Reduced Liver Uptake of Arterially Infused Melphalan during Retrograde Rat Liver Perfusion with Unaffected Liver Tumor Uptake

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Received April 23, 2002; accepted July 22, 2002

ABSTRACT

Isolated hepatic perfusion (IHP) with melphalan is used for patients with nonresectable metastases confined to the liver. To improve the efficacy of IHP and to reduce the toxicity to the liver, reversion (retrograde perfusion) of the bloodstream through the liver in a rat model was studied. For liver tumor induction male WAG/Rij rats were inoculated with CC531 cells, a colorectal tumor cell line. After 11 to 12 days the tumor-bearing rat livers were perfused by single-pass perfusion through either the portal (orthograde) or caval vein (retrograde) for different time periods. During perfusion melphalan (160 μM) was infused in the hepatic artery. Melphalan concentrations were measured by high-performance liquid chromatography. A rapid extraction of melphalan by the liver occurred in the first 5 min, reaching steady state after 10 to 20 min for both perfusion directions. The melphalan concentration of the outflow perfusate was significantly higher in the retrograde perfusion compared with the orthograde perfusion. The melphalan content of the tumor tissue was unaffected by perfusion direction at any time point. To the contrary, the melphalan uptake in liver tissue was strongly influenced: the melphalan content after 40-min retrograde perfusion was 12% of that after orthograde perfusion. The average tumor/liver concentration ratio was 6 for orthograde perfusion and 30 for retrograde perfusion. In conclusion, retrograde IHP with continuous melphalan infusion in the hepatic artery provides a high tumor uptake of melphalan with potentially reduced liver toxicity compared with orthograde IHP.

Isolated hepatic perfusion (IHP) is a treatment for patients with nonresectable metastases confined to the liver, allowing local treatment with high-dose chemotherapy. Because of this high exposure, promising tumor responses and survival rates have been observed (Alexander et al., 1998; Vahrmeijer et al., 2000). However, the percentage of patients with complete remission is still very limited and toxicity remains a major complication. Therefore, new strategies for drug administration during IHP to improve the efficacy of IHP and to reduce the toxicity to the liver are still needed.

Anatomical studies show that metastasizing tumor cells entering the liver via the portal vein develop into liver tumors, which are mainly vascularized by the hepatic artery (Sigurdson et al., 1987). Studies of the blood supply of liver tumors show that the hepatic artery provides up to 95% of their total blood flow (Wang et al., 1994); contrary to normal liver tissue, the portal vein plays a minor role in the blood supply of liver tumors (Taylor et al., 1978).

The liver sinusoids are perfused with blood from both the portal vein and hepatic artery. Studies of the blood supply of the rat liver show that the hepatic arterioles terminate in the first one-third of the sinusoids (zone 1) via an indirect or direct pathway (Fig. 1) (Rappaport, 1980; Watanabe et al., 1994). As a result, the arterial blood reaches all zones of the sinusoid in orthograde perfusion. However, during retrograde perfusion, i.e., using the portal vein for outflow instead of the caval vein, the arterial blood only reaches zone 1, the periportal parts (Pang et al., 1988). Thus, when IHP is performed in the retrograde way, whereas high-dose chemotherapy is only infused via the hepatic artery, the exposure of the whole liver to the cytostatic compound will be reduced, and so probably also liver toxicity. Therefore, retrograde perfusion potentially provides increased safety. As liver tumors obtain most of their blood supply from the hepatic artery, retrograde perfusion is not expected to affect the exposure of liver tumors to arterially administered cytostatic compounds.

To explore the consequences of altered flow direction on the...
treatment of liver tumors, we assessed the distribution and accumulation pattern of melphalan, the drug currently used by us in IHP (Vahrmeijer et al., 2000), infused in the hepatic artery during orthograde and retrograde single-pass liver perfusion in a rat liver model.

Materials and Methods

Chemicals. Melphalan was purchased from GlaxoSmithKline (Zeist, The Netherlands). Just before administration a melphalan solution (16.4 mM) was prepared by dissolving 1 mg of melphalan in 200 µl of 0.09% (w/v) hydrochloric acid, which was subsequently diluted with ice-cold Gelofusine (Vifor Medical, Sempach, Switzerland), a colloid solution of 4% modified gelatin in 0.9% NaCl, to a concentration of 480 µM; pH was adjusted to 7.4 with 1 N NaOH. During the experiment the melphalan solution was kept on room temperature.

Tumor Model. The CC531 tumor cell line used is a carcinoma of the colon, syngeneic for WAG/Rij rats (Marquet et al., 1984). The cells were cultured in medium that consisted of RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 2 mM l-glutamine, 50 µg/ml streptomycin, and 50 IU/ml penicillin (Invitrogen, Breda, The Netherlands). Cells were maintained by serial passage.

For tumor inoculation, exponentially growing cells were harvested by trypsinization, washed twice in phosphate-buffered saline, and adjusted to a suspension containing 1.0 × 10^7 tumor cells/ml. Male WAG/Rij rats with a mean weight of 278 g (range 244–315 g), 95% oxygen, 5% carbon dioxide) and kept at 37°C. The pH was adjusted to 7.4 with sodium hydrogen carbonate.

The liver was perfused from the portal vein (orthograde) or caval vein (retrograde) at a flow rate of 10 ml/min; the hepatic artery was perfused with perfusate at a flow rate of 1.0 ml/min to which melphalan was added by an infusion pump at a flow rate of 0.5 ml/min. Consequently, the melphalan concentration infused in the hepatic artery was 160 µM and 20.9 µM for the total perfusate. During the experiments bile flow remained constant.

Samples of the effluent of the caval vein or portal vein were taken, starting at the moment of infusion of melphalan. At the end of the perfusion a washout was performed with 30 ml of ice-cold saline during 3 min. After the liver was excised, the very solid tumors were excised and weighed. All samples were stored at −70°C until analysis.

High-Performance Liquid Chromatography (HPLC) Analysis. The concentration of melphalan in the effluent samples as well as in tumor and liver tissue samples was measured using an HPLC assay. The HPLC apparatus consisted of a pump (P1000), an autosampler (AS3000), a 100-µl injection loop, and a UV-detector (UV100) (all Spectra Series; Thermo Separation Products, Fremont, CA). Propylparaben was used as the internal standard. Methanol (HPLC-grade) was obtained from Biosolve ( Valkenswaard, The Netherlands), perchloric acid (70% w/v) from Merck (Darmstadt, Germany), and acetic acid (100% v/v, extra pure) from Riedel de Haën (Seelze, Germany). Water was purified by reverse osmosis. The stock solution of melphalan was prepared in 2% (v/v) acetic acid in methanol, and the stock solution of propylparaben was prepared in demi-water.

Injections (25 µl) were made on a Novapak C18 column (100 × 8 mm, d<sub>t</sub> = 4 µm; Waters, Milford, MA), protected by a Novapak C18 precolumn (10 × 8 mm, d<sub>t</sub> = 5 µm; Waters). The column was used at ambient temperature. The eluent comprised 46.5% (v/v) acetonitrile, 53.5% (v/v) water, and 0.03% (v/v) perchloric acid. The eluent flow rate was 2.0 ml/min and the UV detection wavelength was 262 nm. The dynamic range of detection of the HPLC assay was 0.16 to 81.19 µM. The interday coefficients of variation of the assay were 6.8% to 11% for the concentration range detected. Mean extraction efficiency was 95%.

Fifty microliters of perfusate, 25 mg of tumor tissue, or 50 mg of liver tissue was pipetted or weighed into a polypropylene microtube on ice. Next, 25 µl of the internal standard solution (2 µg/ml propylparaben in water) and 100 µl of absolute methanol (−80°C) were added. After vortex mixing all samples for 30 s, an ultrasonic treatment (liver, 10 min; tumor, 15 min) was performed at 0°C for only the tissue samples to extract all drug. After centrifuging at 11,000g for 5 min at 4°C the supernatant was transferred into an injection vial.

Statistical Analysis. All data were analyzed with SPSS statistical software (version 9.0 for Windows; SPSS, Chicago, IL). Correlation coefficients were calculated using the paired Student’s t test. For melphalan in serum and in perfusate we fitted a nonlinear mixed effects model, using the pharmacokinetic function C = (1 − exp(−a · t)) · (1 − exp(−b · t)), with C as random effects for the rats (Lindstrom and Bates, 1990). The parameter C is the asymptote of the function, the ultimate value of melphalan in serum or perfusate. We tested for differences in C between the melphalan concentration of the outflow perfusate after orthograde and retrograde perfusion by also fitting separate values for C for each group and comparing the models with...
the likelihood ratio test. A p value < 0.05 was considered statistically significant.

**Results**

We hypothesized that liver uptake of arterially infused melphalan will be reduced during single-pass retrograde IHP without affecting the tumor uptake compared with orthograde IHP. To test this, tumor-bearing rat livers were perfused in both perfusion modes for different periods of time. We used the same perfusion medium as during our clinical IHP program, Gelofusine, a modified gelatin plasma volume expander. At 10 μg/ml melphalan 29% was protein bound as determined by ultrafiltration. The melphalan concentration in the perfusate was based on the dose currently used in the clinical IHP (Vahrmeijer et al., 2000).

HPLC analysis revealed that tumor uptake of arterially infused melphalan was indeed unaffected by perfusion direction (Fig. 2A): there was no difference in melphalan content of the tumor tissue at any time point. The tumor content seemed to increase roughly linearly for both perfusion directions: approximately 4.5 nmol/min/g tissue (Table 1).

To the contrary, the melphalan uptake by liver tissue was strongly influenced by perfusion direction: the melphalan level after retrograde perfusion was much lower than after orthograde perfusion (Fig. 2B). The liver melphalan content seemed to increase linearly in the orthograde perfusion (0.8 nmol/min/g tissue) (Table 1). In the retrograde perfusion mode maximum melphalan content and steady state in the liver were already reached after 10 min (Fig. 2B).

The uptake by tumor tissue was much higher than by liver tissue in both perfusion directions (Fig. 3). The average tumor/liver uptake ratio from all separate time points was 6 for orthograde perfusion and 30 for retrograde perfusion.

**Table 1**

| Rate of melphalan uptake in tumor and liver tissue for both perfusion directions |
|---------------------------------|------------------|
| Melphalan Uptake                | R                |
| nmol/g tissue/min               |                  |
| Orthograde                      |                  |
| Tumor                           | 4.33             | 0.96          |
| Liver                           | 0.82             | 0.99          |
| Retrograde                      |                  |
| Tumor                           | 4.82             | 0.99          |
| Liver                           | —                | —             |

* No linear relationship between time and the uptake of melphalan.

The extraction of melphalan by the liver in both perfusion modes was determined by analyzing the melphalan concentration in the outflow perfusate (Fig. 4). A rapid extraction of melphalan occurred during the first 5 min, reaching steady state after 10 to 20 min for both perfusion directions. Apparently, the reduced melphalan uptake by the liver in the retrograde perfusion mode resulted in a significantly higher melphalan concentration in the outflow perfusate than for the orthograde perfusion.

When melphalan tissue levels were converted to melphalan concentrations (assuming 1 g of tissue corresponds to 1 ml), the melphalan concentrations in tumor approached the melphalan inflow concentration (160 μM) in the hepatic artery after 40 min (Fig. 5). The melphalan concentrations in the orthograde perfused liver also approached the inflow concentration in the liver sinusoid of 20.9 μM; the melphalan concentrations in retrograde perfused liver remained significantly lower.

**Discussion**

To maximize the effect of tumor treatment, melphalan exposure of the tumor should be as high as possible. However, at the same time toxicity to healthy cells increases with drug dose and therefore is dose-limiting. The concept of IHP is based on local administration of high drug doses to the liver that would be lethal if administered systemically but would not cause fatal hepatotoxicity in IHP. This study was performed to explore possibilities for further improvement of the current clinical IHP with melphalan (Vahrmeijer et al., 2000). The presented results clearly show that retrograde perfusion would not cause fatal hepatotoxicity in IHP. This study was performed to explore possibilities for further improvement of the current clinical IHP with melphalan (Vahrmeijer et al., 2000). The presented results clearly show that retrograde perfusion
perfusion has a major advantage compared with orthograde perfusion, because retrograde perfusion markedly reduces the liver uptake of melphalan, and thus liver toxicity, without affecting the tumor uptake of melphalan.

Melphalan most likely exerts its cytotoxic effect, i.e., cell death, through the formation of interstrand and intrastrand DNA cross-links and DNA-protein cross-links by alkylation (Millar et al., 1986; Hansson et al., 1987). A linear correlation has been observed between the melphalan concentration and the level of DNA adducts: Tilby and colleagues showed this both in vitro as well as in peripheral blood mononuclear cells of patients undergoing high-dose melphalan therapy (Tilby et al., 1993; Frank et al., 1996). Therefore, the cellular content of melphalan may be used as a biomarker for its cytotoxicity.

Our hypothesis that the melphalan uptake by the tumor would be unaffected by perfusion direction as long as the melphalan was infused in the hepatic artery is supported by our current results. We expected the uptake of melphalan by the liver, and thus most likely toxicity, to be less in retrograde single-pass perfusion, because it would probably only reach zone 1. The uptake in liver tissue was indeed strongly affected by flow direction: the level after retrograde perfusion was only 20% that of normal perfusion direction, indicating that melphalan under retrograde flow conditions most likely reaches only the portal branch and hepatocytes of the liver sinusoids. Because these cells account for a maximum of one-third of the liver sinusoid (Watanabe et al., 1994), the tissue uptake ratio probably reflects the mass ratio of the tissue regions that are accessible for arterial input after orthograde or retrograde perfusion. The strongly reduced melphalan uptake in the retrograde perfused liver accounted for the higher melphalan concentration of the outflow perfusate in the retrograde perfusion: much less liver tissue was reached by the melphalan. Therefore, the pattern of melphalan toxicity proportionately affects the melphalan levels in the liver, in contrast to liver tumors, which is strongly affected by flow direction. Thus, our findings confirm that hepatic artery infusion of melphalan leads to a more selective tumor exposure (Chang et al., 1987; Kemeny et al., 1987; Hohn et al., 1989). The melphalan uptake in tumor tissue was much higher than in liver tissue for both perfusion directions.

Melphalan metabolism (conjugation and hydrolysis) was not assessed in this study. Our previous data on rat hepatic glutathione conjugation show that melphalan conjugates accounted for 0.2 to 0.4% of the melphalan dose (Vahrmeijer et al., 1996). In bile and perfusate samples from patients treated by isolated hepatic perfusion with high-dose melphalan (200 mg) for liver metastases none of the melphalan conjugates were present at a detectable level in bile and perfusate samples (Vahrmeijer et al., 1996).

In addition to isolated hepatic perfusion, the cytostatic effects and the pharmacokinetics of melphalan have been assessed extensively in isolated limb perfusion studies. For example, Wu et al. (1997) developed a physiological pharmacokinetic model to describe the concentration-time profile of melphalan in perfusate and tissues in the isolated perfused rat hind limb during perfusion with melphalan.

In conclusion, retrograde IHP with continuous melphalan infusion in the hepatic artery provides a high tumor uptake of melphalan and probably reduced liver toxicity compared with orthograde perfusion. Because selective tumor exposure to melphalan is the sole objective of IHP, these findings justify the further exploration of retrograde liver perfusion. Preclinical studies involving retrograde IHP on pigs have already shown that retrograde IHP is technically feasible (van IJken et al., 1998; Rothbarth et al., 2001). Therefore, a phase I clinical study will be started in our institute in the near future.

Acknowledgments

We thank Dr. K. S. Pang (Department of Pharmacology, University of Toronto, ON, Canada) for teaching the surgical techniques.

References


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