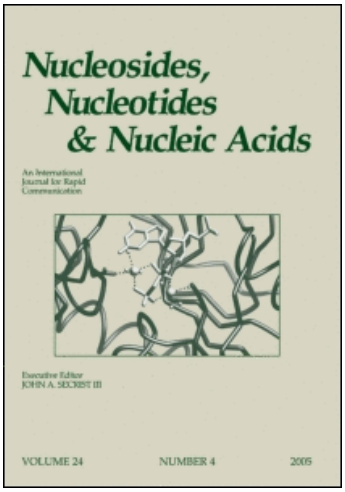

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INHIBITORY MECHANISMS OF 1-(3-*C*-ETHYNYL-β-D-*RIBO*-PENTOFURANOSYL)URACIL (EUrd) ON RNA SYNTHESIS

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▫ *1-(3-C-ethynyl-β-D-ribo-pentofuranosyl)uracil (EUrd) is an antimetabolite that strongly inhibits RNA synthesis and shows a broad antitumor activity in vitro and in vivo. In mouse mammary tumor FM3A cells, EUrd is sequentially phosphorylated to its 5′-triphosphate, EUTP, a major metabolite, and the RNA synthesis is inhibited proportionally to its intracellular accumulation. To study the inhibitory mechanisms of EUrd on RNA synthesis, we have performed the kinetic analysis of EUTP on RNA polymerization using isolated nuclei. RNA synthesis was inhibited competitively by EUTP. The inhibition constant, K_i was much lower than the K_m value of UTP (K_i value of EUTP, 84 nM; K_m value of UTP, 13 μM), indicating that the high affinity of EUTP could contribute to the specific inhibition of RNA synthesis. As a result of RNA synthesis inhibition, EUrd, but not ara-C, induced shrinkage of nucleoli, which are the main sites for RNA synthesis in FM3A cells. Thus, the strong affinity of EUTP to RNA polymerase and specific inhibition of RNA synthesis could contribute to its antitumor effect. EUrd is expected to be a new antitumor drug, possessing a strong inhibitory effect on the synthesis of RNA.*

Keywords EUrd, Inhibitor of RNA Synthesis, Nucleolus

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INTRODUCTION

1-(3-*C*-Ethynyl-β-D-*ribo*-pentofuranosyl)uracil (EUrd) is a uridine analog, which has an acetylene group at the 3′-*C*-carbon (Figure 1). EUrd showed a potent and broad-spectrum antitumor activity against various human tumor cells, both in vitro and in vivo.[1,2] The cytotoxicity of EUrd is caused by the inhibition of RNA synthesis. In FM3A cells, RNA synthesis was decreased to 16.8% of control levels by the treatment with 11 μM EUrd for 4 h. On the other hand, DNA synthesis was not a primary target of EUrd, and slightly decreased to 86.4% of control under the same condition.[2] EUrd is sequentially phosphorylated to its 5′-triphosphate form, a major product, EUrd 5′-triphosphate (EUTP), and then, EUTP is gradually converted to 1-(3-*C*-ethynyl-β-D-*ribo*-pentofuranosyl)cytosine (ECyd) 5′-triphosphate (ECTP). We have found that ECTP has a strong inhibitory activity against RNA polymerase, particularly when CTP is used as a substrate (details will be published elsewhere). We considered that the conversion of EUrd to two different strong inhibitors of RNA synthesis, namely EUTP and ECTP, could contribute to the potent inhibition of RNA synthesis. To evaluate the contribution of EUTP to the inhibition of RNA synthesis, here, we have studied the kinetics of RNA synthesis inhibition by EUTP using isolated nuclei. Moreover, we have analyzed the changes occurring in the nucleoli, which are the main intracellular sites for RNA synthesis, in EUrd treated cells.

MATERIALS AND METHODS

Materials

EUrd was synthesized as described previously.[1] 1-β-D-arabinofuranosylcytosine (ara-C) was purchased from Sigma-Aldrich Co. (St. Louis, MO). ATP, CTP, UTP and azure C were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). [8-^{3}H]GTP was obtained from Amersham Biosciences Corp. (Piscataway, NJ).

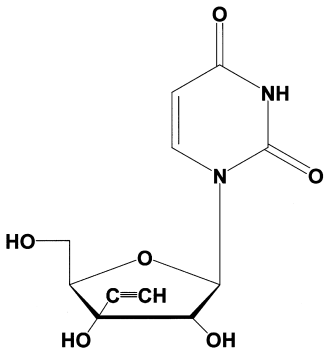

FIGURE 1 Structure of EUrd.

Cell Culture

FM3A (F28-7) mouse mammary tumor cell line was obtained from the Japanese Cancer Research Resources Bank. FM3A cells were maintained in ES medium (Nissui Pharmaceutical Co., Tokyo, Japan) containing 2% heat-inactivated fetal bovine serum (Gibco BRL) and cultured at 37°C in a humidified atmosphere containing 5% CO_2.

Kinetic Analysis of RNA Polymerization in Isolated Nuclei

Nuclei of FM3A cells were prepared as described previously.[2] In brief, FM3A cells (1×10^8) were spun down, washed twice in ice-cold PBS, resuspended in 10 ml of buffer I (0.32 M sucrose, 2 mM $MgCl_2$, 3 mM $CaCl_2$, 0.1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 1 mM spermidine, 0.1% Triton X-100, 10 mM Tris-HCl at pH 8), and homogenized. An equal volume of buffer II (2 M sucrose, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 1 mM spermidine, 10 mM Tris-HCl at pH 8) was added to the homogenates, then one-third volume of buffer II was layered onto them and centrifuged for 45 min at 30,000 × *g*. The pellets were resuspended in buffer III (25% glycerol, 0.1 mM EDTA, 5 mM DTT, 0.1 mM PMSF, 1 mM spermidine, 50 mM Tris-HCl at pH 8), and stored at −80°C. Isolated nuclei (1×10^7 nuclei/ml) were incubated with or without EUTP in the reaction mixture (7 mM Tris, 120 μM KCl, 3.1 mM $Mg(AcO)_2$, 25 μM *S*-adenosylmethionine, 500 μM ATP, 500 μM CTP, 50 μM [8-^{3}H])GTP, and 6 to 18 μM UTP) at 25°C. At indicated time intervals, the aliquots were removed, spotted on 3 mm filters (Whatman), washed with 5% TCA, rinsed twice with acetone and quantitated by liquid scintillation counting. The initial incorporation rates of radioactivity into the acid-insoluble fraction were measured by the method of Marzluff and Huang.[3]

RESULTS

EUTP is a Strong and Competitive Inhibitor of RNA Polymerase

Previously, we found that EUrd was phosphorylated to EUTP and inhibited RNA synthesis without decrease of the ribonucleoside triphosphate pool.[2] Therefore, RNA synthesis was assumed to be inhibited at the RNA polymerization step. To determine the inhibitory mechanism of RNA synthesis more precisely, we have evaluated the inhibition of RNA synthesis by EUTP. The experiments were performed in the presence of isolated nuclei from FM3A cells to kinetically characterize the incorporation of UTP and to determine the inhibitory effect of EUTP. The results presented in the form of Lineweaver-Burk (Figure 2A) indicate that EUTP inhibited UTP incorporation (K_m; 13 μM) into RNA in a competitive manner. The apparent K_i value for the inhibition of RNA polymerization by EUTP was 84 nM as derived form the Dixon plot analysis (Figure 2B). Taking into account the value of apparent K_m for UTP of 13 μM and the K_i for EUTP of 84 nM, EUTP appeared to be a potent competitive inhibitor of RNA synthesis.

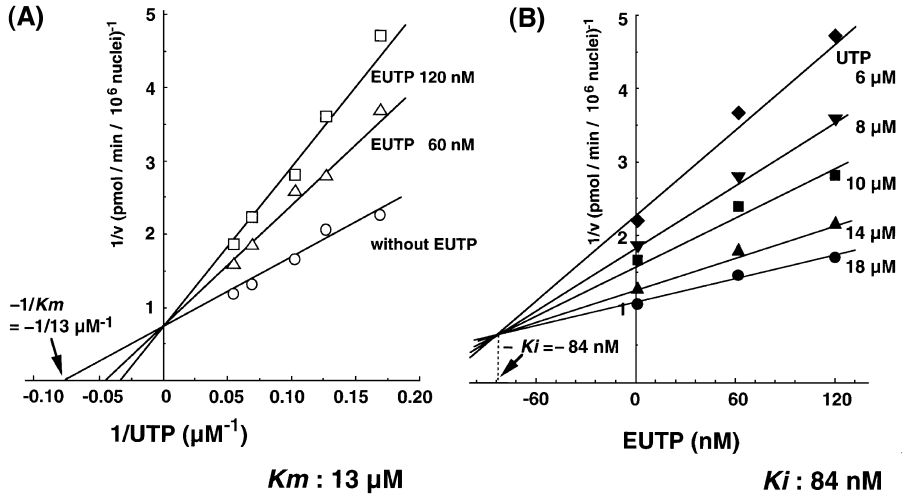

FIGURE 2 Kinetic analysis of the effect of EUTP on RNA polymerase in isolated nuclei. The reaction mixtures contained 500 μM each of ATP and CTP, and 50 μM [8-^{3}H]GTP, and the indicated concentration of UTP and EUTP. The mixtures were incubated with isolated nuclei (1×10^7 nuclei/ml) at 25°C, spotted on 3 mm filters (Whatman), washed with 5% TCA, and rinsed twice with acetone and quantitated by liquid scintillation counting. The K_m value for UTP and the K_i value for EUTP were determined by Lineweaver-Burk plot (A) and Dixon plot (B), respectively. *Points*, means of triplicate analysis with SDs of less than 5%.

EUrd Induced Nucleolar Shrinkage Resulting from the Inhibition of RNA Synthesis

To study the effect of EUrd on cancer cells, we observed nucleoli, which is the main intracellular site for RNA synthesis. By the treatment with 11 μM EUrd, typical nucleolus shrinkage resulting from the inhibition of RNA synthesis was

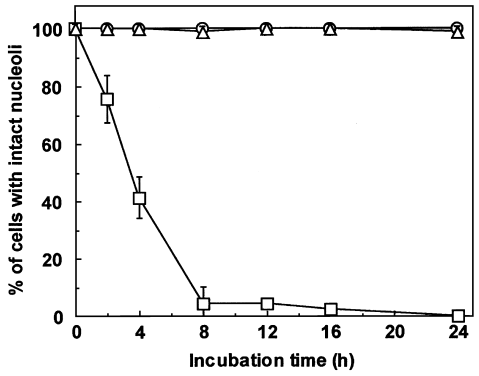

FIGURE 3 EUrd caused nucleolar shrinkage on FM3A cells as a result of RNA synthesis inhibition. Changes of the percentage of the cells with intact nucleoli were evaluated with azure C staining in FM3A cells as described in Ayres.[6] The state of nucleoli was evaluated in more than 1000 cells. Percent of cells with intact nucleoli = A/B × 100, where A is the number of cells with intact nucleoli. B is total cell number excluding mitotic cell number. □, EUrd (11 μM); △, Ara-C (44 μM); ○, Control.

observed within 2 h in FM3A cells. The population of cells with intact nuclei decreased to 60% within 2 h by the treatment with 11 μM EUrd. On the other hand, these morphological changes in the nucleoli were not observed even following 24 h treatment with 44 μM ara-C, a known inhibitor of DNA synthesis (Figure 3).

DISCUSSION

In our experiments, we have found that EUTP appeared to be a potent competitive inhibitor of RNA polymerase. The inhibition constant, K_i, was much lower than the K_m value of UTP (K_i value of EUTP, 84 nM; K_m value of UTP 13 μM) (Figures 2A and 2B), indicating that EUTP has a high affinity to RNA polymerase and, therefore, to exert a potent inhibition of RNA synthesis. When FM3A cells were treated with 11 μM EUrd for 4 h, the intracellular concentration of EUTP was only one ninth of that of UTP; however, it was high enough to inhibit RNA due to a high affinity to a target enzyme.[2] It was shown that the inhibitory manner of EUTP was competitive (Figure 2A). These characteristics could support a hypothesis that EUrd inhibited RNA synthesis without affecting the ribonucleoside triphosphate pool.[2] Moreover, intracellular EUTP is gradually converted in FM3A cells to its cytidine form, ECTP. The conversion of EUTP to ECTP would be mediated by CTP synthase, because both EUTP and ECTP were produced in EUrd treated cells, whereas EUTP was hardly found in ECyd treated cells.[2] ECTP is also a strong competitive inhibitor of RNA synthesis in respect to CTP (K_i value of ECTP, 20 nM; K_m value of CTP 7.6 μM, details will be published elsewhere). We consider EUrd possess a dual inhibitory effect on RNA synthesis resulted from the formation of two distinct and active metabolites, EUTP and ECTP, strongly contributing to the inhibition of overall RNA synthesis (Figure 4). EUrd induced nucleolar shrinkage followed the inhibition of RNA synthesis (Figure 3). Nucleoli are the main sites for RNA synthesis. The potent antitumor activity of EUrd is due to its strong inhibition of RNA synthesis and dysfunction of nucleoli in cancer cells. Both EUrd and ECyd are activated by the same nucleoside kinase, uridine cytidine kinase,[4] which determines their similar inhibitory activity spectrum in vitro.[1] However, their uptake into cancer cells might be different, because uridine is also a

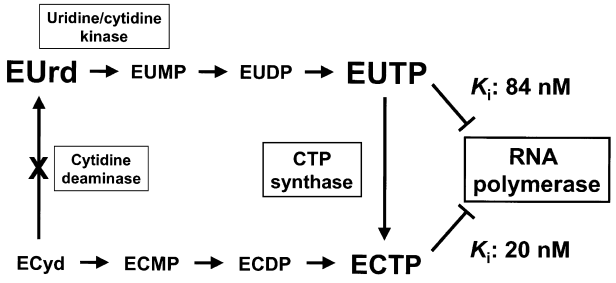

FIGURE 4 The metabolism and mechanisms of EUrd on RNA synthesis inhibition.

good substrate for the purine-selective nucleoside transporter, CNT2.[5] ECyd is now under phase I clinical study against various solid tumors. EUrd may have a potential to overcome resistance for ECyd: therefore, EUrd is expected to be a new antitumor drug that inhibits RNA synthesis.

ABBREVIATIONS

EUrd 1-(3-*C*-ethynyl-β-D-*ribo*-pentofuranosyl)uracil
EUTP EUrd 5′-triphosphate
ECyd 1-(3-*C*-ethynyl-β-D-*ribo*-pentofuranosyl)cytosine
ECTP ECyd 5′-triphosphate
ara-C 1-β-D-arabinofuranosylcytosine

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