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Biliary excretion of melphalan by control and anuric rats

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Melphalan [4-bis(2-chloroethyl)amino-L-phenylalanine] is active against multiple myeloma and several other cancers. The drug appears to be actively transported into cultured cells [1], suggesting that the pharmacokinetics of the amino acid derivative may be influenced by active translocation of the compound in the gut, liver, kidney and other organs. It was reported recently that high concentrations of melphalan are found in the bile and small intestine of dogs treated with the compound by intravenous injection [2, 3]. This paper reports the results of an investigation of the biliary excretion and tissue levels of labeled compounds following the injection of ring-labeled [^{14}C]melphalan to control rats and rats with no renal blood flow.

Materials and Methods

Labeled melphalan. Ring-labeled [^{14}C]melphalan (sp. act. 8 mCi/mmole) was a gift from the Chemical Resources Section, Pharmaceutical Resources Branch Development, The National Cancer Institute, Bethesda, MD. Radiochemical purity of the melphalan preparation was 97 per cent as determined by thin-layer chromatography. Melphalan solutions, prepared by dissolving 4–7 mg of the labeled material in 0.5 ml of ethanol-concentrated HCl (100:0.5, v/v) and diluting to 1 ml with 0.154 M NaCl, were used within 0.5 hr.

Bile collection. Male Sprague–Dawley rats (400–500 g) were anesthetized for the duration of the experiments with sodium pentobarbital (50 mg/kg, i.p.). A carotid artery and jugular vein were cannulated with PE-50 tubing and the bile duct was cannulated with PE-10 tubing. The artery and vein to both kidneys were then tied off to complete the ligated kidney preparation. Rectal temperature was maintained at 37 ± 0.3° during the experiments. Injections were through the venous cannula, and blood samples were taken from the arterial cannula. Bile was pooled for consecutive 10-min periods. Bile volume was determined with an electronic drop counter that delivered 8 μl of bile per drop. Control bile flow was determined for 20 min after completion of the preparations. Bile was stored at −20° until examined by chromatography. Melphalan (30 μmoles/kg) was injected during the first 2 min of the third bile collection period, which corresponds to the first collection period on Fig. 1. Blood samples (0.2 ml) were taken at the midpoint of each bile collection period. Blood lost due to sampling was replaced with 0.154 M NaCl. Samples (0.2 ml) of blood were diluted with 4 vol. of 0.154 M NaCl–2 mM NaEDTA and centrifuged for 5 min at 2000 *g*. The resulting diluted plasma was decanted. After the last bile collection period (1 hr after the melphalan injection), 2 ml of blood was taken to determine whole blood and plasma drug equivalents. A pneumothorax was produced and samples of kidney, liver and skeletal muscle were taken.

Hepatocyte preparation. Hepatocytes were prepared from fed male rats as described elsewhere [4]. Trypan blue was excluded by 95 per cent of the hepatocytes. The rate of oxygen uptake by the cells, suspended in 117 mM NaCl–4.8 mM KCl–1.2 mM $MgSO_4$–1.0 mM KH_2PO_4–20 mM Tris–HCl at pH 7.4, was stimulated less than 20 per cent by the addition of 1 mM succinate [5].

Analytical methods. Samples of plasma and bile were diluted directly with scintillation mixture for counting. Samples of blood, kidney, liver and skeletal muscle were dissolved in Soluene-350 (Packard Instrument Co., Downer Grove, IL), by incubation at 40° for 12 hr. Aliquots of the Soluene solution were diluted with scintillation mixture and stored for 24 hr at room temperature before counting. Thin-layer chromatography was done on glass plates coated with a 250 μm layer of silica gel G. One sample of bile (0.2 ml) was streaked horizontally 1 cm from the bottom of each 10 × 20 cm plate. The plates were developed in the long axis with *n*-butanol–acetic acid–water (7:2:1). After air drying, 1 cm horizontal strips of silica gel were scraped from the plates directly into scintillation vials. Scintillation mix was added, and the vials were shaken mechanically for 12 hr and counted. Bile samples collected from control rats for 0.5 hr after melphalan treatment (Fig. 1) were pooled and examined by chromatography. As a control, melphalan at 1 mg/ml in ethanol-concentrated HCl was diluted 1:1 with control rat bile, incubated for 0.5 hr at 22°, and then examined by thin-layer chromatography.

Calculations and statistics. The concentration of drug equivalents in the samples was calculated from the observed counts and the specific activity of the labeled melphalan. Student's *t*-test was employed with a difference being considered statistically significant when the probability that it had occurred by chance was less than 0.05 ($P < 0.05$).

Results

Figure 1 illustrates the rates of biliary excretion of drug equivalents by control rats and rats with ligated kidneys. Melphalan treatment and kidney ligation had no effect on bile flow, which was 69 ± 2 $\mu l \cdot min^{-1} \cdot kg^{-1}$ (mean ± S.E.M. N = 6). In all bile samples from rats with ligated kidneys, the concentration of drug equivalents was greater than that observed in corresponding bile samples from control rats. The range of drug equivalent concentrations in the bile samples was 0.5–0.8 mM with the maximum concentration occurring in bile collected 10–20 min after melphalan injec-

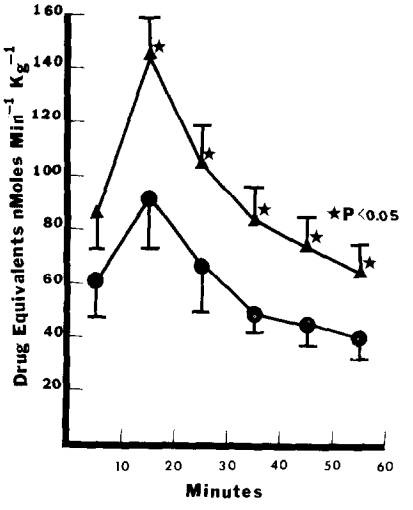

Fig. 1. Rate of biliary excretion of drug equivalents by rats treated with melphalan (30 μmoles/kg, i.v.). Key: (▲) ligated kidney preparation; and (●) control rats. The means ± S.E.M. for three rats are plotted.

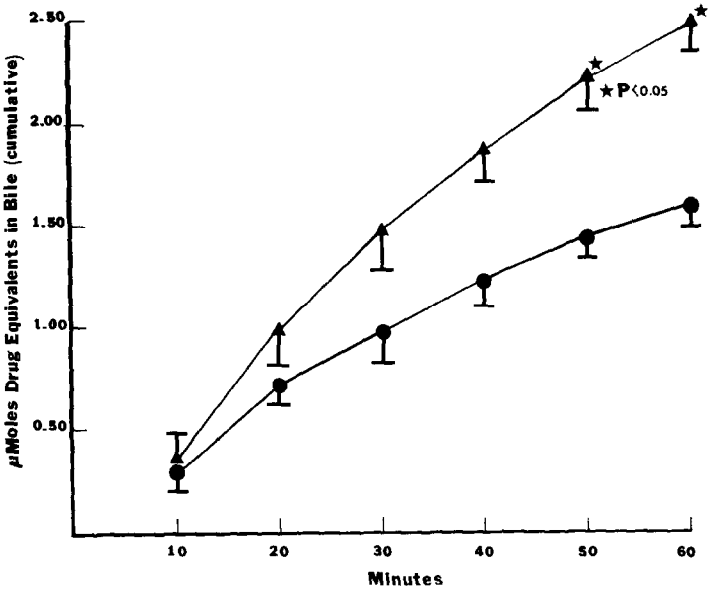

Fig. 2. Cumulative recoveries of drug equivalents in the bile of rats treated with melphalan (30 μmoles/kg, i.v.). Key: (▲) ligated kidney preparation; and (●) control rats. The means ± S.E.M. for N = 3 are plotted.

tion. Maximal bile/plasma drug equivalent concentration ratios, which were 35:1 and 25:1 for the ligated kidney and control preparations, respectively, occurred in samples collected 10–20 min after melphalan injection. In all cases bile/plasma concentration ratios from the ligated kidney preparation were greater than those observed for the control preparation.

The cumulative recoveries of drug equivalents in the bile of the two preparations are seen in Fig. 2. Total recovery of the injected drug equivalents in the bile for 1 hr following melphalan injection was 11 ± 1 and 18 ± 1 per cent ($P < 0.05$) for control and ligated kidney preparations, respectively.

Figure 3 shows the concentration of drug equivalents in plasma for 1 hr following melphalan injection for all six rats since ligation of the kidneys caused no significant change in plasma levels. Blood/plasma ratios in blood collected 1 hr after melphalan injection were 1.9 ± 0.2 and 1.7 ± 0.2 (not significant) for the control and ligated kidney groups, respectively. Thus, renal ligation caused no change in the blood/plasma ratios; however, the data demonstrate that drug equivalents concentrate with blood cells relative to plasma.

Tissue drug equivalents 1 hr after melphalan injection may be seen in Fig. 4. The differences between the control and ligated kidney preparation were statistically significant only in the case of the kidney.

The nature of the labeled compounds in rat bile after melphalan injection was examined by thin-layer chromatography and partition between 1,2-dichloro-ethylene and 10 mM HCl. Thin-layer chromatography of labeled melphalan added to control rat bile showed that 86 per cent of the radioactivity moved from 7 to 10 cm from the origin, as did authentic labeled melphalan. In contrast, chromatography of bile from rats injected with labeled melphalan showed that 81 per cent of the counts were found 0–7 cm from the origin with 23 per cent of the counts recovered 0–3 cm from the origin. The distribution of labeled melphalan between equal volumes of 1,2-dichloroethylene and 10 mM HCl was 34/1 (dichloroethylene/HCl) whereas the radiolabeled material in the bile of the rats treated with labeled melphalan distributed 10/1 (dichloroethylene/HCl).

The excretion of drug equivalents in rat bile suggests that melphalan may be rapidly taken up by isolated rat hepatocytes. Hepatocytes (4×10^6/ml) were incubated in Krebs bicarbonate buffer that was 20 mM in glucose for 15 min at 37° in an atmosphere of 95% O_2–5% CO_2. The assays were then made 0.5 mM in labeled melphalan and 2.5% (v/v) in ethanol. At 5, 10 and 15 min after the addition of melphalan, 0.1-ml aliquots of the mixtures were layered on 0.15 ml of 1-bromododecane [6] which was floating on 0.05 ml of 3 N KOH in a plastic microcentrifuge tube. The tubes were centrifuged for 1 min in a Beckman Microfuge and the lower portion of the tubes containing the KOH and cell pellets was cut off and allowed to fall directly into a scintillation vial. Counting was done after the samples had been shaken overnight with scintillation mixture. Under these conditions, 10^6 hepatocytes took up less than 0.02 nmole melphalan. The amount of melphalan accumulated by the cells was not affected by increasing the incubation time to 20 min or by including 1.0 mM 2,4-dinitrophenol in the assay.

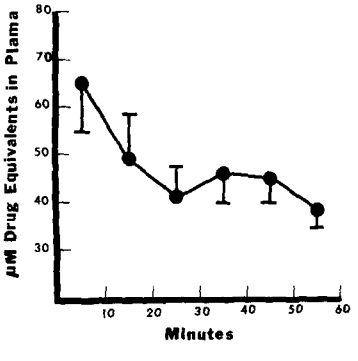

Fig. 3. Plasma drug equivalents in rats treated with melphalan (30 μmoles/kg, i.v.). The means ± S.E.M. for N = 6 are plotted.

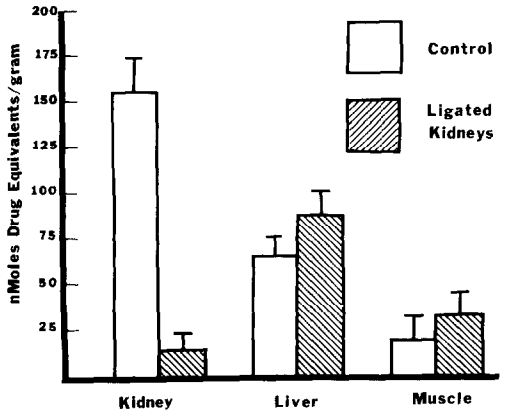

Fig. 4. Tissue drug equivalents 1 hr after treating rats with melphalan (30 μmoles/kg, i.v.). Means ± S.E.M. for N = 3 are plotted. The only statistically significant differences between the control and ligated kidney preparations were in the kidney.

Discussion

As reported for the dog, the present study has demonstrated relatively high concentrations of labeled compounds in rat bile after intravenous dosing with labeled melphalan. In contrast with the dog, the rat appears to excrete largely a melphalan metabolite(s) in its bile. Melphalan does not appear to be chemically unstable in rat bile since the labeled material could be spiked into rat bile *in vitro* and recovered unchanged by thin-layer chromatography. It is possible that species differences in the metabolism of melphalan are significant. Inspection of the data resulting from the application of a chromatography method to the determination of melphalan in rat plasma [7] indicates a plasma half-life of about 0.5 hr following intravenous injection. This compares favorably with the plasma half-life of drug equivalents for the first 0.5 hr that can be estimated from Fig. 3.

Ligation of the kidneys resulted in an immediate increase in the excretion of drug equivalents into rat bile. Although liver levels of drug equivalents were not increased in the ligated kidney preparations 1 hr after melphalan injection, the increased biliary excretion of drug equivalents seen in the ligated kidney preparations suggests that liver drug levels may have been higher than control shortly after melphalan treatment in animals with no renal blood flow.

Maximal rates of biliary excretion (Fig. 1) occurred when plasma levels of melphalan were reduced to about half of the maximal levels observed (Fig. 3). This suggests that hepatic uptake by the liver is fast relative to the rate at which the liver can excrete drug equivalents into the bile. The high bile/plasma ratios and the concentration of drug equivalents in the blood cells relative to plasma observed here suggest that melphalan may be subject to active transport *in vivo*, as is the case in cultured cells [1]. The failure of isolated hepatocytes to accumulate large amounts of label when incubated with melphalan suggests that hepatic mechanisms involved in uptake may have been damaged during isolation of hepatocytes or that the cells rapidly extruded the labeled compounds, thus accumulating only low concentrations of melphalan and/or its metabolites.

In summary, rats given melphalan by intravenous injection quickly excreted labeled compounds in their bile. Animals with no renal blood flow excreted higher concentrations and a larger fraction of the injected dose of melphalan in the bile. Renal ligation had no effect on plasma drug levels. Relative to plasma, melphalan concentrated in blood cells. In control rats, the tissue levels of drug equivalent 1 hr after melphalan injection were: kidney > liver > skeletal muscle. Renal ligation did not have a statistically significant effect on drug equivalents in the liver or muscle 1 hr after treatment.

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