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MACROMOLECULAR INTERACTIONS OF [^{14}C-RING]MELPHALAN IN BLOOD*

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Abstract—The pharmacokinetics and macromolecular interactions of [^{14}C-ring]melphalan (L-PAM) in blood were studied in rats following a single oral dose (20 mg/kg, 0.1 mCi/kg). Radioactivity levels were monitored in blood over a period of 72 hr. The highest levels of radioactivity were observed at 2 hr. The decline of radioactivity from the blood was biphasic with $T_{\frac{1}{2}\alpha}$ = 7 hr and $T_{\frac{1}{2}\beta}$ = 75 hr. The radioactive species in plasma corresponded to unchanged L-PAM and its two known hydrolytic products 4,2-hydroxyethyl 2-chloroethylamino-L-phenylalanine (L-MOH) and 4-[bis(2-hydroxyethyl)amino]-L-phenylalanine (L-DOH). In addition, four other major, previously unknown, metabolites of L-PAM were detected in plasma. At 72 hr, most of the radioactivity was bound to macromolecular components, 26% to plasma macromolecules and 62% in red blood cells. Covalent binding to blood cells was mainly to membrane proteins. Binding to hemoglobin and other soluble components of the red cells was also observed, with a 5000-fold greater affinity for membranes. These studies suggest extensive interaction of melphalan, or its metabolites, with membrane and soluble proteins of red blood cells.

Melphalan (L-phenylalanine mustard, L-PAM)‡ is an antineoplastic alkylating agent used in the treatment of ovarian carcinoma, multiple myeloma and breast cancer [1–3]. Several reports described the pharmacokinetics and distribution of L-PAM and its mono- and dihydroxylated metabolites (L-MOH and L-DOH) in animals [4–7] and humans [8, 9]. In the course of our studies on the disposition of L-PAM in peripheral blood, it was observed that the half-life of the drug in the red blood cell compartment was more than twice as long as the half-life in the plasma. Thus, it appeared that L-PAM may interact with the red blood cells. Such an interaction has been described previously for vinblastine with platelets [10] and more recently for adriamycin [11]. However, the extent of interactions of L-PAM with the macromolecular components of blood has not been fully described. Studies by Linford *et al.* [12] showed the adsorption of L-PAM to red blood cell membranes *in vitro*. Wildenauer and Weger [13] described the binding of the nitrogen mustard TRIS (2-chloroethyl)amine to human erythrocyte membranes *in vitro*. More recently, Barr *et al.* [14], using several anticancer chemotherapeutic agents with alkylating capabilities (e.g. cyclophosphamide), showed increased osmotic fragility and methemoglobin generation when human erythrocytes were exposed to these agents. There has also been one case report of a patient who developed hemolytic anemia during treatment with melphalan [15]. Therefore, it appears that L-PAM may interact with red blood cells or other components of the peripheral blood. This study was undertaken to investigate the interaction of L-PAM with red blood cells and macromolecular components of blood. The pharmacokinetics and distribution of L-PAM in whole blood, plasma and red blood cells are described.

MATERIALS AND METHODS

Reagents. [^{14}C-ring]-L-PAM (sp. act. 8.0 mCi/mmole, 2.48 μCi/mg) was obtained from the National Cancer Institute, NIH. Unlabeled L-PAM, pharmaceutical grade, was provided by the Burroughs Wellcome Co. (Research Triangle Park, NC). Radiochemical purity of each solution administered was verified by HPLC (as described below) where in all cases one single spectrophotometric peak coincided with the radioactivity peak. Hydroxylated L-PAM analogs were synthesized by heating L-PAM in 0.1 M NaOH at 70° for 1 hr, and their structure was confirmed according to the method of Furner *et al.* [5] and Ahmed and Hsu [16]. Solvents used were spectrograde acetonitrile (Burdick & Jackson, Muskegon, MI) and high purity glacial acetic acid (Fisher Scientific Co., Fair Lawn, NJ) diluted to 0.00175 M with double-distilled water. All other reagents were analytical grades obtained from various commercial sources.

Treatments and sample collections. Sprague–Dawley, male, albino rats (Charles River Breeding Laboratories, Wilmington, MA) ranging in weight from 190 to 200 g were used. The animals were divided into groups of three and provided with standard lab chow and tap water *ad lib.* A stock solution of

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‡ Abbreviations: L-PAM, melphalan, L-phenylalanine mustard, 4-[bis(2-chloroethyl)amino]-L-phenylalanine; L-DOH, 4-[bis(2-hydroxyethyl)amino]-L-phenylalanine; L-MOH, 4,2-hydroxyethyl 2-chloroethyl amino-L-phenylalanine; HPLC, high pressure liquid chromatography; and TCA, trichloroacetic acid.

40 mg/ml of L-PAM (containing 20 μCi/ml of [^{14}C-ring]-L-PAM) was freshly prepared prior to administration in 0.12 N HCl. A dose of 20 mg/kg (0.1 mCi/kg) was orally administered to each rat. One group of rats was anesthetized by ether and killed at time intervals of 0.5, 1, 1.5, 2, 4, 8, 12, 24, 48 and 72 hr after drug administration. Blood samples were immediately collected from abdominal venae cavae with heparinized syringes. The blood samples of each group were pooled. About one-half of each blood sample was centrifuged at 2000 rpm for 20 min in a refrigerated centrifuge (Damon/IEC Division CRU-5000 centrifuge) to separate the plasma and red blood cells. Red blood cells were then washed three times with 0.9% NaCl solution.

Determination of radioactivity. The radioactivity of 0.1-ml aliquots of whole blood, red blood cells or plasma was assayed in duplicate by combustion in a sample oxidizer model 306 Tricarb, Packard, Downers Grove, IL. [^{14}C]Carbon dioxide was trapped in scintillation vials each containing 8 ml of Carbo-sorb mixed with 12 ml of Permafluor. The samples were then counted in a liquid scintillation system (Mark IV, 6880, Searle Analytic, Inc.), equipped with an external standard ratio technique to calibrate disintegrations per minute.

Determination of covalently bound radioactivity. Covalently bound radioactivity was determined by the Schmidt–Thannhauser technique [17] as described by Reynolds and Moslen [18] and Gill and Ahmed [19]. Samples were thawed at room temperature and 1-ml aliquots of whole blood, red cells or plasma were extracted twice with 5 ml of 5% trichloroacetic acid (TCA). No radioactivity was detected in subsequent TCA extracts. The level of radioactivity in a 1-ml aliquot of the clear solution of TCA extract of various samples was analyzed using ScintiVerse (Fisher Scientific Co.) as the scintillation media. The TCA precipitate (macromolecular fraction, lipids and proteins) of each sample was dried and combusted in the sample oxidizer, and its total content of radioactivity was determined.

HPLC analysis of L-PAM, L-MOH and L-DOH in plasma. L-DOH, L-MOH, and L-PAM were detected and quantified using a modification of the HPLC analysis developed in our laboratory [16]. Fifty μl of the sample (TCA extract of plasma) was injected into the reversed phase column (10 μBondapak C18, P/N 27324 S/N, Waters Associates, Milford, MA) at room temperature. The initial solvent composition applied was acetonitrile (5%) and 0.00175 M acetic acid (95%). A final composition of 35% and 65% of acetonitrile and 0.00175 M acetic acid, respectively, was automatically attained over a period of 20 min.

The rate of flow across the column was 2.0 ml/min, and chart speed was 1 cm/min. L-PAM, L-MOH and L-DOH were detected at 263 nm and their retention times were 13.5, 8.5 and 4.5 min respectively. The fractions were collected in scintillation vials according to their peaks and retention times, and their radioactivity contents were counted.

Subcellular distribution of melphalan equivalents in red blood cells. The total radioactivity content of red blood cells was determined. Cells were then lysed with 8 mM phosphate buffer (pH 7.2) according to the method of Fuller *et al.* [20]. An aliquot was centrifuged in a Superspeed refrigerated centrifuge CRC-5 (Dupont Inst., Sorvall) at 13,000 rmp for 10 min to separate hemoglobin (soluble fraction) from cell membranes (pellet).

The pellet of ghosts was washed four times with the same buffer. The membrane lipids were extracted from the pellet by the method of Rosenberg and Guidotti [21], and the radioactivity in the extracted lipid was determined. The residue after lipid extraction was dried and weighed, and its content of radioactivity was determined. Membrane protein content in the lipid-free pellet was determined by the method of Lowry *et al.* [22].

An aliquot of the supernatant fraction was counted in ScintiVerse for determination of the radioactivity in the soluble cytoplasmic fraction of the cell. Another aliquot was diluted with 0.1 M Tris buffer (pH 8.7) and applied to a column (60 cm × 2 cm) packed with Sephadex G-100 at a flow rate of 0.7 ml/min according to the method of Wildenauer and Weger [13]. The contents were eluted by 5 mM phosphate buffer (pH = 8). The fractions separated by gel permeation chromatography were then collected and their radioactivity contents were determined. Hemoglobin content of each fraction was determined by the cyanmethemoglobin method using Drabkin's reagent [23]. Covalent binding to both membrane proteins and hemoglobin was determined by TCA extraction as described above.

RESULTS

Concentrations of radioactivity in whole blood, blood cells and plasma over 72 hr are shown in Fig. 1. Two hours after treatment the radioactivities were 6.2×10^4, 5.0×10^4 and 7.8×10^4 dpm/ml in whole blood, blood cells and plasma, respectively. During the first 12 hr the content of radioactivity was higher in plasma than in erythrocytes. The rate of decline of radioactivity from plasma, however, was fast

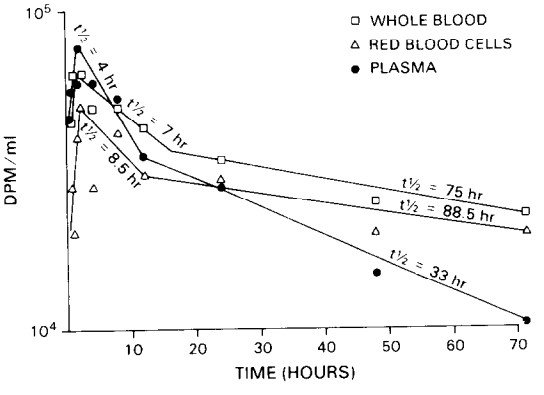

Fig. 1. Uptake and elimination of [^{14}C-ring]-L-PAM equivalence in whole blood, plasma and red blood cells of normal rats. Animals were given [^{14}C-ring]-L-PAM orally (20 mg/kg, 0.1 mCi/kg) and were killed at the times indicated. Each point represents the average level in the blood pooled from three rats. The slope and intercept of the β-phase were calculated by using a Wang-computer program for log-linear regression analysis. The half-lives of the α- and β-phases were calculated as described by Ritschel [24].

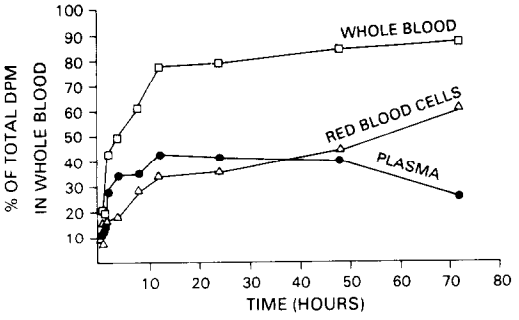

Fig. 2. Irreversibly bound [^{14}C-ring]-L-PAM equivalence in whole blood, plasma and red cells of normal rats. Irreversibly bound radioactivity was calculated as a percent of the total radioactivity in whole blood (□), red blood cells (△) and plasma (●). Irreversibly bound radioactivity was determined by TCA precipitation as described in Materials and Methods. Treatment of animals was the same as described in the legend of Fig. 1.

($T_{\frac{1}{2}\alpha}$ = 4 hr and $T_{\frac{1}{2}\beta}$ = 33 hr) as compared to that of red cells ($T_{\frac{1}{2}\alpha}$ = 8.5 and $T_{\frac{1}{2}\beta}$ = 88 hr). The rate of decline of radioactivity from whole blood was $T_{\frac{1}{2}\alpha}$ = 7 hr and $T_{\frac{1}{2}\beta}$ = 75 hr.

The levels of bound radioactivity in whole blood plasma and red cells are shown in Fig. 2. A gradual increase of radioactivity irreversibly bound to blood macromolecules was observed, and it counted for 88% of the total radioactivity remaining in blood at 72 hr. Irreversibly bound radioactivity associated with red cells tended to increase with time, reaching 62% of the total bound radioactivity by 72 hr. However, the radioactivity bound irreversibly to plasma plateaued by 12–48 hr, after which it tended to decline, reaching only 26% of the total bound radioactivity by 72 hr. A concomitant decline of radioactivity in the acid soluble fraction, unbound drug and its metabolites, was observed (not shown).

The levels of radioactivity corresponding to L-PAM and its hydrolysis products (L-MOH and L-DOH) in plasma are shown in Fig. 3. L-PAM concentration was highest at 0.5 hr (43% of total plasma radioactivity) but decreased sharply within 12 hr with a half-life of 1.5 hr. The concentration of L-DOH increased gradually and ranged from 5 to 15% of the total radioactivity in plasma. L-MOH concentration constituted 2–4% over the 72-hr period.

In addition to L-PAM and its two hydrolysates (L-DOH and L-MOH), four other major, as yet unidentified, metabolites have also been detected in plasma and designated by the letters A, B, C, and D (Fig. 4). Figure 4 shows the relative retention times of these metabolites as separated by the HPLC system mentioned above. The relative concentrations of these metabolites at 2 hr post-treatment are also shown. The highest concentrations for A, B, C, and D were attained at 12, 4, 1 and 8 hr, respectively, after administration (not shown).

Gel permeation chromatography of the soluble red cell fractions at 2 hr post-treatment is shown in Fig. 5. Significant levels of radioactivity were associated with the fractions containing hemoglobin. However, fractions that did not contain hemoglobin also contained high levels of radioactivity. The radioactive contents of this fraction disappeared faster than those associated with hemoglobin fractions (Fig. 6).

The covalent binding of L-PAM to membrane proteins and hemoglobin is shown in Fig. 7. Membrane protein binding increased sharply with time and was highest at 12 hr. Binding to hemoglobin was more

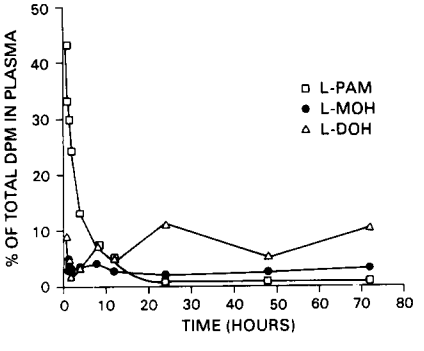

Fig. 3. Levels of [^{14}C-ring]-L-PAM and its hydrolysis products (L-MOH and L-DOH) in plasma of normal rats. L-PAM, L-DOH and L-MOH were estimated by HPLC as described in Materials and Methods. Treatment of animals was the same as for Fig. 1.

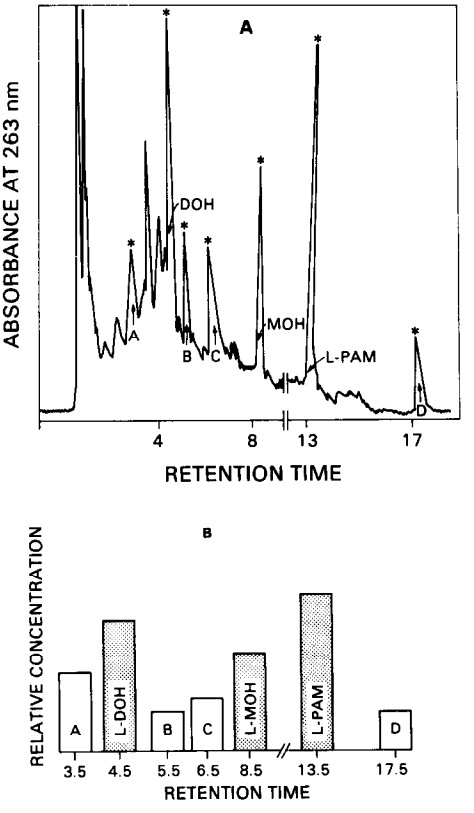

Fig. 4. A typical HPLC chromatogram (A) and relative total concentration of soluble metabolites (B) of [^{14}C-ring]-L-PAM in plasma of normal rats. Animals were given [^{14}C-ring]-L-PAM orally (20 mg/kg, 0.1 mCi/kg) and were killed 2 hr after treatment. Metabolites were measured by HPLC as described in Materials and Methods. An asterisk (*) represents radioactive peaks.

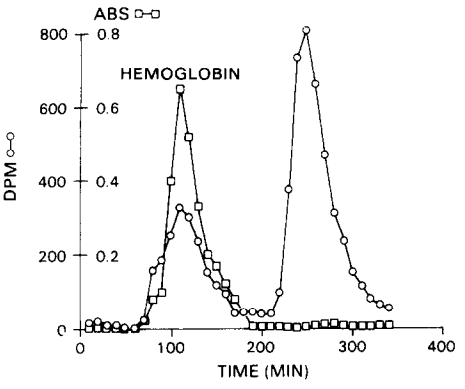

Fig. 5. Gel permeation chromatography of soluble red blood cell fractions. Normal rats were given [^{14}C-ring]-L-PAM orally and killed 2 hr after treatment. Blood was pooled from three animals. Soluble red cell components from lysed cells were separated by gel permeation chromatography by passing through a Sephadex G-100 column as described in Materials and Methods.

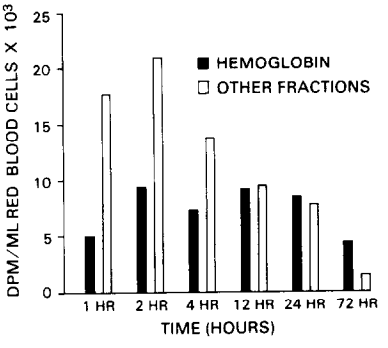

Fig. 6. Levels of [^{14}C-ring]-L-PAM or its equivalence in soluble blood cell components. Treatment of animals was the same as for Fig. 5.

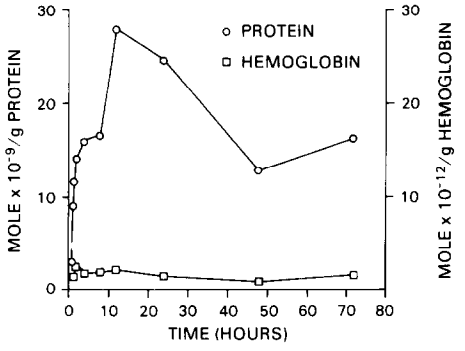

Fig. 7. Covalent binding of [^{14}C-ring]-L-PAM or its equivalence to membrane protein and hemoglobin. Red blood cells were lysed with 8 mM phosphate buffer, and the soluble fraction was separated from the membrane pellets by centrifugation. The soluble red cell components were separated by gel permeation chromatography by passing through a Sephadex G-100 column. Covalent binding to membrane proteins (pellet) and hemoglobin fraction (from column) was determined by TCA extractions.

or less constant with time. The radioactivity content in the membrane proteins varied from 1000- to 5000-fold that of the binding to hemoglobin (an example is shown in Fig. 7).

DISCUSSION

This study describes the pharmacokinetics and macromolecular interactions of orally administered [^{14}C-ring]-L-PAM in rat blood. Peak blood levels were observed at 2 hr after administration. The kinetics of disappearance of radioactivity from whole blood, blood cells and plasma showed a biphasic decline with $T_{\frac{1}{2}\alpha}$ = 7, 8.5 and 4 hr and $T_{\frac{1}{2}\beta}$ = 75, 88 and 33 hr, respectively. The results of this study show that the decline of label from red cells was more than two times as long as the decline of label from plasma. Thus, it appears that the drug is more tightly bound to the red blood cells. This observation was further confirmed by the macromolecular binding studies which showed the extent of binding of L-PAM to both plasma and red cells. In the latter compartment, covalent binding increased with time reaching 62% of the total bound radioactivity in blood, whereas in the plasma compartment covalent binding initially increased, plateaued, and then declined reaching only 26% of the total bound radioactivity by 72 hr.

The binding of label to blood cells appears to be more in the membrane protein. This agrees with the work of Wildenauer and Weger [13] who recently reported the binding of the nitrogen mustard TRIS (2-chloroethyl)amine with human erythrocyte membranes *in vitro*. Linford *et al.* [12] reported adsorption of alkylating agents to RBC membranes *in vitro*. However, they did not determine the type of binding which occurred. Our studies indicate that the binding of L-PAM to red cell membrane proteins is covalent and is 1000- to 5000-fold the binding to hemoglobin.

The consequence of this interaction on red cells or their functions is not yet clear. However, it should be emphasized that such an interaction may alter the efficiency of L-PAM as an antitumor agent. Thus, the amount of free, unbound drug reaching the tumor may be dependent on blood cell count. This has been shown to be the case for the distribution of adriamycin in control and tumor-bearing animals [11]. Broggini *et al.* [11] showed that more than twice as much adriamycin is present in plasma of tumor-bearing animals with lower hematocrits as compared to controls.

The red cell membrane protein appears to be a major target of L-PAM in the peripheral blood. Such an alkylation of the membrane proteins by the nitrogen mustard may lead to alterations in their function. Indeed, Barr *et al.* [14] showed recently that various anticancer alkylating agents cause generation of methemoglobin and increase the osmotic fragility of erythrocytes, *in vitro*. The consequence of alkylation of red cell membrane proteins by L-PAM must await further investigations. In addition, the nature of the alkylating species should be further identified.

The results of this study also reveal the presence of four, previously unknown, metabolites of L-PAM other than L-MOH and L-DOH. These metabolites were designated A, B, C, and D until their respective

structures are elucidated. The peak concentrations of these metabolites in plasma occur at different time periods indicating that they are either formed at different reaction rates, or that one metabolite is further metabolized into another.

In conclusion, our results show that an important aspect of the disposition of L-PAM within the blood, namely that it is bound and conjugated to blood cell macromolecules, with a high affinity to the membrane proteins.

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