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Enzymatic Cleavage of Various Fluorinated Pyrimidine Nucleosides to 5-Fluorouracil and Their Antiproliferative Activities in Human and Murine Tumor Cells

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The antitumor agent 5′-deoxy-5-fluorouridine (5′-dFUrd) is a prodrug from which 5-fluorouracil (5-FUra) is generated mainly by uridine phosphorylase in mice and by thymidine phosphorylase in human tumors. In the present study various derivatives related to 5′-dFUrd were examined to establish the relationship between their susceptibilities to these enzymes, and their *in vitro* antitumor activities against both human and murine tumor cells. These studies divided the compounds into four distinct groups. 1) Compounds (including 5′-dFUrd) which were phosphorolyzed to 5-FUra by extracts from both human tumors and murine sarcoma 180; these compounds inhibited the growth of both murine (sarcoma 180 and B16 melanoma) and human (HeLa and G361 melanoma) cells. 2) Compounds which were phosphorolyzed only by the extract from the murine tumor; these compounds inhibited the growth of only the murine tumor cells. 3) Compounds phosphorolyzed only by the human tumor extracts; these compounds showed activity against the tumor cells of both species, although the relative activity against the human tumor cells appeared to be higher than that of group 1 compounds. 4) Compounds which were not phosphorolyzed by the extracts from the human or murine tumors; these compounds showed no antitumor activity. These results suggest that phosphorolysis of the 5′-dFUrd analogs is essential for antitumor activity, and that the metabolism of pyrimidine nucleosides is different between human and mouse tumors. Future approaches towards the design of prodrugs of 5-FUra and 5′-dFUrd should be aimed at compounds which generate 5-FUra in human tumor cells, or which are substrates for the human enzyme.

Keywords——5-FUra; 5′-dFUrd; fluorinated pyrimidine nucleoside; pyrimidine nucleoside phosphorylase

Introduction

5′-Deoxy-5-fluorouridine (5′-dFUrd) was shown to be a more effective antitumor agent with less toxicity and immunosuppressive activity than 5-fluorouracil (5-FUra), 2′-deoxy-5-fluorouridine (2′-dFUrd) and 1-(2-tetrahydrofuryl)-5-fluorouracil (FT-207).[1,2] 5′-dFUrd is a prodrug from which 5-FUra is generated by pyrimidine nucleoside phosphorylases.[1] The enzyme activity in mice bearing transplantable tumors was reported to be localized preferentially in the tumors as well as in the gastrointestinal tract.[1] Consequently, 5′-dFUrd was preferentially converted to 5-FUra in the tumors after its administration.[3]

Two distinct pyrimidine nucleoside phosphorylases are widely distributed in both prokaryotes and eukaryotes. One is uridine nucleoside phosphorylase [EC 2.4.2.3], which phosphorolyzes both uridine and thymidine, as well as 2′-deoxyuridine and other pyrimidine nucleosides.[4,5] The other is thymidine phosphorylase [EC 2.4.2.4], which is thought to be specific for deoxypyrimidine nucleosides such as thymidine and 2′-deoxyuridine.[6] Recently it was found that different enzymes participate in the metabolism of pyrimidine nucleosides in humans and rodents.[7-9] In mouse tumors, uridine phosphorylase is suggested to be essential

for phosphorolysis of 5′-dFUrd to 5-FUra.[1] On the other hand, thymidine phosphorylase appeared to be responsible for phosphorolysis in human tumors, since only a small amount of uridine could be phosphorolyzed, though 5′-dFUrd was a fairly good substrate.[7-9]

From these observations, one can expect that some analogs of 5-FUra or 5′-dFUrd might be found which could be cleaved to 5-FUra only by the human or by the mouse enzyme. In the present study, we have examined various analogs of 5′-dFUrd for their susceptibilities to both the human and mouse enzymes, and for their antitumor activities against both human and mouse tumor cells. These studies show that such analogs do indeed exist, and suggest that the differences in the metabolic patterns of pyrimidine nucleosides between humans and rodents should be considered in assessing analogs of 5-FUra or 5′-dFUrd in rodents.

Experimental

Animals——Male ddY mice (6 weeks old) were purchased from Shizuoka Agricultural Cooperative for Laboratory Animals, Hamamatsu, Japan.

Extracts of Tumors and Other Tissues——Sarcoma 180 (1×10^6 cells) was transplanted to ddY mice subcutaneously. After two weeks, the solid tumor was excised, and homogenized in 10 mM Tris buffer (pH 7.4) containing 15 mM NaCl, 1.5 mM $MgCl_2$ and 50 μM potassium phosphate. The homogenate was then centrifuged at 130000 × *g* for 90 min, and the supernatant was dialyzed overnight against 0.02 M phosphate buffer (pH 7.4). All procedures were carried out below 4 °C. This preparation was used as the crude pyrimidine nucleoside phosphorylase. Human cancer tissues and normal tissues adjacent to the tumor were obtained by courtesy of Dr. Suga, the Nagoya National Hospital. The preparation of the crude human enzyme was also carried out as described above.

Assay of Pyrimidine Nucleoside Phosphorylases——A typical reaction mixture (0.13 ml) for the assay of the enzyme activity contained 12.2 μmol of potassium phosphate (pH 7.4), 1.83 nmol of ^{14}C-5′-dFUrd (0.05 μCi), ^{3}H-uridine (0.5 μCi), ^{3}H-2′-dFUrd (0.5 μCi), ^{3}H-FUrd (5-fluorouridine, 0.5 μCi) or ^{3}H-thymidine (0.5 μCi), and the crude enzyme from mouse or human tissues. The reaction was carried out at 37 °C for several minutes and then terminated by the addition of 0.13 ml of methanol. After removal of the precipitate, an aliquot of the reaction mixture (20 μl) was applied to a silica gel plate ($60F_{254}$) for thin layer chromatography. The solvent system used was as follows; ethyl acetate : methanol : ammonium hydroxide (40%) = 75 : 25 : 2 (v/v/v). The radioactivity of 5-FUra, uracil or thymine generated from the corresponding nucleosides was measured with a scintillation counter. The *Rf* values of 5-FUra, uracil or thymine were 0.22, 0.35 and 0.42, respectively.

An alternative method in which microorganisms are used is as follows; 5′-dFUrd or its analogs (0.53 μmol) were incubated at 37 °C for 60 min with the crude enzymes and 12 μmol of potassium phosphate (pH 7.4) in the reaction mixture (0.13 ml). Thereafter, the amount of 5-FUra generated from 5′-dFUrd or its analogs was estimated (without separation) from the antibacterial activity against *Micrococcus flavus* BP1202 DR1.[10] This bacterium is deficient in pyrimidine nucleoside phosphorylase, and is consequently resistant to 5′-dFUrd and its analogs used in the present study, but is susceptible to 5-FUra.

Cells and Culture——Sarcoma 180, B16 melanoma, human melanoma G361 and HeLa cell lines were used. For the cell growth–inhibition test, the cells (10^4/well) were cultured at 37°C in a microtest plate with RPMI 1640 medium containing 10% fetal calf serum and serially diluted 5′-dFUrd or its analogs to be tested. After 3 d of culture, the cells were washed with phosphate-buffered saline solution, and the cell growth, estimated from the protein content of the cultured cells, was measured. The 50% inhibitory dose (ID_{50}), at which the cell growth was inhibited by 50% as compared with the control culture, was determined.

Chemicals——5′-dFUrd and its analogs were synthesized as described elsewhere[11-13]. [5-^{3}H]uridine (41.3 Ci/mmol) and [methyl-^{14}C]thymidine (51 Ci/mmol) were purchased from New England Nuclear Co., Boston, Mass. [6-^{3}H]2′-dFUrd (1.26 Ci/mmol) and [2-^{14}C]FUrd (56 Ci/mmol) were obtained from Amersham Corp., Buckinghamshire, England, and Moravek Biochemicals, California, respectively. 2′-Deoxyglucopyranosyl thymine (GPT) was synthesized as described elsewhere.[14]

Results

Substrate Specificities of the Crude Pyrimidine Nucleoside Phosphorylases

The substrate specificities of the crude pyrimidine nucleoside phosphorylases used in this study were examined before investigating the roles of the enzymes in the activation of 5′-dFUrd and its prodrugs. In Table I, the susceptibilities of five pyrimidine nucleosides, 5′-dFUrd, 2′-dFUrd, FUrd, uridine and thymidine, to the extracts from sarcoma 180 and

TABLE I. Substrate Specificities of Pyrimidine Nucleoside Phosphorylases Prepared from Human Tissues and Mouse Sarcoma 180

Enzyme source	Nucleosides phosphorolyzed (nmol/mg protein/h)				
	5′-dFUrd →5-FUra	Urd →Ura	FUrd →5-FUra	dThd →Thy	2′-dFUrd →5-FUra
Exp. 1					
Stomach					
Normal tissue	17.8	9.9	5.2	94.8	57.2
Tumor	52.8	5.8	20.8	231.1	89.0
Oral mucosa					
Normal tissue	0	0	0.2	2.1	1.7
Tumor	4.3	1.7	1.7	23.4	18.6
Bladder					
Normal tissue	0.8	0	0.4	5.1	3.9
Tumor	41.4	0	14.6	199.0	78.5
Mouse sarcoma 180	48.8	202.9	297.0	54.0	61.1
Exp. 2					
Breast cancer	40.9	3.6	90.3	225.0	87.8
Bladder					
Normal	2.7	0	57.5	30.7	13.3
Tumor	45.8	12.1	61.6	267.6	98.5
Brain meningioma	6.0	0	12.3	42.9	27.5
Mouse sarcoma 180	41.8	203.0	444.6	11.8	61.6

TABLE II. Effect of GPT on Phosphorolysis of Pyrimidine Nucleosides by Enzymes Prepared from Human and Mouse Tumors

Enzyme source	Nucleosides phosphorolyzed (nmol/mg protein/h)				
	5′-dFUrd →5-FUra	Urd →Ura	FUrd →5-FUra	dThd →Thy	2′-dFUrd →5-FUra
Human stomach tumor					
−GPT	8.4	N.D.	3.6	57.0	42.0
+GPT	7.8	N.D.	2.4	57.0	32.4
Mouse sarcoma 180					
−GPT	34.2	220.8	294.0	40.2	58.2
+GPT	0.6	0.6	10.2	1.8	2.4

N.D.; not detected.

human tumors are shown. In this study, the amount of 5-FUra, thymine or uracil generated was measured after phosphorolysis of the corresponding nucleosides by the crude enzymes in the presence of inorganic phosphate. The table shows that the substrate specificities of the enzymes are quite different between the two species. The order of relative susceptibility to the enzyme from human tumors was thymidine > 2′-dFUrd > 5′-dFUrd and FUrd, while that to the enzyme from sarcoma 180 was FUrd > uridine > 2′-dFUrd > 5′-dFUrd and thymidine. In the extract from the human tumors, only a small amount of enzyme which phosphorolyzed uridine was detected. These results indicate that the human tumor tissues are deficient in uridine phosphorylase. Since 5′-dFUrd was phosphorolyzed by the extract, enzymes other than uridine phosphorylase must have cleaved 5′-dFUrd to 5-FUra in human tissues. This possibility was confirmed by experiments using a specific inhibitor of uridine phosphorylase, GPT. As shown in Table II, GPT inhibited phosphorolysis by the extract from sarcoma 180 of

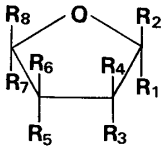

Fig. 1. Structures of the Compounds Tested to Establish the Substrate Specificities of Pyrimidine Nucleoside Phosphorylases, Where R1—R8 are Described in Table III

TABLE III. Substrate Specificities of Pyrimidine Nucleoside Phosphorylases in Extracts from Mouse and Human Tumors

	Substrates[a]								Nucleosides phosphorolyzed (nmol/mg protein/h)		
	R1	R2	R3	R4	R5	R6	R7	R8	Mouse sarcoma 180	Human stomach tumors A	B
Group 1											
5′-dFUrd	H	FU	OH	H	OH	H	H	CH_3	122.3	83.8	27.7
Ro 22-2620	H	FU	OH	H	OH	H	CH_3	H	49.2	26.9	<15.3
Ro 14-9021	H	FU	OH	H	OH	H	H	C_2H_5	56.9	36.9	23.8
Group 2											
Ro 15-6578	H	FU	OH	H	OH	H	H	H	252.3	<13.8	<13.8
Ro 22-3494	H	FU	OH	H	OH	H	H	CH_2NH_2	172.3	<13.8	<13.8
Ro 23-0074	H	FU	OH	H	OH	H	H	CH_2CH_2OH	600.0	<25.3	<25.3
Ro 22-9118	H	FU	OH	H	H	H	H	CH_2OH	92.3	<28.5	<28.5
Group 3											
Ro 08-4960	H	FU	H	H	OH	H	H	CH_3	<13.8	356.2	80.0
Ro 22-4729	H	FU	H	H	H	H	H	CH_3	<13.8	62.3	<13.8
Ro 21-2435	H	FU	H	H	OH	H	H	CH_2I	<13.8	140.0	60.0
Group 4											
Ro 14-6102	FU	H	OH	H	OH	H	H	CH_3	<15.4	<15.4	<15.4
Ro 22-2775	H	FU	H	OH	OH	H	H	CH_3	<15.4	<15.4	<15.4
Ro 22-4552	H	FU	H	OH	H	OH	H	CH_3	<15.4	<15.4	<15.4
Ro 15-3256	H	FU	H	OH	H	OH	CH_3	H	<15.4	<15.4	<15.4
Ro 14-9941	FU	H	H	OH	H	OH	CH_3	H	<15.4	<15.4	<15.4
Ro 22-6493	H	FU	OH	H	H	OH	H	CH_3	<15.4	<15.4	<15.4
Ro 21-7092	H	FU	OH	H	OH	H	H	CH_2I	<15.4	<15.4	<15.4
Ro 22-4425	H	FU	H	H	H	OH	H	CH_3	<15.4	<15.4	<15.4
Ro 22-2844	H	FU	OH	H	OH	H	H	CH_2OCH_3	<15.4	<15.4	<15.4
Ro 15-1288	H	FU	OH	H	OH	H	H	nC_5H_{11}	<15.4	<15.4	<15.4
Ro 22-8363	H	FU	OH	H	OH	H	H	CH_2F	<15.4	<15.4	<15.4
Ro 22-5886	$CH_2OH-\underset{CH_3}{CH}-O-\underset{CH_2OH}{CH}-5FU$								<15.4	<15.4	<15.4
Ro 22-6162	$CH_3CH_2OCH_2-5FU$								<15.4	<15.4	<15.4

a) Structures of the substrates are shown in Fig. 1.

all the nucleosides described above, whereas it did not show any significant inhibitory effect on the activity of the human enzyme. These results again indicate that thymidine phosphorylase predominates in human tumors, whereas uridine phosphorylase predominates in the murine tumor. Thus 5′-dFUrd could be a substrate for both the thymidine and uridine phosphorylases.

Susceptibilities of Analogs of 5′-dFUrd to the Pyrimidine Nucleoside Phosphorylases

Various analogs of 5′-dFUrd were then examined for their susceptibilities to the extracts from the human tumors and sarcoma 180. Each analog was incubated with the extracts, and the amount of 5-FUra generated was measured. The amount of 5-FUra was estimated from

TABLE IV. Antiproliferative Activities of Fluorinated Pyrimidine Nucleosides against Murine and Human Tumor Cells

Compounds	Antiproliferative activity (IC_{50}: μM)				
	Murine tumor cell		Human tumor cell		HeLa/sarcoma 180
	Sarcoma 180	B16	HeLa	G361	
Group 1					
5′-dFUrd	0.72	0.41	17	63	24
Ro 14-9021	9.1	5.4	160	350	18
Group 2					
Ro 22-3494	6.4	4.7	>280	570	>44
Ro 15-6578	2.9	1.6	>600	580	>207
Ro 23-0074	1.9	1.5	>600	135	>316
Group 3					
Ro 08-4960	1.1	0.68	3.9	62	3.5
Ro 21-2435	47	170	24	432	0.51
Ro 22-4729	8.1	8.6	43	23	5.3
Group 4					
Ro 15-3256	>600	>600	>600	>600	—
Ro 22-2775	>600	>600	>600	>600	—
5-FUra	0.11	0.33	2.4	2.3	22

its antibacterial activity against a mutant of *Micrococcus flavus* without separation from the analogs being tested. This bacterium is deficient in pyrimidine nucleoside phosphorylases and is consequently resistant to 5′-dFUrd.[10] None of the analogs of 5′-dFUrd used in the present study showed any inhibition of bacterial growth; the antibacterial activity was only observed after incubation in the presence of both the extract, inorganic phosphate and one of the substrates for the pyrimidine nucleoside phosphorylases. This activity is therefore due to generation of 5-FUra, and in Table III, the susceptibilities thus obtained are compared. The analogs can be divided into 4 groups. 1) Group 1 analogs (including 5′-dFUrd) which were phosphorolyzed by the extracts from both the human and murine tumors. 2) Group 2 analogs which were phosphorolyzed only by the mouse extract. 3) Group 3 analogs which were phosphorolyzed only by the human extract. 4) Group 4 analogs which were not substrates for either human or mouse enzyme. When the extracts from cultured cells, such as sarcoma 180 and HeLa cells, were used as the enzyme source, similar results were obtained (data not shown).

In Vitro Antitumor Activities of the Analogs of 5′-dFUrd

The analogs of 5′-dFUrd were next examined for their *in vitro* antitumor activities against murine (sarcoma 180 and B16 melanoma) and human tumor cells (HeLa). In Table IV, the activities of typical analogs of each group are compared. 5′-dFUrd (group 1 analog) inhibited both human and murine tumor cells, although its activity against the murine tumor cells was relatively higher than that against the human tumor cells. For 5′-dFUrd, the ratio ID_{50} (HeLa cells)/ID_{50} (sarcoma 180) was found to be approximately 24. Group 2 analogs showed activity against the murine tumor cells, whereas the human tumor cells were quite resistant to these analogs, resulting in high ratios of ID_{50} (HeLa cells)/ID_{50} (sarcoma 180). Group 3 analogs, such as those from which 5-FUra was generated only by the human enzyme, inhibited the growth of both human and murine tumor cells, contrary to the expectation that these analogs would be effective only against the human tumor cells. However, when the ratio was compared with group 1 compounds, the relative activity against

the human tumor cells was higher. Enzymes other than pyrimidine nucleoside phosphorylases may also be involved in the activation of group 3 analogs in the human tumor cells. On the other hand, the group 4 analogs did not show any activity against either the human or the mouse tumor cells.

Discussion

The conversion of 5′-dFUrd to 5-FUra by uridine phosphorylase has been shown to be essential for manifestation of the antitumor activity and probably also the toxicity in animal models.[1] However, the enzyme responsible for the conversion of 5′-dFUrd in human tumors has been reported to be thymidine phosphorylase.[7-9] The present study suggests that this anomaly is due to differences in the relative ratios of these two enzymes between humans and mice. Namely, in murine tumor cells, the level of uridine phosphorylase, which can phosphorolyze both uridine and thymidine, is substantially higher than that of thymidine phosphorylase, the latter activity being measured by addition of GPT. On the other hand, the level of thymidine phosphorylase in human cells was substantially higher. The predominance of uridine phosphorylase was also observed in various tissues of mice and dogs, except in the liver, where thymidine cleavage occurred to some extent even in the presence of GPT, whereas the levels of thymidine phosphorylase were overwhelmingly higher in various tissues from baboon and human biopsies, including tumor tissues (unpublished observation). The different proportions of these enzymes between murine and human tumor cells are also supported by the observations that murine and human tumor cells showed different responses to analogs of 5′-dFUrd (Table IV).

The contrasting patterns of pyrimidine nucleoside phosphorylases between rodents and humans have been reported by several investigators. Langen and Etzold, and Krenitsky *et al.* reported that the ratio of uridine phosphorylase to thymidine phosphorylase was characteristically high in rat and mouse tumors and conversely that the ratio of uridine-cleaving enzyme to thymidine phosphorylase was characteristically low in human tumors.[15,16] Kubilus *et al.* showed that only thymidine phosphorylase but not uridine phosphorylase was detected in the homogenate of human amniochorion.[17] Heidelberger and his colleagues found that only thymidine phosphorylase was detected in cytosol preparations of human leukocytes and HeLa cells, as determined by electrofocusing, whereas in Ehrlich ascites tumor and Novikoff hepatoma cells uridine–thymidine phosphorolyzing enzyme, which could be inhibited by GPT, was detected.[18] However, they detected both thymidine and uridine phosphorylases in mouse liver. Yamada also found both enzymes in rat liver with similar contents.[19] In our preliminary experiments the levels of thymidine phosphorylase, the activity of which was estimated in the presence of GPT, were substantially higher in mouse liver than in other tissues (unpublished observation). The proportion of the pyrimidine nucleoside phosphorylases in liver seems different from that of other tissues. On the other hand, Laskin *et al.* reported that extracts from HeLa cells had a capacity to cleave uridine to uracil as well as thymidine to thymine.[20] One possible explanation is that a labile uridine phosphorylase in HeLa cells[21] might be reflected in their experiment, or alternatively the different assay methods for uridine phosphorylase might also have contributed to this discrepancy. They estimated the enzyme activity by measuring the amount of uracil generated from uridine in terms of the characteristic increase of the absorption at 290 nm, whereas in the present study the amount of ^{3}H-uracil generated from ^{3}H-uridine was measured after its separation from uridine by thin layer chromatography.

Various analogs of 5′-dFUrd were tested for their susceptibilities to pyrimidine nucleoside phosphorylases in cell extracts or tissue extracts. Among them, the analogs having 5′- or 3′-deoxyribose, or erythrose as well as ribose were substrates for the mouse enzyme,

probably uridine phosphorylase. On the other hand, the analogs having 5′-deoxyribose or 2′,5′-dideoxyribose as well as 2′,3′,5′-trideoxyribose were substrates for the human enzyme, probably thymidine phosphorylase. These analogs can be divided into the following four groups. 1) Group 1 analogs (including 5′-dFUrd) which are phosphorolyzed by both the mouse and human enzymes. 2) Group 2 analogs which are phosphorolyzed only by the mouse enzyme. 3) Group 3 analogs which are phosphorolyzed only by the human enzyme. 4) Group 4 analogs which are not phosphorolyzed by either the human or the mouse enzyme. It is to be expected that the contrasting patterns of cleavage of the 5′-dFUrd analogs between the two species may lead to differences in the responses of the tumor cells of the two species. The study on inhibition of cell growth by the analogs revealed that this was indeed the case. The analogs which could be phosphorolyzed by the cell extracts were active against the cells from which the extracts were prepared. The only exception is that group 3 analogs were effective against the murine tumor cells as well, although the relative activity of this group against the human tumor cells *versus* the murine tumor cells was generally lower than that of group 1 analogs. One possible explanation for this discrepancy is that enzymes other than pyrimidine nucleoside phosphorylase could generate 5-FUra in the murine tumor cells. Another possible explanation is that group 3 analogs might be hydroxylated at the 2′-position, as observed in the metabolism of FT-207 in rodents,[21-25] so that they could be phosphorolyzed to 5-FUra by uridine phosphorylase.

The present study indicates that some analogs of 5′-dFUrd produce different responses in human and mouse tumor cells. This is due to different patterns of metabolism of pyrimidine nucleosides between these two species. This finding indicates that mouse model systems either *in vitro* or *in vivo* are not adequate for assessment of new analogs of 5-FUra and 5′-dFUrd, and that any future approach to prodrugs of 5-FUra should be aimed towards compounds which generate 5-FUra in human tumor cells as a result of the action of the human enzyme.

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