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ORIGINAL ARTICLE

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Antitumor mechanisms and metabolism of the novel antitumor nucleoside analogues, 1-(3-*C*-ethynyl-β-D-*ribo*-pentofuranosyl)cytosine and 1-(3-*C*-ethynyl-β-D-*ribo*-pentofuranosyl)uracil

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Abstract The antitumor ribonucleoside analogues 1-(3-*C*-ethynyl-β-D-*ribo*-pentofuranosyl)cytosine (ECyd) and 1-(3-*C*-ethynyl-β-D-*ribo*-pentofuranosyl)uracil (EUrd), first synthesized in 1995, have strong antitumor activity against human cancer xenografts without severe side effects. Here, we studied the antitumor mechanisms of ECyd and EUrd using mouse mammary tumor FM3A cells in vitro and the mechanism of selective cytotoxicity of ECyd using human tumor xenografts in nude rats in vivo. In FM3A cells, ECyd and EUrd were rapidly phosphorylated to ECyd 5′-triphosphate (ECTP) and EUrd 5′-triphosphate (EUTP), which strongly inhibiting RNA synthesis. Cells treated with EUrd were later found to contain both EUTP and ECTP, and ECTP accumulated as the final product. Probably the uracil moieties of EUrd derivatives were efficiently converted to cytosine moieties in the cells. EUrd and its derivatives were minor metabolites in the cells treated with ECyd, so cytidine forms probably were not converted to uridine forms at the nucleoside or nucleotide stage. The ultimate metabolite of ECyd and EUrd, ECTP, is stable in cultured cells with a half-life of at least 3 days, so ECyd and EUrd are on a "closed" metabolic pathway to ECTP. These characteristics of ECyd and EUrd may be important for their antitumor activity. ECyd had strong and selective antitumor activity against the human tumor xenografts. ECyd-phosphorylating activity (uridine/cytidine kinase) in the xenografts was higher than that in the organs of the rats. This finding may account for the strong activity with mild side effects. ECyd and EUrd may be a new kind of antitumor nucleoside analogue for clinical use.

Key words 1-(3-*C*-ethynyl-β-D-*ribo*-pentofuranosyl) cytosine · 1-(3-*C*-ethynyl-β-D-*ribo*-pentofuranosyl) uracil · Inhibitors of RNA synthesis

Introduction

Pyrimidine analogues such as 5-fluorouracil and 1-β-D-arabinofuranosylcytosine (Ara-C), which inhibit DNA synthesis, are important antitumor agents used clinically. However, their effects on solid tumors are generally unsatisfactory. In such tumors, the proportion of cells in the S phase (when inhibition of DNA synthesis is possible) is smaller than in rapidly growing tumors. For this reason, our aim is to find and develop inhibitors of RNA synthesis, which occurs throughout the cell cycle except in the M phase. Even when RNA synthesis is only slightly inhibited, the effects are not negligible. For instance, 1-(2-deoxy-2-methylene-β-D-*erythro*-pentofuranosyl)cytosine [14, 23], 2′-deoxy-2′,2′-difluorocytidine (gemcitabine) [4, 8], and 1-(2-*C*-cyano-2-deoxy-β-D-*arabino*-pentofuranosyl)cytosine [2, 13, 22] are effective against a variety of human solid tumors in vitro and in vivo. These nucleosides are potent inhibitors of DNA synthesis, beside inhibiting RNA synthesis, unlike Ara-C [11, 16].

The nucleosides 1-(3-*C*-ethynyl-β-D-*ribo*-pentofuranosyl)cytosine (ECyd; Fig. 1A) and 1-(3-*C*-ethynyl-β-D-*ribo*-pentofuranosyl)uracil (EUrd; Fig. 1B) were designed to inhibit RNA synthesis; both are effective

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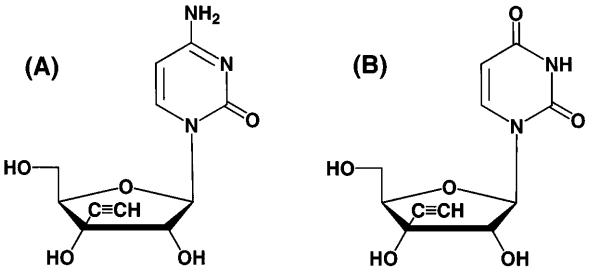

Fig. 1A,B Structures of ECyd (**A**) and EUrd (**B**)

against a variety of tumor cells, with 50% inhibitory concentration (IC_{50}) of 10 to 100 n*M* in vitro [7, 12]. In particular, ECyd has strong antitumor activity with weak toxic effects in vivo [20]. ECyd [7] and EUrd [19] inhibit RNA synthesis in vitro. They must be phosphorylated by uridine/cytidine kinase to have biological activity [6, 12]. In the study reported here, we investigated the antitumor mechanisms and the metabolism of ECyd and EUrd in vitro and in vivo.

Materials and methods

Materials

ECyd and EUrd were synthesized as reported previously [7, 12]. [Cytosine-5(n)-^{3}H]ECyd (200 GBq/mmol) and [uridine-6-^{3}H]EUrd (181 GBq/mmol) were synthesized at Amersham Life Science (Little Chalfont, UK). [8-^{3}H]Guanosine (544 GBq/mmol) was obtained from Moravek Biochemicals (Brea, Calif.). [Methyl-^{3}H]thymidine (740 GBq/mmol) was obtained from Du Pont/NEN Research Products (Boston, Mass.). [8-^{3}H]GTP (211 GBq/mmol) was obtained from Amersham. All other chemicals were of the highest purity available.

Cell culture

Mouse mammary tumor FM3A cells (F28-7) were obtained from the Japanese Cancer Research Resources Bank. The cells were grown at 37 °C in an atmosphere containing 5.0% CO_2 in ES medium (Nissui Pharmaceutical Co., Tokyo, Japan) containing 2% heat-inactivated fetal bovine serum [9]. Cells were counted using a microcell counter (CC-130; Toa Medical Electric Co., Tokyo, Japan). Cell viability during treatment with ECyd or EUrd was estimated by trypan blue exclusion using a hemocytometer. Growth inhibition was monitored as described previously [9]. DNA fragmentation was detected as reported previously [21]. Nucleoli in cells were observed under a microscope with azure C staining [3].

Assays of RNA and DNA synthesis

RNA synthesis

FM3A cells (2×10^5 cells/ml) were incubated at 37 °C in a medium containing 3.0 μ*M* ECyd or 11 μ*M* EUrd. At certain times, duplicate samples were withdrawn and pulse-labeled with 37 kBq/ml [8-^{3}H]guanosine for 30 min. After labeling, the cells were washed twice with ice-cold PBS and suspended in 10% trichloroacetic acid (TCA) at 4 °C for 20 min. The acid-insoluble fraction, which contained both RNA and DNA, was washed with 10% TCA, dissolved in 1 *M* NaOH, and incubated at 37 °C for 16 h to hydrolyse the RNA. The sample was neutralized with 1 *M* HCl, reacidified with TCA, and left on ice for 20 min for precipitation of the DNA [15]. After centrifugation of the sample (14 000 *g*, 4 °C for 10 min), the supernatant was filtered through a nitrocellulose membrane filter cartridge (0.22-μm pores) for removal of any unprecipitated DNA. Radioactivity in the supernatant was measured in a liquid scintillation cocktail (ACSII, Amersham).

Effects of ECTP and EUTP on RNA synthesis in isolated nuclei

Nuclei from exponentially growing FM3A cells were prepared and RNA synthesis was assayed by the method of Marzluff and Huang [10]. The reaction mixture contained 500 μ*M* ATP, CTP and UTP, and 50 μ*M* [8-^{3}H]GTP as substrates for RNA polymerases.

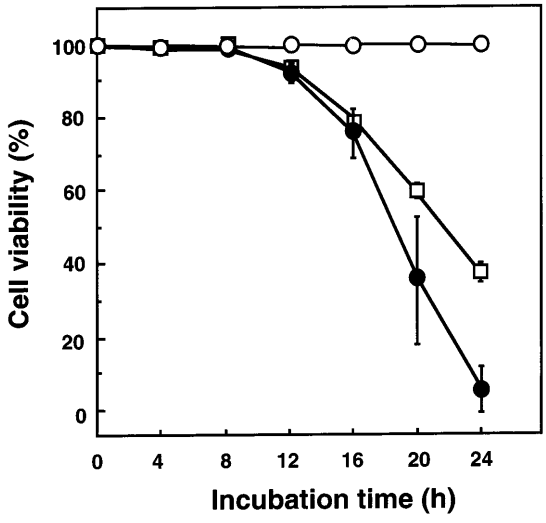

Fig. 2 Cytotoxicity of ECyd and EUrd. FM3A cells growing exponentially were cultured in the presence of 3.0 μ*M* ECyd (●) or 11 μ*M* EUrd (□), or with no additions (○). Cell viability was evaluated by trypan blue exclusion. Points are the means of three separate experiments (*bars* SD)

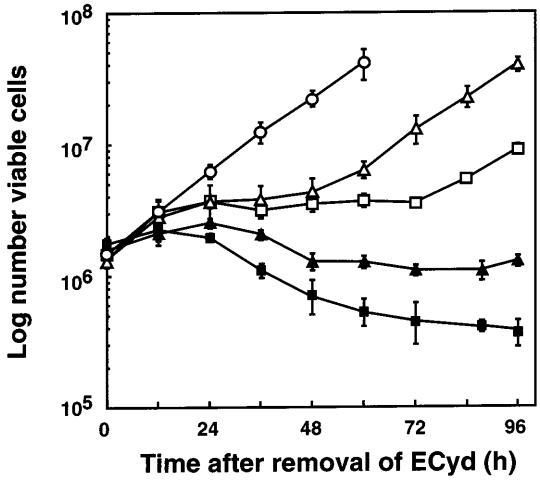

Fig. 3 Effects of short-term incubation with ECyd on growth of FM3A cells. After incubation with 3.0 μ*M* ECyd for 1 (△), 2 (□), 3 (▲) or 4 (■) h, cells were cultivated in fresh medium without ECyd (○ control cells). Points are the means of three separate experiments (*bars* SD)

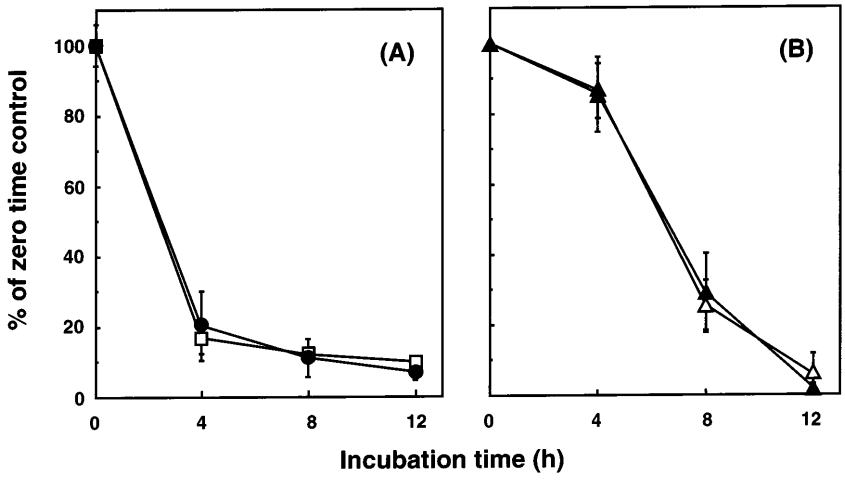

Fig. 4A,B Effects of ECyd and EUrd on incorporation of the labeled precursors [8-^{3}H]guanosine and [methyl-^{3}H]thymidine into the RNA (**A**) and DNA (**B**), respectively, of FM3A cells. **A** incorporation of [8-^{3}H]guanosine during incubation with ECyd (●) or EUrd (□); **B** incorporation of [methyl-^{3}H]thymidine during incubation with ECyd (▲) or EUrd (△). Points are the means of three separate experiments (*bars* SD)

Effects of preincubation with ECTP on RNA synthesis in isolated nuclei

For time-dependent inactivation experiments, nuclei isolated from FM3A cells were incubated with 21–2100 n*M* ECTP in the presence of 500 μ*M* ATP and UTP, and 50 μ*M* [8-^{3}H]GTP. At intervals, 20-μl aliquots were removed and diluted into 80 μl reaction buffer containing 1200 μ*M* CTP, 500 μ*M* ATP, 500 μ*M* UTP and 50 μ*M* [8-^{3}H]GTP. The initial rates of incorporation of radioactivity into the acid-insoluble fraction were measured by the methods of Marzluff and Huang [10].

Effects of ECTP and EUTP on DNA synthesis

At certain times, FM3A cells treated with ECyd or EUrd were labeled with 37 kBq/ml [methyl-^{3}H]thymidine for 30 min as above. The radioactivity in the acid-insoluble fraction was counted as described previously [9].

Metabolism and distribution of ECyd and EUrd in FM3A cells

FM3A cells (4×10^7) treated with tritiated 3.0 μ*M* ECyd or 11 μ*M* EUrd were washed twice with cold PBS and resuspended in more PBS. Cell components were fractionated into acid-soluble, DNA, RNA, lipid, and protein fractions by the method reported by Schneider [18]. The acid-soluble fraction was neutralized with two volumes of a Freon-amine solution (0.5 *M* tri-*n*-octylamine in 1,1,2-trichloro-1,2,2-trifluoroethane) and kept at −80 °C until HPLC (see below). The radioactivity of each fraction was counted.

The acid-soluble fraction was analyzed by HPLC with an anion-exchange column (TSK-gel DEAE-2SW, 4.6 × 250 mm; Tosoh Corporation, Tokyo, Japan). The HPLC apparatus was as described previously [24]. The column was eluted with 100% buffer A (10 m*M* $NH_4H_2PO_4$, 10 m*M* sodium hexanesulfonate, and 20% CH_3CN, pH 3.0) at a flow rate of 1.0 ml/min for 10 min, followed by a linear gradient changing to 60% buffer B (80 m*M* Na_2HPO_4, 10 m*M* sodium hexanesulfonate, and 20% CH_3CN, pH 6.0) for 25 min, and last 100% buffer B for 45 min. This gradient program was controlled by an automated gradient controller (model 680; Waters, Milford, Mass.). The eluate was monitored with a UV detector. Fractions of 1 ml were collected, and the radioactivity of each fraction was measured. Under these conditions, EUTP and UTP eluted simultaneously. The net amount of UTP was calculated by subtraction of the amount of EUTP calculated from its specific activity from the amount of UTP found by calculation of its peak area by HPLC. The concentration of nucleotides in the FM3A cells was calculated by division of the amount of nucleotides by the approximate number of cells ("cell equivalents") and the cell aqueous volume. The volume of the cells was calculated from the mean diameter of cells, and we found 10^6 cells to be equivalent to 1.1 mm^3. We assumed that the extracted nucleotides had been uniformly distributed in the cell aqueous volume.

Intracellular stability of ECTP

The intracellular stability of ECTP was compared with that of Ara-C 5′-triphosphate (Ara-CTP). After incubation with 3.0 μ*M* ECyd or 44 μ*M* Ara-C for 4 h, FM3A cells were washed and cultured in drug-free medium. At specific time-points, acid-soluble fractions were prepared and analyzed using HPLC as described above.

Antitumor experiments

The human tumor cells used in this study were obtained from the Japanese Cancer Research Resources Bank. The experimental groups each consisted of seven or eight nude rats. Human lung (LC-11), pancreas (PAN-12), stomach (AZ-521), and colon (CO-3) tumor xenografts were made by s.c. implantation of a cube of tissue measuring about 8 mm^3 into the area under the right foreleg of male F344/N Jcl-rnu rats. When the volume of tumor (1/2 × major

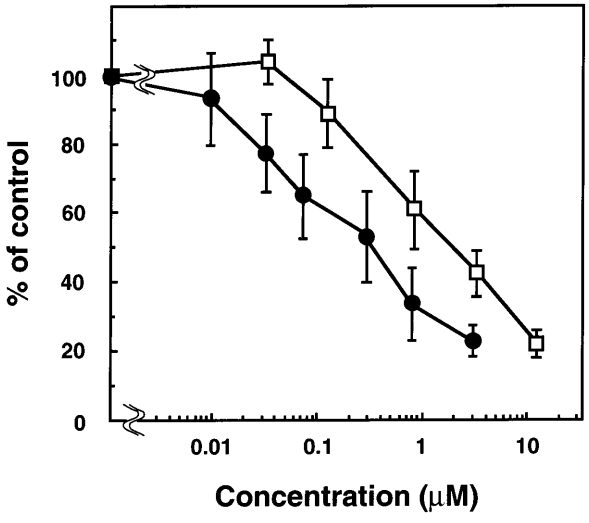

Fig. 5 The relationship between concentration of ECyd (●) or EUrd (□) and incorporation of the labeled precursors [8-^{3}H]guanosine into the RNA of FM3A cells after 4 h of incubation. Points are the means of three separate experiments (*bars* SD)

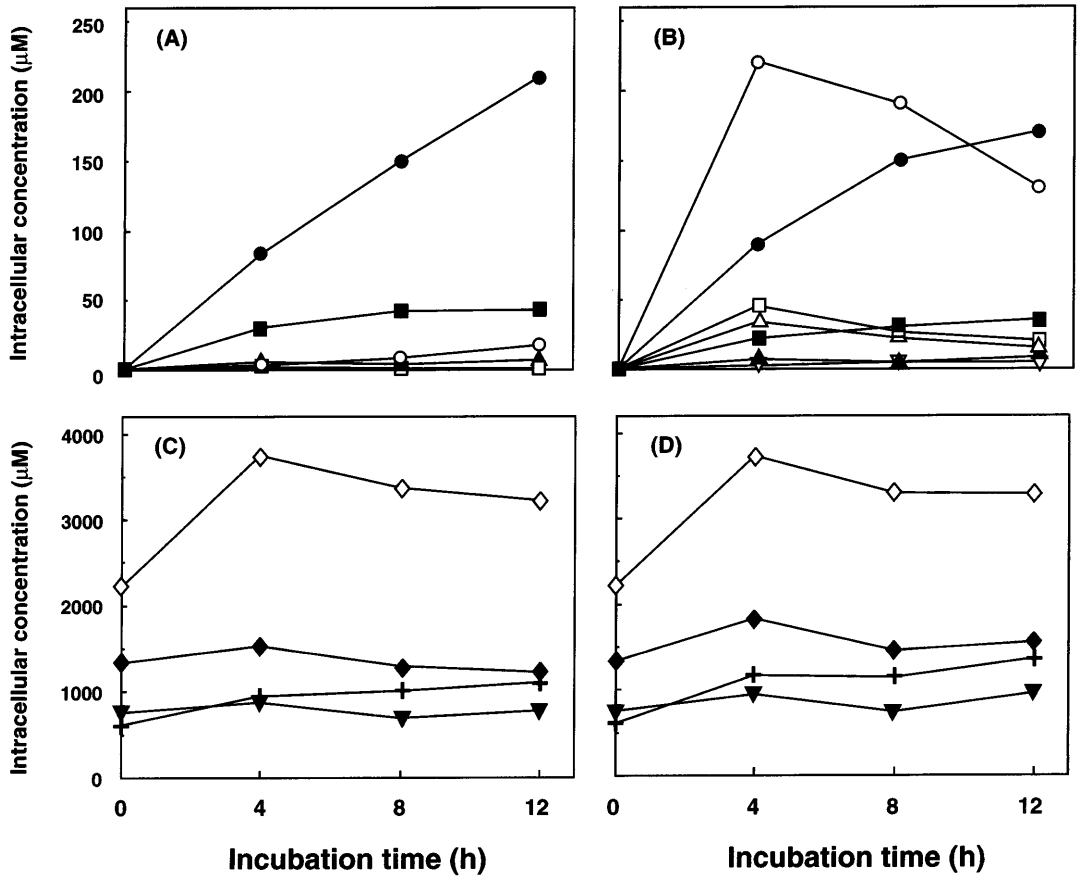

Fig. 6A–D Metabolites of ECyd and EUrd in FM3A cells incubated with ECyd (**A**) or EUrd (**B**), and the effects of ECyd (**C**) or EUrd (**D**) on rNTP in FM3A cells (● ECTP, ■ ECDP, ▲ ECMP, ○ EUTP, □ EUDP, △ EUMP, ▽ metabolite *x*, ◇ ATP, + GTP, ◆ UTP, and ▼ CTP). The retention times of the nucleotides were as follows (min): ECyd, 2.2; EUrd, 2.9; ECMP, 3.5; EUMP, 23.1; ECDP, 33.0; UDP-glucose, 42.3; metabolite *x*, 43.2; EUDP, 52.0; EUTP, 73.3; UTP, 73.5; CTP, 75.5; ECTP, 78.2; ATP, 86.8; GTP, 92.7). Points represent the average of two experiments (Metabolite *x* was an unidentified metabolite eluting near UDP-glucose in HPLC, perhaps an EUDP-sugar derivative)

axis × minor axis^2) reached about 200 mm^3, drug dissolved in saline was injected i.v. for 5 consecutive days. After 2 days rest, the same treatment was repeated for another 5 days. The maximum nontoxic dose was used, defined as the highest dose that caused no decrease in body weight, no gastrointestinal toxicity, and no myelosuppression. This dose was 0.25 mg/kg for ECyd and 5 mg/kg for 5-fluorouridine (FUrd). On day 15, the antitumor activity of the drugs was evaluated as the inhibition of tumor growth in treated rats as a percentage of that of untreated control rats. The care and treatment of rats were in accordance with the guidelines (no. 141, 1987) issued by the Science and International Affairs Bureau of the Japanese Ministry of Education, Science Culture and Sports.

Preparation and assay of uridine/cytidine kinase

All procedures were done at 4 °C. Tissue samples (0.5 to 1.0 g) were minced with scissors and homogenized with four volumes of 50 m*M* Tris-HCl (pH 8.0) containing 10 m*M* 2-mercaptoethanol, 25 m*M* KCl, and 5 m*M* $MgCl_2$. The homogenates were centrifuged at 10 500 *g* for 60 min and the supernatants were used for the assay of ECyd phosphorylating activity (uridine/cytidine kinase).

The reaction mixture, in a total volume of 125 μl, comprised 50 m*M* Tris-HCl (pH 8.0), 10 m*M* NaF, 5 m*M* $MgCl_2$, 0.6 m*M* [cytosine-5(n)-^{3}H]ECyd (37 Bq/tube) and 50 μl enzyme solution. The mixture was incubated at 37 °C for 30 min, heated in a boiling water bath for 2 min, and centrifuged at 2000 *g* for 10 min. Then 20 μl of supernatant was put on a polyethyleneimino-cellulose TLC plate (3 × 10 cm) and developed with water. Phosphorylated compounds at the origin were scraped into vials and extracted with 2 *M* HCl (1 ml), and their radioactivities were measured.

Results

ECyd and EUrd potently inhibited the growth of FM3A cells in vitro. The concentrations of ECyd giving 50% and 90% inhibition of growth (IC_{50} and IC_{90}) were 0.030 and 0.30 μ*M*, respectively. For EUrd, the equivalent concentrations were 0.11 and 4.0 μ*M*, respectively. When cells were incubated with 3.0 μ*M* ECyd or 11 μ*M* EUrd, cell death was first observed 12 h after the start of incubation (Fig. 2). After 24 h of incubation, cell viability was reduced to 11.2% and 41.8% with these concentrations. At that time, DNA fragments, some 100 to 200 kb long and some internucleosomal, were observed in cells treated with either drug [21].

The growth of cells exposed to ECyd for 1 h was inhibited for 48 h (Fig. 3). FM3A cells exposed to ECyd for 3 h or more were irreversibly damaged and died. The cytotoxicity of ECyd after 4 h of exposure was reversed

by the addition of a metabolic competitor, either cytidine (100 μ*M*) or uridine (100 μ*M*), to the drug-free medium (data not shown). Such reversal was also seen in FM3A cells treated with 11 μ*M* of EUrd (data not shown).

In cells treated with ECyd or EUrd, nucleoli shrank before cells began to die. The times required for ECyd at 3.0 μ*M*, EUrd at 11 μ*M*, and actinomycin D at 0.21 μ*M* to reduce the percentage of cells with intact nucleoli to 50% were 3.5, 3.7, and 2.4 h, respectively. In contrast, Ara-C at 44 μ*M* did not affect nucleoli: whether cells were treated or not 99% had intact nucleoli. When cells were incubated with 3.0 μ*M* ECyd or 11 μ*M* EUrd, RNA synthesis was reduced to 20.3% and 16.8% after 4 h of incubation, respectively (Fig. 4A). On the other hand, DNA synthesis was only reduced to 85.5% and 86.4% after 4 h of incubation with these drugs (Fig. 4B). The relationship between concentration of ECyd or EUrd and inhibition of RNA synthesis was analyzed after a 4-h incubation of cells. RNA synthesis was reduced dose-dependently (Fig. 5).

When cells were treated with 3.0 μ*M* ECyd or 11 μ*M* EUrd, their metabolite accumulated in the acid-soluble fraction and RNA. After 12 h of incubation with ^{3}H-ECyd, 96.9% of the total radioactivity was detected in the acid-soluble fraction, and 3.1% was incorporated into RNA. In cells treated with ^{3}H-EUrd for 12 h, 97.8% of the total radioactivity (29 900 dpm/10^6 cells) was detected in the acid-soluble fraction, and 2.2% was incorporated into RNA. ECyd was incorporated into RNA: the amounts of incorporated 3′-ethynyl-pyrimidine nucleosides were 4.0, 6.0, and 9.8 pmol/10^6 cells at 4, 8 and 12 h, respectively. EUrd was also incorporated into RNA: the amounts incorporated were 6.1, 9.3, and 8.6 pmol/10^6 cells at 4, 8 and 12 h, respectively. ECyd and EUrd were not incorporated into DNA, lipid or protein.

Figure 6 shows the results of HPLC of the acid-soluble fraction prepared from cells incubated with ECyd or EUrd. About 75% of the total metabolites in the acid-soluble fraction was ECTP or EUTP and the remainder was their mono- or diphosphate derivatives. During incubation with ECyd, the ECTP accumulated with little EUTP being detected (Fig. 6A). During incubation with EUrd, EUTP was a major metabolite at 4 h of incubation, but by 8 h the amount was decreasing, while the amount of ECTP was still increasing (Fig. 6B). As shown in Fig. 6A, B, while incorporation of ECyd into cells increased time-dependently, that of EUrd had almost ceased by 4 h of incubation. In cells treated with ECyd or EUrd, rNTP pools changed little (Fig. 6C, D). Unphosphorylated ECyd and EUrd and their catabolites, cytosine and uracil, were not detected.

Ara-CTP was eliminated from the cells with a half-life of less than 10 min (Fig. 7). ECTP had a half-life about 81 h. Intracellular ECTP was some 500 times more stable than Ara-CTP.

ECTP and EUTP strongly inhibited RNA synthesis in isolated nuclei. The initial rate in the control was 650 pmol/min per 10^6 nuclei. The IC_{50} values of ECTP and EUTP with respect to the initial rate of RNA synthesis were 3.0 and 10 μ*M*, respectively. Nuclei were preincubated with 21–2100 n*M* ECTP in the absence of CTP. The activity of RNA polymerization was not affected by the preincubation (data not shown).

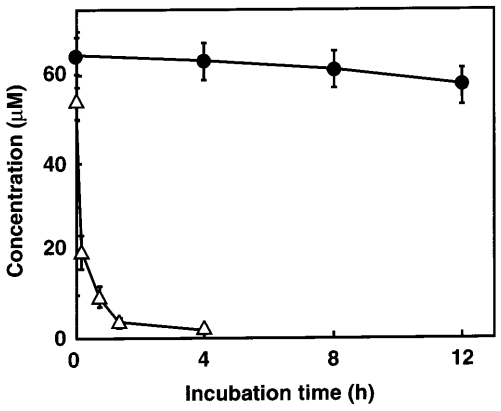

Fig. 7 Changes in ECTP and Ara-CTP with time of incubation in FM3A cells. After incubation of FM3A cells with 3.0 μ*M* ECyd or 44 μ*M* Ara-C for 4 h, the cells were cultivated in fresh medium without ECyd or Ara-C from time zero. At certain times, acid-soluble fractions were prepared. ECTP and Ara-CTP in cells were measured by HPLC (● ECTP, △ Ara-CTP). The retention times of ECTP and Ara-CTP were 78.2 and 81.5 min, respectively. Points are means of three separate experiments (*bars* SD)

The antitumor activity of ECyd was evaluated on human lung (LC-11), pancreas (PAN-12), stomach (AZ-521) and colon (CO-3) cancer xenografts implanted into nude rats. The antitumor efficacy of 5-fluorouridine (FUrd), a fluorinated pyrimidine, was also tested in the same rat model transplanted with LC-11 and PAN-12 because FUrd was known to inhibit RNA metabolism. As shown in Table 1, ECyd (0.25 mg/kg) showed significant effectiveness against all four tumors: the rates of inhibition of tumor growth (IR) were 80.9%, 90.9%, 88.8% and 83.3% for LC-11, PAN-12, AZ-521 and CO-3, respectively. The antitumor activity of FUrd (5 mg/kg) was low, with IR values of 16.6% and 40.4% for LC-11 and PAN-12, respectively. Throughout these therapeutic experiments, ECyd and FUrd produced no severe toxicities such as diarrhea, myelosuppression or loss in body weight (data not shown).

Uridine/cytidine kinase is a key enzyme for the phosphorylation of ECyd and FUrd to exhibit cytotoxicity. The enzyme activities in tumor and normal tissues of nude rats were therefore measured using tritiated ECyd as substrate. As shown in Fig. 8, ECyd phosphorylating activity in LX-1 and CO-3 were 333 and 272 pmol/min per mg protein, respectively, while normal tissues had less activity (below 100 pmol/min per mg protein) than tumor xenografts.

Discussion

The antitumor activity of antimetabolic agents depends greatly on their metabolic profile in tumor cells. For

Table 1 Antitumor activity of ECyd and FUrd against human tumor xenografts in nude rats. Human tumor xenografts (about 8 mm^3 fragments) were implanted into nude rats. The maximal nontoxic dose of ECyd (0.25 mg/kg per day) or FUrd (5 mg/kg per day) was injected i.v. for 5 consecutive days. After 2 days rest, the same treatment was repeated for another 5 days. The antitumor activity of these drugs was evaluated at day 15 after starting the drug treatment. The values are means ± SD (*NT* not tested)

Cancer type	Tumor line	Control RTV[a]	ECyd		FUrd	
			RTV[a]	IR (%)[b]	RTV[a]	IR (%)[b]
Lung	LC-11	4.99 ± 1.82	0.95 ± 0.86**	80.9	4.16 ± 2.09	16.6
Pancreas	PAN-12	2.62 ± 0.96	0.26 ± 0.08**,***	90.9	1.56 ± 0.78*	40.4
Stomach	AZ-521	18.55 ± 5.58	2.08 ± 1.50**	88.8	NT	
Colon	CO-3	3.38 ± 2.09	0.57 ± 0.43**	83.2	NT	

* $P < 0.05$, ** $P < 0.01$ vs control group (Welch's *t*-test); *** $P < 0.05$ vs FUrd group (Welch's *t*-test)
[a] Relative tumor volume (tumor volume on day 15/tumor volume on starting drug administration)
[b] Inhibition rate of tumor growth [(1−RTV of drug treatment/RTV of control) × 100]

example, the antitumor activity of Ara-C is diminished in solid tumors because of the intracellular instability of the active metabolite, Ara-CTP. ECyd and EUrd have more potent antitumor activity against solid tumors than some antimetabolic agents often used clinically [12, 19, 20]. Therefore, we suspected that the antitumor mechanisms and metabolic profiles of these nucleosides might be unusual.

ECyd and EUrd inhibited RNA synthesis in FM3A cells since the nucleoli shrank, as did actinomycin D. ECyd and EUrd were phosphorylated to produce ECTP and EUTP, respectively. Interestingly, ECTP accumulated in cells treated with EUrd. This conversion of EUTP to ECTP may arise from CTP synthase (EC 6.3.4.2; Fig. 9), because EUTP and ECTP were produced in cells treated with EUrd. Small amounts of EUrd derivatives were produced by the cells treated with ECyd perhaps because ECyd and its derivatives resist deamination (Fig. 9) [20]. The efficacy of Ara-C is limited by the rapid elimination of intracellular Ara-CTP from tumor cells [17]. Ara-CTP is dephosphorylated to be deaminated by deoxycytidylate deaminase. ECTP may not be efficiently dephosphorylated in the cells. Thus, we consider that ECyd and EUrd are on a "closed" metabolic pathway accumulating ECTP as a dead-end metabolite. These characteristics might be the reason for the long half-life. With these characteristics, short exposures to ECyd and EUrd could cause the death of cells.

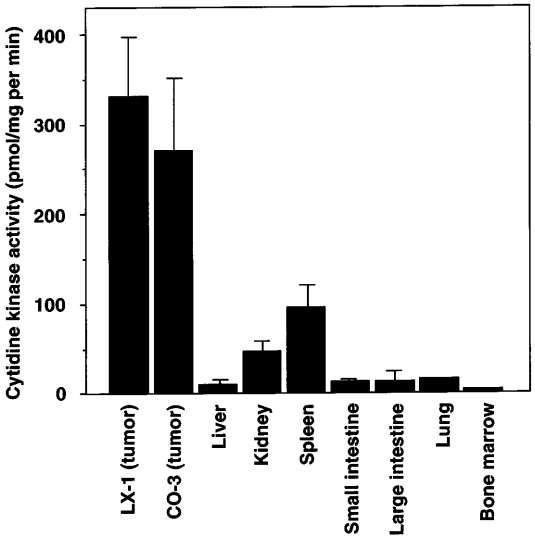

Fig. 8 ECyd-phosphorylating activity in human tumor xenografts and various normal tissues of nude rats. ECyd-phosphorylating activity was assayed as described in Materials and methods. Values are means ± SD for three rats

In cells treated with ECyd, the main metabolites were the dead-end metabolites ECTP, ECDP, ECMP and EUTP. As shown in Fig. 6B, in cells treated with EUrd, an unidentified metabolite, metabolite *x*, which was considered to be an EUDP-sugar, was detected in the acid-soluble fraction, and its concentration as EUrd reached 4 μ*M* after 12 h of incubation. This metabolite may affect the macromolecular synthesis of complex carbohydrates.

Saturation of cells with EUrd may be related to inhibition of its transport by the EUTP formed, and incorporation of the nucleoside analogues into RNA.

Inhibition of RNA synthesis was not accompanied by decreases in pools of rNTP precursors for RNA synthesis. Therefore, the inhibition of RNA synthesis must involve inhibition of RNA polymerization by ECTP and EUTP. It has been reported that T7 RNA polymerase is inhibited competitively by ECTP (Azuma A and Mats-

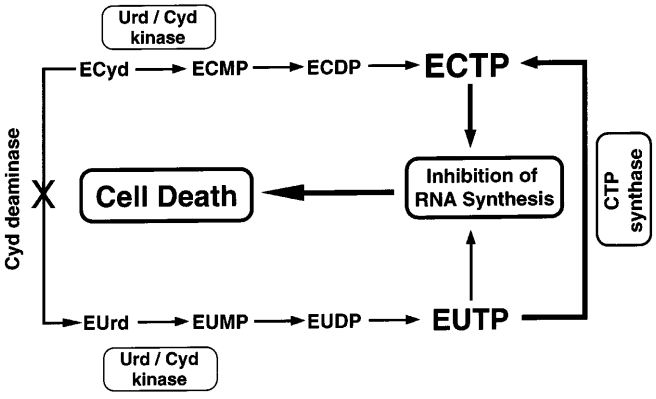

Fig. 9 Metabolism and cytotoxic mechanisms of ECyd and EUrd (*Cyd deaminase* cytidine deaminase, *Urd/Cyd kinase* uridine/cytidine kinase)

uda A, unpublished observation) and EUTP [10]. In our preliminary experiments, RNA polymerase was inhibited competitively by ECTP and EUTP in isolated nuclei of FM3A cells. The Ki values of ECTP and EUTP were 21 n*M* and 84 n*M*, respectively (apparent Km values of RNA polymerase for CTP and UTP were 8.0 μ*M* and 13 μ*M*, respectively; details to be published elsewhere). ECTP is not an irreversible inhibitor of RNA polymerase because ECTP did not inactivate RNA polymerase upon preincubation. These results suggest that the concentrations of ECTP or EUTP in FM3A cells treated with 3.0 μ*M* ECyd or 11 μ*M* EUrd for 4 h were high enough to inhibit RNA polymerization, as shown in Fig. 6. The inhibition of RNA synthesis by ECTP or EUTP in the nuclei isolated from FM3A cells reflected the inhibition by ECyd and EUrd of such synthesis in intact FM3A cells.

The phosphorylation of ECyd in tumor was 20–30 times higher than that in normal organs. It has also been reported that the activity of uridine/cytidine kinase in hepatoma cells is about seven or eight times higher than that in hepatocytes of newborn mice [25]. Furthermore, the biological activity of ECyd and EUrd depends greatly on the first phosphorylation step by uridine/cytidine kinase [6, 19, 20]. The addition of cytidine or uridine to the medium prevented the cytotoxic effects of ECyd and EUrd. Therefore, the first phosphorylation of these nucleosides might be the key step in their selective cytotoxity toward tumor cells. FUrd has been shown to be converted to its active form by uridine/cytidine kinase and incorporated into RNA molecules thus inhibiting RNA metabolic processes such as processing and splicing [1, 5]. Therefore, we compared the antitumor activity of ECyd to that of FUrd at the same minimum toxic doses, and found that ECyd had a higher and more tumor-selective efficacy against human tumor xenografts in nude rats than FUrd, suggesting that ECyd inhibits RNA metabolism in a different manner to FUrd. The inhibition of DNA synthesis by ECyd probably arises from its potent inhibition of RNA synthesis.

The efficient phosphorylation of ECyd and EUrd to ECTP and EUTP and their inhibition of RNA polymerases in tumor cells account for much of the strong and selective antitumor activity of these agents against solid tumors. ECyd and EUrd are candidates for clinical trials. RNA synthesis may be a suitable target in the design of possible antitumor nucleosides with effects on solid tumors.

References

1. Armstrong RD, Takimoto CH, Cadman EC (1986) Fluoropyrimidine-mediated changes in small nuclear RNA. J Biol Chem 261: 21
2. Azuma A, Nakajima Y, Nishizono N, Minakawa N, Suzuki M, Hanaoka K, Kobayashi T, Tanaka M, Sasaki T, Matsuda A (1993) 2′-*C*-Cyano-2′-deoxy-1-β-D-arabinofuranosylcytosine and its derivatives: a new class of nucleoside with a broad antitumor spectrum. J Med Chem 36: 4183
3. Ayres WW (1949) A method of staining nucleoli of cells in fresh benign and malignant tissues. Cancer Res 8: 352
4. Coska K, Liliemark J, Lasson R, Nygren P (1995) Evaluation of the cytotoxic activity of gemcitabine in primary cultures of tumor cells from patient with hematologic or solid tumors. Semin Oncol 22 [Suppl. 11]: 47
5. Doong SL, Dolnick BJ (1988) 5-Fluorouracil substitution alters pre-mRNA splicing in vitro. J Biol Chem 263: 4467
6. Hattori H, Nozawa E, Iino T, Yoshimura Y, Shuto S, Shimamoto Y, Nomura M, Fukushima M, Tanaka M, Sasaki T, Matsuda A (1998) The structural requirements of the sugar moiety for the antitumor activities of new nucleoside antimetabolites, 1-(3-*C*-ethynyl-β-D-*ribo*-pentofuranosyl)cytosine and -uracil. J Med Chem 41: 2892
7. Hattori H, Tanaka M, Fukushima M, Sasaki T, Matsuda A (1996) 1-(3-*C*-ethynyl-β-D-*ribo*-pentofuranosyl)uracil (EUrd) and their nucleobase analogues as new potential multi-functional antitumor nucleosides with a broad spectrum of activity. J Med Chem 39: 5005
8. Hertel LW, Border GB, Kroin JS, Rinzel SM, Poore GA, Todd GC, Grindey GB (1990) Evaluation of the antitumor activity of gemcitabine (2′,2′-difluoro-2′-deoxycytidine). Cancer Res 50: 4417
9. Hirota Y, Yoshioka A, Tanaka S, Watanabe K, Otani T, Minowada J, Matsuda A, Ueda T, Wataya Y (1989) Imbalance of deoxyribonucleoside triphosphates, DNA double-strand breaks, and cell death caused by 2-chlorodeoxyadenosine in mouse FM3A cells. Cancer Res 49: 915
10. Marzluff WF, Huang RCC (1984) Transcription of RNA in isolated nuclei, In: Hames BD, Higgins SJ (eds) Transcription and translation. IRL Press, Oxford, p 89
11. Matsuda A, Azuma A (1995) 2′-*C*-Cyano-2′-deoxy-1-β-D-arabinofuranosylcytosine (CNDAC): a mechanism-based DNA-strand-breaking antitumor nucleoside. Nucleosides Nucleotides 14: 461
12. Matsuda A, Hattori H, Tanaka M, Sasaki T (1996) 1-(3-*C*-ethynyl-l-β-D-pentofuranosyl)uracil as a broad spectrum antitumor nucleoside. Biomed Chem Lett 6: 1887
13. Matsuda A, Nakajima Y, Azuma A, Tanaka M, Sasaki T (1991) 2′-*C*-Cyano-2′-deoxy-1-β-D-arabinofuranosylcytosine (CNDAC): design of potential mechanism-based DNA-strand-breaking antineoplastic nucleoside. J Med Chem 34: 2917
14. Matsuda A, Takenuki K, Tanaka M, Sasaki T, Ueda T (1991) Synthesis of new broad spectrum antineoplastic nucleosides, 2′-deoxy-2′-methylidenecytidine (DMDC) and its derivatives. J Med Chem 34: 812
15. Rosenkranz HS, Pollak RD, Schmidt RM (1969) Biologic effects of isohydroxy urea. Cancer Res 29: 209
16. Ruiz VW, Haperen V, Veerman G, Vermorken JB, Peters GJ (1993) 2′,2′-Difluoro-2′-deoxycytidine (gemcitabine) incorporation into RNA and DNA of tumor cell lines. Biochem Pharmacol 46: 762
17. Rustum YM, Preisier HD (1979) Correlation between leukemic cell retention of 1-β-D-arabinofuranosylcytosine 5′-triphosphate and response to therapy. Cancer Res 39: 42
18. Schneider WC (1946) Phosphorus compounds in animal tissues. III. A comparison of methods for the estimation of nucleic acids. J Biol Chem 164: 747
19. Tabata S, Tanaka M, Endo Y, Obata T, Matsuda A, Sasaki T (1997) Anti-tumor mechanisms of 3′-ethynyluridine development and characterization of 3′-ethynyluridine-resistant cells. Cancer Lett 116: 225
20. Tabata S, Tanaka M, Matsuda A, Fukushima M, Sasaki (1996) Antitumor effect of a novel multifunctional antitumor nucleoside, 3′-ethynylcytidine, on human cancers. Oncol Rep 3: 1
21. Takatori S, Tsutsumi S, Hidaka M, Kanda H, Matsuda A, Fukushima M, Wataya Y (1998) The characterization of cell death induced by 1-(3-*C*-ethynyl-β-D-*ribo*-pentofuranosyl) cytosine (ECyd) in FM3A cells. Nucleosides Nucleotides 17: 1309
22. Tanaka M, Matsuda A, Terao T, Sasaki T (1992) Antitumor activity of a novel nucleoside, 2′-*C*-cyano-2′-deoxy-1-β-D-ar-

abinofuranosylcytosine (CNDAC) against murine and human tumors. Cancer Lett 64: 67
23. Yamagami K, Fujii A, Arita M, Okumoto T, Sakata S, Matsuda A, Ueda T, Sasaki T (1991) Antitumor activity of 2′-deoxymethylidenecytidine, a new 2′-deoxycytidine derivative. Cancer Res 51: 2319
24. Yamato M, Hirota Y, Yoshida S, Tanaka S, Morita T, Sakai J, Hashigaki K, Hayatsu H, Wataya Y (1992) Imbalance of deoxyribonucleoside triphosphates and DNA double-strand breaks in mouse mammary tumor FM3A cells treated in vitro with an antineoplastic tropolone derivative. Jpn J Cancer Res 83: 661
25. Weber G (1983) Biochemical strategy of cancer cells and the design of chemotherapy: G.H.A. Clowes Memorial Lecture. Cancer Res 43: 3466