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Unexpected Synergy Between *N*-Phosphonacetyl-L-aspartate and Cytidine Against Human Tumor Cells*

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Abstract—*Cytidine, a non-toxic endogenous nucleoside, was found unexpectedly to augment the cytotoxicity of a pyrimidine antimetabolite* N*-phosphonacetyl-*L*-aspartate (PALA) in human ovarian carcinoma cells. The PALA/cytidine synergy is confirmed here in other human tumor cells (T242 melanoma, HL60 promyelocytic leukemia and SKOV3 ovarian carcinoma) in the cytidine concentration range of* 1–10 *micromolar. The synergy was not observed in Chinese hamster ovary (CHO) cells. Exogenous uridine* (5–50 μM) *completely reversed the PALA/cytidine cytotoxicity in a concentration-dependent manner. Measurements of cellular ribonucleotide levels revealed that the PALA treated cells had reduced UTP and CTP pools* (10% *and* 40% *of control respectively); and the PALA/cytidine treated cells had elevated CTP and GTP levels while their UTP levels remained at* 10% *of control. Deoxyribonucleotide levels were unremarkable except for a slight elevation of dCTP in the PALA/cytidine treated cells. Uridine competitively inhibited radioactive cytidine transport into* 2008 *cells, which may explain its ability to antagonize the PALA/cytidine synergy. These results suggest that the ribonucleotide biosynthetic mechanism is the primary cellular target for PALA/cytidine activity, and that the ratio of ribonucleotides to each other is an important determinant of tumor cell viability. The use of non-cytotoxic nucleosides to augment the activity of antimetabolites may have clinical relevance in cancer therapy.*

INTRODUCTION

WE HAVE REPORTED that the membrane nucleoside transport inhibitor dipyridamole can dramatically augment the anti-tumor activity of the pyrimidine antimetabolite PALA [1, 2]. Other workers have documented similar augmentation using different antimetabolite/dipyridamole combinations in different tumor model systems [3–5]. In the human ovarian carcinoma cell line 2008, PALA potently inhibited the *de novo* synthesis of UTP and CTP, but was associated with modest cytotoxicity [2]. Our observations were in agreement with earlier studies reporting that the profound inhibition of pyrimidine biosynthesis by PALA did not result in appreciable anti-cancer activity in clinical trials. It has been demonstrated in cultured tumor cells that the presence of preformed nucleosides in the medium, specifically uridine in the case of PALA, allowed salvage metabolism to proceed and negated the effects of the *de novo* blockage [2, 6]. In experiments designed to screen the ability of various nucleosides to antagonize the cytotoxicity of PALA and PALA combined with dipyridamole, we observed that the presence of cytidine markedly potentiated the activity of PALA against 2008 cells. Under the same conditions, cytidine alone was completely non-cytotoxic even at millimolar concentrations [7]. We have since confirmed the PALA/cytidine synergism in other human tumor cell lines and have further characterized the underlying mechanisms in human ovarian tumor cells. The mechanism of synergy between cytidine and PALA seems to be related to disruptions of cellular nucleoside triphosphate (NTP) pools and the subsequent unbalanced growth resulting from the disruption.

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MATERIALS AND METHODS

Drugs and chemicals

All nucleosides and nucleotides were purchased from Sigma Chemical Company (St. Louis, MO). PALA.disodium was obtained from the Division of Cancer Treatment, National Cancer Institute

(Bethesda, MD) and all other chemicals were purchased from Fisher Scientific (Fairlawn, NJ). Tissue culture media (RPMI 1640 and DME) were purchased from Irvine Scientific (Santa Ana, CA), all bovine sera and other reagents for cell culture work were obtained from Gibco (Grand Island, NY). Cytidine (5-^{3}H) was purchased from New England Nuclear (Boston, MA).

Cell lines and culture conditions

The human tumor lines: 2008 (ovarian carcinoma), T242 (malignant melanoma) and HL60 (promyelocytic leukemia) were maintained in exponential growth in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% L-glutamine. SKOV3 (ovarian carcinoma) cells were maintained in DME medium supplemented with 10% newborn calf serum and 1% L-glutamine; and CHO (Chinese hamster ovary) cells were grown in DME medium supplemented with 5% newborn bovine serum and 1% each of non-essential amino acids and L-glutamine. All cell lines were incubated at 37°C under 5% CO_2 atmosphere in humidified incubators.

Cytotoxicity assays

The synergy between PALA and cytidine was tested in T242, HL60, SKOV3 tumor cells and CHO cells using a 72-h growth rate assay. Initial seeding densities of 1×10^4 cells per well of CHO, 2008 and SKOV3; and 2×10^4 cells per well of T242 and HL60 were plated onto Linbro type 24-well plates (Flow Lab., VA). Details of this assay have been published previously [2]. The concentrations of PALA used in these assays were 10, 100 and 1000 μM with or without cytidine (1, 10, 100 μM). Further characterization of the PALA plus cytidine cytotoxicity was performed on 2008 cells using clonogenic assays on plastic culture dishes. Briefly, exponentially dividing cells were harvested by trypsinization, washed and plated in triplicates onto 60 mm plastic culture dishes (Falcon Plastics, MD) at a cell density of 300 cells per plate. Varying concentrations of PALA and nucleosides (cytidine 5 μM, uridine 1–100 μM) were added to the plates with the control cultures receiving aliquots of normal saline. The plates were then incubated under 7% CO_2 at 37°C for 10–14 days. Cells were then washed once with phosphate buffered saline (PBS), fixed in methanol and stained with Giemsa stain. Each cluster of greater than 50 cells was counted as one colony, and the control plates usually have 100–120 colonies with less than 5% variation in triplicates. Nucleoside concentrations in the media were routinely monitored using a reverse-phase HPLC method developed in our laboratory [2, 8].

Cytidine uptake in 2008 cells

Since we have collected an abundance of biochemical data on 2008 cells, we decided to continue our mechanistic investigations using this cell line. Freshly harvested cells were suspended in 'uptake medium' consisting of normal growth medium supplemented with dialyzed serum in the presence and absence of uridine. Cytidine (5-^{3}H, 30 Ci/mM) was added to the cell suspensions to achieve a final concentration of 10 μM (10 μCi/ml) and the suspensions (5×10^6 cells in 2.0 ml) were incubated at 37°C with constant shaking. Aliquots of cells were removed at 1, 5, 10, 20 and 30 min and diluted into 10 volumes of chilled PBS and centrifuged at 4°C for 5 min at 1000 ***g***. The cell pellets were washed twice with 1 ml chilled PBS and then resuspended in 0.9 ml of 0.1 M NaOH. After 10 min of alkaline digestion on ice, a 500 μl aliquot from each sample was removed and added to 9.5 ml of Scintiverse BioHP (Fisher) and the radioactivity quantified in a Beckman liquid scintillation counter [2]. Typical loss of radioactive cytidine from the cells after loading was less than 2% per hour in chilled PBS and cells preincubated for 1 h in 50 μM PALA before the uptake experiments had cytidine uptake rates similar to untreated control cells.

Cytidine transport into 2008 cells was measured using a modified oil-stop method [9]. Briefly, 100 μl of the uptake medium containing radiolabeled cytidine was layered carefully onto 100 μl of an oil mixture of 9:1 silicon oil (Aldrich 17563-3):parafin oil (Fisher 0-119) in a 1.5 ml Eppendorf microcentrifuge tube. Transport measurements were initiated by the addition of 100 μl of cell suspension and the reactions were stopped at timed intervals between 0 and 60 s by pelleting the cells through the oil cushion at 12,000 ***g*** for 30 s. The oil was then aspirated and the cell pellet digested with 0.1 M NaOH and the radioactivity quantified using liquid scintillation counting. The amount of radioactivity in the cell pellet not associated with transport was estimated by pre-incubating cells in medium containing 10 μM dipyridamole and initiating the reaction with the transport inhibitor present.

Cellular ribonucleoside triphosphates (NTPs) measurement

Cells (2008 and CHO) were seeded into T-75 (Corning) flasks containing growth medium with predetermined concentrations of drugs and nucleosides and incubated from 1 to 24 h. They were then harvested and washed twice in fresh medium and an aliquot from each sample was removed for cell number determination on a Coulter ZF counter (Hialeah, FL). Perchloric acid (0.4 N) extraction of cells was carried out on ice for 10 min and the resultant precipitates were removed by centrifug-

ation. The supernatants were neutralized with 2.2 M potassium bicarbonate, and after a second centrifugation, measured volumes of the clear supernatants were injected into the HPLC for NTP analysis. The HPLC system consisted of the following Waters Associates equipment: two Model 6000A pumps, Model 660 solvent programmer, Model 440 dual-channel u.v. detector and Model 730 data module. The column used was a Waters SAX-10 μm radial compression cartridge in a Z-module®. Elution was effected using the following mobile phase: Buffer A, 0.1 M KH_2PO_4/0.1 M KCl, pH 4.0; and Buffer B, 0.25 M KH_2PO_4/0.5 M KCl, pH 5.0. The initial conditions were 80% A and 20% B, and a hyperbolic gradient (curve 4 of the controller) to 100% B was generated over 30 min at a flow rate of 2.0 ml/min (retention times: UTP, 19.0; CTP, 23.2; ATP, 30.0 and GTP 34.8 min). Nucleotide concentrations were extrapolated from A_{254} using standard curves constructed before the analysis run, and values were normalized to picomoles/10^6 cells. The identity of each nucleotide was confirmed by its 254/280 absorbance ratio and co-elution of cellular extract with nucleotide standards [2].

Measurements of deoxyribonucleoside triphosphates (dNTPs)

The levels of cellular dNTP after PALA and cytidine treatments were measured using a modified periodate/methylamine method first reported by Garrett and Santi [10]. Briefly the cells were harvested after being treated with the various combinations of drugs, extracted with perchloric acid and neutralized with potassium bicarbonate as outlined above. Fifty microliters of 0.5 M sodium periodate were added to 0.85 ml of neutralized cell extract and left at room temperature for 5 min. Then 50 μl of freshly prepared methylamine (pH adjusted to 7.5 with H_3PO_4) were added to each sample, vortexed vigorously, and the samples were incubated at 37°C for 30 min. Fifty microliters of 0.2 M rhamnose were added to each tube at the end of the reaction and the samples were immediately put on ice until analysis. The HPLC column used was a Waters SAX 10 μm radial compression cartridge in a Z-module, and an isocratic solvent of 0.35 M ammonium phosphate (pH 3.5) and acetonitrile at a ratio of 10:1 was employed at a flow rate of 2.5 ml/min to elute the dNTPs (retention times: TTP, 18.5 min; dCTP, 22.1 min; dATP, 31.7 min; dGTP, 42.6 min). Cellular dNTP levels were calculated from their A_{254} values and extrapolation from standard curves constructed under the same conditions. Values were normalized to picomoles per 10^7 cells. This procedure routinely removed 98% of the interfering NTPs and degraded less than 10% of the dNTPs of interest in the cell extracts.

RESULTS

Cytotoxicity of cytidine and PALA

Initial experiments documenting the augmentation of PALA cytotoxicity by cytidine were performed using 2008 ovarian carcinoma cells in a standard 72 hour growth rate assay [7]. The synergism between these two compounds against other human tumor cell lines was confirmed here using the same assay and the results are summarized in Table 1. PALA alone at 100 μM was only mildly effective in inhibiting the growth of all the cell lines tested. The inclusion of 10 μM cytidine, however, augmented the activity of PALA against all the human tumor cell lines but not the CHO cells. Cytidine alone was non-cytotoxic at concentrations up to 1 mM in all of the cell lines tested here. To further characterize the cytotoxicity of PALA plus cytidine, we evaluated the dose–response characteristics of these two compounds in clonogenic assays using the 2008 cells. Figure 1 summarizes the results of the clonogenic assays and depicts the dose–response relationships of these two compounds against the 2008 cells. PALA at 50 μM resulted in less than 30% reduction in clonal survival, while the addition of 5 μM of cytidine to the treatment regimen reduced the clonal survival by 99%.

Uridine antagonism of PALA and cytidine toxicity

The ability of uridine to antagonize the cytotoxic activity of PALA and PALA plus cytidine in a concentration-dependent fashion is illustrated in Fig. 2. The presence of 50 μM of exogenous uridine completely reversed the cytotoxicity of 50 μM PALA and PALA plus 5 μM cytidine. This entire dose–response curve for cytidine/PALA treated cells was shifted to the right when 10 μM of cytidine was used, suggesting competition between cytidine

Table 1. Growth inhibitory activities of PALA and PALA plus cytidine against human tumor cells in 72 h growth assays

Cell line	Percentage of control (mean ± S.E.M., $n = 4$)*	
	PALA (100 μM)	PALA (100 μM) + cytidine (10 μM)
1. 2008	82 ± 6	15 ± 4
2. T242	85 ± 8	33 ± 7
3. HL60	86 ± 5	51 ± 3
4. SKOV3	76 ± 7	53 ± 3
5. CHO	90 ± 5	86 ± 6

*Values are mean cell numbers normalized to percentage of control values (without drug treatments).

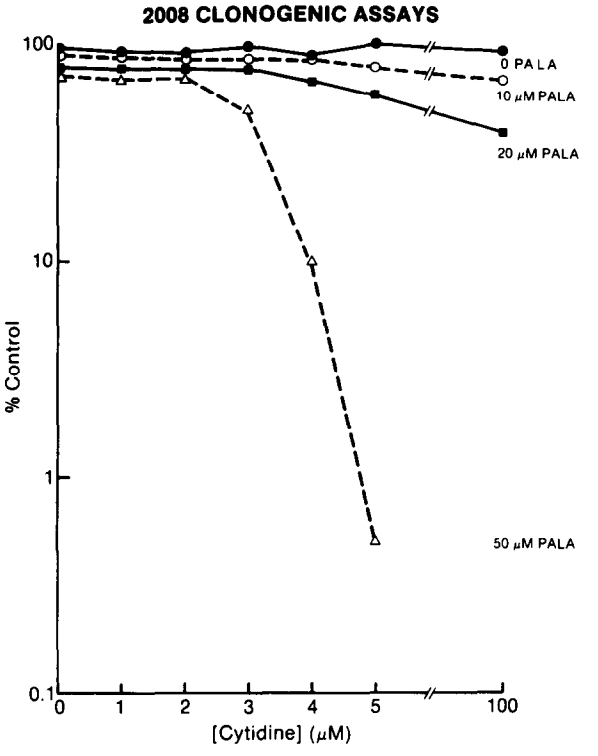

Fig. 1. Dose–response characteristics of 2008 clonal survival to different concentrations of PALA and cytidine. Only clusters of greater than 50 cells were counted as one colony. The control plates contained 122 ± 5 colonies. Each point represents the mean of five experiments, and the usual standard deviations were less than 5% above and below the mean values in all cases. The colony count for 10 μM PALA was 116, for 20 μM PALA was 103, and for 50 μM PALA was 90.

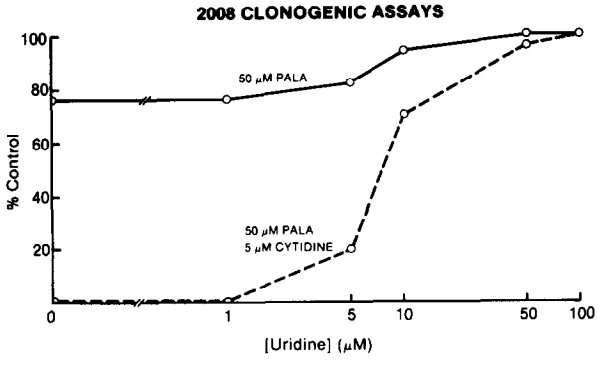

Fig. 2. Rescue of 2008 cells from PALA and PALA/cytidine cytotoxicity by uridine. Each point represents the mean of five experiments and the standard deviations were less than 5% above and below the mean values. The respective ED_{50} values for reversal of PALA and PALA plus cytidine cytotoxicity were the same (8 μM uridine). The control colony count was 116, for 50 μM PALA the count was 78, and for 50 μM PALA plus 5 μM cytidine was 0.

and uridine for cellular effector sites. The nature of such competition is currently being investigated.

Cellular NTP and dNTP levels

The levels of cellular NTPs and dNTPs in 2008 and CHO cells treated with PALA plus cytidine were measured using HPLC. Table 2 summarizes the NTP levels in cells subjected to various combinations of drug treatment. Cytidine and uridine at concentrations up to 1 mM did not alter the NTP levels in the 2008 cells as previously reported [2]. PALA at 50 μM reduced the UTP and CTP pools to 10% and 40% of control respectively, and elevated the GTP pool slightly to 105% of control. PALA plus cytidine specifically raised the CTP levels back to 108% of control and raised the GTP pool further to 129% of control while leaving the rest of the NTPs unchanged from those measured in cells exposed to PALA alone. These alterations in nucleotide levels were associated with pronounced cytotoxicity (Fig. 1). The addition of uridine to PALA plus cytidine treated cells elevated UTP back to 89% of control and reduced CTP to 90% of control, therefore normalizing the UTP to CTP ratio. The GTP pool was also reduced towards control values and these changes were associated with near normal growth in the 2008 cells. The UTP and CTP pools in CHO cells were comparably depressed as documented in the 2008 by PALA treatment alone, but the CTP pool did not rise in these cells after PALA plus cytidine treatment. It is important to note that cytidine did not augment PALA cytotoxicity in these cells (Table 1).

The dNTP pools in cells treated with the various combinations of drug and nucleosides are summarized in Table 3. There were minimal disruptions in the dNTP pools under the various treatments. In the PALA/cytidine treated cells, the dCTP levels were slightly elevated and the dATP levels were marginally depressed. Overall, the dNTP pools were less perturbed by PALA and PALA plus cytidine than the NTP pools at the drug concentrations used here.

Cytidine uptake and transport into 2008 cells

The data from the NTP and dNTP pools suggest that the availability of cytidine for salvage during PALA exposure specifically increased the cytidylate and deoxycytidylate pools in the 2008 cells. The kinetics of cytidine transport and uridine's influence on this process were studied to gain a better understanding of the mechanism by which uridine prevented the cytotoxicity of the PALA/cytidine combination. The first series of experiments measured total cellular radioactivity over a relatively long period, therefore the uptake rate represents cytidine transport and its subsequent metabolism within the 2008 cells. Cytidine uptake was linear for the first 60 min, but at a slower rate than in our previously reported study for uridine performed under the same conditions [2]. Table 4 summarizes the reduction of cytidine uptake into 2008 cells in the presence of varying concentrations of uridine. The intracellular radioactivity (cpm/10^6 cells) was normalized as percentage control value at the respective time-points when no uridine was present in the medium.

Table 2. Ribonucleoside triphosphate levels in 2008 cells treated with combinations of PALA and nucleosides

Treatment groups (n = 6)	Ribonucleotides*			
	UTP	CTP	ATP	GTP
1. Control	272 ± 55	63 ± 12	2198 ± 311	360 ± 33
2. Cytidine (100 μM)	265 ± 43	65 ± 15	2213 ± 292	354 ± 27
3. PALA (50 μM)	28 ± 10†	26 ± 11†	2133 ± 304	379 ± 42
4. PALA (50 μM)/cytidine (5 μM)	33 ± 9†	68 ± 14	1976 ± 287	467 ± 45†
5. PALA (50 μM)/cytidine (5 μM)/uridine (100 μM)‡	243 ± 61	57 ± 18	2205 ± 299	366 ± 39

*Ribonucleoside triphosphate levels in cells exposed for 24 h to the treatments as listed. Values are mean ± S.D. for six experiments, in picomoles/10^6 cells. Additional experiments with treatment periods up to 72 h were performed and the results were similar to the 24 h exposure.
†Significantly different from control values, $P < 0.05$, paired t test.
‡Treatment with 100 μM uridine results in NTP levels similar to control values.

Table 3. Deoxyribonucleoside triphosphate levels in 2008 cells treated with combinations of PALA and nucleosides

Treatment groups	Deoxyribonucleotides*			
	TTP	dCTP	dATP	dGTP
1. Control	38 ± 14	86 ± 15	41 ± 17	35 ± 11
2. PALA (50 μM)	35 ± 12	83 ± 10	36 ± 13	42 ± 12
3. PALA (50 μM)/cytidine (5 μM)	32 ± 10	102 ± 13†	35 ± 14	45 ± 10
4. PALA (50 μM)/cytidine (5 μM)/uridine (100 μM)	40 ± 11	85 ± 14	38 ± 12	39 ± 14

*Deoxyribonucleotide levels in cells treated for 24 h by groups. Values are mean ± S.E.M. of three experiments, in picomoles/10^7 cells.
†Significantly different from control values, $P \leq 0.05$, paired t test.

Table 4. The effects of uridine and dipyridamole on radioactive cytidine uptake into 2008 cells

Treatment (n = 4)	Percentage of control* (Mean ± S.E.M.)	
	10 min	30 min
Control	100	100
10 μM uridine	65 ± 7	70 ± 6
100 μM uridine	15 ± 8	18 ± 5
1000 μM uridine	9 ± 5	12 ± 7
1 μM dipyridamole	10 ± 4	12 ± 6

*Radioactivity (cpm/10^6 cells) was normalized to a percentage of control values at the respective time points. The control radioactivity uptake rate was calculated to be 1180 ± 210 cpm/million cells/min. Using a specific activity quoted by the supplier (30 Ci/mmole) and assuming a counting efficiency of 50% for tritium, the cytidine uptake rate is calculated to be 3 picomoles/million cells/min in the 2008 cells.

As shown in Table 4, 100 μM uridine inhibited radioactive cytidine uptake completely in the 2008 cells. Dipyridamole was included in our experiments to serve as a positive control because it is a potent and non-specific inhibitor of nucleoside uptake. Under the same conditions, cytidine uptake into CHO cells was less than 50% of the rate measured in 2008 cells.

The transport data from the oil-stop experiments during the first 60 s of radioactive cytidine exposure essentially paralleled the data from the uptake experiments of longer durations. The baseline transport rate was 3.23 ± 0.48 pmoles/min/10^6 2008 cells (n = 5), and uridine inhibited the transport in a concentration-dependent manner so that the transport rate of cytidine in the presence of 100 μM uridine was 0.28 ± 0.06 pmoles/min/10^6 cells (n = 5). The baseline cytidine transport rate for the CHO cells was 1.26 ± 0.54. These data confirm that uridine affects cytidine influx at the membrane transporter level and probably not at the subsequent metabolic conversions.

DISCUSSION

PALA is a specific and potent inhibitor of *de novo* pyrimidine synthesis and it has been shown to inhibit the growth of mammalian cells in culture [11–13]. The *in vitro* cytotoxicity of this drug, however, did not translate into meaningful clinical antitumor activity [14, 15]. We and others have reported that tumor cells can salvage uridine from their environment to circumvent the blockage of *de novo* pyrimidine synthesis by PALA [2, 6]. Data are presented here to support a novel observation that

cytidine, a non-cytotoxic nucleoside, can enhance the *in vitro* activity of PALA in several human tumor cell lines, although the degree of augmentation varied from one tumor type to another. CHO cells were the only phenotype resistant to cytidine augmentation within the concentrations of drugs tested. While these cells may not be 'normal', they nonetheless served the role of being a non-malignant cell type in our assays. Their resistance to PALA plus cytidine may be related to their reduced cytidine uptake since this drug combination failed to increase the depressed CTP pool toward control levels in these cells. There is evidence in the literature suggesting that non-malignant cells tend to have lower salvage activity when compared to tumor cells [16].

Although the cytotoxicity of PALA plus cytidine led us to expect profound disruptions in the NTP pools, the only significant difference between the PALA treated and the PALA plus cytidine treated cells was the elevation of the CTP pool to control levels while the UTP pool remained depressed (Table 2). This observation is surprising since one expects that the cytidine/deoxycytidine deaminases in the cells would convert cytidine into uridine and replenish the UTP pool. There is some evidence that only low levels of this enzyme activity are present in solid tumor cells [17], however, and we are currently measuring deaminase activities in viable 2008 cells in the absence and presence of PALA. Although the PALA plus cytidine treated cells also had elevated GTP pools when compared to control cells, the difference in GTP pools between the PALA treated cells and the PALA plus cytidine treated cells was not statistically significant. It has been reported that 3–5-fold elevations in the GTP pool were associated with inhibition of proliferation in lymphoid cells [18]. The ratio of one NTP to another seems to be critical for cell proliferation, since PALA alone induced drastic drops in the UTP and CTP levels (without disrupting their ratio) and was associated with only mild cytotoxicity. A slight elevation of the dCTP pool was the only notable difference in the dNTP pools of the PALA/cytidine treated cells compared to those treated with PALA alone. Therefore our data also suggest ribonucleotide metabolism as a cellular target/effector for PALA/cytidine cytotoxicity. It has been proposed that 'unbalanced growth' resulting from the specific blockade of one of the cellular intermediary metabolic pathways without affecting others can result in cell death [19–22]. Our data are supportive of this phenomenon being responsible for the PALA plus cytidine toxicity.

Uridine reversed the PALA/cytidine cytotoxicity and normalized the cellular NTP pools, especially their relative ratios (Table 2). Taken together with the observation that uridine also inhibited radioactive cytidine transport into 2008 cells, our results suggest that uridine may be simply competing with cytidine for the membrane nucleoside transporter, thereby excluding cytidine from the cell. Jarvis *et al.* [23] and Plagemann and Wohlheuter [24] have reported that cytidine has a 10-fold lower affinity for the transporter than uridine, which may explain the ability of uridine to exclude cytidine from the cells so readily.

If preclinical trials confirm the tumoricidal activity of PALA in combination with cytidine in animal tumor models, new therapeutic regimens such as localized infusion of cytidine or co-administering a cytidine deaminase inhibitor (tetrahydrouridine) to elevate cytidine levels [25] during PALA therapy may improve tumor response rates. The use of non-cytotoxic nucleosides to augment the activity of other antimetabolites can open up new areas of research in cancer pharmacology. There are preliminary data showing that cytidine (100 μM) is synergistic with pyrazofurin in killing human lymphoma cells [26]. The utility of this type of cytotoxicity modulation awaits confirmation from preclinical screening in animal tumor models.

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