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MODULATION OF FLUOROPYRIMIDINE METABOLISM IN L1210 CELLS BY L-ALANOSINE*

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Abstract—L-Alanosine, an analogue of aspartic acid which inhibits the conversion of inosine monophosphate to adenosine monophosphate (AMP), was evaluated in L1210 cells as a modulator of 5-fluorouracil (FUra) and 5-fluorouridine (FUrd) metabolism. L-Alanosine resulted in increased intracellular levels of 5-phosphoribosyl-1-pyrophosphate (PRPP), enhanced FUra metabolism to ribonucleotide derivatives, and resulted in more FUra residues incorporated into RNA. Sequential addition of L-alanosine and FUra also resulted in synergistic cytotoxicity as determined by soft agar cloning. Adenine antagonized these biochemical and biological effects of L-alanosine. L-Alanosine also augmented the rate at which FUrd was metabolized and was also associated with a greater incorporation of the FUra residues into RNA. Cytotoxicity after sequential L-alanosine and FUrd was also synergistic. The mechanism by which L-alanosine altered the metabolism of FUrd, however, was different from the way in which it enhanced FUra metabolism in that aspartic acid and not adenine was able to reverse the effects of L-alanosine on FUrd metabolism and cytotoxicity. L-Alanosine appeared to augment the RNA-directed activity of FUra and FUrd in that there was no correlation between the enhanced metabolism and cytotoxicity of these two fluoropyrimidines and either levels of fluorodeoxyuridylate (FdUMP) which inhibits thymidylate (TMP) synthetase or inhibition of the ability of cells to incorporate deoxyuridylate into acid-precipitable material.

5-Fluorouracil (FUra) has been widely used in clinical chemotherapy. The primary locus of its cytotoxicity has been generally thought to be the inhibition of thymidylate synthetase by the metabolite 5-fluorodeoxyuridylate (FdUMP) [1]. Recently, rescue experiments using thymidine and uridine [2–4], and biochemical studies undertaken by Wilkinson [5, 6], Randerath [7], Glazer [8–12] and Martin [13–15] have shown that incorporation of 5-fluororibonucleotides into RNA may also be cytotoxic. 5-Fluorouridine (FUrd) when compared to equimolor FUra, resulted in higher analogue ribonucleotide pools [4], greater gross changes in mitotic chromosome structure [16, 17], more extensive inhibition of ribosomal RNA maturation [5], and greater cytotoxicity [4]. However, from clinical trials with FUrd alone, it has been concluded that administering FUrd rather than FUra is not advantageous [18]. It is possible that the modulation of normal metabolic pathways by other drugs may provide a mechanism for selective metabolism and cytotoxicity of these fluoropyrimidines.

FUra and FUrd are anabolized to nucleotides by different routes and provide the means by which selective modulation might be possible. FUra can be converted to FUMP by orotate phosphoribosyltransferase in the presence of the ribose and phosphate donor, 5-phosphoribosyl-1-pyrophosphate (PRPP) [19, 20]. FUra can also be converted to FUrd by pyrimidine nucleoside phosphorylase [21] which then can be phosphorylated by uridine-cytidine kinase to FUMP. FUMP is then metabolized to the di- and triphosphate ribonucleoside derivatives and also to the deoxyribonucleotide FdUMP. Finally, FUra can be donated a deoxyribose by pyrimidine deoxynucleoside phosphorylase forming 5-fluorodeoxyuridine (FdUrd), which can be directly phosphorylated to FdUMP by thymidine kinase [22]. Our work has shown that for a variety of cell lines from both murine and human sources the predominant pathway for FUra metabolism is phosphoribosyl addition from PRPP by orotate phosphoribosyltransferase [20, 23–25].

Exogenous FUrd can either be phosphorylated by uridine-cytidine kinase or it can be catabolized first to the FUra and then become a substrate for any one of three possible pathways above. Our attempts to modulate the metabolism of fluoropyrimidines in L1210 cells have demonstrated that agents that promote salvage of preformed pyrimidine nucleosides enhance FUrd metabolism while those that either decrease orotic acid or increase PRPP will enhance FUra. Pyrazofurin, which, as the monophosphate, inhibits orotidylate decarboxylase and increases orotate pools, will increase the rate of FUrd accumulation and decrease the rate of FUra accumulation [26]. Methotrexate increases PRPP pools which results in enhanced FUra phosphorylation to FUMP and, therefore, increased total intracellular FUra accumulation as nucleotide derivatives. FUrd phos-

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phorylation, however, is reduced in methotrexate-treated cells presumably from increased inhibition of uridine-cytidine kinase by elevated UTP pools. Agents that inhibit pyrimidine metabolism proximal to orotate formation, for instance *N*-(phosphonacetyl)-L-aspartate (PALA), increase both FUra and FUrd metabolism [20, 26].

This report extends these concepts of biochemical modulation of fluoropyrimidine metabolism by evaluating the effects of L-alanosine (2-amino-3-*N*-nitrosohydroxylamino-propionic acid), an inhibitor of *de novo* adenosine monophosphate synthesis [27] and a naturally occurring analogue of aspartate [28]. The antiproliferative activity of L-alanosine is the result of the metabolite, 5-amino-4-carboxyl-(2-*N*-alanosine)-imidazole-1-ribonucleotide (Alan-AICAR) [29]. Alan-AICAR has a K_i for adenylosuccinate synthetase which is below 1 μM in L1210 cells [30]. The pivotal nucleotides regulating PRPP synthesis are ADP and ATP [31]. Since Alan-AICAR inhibits the formation of adenosyl nucleotides without lowering the levels of guanosyl nucleotides, we felt that this drug would provide a more defined framework in which to evaluate the effect that adenosyl nucleotides have in regulating PRPP levels and modulating fluoropyrimidine metabolism.

MATERIALS AND METHODS

Drugs and cells. L-Alanosine was a gift of Dr. David A. Cooney (National Cancer Institute, Bethesda, MD). FUra, FUrd, adenine and nucleotide diphosphates and triphosphates were purchased from the Sigma Chemical Co. (St. Louis, MO). PRPP was purchased from Calbiochem (La Jolla, CA). [1-^{14}C]Glycine (20 mCi/mmole) [2-^{3}H]adenine (25.5 Ci/mmole), and [6-^{3}H]deoxyuridine (21.9 Ci/mmole) were purchased from the New England Nuclear Corp. (Boston, MA). [6-^{3}H]FUra (20 Ci/mmole) and [6-^{3}H]FUrd (18 Ci/mmole) were obtained from Moravek Biochemicals (City of Industry, CA).

L1210 cells were maintained as suspension cultures in Fischer's medium plus 10% horse serum and kept at 37° in a 5% CO_2 incubator. All experiments were performed with cells which were in mid-logarithmic growth at a concentration of between 0.8 and 2×10^5 cells/ml.

Cloning. Cell viability was determined by a modification of a method first described by Chu and Fischer [32] and reported in detail by us [26].

In brief, treated and control cultures were washed twice in Fischer's medium to remove any intracellular drug and then diluted appropriately such that twenty-five cells in 2 ml of Fischer's medium + 15% horse serum were pipetted into 15 ml culture tubes which contained 3 ml of liquified agar and drug-free Fischer's medium plus 15% horse serum. The tubes were capped and placed upright and incubated at 37° in a 5% CO_2 atmosphere. After 10 days, macroscopic colonies were counted. The percent viability is the ratio of clones formed from drug-treated cultures to clones formed from untreated cultures multiplied by 100. The cloning efficiency of control L1210 cells in this system was 85–90%. All experiments were done in quadruplicate on at least two separate occasions.

Intracellular accumulation of fluoropyrimidine metabolites. The intracellular accumulation of radiolabeled fluoropyrimidine metabolites was measured by the microfuge method which we have reported previously in detail [20, 26]. This method allows for rapid separation of cells from medium to which drug has been added.

Incorporation of pyrimidine metabolites into macromolecules. This was measured in two ways for accuracy. In the first, cells were concentrated by centrifugation and, as for measurement of intracellular accumulation, resuspended in their own culture medium supplemented with 0.01 M 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES). The addition of HEPES was necessary only to compensate for the loss of HCO_3-buffering that occurs outside of a 5% CO_2 incubator. [^{3}H]FUra was added at 3 μM. [^{3}H]FUrd was added at a range from 0.1 to 10 μM. [^{3}H]Cyd or [^{3}H]Urd was added at approximately 0.1 μM. Following the addition of the radiolabeled drug, 0.025 ml, containing 4–8×10^4 cells, was deposited in wells of harvesting plates (Limbro Scientific, Inc., Hamden, CT) and aspirated onto Reeve-Angel 924 AH glass fiber filter paper strips (Whatman Inc., Clifton, NJ) using a MASH II cell harvester (Microbiological Associates, Walkersville, MD). Filters were washed with at least 10 vol. of 10% trichloroacetic acid (TCA) and distilled H_2O and dried. The regions containing the acid-precipitable pellet were added to 3 ml of scintillant [15 g 2,5-diphenyloxazole (PPO), 0.3 g 1,4-bis-[2-(4-methyl-5-phenyloxazolsyl)]benzene (POPOP), 1 liter Triton X-100, 2 liters toluene] and counted in a Packard Tri-Carb scintillation counter (Packard Instrument Co., Downers Grove, IL).

Since fluoropyrimidines are incorporated almost exclusively in RNA, this method of determining the amount of radioactivity incorporated into acid-precipitable material is a close approximation of the amount of drug incorporated into RNA. The second method utilized alkaline hydrolysis to specifically isolate more accurately the RNA. Radiolabeled cells were collected by centrifugation (8 min, 1200 rpm) and lyzed in ice-cold 0.5 M $HClO_4$. After 20 min on ice, the cell lysate was centrifuged at 2000 rpm for 10 min and repeatedly washed in ethanol until 0.2 ml of supernatant fluid did not exceed background radioactivity. The pellet was then resuspended in 1 N KOH, and the RNA was hydrolyzed for 5 hr at 37°. Control studies demonstrated that this technique hydrolyzed >95% of the RNA while >98% of the DNA remained intact. While this treatment will result in some loss of ^{3}H from the [^{3}H]fluorouracil residues in RNA [33], the total radioactivity made soluble by alkaline hydrolysis was counted to minimize the error this might introduce into the quantitation. The quantity of RNA in the hydrolysates was measured by the orcinol colorimetric assay [34].

Incorporation of [^{3}H]dUrd into acid-precipitable material. Cells were prepared as above, and, at designated times after adding [6-^{3}H]dUrd to a concentration of 0.1 μM, 0.025 ml of the cell suspension was placed in harvesting plates and processed with the MASH cell harvester. The radioactivity was determined as just described. This is a standard method to evaluate the ability of cells to convert

dUMP to dTMP which is incorporated into DNA as dTTP. Multiple enzymatic processes are involved in the ultimate incorporation of label into DNA from exposing cells to [^{3}H]dUrd. Therefore, the results from these experiments can only be estimates of thymidylate synthesis.

Nucleotide pools. The acid-soluble supernatant fraction of cells lyzed with 0.5 M $HClO_4$ was examined by high performance liquid chromatography (HPLC) analysis after the pH was adjusted to approximately 7 to 8.5 with KOH. Samples were frozen at −21° for up to 4 weeks and then analyzed consecutively on an SAX-10 anion exchange column using an isocratic elution at 1 ml/min with 0.35 M NaH_2PO_4, pH 3.7. Elution times were 20.5 min for CTP, 25 min for UTP, 31 min for ATP, and 62.5 min for GTP.

To separate the nucleoside diphosphates, the concentration of NaH_2PO_4 was reduced to 0.1 M, pH 3.85. For the first 25 min an isocratic elution was used which was followed by a linear gradient to 0.45 M over 25 min. Nucleosides appeared in the void volume, and the nucleoside monophosphates eluted within 14 min but were not resolved by this method. CDP eluted at 17 min, UDP at 25 min, ADP at 32–33 min, and GDP at 45 min. Elution times for CTP, UTP, ATP, and GTP were 58, 61, 67, and 84 min respectively. This method was used to separate the [6-^{3}H]FUra and [6-^{3}H]FUrd nucleotide derivatives. One-minute fractions were collected and the eluted radionuclide was quantitated. The base or nucleoside appeared in the void volume. FUMP, FUDP, and FUTP radioactivity peaked in fractions 11, 27, and 62. There was no radioactivity associated with cytidine nucleotides.

De novo *purine synthesis.* To evaluate the rate of *de novo* purine synthesis, the incorporation of [1-^{14}C]glycine into acid-soluble and macromolecular purines was determined. Drug was added to 100 ml of suspension cell cultures for 2 hr before [1-^{14}C]glycine (~20 mCi/mmole). Following an additional 2 hr of incubation, the cells were washed by centrifugation and two rinses with phosphate-buffered saline (PBS) containing 1 mM cold glycine. Cells were then lyzed in 1.5 ml of 1 N $HClO_4$ and the nucleic acids were depurinated by placing samples in a boiling water bath for 1 hr. The soluble extract was then analyzed on HPLC using an ODS-2 column (Whatman Inc.) eluting with 0.1 M sodium acetate, pH 5.5, and an acetonitrile gradient from 0 to 7.5% over 30 min. Non-radiolabeled adenine and guanine were used as markers, absorbance was recorded at 254 and 280 nm, 1 ml fractions were collected, and the radioactivity was quantitated. Glycine appeared in the void volume.

PRPP determination. PRPP was measured by converting [2-^{3}H]adenine to [^{3}H]AMP by adenine phosphoribosyltransferase as previously reported [20, 26, 35]. The assay mixture contained 100 μl of a cell extract corresponding to 5×10^5 cells prepared by boiling for 90 sec or a known amount of PRPP in 0.2 M Tris, pH 7.4, as a standard to which was added 20 μl of 20 μM $MgCl_2$, 5 μl [2-^{3}H]adenine (sp. act. 7.7 Ci/mmole) at 33 μM, and 100 μl of an extract containing adenine phosphoribosyltransferase prepared from one ammonium sulfate precipitation fraction of L1210 cells which was then eluted on a Sephadex G150 column. The addition of PRPP standards to extracts of control cells did not result in appreciable alteration of values obtained from PRPP in Tris buffer. An overestimate was observed which accounted for endogenous PRPP. The slopes of the control curves were identical. These were mixed in tubes on ice, and the tubes were incubated for 20 min at 19°. The reaction was stopped by placing the tubes on ice and adding 0.275 ml of 3 mM citric acid in absolute methanol to each; 0.050 ml of this reaction mixture was spotted onto Whatman DE81 filter paper discs washed twice with 2 mM citric acid, 60% methanol (10 ml/disc), and then washed once with absolute methanol (10 ml/disc). The amount of intracellular adenine contained in 5×10^5 cells would not appreciably alter the adenine concentration of the reaction mixture and, therefore, alterations of intracellular adenine due to drug treatment would have a minimal effect on the PRPP determinations. After the discs were dried at room temperature and placed in scintillation vials, the [^{3}H]AMP was eluted with 1 ml of 0.5 M NaCl in 1 N HCl, 10 ml of the PPO/POPOP scintillant was added, and the vial was agitated until the solution was clear and the radioactivity was quantitated.

RESULTS

Effect of L-*alanosine on cell growth.* The effect of continuous exposure to various concentrations of L-alanosine on the culture growth of L1210 cells was ascertained (Fig. 1). Adenine at 30 μM could completely reverse the growth inhibitory effect of 10 μM L-alanosine. Aspartate at concentrations as high as 1 mM did not ameliorate this L-alanosine effect.

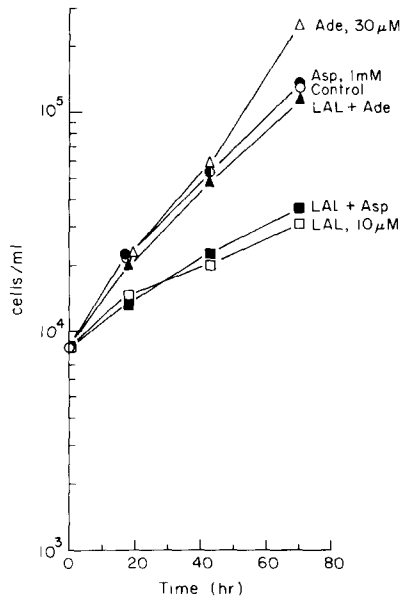

Fig. 1. Growth of L1210 cells and the effect of L-alanosine (LAL), adenine (Ade). L-aspartate (Asp) and the combination of LAL and Ade or Asp. The drugs were added to the suspension cultures at the indicated concentrations at time 0. N = two experiments, each in duplicate.

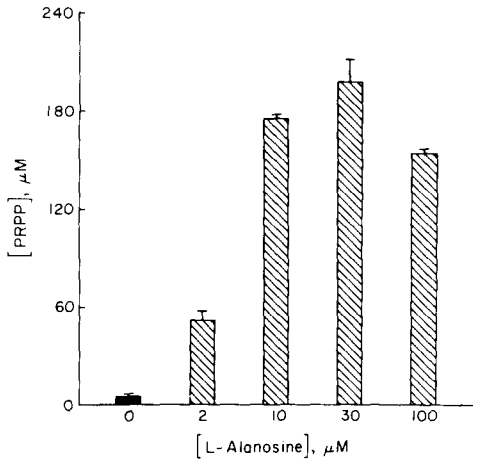

Fig. 2. PRPP concentrations in L1210 cells and the effect of various concentrations of L-alanosine for 4 hr. Values are means ± S.D.; N = 3.

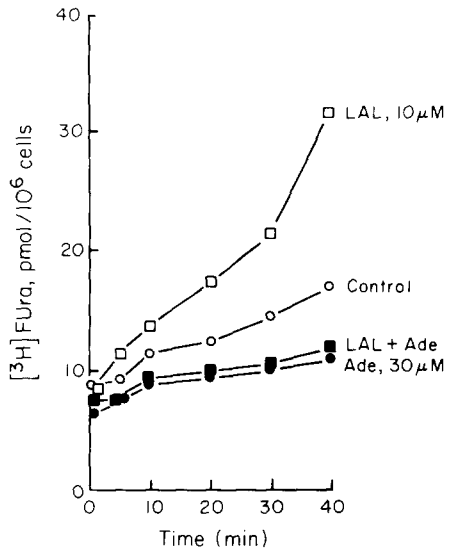

Fig. 3. Intracellular accumulation of FUra metabolites into L1210 cells following a 4-hr exposure to L-alanosine (LAL), adenine (Ade) or the two agents added simultaneously. N = 3.

De novo *purine synthesis and PRPP pools.* The incorporation of [1-^{14}C]glycine into adenine was 20 and 7.8% of control following 10 and 30 μM L-alanosine respectively. This inhibition of *de novo* adenylate synthesis was associated with increased PRPP pools which were also dependent on L-alanosine dose (Fig. 2). This supported *in vitro* data which suggested that levels of adenosine nucleotides were important in regulating the rate of PRPP synthesis [31] and that decreases in adenosine nucleotides increase the rate at which PRPP is synthesized [36].

Nucleotide pools. Following a 4-hr exposure to 10 μM L-alanosine, changes in the nucleoside diphosphates and triphosphates were consistent with observed alterations in *de novo* purine synthesis. ATP levels were halved, GTP was increased by 20%, and no consistent alteration was observed for UTP and CTP (Table 1).

Fluoropyrimidine metabolism. Cells pretreated with L-alanosine at various doses for 4 hr had an enhanced intracellular accumulation of [^{3}H]FUra metabolites which was linear to 90 min. Following L-alanosine concentrations from 2 to 30 μM, the rate of FUra metabolite accumulation was enhanced from 58 to 271% respectively. FUra accumulation following 10 μM L-alanosine is represented in Fig. 3. The magnitude of the enhanced [^{3}H]FUra metabolite accumulation correlated with the increased PRPP pools. Analysis of the fluoropyrimidine nucleotides by HPLC revealed an increase in the FUTP pool following 10 μM L-alanosine pretreatment for 4 hr (Fig. 4A). Since FUTP was increased following L-alanosine pretreatment, the incorporation of [^{3}H]FUra into RNA was evaluated. Following 10 μM L-alanosine for 4 hr, 3 μM [^{3}H]FUra was added to the cell culture for 2 more hr before extracting the RNA and hydrolyzing it in KOH. There were 4675 cpm/μg RNA in control cultures compared to 7850 cpm/μg RNA in treated cultures. This was a 51% increase and correlated with the 65% increase in FUTP in the L-alanosine-treated cell cultures. Alterations in *de novo* purine synthesis which resulted in increased FUra metabolism may explain the better than additive cytotoxicity of the L-alanosine–FUra combination observed in the cloning assays (Table 2A).

Although UTP and CTP levels were unchanged in cultures treated with L-alanosine for 4 hr (Table 1), there was a 103% increase in cytidine nucleotide

Table 1. Nucleoside triphosphate pools in L1210* cells

Condition	ATP	CTP	UTP	GTP
		(pmoles/10^6 cells)		
Control	1.973 ± 0.26	0.329 ± 0.043	0.576 ± 0.203	0.315 ± 0.021
10 μM L-Alanosine, 4 hr	0.956 ± 0.354	0.369 ± 0.076	0.494 ± 0.127	0.384 ± 0.077
30 μM Adenine, 1 hr	2.110	0.265 ± 0.054	0.808 ± 0.408	0.300
1 mM Aspartic acid, 1 hr	2.305	0.298	0.527	0.302 ± 0.059
10 μM L-Alanosine, 4 hr + 30 μM adenine, 1 hr	2.070	0.301	0.399 ± 0.082	0.294
10 μM L-Alanosine, 4 hr + 1 mM aspartic acid, 1 hr	1.335 ± 0.092	0.299	0.533	0.407 ± 0.058

* Values are means ± S.D. For the control and the 10 μM L-alanosine alone experiments, N = 3; for other cases with S.D., N = 2.

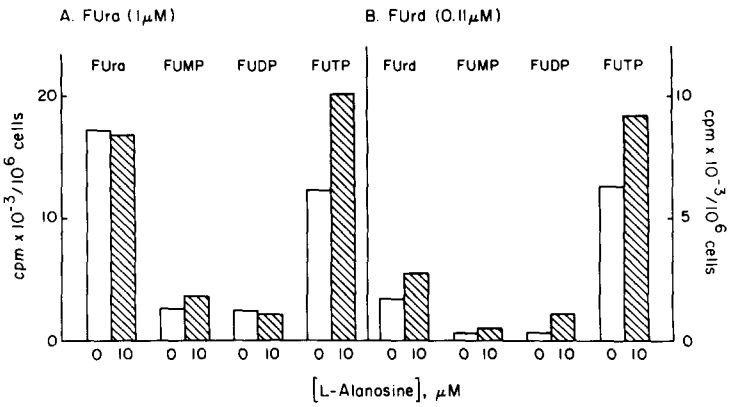

Fig. 4. Fluoropyrimidine ribonucleotide levels in L1210 cells exposed for 1 hr to equal toxic concentrations of FUra (1 μM) and FUrd (0.11 μM). Cells exposed to 10 μM L-alanosine for 4 hr prior to the addition of fluoropyrimidines are represented by the hatched areas. For FUra experiments, N = 3; for FUrd, N = 2.

and a 41% increase in uridine nucleotide accumulation within cells pretreated with 10 μM L-alanosine for 4 hr when 0.1 μM radiolabeled cytidine or uridine was added to the culture medium. These data suggested that L-alanosine might exert an influence on pyrimidine synthesis by interfering with aspartate metabolism and with aspartate transcarbamoylase. Therefore, FUrd metabolism after L-alanosine pretreatment was evaluated. Enhanced FUrd intracellular accumulation and incorporation into the acid-precipitable cell fraction did occur. [^{3}H]FUrd accumulation, unlike FUra accumulation, was not linear much beyond 10 min. When FUrd was 0.1 μM, 30-min accumulation of FUrd metabolites was increased from 7.76 pmoles/10^6 cells to 11.21 pmoles/10^6 cells in L-alanosine-treated cells. Incorporation of [^{3}H]FUrd into acid-precipitable material was increased from 4.35 to 7.69 pmoles/10^6 cells under similar conditions. Figure 4B describes the distribution of intracellular, acid-soluble [^{3}H]FUrd metabolites after 1 hr with and without 10 μM L-alanosine pretreatment. Removal of L-alanosine from medium before addition of [^{3}H]FUrd resulted in loss of this enhancement.

Effect of L-alanosine and fluoropyrimidine on FdUMP formation and [^{3}H]dUrd incorporation. Levels of total intracellular FdUMP were measured with and without 10 μM L-alanosine pretreatment for 4 hr. After 1 hr of 1 μM [^{3}H]FUra, FdUMP was increased from 114 ± 13 to 158 ± 17 fmoles/10^6 cells ($P < 0.05$). After 1 hr of 0.1 μM [^{3}H]FUrd, FdUMP was decreased from 101 ± 19 to 78.7 ± 31.6 fmoles/10^6 cells ($P > 0.10$).

Treatment of cells with L-alanosine alone had little effect on [^{3}H]dUrd incorporation. The incorporation of [^{3}H]dUrd in cells treated with fluoropyrimidine with or without L-alanosine, therefore, probably reflects the L-alanosine-induced alteration of fluoropyrimidine metabolism and the effects of these changes on the metabolism of [^{3}H]dUrd. Decreased

Table 2. Effect of adenine and aspartate on the cytotoxicity of L-alanosine and the combination of L-alanosine and 5-fluorouracil and 5-fluorouridine

(A)	FUra study*		(B)	FUrd study†	
	Condition	Viability (% of control)		Condition	Viability (% of control)
1.	Control	100	1.	Control	100
2.	Ade	99	2.	Ade	97
3.	Asp	105	3.	Asp	98
4.	FUra	70	4.	FUrd	84
5.	FUra + Ade	112	5.	FUrd + Ade	87
6.	FUra + Asp	95	6.	FUrd + Asp	78
7.	LAL	102	7.	LAL	89
8.	LAL + Ade	88	8.	LAL + Ade	102
9.	LAL + Asp	105	9.	LAL + Asp	84
10.	LAL + FUra	14	10.	LAL + FUrd	52
11.	LAL + Ade + FUra	99	11.	LAL + Ade + FUrd	51
12.	LAL + Asp + FUra	13	12.	LAL + Asp + FUrd	69

* Average of two experiments. Key: Ade = adenine, 30 μM for 4 hr; Asp = aspartic acid, 1 mM for 4 hr; FUra = 5-fluorouracil, 10 μM for 1.5 hr; LAL = L-alanosine, 10 μM for 4 hr; and FUrd = 5-fluorouridine, 0.11 μM for 1 hr.

† Average of three experiments. Maximum standard deviation was ± 3%.

Table 3. Effects of L-alanosine on the inhibition of [^{3}H]dUrd incorporation by FUra and FUrd

Condition	Without alanosine*	Alanosine* (4 hr, 10 μM)	Ratio of treated/control
Control†	0.169 ± 0.101	0.154 ± 0.066	0.911 ± 0.365
1 μM FUra‡	0.152 ± 0.035	0.153 ± 0.040	1.007
3 μM FUra	0.092	0.094	1.022
10 μM FUra‡	0.065 ± 0.004	0.051 ± 0.027	0.785
0.3 μM FUrd‡	0.064 ± 0.013	0.100 ± 0.054	1.563
1 μM FUrd‡	0.037 ± 0.008	0.062 ± 0.003	1.676
3 μM FUrd‡	0.036 ± 0.013	0.040 ± 0.007	1.111

* Expressed as pmoles 10^6 cells per min. Values are means ± S.D.
† Average of six experiments.
‡ Average of two experiments.

FdUMP from FUrd after L-adenosine treatment was correlated to increased incorporation of dUrd while increased FdUMP accompanying FUra in L-alanosine-treated cells was associated with a reduced incorporation of [^{3}H]dUrd (Table 3).

Effect of aspartate and adenine on L-alanosine. In L-alanosine-treated cells (10 μM for 4 hr), the addition of 30 μM adenine restored the adenine nucleotide levels to control values within 30 min (Table 1). The administration of adenine was sufficient to completely reverse the cytotoxicity of continuous L-alanosine exposure in suspension cultures (Fig. 1). The addition of adenine to L-alanosine-pretreated cells resulted in rapid intracellular accumulation of adenine metabolites and in decreases in PRPP to control levels within 30 min. The consequences of these adenine effects were also seen on the accumulation and clonogenicity of cells given FUra. FUra metabolites were reduced below control values (Fig. 3), and enhanced cytotoxicity of the sequential combination of L-alanosine and FUra was completely reversed with the addition of adenine (Table 2A). This is not unexpected since exogenous adenine requires PRPP for the conversion to AMP.

L-Aspartic acid, even at millimolar concentrations, had no effect on the L-alanosine cytotoxicity, nor did it influence the L-alanosine-induced modulations in nucleotide pools (Table 1), PRPP levels, or FUra accumulation (Fig. 5). These observations are similar to those of Graff and Plagemann [37]. However, when 1 mM aspartate was administered to L-alanosine-treated cultures 1 hr before [^{3}H]FUrd was added, incorporation of the FUrd into the acid-precipitable fraction was lowered to control levels (Fig. 6). The administration of adenine, at a concentration capable of negating the enhanced accumulation of FUrd (30 μM), was without effect on [^{3}H]FUrd metabolism following L-alanosine pretreatment.

The cloning of the sequential combination of L-alanosine and FUrd resulted in greater than additive

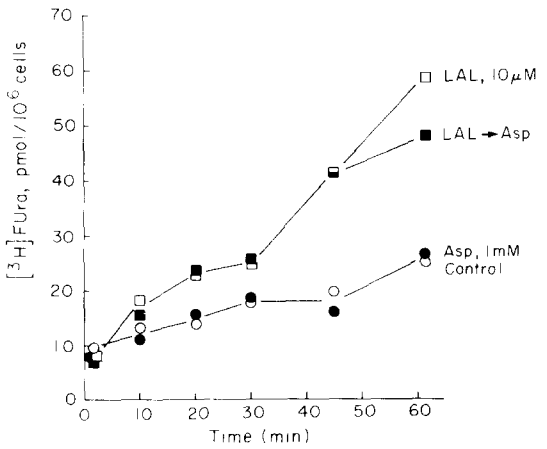

Fig. 5. Inability of L-aspartate (Asp) to reverse the L-alanosine (LAL) enhanced intracellular accumulation of FUra into L1210 cells. Following a 3-hr exposure to LAL, Asp was added for 1 hr. [^{3}H]FUra (3 μM) was added, and the intracellular accumulation of radioactivity was determined. The delay in Asp administration was to reduce the possibility of competition with LAL for transport within the cell. N = 2.

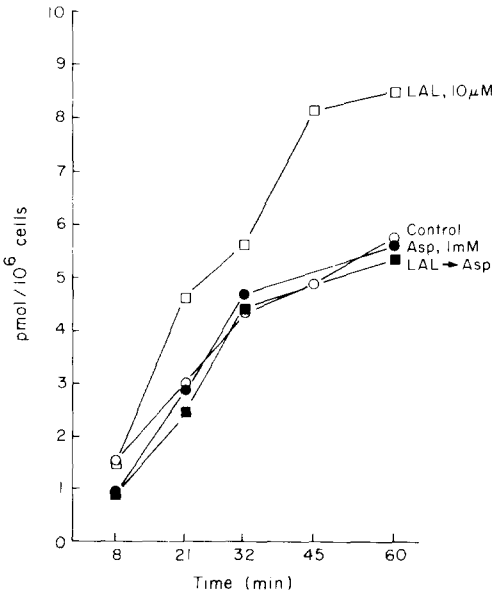

Fig. 6. Ability of L-aspartate (Asp) to reverse the effects of L-alanosine (LAL) on FUrd intracellular accumulation into L1210 cells. This study was performed as that described for Fig. 5 except that 0.11 μM [^{3}H]FUrd was given and the intracellular accumulation of radioactivity was determined. N = 4.

cytotoxicity at the doses tested. If the two drugs acted additively, 75% survival should have resulted. The combination killed twice as many cells as expected (Table 2B). The cytotoxicity was partially overcome by L-aspartate, but not at all by adenine, although in the presence of adenine there was no cytotoxicity that resulted from L-alanosine. This suggests that the reason for the heightened toxicity of the combination of L-alanosine and FUrd was enhanced accumulation and incorporation of FUrd nucleotides.

DISCUSSION

When L-alanosine preceded FUra or FUrd, the anabolism of the fluoropyrimidines was promoted and the cytotoxicity was increased. As an inhibitor of thymidylate synthetase, FdUMP is a major factor in cytotoxicity of fluoropyrimidines. But, there was less direct correlation between cytotoxicity of FUrd and FUra and levels of FdUMP than between cytotoxicity and appearance of fluorouridylate residues in RNA. This has been shown in gastrointestinal tissues [4] and Novikoff hepatoma cells [38] and is suggested by our previous studies with methotrexate-treated L1210 cells [20, 26]. There was no association of levels of total intracellular fluoropyrimidine accumulation before and after L-alanosine treatment with inhibition of the incorporation of dUrd into acid-precipitable material. Since dUrd is transported in the cells and rapidly phosphorylated to dUMP, it has often been used to assess the ability of cells to synthesize TMP from dUMP. Only after TTP is formed and incorporated into DNA can [^{3}H]dUrd metabolites be found in acid-precipitable material. Since L-alanosine itself had no effect on dUrd incorporation, the effects seen after fluoropyrimidines and L-alanosine probably result from the ability of FdUMP to inhibit TMP synthetase. These data do suggest that L-alanosine does not increase the DNA-directed effects of fluoropyrimidines in the L1210 cells used in this study. The increased total fluoropyrimidine accumulation and increased incorporation of FUTP into RNA were, however, associated with the synergistic cytotoxicity following sequential combinations of L-alanosine and FUra or FUrd.

The results of combining L-alanosine and FUra were anticipated. The primary antimetabolic effect of L-alanosine on *de novo* purine synthesis would result in increased PRPP levels [39]. The higher concentrations of PRPP, which are normally below the K_m of FUMP formation, would hasten the rate at which the orotate phosphoribosyltransferase would convert FUra to FUMP [26]. The fact that linear enhancement of FUra accumulation and metabolic conversion does not occur with increasing PRPP concentrations is a reflection that the K_m for FUMP formation is probably exceeded at the higher PRPP levels. Rescue of cells from L-alanosine effects by adenine corrobated the hypothesis that PRPP is important in metabolizing FUra.

The results with FdUrd were unforeseen. The enhancement of FUrd accumulation into nucleotide pools and into RNA, and the synergy of the combination, clearly demonstrated that L-alanosine can alter normal pyrimidine metabolism also. This enhancement did not occur via a conversion of FUrd to FUra. This would require pyrimidine nucleoside phosphorylase activity. Previous work in our laboratory has shown that there is very little pyrimidine phosphorylase activity in L1210 cell cultures [40]. Furthermore, the inability of adenine to rescue cells or to reduce accumulation of FUrd militated against the conversion of FUrd to FUra.

Pyrimidine ribonucleotide pool size changes result from many factors. Consumption of nucleotides can occur either by catabolism or by the synthesis of macromolecules. Net synthesis is a summation of contributions by *de novo* biosynthesis, salvage of nucleotides and aglycone pyrimidines, and rephosphorylation of the monophosphates created by nuclease digestion of RNA. The constancy of CTP and UTP pools is not necessarily a reflection of stable pools; the rate of flux into and out of these pools could by very dynamic. Intuitively, the increased PRPP levels, which enhance the rate of carbamoyl phosphate synthetase II [41–43], should result in greater pyrimidine ribonucleotide pools. Failure of L-alanosine to expand pyrimidine nucleotide pools might be explained if, in this context, L-alanosine acted as an analogue of L-aspartate, and if aspartate transcarbamoylase functioned at less than its normal rate through competitive inhibition from the drug. Part of the competition may be at the level of aspartate transport into the cells [37] which could result in a relative undersaturation of aspartate transcarbamoylase. L-Alanosine does exhibit greater inhibition of this enzyme in the presence of nonsaturating concentrations of aspartate [44]. L-Alanosine may be transcarbamoylated [43] to an analogue of carbamoylaspartate which could affect other enzymes of pyrimidine biosynthesis also. If L-alanosine does inhibit *de novo* pyrimidine biosynthesis, failure of L-alanosine to lower the pyrimidine nucleotide pools may result if salvage of pyrimidine nucleosides is increased or consumption of pyrimidine nucleotides is decreased. Heightened incorporation of FUrd, uridine, and cytidine after L-alanosine may be the result of compartmentalization of the total pools [45, 46]. In such a circumstance, nucleotides that enter the cell through the salvage pathways may enter directly into the small nuclear pool involved in RNA synthetis [47]. This would result in a greater proportional incorporation into RNA compared to the total intracellular pool which would be proportionately less. While these explanations are speculative, it is nonetheless clear that the effects of L-alanosine on the metabolism of FUrd were abrogated by L-aspartate and that its effects on the cytotoxicity were at least partially reversed by L-aspartate. It appears, therefore, that the mechanism by which these events occurred is different from that which modulated FUra metabolism.

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