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MOLECULAR PHARMACOLOGY, 30:136–141

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Uracil Nucleotide Synthesis in a Human Breast Cancer Cell Line (MCF-7) and in Two Drug-Resistant Sublines that Contain Increased Levels of Enzymes of the *de Novo* Pyrimidine Pathway

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Received October 15, 1985; Accepted April 29, 1986

SUMMARY

Cultured wild-type MCF-7 human breast cancer cells and two MCF-7 sublines that overproduce enzymes of the *de novo* pyrimidine biosynthetic pathway were compared with regard to: rate of *de novo* biosynthesis of uracil nucleotides, sensitivity of the *de novo* and salvage pathways to the concentration of intracellular uracil nucleotides, and potential of exogenous uridine at concentrations equivalent to plasma levels to affect *de novo* pyrimidine biosynthesis. The PALA^R MCF-7 subline, which is resistant to *N*-(phosphonacetyl)-L-aspartate and has 5.2 times the activity of the first *de novo* enzyme as the wild-type MCF-7 cells, synthesizes uracil nucleotides via the *de novo* pathway at a rate that is 5.8 times that of the wild type MCF-7 cells. The PYR^R MCF-7 subline, which is resistant to pyrazofurin and has 15.1 times the activity of orotate phosphoribosyltransferase as the wild-type MCF-7 cells, synthesizes uracil nucleotides via the *de novo* pathway at a rate that is 1.4 times that of wild-type MCF-7 cells. These results are consistent with carbamyl phosphate synthetase being the rate-controlling step of *de novo* pyrimidine biosynthesis. In the presence of exogenous uridine at concentrations equivalent to that found in plasma (4.4–8.6 μM), the uracil nucleotide pool of wild-type MCF-7 cells was expanded by 20% and *de novo* synthesis was inhibited by 55%. Incubation of PALA^R MCF-7 cells with uridine at concentrations between 7.3 and 16.8 μM caused a 40% increase in the uracil nucleotide pool and a 30% inhibition of *de novo* synthesis. *De novo* synthesis of uracil nucleotides in PYR^R MCF-7 cells was not affected by a >10-fold increase in the uracil nucleotide pool. Salvage of [^{14}C] uridine was inhibited by an expanded uracil nucleotide pool in the wild-type and PYR^R MCF-7 cells but was not inhibited in the PALA^R MCF-7 cell line. These results demonstrate that, although the overproduced enzymes exhibit substrate affinities and specificities in cell-free preparations similar to those of the wild-type enzymes, in intact cells the resistant cell lines exhibit marked differences in the control of *de novo* and salvage pyrimidine biosynthetic pathways by intracellular uracil nucleotides.

The enzymes responsible for *de novo* pyrimidine biosynthesis in mammalian cells include the cytosolic multienzyme *pyr1–3*, the mitochondrial enzyme dihydroorotate dehydrogenase, and the final cytosolic multienzyme *pyr5,6*. Animal cell lines resistant to inhibitors of the *de novo* pathway have been isolated that overproduce either multienzyme *pyr1–3* (1–3) or multienzyme *pyr5,6* (4, 5) depending on the inhibitor used. Since the *de novo* pyrimidine pathway is tightly controlled by end-products of the pathway, it is important to consider pathway control in resistant cell lines. Levinson *et al.* (4) measured the rate of *de novo* pyrimidine synthesis in murine cells that overproduce *pyr5,6* and found it to be the same as in the wild-type cell. Flux through the *de novo* pathway in cells that overproduce *pyr1–3* has not been reported. Since carbamyl phosphate synthetase is generally considered to be the rate-controlling step of the pathway, *de novo* pyrimidine synthesis might be expected to increase in cells with elevated *pyr1–3*, thus making them less dependent on salvage pathways to satisfy cellular pyrimidine requirements. Such a change in relative dependency on *de novo* versus salvage pathways could have important implications in cancer chemotherapy in that such cells may have an altered sensitivity to cytotoxic pyrimidine analogues that require activation by the salvage pathway.

Two sublines of the human breast cancer cell line MCF-7 cells have been developed: a PALA^R MCF-7 cell line (6), which is resistant to 10 mM PALA [a transition state inhibitor of aspartate transcarbamylase (7, 8)] and contains a 5.2-fold increase in the level of the multienzyme *pyr1–3* over that present in the wild-type cell, and a PYR^R MCF-7 cell line (9), which is resistant to 2 mM pyrazofurin [as its monophosphate, an inhib-

ABBREVIATIONS: *pyr1–3*, the multifunctional polypeptide which comprises carbamyl-phosphate synthetase (EC 2.7.2.5), aspartate transcarbamylase (EC 2.1.3.2), and dihydroorotase (EC 1.3.3.1); *pyr5,6*, enzyme complex comprising orotate phosphoribosyltransferase (EC 2.4.2.10) and orotidine-5′-phosphate decarboxylase (EC 4.1.1.23); PALA, *N*-(phosphonacetyl)-L-aspartate; PALA^R, MCF-7 cell line resistant to PALA; PYR^R, MCF-7 cell line resistant to pyrazofurin; EDTA, ethylenediaminetetraacetate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, a 0.9% NaCl solution containing 0.075% K_2HPO_4 and 0.01% KH_2PO_4; PRPP, phosphoribosylpyrophosphate.

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itor of orotidine-5′-phosphate decarboxylase (10–13)], cross-resistant to 1 mM 6-azauridine, and contains a 15.1-fold increase in the multienzyme *pyr5,6* over that present in the wild-type cell. The increased level of enzyme activity in both sublines remains stable for at least 24 weeks without the presence of inhibitor. As in the previously derived resistant murine and hamster cell lines, these drug-resistant human cells display coordinate increases in the enzymatic activity of each enzyme within a complex with no apparent alteration in the K_m values for substrates between the wild-type and resistant cell lines.

In this paper we report the results of studies designed to compare the three human cell lines (i.e., MCF-7, PALA^R MCF-7, and PYR^R MCF-7) with regard to: (a) rate of *de novo* synthesis of uracil nucleotides; (b) sensitivity of *de novo* and salvage pathways to intracellular uracil nucleotide pool size; and (c) the capacity of extracellular uridine concentrations equivalent to those found in plasma to affect *de novo* pyrimidine biosynthesis.

Experimental Procedures

Materials. Nucleosides, nucleotides, enzymes, and tri-*n*-octylamine were purchased from Sigma. Sodium [^{14}C]bicarbonate (9.2 mCi/mmol) and [U-^{14}C]uridine (522 mCi/mmol) were obtained from New England Nuclear. L-Glutamine, penicillin, and streptomycin were supplied by the National Institutes of Health Media Unit. Trypsin:EDTA (1×) solution and minimal essential medium (Eagle's) with 25 mM HEPES buffer and Earle's salts were purchased from Grand Island Biological Co. Fetal calf serum and Dulbecco's (modified Eagle's) medium with 4.5 g of glucose/liter were obtained from HEM Research, Inc. Ammonium dihydrogen phosphate (HPLC grade) was purchased from Fisher Scientific.

The MCF-7 cells, a line of human breast cancer cells (originally a gift of Dr. Marvin Rich, Michigan Cancer Foundation), PALA^R cells (6), and PYR^R cells (9) were grown as monolayers in 75-cm^2 flasks under a 5% CO_2 atmosphere in Dulbecco's medium supplemented with 10% fetal calf serum, 4 mM L-glutamine, penicillin (50 units/ml), and streptomycin (50 μg/ml). The cells were subcultured once a week after resuspension in trypsin:EDTA solution. PALA^R and PYR^R cells were produced by growing MCF-7 cells initially in 50 μM PALA and 0.1 μM pyrazofurin and increasing the concentration of the drugs in a 1.5–2-fold stepwise manner until the cells were able to grow in 10 mM PALA and 2 mM pyrazofurin, respectively.

Comparison of rate of *de novo* synthesis between wild-type MCF-7 and resistant lines. When cells approached confluency in a 75-cm^2 flask, they were suspended in 5 ml of trypsin:EDTA (1×) solution. Ten ml of Eagle's minimal essential medium with HEPES buffer (supplemented with 10% fetal calf serum, 4 mM-L-glutamine, 50 units/ml of penicillin, and 50 μg/ml of streptomycin) were added, and the cells were pelleted at 200 × *g* for 10 min. The supernatant was discarded, the cells were resuspended in 15 ml of HEPES containing medium, and distributed into three 15-ml culture tubes, and the tubes were immersed horizontally in a 37° shaking water bath. Following incubation for 2 hr with 50 μCi of NaH$^{14}CO_3$, all intracellular uracil nucleotides and phosphodiesters were converted to uridine (14) and were analyzed by high pressure liquid chromatography (14, 15) using a Waters 8 mm × 10 cm C18 Radial Pak Cartridge (5-μm particle size). The samples were eluted with 0.01 M sodium acetate, 0.01 M acetic acid, 0.05% triethylamine, and 2% methanol at 2 ml/min. Measurements of the intracellular protein content were performed on each sample using the Bio-Rad Protein Assay.

Effect of exogenous uridine on *de novo* biosynthesis. Either wild-type or PALA^R cells were distributed into 12 tubes (6 ml/tube) as described above. After 15 min of incubation at 37°, 125 μl of a 0.4 mM uridine solution were added to all but the three control tubes. The tubes were connected to an infusion pump (14) with the control tubes being infused with PBS and the remaining tubes being infused with uridine in the range of 1.25–10 mM. The pump delivered 10.9 μl/hr. One hr later, 1 ml from each tube was removed for measurement of the media uridine concentrations (14). At this time 50 μCi of NaH$^{14}CO_3$ were added to each tube which was then reattached to the pump. After 2 hr, the cells were pelleted, and 1 ml of the medium was saved for analysis. The intracellular uracil nucleotides were isolated and analyzed as described in the previous section.

Measurement of [^{14}C]uridine salvage. Each cell line was distributed into 12 tubes (5 ml/tube) as described above. Six tubes were made 1 mM in uridine, and all tubes were incubated at 37°. After 2 hr, the cells from three control tubes and three tubes containing added uridine were pelleted, and the intracellular uracil nucleotides were isolated and measured as previously described. The cells in the other six tubes were washed twice with medium and incubated at 37° for 1 hr with [U-^{14}C] uridine (2.5 μM, 0.25 μCi). The cells were then centrifuged, the pellet was resuspended in 2 ml of H_2O, the sample was recentrifuged, and two 50-μl aliquots were removed for protein determinations. The sample was filtered through Whatman DE81 filter disks, the disks were rinsed with 30 ml of H_2O, and the phosphorylated anabolites of uridine adsorbed onto the disks were quantitated by scintillation counting.

Quantitation of nucleotides. Each cell line was grown in 75-ml cell culture flasks until reaching 3–5 × 10^6 cells/flask. Each flask was rinsed twice with 15 ml of 0.9% NaCl solution. Then 3 ml of an ice-cold 10% trichloroacetic acid solution were pipetted into the flasks, and the flasks were placed into a 4° refrigerator for 5 min. Following refrigeration, the solution was transferred to a 15-ml centrifuge tube kept at 4°. An additional 3 ml of ice-cold 10% trichloroacetic acid was added to each flask, each flask was again refrigerated for 5 min, and the solution in each flask was added to the first trichloroacetic acid solution. The combined solutions were centrifuged at 4° for 10 min at 2500 × *g*. The resulting supernatant was extracted with 6 ml of an ice-cold solution of trichlorotrifluoroethane:tri-*n*-octylamine solution (2:1), and the organic layer was discarded. The resulting aqueous solution was lyophilized and resuspended in 400 μl of water. The equivalent of 5 × 10^5 cells was injected into the high pressure liquid chromatograph.

The samples were chromatographed on an Altex high pressure liquid chromatograph equipped with a Whatman (Clifton, NJ) Partisil 10 SAX column, a C18 guard column, a Gilson (Middleton, WI) 111 UV detector, and an LDC/Milton Roy (Rivera Beach, FL) CI-10 integrator. The samples were run at a flow rate of 2.2 ml/min with the following gradient system: 8 min of buffer A, followed by a linear gradient to 40% buffer B for 34 min, followed by a linear gradient to 65% buffer B for 1 min, followed by a linear gradient to 100% buffer B for 8 min, and finally 100% buffer B for 12 min. The column was reequilibrated for 12 min with buffer A prior to the next injection. Buffer A is 7 mM ammonium dihydrogen phosphate (pH 3.8), and buffer B is 500 mM KCl plus 250 mM ammonium dihydrogen phosphate (pH 4.72).

Results

Comparison of the rate of *de novo* synthesis and the pool size of uracil nucleotides between wild-type MCF-7 cells and the resistant lines. The rate of formation of uracil nucleotides via the *de novo* pathway is 5.8 times greater in the PALA^R cells than in the wild-type MCF-7 cells, and the pool of uracil nucleotides in the PALA^R cells is 1.8 times larger than in the wild-type MCF-7 cells (Table 1). Although the rate of *de novo* synthesis of uracil nucleotides in the PYR^R cells is slightly greater (34%) than that of the wild-type MCF-7 cells, the uracil nucleotide pool is 30% smaller. Thus, the increase in *pyr1–3* activity results in an increased production of UMP and a larger uracil nucleotide pool as compared to wild-type cells. In contrast, an increase in *pyr5,6* activity led to only a slight

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TABLE 1

Comparison of rate of *de novo* synthesis and uracil nucleotide pool size

	Wild-type MCF-7	PALA^R MCF-7	PYR^R MCF-7
Drug resistance		10 mM PALA	2 mM pyrazofurin
De novo enzymatic activity			
carbamyl-P-synthetase II[a]	1.3	6.7	0.9
ORP transferase[a]	38.8	ND[b]	585.6
Uridine kinase activity[c]	2.57 ± 0.17	3.11 ± 0.24	3.46 ± 0.10
Rate of *de novo* synthesis of uracil nucleotides[d]	10.0 ± 0.5	58.4 ± 4.8	13.4 ± 1.4
Pool size of uracil nucleotides (nmol/μg of protein)	0.20 ± 0.04[e]	0.36 ± 0.06[e]	0.14 ± 0.05[f]

[a] Cell-free enzymatic activity of carbamyl-P (phosphate)-synthetase II and orotate phosphoribosyltransferase (ORP) is expressed in nmol/mg/hr. For each cell line, the variance in the measurements was less than 10%. All three enzymes were assayed as described in Ref. 4.

[b] ND, not determined.

[c] Cell-free uridine kinase activity is expressed in nmol/mg/min. The numbers represent the mean ± standard deviation (n = 3–6). The enzyme was assayed as described in Ref. 31.

[d] Measured as dpm of $NaH^{14}CO_3$ incorporated into the uracil nucleotide pool of the wild-type, PALA^R, or PYR^R MCF-7 cells per μg of protein following a 2-hr exposure to $NaH^{14}CO_3$. Values are means ± standard error (n = 12–14).

[e] Mean ± standard deviation (n = 9).

[f] Mean ± standard deviation (n = 15).

increase in UMP production and a decrease in the pool of uracil nucleotides.

Concentration of individual nucleotides in the wild-type MCF-7 cells and in the resistant lines. The concentration of each individual nucleotide was measured in dividing cells for all three cell lines by high pressure liquid chromatography. A chromatogram of standards and of a typical sample are illustrated in Fig. 1. The nucleotide concentrations for each cell line expressed as nmol/10^6 cells are detailed in Table 2. Under the chromatographic conditions used, UDP *N*-acetylglucosamine and UDP *N*-acetylgalactosamine (the UDP *N*-sugars) are not separated, and the UDP glucose and the UDP galactose also do not separate. All of the cell lines exhibit a total uracil nucleotide pool that is at least 10-fold higher than their total cytosine nucleotide pool. Each cell line also contains a relatively large pool of UDP *N*-sugars. Some differences between the cell lines are evident. The UTP concentration in both of the resistant lines is twice the UTP concentration of the wild-type cells. The concentrations of GTP and ATP in the PYR^R cells are at least 60% and 30% higher, respectively, than in the wild-type and PALA^R cells; however, the level of UDP *N*-sugars in the PYR^R cell line is 40% lower than in the other two cell lines.

Effect of expansion of uracil nucleotide pool on *de novo* synthesis of uracil nucleotides. Carbamyl phosphate synthetase II is regulated allosterically by end-products of *de novo* synthesis such as UTP, UDP, UDP glucose, UMP, and sometimes CTP (16–20). In colon cancer cells and normal mucosa, UMP, UDP, and UTP are equally effective; however, CTP is ineffective (21). An increase in the concentration of the uracil nucleotides would, therefore, be expected to inhibit *de novo* synthesis. Since the concentration of these uracil nucleotides is more than 4–5-fold greater than the concentration of CTP (Table 2) and, since CTP is not as effective an inhibitor as UTP, the uracil nucleotide pool was chosen for examination. The rate of *de novo* synthesis of uracil nucleotides, as measured

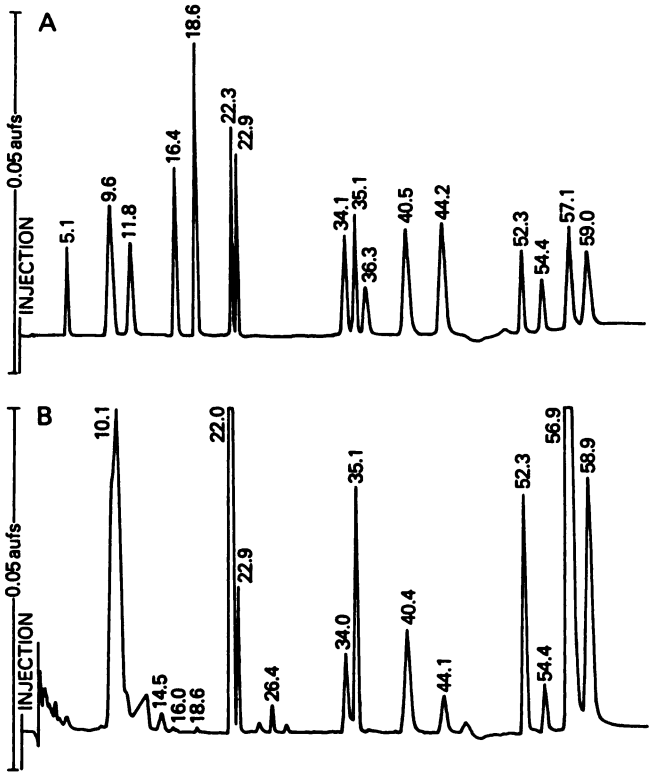

Fig. 1. Chromatogram of nucleotide standards (A) and of the wild-type MCF-7 cells (B). Both chromatograms were analyzed by UV at 254 nm. Each peak in the standards chromatogram represents 2 ng. The standards at the following times (min) post-injection are: 5.1, CMP; 9.6, AMP; 11.8, UMP; 16.4, GMP; 18.6, IMP; 22.3, UDP *N*-acetylgalactosamine; 22.9, UDP glucose; 34.1, UDP; 35.1, UDP glucuronide; 36.3, CDP; 40.5, ADP; 44.2, GDP; 52.3, UTP; 54.4, CTP; 57.1, ATP; and 59.0, GTP. The MCF-7 cells were prepared and chromatographed as described in Experimental Procedures. The injection represents nucleotides from approximately 5 × 10^5 cells.

by sodium [^{14}C]bicarbonate incorporation, in cells with an expanded uracil nucleotide pool was compared to the rate of *de novo* synthesis in cells which had not been preincubated with uridine. In the wild-type MCF-7 cells, an increase in uracil nucleotides of 1.8-fold resulted in a decrease of *de novo* synthesis of 72%, whereas a 6.5-fold expansion reduced the rate of synthesis 85% (Table 3). *De novo* synthesis in PALA^R cells also decreased when the uracil nucleotide pool was expanded; however, a maximum inhibition of only 30% was achieved when the pool was expanded 6-fold. In contrast, *de novo* synthesis of uracil nucleotides in PYR^R cells was essentially unaffected by even larger expansions, greater than 30-fold, of the uracil nucleotide pool.

Effect of plasma concentrations of uridine on *de novo* synthesis of uracil nucleotides. After establishing that *de novo* biosynthesis of uracil nucleotides in wild-type MCF-7 and PALA^R cells is inhibited in the presence of high concentrations of exogenous uridine, we examined the level of expansion of the uracil nucleotide pool and the degree of inhibition of *de novo* synthesis in cells exposed to uridine at concentrations similar to that found in plasma [1–10 μM (15)]. Since uridine is depleted from the medium of wild-type and PALA^R cells,[1] uridine was infused into the culture tubes during the 2-hr

[1] Approximately 55% of the medium uridine disappeared when twice the number of cells used in Table 3 were incubated 2 hr with 100 μM uridine.

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TABLE 2

Individual nucleotide concentrations for the wild-type MCF-7 cells and the PALA^R and PYR^R MCF-7 cell lines

Nucleotide	Concentration		
	Wild type	PALA^R	PYR^R
	nmol/10^6 cells		
CMP	–[a]	–[a]	–[a]
CDP	–[a]	0.3	0.4
CTP	3.3	5.2	6.0
Total	3.3	5.5	6.4
UMP	–[b]	–[b]	–[b]
UDP *N*-acetylglucosamine + UDP *N*-acetylgalactosamine	45.2	46.5	27.4
UDP glucose + UDP galactose	3.0	4.9	4.6
UDP glucuronide	7.0	5.5	3.8
UDP	2.4	3.7	2.6
UTP	10.3	20.7	21.9
Total	67.9	81.2	60.3
GMP	0.06	0.1	0.1
GDP	1.1	1.2	2.3
GTP	12.5	9.9	20.5
Total	13.7	11.2	22.9
AMP	–[b]	–[b]	–[b]
ADP	3.4	4.7	6.7
ATP	45.8	37.9	61.4
Total	49.2	42.6	68.1

[a] –, less than 0.07 nmol/10^6 cells.
[b] –, under interfering peaks.

TABLE 3

Effect of an expanded uracil nucleotide pool on *de novo* synthesis of uracil nucleotides

Cells were incubated for 2 hr with 1 mM uridine followed by a 2-hr incubation with NaH$^{14}CO_3$ as described in Experimental Procedures. The amount of NaH$^{14}CO_3$ incorporation into uracil nucleotides of cells with an expanded uracil nucleotide pool is expressed as a percentage of the amount of NaH$^{14}CO_3$ incorporated into uracil nucleotides of cells not incubated with uridine. Each cell line served as its own control. Two separate experiments for each cell type are recorded. NaH$^{14}CO_3$ incorporation into uracil nucleotides of controls ranged from 1000 to 3000 dpm/sample. The specific incorporation of NaH$^{14}CO_3$ for each cell line is given in Table 1.

MCF-7 cell line	Uracil nucleotide pool		NaH$^{14}CO_3$ incorporation into uracil nucleotides
	Control	Expanded	
	nmol/μg of protein		*% of control*
Wild type	0.20 ± 0.04[a]	0.37 ± 0.02[b]	27.7 ± 5.2[c]
		1.31 ± 0.12	15.5 ± 1.0
PALA^R	0.36 ± 0.06	0.50 ± 0.03	68.7 ± 7.0
		1.68 ± 0.06	68.5 ± 11.5
PYR^R	0.14 ± 0.05	1.45 ± 0.10	81.7 ± 6.4
		4.99 ± 0.09	102.8 ± 10.4

[a] Values are means ± standard deviation (n = 9–15).
[b] Values are means ± standard deviation (n = 3).
[c] Values are means ± standard error (n = 3).

incubation with sodium [^{14}C]bicarbonate in order to maintain relatively constant medium uridine concentrations.

The results in Table 4 show that, in the presence of plasma concentrations of uridine, *de novo* synthesis of uracil nucleotides in wild-type MCF-7 cells is inhibited by approximately 50%. A 20–22% increase in the intracellular uracil nucleotide pool was observed under these conditions. Although a larger increase (40%) in the uracil nucleotide pool was observed with the PALA^R cells, the inhibition of *de novo* synthesis was lower, ranging from 11 to 30%. These results are consistent with the data in Table 3 showing a greater effect in wild-type cells than in PALA^R cells.

Effect of expansion of the uracil nucleotide pool on salvage of exogenous uridine. Since UTP also acts as an allosteric regulator of the salvage enzyme uridine/cytidine kinase (EC 2.7.1.48) *in vitro* (22), wild-type MCF-7 cells and the two resistant cell lines were preincubated with 1 mM uridine to expand the uracil nucleotide pool. The effect of the subsequently expanded uracil nucleotide pool on pyrimidine salvage was examined using 2.5 μM [^{14}C]uridine as described in Experimental Procedures. The concentration of [^{14}C]uridine was chosen to be equivalent to plasma uridine concentrations. As shown in Table 5, inhibition of uridine salvage in MCF-7 and PYR^R cells correlated with expansion of the uracil nucleotide pool. However, a 9.4-fold expansion of the uracil nucleotide pool in the PALA^R cells caused only slight (8%) inhibition of uridine salvage.

TABLE 4

Effect of plasma concentrations of uridine on *de novo* synthesis of uracil nucleotides

Wild-type or PALA^R cells were incubated with NaH$^{14}CO_3$ for 2 hr while uridine at varying concentrations was being constantly infused as described in Experimental Procedures. The medium uridine concentration of triplicate samples was measured at the beginning and end of the 2-hr incubation. The uracil nucleotide pool expanded from 0.20 ± 0.04 (SD, n = 12) to 0.25 ± 0.01 (n = 8) in the wild-type cells and from 0.36 ± 0.06 (n = 12) to 0.51 ± 0.06 (n = 9) in the PALA^R cells. The amount of incorporation of NaH$^{14}CO_3$ into the uracil nucleotide pool of the uridine-infused samples was compared to the corresponding values obtained from samples infused with PBS. Each cell line served as its own control. Three separate experiments for each cell type are recorded. NaH$^{14}CO_3$ incorporation into uracil nucleotides of controls ranged from 1000 to 3000 dpm/sample. The specific incorporation of NaH$^{14}CO_3$ for each cell line is given in Table 1.

MCF-7 cell line	Medium uridine concentration		NaH$^{14}CO_3$ incorporation into uracil nucleotides
	Initial	Final	
	μM		*% of control*
Wild type	4.4 ± 0.3[a]	8.6 ± 1.4[a]	45 ± 3[a]
	5.9 ± 0.6	26.3 ± 1.7	39 ± 3
	5.8 ± 0.6	32.2 ± 4.7	47 ± 1
PALA^R	7.3 ± 0.3	9.4 ± 1.2	89 ± 9
	7.6 ± 0.2	13.8 ± 0.4	70 ± 9
	8.5 ± 1.0	16.8 ± 1.2	80 ± 7

[a] Values are means ± standard error (n = 3).

TABLE 5

Effect of an expanded uracil nucleotide pool on [^{14}C]uridine uptake by intact cells

Cells from each cell line were incubated for 2 hr with uridine, washed, and incubated for 1 hr with 2.5 μM [U-^{14}C]uridine as described in Experimental Procedures. The amount of intracellular phosphorylated products of [^{14}C]uridine found in the cells preincubated with uridine is expressed as a percentage of the corresponding values for cells which had not been preincubated with uridine. Each cell line served as its own control. Incorporation of [^{14}C]uridine into the uracil nucleotide pool of controls was 1.7, 0.4, and 0.7 nmol/mg of protein/hr for the wild-type, PALA^R, and PYR^R cells, respectively.

MCF-7 cell line	Uracil nucleotide pool		[^{14}C]Uridine incorporation into uracil nucleotides
	Control	Expanded	
	nmol/μg of protein		*% of control*
Wild type	0.20 ± 0.04[a]	0.83 ± 0.08[b]	42.5 ± 1.2[c]
PALA^R	0.36 ± 0.06	3.40 ± 0.22	99.4 ± 1.6
PYR^R	0.14 ± 0.05	0.23 ± 0.01	53.1 ± 5.9

[a] Values are means ± standard deviation (n = 9–15).
[b] Values are means ± standard deviation (n = 3).
[c] Values are means ± standard error (n = 3).

Discussion

Overproduction of a target enzyme is a form of drug resistance *in vitro* (23) and has been observed *in vivo* (24–26). In most drug-resistant cell lines in which target enzyme production is increased, the overproduced enzyme is similar to the enzyme from the normal wild-type cell with respect to substrate

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affinity and specificity. A change in pathway flux and regulation could make such a resistant cell population vulnerable to the cytotoxic effects of another agent and, therefore, might be important to the rational use of multidrug chemotherapy. The rate of biosynthesis of uracil nucleotides in mammalian cells has been shown to be affected by various factors (27, 28): 1) the level of PRPP, 2) the level of ATP, 3) the level of uracil and cytosine nucleotides, 4) enzyme deficiency as in orotic aciduria, and 5) pyrimidine antimetabolites. The major objective of the current study was to compare the control of *de novo* and salvage pathways for pyrimidine biosynthesis by intracellular uracil nucleotide pools in a wild-type human cell line and in two drug-resistant sublines that overproduce either the *pyr1–3* or *pyr5,6* enzyme complexes of the *de novo* pyrimidine pathway.

The data in Tables 3–5 show that there are marked differences in the control of *de novo* and salvage pathways in the three human cell lines. In these cells the level of multienzyme *pyr1–3* activity may be a factor in the extent of allosteric regulation of *de novo* synthesis by uracil nucleotides, since greater inhibition of *de novo* synthesis is observed in the wild-type cells than in the PALA^R cells, even though the concentration of uracil nucleotides was higher in the PALA^R cells. The insensitivity of the *de novo* pathway in the PYR^R cells to an expanded uracil nucleotide pool indicates additional changes in the PYR^R cells other than in the multienzyme *pyr5,6*. These changes do not include an increase in the baseline level of enzymatic activity of carbamyl phosphate synthetase II in the PYR^R cells from wild-type cells (Table 1). With regard to pyrimidine salvage, the control of [^{14}C]uridine phosphorylation by intracellular uracil nucleotides was similar in the MCF-7 and PYR^R cell lines, although the rate of uridine salvage differed; in contrast, the salvage of [^{14}C]uridine by PALA^R cells was insensitive to expansion of the uracil nucleotide pool, although baseline uridine kinase activity in cell-free preparations for all three cell lines was similar (Table 1).

The wild-type MCF-7 human breast cancer cells behaved similarly to the murine L1210 tumor cells previously examined in our laboratory (14). In both cell lines, an increase in the intracellular uracil nucleotide concentration resulted in inhibition of uracil nucleotide synthesis by *de novo* and salvage pathways, the *de novo* pathway exhibiting greater sensitivity to the intracellular uracil nucleotide concentration than the salvage pathway. Extracellular uridine concentrations equivalent to those found in plasma markedly reduce *de novo* pyrimidine synthesis in both cell lines, indicating a preference for nucleoside salvage over *de novo* synthesis when these cell lines are exposed to physiologic concentrations of uridine. In the uridine uptake experiments shown in Table 5, the wild-type MCF-7 cells incorporated exogenous uridine into uracil nucleotides at a faster rate than did the resistant sublines, suggesting less dependence on uridine salvage by the resistant sublines than by the wild-type MCF-7 cells.

Both multienzyme *pyr1–3* and multienzyme *pyr5,6* have been proposed as the rate-limiting enzyme of *de novo* pyrimidine biosynthesis (18, 29, 30). In a recent review, Jones (27) states that multienzyme *pyr1–3* is rate-limiting except under circumstances in which cellular ATP levels rise at a time when both uracil nucleotide and PRPP pools are low. Our results are consistent with multienzyme *pyr1–3* being rate limiting, as an amplification of multienzyme *pyr1–3* activity of 5.2-fold produced a corresponding increase in the rate of *de novo* biosynthesis of uracil nucleotides of 5.8-fold (Table 1). By comparison, there was only a 44% increase in the rate of *de novo* biosynthesis in cells with a 15.1-fold increase in multienzyme *pyr5,6* activity. The results obtained for the PYR^R cells are similar to those obtained by Levinson *et al.* (4), who observed little change in the rate of *de novo* pyrimidine biosynthesis in cultured mouse T-lymphocytes with altered multienzyme *pyr5,6* activity.

Thus, although the enzymes that are overproduced in the PALA^R and PYR^R cells exhibit substrate affinities and specificities similar to those of the enzymes from the wild-type MCF-7 cells, the resistant cell lines exhibit marked differences in the control of *de novo* and salvage pathways by intracellular uracil nucleotides. The results of the present study indicate that uracil nucleotide synthesis is not tightly regulated by the uracil nucleotide end-products in the resistant cells. Overproduction of an enzyme complex may cause disruption of the normal physical arrangement of enzymes or the controlled flow of substrates and products through the reaction pathway. Since pyrimidine production in the resistant cells is not under the same controls as wild-type cells, restricting the pyrimidine pool as a desired result of chemotherapy may be much more difficult in the resistant cells. Conversely, knowledge of the lack of control might be used to advantage with chemotherapeutic agents that require enzyme activation such as 5-fluorouracil. Thus, this information could be of value in selecting the appropriate agent to use against a drug-resistant cell population.

Acknowledgments

We wish to acknowledge Robin Wolfe and Elizabeth Rubalcaba for their excellent technical assistance.

References

1. Kempe, T. D., E. A. Swyryd, M. Bruist, and G. R. Stark. Stable mutants of mammalian cells that overproduce the first three enzymes of pyrimidine nucleotide biosynthesis. *Cell* **9**:541–550 (1976).
2. Padgett, R. A., G. M. Wahl, P. F. Coleman, and G. R. Stark. *N*-(Phosphonacetyl)-L-aspartate-resistant hamster cells overaccumulate a single mRNA coding for the multifunctional protein that catalyzes the first steps of UMP synthesis. *J. Biol. Chem.* **254**:974–980 (1979).
3. Wahl, G. M., R. A. Padgett, and G. R. Stark. Gene amplification causes overproduction of the first three enzymes of UMP synthesis in *N*-(phosphonacetyl)-L-aspartate-resistant hamster cells. *J. Biol. Chem.* **254**:8679–8689 (1979).
4. Levinson, B. B., B. Ullman, and D. W. Martin. Pyrimidine pathway variants of cultured mouse lymphoma cells with altered levels of both orotate phosphoribosyltransferase and orotidylate decarboxylase. *J. Biol. Chem.* **254**: 4396–4401 (1979).
5. Suttle, D. P., and G. R. Stark. Coordinate overproduction of orotate phosphoribosyltransferase and orotidine-5′-phosphate decarboxylase in hamster cells resistant to pyrazofurin and 6-azauridine. *J. Biol. Chem.* **254**:4602–4607 (1979).
6. Cowan, K., and M. Lippman. Human breast cancer cells resistant to PALA (*N*-(phosphonacetyl)-L-aspartate). *Proc. Am. Assoc. Cancer Res.* **21**:41 (1980).
7. Hoogenraad, N. J. Reaction mechanism of aspartate transcarbamylase from mouse spleen. *Arch. Biochem. Biophys.* **161**:76–82 (1974).
8. Swyryd, E. A., S. S. Seaver, and G. R. Stark. *N*-(phosphonacetyl)-L-aspartate, a potent transition state analog inhibitor of aspartate transcarbamylase, blocks proliferation of mammalian cells in culture. *J. Biol. Chem.* **249**:6946–6950 (1974).
9. Cowan, K. H., J. Levin, E. Trautmann, E. Rubalcaba, and R. Klecker. Pyrazofurin resistant human breast cancer cells (Pyr^R MCF-7) containing increased levels of OPTase and OMPdecase are collaterally sensitive to 5-FU. *Proc. Am. Assoc. Cancer Res.* **25**:337 (1984).
10. Gutkowski, G. E., M. J. Sweeney, D. C. DeLong, R. L. Hamill, K. Gerzon, and R. W. Dyke. Biochemistry and biological effects of the pyrazofurins (pyrazomycins): initial clinical trial. *Ann. N. Y. Acad. Sci.* **25**:544–551 (1975).
11. Cadman, E. C., D. E. Dix, and R. E. Handschumacher. Clinical, biological, and biochemical effects of pyrazofurin. *Cancer Res.* **38**:682–688 (1978).
12. Cadman, E., F. Eiferman, R. Heimer, and L. Davis. Pyrazofurin enhancement of 5-azacytidine antitumor activity in L5178Y and human leukemia cells. *Cancer Res.* **38**:4610–4617 (1978).
13. Dix, D. E., C. P. Lehman, A. Jakubowski, J. D. Moyer, and R. E. Handschumacher. Pyrazofurin metabolism, enzyme inhibition, and resistance in L5178Y cells. *Cancer Res.* **39**:4485–4490 (1979).

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14. Karle, J. M., L. W. Anderson, and R. L. Cysyk. Effect of plasma concentrations of uridine on pyrimidine biosynthesis in cultured L1210 cells. *J. Biol. Chem.* **259**:67–72 (1984).
15. Karle, J. M., L. W. Anderson, D. D. Dietrick, and R. L. Cysyk. Determination of serum and plasma uridine levels in mice, rats, and humans by high-pressure liquid chromatography. *Anal. Biochem.* **109**:41–46 (1980).
16. Ito, K., S. Nakanishi, M. Terada, and M. Tatibana. Control of pyrimidine biosynthesis in mammalian tissues. II. Glutamine utilizing carbamoyl phosphate synthetase of various experimental tumors: distribution, purification, and characterization. *Biochim. Biophys. Acta.* **220**:477–490 (1970).
17. Levine, R. L., N. J. Hoogenraad, and N. Kretchmer. A review: biological and clinical aspects of pyrimidine metabolism. *Biochemistry* **10**:3694–3699 (1971).
18. Tatibana, M., and K. Shigesada. Two carbamyl phosphate synthetases of mammals: specific roles in control of pyrimidine and urea biosynthesis. *Adv. Enzyme Regul.* **10**:249–271 (1971).
19. Aoki, T., H. P. Morris, and G. Weber. Regulatory properties and behavior of activity of carbamoyl-phosphate synthetase II (glutamine-hydrolyzing) in normal and proliferating tissues. *J. Biol. Chem.* **257**:432–438 (1982).
20. Tatibana, M., and K. Shigesada. Control of pyrimidine biosynthesis in mammalian tissues. V. Regulation of glutamine-dependent carbamyl phosphate synthetase: activation by 5-phosphorylribosyl 1-pyrophosphate and inhibition by uridine triphosphate. *J. Biochem.* **72**:549–560 (1972).
21. Sebolt, J. S., T. Aoki, J. N. Eble, J. L. Glover, and G. Weber. Inactivation by acivicin of carbamoyl-phosphate synthetase II of human colon carcinoma. *Biochem. Pharmacol.* **34**:97–100 (1985).
22. Anderson, E. P., and R. W. Brockman. Feedback inhibition of uridine kinase by cytidine triphosphate and uridine phosphate. *Biochim. Biophys. Acta* **91**:380–386 (1964).
23. Schimke, R. T. (ed.). *Gene Amplification.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982).
24. Curt, G. A., D. N. Carney, K. H. Cowan, B. D. Bailey, K. C. Drake, C. S. Kao-Shan, J. D. Minna, and B. A. Chabner. Unstable methotrexate resistance in human small-cell carcinoma associated with double minute chromosomes. *N. Engl. J Med.* **308**:199–202 (1983).
25. Cardman, M. D., J. H. Schornagel, R. S. Rivest, S. Rsimatkandada, C. S. Portlock, T. Duffy, and J. R. Bertino. Resistance to methotrexate due to gene amplification in a patient with acute leukemia. *J. Clin. Oncol.* **2**:16–20 (1984).
26. Horns, R. C., W. J. Dower, and R. T. Schimke. Gene amplification in a leukemic patient treated with methotrexate. *J. Clin. Oncol.* **2**:2–7 (1984).
27. Jones, M. E. Pyrimidine nucleotide biosynthesis in animals: genes, enzymes, and regulation of UMP biosynthesis. *Annu. Rev. Biochem.* **49**:253–279 (1984).
28. Keppler, D., and A. Holstege. Pyrimidine nucleotide metabolism and its compartmentation, in *Metabolic Compartmentation* (H. Sies, ed.). Academic Press, London, 147–203 (1982).
29. Hoogenraad, N. J., and D. C. Lee. Effect of uridine on *de novo* pyrimidine biosynthesis in rat hepatoma cells in culture. *J. Biol. Chem.* **249**:2763–2768 (1974).
30. Hoffman, D. H., and M. J. Sweeney. Orotate phosphoribosyltransferase and orotidylatic acid decarboxylase activities in liver and Morris hepatoma. *Cancer Res.* **33**:1109–1112 (1973).
31. Cysyk, R. L., P. E. Gormley, M. E. D'Anna, and R. H. Adamson. The disposition of 3-deazauridine in mice. *Drug Metab. Dispos.* **6**:125–132 (1978).

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