Long-Term Association of \( N\)-(Phosphonacetyl)-L-aspartate with Bone

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ABSTRACT

By means of enzymatic and autoradiographic techniques, it has been demonstrated that, 24 hr after a single dose of the antitumor amino acid \( N\)-phosphonacetyl-L-aspartic acid (PALA), (400 mg/kg i.p.; 1.15 mmol/kg) to C57BL x DBA/2 F, mice, the agent accumulates in bone to a concentration of approximately 400 \( \mu \)m; this is 3000 times greater than the \( K_{i} \) of PALA for its target enzyme, aspartate carbamoyltransferase. However, disproportionately low inhibition of enzyme activity was demonstrated in homogenates of bone from these recipients, suggesting that the drug was sequestered from its target in this tissue. Autoradiography of sections of femoral shafts from mice treated with \( ^{14} \)C-labeled drug demonstrated that autoradiogram density due to \([^{14}C]\) PALA equivalents was confined to the bony matrix, with no label above background resolvable in bone marrow. Following in vivo administration of PALA (400 mg/kg i.p.), the half-life of the drug in the bone was approximately 23 days. In vitro, with equilibrium dialysis at pH 7.4, it was demonstrated that: (a) normal pulverized and decalcified bone bound PALA with capacities of 3.5 nmol/mg and 0.1 nmol/mg bone, respectively, at a PALA concentration of 5 mm; (b) binding of PALA to normal bone reached saturation at a concentration of 200 \( \mu \)m; and (c) PALA functions as a solubilizer of bone at concentrations above this. Since administration of PALA was shown to produce long-lasting inhibition of aspartate carbamoyltransferase in liver and tumor and since its ultimate half-life in the plasma of mice, following a single 400-mg/kg administration of the drug, is 8 days, it is suggested that bone serves as a reservoir from which PALA is released at a slow rate into plasma and other tissues.

INTRODUCTION

PALA is a powerful inhibitor of ACTase (EC 2.1.3.2), from microbial (9) and mammalian cells (10, 14, 24, 25). By inhibiting ACTase, PALA blocks de novo pyrimidine biosynthesis and is cytotoxic to many types of cells in culture; PALA also exhibits chemotherapeutic activity against an unusual spectrum of solid tumors in rodents; however, it demonstrates no activity against transplantable murine leukemias (1, 10-13, 23). Presently, PALA is undergoing Phase II trials at several cancer centers (8, 9). In this report, studies on the fate and distribution of PALA will be presented. These document that, 24 hr following PALA administration, kidney and skeleton were the principal sites of persistence of the drug (3, 4). The current study also quantitates the persistence of PALA in calcified tissue, describes the nature and saturability of the sites responsible for this binding, and suggests the possibility that the slow release of PALA from bone contributes to the persistence of PALA in plasma and tissues (15).

MATERIALS AND METHODS

\( L\)-(U-\( ^{14} \)C) Aspartic acid (184 mCi/mmol) was purchased from New England Nuclear (Boston, Mass.). \( N\)-(Phosphonacetyl)-L-aspartate-[U-\( ^{14} \)C]-tetrasodium salt for the whole-animal autoradiographic technique was prepared according to the method of Swyryd et al. (24). The radioactive compound was shown to be 95% pure by gas-liquid chromatography (2). For the equilibrium dialysis studies and the isolated bone autoradiographic methods, \([\text{acetyly}-^{14} \)C\]PALA (23 mCi/mmol) was supplied by Stanford Research Institute, Palo Alto, Calif. Prior to use, \([^{14} \)C\]PALA was chromatographed on a 1- x 10-cm column of Aminex A-14 (Bio-Rad, Richmond, Calif.) eluted with a 0 to 1 M gradient of ammonium bicarbonate. The major peak was pooled and desalted by exhaustive lyophilization. Greater than 99% radiochemical purity was achieved as judged by chromatography on a Jeol JLC-5AH amino acid analyzer equipped with an 8- x 150-mm column packed with Hamilton HA-X4 resin; \([^{14} \)C\]PALA eluted at 200 min with a series of 5 lithium citrate buffers (pH 2.72), ranging from 0.15 to 0.225 M (26). The tetrasodium salt of PALA was provided by Dr. Harry Wood of the National Cancer Institute, NIH, Bethesda, Md. All other reagents and chemicals were of the highest quality available commercially.

Autoradiographic Studies

\([^{14} \)C\]PALA was administered to male C57BL x DBA/2 F, (hereafter called BD2F,) mice at 400 mg/kg (650 \( \mu \)Ci/kg) via the caudal vein in 0.9% NaCl solution. Mice were housed in conventional cages and allowed food and water ad libitum. At Days 1, 4, 8, and 12, mice were sacrificed by immersion in a dry ice-hexane bath and stored at -25°. Whole-body dorsoventral sections were taken and autoradiograms were generated by conventional techniques (20). Film was exposed to sections for approximately 120 days.

Twenty-four hr following the i.p. administration of an admixture of \([^{14} \)C\]PALA (10 \( \mu \)Ci) and PALA (400 mg/kg) to a mouse, the animal was sacrificed; sternum, femur, and vertebrae were excised, fixed overnight in Perifix, and embedded in methacrylate without decalcification. Sections of bones (1 to 2 \( \mu \)m) were processed for autoradiographs by immersion in NTB 2 nuclear emulsion (Eastman Kodak Co., Rochester, N. Y.) and exposed for 10 days. The autoradiographs were developed and stained with hematoxylin-eosin as described previously (5).
Quantitative Measurement of PALA in Bone

Groups of 8 to 10 male BD2F1 mice were given i.p. injections of 0.9% NaCl solution or PALA at doses ranging from 25 to 800 mg/kg; 24 hr later, the animals were sacrificed, and femurs were quickly removed. The ends of the bones were excised with a single-edged razor blade, and the marrow was flushed out with 1 ml of 0.9% NaCl solution. The shafts were frozen on dry ice and pulverized with a percussion hammer maintained at -80°. Five-mg aliquots of the resultant powder were suspended in 500 ìl of 0.1 N HCl and incubated at 37° overnight. Aliquots (50 to 200 ìl) of the supernatant were then layered onto individual 8- x 40-mm columns of Dowex 50 (H+), 200 to 500 mesh; calcium was quantitatively retained on the resin, whereas PALA was quantitatively eluted with 1500 ìl of water. The eluates were lyophilized to remove acid and reconstituted to their original volume. Measurement of PALA was then conducted using an enzyme inhibition assay (16).

Determination of ACTase

ACTase activity was measured radiometrically (7). For osseous specimens, the pulverized bone (marrow removed) from one femur was suspended in 200 ìl of the homogenization medium and shaken at 4° for 1 hr; the extracts were centrifuged at 12,000 x g for 3 min; then, 5 ìl of the resultant supernatants were used for the analyses. To rule out inhibition of ACTase by calcium, selected extracts were divided into 2 parts, one of which was dialyzed against 1 liter of homogenization medium before assay. The specific activity of ACTase was diluted some 6- to 10-fold in the assay method used.

Dose Response of PALA and ACTase

BD2F1 mice were given single i.p. injections of 0.9% NaCl solution or PALA at doses ranging from 25 to 800 mg/kg; 24 hr later, the animals were sacrificed by cervical dislocation, and the femurs were removed, cleaned, pulverized, and extracted as described above.

PATA Time Course

PALA (400 mg/kg) was administered i.p. to 6 groups of 5 to 10 mice; on Days 1, 7, 14, 15, 17, and 35, the recipients were sacrificed; femurs were removed, cleaned, pulverized; and PALA was extracted as described above.

Measurement of PALA in Plasma

BD2F1 mice were given i.p. injections of PALA at a single dose of 400 mg/kg or 80 mg/kg or at 5 daily doses of 80 mg/kg. At designated time periods (Days 1, 2, 3, 4, 5, 6, 8, 10, 12, and 14), blood was collected in capillary tubes from the tails of the mice. PALA was measured in the plasma by a radiometric enzyme inhibition assay (16).

Equilibrium Dialysis

Preparation of Bone. Mice were sacrificed by cervical dislocation, and the femurs were removed and cleaned. The ends of the bones were excised with a single-edged razor blade, and the marrow was flushed out with 1 ml of 0.9% NaCl solution. One hundred sixty femoral shafts were divided into 4 groups and incubated overnight at 4° in 4 liters of 0.9% NaCl solution-0.1 M EDTA-0.1 M HCl-0.1 M NaOH; at which time the femoral shafts were washed with water for 24 hr to remove any interfering substances. The shafts were frozen on dry ice and pulverized with a percussion hammer maintained at -80°. The pulverized bones were kept at -80° prior to dialysis studies.

Equilibrium Dialysis. Equilibrium dialysis experiments were performed in Lucite chambers (Gateway Immunosera, Chanhokia, Ill.) at atmospheric pressure using a constant sample and bath volume of 100 ìl. Five mg of pulverized bone were accurately weighed and placed into one side of the equilibrium chamber; dialysis membrane was interposed, and then 100 ìl of 0.05 M potassium phosphate buffer (pH 7.4) were added to the pulverized bone. To the other side of the equilibrium chamber, 100 ìl of [14C]PALA (0.05 to 400 ìCi) were contained in 0.05 M potassium phosphate buffer (pH 7.4). One hundred ìl of 10 N HCl were then added and incubated at 95° for 5 min to solubilize the bone, and the radioactivity was determined by scintillation spectrometry.

Determination of Total Calcium of the Media in Equilibrium Dialysis. Experiments were set up similar to those for the determination of binding of PALA to normal pulverized bone. The Lucite chambers were equilibrated at 4° with constant gentle shaking for 18 hr. Aliquots from the sides of the dialysis chamber were then taken for the measurement of radioactivity. The sample of bone from the equilibrated chamber was transferred into Eppendorf tubes and washed 3 times with 1 ml of 0.05 M potassium phosphate buffer (pH 7.4). One hundred ìl of 10 N HCl were then added and incubated at 95° for 5 min to solubilize the bone, and the radioactivity was determined by scintillation spectrometry.

RESULTS

 Autoradiograms. Fig. 1 represents autoradiograms of whole-body dorsoventral sections of a mouse given [14C]PALA, (400 mg/kg, 650 ìCi/kg). The labeling of bone and kidney is approximately equal on Day 1, but the skeleton is the only site in which detectable PALA remains by Day 12. Fig. 2 represents autoradiographs of selected bones from a BD2F1 mouse treated with PALA (400 mg/kg) and [14C]PALA (10 ìCi). Microscopically, most labeling was confined to the body matrix. In the epiphyseal areas, low concentrations of [14C]PALA equivalents were noted in either the bone marrow or the cartilage. Within the bony matrix, labeling was generally intense at the edge of bony trabeculae and at the periphery of compact bone.

PATA Dose Response. To quantify these autoradiographic observations, enzymatic assays were performed on extracts of pulverized bones for quantitation of PALA following administration of the drug at doses of 25, 50, 120, 250, 400, and 800 mg/kg i.p. Chart 1 demonstrates that the specific activities of the recipients achieved remarkably high concentrations of PALA (0.08 to 0.3 nmol per mg protein per hr). Furthermore, following i.p. administration of the drug, there was a limit of the capacity of the bone to retain PALA; at 400 mg/kg, the bony sites of the mouse appear to have been fully saturated (Chart 1).

ACTase Dose Response. PALA demonstrated a suboptimal inhibition of bone ACTase with a basal specific activity of 26 nmol per mg protein per hr (Chart 2). Twenty-four hr following administration of PALA (400 mg/kg) in a susceptible organ such as spleen, there is a near total inhibition of ACTase (7).
Fig. 1. Whole-body autoradiographs with $^{14}$C]PALA. $^{14}$C]PALA (400 mg/kg; 650 µCi/kg), was administered i.v. to BD2F, mice. At various times, mice were immersed in dry ice-hexane, serial sections were taken, and autoradiograms were generated. The labeling of mouse bone and kidney is apparently equal in Day 1, (top), but the skeleton is the only site in which detectable $^{14}$C]PALA remains by Day 12 (bottom). Arrows, body structures.

This can be partially explained by activation of pyrimidine salvage enzymes and the mechanical barrier between PALA and its target cells.

**PALA Time Course in Bones.** Chart 3 demonstrates that the half-life of PALA in the femoral shafts of mice given a single i.p. injection of the drug (400 mg/kg) approximates 23 days; this is comparable to the half-life of other mono- and diphosphonates (21, 22).

**Measurement of PALA in Plasma.** Pharmacokinetic studies of PALA in mice show the drug to be present in the plasma at detectable concentrations for up to 1 month following single-dose administration (16). It is thought that this persistence reflects a long-term sequestration of the drug in sites such as the bone. To assess the relationship between plasma and bone PALA concentrations, mice were given either single i.p. doses of 400 or 80 mg/kg or 5 daily doses of 80 mg/kg. As shown in Chart 4, plasma PALA levels were determined over a 14-day period, at which time the animals were sacrificed and PALA concentrations in the femurs were measured. Plasma PALA concentrations were 80, 30, and 45 nm at Day 14 in animals receiving 400, 80, and 80 mg/kg daily in 5 daily doses.
respectively. Correspondingly, in these animals, bone PALA concentrations were 300, 60, and 100 µM. Since Chart 1 shows that bone uptake of PALA is saturated at a 400-mg/kg dose, it was of interest to see whether the accumulation of PALA in bone was comparable in animals receiving a single 400-mg/kg dose or the same total dose given as 80 mg/kg in 5 daily doses. Clearly, there is less retention of PALA in the bone when the drug is given in a sequential manner; presumably, this reflects the rapid primary phase of PALA clearance shown in Chart 4, where the half-life is approximately 3 hr.

**Equilibrium Dialysis: Binding of PALA to Bone.** Chart 5A illustrates the influence of treating the bone with acid, alkali, and EDTA on the binding of PALA. Pulverized normal bone binds PALA in a linear fashion to a concentration of 200 mM (Chart 5B). However, treatment with HCl, NaOH, or EDTA almost totally abolished the binding of PALA to the bone. Chart 5B illustrates the binding of PALA to a normal pulverized bone. There is a linear binding of PALA to bone up to a concentration of about 200 mM; thereafter, increasing the concentration of PALA leads to a decrease in the binding capacity of this agent to the calcified bone. As illustrated in Chart 5B, binding of PALA to the normal bone is associated with a release of calcium from the bone to the medium indicating that PALA may be solubilizing calcium from bone. This phenomenon was observed at all the concentrations of PALA examined; furthermore, the release of calcium from normal bone was linear over 100 to 250 mM PALA.

**DISCUSSION**

It is apparent that PALA, in common with the other phosphonates, e.g., disodium ethane-1-hydroxyl-1,1-diphosphonate, exhibits a distinct and durable affinity for the inorganic elements of the bone (19, 21). We have demonstrated in Chart 1 that, 24 hr following i.p. administration of PALA at doses of 20 to 400 mg/kg, the concentration of PALA in the femoral bone increased in a linear fashion. At a PALA dose of 400 mg/kg,
kg, total PALA binding to the bone approximates 0.3 μmol per 25 g mouse which is approximately 1% of the PALA administered. Moreover, even at these extremely high concentrations of PALA bound to calcified bone, the target enzyme of the agent is only subtotally inhibited; therefore, much of the PALA bound to bone is effectively sequestered and remains for a time chemically inert. Furthermore, the bony sites are saturable; e.g., at a PALA dose of 800 mg/kg i.p. to BD2F mice and in femoral bone examined 24 hr later, the PALA content was identical to that in those animals in whom PALA (400 mg/kg) was administered. In BD2F mice, the half-life for bone retention of PALA was approximately 23 days (Chart 3) which is similar to the retention time (2 to 4 weeks) of the diphosphonates of disodium ethane-1-hydroxy-1,1-diphosphonate (18, 22). From this bony compartment, circulating PALA can experience at least 3 fates: (a) it can be excreted by the kidney (20); (b) it can be reabsorbed with the skeleton; or (c) it can enter cytoplasmic sites and there be associated with or inhibit ACTase. In support of the latter hypothesis, we have demonstrated that, following single or multiple high or low doses of PALA, there is a finite plasma PALA concentration at Day 14. Thus, PALA demonstrated a strong affinity for bone and is responsible for the reservoir effect of PALA seen in the studies reported here.

In the subsequent in vitro studies with equilibrium dialysis, we have attempted to define the exact position of this bound PALA. Chart 5A demonstrates that any modulation which reduces the calcium content of bone brought about a reduction in the quantity of PALA which could be bound to bone. Thus, overnight treatment of intact bones at 4° with 0.1 M HCl, 0.1 M NaOH, or 0.1 M EDTA and subsequent pulverization at -80° reduced PALA binding to the treated femoral bone to negligible quantities.

This study supports the hypothesis that the calcified compartment of bone plays a large role in its ability to bind PALA. In our in vitro studies, it was interesting to observe that binding of PALA to normal pulverized bone increased in a linear manner when the PALA concentration in the media ranged from 0 to 200 mM. However, above this concentration, the quantity of PALA bound to bone began to decrease. This phenomenon suggests that PALA may function as a solubilizer of bony calcium. Other phosphonates are known to behave in a similar manner (18). Thus, we attempted to measure the calcium content of the dialysate in our in vitro studies. Chart 5B demonstrates that PALA acts as a solubilizer of calcium at all the nonpharmacological concentrations of PALA, and exposure of bone to PALA in mM range results in release of calcium from this site.

Further support as to the localization of PALA in murine bone is demonstrated by the autoradiographs of the femoral shafts. Fig. 2 clearly demonstrates that the PALA-derived radioactivity was confined almost exclusively to the bony matrix whereas the bone marrow is nearly free of any autoradiogram density induced by [1^4]C]PALA equivalents. This observation could be a partial explanation as to why this antimetabolite is not bone marrow suppressive. However, since the cortical blood supply is independent of that of the bone marrow, one can argue that the bone marrow-sparing effect of PALA cannot be a simple process of strong affinity of bone for PALA. We are currently exploring the biochemical loci leading to the differential enzymatic inhibition of bone marrow cells versus known sensitive target cells to PALA, i.e., spleen cells.

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**REFERENCES**


Fig. 2. Isolated bone autoradiographs with \[^{14}C\]PALA. \[^{14}C\]PALA (10 μCi; specific activity, 21.3 mCi/mmol) was administered i.p. to a BD2F1 mouse; 24 hr later, the animal was sacrificed; sternum, femurs, and selected vertebrae were removed, fixed, and embedded in plastic without decalcification. Autoradiograms of bone sections were made as outlined in "Materials and Methods." A, sternum. \[^{14}C\]PALA equivalents were present in bone matrix and not in bone marrow. × 800. B, femur. PALA-derived \[^{14}C\]-density was noted in the growth plate and along the bone matrix; however, no \[^{14}C\]PALA equivalents were evident in the hyaline cartilage matrix. × 490. C, vertebra. Autoradiogram density induced by \[^{14}C\]PALA equivalents was evident throughout bone trabeculae. × 490. D, vertebra. A high-resolution photomicrograph demonstrating that \[^{14}C\]PALA-derived radiodensity was associated with matrix and not with osteocytes. Note a mitotic cell in upper part of figure. × 2000.
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