Kinetics of N-(Phosphonacetyl)-L-aspartate and Pyrazofurin Depletion of Pyrimidine Ribonucleotide and Deoxyribonucleotide Pools and Their Relationship to Nucleic Acid Synthesis in Intact and Permeabilized Cells

James D. Moyer, Patricia A. Smith, Emily J. Levy, and Robert E. Handschumacher

Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510

ABSTRACT

Pools of uridine triphosphate and cytidine triphosphate are greatly (90%) reduced in cultured L1210 cells exposed to N-(phosphonacetyl)-L-aspartate (PALA) or pyrazofurin; the concentration of the deoxynucleotides deoxycytidine triphosphate, deoxythymidine triphosphate, and deoxyguanosine triphosphate also decreases, but deoxyadenosine triphosphate pools are enlarged. Associated with these pool depletions is a pronounced inhibition of DNA synthesis even when pools are only moderately reduced; RNA synthesis is only slightly inhibited under these same conditions. DNA synthesis in permeabilized preparations of L1210 cells was also more sensitive than was RNA synthesis when the concentrations of ribonucleotide and deoxyribonucleotide triphosphates presented were equivalent to those found in PALA- or pyrazofurin-treated cells. The specific sensitivity to depletion of DNA precursors was also seen in protection of both DNA synthesis and growth of L1210 cells by deoxycytidine and thymidine. This supplement restored deoxycytidine triphosphate, deoxythymidine triphosphate, and deoxyguanosine triphosphate pools to normal but of course did not affect the marked depletions of uridine triphosphate and cytidine triphosphate or the less marked effect of PALA on RNA synthesis.

The relative ability of PALA to reduce uridine triphosphate and cytidine triphosphate pool size in L1210 ascites and Lewis lung carcinoma in vivo correlates with the intrinsic sensitivity to this agent.

INTRODUCTION

The aspartate transcarbamylase inhibitor PALA is effective as an antineoplastic agent in several murine tumor systems (10) and has produced occasional objective responses in patients (6, 14). Several ongoing clinical studies are examining the use of PALA in combination with other drugs (2, 5).

Initial studies indicated that PALA strongly but incompletely inhibited aspartate transcarbamylase in vitro and depleted pyrimidine ribonucleotide pools in tumors (13, 18). This depletion has been considered to be essential for effective antitumor activity, since administration of uridine to restore these pools abolishes the antitumor effects (9). However, such depletions may not be sufficient for antitumor effectiveness, since UTP and CTP pools in resistant variants of Lewis lung tumors are reduced to almost the same degree after PALA treatment as is seen with the sensitive parent tumor (13). Although the reduced availability of pyrimidine nucleotides could possibly limit cell replication simply by decreasing the rates of nucleic acid synthesis, other processes may also be affected (i.e., pyrimidine nucleotide-sugar and CDP-choline reactions). The present study attempts to correlate the effects of PALA on ribonucleotide and deoxynucleotide pools and the consequent alterations in nucleic acid synthesis in intact and permeabilized cells. In addition, it demonstrates that in vivo the pyrimidine nucleotide pools in the sensitive Lewis lung tumor are reduced at doses of PALA that only slightly perturb these pools in the resistant L1210 tumor.

MATERIALS AND METHODS

Measurement of Nucleotide Pools. [8-3H]dATP (7 to 9 Ci/mmol), [methyl-3H]dTTP (18 to 22 Ci/mmol), [8-3H]dGTP (13 to 14 Ci/mmol), and [5-3H]dCTP (21 Ci/mmol) were obtained from Becton-Dickinson Immunodiagnostics, Orangeburg, N. Y. Unlabeled deoxynucleotides were purchased from P-L Biochemicals, Inc., Milwaukee, Wis., and Sigma Chemical Co., St. Louis, Mo. Stock solutions of deoxynucleotides were prepared in 0.1 M Tris-HCl (pH 7.5) every 6 to 8 weeks. Concentrations were determined from UV spectrophotometric measurements, and the purity of the deoxynucleotides was checked on high-pressure liquid chromatography. Lyophilized DNA polymerase I (EC 2.7.7.7) from Escherichia coli with a specific activity of 5000 units/mg protein was obtained from Worthington Biochemical Corp., Freehold, N. J. It was reconstituted in 0.1 M potassium phosphate buffer (pH 7.0) containing 1 mg bovine serum albumin (Fraction V powder) per ml from Miles Laboratories, Kankakee, III. Micrococcus lysodeikticus DNA polymerase I (specific activity, 524 units/mg protein) was purchased from Miles Laboratories, Inc., Elkhart, Ind. A solution of 0.5 M Tris-HCl (pH 7.5), 0.01 M 2-mercaptoethanol, and 1 mg bovine serum albumin per ml was used as the enzyme diluent. Freeze-dried poly(dA-dT), sodium salt, and freeze-dried poly(dC-dC), double-stranded sodium salt, were obtained from Miles Laboratories, Inc., and were each dissolved in 0.1 M NaCl (pH 7.0). All of the above materials were stored at –20°.

Deoxyribonucleoside triphosphates were determined by a modification of previously reported methods (15, 22). The assay was performed in 96-well plates (Linbro Division, Flow Laboratories, Inc., Hamden, Conn.) in a final volume of 0.1 ml, consisting of 60 μl of sample or limiting deoxynucleotide and 40 μl of reaction mixture. To determine dATP and dTTP, each well contained: 5.6 μmol glycine-NaOH (pH 9.2); 1.5 μg poly(dA-dT); 0.14 units DNA polymerase I (E. coli); 0.57 μmol...
J. D. Mayer et al.

**RESULTS**

**Effect of Nucleotide Pool Depletions on RNA and DNA Synthesis.** The effects of inhibition of pyrimidine biosynthesis de novo on the ribonucleotide and deoxyribonucleotide pools as well as the incorporation of radiolabeled precursors into RNA and DNA were measured in L1210 cultures exposed to PALA. Exposure of cells to 1 mM PALA for 5 hr decreased the pools of UTP and CTP to 10% of control values, while ATP increased slightly, and GTP was essentially unchanged (Chart 1). Simultaneous measurement of dNTPs revealed a decrease of 70 to 80% in dCTP, dTTP, and dGTP, while dATP increased almost 2-fold.

The effect of these pool changes on nucleic acid synthesis was assessed using guanosine incorporation as a measure of synthesis, since the changes in the pool of GTP were negligible. After 5-hr exposure to PALA, DNA synthesis was 80% inhibited as measured by pulse labeling with [3H]guanosine, while RNA synthesis appeared to be only slightly (20%) inhibited at this time despite the greatly reduced pools of pyrimidine ribonucleoside triphosphates (Chart 1).

To examine the relationship of pool depletions to nucleic acid synthesis, parallel experiments were performed utilizing PF as the inhibitor of pyrimidine synthesis de novo. PF at a concentration of 2 μM depletes UTP and CTP pools of L1210 cells more rapidly than does 1 mM PALA (Chart 2). The depletion of dCTP, dTTP, and dGTP pools is also more rapid and extreme than those depletions produced by PALA. Under these conditions, the incorporation of [3H]guanosine into DNA again decreased in parallel with pools, whereas incorporation of label into RNA was less sensitive (40% of the control rate) despite a 97% reduction in pyrimidine ribonucleotides. Thus, the effects of PF on nucleic acid synthesis are similar to those of PALA, but PF is about 100-fold more potent in its ability to deplete nucleotide pools in these cells. Similarly, the growth of L1210 cells in culture is very much more sensitive to PF (IC50 = 0.1 μM) than to PALA (IC50 = 120 μM).

Since any measurement of macromolecular synthesis based on the incorporation of a labeled precursor would incorrectly imply a decreased rate of nucleic acid synthesis if drug treatment caused a decrease in the specific activity of the precursor pool, the uptake of [3H]guanosine into acid-soluble nucleotides was determined. The specific activity of GTP pools in treated cells was found to be approximately equal to that of controls (150 cpm/μmol) despite the slight increase in GTP pool size. Therefore, the apparent inhibition of RNA and DNA synthesis cannot be explained as a decrease in the specific activity of the precursor pool. As a second measure of nucleic acid
Nucleotide Depletion and DNA Inhibition by PALA

Chart 1. Effect of PALA on nucleotide pools and nucleic acid synthesis. L1210 cultures of 150 ml (250,000 cells/ml) were exposed to PALA at 1 mM. At the indicated intervals, 40 ml of the culture were removed for determination of nucleotide pools, and 10 ml were used for determination of incorporation of \(^{3}H\)guanosine into RNA and DNA as described in "Materials and Methods." A, incorporation into nucleic acids. Control values of incorporation into RNA, 400 \(\times 10^{3}\) cpm/10\(^6\) cells, and DNA, 35 \(\times 10^{3}\) cpm/10\(^6\) cells, were observed. B, ribonucleotide pools. The ribonucleotide control values were: CTP, 0.8 nmol/10\(^6\) cells; UTP, 2.0 nmol/10\(^6\) cells; ATP, 5.9 nmol/10\(^6\) cells; and GTP, 1.3 nmol/10\(^6\) cells. C, deoxyribonucleotide pools. The dNTP control values were: dCTP, 51 pmol/10\(^6\) cells; dTTP, 52 pmol/10\(^6\) cells; dATP, 36 pmol/10\(^6\) cells; and dGTP, 25 pmol/10\(^6\) cells. Cell size was determined by a Coulter Channelizer calibrated with 10-\(\mu\)m-diameter polystyrene beads, and mean cell volume was 1.3 \(\mu\)l.

Chart 2. Effect of PF on nucleotide pools and nucleic acid synthesis. L1210 cells at 250,000 cells/ml exposed to PF at 2 \(\mu\)M and nucleotide pools and incorporation of \(^{3}H\)guanosine into DNA and RNA were determined as described in "Materials and Methods." Control values are given in Chart 1. A, incorporation into nucleic acids; B, ribonucleotide pools; C, deoxyribonucleotide pools. The results of 2 experiments are averaged for measurements of \(^{3}H\)guanosine incorporation, and 3 experiments are averaged for the pool measurements.

synthesis, \([8-\text{H}]\)adenosine was substituted for \([8-\text{H}]\)guanosine in the pulse. The percentage of inhibition of RNA and DNA synthesis indicated by this method was similar to that found in experiments using \(^{3}H\)guanosine as precursor. For example, RNA and DNA syntheses were inhibited 32 and 72\% at 3 hr and 54 and 89\% at 5 hr after exposure to 1 mM PALA, a result comparable to that with \(^{3}H\)guanosine (Chart 1). By contrast, incorporation of [methyl-\(^{3}H\)]thymidine into DNA was only slightly inhibited (10\%) at 5 hr. In this case, the decreased size of the dTTP pool presumably masks the actual effect on DNA synthesis.

Effect of Deoxynucleosides on Inhibition of Cell Nucleic Acid Synthesis and Replication by PALA and PF. Since inhibition of DNA synthesis might be attributed to the decreased availability of CDP and UDP for conversion to deoxyribonucleotides and the resulting decreased dNTP pools, this possibility was examined by measuring pool depletion and nucleic acid synthesis in the presence of added deoxycytidine and thymidine (Chart 3). As expected, deoxycytidine and thymidine could not prevent the rapid ribonucleotide depletion of the UTP and CTP pools by PALA (1 mM). The limited inhibition of RNA synthesis was also similar to that seen in the absence of deoxynucleotides. DNA synthesis, however, was dramatically restored. Similar reversal of the inhibition of DNA synthesis without reversal of the inhibition of RNA synthesis was observed in cells treated with PF (2 \(\mu\)M) in medium containing 10
μM deoxycytidine and thymidine. The depletions of dGTP and dTTP pools were completely reversed, and dGTP pools were restored to even greater than control values by the combination of deoxycytidine and thymidine in cells treated with PALA (Table 1).

It has been reported previously that uridine completely reverses the growth-inhibitory effects of PALA or PF (9, 10) in cell culture. Since DNA synthesis is more sensitive than RNA synthesis, the possibility of protecting cells from growth inhibition by thymidine and deoxycytidine was examined (Chart 4). A combination of these deoxynucleosides protected the cells from the growth-inhibitory effects of both PALA and PF at lower drug concentrations, but a parallel response was seen with a 7-fold-greater concentration of the inhibitors. It should be noted that uridine (50 μM) could protect cells from much greater concentrations of PF or PALA.

The protection afforded by deoxycytidine and thymidine reinforces the hypothesis that reduction in DNA synthesis is the primary limitation on replication for cells grown in the presence of PALA or PF. At higher concentrations of drug, however, inhibition of RNA synthesis may become growth limiting.

Effect of Pool Changes on Nucleic Acid Synthesis in Permeabilized Cells. The lack of sensitivity to RNA synthesis to pretreatment of cells with PF was also examined by measuring the incorporation of ribonucleotide triphosphates into RNA by permeabilized cells with concentrations of the triphosphates that mimic those found in control and PF-treated cells. In these experiments, there was no difference in the rate of RNA synthesis (25 pmol/10⁶ cells/10 min) at control ribonucleotide triphosphate concentrations versus those found in PF-treated cells when compared to the cells observed in the presence of an excess of the 4 ribonucleotide triphosphates (200 μM). However, in this system, the complete elimination of CTP and dTTP from the incubation mixture only inhibited incorporation of GTP to approximately 40% of that of the maximum rate.

In similar experiments, the rate of DNA synthesis was determined using [³H]dTTP. With excess concentrations (200 μM) of each dNTP, synthesis was between 20 and 40% of that calculated for exponentially growing cells with a generation time of approximately 12 hr. When the concentrations of dNTPs were reduced to those found in cells from control cultures, there was a modest reduction in the rate of incorporation of dNTPs into DNA (Chart 5). In permeabilized cells incubated with the concentrations of dNTPs present in cells 5 hr after exposure to 2 μM PF, the rate of DNA synthesis was approximately 40% of that in the presence of normal cellular dNTP concentrations. Although this degree of inhibition of DNA synthesis by limiting dNTPs could have profound effects on cell growth, it is not quantitatively as great as that seen in whole cells by precursor incorporation. Since this inhibition of cell growth also may have reflected an effect on the cellular apparatus responsible for synthesis of DNA, cells were pretreated...
with PF (2 μM) for 4 hr before permeabilization. However, the rates of DNA synthesis in these preparations were identical with those seen with control cells.

Effects of PALA on Nucleotide Pools in Vivo. The effect of high doses of PALA (400 to 500 mg/kg) on the total pyrimidine nucleotide content of Lewis lung tumors has been reported earlier by this group and others (13, 18). The current study was performed to compare the pool reductions seen in vivo to those seen in cultured cells and to examine the effects at a low dose that inhibits the naturally sensitive Lewis lung tumor. These data are also compared to experiments with the naturally resistant L1210 tumor. The results are presented in Tables 2 and 3.

The values in Table 2 should be used only for comparison of treated tumors to controls rather than as absolute in situ concentrations, since in the process of removing and freezing the tumor in liquid nitrogen, some conversion of tri- to diphosphate probably occurs after blood supply is interrupted. In these experiments, the ratios of ATP to ADP in Lewis lung tumors determined by high-pressure liquid chromatography (18) were from 1.7 to 2.5 but were not different in treated versus controls in 4 pairs examined. Since the ratio in most normal tissues is often greater than 10, the values reported in this table may underestimate the absolute physiological concentrations. Comparison of the concentration in treated and control Lewis lung tumors reveals that PALA at 25 or 250 mg/kg produces large decreases in UTP and CTP concentrations within 3 hr without depleting ATP or GTP pools and that the changes persist for at least 48 hr. Thus, the effect on UTP and CTP pools in vivo is qualitatively and quantitatively similar to the effect observed in cultured cells (18).

Although L1210 cells grown as an ascites had depleted UTP and CTP pools after administration of PALA, the rate of decrease and total reductions were smaller than those observed in Lewis lung cells (Table 3). It was not feasible to extend these studies to 48 hr since control mice were dying of leukemia by this time. The ratio of ATP to ADP (3.5 to 1) of these treated and control cells was higher than that of Lewis lung tumors, possibly because as ascites tumors they can be rapidly chilled on harvesting and are less dependent on vascularization for supply of oxygen. The relative resistance of L1210 ascites cells to pool depletions produced by PALA is particularly notable, since the drug was injected directly into the fluid surrounding the tumor in this case.

Among normal tissues examined, the small intestine was the only tissue that had significant (p < 0.02) decreases in CTP and UTP pools 24 hr after 1000 mg PALA per kg. CTP decreased from 0.07 ± 0.008 (S.D.) to 0.04 ± 0.015 μmol/g, and UTP decreased from 0.17 ± 0.03 (S.D.) to 0.08 ± 0.04 μmol/g. We have reported previously that total pyrimidine nucleotide pools of liver and spleen were not depleted after administration of PALA (18).

**DISCUSSION**

Both PALA and PF produce marked depletions of pyrimidine ribonucleoside and deoxyribonucleoside triphosphates in cultured cells as might be expected for such potent inhibitors of
pyrimidine synthesis de novo. The simultaneous and essentially equal reduction in dGTP pools following exposure was unexpected. This decrease is probably an indirect effect of these drugs attributable to the reduction in dTTP pools, since dTTP is a necessary activator for the reduction of GDP (17). Furthermore, when dTTP pools are elevated by administering thymidine, dGTP pools increase (7). The restoration of dGTP concentrations when thymidine and deoxycytidine are provided to drug-treated cells is also consistent with this explanation (Table 1). Since dGTP is present in cells at the lowest concentration of all the dNTPs, its depletion may be the rate-limiting factor in DNA synthesis. The failure of deoxycytidine to restore CTP pools is consistent with the inability of this cell line to cleave pyrimidine deoxynucleosides.

The greater susceptibility of DNA synthesis to inhibition by PF or PALA (Charts 1 and 2), together with the substantial but incomplete protection afforded cells by thymidine and deoxycytidine (Chart 4), suggests that inhibition of DNA synthesis subsequent to dNTP pool reductions may be the critical lesion produced by both PF and PALA. This inhibition of DNA synthesis probably results from decreased substrate (dNTP) concentrations, since restoration of these pools removes the inhibition (Chart 3). Plagemann and Behrens (21) had also observed a restoration of DNA synthesis in PF-treated hepactoma cells when deoxynucleosides were supplied, although pool sizes were not examined. The intracellular concentrations of dCTP, dTTP, and dGTP for L1210 cells can be calculated as 42, 42, and 19 µM, respectively, from the data of Chart 1. For comparison, the $K_m$ of DNA polymerase for dNTPs has been estimated at 2 to 30 µM in experiments with isolated enzymes (3, 23, 26).

The permeabilized cell system permits direct manipulation of nucleotide concentrations in a model approximating the intact cell and thus provides a new approach for determining the substrate concentrations required for nucleic acid synthesis. Although a $K_m$ of 50 µM was reported for permeabilized baby hamster kidney cells (1), experiments in this laboratory suggest the value may be much lower for individual dNTPs when physiologic concentrations of the other precursors are present in permeabilized Lewis lung cells. In this same system, complete elimination of one or two dNTPs reduced DNA synthesis to 16 and 9% of control values. Thus, the rate of DNA synthesis should be sensitive to decreased dNTP concentrations over the range reported here, but the whole-cell data suggest a greater sensitivity than that seen in the permeabilized cells. It is also possible that the increased dATP pools may contribute to the inhibition by generating an imbalance in the dNTP pools or possibly by interaction with a putative regulatory protein (24). Further studies are necessary to determine if decreases in all 3 dNTPs contribute to the inhibition or if only one of these is limiting for DNA synthesis in treated cells.

Some inhibition of RNA synthesis occurs in treated cells, although the reduced UTP and CTP concentrations remain well above the $K_m$ concentrations reported for RNA polymerase (13 to 20 µM, Ref. 25), and no significant inhibition was seen in permeabilized cells. Possibly, conditions in the intact cells generate a higher apparent $K_m$, or the measured pools may not reflect the concentration available to the polymerase because of pool compartmentation that may be lost in permeabilized cells. In addition, measurement of incorporation of nucleosides into total RNA gives a combined rate of synthesis of 3 classes of RNA, each synthesized by a separate polymerase. Each polymerase may have a different substrate concentration dependence, and thus, a different extent of inhibition may be seen for each class of RNA.

PALA seems to be remarkably tumor and possibly tissue specific in its ability to reduce pyrimidine pools in vivo, as evidenced by Tables 2 and 3 as well as in our earlier report (18). Particularly notable is the markedly greater effect of PALA on the Lewis lung tumor compared to L1210 leukemia. This is even more remarkable because the drug is injected directly into the ascites tumor site. This differential response of the nucleotide pools is consistent with the difference in antitumor activity reported by Johnson et al. (10). The extent of UTP and CTP pool depletion seen may be related to levels of aspartate transcarbamylase, which are lower in Lewis lung than in L1210 (11). However, these differences may also reflect differences in the extent to which salvage can compensate for reduced de novo synthesis. Although uridine and cytidine are present only at micromolar concentrations in murine or human plasma (5, 12, 19), a very rapid exchange of uridine between blood and tissues has been demonstrated recently (19). Furthermore, deoxycytidine was shown recently to be present in ascitic fluid of L1210-bearing mice at a relatively high concentration (20 µM, Ref. 4). Salvage of this nucleoside may therefore be a factor in the resistance of L1210 and other tumors to PALA. Effective inhibitors of the salvage process would be invaluable to permit definition of the in vivo role of circulating nucleosides.

REFERENCES


Kinetics of N-(Phosphonacetyl)-l-aspartate and Pyrazofurin Depletion of Pyrimidine Ribonucleotide and Deoxyribonucleotide Pools and Their Relationship to Nucleic Acid Synthesis in Intact and Permeabilized Cells

James D. Moyer, Patricia A. Smith, Emily J. Levy, et al.


Updated version  Access the most recent version of this article at:  
http://cancerres.aacrjournals.org/content/42/11/4525

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, use this link  
http://cancerres.aacrjournals.org/content/42/11/4525.  
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.