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ENHANCEMENT OF 5-FLUOROURACIL INCORPORATION INTO HUMAN LYMPHOBLAST RIBONUCLEIC ACID*

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Abstract—Various drug combinations have been explored as a means of enhancing the incorporation of 5-fluorouracil (5-FU) into RNA from the human T-lymphoblast CEM line. The relative incorporation of [^{3}H]FU into newly synthesized alkali-sensitive RNA was determined by concurrent ^{32}P-incubation and calculation of the ratio of [^{3}H]FU/^{32}P. *N*-(Phosphonacetyl)-L-aspartate (PALA) was employed at concentrations of 0.1 mg/ml for 16 hr prior to the addition of [^{3}H]FU; it enhanced the formation of (5-FU)RNA by 3- to 5-fold. The effects of methotrexate (MTX) and 6-methylmercaptopurine riboside (MMPR) on the formation of (5-FU)RNA and phosphoribosyl-1-pyrophosphate (PRPP) were also monitored. Increases of 10- to 15-fold in PRPP occurred following a 6-hr exposure to either MTX or MMPR at concentrations of 1 μM and resulted in up to a 5-fold enhancement in the [^{3}H]FU/^{32}P ratio. Exposure of cells to combinations of PALA with MTX or MMPR resulted in up to 20-fold increases in (5-FU)RNA formation. These drug combinations were more than additive with 5-FU in their inhibitory effects as determined by assays monitoring cell growth. These studies should be applicable in expanding ongoing clinical trials of combination chemotherapy with PALA and 5-FU.

The fluorinated pyrimidine 5-fluorouracil (5-FU) has been used extensively in the palliative therapy of a variety of human tumors. Despite widespread clinical use, the precise mechanism of action of 5-FU remains unclear. Experimental evidence indicates that the following two mechanisms of action may be responsible for the effects of 5-FU: (1) the conversion of 5-FU to FdUMP which binds irreversibly to thymidylate synthetase and thereby inhibits *de novo* synthesis of dTMP and, consequently, DNA synthesis, and (2) the conversion of 5-FU to FUTP which can be incorporated into RNA and thereby disrupt RNA synthesis and function [1–4]. Although either, or both, of these mechanisms may be responsible for the effects of 5-FU, the antitumor activity of this agent has been directly correlated with the incorporation of FUTP into tumor cell RNA [5–8].

One approach designed to enhance the incorporation of 5-FU ribonucleotides into RNA is through the reduction of uracil nucleotide pools. *N*-(Phosphonacetyl)-L-aspartate (PALA), a transition state analog inhibitor of aspartate transcarbamylase, has been shown to cause marked reductions in the uracil nucleotide pools by inhibiting *de novo* pyrimidine biosynthesis [9, 10]. The combination of PALA with 5-FU results in a relative increase in incorporation of 5-FU into RNA that correlates directly with antitumor activity [11, 12]. This combination has been extended clinically and has resulted in responses in patients previously resistant to 5-FU alone [13].

Another approach has been used to enhance the formation of (5-FU)RNA by increasing intracellular levels of phosphoribosyl-1-pyrophosphate (PRPP) and thereby phosphoribosyl transfer to 5-FU. Methotrexate (MTX) has been shown to increase levels of PRPP and thus enhance the incorporation of 5-FU into RNA [8]. This work demonstrates the importance of sequencing MTX prior to the administration of 5-FU as has been shown in a number of *in vivo* systems [14–16] and emphasizes the relevance of 5-FU incorporation into RNA as a major determinant of cytotoxicity. Similar increases in PRPP levels have been observed as a result of cellular exposure to 6-methylmercaptopurine riboside (MMPR) and thus serves as another means of enhancing the formation of 5-FU ribonucleotides [8]. MMPR has also been shown to potentiate the antitumor activity of 5-FU in an animal tumor model [12].

In the investigation reported here several approaches directed at enhancing the incorporation of 5-FU into human tumor cell RNA were employed. PALA was used as a means of reducing uracil nucleotide pools, while MTX and MMPR were used to enhance the formation of 5-FU nucleotides through increased intracellular levels of PRPP. Our results indicate that these approaches can be employed simultaneously and lead up to nearly 20-fold increases in (5-FU)RNA formation. The incorporation of 5-FU into tumor cell RNA is accompanied by enhanced cytotoxicity and supports the conclusion that this mechanism is relevant in determining antitumor effectiveness.

MATERIALS AND METHODS

Cell culture. The CEM human lymphoblast cells were cultured in S-MEM medium supplemented with

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10% heat-inactivated, dialyzed fetal calf serum, 100 units of streptomycin/ml, 100 μg penicillin/ml and 1% L-glutamine [17].

The CEM cells were in log phase growth at the time of drug exposures. PALA (Division of Cancer Treatment, NCI, Bethesda, MD) was added to the cells over a concentration range of 0.01 to 1.0 mg/ml. MTX (Lederle Laboratories Division, Pearl River, NY) and MMPR (Sigma Chemical Co. St. Louis, MO) were both freshly prepared in S-MEM medium at concentrations of 10^{-4} M. The drugs were sterilized by Millipore filtration, and cells were exposed to concentrations of 10^{-5} to 10^{-7}M.

Measurement of (5-FU)RNA formation. Control and drug-exposed CEM cells were adjusted to 1×10^7/ml in complete medium (HEPES* buffer) prior to the labeling procedure. The cells were then incubated with 10^{-5} M [^{3}H]FU (New England Nuclear, Boston, MA) (sp. act. 5 Ci/mmole) or 10^{-4} M [^{3}H]FU (sp. act. 0.5 Ci/mmole), and 10 μCi/ml of ^{32}P (New England Nuclear), carrier-free, for 3 hr at 37°. After labeling, the cells were washed three times with 5 ml of phosphate-buffered saline (PBS). The final cell pellet was resuspended in 1 ml PBS and incubated at 37° for 3 hr with occasional vortexing after the addition of 2.5 mg of pronase B and 2 ml of 0.01 M Tris (pH 7.4), 0.01 M EDTA, and 0.5% sodium dodecylsulfate (SDS). The nucleic acids were precipitated by the addition of 1/10 volume 4 M NaCl and 2 volumes of absolute ethanol. After overnight incubation at −20°, the samples were spun at 10,000 *g* for 30 min, and the pellets were resuspended in 0.005 M EDTA. The $A_{260/280}$ ratio was then determined as a measure of nucleic acid concentration and purity (range: 1.9 to 2.0). The samples were then divided into two equal aliquots, and one was subjected to alkaline digestion with 0.3 M NaOH and incubation overnight at 37°. The untreated and alkaline-digested nucleic acid preparations were then adjusted to 50% formamide and incubated at 80° for 15 min to denature hybrid complexes. After quick chilling to 0°, the samples were added to 5.4 ml of 0.005 M EDTA, mixed with an equal volume of saturated Cs_2SO_4 to yield a starting density of 1.52 g/ml, and centrifuged at 44,000 rpm in a Spinco 50 Ti rotor for 60 hr at 20°. Fractions (0.4 ml) were collected from the bottom of the tube and assayed for radioactivity precipitable by trichloroacetic acid.

Measurement of intracellular PRPP levels. The PRPP levels were determined by a method described previously [18].

Studies on inhibition of cellular growth. The effects of PALA, MTX, and MMPR alone and in combination with 5-FU on cells in logarithmic growth phase were studied. 5-FU (Roche Laboratories, Nutley, NJ) was freshly prepared in S-MEM medium at a stock concentration of 10^{-3} M. Cells were exposed to 5-FU at a concentration of 10^{-5} M or 10^{-4} M for a period of 3 hr. Following the drug exposure, the cells were harvested and counted in a model Z Coulter counter and then reseeded in fresh medium without drug at a density of 2×10^5 cells/ml. Growth inhibition was measured after 72 hr and is expressed as a percentage of control growth, after correction for the initial seeding density. Values represent the mean ± standard deviation of three determinations.

* HEPES, 4-(2-hydroxyethyl)-l-piperazine-ethanesulphonic acid.

RESULTS

Figure 1A is a representative Cs_2SO_4 density profile of CEM RNA (banding between densities 1.62 and 1.68 g/ml) and DNA (banding between densities 1.42 and 1.48 g/ml) after labeling with [^{3}H]FU and ^{32}P. The ^{32}P-labeling serves as a measure of newly synthesized RNA or DNA and permits a determination of the relative incorporation of [^{3}H]FU, by calculating the [^{3}H]FU/^{32}P ratio. The Cs_2SO_4 profile obtained after alkali digestion is shown in Fig. 1B with loss of the RNA labeling while there is little if any effect on incorporation into DNA. Thus, the incorporation of [^{3}H]FU and ^{32}P into the alkali sensitive RNA fraction can also be determined, without Cs_2SO_4 analysis, by the difference in radioactivity obtained before and following alkali digestion. Further, a calculation of the RNA [^{3}H]FU/^{32}P ratio permits a comparison of the amount of [^{3}H]FU incorporation into newly synthesized nucleic acid under various experimental conditions.

Figure 2 illustrates the effects of various concentrations of PALA on the incorporation of [^{3}H]FU into CEM RNA. These cells were incubated with PALA at concentrations ranging from 0.01 to 1.0 mg/ml for 19 hr prior to the addition of 10^{-5} M [^{3}H]FU and ^{32}P. The incorporation of [^{3}H]FU into alkali sensitive RNA was then determined and adjusted for newly synthesized RNA by the ratio of [^{3}H]FU/^{32}P. This ratio increased slightly above control after exposure to concentrations of PALA at 0.01 mg/ml and reached a plateau at concentrations of 1.0 mg/ml. Similar data has been obtained following incubations of at least 6 hr in duration, and this effect persists at least through 24 hr of PALA exposure. Subsequent experiments with drug combinations were therefore performed with PALA at a concentration of 0.10 mg/ml for 22 hr.

The CEM cells were also exposed to MTX or MMPR at concentrations of 0.1, 1.0 and 10 μM for periods up to 9 hr. Maximum increases in PRPP levels were achieved following exposure to concentrations of 1 μM for each drug. Figure 3 illustrates the maximum increases in PRPP after 3 hr of drug exposure and the plateau levels reached at 6 hr and 9 hr. Under these conditions, MTX resulted in increases in PRPP levels of approximately 10-fold, whereas exposure to MMPR raised these levels by approximately 15-fold.

Table 1 lists the effects of combinations of PALA, MTX, and MMPR on intracellular PRPP levels and on the relative incorporation of [^{3}H]FU into RNA. In these experiments, PALA had little if any effect on PRPP levels, but it resulted in enhancement of the [^{3}H]FU/^{32}P ratio by up to 5-fold. Enhancement of the [^{3}H]FU/^{32}P ratio was also achieved with MTX, MMPR, and the combination MTX/MMPR, while these experimental conditions resulted in increases in PRPP levels. It should be noted that the combination of MTX and MMPR was not additive in terms

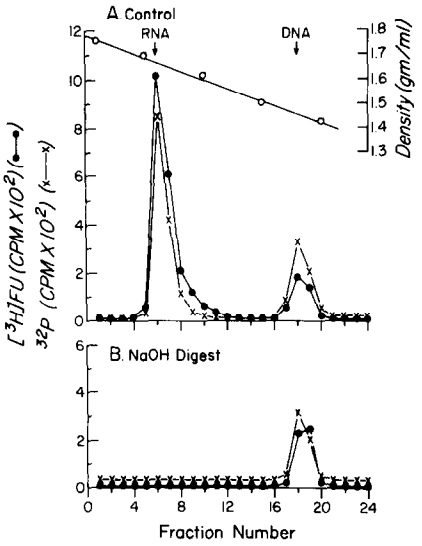

Fig. 1. Incorporation of [^{3}H]FU and ^{32}P into CEM nucleic acids. Cs_2SO_4 density gradient profiles of CEM (1×10^7 cells) nucleic acids, incubated with [^{3}H]FU (50 μCi/ml) and ^{32}P (10 μCi/ml) for 3 hr were measured before (A) and after (B) alkaline digestion.

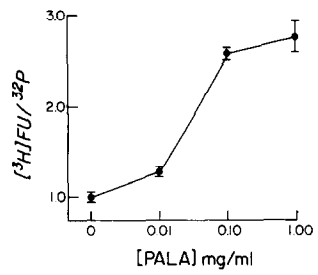

Fig. 2. Effect of PALA on the incorporation of 10^{-5} M [^{3}H]FU into newly synthesized CEM RNA as determined by the radioactivity obtained before and after alkali digestion. The ratio of [^{3}H]FU/^{32}P for the controls not exposed to PALA was 2.2. The values plotted are the means ± S.D. of four determinations normalized to the control ratio.

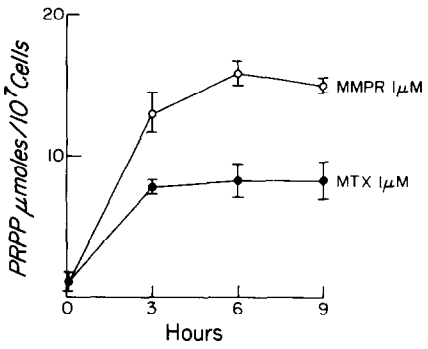

Fig. 3. Effect of MMPR and MTX on PRPP levels in CEM cells as determined by the method described previously [18]. MTX and MMPR were employed at concentrations of 1 μM, and the values are the means ± S.D. of two separate determinations.

of PRPP levels or the incorporation of 5-FU into RNA. Thymidine (10^{-4} M) was also employed in these studies, but failed to yield a significant increase in either PRPP or 5-FU incorporation into RNA.

The effects of combining PALA with MTX, MMPR, or MTX/MMPR are also listed in Table 1. The combination PALA/MTX resulted in lower PRPP levels than MTX alone; however, the combination was approximately additive in terms of enhancing the [^{3}H]FU/^{32}P ratio. The highest increases in PRPP levels were achieved with

Table 1. Effects of various drug combinations on intracellular PRPP levels and on incorporation of [^{3}H]FU into CEM RNA*

Drug exposure	PRPP	[^{3}H]FU/^{32}P	
		10^{-5} M [^{3}H]FU	10^{-4} M [^{3}H]FU
Control	1.0 ± 0.5	1.0	10.0
PALA	2.1 ± 0.9	5.6 ± 1.3	39.7 ± 4.0
TdR	1.9 ± 0.08	1.1 ± 0.1	ND†
MTX	10.7 ± 1.4	2.7 ± 1.1	34.1 ± 4.2
MMPR	15.3 ± 1.8	5.5 ± 2.3	39.5 ± 6.5
MTX/MMPR	13.8 ± 5.2	4.8 ± 1.7	33.7 ± 5.8
PALA/MTX	4.2 ± 1.0	10.3 ± 3.6	47.4 ± 5.9
PALA/MMPR	18.8 ± 2.7	20.1 ± 4.4	69.8 ± 9.2
PALA/MTX/MMPR	14.3 ± 1.2	19.4 ± 4.4	69.4 ± 4.0

* CEM cells were exposed to various drug combinations employing PALA: 0.1 mg/ml (22 hr), thymidine: 10^{-4} M (22 hr), MTX: 1 μM (6 hr) and MMPR: 1 μM (6 hr). The PRPP levels were determined by the method described previously [18], and the ratio [^{3}H]FU/^{32}P was determined from the radioactivity obtained before and after alkali digestion. Each value is the mean ± S.D. of at least three determinations. The PRPP levels represent nmoles/10^7 cells. The [^{3}H]FU/^{32}P ratios are normalized to a control ratio of 1.6 for the 10^{-5} M [^{3}H]FU (sp. act. 5 Ci/mmole) exposures and 1.8 for the 10^{-4} M [^{3}H]FU (sp. act. 0.5 Ci/mmole) exposures.

† Not determined.

Table 2. Effects of various drugs alone and in combination with 5-FU on CEM cellular growth*

Drug exposure	% Control growth		
	− 5-FU	+ 5-FU (10^{-5} M)	+ 5-FU (10^{-4} M)
Control		103.4 ± 2.8	89.1 ± 7.3
MTX	85.7 ± 5.0	68.7 ± 2.0	19.0 ± 2.1
MMPR	91.7 ± 1.8	89.1 ± 3.8	21.9 ± 1.3
MTX/MMPR	89.0 ± 8.5	61.6 ± 2.5	23.9 ± 1.0
PALA	74.3 ± 1.0	67.7 ± 8.0	22.4 ± 1.3
PALA/MTX	31.3 ± 2.3	24.7 ± 2.3	2.2 ± 0.6
PALA/MMPR	71.5 ± 1.0	19.7 ± 3.8	4.3 ± 2.2
PALA/MTX/MMPR	20.7 ± 2.3	14.2 ± 1.6	4.2 ± 1.6

* Experimental conditions for PALA, MTX, and MMPR are identical to those employed in the legend to Table 1. Cells were also exposed to 5-FU at concentrations of 10^{-5} M and 10^{-4} M for 3 hr. Following drug exposure, the cells were reseeded and counted after 72 hr. The results (mean ± S.D. for four determinations) are expressed as percentage of control growth.

PALA/MMPR, and this correlated with the greatest enhancement of the [^{3}H]FU/^{32}P ratios upon exposure to both 10^{-5} M and 10^{-4} M [^{3}H]FU. Similar increases in [^{3}H]FU/^{32}P ratios were also observed with the PALA/MTX/MMPR combination.

The effects of these various drug combinations on growth of CEM cells are listed in Table 2. The conditions of drug exposure were identical to those employed in the biochemical studies with exposure to 5-FU at concentrations of 10^{-5} and 10^{-4} M for periods of 3 hr. The cells were exposed to the various drugs, reseeded in fresh medium, and then counted 72 hr after drug exposure. At the lower concentration of 5-FU (10^{-5} M), minimal effects were observed with the various drug combinations with the exception of PALA/MMPR which showed more than additive effects. In studies employing 5-FU at concentrations of 10^{-4} M, there was more than additive activity with each of the drug combinations and virtually complete inhibition of growth with combinations of PALA with MTX or MMPR.

DISCUSSION

Several approaches using PALA or thymidine have been shown to be effective as a means of enhancing the incorporation of 5-FU into tumor cell RNA and thereby increasing therapeutic efficacy [5–7, 19–21]. Thymidine bypasses 5-FU inhibition of thymidylate synthetase, whereas PALA inhibits *de novo* pyrimidine synthesis and reduces uridine nucleotide pools. Each of these modulations results in increases in 5-FU incorporation into cellular RNA. The enhancement of (5-FU)RNA formation has also been shown to result from increased intracellular levels of PRPP, a substrate involved in the conversion of 5-FU to FUMP [8, 22].

The current investigation was initiated to determine whether (5-FU)RNA formation could be enhanced in human cells using either PALA to reduce the uridine nucleotide pools or agents that have been employed to raise PRPP levels in animal cells. Further, it was of interest to determine whether both mechanisms could be exploited simultaneously to provide even further enhancement of 5-FU incorporation into tumor cell RNA. The results indicate that modulation directed at increasing PRPP levels while concomitantly reducing endogenous uridine nucleotide pools can enhance the formation of (5-FU)RNA even further.

PALA was employed at a concentration of 0.1 mg/ml based upon results indicating that near maximal incorporation of [^{3}H]FU incorporation into newly synthesized RNA occurred at this concentration between 6 and 24 hr of exposure. PALA alone had little effect on PRPP levels, while it did provide enhancement in the [^{3}H]FU/^{32}P ratios. In contrast, thymidine also had little effect on PRPP levels and, under our experimental conditions, failed to enhance the formation of (5-FU)RNA. The most marked increases in PRPP levels were observed with either MTX or MMPR. Similar increases have been observed upon exposure of L1210 cells under similar experimental conditions [8, 22]. In the CEM line, MTX and MMPR also increased 5-FU incorporation into RNA by approximately 3- to 5-fold. The combination of MTX and MMPR had effects similar to those obtained with MMPR alone, indicating the absence of an additive effect with these two agents.

Combinations of PALA with MMPR resulted in the most marked increases in both PRPP levels and in 5-FU incorporation into RNA. The combination of PALA with MTX, however, resulted in PRPP levels that were less than additive and an incorporation of [^{3}H]FU into RNA that was half that achieved with PALA/MMPR. Further, the incorporation of [^{3}H]FU into RNA with the combination of PALA/MTX/MMPR was similar to that with PALA/MMPR alone.

The incorporation of 5-FU into RNA is certainly one of two possible explanations for the effects of 5-FU, and the conversion to FdUMP with inhibition of thymidylate synthetase might also be explored, in view of the effects of these drug combinations. There is evidence that suggests that different mechanisms may be responsible for the biologic effects seen in various tissues [1]. Depression of DNA synthesis via inhibition of thymidylate synthetase may predominate in bone marrow toxicity, whereas gastrointestinal toxicity may be more closely related to FUTP incorporation into RNA. Our data suggest that increased (5-FU)RNA formation is associated

with enhanced biologic effect as each of the drug combinations was more than additive with 5-FU in cell growth experiments. These findings should be applicable in providing a biochemical rationale for further clinical studies of combinations with 5-FU.

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