Lack of Glutathione Conjugation of Melphalan in the Isolated in Situ Liver Perfusion in Humans

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ABSTRACT

Tumor cell resistance against melphalan (LPAM) has been associated with increased cellular reduced glutathione (GSH) levels and glutathione S-transferase activity. Therefore, GSH conjugation of LPAM has been hypothesized to be a key factor in tumor cell resistance. In the present study, we evaluated GSH conjugation of LPAM by the perfused liver in patients with colorectal cancer metastases undergoing a Phase II study of isolated liver perfusion as well as in the rat.

To evaluate whether LPAM-GSH conjugates were synthesized in the rat in vivo, LPAM was infused i.v. at a rate of 2.0 µmol/kg/min. In bile samples obtained during the infusion, two major GSH conjugates were identified by mass spectrometry: mono-hydroxy-mono-GSH-LPAM and di-GSH-LPAM. The maximum biliary excretion rate of these two conjugates accounted for only 1.3% of the LPAM infusion rate.

In bile or perfusate samples from patients treated for 60 min initially with 0.3 mg LPAM in the perfusion medium via isolated liver perfusion (200 mg LPAM in approximately 2 liters perfusion medium), none of the above-mentioned conjugates were detected. When comparable rat liver perfusions were performed initially with 66 µm or 0.66 mM LPAM in the perfusion medium, bile samples did contain GSH-LPAM conjugates; the cumulative biliary excretion of the two conjugates amounted to 0.4 and 0.2% of the LPAM dose, respectively.

These data suggest that both in rats and humans, hepatic GSH conjugation plays a very minor (if any) role in the elimination of LPAM and, therefore, that modulation of GSH levels is unlikely to affect the rate of elimination of this drug.

INTRODUCTION

LPAM is a clinically important nitrogen mustard in the treatment of solid tumors and hematological malignancies (1) as a bifunctional alkylating agent (2, 3). Unfortunately, the efficacy of LPAM as an anticancer agent is severely restricted due to the development of resistance to this drug, a phenomenon in which GSH conjugation has been implicated (4–6). In vitro studies have shown that LPAM can be conjugated to GSH to form either a monoglutathione or diglutathione conjugate (Fig. 1), a reaction catalyzed by GST isoenzymes of the α class, which are expressed at high levels in the liver (5–9).

In various tumor types resistant to nitrogen mustards, increased levels of GSH (10), elevated GST activities (11), and increased transcription of α-GST genes have been observed (7). Furthermore, it has been shown in vitro and in vivo that either depletion of tumor cell GSH by the GSH biosynthesis inhibitor buthionine sulfoximine or inhibition of GST activity by ethacrynic acid (which also depletes GSH) renders resistant tumor cells more susceptible to several alkylating agents (12–19). Based on these data, clinical Phase I studies combining chemotherapy treatment with GSH depletion or GST inhibition were initiated (20–22). However, the hypothesis that an increased rate of LPAM detoxification by GSH conjugation is a major mechanism of drug resistance has, to our knowledge, never been proven directly by demonstrating in vivo formation of LPAM-GSH conjugates. Therefore, in the present study, we evaluated the capacity of rat and human liver, which have a high intracellular GSH level and α-GST activity, to form LPAM-GSH conjugates.

Recently, we evaluated the pharmacokinetics and antitumor effect of LPAM in a model of colorectal cancer hepatic metastases using ILP in the rat (23). Having obtained promising data of complete remissions from this animal study, the Department of Surgery is now engaged in a study in which patients suffering from liver metastases derived from colorectal cancer are treated by a 1-h perfusion of their livers, isolated from the peripheral circulation, with very high doses of LPAM. With this technique, complete vascular isolation of the in situ-perfused liver is established to expose only the liver and liver metastases to LPAM. Analysis of bile and perfusate samples obtained during this human ILP treatment offered a unique opportunity to evaluate the hepatic GSH conjugation of LPAM in humans. Similar perfusions with LPAM were performed in rats at doses comparable to those used in the patients. The results show that, contrary to the rat liver, in the human liver we could not detect GSH conjugation of LPAM.

MATERIALS AND METHODS

Chemicals. Melphalan for the human ILP and rat experiments was provided by Wellcome Pharmaceuticals B.V. (Utrecht, the Netherlands). [3H]-GSH ([γ-glutamyl-cysteinyl]−[3H]-glycine) was from New England Nuclear (Dreieich, Germany). BSA fraction 5, γ-GT, GSH reductase, and NADPH were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine blood was kindly provided by a slaughterhouse (Slachthuis Leiden B.V., Leiden, the Netherlands). All other reagents were of analytical grade.

Animals. Male SPF Wistar/Wu rats (Charles River Breeding Laboratories, Sulzfeld, Germany) weighing 245–250 g were used. Animals were housed in Macrolon cages with hardwood bedding. The rats were fed laboratory chow pellets and water ad libitum. An alternating 12-h light and dark cycle was maintained in the animal rooms.

Identification of LPAM-GSH Conjugates. The LPAM-GSH conjugates were synthesized as follows. Freshly prepared solutions of 0.5 mM LPAM and 2.0 mM GSH in 100 mM sodium phosphate buffer (pH 7.4) were incubated for 2 h at 37°C. The reaction was terminated by mixing the samples with ice-cold stop buffer, an aqueous solution of 10% (w/v) trichloroacetic acid in 0.1 M NaNO3, 0.01 M KBr, 0.01 M citric acid, 0.1 mM Na2-EDTA, and 0.1 mM 1-decanesulfonic acid sodium salt. This stop buffer was used because GSH conjugation with LPAM continued in collected bile samples and deproteinated incubation samples. After diluting the samples with stop buffer, no further
conjugation of LPAM occurred, and no degradation of conjugates was observed. The conjugates were separated by HPLC using ECD, as described below. To identify the GSH conjugates in the chromatogram, the conjugate mixture was treated with γ-GT. For MS analysis, the three γ-GT-sensitive peaks were isolated using the analytical HPLC system (see below) with indirect electrochemical detection at 254 nm instead of ECD to avoid oxidation of GSH conjugates by bromine. The conjugate-containing fractions were desalted by solid-phase extraction, using Sep-Pak C18 cartridge columns (Waters Millipore Co., Milford, MA). The pooled fractions were loaded onto the columns, preconditioned with 1% (v/v) acetic acid, and rinsed with a 1% (v/v) acetic acid solution (10 retention volumes), followed by methanol (5 retention volumes) to elute the conjugate. The eluate was lyophilized and stored at −20°C. After isolation, they were subjected to FAB-MS analysis for structure confirmation (4, 8).

**Quantitation of LPAM-GSH Conjugates.** GSH conjugates of LPAM in bile and perfusate (ILP in humans and rats) were quantified by a HPLC method with indirect electrochemical detection, as described previously (24). Briefly, conjugates were separated on a C18 (Spherisorb ODS-2) column (200 × 3-mm inside diameter, 5-μm particles) in combination with a Vydac SC-201 RP pre-column (22 × 2-mm inside diameter, 30-40-μm particles). Isocratic elution was performed at a constant flow of 0.45 ml/min. The eluent consisted of a mixture of 0.1 M NaNO3, 0.01 M KBr, 0.01 M citric acid, 0.1 mM Na2-EDTA, 710 mass spectrometer in the positive ion mode. Glycerol or thioglycerol was used as the liquid matrix.

**GSH and LPAM Analysis.** GSH concentrations in perfusate and blood, as well as the amount of GSH in liver biopsies, were assayed as described (27). Perfuse samples obtained during the ILP treatment were analyzed for LPAM concentrations by a HPLC assay (28).

**LPAM Infusion in the Rat in Vivo.** Rats were anesthetized by an i.p. injection of sodium pentobarbital (60 mg/kg). Throughout the experiment, the body temperature was kept at 37°C with an electric heating pad. LPAM was first dissolved in acid ethanol [ethanol:36% (w/v) HCl = 95:5 (v/v)] to a concentration of 192 mM and then diluted with a 60:40 (v/v) mixture of propylene glycol and an aqueous solution of 0.17 M K2HPO4 to obtain a final LPAM concentration of 32 mM. After cannulating the bile duct and external jugular vein (29), the LPAM i.v. infusion was started. The solution was infused at a rate of 0.94 ml/h (500 nmol/min). Bile was collected at 15-min intervals into preweighed cups containing 600 μl ice-cold stop buffer. Bile samples were immediately frozen in liquid nitrogen and stored at −80°C until analysis.

**Human Liver Perfusion.** Patients with nonoperable liver metastases from colorectal cancer were treated in a Phase II study with a 200-mg (0.66 mmol) total dose of LPAM. This study was approved by the Medical Ethical Committee of the Leiden University Hospital, and written informed consent was obtained from all patients. The ILP scheme is outlined in Fig. 2. The perfusion medium consisted of intrahepatically trapped blood and 1 liter Gelfusinse (Vifor Medical SA, Sempach, Switzerland) to yield a final volume of approximately 2 liters. The perfusate was kept at 37°C and oxygenated using a heart-lung machine. LPAM (200 mg) was first dissolved in 40 ml Wellcome diluent [a 60:40 (v/v) mixture of propylene glycol containing 5.2% (v/v) ethanol and 0.068 mM sodium citrate], which was subsequently diluted with 60 ml sterile saline. LPAM was administrated as a bolus in the isolated circuit. From six patients treated by ILP, perfusate samples were collected before and after drug administration. For GSH analysis, to 100 μl perfusion medium (including RBC) 1.9 ml of 1 mM EDTA were added, followed by 2 ml of 0.8 M perchloric acid. Heparinized samples were centrifuged for 10 min at 750 × g, and one part of the supernatant was diluted (1:1) with 0.8 M perchloric acid for perfusate plasma GSH measurement; the remaining plasma sample was used for LPAM analysis. To obtain samples for LPAM conjugate analysis, 0.5 ml perfusion medium was diluted (1:1) with stop buffer. During the perfusion period, the common bile duct was clamped, and a bile sample was taken before LPAM addition. Subsequently, bile was collected until the end of the perfusion (60 min). Liver biopsies for GSH analysis were taken within 30 min after ILP treatment.

**Recirulating Rat Liver Perfusion.** The surgical procedure was identical as described previously for the single-pass in situ rat liver perfusion (30); the outflow of the liver was collected in the reservoir-oxygenator and then recirculated into the portal vein. The liver was perfused for 2 h at a constant flow rate of 10 ml/min. The LPAM solution was prepared by dissolving 3.3 or 33 μmol LPAM in 100 μl acid ethanol (ethanol:36% (w/v) HCl = 95:5 (v/v)), which was subsequently diluted with 4900 μl Krebs-Henseleit bicarbonate solution. The perfusion medium (50 ml) consisted of 20% (v/v) washed bovine RBC in Krebs-Henseleit bicarbonate solution, buffered to pH 7.4. The perfusate containing 1% (w/v) BSA, 0.3% (w/v) glucose, and initially 66 μM or 0.66 mM LPAM was oxygenated (95% oxygen:5% carbon dioxide) and kept at 37°C. Bile was collected as described above. Samples for LPAM and LPAM conjugate analysis were taken from the reservoir and pretreated as described.
Identification of LPAM-GSH Conjugates. To find out whether in rats LPAM-GSH conjugates are synthesized and subsequently excreted in bile, high-dose LPAM infusion experiments were performed. HPLC analysis of rat bile samples obtained during LPAM infusion revealed the presence of three metabolites, referred to as 1–3 (retention times 7, 8.5, and 29 min, respectively; Fig. 3), which were not observed in blank bile.

The same three metabolites were observed after analyzing in vitro incubation media; when LPAM was incubated with [3H]GSH, the radiolabel was incorporated into these three metabolites. Furthermore, all three metabolites were γ-GT sensitive, indicating that they contained a γ-glutamyl moiety and are likely LPAM-GSH conjugates.

FAB-MS analysis of the metabolite 1 yielded a base peak of m/z 580, most likely corresponding to the sodium adduct of mono-OH-mono-GSH-LPAM. However, the protonated molecular ion of mono-OH-mono-GSH-LPAM itself (m/z 558) was not observed. This peak, therefore, is assumed to be the mono-OH-mono-GSH-LPAM conjugate (6, 8).

Analysis of metabolite 3 yielded a base peak of m/z 847, corresponding to the protonated molecular ion of intact di-GSH-LPAM. Sodium and potassium adducts (m/z 869 and 885, respectively) were also observed. This spectrum is very similar to a previously published spectrum for di-GSH-LPAM by Dulik et al. (6). Therefore, this peak was identified as the di-GSH-LPAM conjugate.

Metabolite 2 could not be positively identified but may represent mono-GSH-LPAM. We have further concentrated on the mono-OH-mono-GSH-LPAM and di-GSH-LPAM conjugates.

Biliary Excretion of LPAM-GSH Conjugates during LPAM Infusion in the Rat in Vivo. The biliary excretion rates of the two LPAM-GSH conjugates were monitored during a 4-h i.v. infusion of LPAM (2.0 μmol/kg/min). The excretion rate of mono-OH-mono-GSH-LPAM increased continuously in the course of the LPAM infusion, whereas the excretion rate of di-GSH-LPAM reached a maximum of 4 nmol/min and then gradually declined to 2.7 nmol/min toward the end of the experiment (Fig. 4). During the course of the infusion, GSH levels decreased to approximately 50% of control values (data not shown), indicating that there is still enough GSH left by the end of the experiment. The maximum biliary excretion rate of mono-OH-mono-GSH-LPAM plus di-GSH-LPAM amounted to approximately 6.5 nmol/min, which corresponded to 1.3% of the LPAM infusion rate.

Human ILP. In a related project, a Phase II study of ILP with LPAM was done in patients with colorectal cancer confined to the liver. From six of these patients with otherwise normal liver function, samples were taken from the perfusate during the perfusion, and bile was collected during that 1-h period.

Pharmacokinetic analysis of LPAM showed a biphasic decline in the perfusate, suggesting a rapid uptake phase followed by a much slower elimination phase. Analysis of LPAM samples obtained simultaneously from the inflow and outflow catheter during ILP demonstrated that after the rapid uptake phase, the inflow and outflow LPAM concentrations were similar (Fig. 5), indicating that the liver removed only a little LPAM after the initial uptake phase. Furthermore, these data demonstrate that throughout the whole perfusion period, LPAM was available for conjugation.

![Figure 3](https://example.com/fig3.png)

Fig. 3. Representative chromatograms of the HPLC analysis of rat and human bile and human perfusate. Three LPAM-GSH conjugates were observed in the LPAM-GSH standard and in rat bile, designated 1–3, with retention times of 7, 8.5, and 29 min, respectively.
Despite extensive analyses of bile and perfusion medium samples obtained during the 1-h perfusion of six human livers, the GSH-LPAM conjugates could not be detected (Fig. 3). This result was rather surprising, because the HPLC was sensitive enough to detect 0.5 μM LPAM-GSH conjugate. Bile and perfusion medium samples from three patients were spiked with a known amount of in vitro-synthesized LPAM-GSH conjugates; subsequent HPLC analysis of the spiked samples demonstrated the presence of LPAM-GSH conjugates in concentrations corresponding to the added amounts, indicating that it was possible to detect them in bile and perfusion medium.

We observed no significant decrease in GSH concentrations in perfusion medium and perfusate plasma during ILP (data not shown), indicating that GSH in both plasma and RBC was not depleted during the perfusion. The content of GSH in liver biopsies taken within 30 min after ILP ranged from 2.7 to 3.0 nmol/g (n = 6).

Rat ILP. When we did similar perfusions with rat livers, we found GSH-LPAM conjugates in bile (Fig. 3) but again failed to detect any conjugate in the perfusion medium.

The biliary excretion profile of both conjugates showed that the di-GSH-LPAM conjugate was the predominant conjugate excreted in bile at the low LPAM dose, whereas mono-OH-mono-GSH-LPAM conjugate excretion was greater in the high LPAM dose (Fig. 6). In addition, the time to the peak excretion rate was shorter with the high dose compared with the low dose. The biliary excretion of both conjugates reached a maximum of 0.21 and 1.8 nmol/min within 40 min after drug administration in the 3.3 and 33 μmol LPAM-treated rats, respectively, and then gradually declined during the course of treatment. The cumulative amount of both conjugates in rat bile during the 2-h perfusion accounted for only 0.2 or 0.4% of the 3.3- or 33-μmol LPAM dose, respectively, indicating that in rats, LPAM-GSH conjugation is only a minor route in the hepatic elimination of LPAM.

Pharmacokinetic analysis of LPAM during the recirculating perfusion with 3.3 or 33 μmol LPAM added as a bolus to the perfusion medium showed that the disappearance of LPAM was similar to that in the human liver perfusions (Fig. 7) at the low dose of LPAM. To study the disappearance of LPAM in the absence of the liver, we performed LPAM perfusions at the low dose without the liver. Under these conditions, the rate of LPAM disappearance was similar to the elimination of LPAM after the initial uptake phase in perfusions with the rat liver (Fig. 7).

DISCUSSION

In bile samples from LPAM-treated rats as well as from the perfused rat livers, three LPAM-GSH conjugates were found. In accordance with findings reported by Bolton et al. (8), the two major conjugates were identified as mono-OH-mono-GSH-LPAM and di-GSH-LPAM. The maximum biliary excretion rate of these conjugates in the rat in vivo accounted for only 1.3% of the LPAM infusion rate. Because we have no data on urinary excretion products of LPAM, this provides a minimum estimate of LPAM-GSH conjugation in the rat in vivo; under such conditions, part of the GSH conjugates formed may have been converted to mercapturates and excreted in urine.

Because in rats readily detectable levels of unchanged LPAM were excreted in bile throughout the infusion (data not shown), uptake of LPAM by the liver does not seem to be rate limiting. Biliary excretion rates of LPAM-GSH conjugates will only reflect the GSH conjugation rate, if the transport of these conjugates from the hepatocytes into bile is not rate limiting. Rate limitation by transport processes seems unlikely, because the maximum biliary excretion of LPAM-GSH conjugates during ILP were 0.2 and 1.8 nmol/min in rats treated with...
3.3 and 33 μmol LPAM, respectively, indicating that a 10-fold higher dose of LPAM resulted in a similar increase in the biliary excretion rate of LPAM-GSH conjugates.

Because the human liver has a high GSH content and high activity of the α-GST isoenzymes, which have been reported to catalyze the conjugation of LPAM (5–9), we expected to find LPAM-GSH conjugates in human bile. However, when we evaluated hepatic GSH conjugation of LPAM in humans during our unique ILP system, HPLC analysis of perfuse and bile samples showed that during the 1-h liver perfusion, none of the LPAM-GSH conjugates were present at a detectable level. This result was rather surprising, because throughout the perfusion both GSH in hepatocytes, RBC, and perfusate plasma and LPAM were continuously available. In this clinical Phase II study, patients were treated with 0.66 mmol LPAM administered as a bolus in the ILP circuit, resulting in a 0.3 mm LPAM concentration. A concentration of only 0.5 μmol LPAM-GSH conjugate was needed for a positive identification; therefore, even if 0.005% of LPAM was conjugated to GSH during ILP and subsequently excreted in bile (approximately 50 ml bile production during the 1-h ILP treatment), our HPLC system would have been sensitive enough to identify the conjugates.

Although we cannot rigorously exclude the possibility that during the perfusion LPAM-GSH could have been further metabolized to mercapturates, this seems unlikely to be a quantitatively important event for two reasons. In the first place, such further metabolism takes place mainly in the kidney and has never been observed in the perfused rat liver. In the second place, our ECD method also detects mercapturates (and cysteine conjugates) quite sensitively; therefore, such further metabolites easily could have been detected if they had been present in bile or perfusion medium. Furthermore, we tested a number of colorectal cancer metastases for the presence of γ-GT by enzyme histochemistry. We could not detect γ-GT activity in any of these tumors. Therefore, it is unlikely that the tumors would break down the LPAM-GSH conjugates in the perfused liver.

The discrepancy between data from i.v. infusion of LPAM in rats on the one hand and human liver perfusion on the other could be due to extrahepatic conjugation of LPAM in the former, because it has been shown that several extrahepatically formed GSH conjugates (e.g., bromosulphathalein-GSH and 1-chloro-2,4-dinitrobenzene-GSH conjugates) were indeed taken up by the liver and subsequently excreted in bile (31). To rule out this possibility, we did rat liver perfusions at LPAM concentrations comparable to those in the clinical ILP study. Again, we detected GSH-LPAM conjugates in bile samples, although the cumulative biliary excretion amounted to only 0.2–0.4% of the LPAM dose (3.3 or 33 μmol LPAM, respectively). These data confirm that in the rat, GSH-mediated detoxification of LPAM is not an important mechanism in LPAM elimination. Alberts et al (32, 33) demonstrated that in humans, i.v.- or p.o.-administered LPAM is primarily eliminated by hydrolysis. This is also substantiated by our data on LPAM kinetics during ILP in cancer patients. Hydrolysis in the perfusion circuit most likely is the major mechanism for LPAM removal after the initial rapid uptake phase. Indeed, after the initial uptake, the LPAM disappearance rate in the rat liver perfusion was not greater than in the perfusion system without a liver (Fig. 7).

These findings make it unlikely that GSH conjugation of LPAM plays a role in tumor resistance against LPAM. Recently, other mechanisms for the cytotoxic protective effect of GSH have been postulated. GSH may: (a) preserve the integrity of DNA repair enzymes and thereby enhance the repair of LPAM-induced DNA damage (34); (b) scavenge lipid peroxidation products generated by LPAM (35, 36); and (c) enhance the hydrolytic dechlorination of LPAM, leading to the formation of inactive hydroxylated metabolites (37). Therefore, even if conjugation of LPAM to GSH would not be a relevant factor in drug resistance, GSH modulation may still have a significant impact on the efficacy of this drug.

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REFERENCES


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