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ANTITUMOR ACTIVITY OF *N*-PHOSPHONACETYL-L-ASPARTIC ACID IN COMBINATION WITH NITROBENZYLTHIOINOSINE*

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Abstract—*N*-Phosphonacetyl-L-aspartic acid (PALA) resistance may be due to the ability of tumor cells to utilize preformed circulating pyrimidine nucleosides, thereby overcoming the block of *de novo* pyrimidine biosynthesis which PALA causes. To test this hypothesis we examined the effects of PALA and nitrobenzylthioinosine (NBMPR) alone and in combination on B16 melanoma cells *in vitro* using a clonogenic assay and *in vivo* using growth delay. In medium containing purine and pyrimidine nucleosides at a final concentration of 28 μM, exposure to PALA (100 μM) alone or to NBMPR (10 μM) alone for periods up to 72 hr did not result in any cytotoxicity. However, exposures to PALA (100 μM) plus NBMPR (10 μM) resulted in a decrease in clonogenic survival to 0.011 at 72 hr. In medium without nucleosides, PALA (100 μM) exposure for 72 hr caused a similar decrease in survival to 0.015, whereas NBMPR (10 μM) had no effect on survival. The addition of uridine resulted in a concentration-dependent reversal of the cytotoxic effects of PALA. C57 Bl female mice bearing B16 melanoma were treated intraperitoneally daily for 4 days with PALA, the phosphate of NBMPR (NBMPR-P), or PALA plus NBMPR-P. PALA, 300 mg/kg daily × 4, resulted in a 6-day tumor growth delay but NBMPR-P, 100 mg/kg daily × 4, had no effect. PALA, 150 mg/kg daily × 4, plus NBMPR, 50 or 100 mg/kg daily × 4, resulted in a 6-day tumor growth delay also. These studies demonstrate that: (1) circulating pyrimidine nucleosides are determinants of the cytotoxic effects of PALA; (2) *in vitro* PALA and NBMPR combine to cause significant cytotoxicity whereas either agent alone has no effect; (3) *in vivo* the combination of PALA and NBMPR-P results in the same antitumor affect as PALA alone at twice the dose; and (4) due to an increase in animal toxicity, no therapeutic advantage could be demonstrated for the combination over PALA alone *in vivo*. We conclude that the cytotoxic effect of PALA is modulated by the levels of the preformed circulating nucleosides and that combining PALA with an inhibitor of salvage pyrimidine uptake would not increase the therapeutic efficacy of PALA because of an increase in toxicity.

Pyrimidine nucleotides are necessary for DNA synthesis, and cells are capable of maintaining intracellular levels of pyrimidine nucleotides by three mechanisms: (1) *de novo* synthesis, (2) salvage synthesis, and (3) intracellular interconversion [1]. *De novo* synthesis utilizes the basic building blocks of glutamine and carbon dioxide and consists of six enzymes, some of which are regulated through feedback inhibition by intracellular pyrimidine nucleotide pools. The salvage pathway utilizes preformed circulating pyrimidine nucleosides and converts them to nucleotide monophosphates. Intracellular interconversion of pyrimidine nucleotides enables cells to convert uridine triphosphate to cytidine triphosphate. This source of pyrimidine nucleotides is limited by the overall supply of total intracellular pyrimidines and becomes important if *de novo* and salvage synthesis are inhibited completely. Studies of antitumor drugs which inhibit pyrimidine nucleotide biosynthesis must take these compensatory mechanisms into account.

N-Phosphonacetyl-L-aspartic acid (PALA‡) was synthesized by Collins and Stark [2] as a tight-binding inhibitor of aspartate transcarbamylase, the second enzyme in *de novo* pyrimidine biosynthesis [3]. PALA was found to be active against several experimental murine tumors including B16 melanoma and Lewis lung carcinoma but inactive against L1210 leukemia and P388 leukemia [4]. Resistance to PALA in experimental systems correlates with intracellular levels of aspartate transcarbamylase [5]. Resistant mutants have been derived which have markedly increased levels of aspartate transcarbamylase associated with amplification of the genes which encode this enzyme [6]. The administration of uridine may rescue tumors from the

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‡ Abbreviations: PALA, *N*-phosphonacetyl-L-aspartic acid; NBMPR, nitrobenzylthioinosine; NBMPR-P, nitrobenzylthioinosine-phosphate; PBS, phosphate-buffered saline; α-MEM⁺, alpha minimal essential medium containing uridine, cytidine, adenosine, guanosine, deoxyadenosine, deoxycytidine, deoxyguanosine and deoxythymidine, each at a final concentration of 28 μM; and α-MEM⁻, alpha minimal essential medium containing no nucleosides.

cytocidal effects of PALA [7] by replenishment of the PALA-depleted intracellular pyrimidine nucleotide pools via salvage pathways.

Pyrimidine and purine nucleosides may be found circulating in plasma and are believed to be derived from normal tissue breakdown and hepatic synthesis. Levels of uridine may be in the range which would enable cells to adequately salvage pyrimidine biosynthesis should *de novo* biosynthesis be inhibited [8]. An effective inhibitor of pyrimidine salvage biosynthesis has not been found, but several agents exist which are effective inhibitors of the facilitated diffusion process of nucleoside transport across cell membranes [9]. Among these, nitrobenzylthionosine (NBMPR) appears to be the most specific, binding tightly (K_D = 1 nM) to cell membrane receptors in those cell lines examined [10]. The uptake of both purine and pyrimidine nucleoside is effectively blocked by this agent. A major drawback of NBMPR is its limited solubility in aqueous solutions (approximately 50 μM), making *in vivo* administration difficult. However, the phosphate form of this drug, NBMPR-P, has significantly greater solubility and acts as a prodrug of NBMPR. NBMPR is released *in vivo* and inhibits nucleoside uptake.

PALA has undergone clinical trials in the treatment of patients with various malignancies but was found to be ineffective. Whereas the drug significantly inhibited aspartate transcarbamylase activity in many tissues of patients receiving it, no antitumor effect was demonstrated [11]. We hypothesized that such resistance might be due to the ability of tumor cells to circumvent the block induced by PALA by utilizing preformed circulating pyrimidine nucleosides normally found in human plasma. If this were so, then limiting access to the preformed pyrimidine nucleosides should increase PALA cytotoxicity. To test this hypothesis, we examined the effects of PALA and NBMPR alone and in combination in the treatment of B16 melanoma cells *in vitro* and *in vivo*.

MATERIALS AND METHODS

Chemicals. PALA was obtained from Dr. V. L. Narayanan, Drug Synthesis and Chemistry Branch, N.C.I. (Bethesda, MD). NBMPR and NBMPR-P were provided by Dr. A. R. P. Paterson, McEachern Laboratory (Edmonton, Alberta). Uridine and cytidine were purchased from the Sigma Chemical Co. (St. Louis, MO.). All drugs for *in vitro* studies were prepared in Dulbecco solution A (PBS). Drugs for *in vivo* studies were prepared in 0.15 M saline. Because of the limited solubility of NBMPR, excess drug was added to PBS and agitated at 37° for 18 hr prior to use. The concentration of dissolved drug was determined spectrophotometrically [12].

Cell cultures. The B16-F1 melanoma cell line was obtained from Dr. I. Fidler, Frederick Cancer Research Center (Frederick, MD) [13]. The cells were maintained in α-MEM [14] supplemented with 10% fetal calf serum (v/v) (Flow Laboratories Inc., Rockville, MD). For clonogenic studies cultures were initiated in plastic flasks (Costar, Cambridge, MA) at an initial cell density of 5×10^3 cells/ml. Twenty-four hours after initiation, when the cells were growing exponentially, drug exposure was commenced. The doubling time under these conditions was 12 hr. For drug exposure, cells were washed with 10 ml of PBS twice, and drug containing medium was added to the final concentrations designated. For all drug exposures, 10% dialyzed fetal calf serum was used. In those cases where α-MEM$^+$ was used, the final concentration of all nucleosides was 28 μM. In α-MEM$^-$ all nucleosides were absent. Cells were exposed for 24, 48, or 72 hr, and then the drug containing medium was removed, the cells were washed with 2 vol. of calcium-free PBS, and a single cell suspension was prepared using trypsin (0.05%, v/v) with EDTA (0.02%, w/v). The cells were counted and plated at various dilutions between 10^2 and 10^5 cells/petri dish (Falcon, Oxnard, CA). The plates were incubated for 10 days at 37° with 5% CO_2; then the medium was removed, colonies visible to the eye were counted, and the surviving fraction was calculated as the ratio of colonies formed in drug-treated plates to those in control flasks. The plating efficiency of untreated control cells was 70–80% in all experiments described.

Animal studies. C57 black female mice (Jackson Laboratories, Bar Harbor, ME), weighing between 20 and 25 g, were injected with 2×10^5 B16 melanoma cells intramuscularly in the hind leg. Eight or nine mice were allocated to each treatment group. Tumor weight doubling time for control animals was 2 days. Intraperitoneal injections daily for 4 consecutive days with PALA, NBMPR-P or PALA and NBMPR-P together were started 6 days after tumor inoculation, i.e. when tumors were just detectable. Drug was administered in a total volume of 0.2 ml. Tumors were measured by passing the hind leg through lucite with holes which had been calibrated previously to tumor weight.

RESULTS

In α-MEM$^+$, exposure of B16 melanoma cells to PALA (100 μM) alone or NBMPR (10 μM) alone for 24, 48, or 72 hr did not reduce clonogenicity (Fig. 1), but the combination of PALA and NBMPR caused a marked time-dependent decrease in clonogenic survival (Fig. 1) to 0.011. In α-MEM$^-$, a time-dependent decrease in clonogenic survival was observed when these cells were exposed to PALA (100 μM) alone (Fig. 2), reaching a surviving fraction of 0.015. However, NBMPR (10 μM) alone had little effect on survival in α-MEM$^-$ (Fig. 2). The combination of PALA and NBMPR at the same drug concentrations had an effect similar to that of PALA alone when exposures were carried out in α-MEM$^-$.

To determine whether these effects could be reversed by adding uridine or cytidine to the medium, cells were exposed to PALA (100 μM) for 72 hr in media containing various concentrations of each of these nucleosides. The dependency of PALA cytotoxicity on uridine and cytidine concentrations is demonstrated in Fig. 3. The concentrations of uridine and cytidine were varied from 0 to 40 μM. Recovery from PALA toxicity was evident at a uridine or cytidine concentration of 5 μM. The recovery of B16 melanoma was 80% of controls at 30 μM uridine. But cytidine at concentrations up to 40 μM

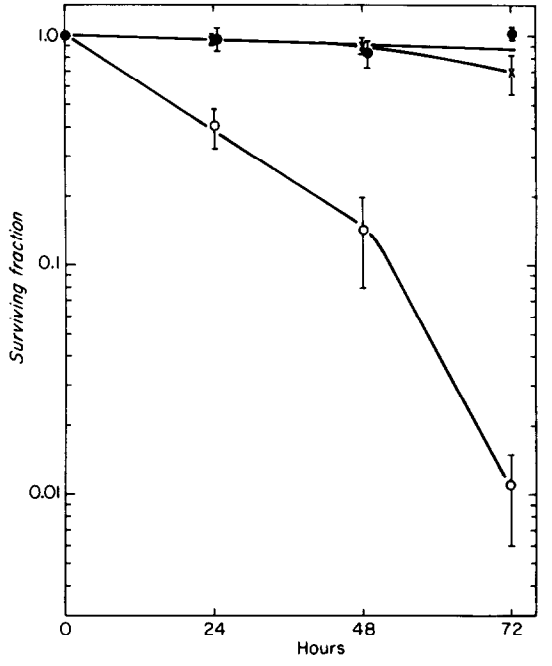

Fig. 1. Clonogenic survival of B16 melanoma cells exposed to PALA (100 μM) alone (●), NBMPR (10 μM) alone (×), and a combination of PALA (100 μM) and NBMPR (10 μM) (○) in α-MEM$^+$ for 24, 48, or 72 hr. Each point represents the mean of at least four experiments. Error bars represent standard error of the mean.

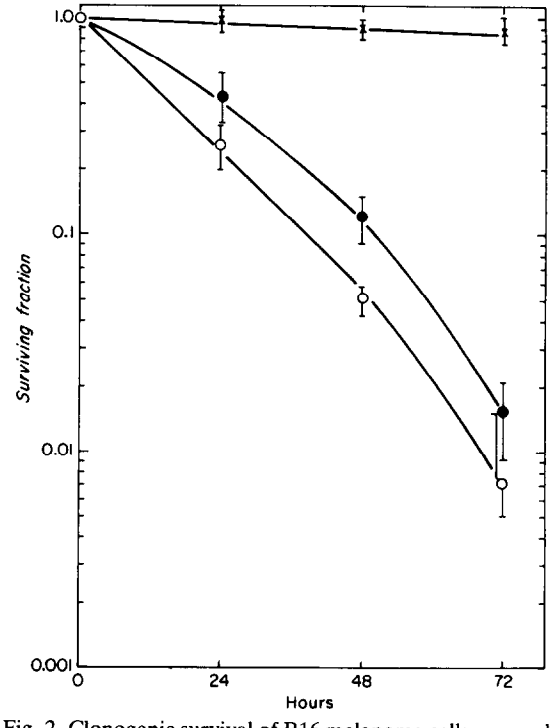

Fig. 2. Clonogenic survival of B16 melanoma cells exposed to PALA (100 μM) alone (●), NBMPR (10 μM) alone (×), and a combination of PALA (100 μM) and NBMPR (10 μM) (○) in α-MEM$^-$ for 24, 48, or 72 hr. Each point represents the mean of at least four experiments. Error bars represent standard error of the mean.

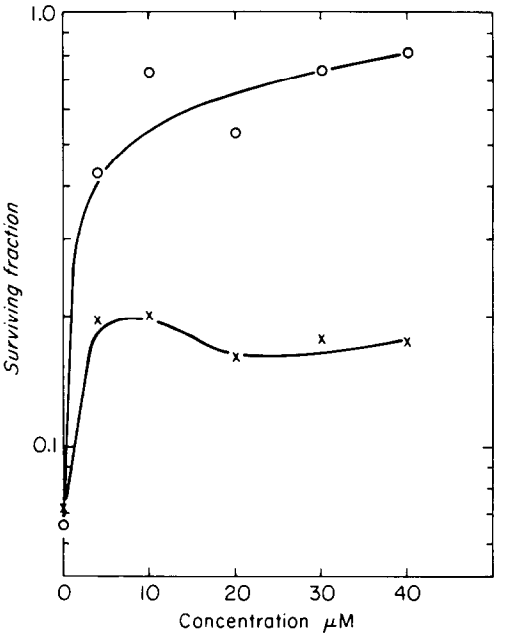

Fig. 3. Dependence of PALA cytotoxicity on extracellular concentrations of uridine (○) or cytidine (×). Cells were exposed to PALA (100 μM) for 72 hr. Each point represents the mean of two experiments.

produced recovery from PALA cytotoxicity to 10% of controls only.

In view of these *in vitro* results, we undertook to examine the effects of the nucleoside transport inhibitor NBMPR-P on the toxicity and antitumor activity of PALA in C57 Bl female mice bearing B16 melanoma cells inoculated intramuscularly. In toxicity experiments, the LD_{50} for PALA was 350 mg/kg/day × 4 days. NBMPR-P at doses up to 200 mg/

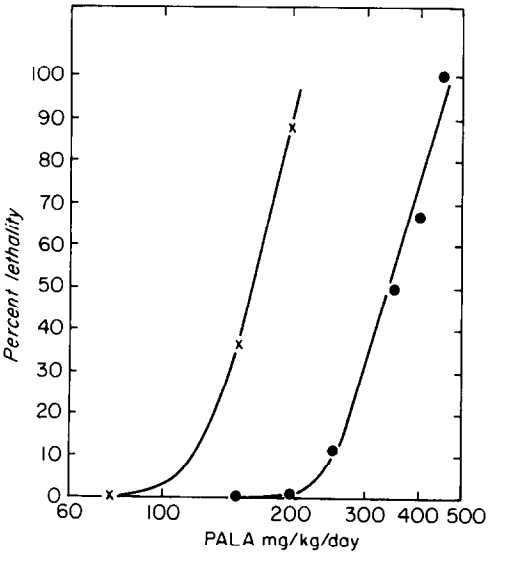

Fig. 4. Dose-survival curve for C57 black female mice treated with PALA (●) or PALA and NBMPR-P (100 mg/kg/day) (×) intraperitoneally daily for 4 days.

kg/day × 4 did not produce any deaths. NBMPR-P (100 mg/kg/day × 4) shifted the toxic dose–response curve of PALA to produce an LD_{50} of 160 mg/kg/day (Fig. 4). To compare the therapeutic effects of PALA alone and in combination with NBMPR-P, we chose equitoxic doses of the single agent (300 mg/kg/day × 4) and the combination (PALA 150 mg/kg/day × 4, NBMPR-P 50 or 100 mg/kg/day × 4). These doses were LD_{15} doses in tumor-bearing animals. The results of these studies are shown in Fig. 5. The growth delay for PALA at 300 mg/kg/day × 4 was 6 days relative to control. PALA at 150 mg/kg/day × 4 did not cause a significant growth delay. NBMPR-P at 100 mg/kg/day × 4 did not cause any growth delay. The growth delay for PALA, 150 mg/kg/day × 4, plus NBMPR-P, 50 or 100 mg/kg/day × 4, was restored to 6 days. The regrowth curves were shifted to the right and were parallel to the control curve in each case.

DISCUSSION

Many *in vitro* studies with PALA and other antimetabolites have been carried out in medium which was devoid of pyrimidine or purine nucleosides. Such studies have demonstrated that the antitumor effects of PALA are probably due to the effects of inhibition of *de novo* pyrimidine biosynthesis [15]. However, *in vivo* preformed circulating pyrimidine nucleosides are available for utilization by the pyrimidine salvage pathway. Pyrimidine salvage could, therefore, overcome any inhibitory effects of PALA *in vivo*. Our *in vitro* studies using B16 melanoma cells verify that levels of preformed pyrimidine nucleosides are important determinants of PALA cytotoxicity. In those situations where cells were exposed to PALA in the presence of nucleosides, no cytotoxicity was apparent. In the absence of all nucleosides, PALA had a marked cytotoxic effect.

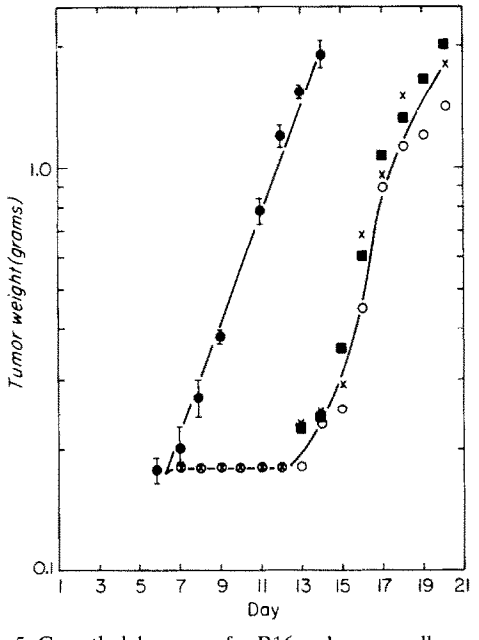

Fig. 5. Growth delay curve for B16 melanoma cells growing intramuscularly in C57 black female mice. Day 1 is day of tumor inoculation. Treatment was started on Day 6 and given daily for 4 days intraperitoneally. Key: Controls (●); PALA, 300 mg/kg/day (○); PALA, 150 mg/kg/day, plus NBMPR-P, 100 mg/kg/day (×); PALA, 150 mg/kg/day, plus NBMPR-P, 50 mg/kg/day (■). NBMPR-P alone at 50 or 100 mg/kg/day resulted in the same curve as controls and is not shown. Error bars on control are S.E.M. for eight mice. No error bars are shown for drug curves due to overlap for all data points.

We employed NBMPR as a specific inhibitor of nucleoside uptake to overcome the effects of pyrimidine nucleoside salvage. This drug was chosen because no effective inhibitor of uridine kinase is currently available. Alone, this drug had no cytotoxic effect in medium containing nucleosides or in medium devoid of them. The absence of cytotoxicity would be anticipated if *de novo* pyrimidine biosynthesis continued uninterrupted. But when this agent was combined with PALA in medium containing pyrimidine nucleosides, it enabled PALA to produce a cytotoxic effect similar to that observed when PALA was used in nucleoside-free medium. In medium devoid of nucleosides, the combination of PALA and NBMPR was no more effective than PALA alone. These two observations, taken together, would suggest that NBMPR is inhibiting pyrimidine salvage in α-MEM$^+$ and is not acting by another mechanism, heretofore unanticipated. Furthermore, since PALA cytotoxicity was observed in α-MEM$^-$ in the presence of NBMPR, it is unlikely that NBMPR inhibits PALA uptake.

The dependence of PALA cytotoxicity on the concentration of uridine and cytidine is shown in Fig. 3. The concentration range in which the "rescue" effect was evident is comparable to that normally observed in plasma in mice and is similar to that observed in man [8]. In the B16 melanoma cell line, uridine completely reversed the effects of PALA whereas cytidine in the same concentrations did not. Since the effect of increasing cytidine results in a leveling off of the recovery from PALA cytotoxicity, there may be a limited capacity for conversion of cytidine nucleotides to uridine nucleotides present in these cells. Among five patients treated with PALA, a clinical response was observed only in one patient who demonstrated a consistent and sustained decrease in plasma uridine levels after PALA administration [16]. Although this observation is consistent with our experimental data, too few patients were studied to allow any firm conclusions. Furthermore, levels of other pyrimidine nucleosides such as cytidine were not measured.

In vivo studies of PALA and NBMPR-P were performed to determine whether the effects of nucleosides *in vitro* were operative *in vivo* and to determine whether the combination resulted in an improved therapeutic index. PALA alone produced a growth delay in B16 melanoma, suggesting that for this cell line inhibition of *de novo* pyrimidine synthesis is sufficient for cytotoxic effects. NBMPR-P had no antitumor effect when administered alone in these animals. The toxicity studies demonstrated that the PALA dose which one could administer had to be decreased by 50% if drug lethality was to be maintained at an acceptable level. Therefore, the drug combination had much greater normal tissue

toxicity than the single agent alone at lower doses. Using maximum doses of PALA or PALA and NBMPR-P, a growth delay of 6–7 days was observed in both cases. Although a similar growth delay was observed with the combination using 50% of the dose of PALA as compared to the single agent treatment, we could not improve this because of increased animal toxicity. These studies demonstrated that the salvage effect of preformed circulating nucleosides on PALA antitumor activity can be overcome by NBMPR-P, but the therapeutic index for PALA plus NBMPR-P was no greater than for PALA alone.

These experiments illustrate two important points with regard to experimental studies with antimetabolites. First, there is a need to carry out the assessment of *in vitro* cytotoxicity of antimetabolites in medium containing preformed nucleosides in addition to the common practice of performing such studies in nucleoside-free medium. Such complete studies are necessary because preformed nucleosides which circulate normally influence the antitumor effect of such agents *in vivo*. Second, an assessment of therapeutic index of drug combinations *in vivo* is imperative. Whereas PALA and NBMPR were synergistic *in vitro* in medium containing nucleosides, the combination did not contribute significantly *in vivo* because the toxicity of the drug combination was too great to be administered at maximum doses simultaneously. The need to decrease the dose of PALA in the combination in order to decrease animal toxicity compromised any increased antitumor effect which the combination might have achieved. Such studies are important in initially defining the utility of drug combinations in man.

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