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Hematologic and Histopathologic Evaluation of *N*-(Phosphonacetyl)-L-Aspartate (PALA) in Mice

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Summary. *The study reported here was designed to provide a preliminary indication of the qualitative and quantitative toxicity of N-(phosphonacetyl)-L-aspartate (PALA). PALA was administered IP to male B6D2F1 mice (22–24 g) daily for 9 days in dosages of 180, 220, and 290 mg/kg. These dosages were chosen to approximate 0.8 LD_{10}, LD_{10}, and LD_{50}, based on historical 30-day lethality data. Five mice from each dosage group were killed on days 6, 10, 16, and 30 (day of first treatment = day 1) for hematologic and histopathologic evaluation. Only minimal changes were detected in peripheral hematologic values. PALA produced reticulocytopenia with a nadir on day 10 (24 h after last treatment). Reticulocyte counts were only marginally reduced by 180 mg/kg/day; nadirs were 33% and 23% of control following 220 and 290 mg/kg/day. Lymphopenia was observed on day 10, but it was not as severe in survivors of 290 mg/kg as it was in recipients of the lower dosages. Thrombocytopenia was not observed, and total marrow cell counts did not reflect hematotoxicity. Peripheral hematology was normal in survivors of 180 and 220 mg/kg/day by day 16. Reticulocytopenia in survivors of 290 mg/kg/day persisted, however, and no survivors of this dosage were available for assessment of recovery on day 30. Mild, diffuse hypertrophy of the gastrointestinal epithelium was the most consistent lesion observed. These lesions were most evident on day 10, and they were comparable in severity across the dosage range studied. The mice treated with 180 mg/kg/day exhibited no evidence of gastrointestinal toxicity on day 16; survivors of 220 and 290 mg/kg/day had residual lesions that generally were less severe than on day 10, although the effects of 290 mg/kg were more distinct. No survivors exhibited lesions on day 30. These results suggest that gastrointestinal toxicity may be dose-limiting, but, unlike most other anticancer drugs of the antimetabolite class, PALA may be only mildly hematotoxic.*

Introduction

N-(Phosphonacetyl)-L-aspartate[1] inhibits aspartate transcarbamylation, which is required for pyrimidine nucleotide biosynthesis [2]. It has therapeutic activity against experimental murine tumors [7, 8, 12, 13] (D. P. Griswold, Jr. and T. H. Corbett, Southern Research Institute, personal communication), including mammary adenocarcinoma 13/C, colon adenocarcinoma 38, B16 melanoma, and Lewis lung carcinoma. The activity against early Lewis lung carcinoma is marked; few other compounds can effect cures of this tumor [13]. In contrast, murine leukemia L1210 and P388 populations increase by two or more orders of magnitude under treatment with doses up to LD_{10} [8, 9] (F. M. Schabel, Jr., personal communication). This observation prompted the question as to whether PALA might lack hematotoxicity. If so, PALA would be unusual among anticancer drugs of the antimetabolite class, and it might be used to advantage in combination chemotherapy. These questions resulted from experimental chemotherapy trials completed before any information was available about the target organ for PALA toxicity. We were interested in a timely assessment of the qualitative and quantitative toxicity of PALA, and we were also interested in the predictive accuracy of the mouse model, since we were confident that dog and monkey data would ultimately be available for comparison. The results reported here were obtained in two experiments: an initial pilot study to provide an early answer for the question of hematotoxicity and a more extensive study to determine the effects of increasing dosage and to determine the likely target organ for dose-limiting toxicity of PALA.

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1 Abbreviation: PALA (NSC 224131)

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Materials and Methods

PALA was supplied by the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, NIH, Bethesda, Maryland. The drug was supplied as a yellow, aqueous solution of PALA, 200 mg/ml, in 2-ml ampoules (Lot UI-76-010). This solution was diluted with aqueous NaCl (0.9 g/100 ml) to provide 0.1 ml/10 g body weight for injection to mice.

Male C57BL/6 × DBA/2 (hereafter called B6D2F1) mice (Simonsen Laboratories, Gilroy, California) were used in these studies. In the pilot experiment, the mice weighed 16–19 g each. In the second experiment, they weighed 22–24 g each. All the mice were apparently healthy. They were housed in individual stainless steel cages with hardwood bedding (Betta-Chip™, Northeastern Products Corp., Warrensburg, New York) and received Wayne Lab-Blox® F6 (Allied Mills, Inc., Chicago, Illinois) and tap water ad libitum.

Ten mice were used in a pilot experiment. Five were treated with PALA, 178 mg/kg IP once a day for 9 days, and five received no treatment. The mice were killed on day 10 (1 day after the last treatment), and blood was collected for hematologic evaluation as described below.

A second experiment was carried out with 150 mice. Two groups of 35 mice each were treated with 180 and 220 mg/kg of PALA. Fifty mice were treated with 290 mg/kg, and 30 mice were treated with aqueous NaCl (0.9 g/100 ml) to serve as controls. These dosages were chosen to approximate the 0.8 LD_{10}, LD_{10}, and LD_{50} of PALA [13]. All treatment was IP once a day for 9 days, on the basis of individual body weights. All mice were predesignated for study on a specific day. Five mice from each dosage group including controls were killed on days 6, 10, 16, and 30. Ten mice from each dosage group were observed for 45 posttreatment days, and body weights and incidents of mortality were recorded.

Mice were killed by cardiac puncture and exsanguination under chloroform anesthesia. Blood was collected with needles and syringes rinsed with aqueous lithium fluoride solution (0.2 g/100 ml) containing lithium heparin (about 1000 units/ml). Blood samples for hematology were transferred to vials containing dipotassium salt of EDTA. Hematologic measurements included erythrocyte, leukocyte, platelet, differential leukocyte, and reticulocyte counts. Hemoglobin concentration and packed cell volume were determined for each specimen. The right femur of each mouse was cleaned of attached tissues and the epiphyses removed; the shaft was split longitudinally with a scalpel blade and placed in 0.2 ml Eagle's medium. The Eagle's medium was agitated to free the marrow elements. This suspension provided total and differential marrow cell counts [3]. Samples of liver, kidney, spleen, stomach, duodenum, ileum, jejunum, colon, heart, lungs, adrenals, gonads, and marrow were collected and preserved in buffered formalin (10 g formaldehyde/100 ml phosphate buffer, pH 7). The samples were embedded in Paraplast® (Sherwood Medical Industries, St. Louis, Missouri), sectioned at 4–6 μm, and stained with Harris' hematoxylin and eosin [11].

Results

In the pilot experiment, mice treated IP with a sublethal ($\leq LD_{10}$) dosage of PALA (178 mg/kg/day for 9 days) exhibited no clearly distinguishable change in their peripheral hematology. Our experience with hematology of the B6D2F1 mouse [5] and our qualitative and quantitative data on the toxicity of 5-fluorouracil [6], another drug of the antimetabolite class, supported our tentative conclusion that PALA was not appreciably toxic to the marrow or to the formed elements of peripheral blood. These results required confirmation, however, and we were even more interested to determine what the target of dose-limiting toxicity of PALA might be.

Our hematologic studies were confirmed and extended by the data presented in Table 1. In this particular study, the number of dead/total mice in the 45-day observation groups were 1/9, 5/10, and 9/10 for 180, 220, and 290 mg/kg, respectively. Since the two higher dosages exceeded the LD_{10}, this experiment serves as a relatively vigorous test of the sensitivity of the hematopoietic system of mice to PALA.

Mice treated with 290 mg/kg (LD_{90} in this experiment) exhibited reticulocytopenia that became progressively severe through day 16. None of the mice predesignated for evaluation on day 30 survived in this dosage group. In the lower dosage groups, the reticulocyte nadir occurred on day 10. Even at the nadir however, the reticulocytes of mice treated with 180 mg/kg were not clearly reduced in number below the reference range. Reticulocytosis was evident in survivors on day 30. Erythrocytes, packed cell volumes, and hemoglobin concentrations were not affected by PALA treatment.

Total leukocyte counts remained within the established reference range [5] throughout the course of study. Granulocytes were also apparently unaffected. Mild lymphopenia with a nadir on day 10 was observed in mice treated with 180 and 220 mg/kg. This observation did not extend to the highest dosage, however, and it was not predicted by the pilot study. The effect, if treatment-related, was clearly not severe, and recovery was apparent by day 16. Thrombocytopenia was not observed. Total marrow cell counts provided no indication of hematotoxicity.

The principal microscopic findings in mice killed at predesignated times are summarized in Table 2. The predominant lesions were found in the gastrointestinal tract on days 6, 10, and 16. Principal characteristics of gastrointestinal lesions were mild to moderate epithelial hypertrophy, hyperplasia, and macronucleosis. Other characteristics described were cystic dilatation and atrophy of intestinal glands, subchronic colitis, submucosal edema of the colon, cystic gastric glands, epithelial degeneration and necrosis of the small intestine and interstitial fibrosis of the colon. These changes are typical of attempted repair following injury of intestinal mucosae, and they have been observed consistently following administration of a variety of anticancer drugs, including cytosine arabinoside [10], methyl-CCNU [3], 5-fluorouracil [6], and adriamycin (E. P. Denine, unpublished data). Lesions likely to be directly or indirectly drug-related and occurring in other organs were lymphoid atrophy of the spleen, diffuse hepatocyte hyperplasia (6 mice), lymphoid atrophy of the mesenteric

Table 1. Effects of PALA[a] on hematology and body weight of B6D2F1 mice

Test (units) (reference range)	Dosage (mg/kg/day)	Day 6	Day 10	Day 16	Day 30
Reticulocytes (10^4/mm^3) (8.0–26.0)	–	16.5 ± 4.8	18.3 ± 5.9	23.0 ± 7.3	8.5 ± 4.1
	180	10.0 ± 5.5	7.9 ± 6.3	12.1 ± 9.9	24.6 ± 23.1
	220	6.9 ± 3.1	2.6 ± 3.2	8.2 ± 10.3	40.1 ± 10.5
	290	6.0 ± 2.3	4.2 ± 4.8	1.8 ± 1.2	–
Erythrocytes (10^6/mm^3) (7.3–9.7)	–	9.1 ± 0.1	8.9 ± 0.4	9.3 ± 0.3	7.8 ± 1.5
	180	9.0 ± 0.2	9.5 ± 0.4	8.8 ± 0.6	9.0 ± 0.6
	220	9.2 ± 0.7	10.3 ± 1.3	8.9 ± 0.5	8.4 ± 0.7
	290	9.5 ± 0.6	10.0 ± 0.4	9.5 ± 0.8	–
Packed cell volume (%) (35.0–47.0)	–	43.9 ± 1.7	44.4 ± 1.1	45.5 ± 1.0	39.1 ± 7.3
	180	43.8 ± 0.8	45.9 ± 3.2	45.1 ± 1.8	42.8 ± 0.8
	220	44.6 ± 2.2	45.2 ± 4.6	43.6 ± 2.2	41.9 ± 2.4
	290	45.1 ± 1.9	46.0 ± 2.6	40.5 ± 0.7	–
Hemoglobin (g/100 ml) (12.9–15.9)	–	14.9 ± 0.5	15.2 ± 0.2	15.4 ± 0.5	13.4 ± 2.6
	180	14.9 ± 0.2	15.7 ± 0.6	15.3 ± 0.8	14.7 ± 0.7
	220	15.3 ± 0.8	17.0 ± 1.7	14.5 ± 0.6	14.4 ± 0.9
	290	15.4 ± 0.5	16.3 ± 0.9	14.5 ± 0.3	–
Platelets (10^5/mm^3) (1.2–9.0)	–	4.5 ± 1.1	5.5 ± 0.7	5.0 ± 0.2	5.1 ± 0.9
	180	4.6 ± 0.5	4.5 ± 0.8	5.1 ± 1.5	6.0 ± 2.1
	220	4.4 ± 0.2	2.2 ± 1.3	3.8 ± 2.5	7.2 ± 2.1
	290	4.9 ± 1.5	5.5 ± 1.0	4.4 ± 0.3	–
Leukocytes (10^3/mm^3) (5.9–15.6)	–	7.5 ± 0.6	7.6 ± 1.5	11.7 ± 1.2	8.5 ± 0.7
	180	8.1 ± 2.7	4.8 ± 1.4	11.9 ± 2.4	9.2 ± 0.8
	220	9.2 ± 3.4	5.6 ± 1.9	15.2 ± 5.4	7.0 ± 1.0
	290	7.9 ± 1.4	6.9 ± 1.2	11.0 ± 5.6	–
Granulocytes (10^3/mm^3) (0.9–4.8)	–	1.5 ± 0.9	1.9 ± 1.0	1.9 ± 0.7	2.3 ± 0.9
	180	1.6 ± 0.7	1.6 ± 0.6	3.5 ± 2.4	2.3 ± 1.1
	220	2.0 ± 0.8	3.0 ± 1.6	7.4 ± 1.5	2.3 ± 0.9
	290	3.9 ± 1.7	2.6 ± 1.0	5.7 ± 4.4	–
Lymphocytes (10^3/mm^3) (4.0–12.2)	–	6.1 ± 1.1	5.7 ± 0.8	9.7 ± 1.8	6.2 ± 0.9
	180	6.5 ± 2.1	3.2 ± 1.0	8.4 ± 3.6	6.9 ± 1.8
	220	7.2 ± 3.2	2.6 ± 0.6	7.5 ± 6.0	4.7 ± 1.6
	290	4.0 ± 1.3	4.3 ± 1.2	5.3 ± 1.2	–
Marrow cell total (10^6) (1.8–15.5)	–	10.2 ± 2.2	10.0 ± 3.2	7.4 ± 2.6	4.5 ± 2.2
	180	10.2 ± 1.1	8.2 ± 2.5	6.2 ± 0.9	5.2 ± 2.0
	220	9.5 ± 1.5	5.5 ± 1.8	9.2 ± 3.6	5.9 ± 3.2
	290	6.9 ± 2.3	7.0 ± 1.7	3.8 ± 4.2	–
Body weight (g)	–	24 ± 2	25 ± 2	25 ± 2	26 ± 2
	180	21 ± 1	19 ± 2	22 ± 1	26 ± 2
	220	20 ± 1	18 ± 2	21 ± 2	26 ± 1
	290	19 ± 1	17 ± 1	17	–

[a] Mice received the indicated PALA dosages IP once a day for 9 days. Dosages of 180, 220, and 290 mg/kg correspond to 540, 660, and 870 mg/m^2. In this experiment, 180 mg/kg was the LD_{10}. The day of first treatment was day 1. Tabulated data are the mean ± SD of five individual values for each test. The reference ranges listed have been described previously [5]

lymph node, and individual hepatocyte necrosis (2 mice).

The gastrointestinal lesions were apparently in a repair or recovery phase on the day of earliest observation (day 6), although treatment was in progress at that time. This observation suggests that gastrointestinal damage occurs early, perhaps during the first 24 h posttreatment. Gastrointestinal epithelial hypertrophy was most pronounced on day 10. This effect was reflected in the modest loss and recovery of body weight (Table 1). The severity of lymphoid atrophy in the spleen and mesenteric lymph nodes was also greatest on day 10 (data not shown). The time needed for recovery from these drug-related lesions was dose-dependent. All the mice had recovered from sublethal gastrointestinal and splenic lesions by day 30.

Table 2. Principal microscopic findings[a] in B6D2F1 mice treated with PALA

Organ: lesion	Dosage (mg/kg/day)	Day 6	Day 10	Day 16	Day 30
Stomach:	–	0/5	0/5	0/5	0/5
Epithelial hypertrophy,	180	3/5 (0.6)	5/5 (1.0)	0/5	0/5
hyperplasia, and macro-	220	5/5 (1.0)	1/5 (0.2)	1/5 (0.2)	0/5
nucleosis	290	4/5 (0.8)	5/5 (1.0)	0/2	–
Small intestine:	–	0/5	0/5	0/5	0/5
Epithelial hypertrophy,	180	4/5 (0.8)	5/5 (1.0)	0/5	0/5
hyperplasia, and macro-	220	5/5 (1.0)	5/5 (1.0)	1/5 (0.2)	0/5
nucleosis	290	5/5 (1.0)	5/5 (1.0)	1/2 (0.5)	–
Large intestine:	–	0/5	0/5	0/5	0/5
Epithelial hypertrophy,	180	0/5	3/5 (1.2)	0/5	0/5
hyperplasia, and macro-	220	1/5 (0.2)	4/5 (1.2)	3/5 (0.6)	0/5
nucleosis	290	2/5 (0.4)	4/5 (1.2)	2/2 (1.5)	–

[a] The numerators are the numbers of affected mice, the denominators, the numbers of mice examined, and the numbers in the parentheses give the mean degree of severity in each case: 0, no lesion; 1, slight; 2, moderate; and 3, severe

Discussion

The choice of daily administration of PALA for 9 days was based on the success of this schedule for curative chemotherapy of early Lewis lung carcinoma [13]. The pharmacokinetics of PALA support this choice. PALA is rapidly eliminated in the urine in mice, rats, dogs, and monkeys [1]. Residual PALA equivalents that may reflect the release of drug from enzyme binding are eliminated at a much slower rate; nevertheless, repeated doses are clearly required to maintain cytotoxic concentrations of PALA in plasma.

With the exception of mild, reversible reticulocytopenia and lymphopenia, we found no evidence of hematotoxicity in mice treated with dosages of PALA equal to or exceeding the LD_{10}. These results are in excellent agreement with data published recently by Johnson et al. [9]. We have added the reticulocyte determinations; their experiment did not include them.

The histopathologic evaluations presented here and reported previously[2] conflict considerably with an earlier report [9]. The reason for the discrepancy is not clear. The present study indicates that PALA-induced damage to the gastrointestinal epithelium may be dose-limiting. Dose-limiting hepatotoxicity, which has been reported by others [9], was not observed in this experiment. Moreover, an extensive preclinical toxicologic evaluation of PALA conducted in dogs and monkeys since completion of the present study revealed no important hepatotoxicity in any of these species [4], but confirms the importance of the gastrointestinal epithelium as a principal target of PALA toxicity.

In our laboratories, the mouse has proved repeatedly to provide data qualitatively and quantitatively comparable to the results obtained with dogs and monkeys in the evaluation of anticancer drug toxicity. Until now, most of our comparisons of the animal models have been retrospective. When questions about PALA toxicity were raised, no large animal data had been generated, so we had an opportunity to test the predictive reliability of the mouse model in a prospective way. The data presented here and reported in part previously[2] have been confirmed by studies in larger animals [4]. Clearly, the mouse can provide extensive data in addition to LD_{10}, LD_{50}, and LD_{90} values, on which the more expensive and traditionally more critical studies in dogs and monkeys can be based.

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2 A preliminary report of some of these results was presented at the 17th Annual Meeting of the Society of Toxicology, San Francisco, CA, USA, March 12–16, 1978

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