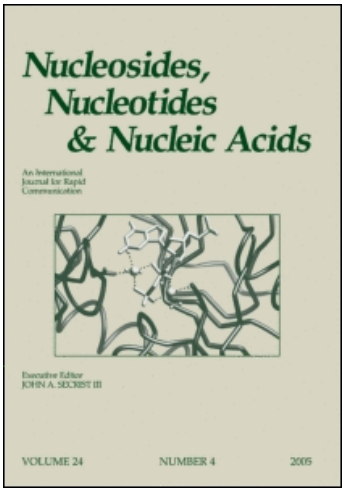

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1-(3-*C*-ETHYNYL-β-D-*RIBO*-PENTOFURANOSYL)CYTOSINE (ECYD, TAS-106)1: ANTITUMOR EFFECT AND MECHANISM OF ACTION

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1-(3-*C*-ETHYNYL-β-D-*RIBO*-PENTOFURANOSYL)CYTOSINE (ECYD, TAS-106)1: ANTITUMOR EFFECT AND MECHANISM OF ACTION

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ABSTRACT

The antitumor activity, cellular metabolism and mechanism of action of the antitumor nucleoside analog, 1-(3-*C*-ethynyl-β-D-*ribo*-pentofuranosyl)cytosine (ECyd) are described.

INTRODUCTION

Nucleoside analogs such as 1-β-D-arabinofuranosylcytosine (ara-C) and 2′,2′-difluorodeoxycytidine (gemicitabine) are used clinically as antitumor agents (1,2,3). The main mechanism of action of these nucleosides is believed to be inhibition of cellular DNA synthesis, due to incorporation of their 5′-triphosphates into DNA by DNA polymerases in cells (4,5). Furthermore, gemcitabine 5′-diphosphate, one of the intracellular metabolites of gemcitabine, inhibits ribonucleoside diphosphate reductase resulting in an imbalance of dCTP pool, and this inhibition enhances incorporation of gemcitabine 5′-triphosphate into cellular DNA (6). The inhibition of DNA synthesis with therapeutic nucleoside analogs causes cell cycle blockade

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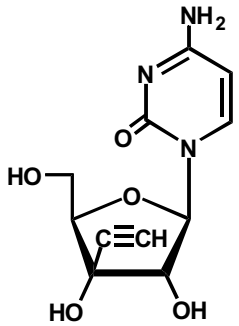

Figure 1. Structure of ECyd.

mainly in the S phase. On the other hand, it has been reported that some therapeutic nucleoside analogs also inhibit RNA synthesis (7), but this would account for only part of the mechanism underlying these antitumor effects.

To investigate more potent antitumor nucleosides, 1-(3-*C*-ethynyl-*β*-D-*ribo*-pentofuranosyl)cytosine (ECyd, Fig. 1) was designed as an RNA synthesis inhibitor (8–10). ECyd showed potent antitumor activity against various human tumors *in vitro* and *in vivo* (11), and has been used in clinical studies in the US. In this report, we describe the antitumor activity, metabolism and drug action of ECyd in human tumor cells.

ANTITUMOR ACTIVITY

The inhibitory activity of ECyd on *in vitro* tumor cell growth was investigated using 27 human tumor cell lines employing the MTT assay (Table 1). ECyd was more potent against most of lines in this panel than other antitumor reagents, such as 5-FU, gemcitabine, and CDDP, and was effective with IC_{50} values in the nano-molar range on all cell lines.

The antitumor effects of ECyd on 19 human tumors including pulmonary, pancreatic, colon, gastric, hepatic, cervical, head and neck, renal and bladder tumors as xenografts in nude rats were also evaluated (Table 2). ECyd was intravenously administrated at a dose of 6 mg/kg (once a week for two weeks), or 1 mg/kg (three times a week for two weeks). As shown in Table 2, tumor growth inhibition rates with ECyd in these tumors were between 44 and 99%, and there was no detectable toxicity to nude rats at these doses. The antitumor effects of ECyd were compared with those of gemcitabine (15 mg/kg, once a week for two weeks) and CDDP (5 mg/kg, once in two weeks) on most of the tumors in this panel. Tumor growth inhibition rates with gemcitabine in these tumors were 35 to 88%, and those of CDDP were 14 to 86%. The antitumor activity of ECyd with both dose schedules appeared to be more potent than those of gemcitabine and CDDP on most of the tumors in this panel.

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Table 1. Cytotoxicities of ECyd Against Human Cancer Cells

	IC_{50} values (μM)			
Cell Line	TAS-106	5-FU	Gemcitabine	CDDP
Lung				
A549	0.022	6.80	0.321	31.6
EBC-1	0.231	67.7	0.005	50.5
LC-11	0.334	>1000	>1000	14.6
Lu-99	0.027	5.49	0.003	1.16
Lx-1	0.036	33.9	0.028	1.92
Colon				
DLD-1	0.039	21.7	–	–
H630	0.150	377	–	–
HCT-15	0.022	12.1	–	–
HT-29	0.034	5.03	–	–
SNU-C2A	0.020	4.99	–	–
Stomach				
AGS	0.063	37.5	–	–
AZ521	0.017	6.83	–	–
MKN74	0.086	24.0	–	–
NUGC-3	0.050	19.4	–	–
NUGC-4	0.034	10.3	–	–
Pancreas				
ExPC-3	0.081	138	0.021	0.554
MIAPaCa-2	0.027	22.0	0.023	4.92
Liver				
Li-4	0.080	30.0	0.079	0.854
Li-7	0.084	22.5	0.008	0.394
Esophagus				
T.T.	0.423	167	2.72	4.83
T.Tn	0.127	52.3	0.014	0.855
Cervix				
ME-180	0.114	7.71	0.014	0.367
OMC-1	1.370	>1000	>1000	6.49
Prostate				
DU-145	0.056	20.0	0.021	0.960
PC-3	0.109	131	1.640	9.51
Renal				
VMRC-RCW	3.11	>1000	502	7.44
VMRC-RCZ	3.68	>1000	20.0	5.65

METABOLISM AND DRUG ACTION

To clarify the intracellular metabolism and mechanism of action of ECyd, human fibrosarcoma HT-1080 cells were used, on which ECyd showed potent cytotoxicity with an IC_{50} value of 27 nM with 72 h treatment. The intracellular metabolism of ECyd in HT-1080 cells was studied using [^{3}H]ECyd (Fig. 2). After treatment with 1 μM [^{3}H]ECyd for 4 to 8 h, an acid-soluble fraction of the cells was extracted and analyzed by anion-exchange HPLC. The major metabolite of ECyd

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Table 2. Antitumor Effects of ECyd on Human Tumor Xenografts in Nude Rats

Tumor		Tumor Growth Inhibition Rate (%)			
		TAS-106		Gemcitabine	CDDP
		6 mg/kg × 2 (1/W)	1 mg/kg × 6 (3/W)	15 mg/kg × 2 (1/W)	5 mg/kg × 1
Lung	LX-1	92.4	94.5	–	64.9
	LC-11	99.1	90.5	75.2	83.4
	Lu-61	56.3	53.0	35.7	78.2
Pancreas	PAN-4	89.1	89.2	85.3	66.6
	PAN-12	91.0	88.7	80.0	81.1
	H-48	44.0	39.9	48.9	14.2
Colon	Co-3	89.0*	94.6	–	–
	KM12C	54.6**	78.4	77.0	70.3
	KM20C	96.7	88.9	88.4	50.3
Stomach	SC-2	76.9	74.7	–	–
	St-40	86.9	92.8	–	82.7
Liver	Li-4	98.6	98.7	80.6	59.9**
	Li-7	72.2	63.6	34.6	46.0
Cervix	OMC-1	40.3	77.9	–	55.9
	ME-180	65.4	75.1	74.1	67.9
Head & Neck	PNC-1	66.9	69.8	68.3**	74.4
	PHA-1	86.8	83.8	36.6**	86.5
Renal	VMRC-RCW	72.9	70.0	55.5	52.5**
Bladder	EJ-1	84.1	83.9	78.6	48.2

*1/7 death, **1/8 death.

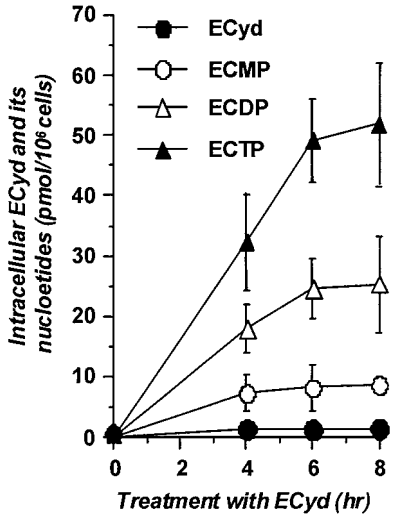

Figure 2. ECyd metabolism in HT-1080 cells.

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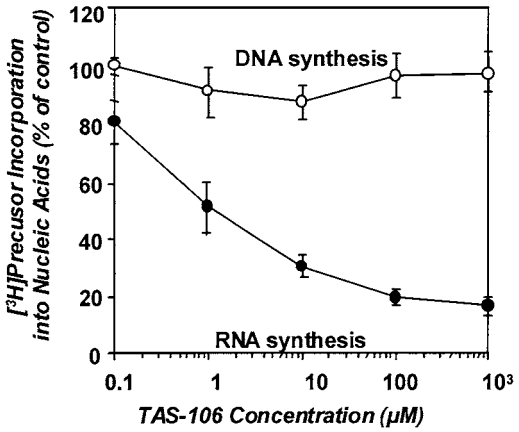

Figure 3. The effects of ECyd on DNA and RNA synthesis in HT-1080 cells.

was a 5′-triphosphate of ECyd (ECTP). The cellular concentration of ECTP with 4 h ECyd treatment reached 32 pmol/10^6 cells, accounting for 55% of total cellular ECyd uptake in HT-1080 cells, and its accumulation increased time-dependently. ECyd, its 5′-monophosphate (ECMP), and 5′-diphosphate of ECyd (ECDP) were minor metabolites in whole cells, and their cellular concentrations and the ratio of total cellular ECyd uptake were 1.3 pmol/10^6 cells, 7.2 pmol/10^6 cells, 18 pmol/10^6 cells, and 2%, 12%, and 31%, respectively.

To investigate of the effects of ECyd on DNA and RNA synthesis in HT-1080 cells, [^{3}H]thymidine and [^{3}H]guanosine were used as precursors, respectively (Fig. 3). Guanosine incorporation into cellular RNA was inhibited by ECyd treatment, and its IC_{50} value was 1.8 μM with 4 h ECyd treatment, whereas thymidine incorporation into cellular DNA was not inhibited by ECyd treatment in whole cells. RNA synthesis of T7 RNA polymerase and human RNA polymerase II was reported to be inhibited by ECTP in a cell-free system (12), and all cellular transcription by each of the RNA polymerase I, II, and III in whole cells was inhibited equally by ECyd treatment (13). Therefore, the intracellular RNA synthesis inhibition by ECyd would be attributable by the inhibition of RNA polymerase by cellular ECTP, a major cellular metabolite of ECyd.

ESTABLISHMENT AND CHARACTERIZATION OF ECyd RESISTANT CELLS

To determine in greater detail the mechanistic action of ECyd, we established sublines of HT-1080 resistant to ECyd (HT/ECyd cells) or 3′-*C*-ethynyluridine (EUrd) (HT/EUrd cells). The IC_{50} values of HT/ECyd cells and HT/EUrd cells were 8.3 μM and 30 μM, respectively, for 72 h treatment, as detected by MTT assay, and these values indicated 300- and 1100-fold resistance to ECyd as compared to parental HT-1080 cells, respectively. We evaluated the inhibitory effects of ECyd

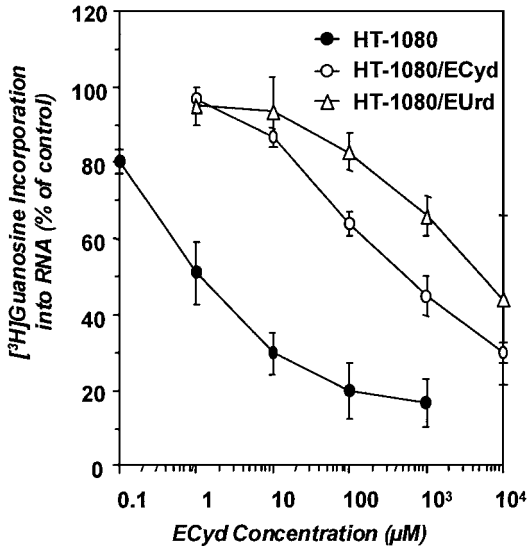

Figure 4. RNA synthesis inhibition of ECyd.

on RNA synthesis in these ECyd resistant cell lines for comparison with parent HT-1080 cells (Fig. 4). ECyd inhibited guanosine incorporation into cellular RNA in HT/ECyd cells and HT/EUrd cells, and these IC_{50} values for 4 h ECyd treatment were 0.6 mM and 6.5 mM, respectively, whereas inhibition of thymidine incorporation into cellular DNA was not observed with ECyd treatment in either cell line. The resistance to cellular RNA synthesis inhibition with ECyd as compared to that of parental HT-1080 cells, was calculated to be 350- for HT-1080/ECyd cells and 3700-fold for HT-1080/EUrd cells, these degrees tended to be close to the ECyd cytotoxicities against these resistant cell lines.

Cellular uptake and metabolism of ECyd in these ECyd resistant cell lines were studied by 1 μM [^{3}H]ECyd treatment (Fig. 5). Total cellular uptakes of ECyd into HT/ECyd cells and HT/EUrd cells, with 4 h treatment, were 1 and 0.4 pmol/10^6 cells, respectively, and these values were remarkably lower than that of HT-1080 cells (60 pmol/10^6 cells). Furthermore, most cellular ECyd was not metabolized to its nucleotides in HT/ECyd cells, and the cellular concentration and ratio of ECyd from total ECyd uptake, with 4 h treatment, were 0.7 pmol/10^6 cells and 60%. The cellular concentrations of other ECyd metabolites, ECMP, ECDP, and ECTP in HT/ECyd cells were 0.02, 0.13, and 0.25 pmol/10^6 cells, respectively. Especially, the cellular accumulation of ECTP, which is considered to be an active metabolite of ECyd in the inhibition of cellular RNA synthesis, was 150 times less in HT/ECyd cells than in HT-1080 cells. This ratio of ECTP accumulation between HT/ECyd cells and HT-1080 cells tended to approximate the degree of resistance to cellular RNA synthesis inhibition by ECyd, which was 350. On the other hand, ECyd was detectable only in HT/EUrd cells, and cellular accumulations of ECMP, ECDP, and ECTP were too low to be detectable with this HPLC detection system.

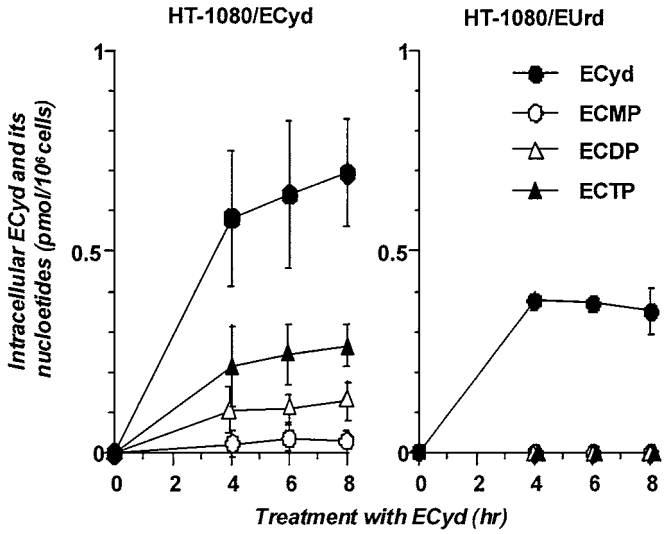

Figure 5. ECyd metabolism in ECyd resistant cells.

The ratios of intracellular ECyd to total ECyd uptake in both HT/ECyd cells and HT/EUrd cells were remarkably higher than that of parental HT-1080 cells, suggesting that intracellular phosphorylation of ECyd, especially ECyd to ECMP, would be downregulated in ECyd resistant cell lines. This first phosphorylation step of ECyd in whole cells would be expected to be carried out by uridine/cytidine kinase (UCK), because the cytotoxic effects of ECyd *in vitro* were blocked by the presence of cytidine or uridine (11). Therefore, ECyd-phosphorylating activities of HT-1080 cells, HT/ECyd cells, and HT/EUrd cells were determined by UCK assay using the corresponding cell extracts. ECyd-phosphorylation activities of HT/ECyd cells and HT/EUrd cells were 1.5 nmol/mg/min and 0.2 nmol/mg/min, respectively, indicating marked downregulation from the parental HT-1080 cells that was 4.6 nmol/mg/min.

These results suggested cellular uptake of ECyd and cellular ECTP accumulation, especially the first phosphorylation step of ECyd by UCK, to be downregulated. This reflected the low sensitivity of ECyd to HT/ECyd cells and HT/EUrd cells. Furthermore, these two factors would determine the sensitivity of ECyd in clinical usage.

DISTRIBUTION AND METABOLISM OF ECyd *IN VIVO*

Concerning the toxicity of antitumor drugs, the *in vivo* drug distribution is very important. Therefore, we demonstrated the distribution of ECyd in LX-1 human lung tumor bearing nude rats. [^{3}H]ECyd was i.v. administrated into LX-1 human lung tumor bearing nude rats at a dose of 6 mg/kg, and the distributions of ECyd in normal and tumor tissues from rats were determined until 24 h after administration

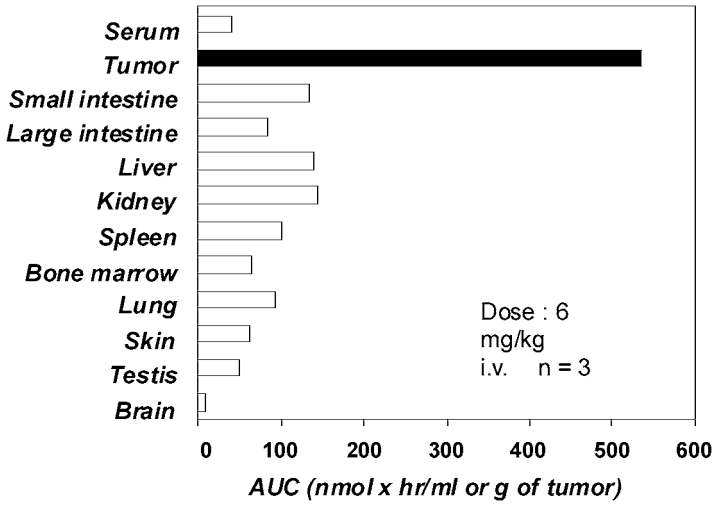

Figure 6. Distribution of ECyd in LX-1 human lung tumor bearing nude rats.

by detection of radioactivity. The distribution of ECyd and its nucleotides in each tissues was indicated by area under the concentration time curve (AUC), as shown in Figure 6. ECyd was highly distributed in tumor tissues, and was at least 5 times more amount than in any normal organs or serum.

Furthermore, metabolism of ECyd in its LX-1 tumor tissue was determined (Fig. 7). Tumor tissues were homogenized with 4% of perchloric acid (PCA) to extract an acid-soluble fraction that contained ECyd and its nucleotides, and to

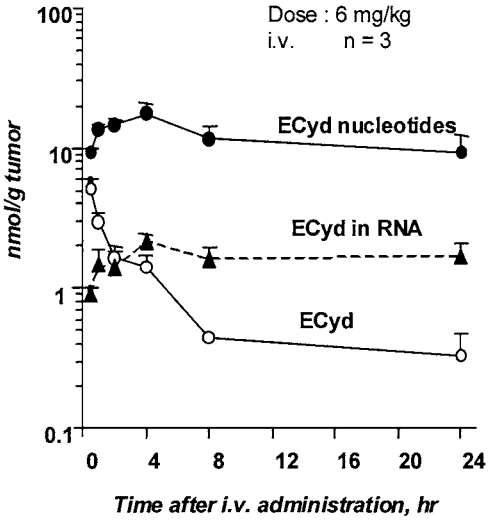

Figure 7. ECyd metabolism in tumor tissues from nude rats.

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separate an acid-insoluble fraction that contained DNA and RNA, and the corresponding radioactivities were determined. The radioactivity of the acid-soluble fraction was much higher than that of the acid-insoluble fraction, and was mainly ECyd nucleotides including ECMP, ECDP, and ECTP. ECyd nucleotides were retained in the tumor tissues at almost the same level from 1 h to 24 h after ECyd administration. In contrast, the ECyd concentration in tumor tissues decreased time-dependently. Generally, nucleoside analogs are known to be able to penetrate through the cell membranes via specific transporters. This is not the case, however, for nucleotides. Therefore, not only intracellular phosphorylation in tumor tissues and stability of these phosphorylated analogs, but also membrane transport are presumably important factors in tumor accumulation of nucleoside analogs to be effective against tumor growth. Long retention of ECyd nucleotides in tumor tissues suggests that ECyd nucleotides are not readily decomposed to ECyd in tissues. On the other hand, radioactivity was detected only in the RNA, and not the DNA in the acid-insoluble fraction of the cells. ECyd was metabolized to ECTP, which caused inhibition of RNA polymerases. At that time, slight ECyd was detected from RNA but not from DNA *in vitro* (data not shown). These data suggested that ECyd predominantly affect RNA synthesis but not DNA synthesis, *in vivo*, as well.

ECyd phosphorylation steps are important for long retention of ECyd in tissue, and especially the first step, that is from ECyd to ECMP, is carried by UCK. UCK activities in normal and LX-1 tumor tissue homogenates from nude rats were determined using [^{3}H]ECyd as a substrate (14) (Fig. 8). UCK activity in tumor tissues was higher than in any normal tissues. UCK activities and the ECyd

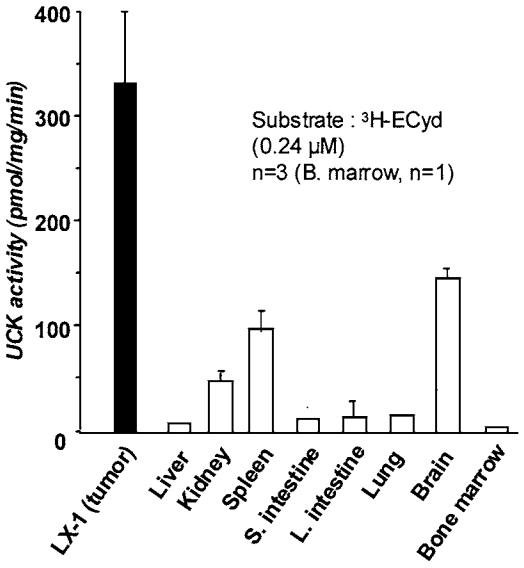

Figure 8. ECyd phosphorylation activity in normal and tumor tissues.

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distribution in normal and tumor tissues showed a good correlation, suggesting the first step of ECyd phosphorylation to have an important role in ECyd distribution, as expected. Furthermore, it should be an issue that relatively selected distribution of ECyd in tumor tissues is strongly related to strong antitumor effects and low toxicity of ECyd *in vivo*. Actually, we have not found any serious toxicity in our toxicological study using nude rats with the schedules we used. In normal organs, we found high UCK activity in brain (Fig. 8). However, the labeled ECyd distribution was very low in brain (Fig. 6). Therefore, we speculated that ECyd passage through the blood-brain-barrier is minimal.

CONCLUSION

ECyd has potent antitumor activity against various human tumor models *in vitro* and *in vivo*. ECyd is phosphorylated in whole cells to ECMP, ECDP, and finally ECTP, and the first step from ECyd to ECMP is carried by uridine/cytidine kinase. ECTP is an active metabolite of ECyd and inhibits RNA polymerase I, II, and III without selectivity to cause RNA synthesis inhibition. In the ECyd resistant cell lines, the cellular uptake of ECyd and the ECTP accumulation are downregulated as compared to these parental cells. ECyd is highly distributed in tumor tissues *in vivo* where UCK activity is higher than in normal tissues, and these data would explain high activity and low toxicity of ECyd against various tumor models.

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