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Reduced cellular transport and activation of fluoropyrimidine nucleosides and resistance in human lymphocytic cell lines selected for arabinosylcytosine resistance

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Abstract

Arabinofuranosylcytosine (araC) resistant H9-araC0.05 and H9-araC0.5 sublines were obtained following in vitro exposure of H9 cells to 0.05 and 0.5 μM araC, respectively. These cell lines were 83.3- and 266.7-fold, 21- and 80-fold, and 2.4- and 4.0-fold more resistant to 5-fluorouridine (FUR), 5-fluoro-2′-deoxyuridine (FdUR), and 5-fluorouracil (FU), respectively. Compared with H9 cells, the cellular accumulation of FUR was 2.2 and 0.2%, FdUR 15.6 and 0.9%, and FU 56.9 and 66.5% in H9-araC0.05 and H9-araC0.5 cells, respectively. An araC resistant HL60 cell line (promyelocytic cell line) was 5.0- and 1.7-fold resistant to FUR and FdUR, respectively, but displayed no resistance to FU. The lower FUR and FdUR nucleotide levels in the resistant cells were a result of reduced cellular transport and uridine kinase (UR kinase) and thymidine kinase (TK) activities. Compared with the parental cell line, the *p*-nitrobenzyl thioinosine (an inhibitor of nucleoside transport) binding sites also were lower in the araC resistant cells. There was no difference in the expression of multidrug-resistant protein and thymidylate synthase mRNA in the parental and the resistant cell lines. Data presented here suggest that araC exposure of H9 cells, in addition to araC resistance, induced/selected cells that were resistant to FUR and FdUR. These cells had altered cellular drug transport and lower TK and UR kinase activities. Further studies to understand molecular mechanisms of this phenomenon are warranted.

Keywords: Arabinosylcytosine; Fluoropyrimidines; Fluorouracil; Fluorouridine; Fluorodeoxyuridine; Cross-resistance; Arabinosylcytosine resistance; Human lymphocytic cell lines; Promyelocytic cell lines; H9 cells; HL60 cells; Nucleoside transport

1. Introduction

FPyr (FU, FUR, and FdUR) play a crucial role in the treatment of colorectal cancers [1–3]. Permeation and cellular activation are essential prerequisites for their therapeutic activity. While all these drugs appear to be transported by facilitated transport systems, differences do occur in their permeation [3–6]. For example, while FU influx is rapid, FUR and FdUR enter cells by a rate-limiting transport system [6]. Once inside cells, FU is activated by orotate phosphoribosyltransferase to FUMP, FUR by UR kinase to FUMP, and FdUR to FdUMP by TK. The monophosphates FUMP and FdUMP may be phosphorylated further by cellular kinases to FUTP and FdUTP, as shown in Scheme 1. These nucleotides then interfere with the synthesis of RNA and DNA, inhibit TS, a key enzyme necessary for *de novo*

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*Abbreviations: ara-C, 1-β-D-arabinofuranosylcytosine; ara-CTP, 1-β-D-arabinofuranosylcytosine 5′-triphosphate; dCK, deoxycytidine kinase; dCMPK, deoxycytidine monophosphate kinase; dCR, 2′-deoxycytidine; FPyr, fluoropyrimidines; FdUR, 5-fluoro-2′-deoxyuridine; FU, 5-fluorouracil; FUMP, fluorouridine monophosphate; FUDP, fluorouridine diphosphate; FUTP, fluorouridine triphosphate; FdUMP, 5-fluoro-2′-deoxyuridine monophosphate; FdUDP, 5′-fluoro-2′-deoxyuridine diphosphate; FdUTP, 5-fluoro-2′-deoxyuridine triphosphate; FUR, 5-fluorouridine; MDR, multidrug resistance; NBMPR, p-nitrobenzyl thioinosine; NDPK, nucleoside diphosphate kinase; R, resistance factor (ratio of IC_{50} values of resistant and sensitive cells); RPMI 1640, Roswell Park Memorial Institute 1640; RT-PCR, reverse transcription-polymerase chain reaction; TdR, thymidine; TK, thymidine kinase; TS, Thymidylate synthase; UR, uridine; and UR kinase, uridine kinase.

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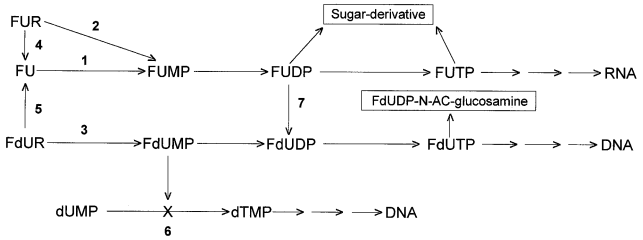

Scheme 1. Simplified scheme of FPyr anabolism and major enzymes involved. (1) Orotate phosphoribosyltransferase (OPRT); (2) uridine kinase (UK); (3) thymidine kinase (TK); (4) uridine phosphorylase; (5) thymidine phosphorylase; (6) thymidine synthase (TS); and (7) nucleoside diphosphate dehydrogenase.

TMP synthesis, and form FU–sugar derivatives [3,7–9]. The major effect of FdUR is considered to be through inactivation of TS, resulting in the inhibition of DNA synthesis [8]. The effects of FUR are mostly through incorporation into RNA [10,11]. FU affects both RNA and DNA syntheses [3]. Thus, the effects of FUR are independent of the cell cycle, in contrast to many other antimetabolite anticancer agents like FdUR. While FU and FdUR are widely used in the treatment of gastrointestinal tumors, the use of FUR has been limited due to its systemic toxicity. The differences in metabolism, mechanism of action, and favorable pharmacokinetics of FUR have renewed interest in this drug for the treatment of superficial bladder cancers that represent about 80% of the bladder cancers [12–14]. However, the emergence of drug-resistant cells following drug exposure mitigates their therapeutic potential and poses a major problem in chemotherapy. Various biochemical mechanisms have been reported that are responsible for the drug resistance, including increased and altered TS activity [15,16], decreased drug transport across the cell membrane [17], decreased accumulation and retention of active nucleotides, decreased activities of activating enzymes, TK and UR kinase, and increased catabolism of the drugs and their nucleotides [9,18,19].

Another pyrimidine nucleoside analog, araC, is an important anticancer agent used in the treatment of acute nonlymphocytic leukemias, acute lymphocytic leukemias, and lymphomas [20–22]. We have isolated araC resistant H9-araC0.05 and H9-araC0.5 cell lines following in vitro exposure of human lymphocytic H9 cells to 0.05 and 0.5 μM araC [23]. Examination of these sublines revealed that they were cross-resistant to FUR and FdUR. The studies presented here explore the mechanism(s) responsible for this unexpected phenomenon.

2. Materials and methods

2.1. Materials

[6-^{3}H]FdUR (17 Ci/mmol), [6-^{3}H]FU (15.3 Ci/mmol), [6-^{3}H]FUR (16.6 Ci/mmol), [5,6-^{3}H]UR (39.7 Ci/mmol), and [G-^{3}H]NBMPR (20.5 Ci/mmol) were purchased from Moravek Biochemicals. RPMI 1640 culture medium and PBS were from Life Technologies. All other chemicals were obtained from the Sigma Chemical Co.

2.2. Cells

H9 cells were maintained in culture in RPMI 1640 medium as described earlier [24]. Exposure of H9 cells to 0.05 and 0.5 μM araC for 3–4 weeks resulted in the generation of araC resistant cells. Once established, the resistant cells were washed and maintained in a drug-free medium. The resulting sublines, H9-araC0.05 and H9-araC0.5 cells (27.8- and >294-fold more resistant to araC), have reduced dCK activity and araC anabolism [23]. HL60 (promyelocytic human leukemia cell lines) and its araC resistant subclone, HL60-araC cells, were supplied by Dr. K. Bhalla of our institution and maintained in RPMI 1640 medium containing 1% non-essential amino acids, 1 mM sodium pyruvate, and 10% heat-inactivated fetal bovine serum [25].

2.3. Drug sensitivity assays

The cell growth inhibitory effects of FU, FUR, and FdUR were determined in triplicate by seeding 5×10^4 cells/mL in 24- or 96-well Costar plates. Increasing concentrations of the drugs were added, and the trypan blue excluding cells were counted after 72 hr of incubation at 37°. The IC_{50} values (inhibitory concentrations at 50%) were calculated from the growth inhibition versus drug concentration curves.

2.4. Cellular accumulation of FU, FUR, and FdUR

The cellular accumulation of FPyr was studied by incubating triplicate cell suspensions (1.0 x 10^6 cells/mL) with 1 μM [^{3}H]FUR or [^{3}H]FdUR or 10 μM [^{3}H]FU (2 μCi/mL) for 3 hr at 37°. The cells were harvested, washed twice with PBS, and extracted overnight at −20° with 0.5 mL of 65% (v/v) methanol. The methanol "soluble" fractions were collected by centrifugation in a refrigerated centrifuge. Aliquots of the "soluble" fractions were counted directly and/or analyzed by HPLC as described earlier [26]. The eluates were monitored at 254 nm, and 1.5-mL samples were collected. Aliquots (1 mL) were mixed with 10 mL of Universol scintillation fluid (ICN) and counted for radioactivity in a Beckman scintillation counter (model LS5000TD). The methanol "insoluble" fractions were washed once with ice-cold 65% methanol and dissolved in 0.5 mL of 0.5 N NaOH. Fractions (0.2 mL) were placed in scintillation vials, neutralized with 0.5 N HCl, mixed with 5–10 mL of the scintillation fluid, and counted for radioactivity.

2.5. FUR and FdUR influx

To determine drug influx, triplicate cell suspensions (0.15 mL each) containing 1×10^6 cells and 5.5 μM [^{3}H]FdUR or [^{3}H]FUR (0.7 μCi/mL) were incubated at room temperature in sodium-containing transport buffer (20 mM Tris–HCl, 3 mM K_2HPO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM glucose, and 130 mM NaCl, pH 7.4) as described by Dumontet et al. [27]. Reactions were terminated at 0 and 60 sec by adding 30 μL of 1 mM unlabeled drugs. The reaction mixture was layered over 0.7 mL of an oil mixture (dibutyl phthalate:dioctyl phthalate in a 4:1 ratio) and centrifuged at 8000 g for 1 min. After removing the aqueous and oil layers, the cell pellets were dissolved in 0.5 mL of 0.5 N NaOH for 30 min, neutralized with 0.5 N HCl, and counted for radioactivity as described above.

2.6. Measurement of [^{3}H]NBMPR binding

[^{3}H]NBMPR binding was determined by a slight modification of a method described by Wiley et al. [28]. Briefly, triplicate cell suspensions (10^6 cells in 150 μL of sodium-containing transport buffer described above) were incubated with 5.0 nM [^{3}H]NBMPR at room temperature. After 5 min of incubation, the reaction mixtures were layered on the oil mixture, and the cells were separated by centrifugation as described above. The non-specific binding and [^{3}H]NBMPR trapped in the extracellular space of the cell pellets were determined in parallel by incubations of cells first with unlabeled NBMPR (10.0 μM) followed by 5.0 nM [^{3}H]NBMPR 5 min later. The cell pellets were dissolved in 0.5 N NaOH and counted as described above. The specific binding was taken as the difference between the isotope binding to cells incubated with [^{3}H]NBMPR and those incubated with unlabeled NBMPR plus [^{3}H]NBMPR.

2.7. Drug retention

FdUR nucleotide retention was determined by first allowing the cells to accumulate radiolabeled FdUR as described above and then following their retention by incubating in drug-free medium. Briefly, 0.5×10^6 cells/mL (6 mL total) were washed twice with PBS after incubation with 1 μM FdUR (2 μCi/mL) for 3 hr at 37°, resuspended in 6 mL of drug-free culture medium, and divided into six 1-mL aliquots. Then duplicate samples were centrifuged at 0, 1, and 2 hr of incubation at 37°. The cell pellets were solubilized in NaOH and counted as described above. The cellular medium was also counted for the released radioactivity.

2.8. MDR phenotype

The MDR phenotype was evaluated by flow cytometry [29] using rhodamine-123 or daunorubicin in the presence and absence of the MDR inhibitor verapamil as described earlier [26].

2.9. Enzyme assays

The UR kinase and TK activities were determined in the cell-free extracts using radiolabeled FUR and FdUR as substrates, respectively, by a method described earlier [24]. Protein concentrations were determined using the Bio-Rad protein assay.

2.10. RT–PCR for TS

TS mRNA levels were measured by RT–PCR. Exponentially growing H9, H9-ara0.05, and H9ara-0.5 cells (1×10^7 cells) were harvested and lyzed by mixing with 1 mL of Trizol reagent and repeated pipetting and vortexing. After a 5-min incubation at room temperature, 0.2 mL chloroform was added, and the samples were vortexed vigorously and then centrifuged at 12,000 *g* for 15 min at 4°. The RNA was precipitated from the aqueous phase by mixing with 0.5 mL of isopropanol. After 10 min at room temperature, the samples were centrifuged at 12,000 *g* for 10 min at 4°. The RNA pellet was washed once with 75% ethanol, briefly air-dried, and dissolved in diethyl pyrocarbonate-treated water.

RT–PCR was done using 1 μg RNA ($A_{260:280} > 1.8$) and the Superscript Pre-amplification system (Gibco BRL) [30]. The PCR mixture contained 4 μL of cDNA reaction mixture, 0.1 μM of either the TS or actin forward and reverse primers, deoxynucleoside triphosphates (1 mM) and 2.5 U of Taq DNA polymerase (Sigma). The sequence of the forward primer for TS was ATG CCT GTG GCC GGC TCG GAG CTG and the reverse primer was TAA TAA CTG ATA GGT CAC GGA CAG. For actin, the sequence of the forward primer was TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA and the reverse primer was CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG. PCR was carried out for 35 cycles as follows: denaturation at 94° for 1 min, annealing at 68° for 2 min, and extension at 72° for 3 min. The PCR products were separated on an ethidium bromide-containing agarose gel.

3. Results

araC sensitive and resistant H9 subclones were tested for their sensitivity to FPyr (Fig. 1). As shown in Fig. 1A, the responses of FUR and FdUR in araC resistant cells were markedly different from those in H9 cells, i.e. the araC resistant cells were highly cross-resistant to FdUR and FUR. The cells also displayed cross-resistance to FU but to a lesser extent. The IC_{50} values determined from these curves (Table 1) indicated that while the resistance to FU in H9-araC0.05 and H9-araC0.5 cells was 2.4- and 4.0-fold, the resistance to FUR (83.3- and 266.7-fold) and FdUR (21- and 80-fold) was significant. To determine whether these observations were limited to H9-araC resistant cells, another cell line, an HL60 cell line resistant to araC, was examined (Fig. 1B). HL60-araC cells displayed a somewhat

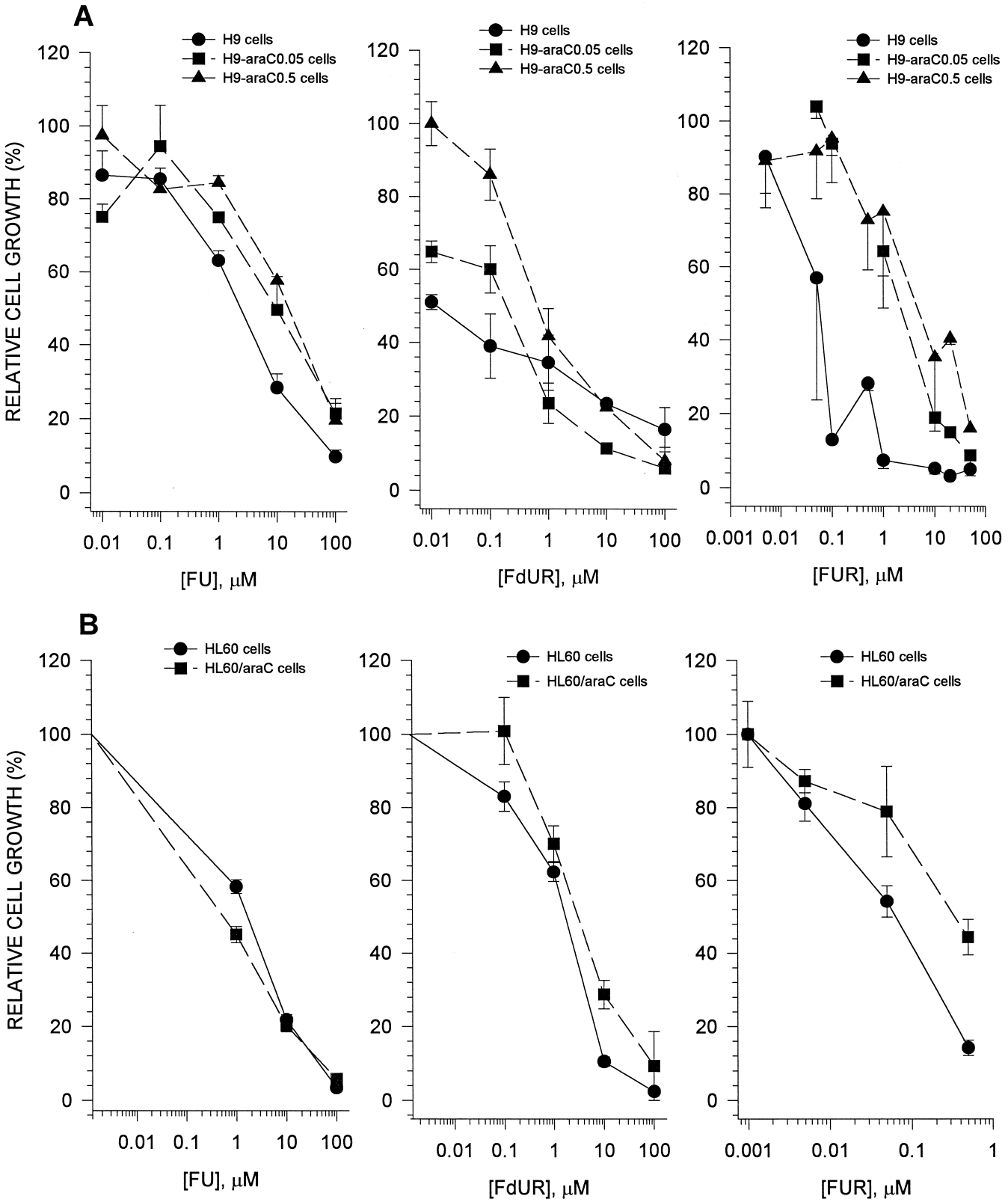

Fig. 1. (A and B) Effects of FU, FUR, and FdUR on the growth of araC resistant and sensitive cells. Triplicate cell suspensions (5×10^4 cells/mL) were incubated with various concentrations of the drugs. After 72 hr of incubation at 37°, the trypan blue excluding cells were counted. Values are means ± SEM of triplicate determinations.

Table 1
IC_{50} Values of FU, FUR, and FdUR in araC resistant and parental H9 and HL60 cell lines

Cells	IC_{50} (μM)					
	FU	(R)[a]	FUR	(R)	FdUR	(R)
H9	2.5	(1.0)	0.03	(1.0)	0.01	(1.0)
H9-araC0.05[b]	6.0	(2.4)	2.5	(83.3)	0.2	(21)
H9-araC0.5[b]	10.0	(4.0)	8.0	(266.7)	0.8	(80)
HL60	1.8	(1.0)	0.07	(1.0)	1.8	(1.0)
HL60-araC	0.7	(0.4)	0.35	(5.0)	3.0	(1.7)

The effects of FU, FUR, and FdUR on cell growth were measured as described in section 2. The values represent the concentration of the drugs required to give 50% inhibition of cell growth.

[a] R, resistance factor; the ratio of IC_{50} values of the resistant and sensitive cell lines is given parentheses.

[b] Compared with H9 cells, H9-araC0.05 and H9-araC0.5 cells were 27.8- and > 294-fold more resistant to araC [23].

similar pattern but the R values of FdUR and FUR in HL60-araC cells were much lower (2- to 5-fold) than those observed in the H9-araC resistant cells (Fig. 1 and Table 1).

A series of investigations were performed to determine the mechanism(s) of cross-resistance of araC resistant subclones to FPyr. The total cellular accumulation of the drugs is shown in Table 2. H9, H9-araC0.05, and H9-araC0.5 cells achieved total concentrations of 28.1, 16.0, and 18.7 pmol of FU/10^6 cells following a 3-hr incubation of cells with a 10 μM concentration of the drug. Of the total incorporation, 39–46% of FU was incorporated into the nucleic acid fractions. FdUR incorporations of 8.2 and 0.5 pmol/10^6 cells in H9-araC0.05 and H9-araC0.5 cells were 15.6 and 0.9%, respectively, of the incorporation of 52.4 pmol/10^6 cells in H9 cells. Amounts of FdUR-derived radioactivity in the nucleic acid fractions of the cells (methanol-insoluble fractions) were 1.0, 0.7, and 0.2 pmol/10^6 cells, respectively, and lacked correlation with the decrease in total accumulation. FUR accumulations of 6.2 and 0.5 pmol/10^6 cells in H9-araC0.05 and H9-araC0.5 cells were reduced to 2.2 and 0.17% from the incorporation of 282 pmol/10^6 cells in H9 cells. Of the total FUR accumulated, 36–42% was incorporated into the nucleic acid fraction (methanol-insoluble fraction). Similarly, the FdUR and FUR incorporations examined in HL60-araC cells were found to be diminished to 80.2 and 246.9 pmol/10^6 cells, respectively, compared with 133.5 and 429.8 pmol/10^6 cells in HL60 cells.

The distribution of FPyr in the cellular nucleotide pool was determined by HPLC analysis of methanol-soluble fractions. The radioactivity was found in three major peaks corresponding to mono-, di-, and triphosphates of the drugs. As shown in Table 3, FU distributed well among the three nucleotides. Triphosphates of FU, however, were diminished to about 46% in the araC resistant cells. The monophosphate (FdUMP) was the major metabolite (>98%) of FdUR. However, the concentrations of FdUMP (considered to be an active metabolite of FdUR) in the H9-araC0.05 and H9-araC0.5 cells were decreased to 12.2 and 0.3% of H9 cells. Concentrations of FdUDP and FdUTP were very low. In contrast, FUR was metabolized almost equally to FUMP, FUDP, and FUTP. However, compared with the value of 51.5 pmol/10^6 H9 cells, the concentrations of FUTP (considered the cytotoxic metabolite of FUR) achieved in H9-araC0.05 and H9-araC0.5 cells were very low, i.e. 2.9 and 0.06 pmol/10^6 cells, respectively.

To determine the cause of diminished cellular FUR and FdUR accumulation and activation in the resistant cells, the metabolism of the drugs was examined. As shown in Table 4, the values of FUR influx in H9-araC0.5 and H9-araC0.05

Table 2
Cellular accumulation of FU, FUR, and FdUR in H9, H9-araC0.05, H9-araC0.5, HL60, and HL60-araC cells

	Incorporation (pmol/10^6 cells)				
	H9	H9-araC0.05	H9-araC0.5	HL60	HL60-araC
FU					
Soluble	15.4 ± 4.0	9.8 ± 0.8	10.2 ± 0.9	ND[a]	ND
Insoluble	12.7 ± 0.8	6.2 ± 0.5	8.5 ± 0.6	ND	ND
Total	28.1	16.0	18.7	ND	ND
FUR					
Soluble	181.2 ± 22.0	3.6 ± 0.3	0.3 ± 0.07	283.9 ± 39.4	168.3 ± 29.7
Insoluble	100.8 ± 22.9	2.6 ± 0.2	0.2 ± 0.05	145.9 ± 31.6	78.6 ± 13.6
Total	282.0	6.2	0.5	429.8	246.9
FdUR					
Soluble	51.4 ± 13.3	7.5 ± 1.4	0.3 ± 0.2	132.7 ± 5.0	79.1 ± 1.0
Insoluble	1.0 ± 0.3	0.7 ± 0.1	0.2 ± 0.2	0.8 ± 0.03	1.1 ± 0.04
Total	52.4	8.2	0.5	133.5	80.2

Triplicate cell suspensions (1.0×10^6 cells/μL) were incubated with 1 μM FUR or FdUR or 10 μM FU (2 μCi/mL) for 3 hr at 37°. After incubation, the cells were harvested, washed, and extracted with 65% ice-cold methanol as described in section 2. Radioactivity was determined in the methanol "soluble" and insoluble" fractions. Values are means ± SEM of triplicate estimations.

[a] ND, not determined.

Table 3
FU, FUR, and FdUR nucleotides in cellular pool

Cells	Monophosphate			Diphosphate			Triphosphate		
	FU	FUR	FdUR	FU	FUR	FdUR	FU	FUR	FdUR
	(pmol/10^6 cells)								
H9	3.5	59.3	54.8	1.2	74.3	0.11	6.1	51.5	0.34
H9-araC0.05	3.4	3.5	6.7	0.6	2.9	0.04	2.8	2.9	0.03
H9-araC0.5	3.5	0.3	0.15	0.9	0.1	0.12	2.9	0.06	0.09

Triplicate cell suspensions (1×10^6 cells/mL) were incubated with 1 μM [^{3}H]FUR or [^{3}H]FdUR or 10 μM [^{3}H]FU (2.0 μCi/mL). After a 3-hr incubation at 37°, the cells were harvested, washed, and extracted with 65% methanol. Aliquots from triplicate samples were pooled, dried by speed-vac, reconstituted in a small volume of water, and analyzed by HPLC.

cells were 8.5 to 10.6% of the value of H9 cells. The influx of FdUR was also reduced but to 33.5 and 60.5%.

NBMPR is a tight-binding inhibitor of the nucleoside transport system [6]. As shown in Table 4, H9-araC0.05 and H9-araC0.5 cells bound 12.8 and 9.3 fmol of [^{3}H]NBMPR/10^6 cells compared with the 24.4 fmol of the H9 cells. The decreases in NBMPR binding in H9-araC0.05 and H9-araC0.5 cells were markedly lower than those of the parental cells.

UR kinase and TK are considered to catalyze the first step of phosphorylation of FUR and FdUR, respectively. To affirm the involvement of these enzymes in the activation, the effects of dCR, TdR, and UR on the total cellular incorporation of FUR and FdUR were examined (Fig. 2; see also Ref. [31]). The incorporation of FUR was reduced markedly to 29.9% by UR, whereas TdR and dCR had no effect. On the other hand, TdR inhibited the incorporation of FdUR [31] and phosphorylation to 1.0% of control, while UR had no effect (Fig. 3). The observations suggest that FUR and FdUR are transported and activated by different mechanisms. Activities of these enzymes in the sensitive and the resistant cell lines also were examined in cell-free extracts using FUR and FdUR as substrates. H9-araC0.05 and H9-araC0.5 cells activated FUR at the rates of 63 and 58.6% and FdUR at the rates of 76.1 and 66.9% of the parental cell line (Table 4).

To determine if the expression of TS was different in the resistant cell lines, TS mRNA levels were determined as shown in Fig. 4. It is evident from the figure that there was no difference in the TS mRNA levels between the parental and the resistant cell lines. Similarly, there was no evidence of an abnormal expression of MDR protein or FdUR nucleotide efflux in the resistant cells (data not shown).

4. Discussion

FPyr and araC are an integral part of cancer chemotherapy [1–3,20–22]. The evidence presented here clearly demonstrates collateral resistance of araC resistant H9 cell lines to FUR and FdUR. Emergence of resistance following treatment with these drugs (*in vitro* and *in vivo*) is well known [9,18,19,25]. The cross-resistance to FPyr following exposure to araC, to our knowledge, has not been reported earlier. It should be noted that FPyr and araC are different types of drugs. They are metabolized differently, and affect different cellular target sites [3,7–9,19,32]. araC (a dC analog) is phosphorylated by dCK, dCMPK, and NDPK to araCTP and incorporated into DNA resulting in DNA chain termination [32,33]. FPyr are also activated by intracellular phosphorylation but by different enzymes (Scheme 1) and affect different cellular target sites, e.g. TS, RNA, and DNA. The collateral resistance to FUR and FdUR, therefore, could have been caused due to an alteration in the activity of TS and/or a decrease in cellular accumulation of FdUMP and FUTP. Although the activity or altered affinity of TS was not determined here, the TS mRNA levels did not differ between the parental and the resistant cell lines.

Table 4.
FUR and FdUR influx, NBMPR binding, and TK and UR kinase activities

Cells	Influx[a] (cpm/10^6 cells · 60 sec)		[^{3}H]NBMPR binding[b] (fmol/10^6 cells)	Enzyme activities[c] (pmol/mg protein · min)	
	FUR	FdUR		TK	UR kinase
H9	2460 ± 125	670 ± 111	24.4 ± 4.3	163 ± 9.6	195 ± 28
H9-araC0.05	260 ± 45	406 ± 109	12.8 ± 0.4	124 ± 5.0	123 ± 48
H9-araC0.5	210 ± 45	225 ± 108	9.3 ± 3.0	109 ± 5.8	115 ± 57

[a] Means ± SEM of three experiments.
[b] Averages ± range of two experiments.
[c] The phosphorylation of [^{3}H]FUR or [^{3}H]FdUR (50 μM, 40 μCi/μmol) was determined in cell-free extracts. Values are averages ± range of two experiments.

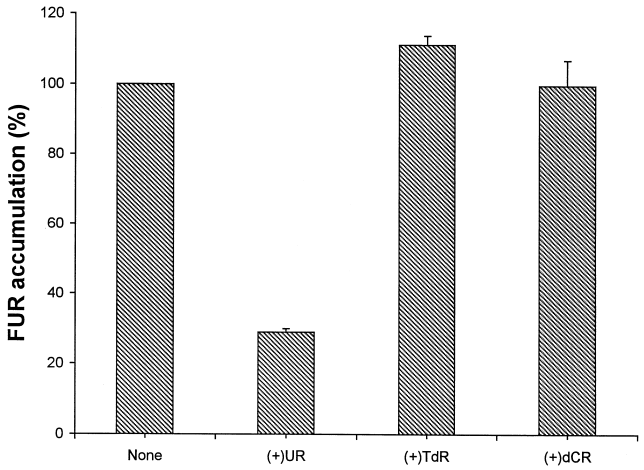

Fig. 2. Effects of uridine, thymidine, and deoxycytidine on FUR accumulation in H9 cells. Duplicate cell suspensions of H9 cells (1×10^6 cells/mL) were incubated with 1 μM FUR (2 μCi/mL) in the absence (control) or presence of 10 μM uridine (UR), thymidine (TdR), or deoxycytidine (dCR). After 3 hr of incubation at 37°, the cells were harvested, washed, and extracted with 65% ice-cold methanol and counted for radioactivity. The data are expressed as averages ± range of duplicate estimations.

Therefore, significantly lower amounts of FdUMP and FUTP may have contributed to the observed resistance.

Increased catabolism and decreased anabolism (cellular transport and activation) of the drugs and their nucleotides can reduce pharmacologically active FPyr nucleotide concentrations leading to resistance [3]. The catabolic reactions include conversion of FUR and FdUR to FU and increased dephosphorylation of FdUMP and FUTP to inactive nucleo-

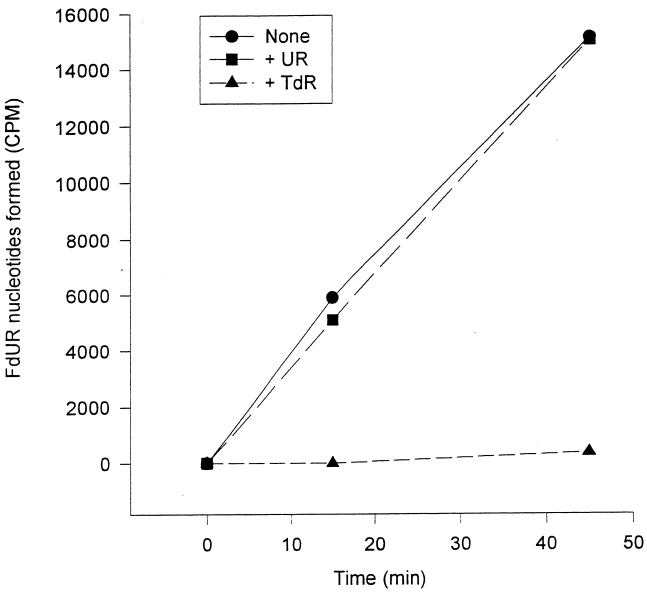

Fig. 3. Effects of TdR and UR on FdUR phosphorylation in cell-free extracts. The phosphorylation of FdUR (50 μM, 40 μCi/μmol) in the cell-free extracts of H9 cells was followed in the absence or presence of 10 μM UR or TdR. This experiment was performed once.

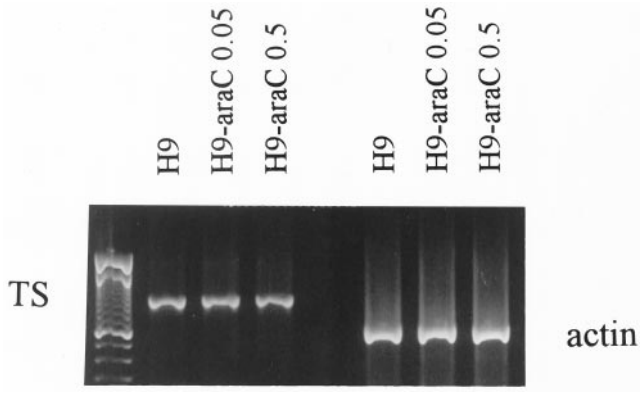

Fig. 4. RT–PCR analysis of TS and β-actin (control) gene expression in H9, H9-araC0.05, and H9-araC0.5 cells. The 1079-bp product for TS and the 610-bp product for actin are shown beside the 100 bp Hind III size marker in the first lane.

sides. Our observations that the resistance to FU was only 2- to 4-fold compared with FUR (83- to 267-fold) and FdUR (21- to 80-fold) suggest that the catabolism of FUR and FdUR to FU would have rendered the cells more sensitive rather than resistant. Furthermore, our preliminary studies on the cellular retention of FdUMP failed to show any difference between the parental and the resistant cells (data not shown here) making this mechanism(s) of resistance unlikely. The decreased anabolism therefore appears to be responsible for lower levels of FUR and FdUR nucleotides in the resistant cells.

A decrease in drug anabolism could be due to reduced cellular transport and/or activation. The cellular uptake of nucleosides including that of FPyr is essential for their effects on nucleic acid synthesis [4,5,17]. Nucleoside influx in mammalian cells is mainly of two types: equilibrative and concentrative [6]. The equilibrative nucleoside transport system that is common among the lymphocytic cells is both sensitive and insensitive to NBMPR [6]. Irrespective of the mechanisms, the diminished drug permeation into cells should lower their intracellular nucleotide concentrations and consequently cause drug resistance. Our findings that, compared with the sensitive cells, FUR permeated H9-araC0.05 and H-araC0.5 cells at the rates of 10.6 and 8.5% and FdUR at 60.5 and 33.5%, respectively, are consistent with this hypothesis. Although additional studies are required to understand the mechanisms of FPyr influx, the reduction in NBMPR binding sites in the resistant cells suggests that the NBMPR-sensitive equilibrative transport system may have played a role in the influx of FUR and FdUR in these cell lines.

The data in Table 4 suggest that in the resistant cells the influx of FUR was affected much more than the influx of FdUR. Although the reason for this difference is not clear, it is possible that lack of an OH group in FdUR may have rendered it more lipophilic than FUR and affected its permeability.

In addition, the activities of the FUR and FdUR activat-

ing enzymes, UR kinase and TK, were much lower in the resistant cell lines than in the sensitive cells (Table 4). Thus, the combined effect of reduced permeation and activation caused reduction in nucleotide formation in the resistant cells. These findings are consistent with earlier reports on resistance to these drugs [3,9]. However, the mechanism by which araC affected the activities of UR kinase and TK is not clear. araC is known to induce drug resistance by decreasing cellular dCK activity [25] and dCK isozymes having TK activity [34,35]. Therefore, it is possible that araC altered TK2 (a TK isozyme capable of phosphorylating dC), consequently decreasing FdUR activation. We have shown previously that TdR strongly inhibits cellular FdUR accumulation in H9 cells, whereas dC has no effect [31]. Furthermore, FdUR phosphorylation in cell-free extracts of H9 cells was strongly inhibited by TdR (Fig. 3), reaffirming that FdUR is activated by TK. Strong inhibition of cellular accumulation of FUR by uridine indicates that FUR is activated by UR kinase. The lack of an effect of dCR and TdR on cellular accumulation of FUR suggests that the pathways of FUR influx and activation are different from those of dCR and TdR. Even though mechanisms of decreased UR kinase and TK by araC remain elusive, araC is known to cause hypermethylation of DNA, leading to inhibition of nucleoside kinase expression [36]. The failure of azacytidine and cyclophosphamide (DNA demethylating agents) to affect dCK and TK activities (our unpublished data) casts doubt on this mechanism. However, more work is needed to rule out this possibility. Even though the mechanism(s) is not fully understood, these results and our earlier observations of cross-resistance of ddC resistant H9 cells to azidothymidine and FdUR [26, 31] suggest that deoxycytidine analogs, araC and ddC, are capable of inducing genetic modifications that affect multiple genes. The availability of new technology such as DNA array analysis may help in identifying other genes, yet unknown, whose expression may be affected by araC and ddC. Studies in our laboratory are underway to explore these mechanisms. However, an important implication of our findings is that if this phenomenon occurs in vivo it may affect the therapeutic potential of FPyr and other related drugs.

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