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Alexander L. Vahrmeijer · Jan Hein van Dierendonck
Jan Schutrups · Cornelis J.H. van de Velde
Gerard J. Mulder

Potentiation of the cytostatic effect of melphalan on colorectal cancer hepatic metastases by infusion of buthionine sulfoximine (BSO) in the rat

Enhanced tumor glutathione depletion by infusion of BSO in the hepatic artery

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Abstract *Purpose*: Glutathione (GSH) plays an important role in the resistance of tumors to cytostatics. Therefore, depletion of GSH by the GSH synthesis inhibitor buthionine sulfoximine (BSO) has been proposed to enhance the efficacy of certain anticancer agents. We studied the effect of BSO in rats bearing intrahepatically implanted tumors of the CC531 colorectal cancer cell line on the antitumor activity of melphalan (L-PAM). Since these liver tumors tend to derive most of their blood supply from the hepatic artery, we evaluated whether delivery of BSO into the hepatic artery would more selectively decrease GSH levels in the implanted tumor tissue as compared with normal liver and extrahepatic tissues. *Methods*: Tumor-bearing rats were treated with a 24-h continuous infusion of 0.375 mmol/kg BSO via the jugular vein, immediately followed by a bolus L-PAM (15 μmol/kg; 4.5 mg/kg) infusion via the hepatic artery. Laparotomy was performed on day 14 and 28 after treatment for measurement of the liver tumors. For the evaluation of locoregional administration of BSO, a 24-h continuous infusion of 0.375 mmol/kg BSO was delivered into either the hepatic artery, the portal vein, or the jugular vein in freely moving rats and GSH levels in the tumor, liver, kidney, lung, heart, bone marrow, and blood were measured. *Results*: BSO infusion via the jugular vein increased the antitumor efficacy of L-PAM injected into the hepatic artery 2-fold as determined at 14 days after treatment. Although infusion of BSO via the hepatic artery depleted GSH more severely in the tumor as compared with jugular vein or portal vein administration, the additional effect was only slight (10%). No difference was observed in any other tissue. *Conclusion*: GSH depletion increased the cytostatic efficacy of L-PAM 2-fold in vivo as determined at 14 days after treatment. Hepatic artery infusion of BSO translated into a statistically significant, but probably not therapeutically relevant, increase in tumor GSH depletion as compared with the other routes of BSO administration.

Key words Drug resistance · Melphalan · Extrahepatic organs

Abbreviations *GSH* Glutathione · *BSO* Buthionine sulfoximine · *L-PAM* Melphalan · *FUdR* Fluorodeoxyuridine

A.L. Vahrmeijer (✉) · J.H. van Dierendonck
J. Schutrups · C.J.H. van de Velde
Department of Surgery, Leiden University Medical Center,
Building P1-Q, P.O. Box 9600, 2300 RC,
Leiden, The Netherlands
Tel.: +31-71-5262309; Fax: +31-71-5248213
E-mail: vahrmeyer@surgery.azl.nl

A.L. Vahrmeijer · J. Schutrups · G.J. Mulder
Division of Toxicology,
Leiden/Amsterdam Center for Drug Research,
Leiden University, Leiden, The Netherlands

Introduction

The tripeptide glutathione (GSH) is involved in many cellular processes, several of which are important in the resistance of tumors to cytostatics [17, 18]. Thus, conjugation of GSH to a variety of potentially harmful compounds, catalyzed by GSH transferases, results in conjugates that are easily excreted, for instance, via the kidneys, as mercapturates [27]. The GSH level also determines the activity of several proteins such as the multidrug-resistance protein (MRP), which is involved in the transport of (chemotherapeutic) drugs or their GSH conjugates [30]. A major difference between tumor cell lines that are sensitive or resistant to various anticancer agents in vitro is indeed their cellular GSH concentration [4, 11, 21]. Moreover, in most of these resistant cell lines, drug sensitivity can be restored by treatment with buthionine sulfoximine (BSO), a relatively nontoxic inhibitor of GSH synthesis [10]. Therefore, GSH depletion by BSO pretreatment has been

proposed as a strategy to increase the cytotoxic efficacy of various anticancer drugs, such as alkylating agents and platinum compounds.

At the Leiden University Medical Center, selected patients with inoperable hepatic metastases from colorectal cancer are treated by a 1-h recirculating perfusion of their livers with a dose of melphalan (L-PAM) that would be fatal if given systemically [28, 29]. Although impressive tumor responses are obtained, complete remissions have thus far been rare. Therefore, for further improvement in the tumor response a reduction in tumor GSH levels by pretreatment with BSO prior to perfusion with L-PAM seems a logical choice. Using clinically relevant concentrations of BSO, we found that GSH depletion increased the cytotoxicity of L-PAM approximately 2- to 3-fold in five human (HT-29, LS-180, LOVO, SW837, SW1116) colorectal cancer cell lines in vitro (Vahrmeijer et al., submitted for publication). Sufficient depletion of tumor GSH in vivo or in an organ is reached only when the block of GSH synthesis is sustained for at least 24 h, i.e., after continuous or repeated systemic delivery of BSO [9]. However, this may result in GSH depletion in the normal liver tissue and in critical organs such as the kidney and bone marrow. GSH depletion in the latter organs by itself does not seem to cause serious complications. Recently, in two phase I clinical trials, patients with various malignancies were treated first with BSO alone (given as a 30-min infusion every 12 h for 3 days); 1 week later the same BSO schedule was combined with a fixed dose of 15 mg/m^2 L-PAM given at 48 h [2, 20]. In both studies the BSO doses tested ranged from 1.5 to 17 g/m^2 and the only toxicity attributable to BSO was grade I or II nausea/vomiting in 50% of the patients. However, in combination with L-PAM, at a dose as low as 7.5 g/m^2, dose-related neutropenia required an L-PAM dose reduction to 10 mg/m^2. Therefore, in combination with the stress of the liver perfusion surgery as well as the extremely high dose of L-PAM used in our human perfusion treatment, more serious complications could eventually occur in BSO-pretreated patients.

Because liver metastases tend to derive their blood supply mainly from the hepatic artery [1, 31], the question arose as to whether a more selective GSH depletion in these metastases could be obtained by direct delivery of BSO into the hepatic artery. This seems to be supported by the findings of Sigurdson and co-workers [23], who reported a 15.5-fold increase in tumor concentrations of fluorodeoxyuridine (FUdR) after infusion of this drug into the hepatic artery as compared with the portal vein. Therefore, in a rat model for hepatic metastases established by subcapsular inoculation of cultured CC531 rat colorectal cancer cells we evaluated whether a continuous BSO infusion delivered via the hepatic artery could more or less selectively deplete tumor GSH as compared with jugular or portal vein infusions. A dose of BSO was used that was shown to increase significantly the antitumor effect of L-PAM when given via the jugular vein.

Materials and methods

Tumor model

The CC531 tumor used is a dimethylhydrazine-induced adenocarcinoma of the colon, syngeneic for WAG/Rij rats [16]. An established cell line was maintained in culture in RPMI 1640 (Dutch modification) medium supplemented with 10% (v/v) fetal calf serum, 2 m*M* L-glutamine, streptomycin at 50 μg/ml, and penicillin at 50 IU/ml (all from Gibco, Life Technologies, Breda, The Netherlands). Exponentially growing cells were harvested by trypsinization, and 5×10^5 cells in 50 μl 0.9% NaCl (saline) were subcapsularly injected into the left and right main lobes and into the right accessory lobes of the liver of inbred male WAG/Rij rats (Harlan/CPB, Zeist, The Netherlands). At the time of tumor cell inoculation the weight of the rats was approximately 250 g. All animals had free access to food and water, and an alternating 12-h light/dark cycle was maintained in the animal room. Treatment of the rats with BSO was started at 9 days after tumor cell inoculation; at that time point the mean tumor cross-sectional area was 22 mm^2 (estimated by caliper measurements and calculated as $\pi \times 0.25 \times$ *largest diameter* $\times$ *pendicular diameter*). The animal welfare committee of the Leiden University Medical Center approved the animal experiments. All surgical procedures were carried out using diethyl ether anesthesia.

Effect of L-PAM on CC531 liver metastases

To evaluate whether pretreatment with BSO (L-buthionine-[*S*,*R*]-sulfoximine; Sigma, St. Louis, Mo., USA) increased the efficacy of L-PAM, CC531 tumor-bearing rats were treated with a 24-h continuous infusion of 0.375 mmol/kg BSO via the external jugular vein as described below, immediately followed by a bolus L-PAM (Wellcome Pharmaceuticals B.V. Utrecht, The Netherlands) infusion via the hepatic artery. The dose of 15 μmol/kg (4.5 mg/kg) L-PAM was given over 3 min into the hepatic artery ($n = 20$) at 10 days after tumor cell inoculation; controls received saline ($n = 3$). After we had made a midline abdominal incision, a catheter was inserted into the gastroduodenal artery with the tip in the hepatic artery. During the 3-min bolus L-PAM or saline (control) infusion the common hepatic artery was clamped to prevent retrograde flow into the coeliac axis and the aorta [15]. Eight rats were pretreated with a 24-h continuous infusion of 0.375 mmol/kg BSO via the jugular vein immediately before L-PAM infusion as described above, and eight rats received saline according to the same procedure. After the L-PAM infusion, BSO-infusion minipumps and catheters were removed and the abdomen was closed. Rats were weighed twice a week and on day 14 after L-PAM treatment, laparotomy was performed for measurement of the liver metastases. Rats were eutharized at day 28 after treatment and liver metastases were measured. The tumor growth index was defined as the tumor cross-sectional area at day 14 or 28 after L-PAM treatment divided by the tumor cross-sectional area at day 0 (day of L-PAM treatment).

Effect of BSO on tissue GSH content in vivo

For determination of the effect of prolonged BSO treatment on intracellular GSH content, rats were treated for 24 h with 0.375 mmol/kg BSO given as a continuous infusion via the jugular vein ($n = 8$), hepatic artery ($n = 8$), or portal vein ($n = 7$). For the continuous infusion, Alzet osmotic minipumps (model 2001D, Alza Corporation, Palo Alto, Calif., USA) were used and filled with BSO dissolved in heparinized saline. The pump was placed subcutaneously on the back of the rat and attached to a cannula, which was inserted into the gastroduodenal branch of the hepatic artery, into the pyloric branch of the portal vein, or into the external jugular vein [15]. Control rats received a sham operation ($n = 8$). Blood was collected from the abdominal aorta at 24 h

after the start of the BSO infusion using diethyl ether anesthesia, after which the rats were killed by opening of the thoracic cavity. For GSH analysis in whole blood, 1.9 ml 1 m*M* ethylenediaminetetraacetic acid (EDTA) was added to 0.1 ml blood and this was subsequently mixed with 2 ml 0.8 *M* perchloric acid. Lung, heart, kidney, liver, and tumor tissue samples were taken and immediately frozen in liquid nitrogen. Femurs were excised and bone marrow was collected by flushing of the femurs with ice-cold saline. The collected cell suspension was centrifuged for 10 min at 250 *g*; after lysis of red blood cells by resuspension of the pellet at 37 °C for 5 min in lysis buffer (150 m*M* NH_4Cl, 10 m*M* $KHCO_3$, and 0.1 m*M* EDTA, pH 7.4) the suspension was divided into halves. After centrifugation the pellets containing the bone marrow cells were either lysed in 5% (w/v) 5-sulfosalicylic acid (Janssen Chimica, Geel, Belgium) for GSH analysis or lysed in 1 *M* NaOH for protein analysis. All samples were stored in liquid nitrogen until analysis.

GSH analysis

GSH in tissue biopsies and bone marrow samples was assayed by the colorimetric method of Ellman [6], with some previously reported modifications [26]. The GSH content was expressed in micromoles per gram of tissue or in micromoles per gram of protein in the case of bone marrow. Total protein was determined by the method of Lowry et al. [14]. GSH concentrations in blood samples were determined using a high-performance liquid chromatography assay as previously reported [19]. Blood GSH was expressed in millimoles per mole of hemoglobulin (Hb). Hb was measured in heparinized blood samples using a Ciba Corning 288 blood-gas analyzer.

Results

Antitumor activity of L-PAM in vivo

After inoculation into the liver the CC531 cells grow very rapidly into large solid tumors, which reach a cross-sectional area of 22 mm^2 within 9–10 days [15]. Treatment of these experimental liver metastases with 15 μmol/kg (4.5 mg/kg) L-PAM given as a 3-min infusion via the hepatic artery resulted in retardation of tumor growth. GSH depletion by a continuous infusion of 0.375 mmol/kg BSO over 24 h into the jugular vein alone had no effect on tumor growth (data not shown); however, pretreatment of the rats with BSO resulted in a 2-fold increase in the antitumor efficacy of L-PAM as measured at 14 days after treatment (Table 1). At day 28 there was no longer a statistically significant difference between the antitumor effect of L-PAM with versus without BSO pretreatment. The body weight decreased after L-PAM treatment in both control and BSO-pretreated rats (Fig. 1). However, BSO pretreatment in the L-PAM-treated rats resulted in an (statistically significant) additional decrease (maximally 22 g at day 8 after L-PAM treatment) in body weight.

Table 1 Effect of a 3-min infusion of L-PAM into the hepatic artery on established CC531 tumors in the liver of control or GSH-depleted rats (pretreatment with a 24-h infusion of 0.375 mmol/kg BSO via the jugular vein). The tumor growth index was defined as the tumor cross-sectional area at day 14 or 28 after L-PAM treatment divided by the tumor cross-sectional area at day 0. Data represent mean values ± SD

Treatment	Number of rats	Tumor growth index	
		Day 14	Day 28
Control	3	5.6 ± 3.5	9.3 ± 3.6
L-PAM	4	2.5 ± 1.1*	3.6 ± 2.1*
L-PAM, v. Jug pump NaCl	8	2.4 ± 1.9*	2.5 ± 4.2*
L-PAM, v. Jug pump BSO	8	1.2 ± 1.0**	2.2 ± 2.1*

* $P < 0.05$ versus the control group; ** $P < 0.05$ versus all other groups on the same day (LSD, one-way ANOVA)

GSH levels in tumor and normal tissues after BSO infusion via different routes

In an attempt selectively to decrease GSH levels in colorectal cancer hepatic metastases we compared the effect of a 24-h continuous infusion of BSO via the hepatic artery, portal vein, or jugular vein, respectively, on GSH levels in tumor and normal tissues using implantable osmotic minipumps. Interestingly, the GSH content of the tumors was much lower than that of the liver (1.24 versus 5.9 μmol/g tissue). BSO treatment decreased the tumor and hepatic GSH content by about the same extent, approximately 60%. However, since the hepatic content was much higher, the minimal level also differed considerably during maximal depletion (0.47 μmol/g in the tumor versus 2.3 μmol/g in the liver). For tumor GSH there was a more pronounced decrease when BSO was infused via the hepatic artery as compared with portal or jugular vein infusion; hepatic artery infusion resulted in a statistically significant enhancement of only tumor GSH depletion by an additional 0.12 μmol/g, which was 10% of the control value ($P = 0.02$; Table 2, Fig. 2). The degree of GSH depletion after BSO infusion in the extrahepatic organs was greatest for the kidney (67%), tumor (62%), and liver (61%), followed by the bone marrow (47%), lung (36%), and heart (29%; Table 2). No GSH depletion was observed in whole blood.

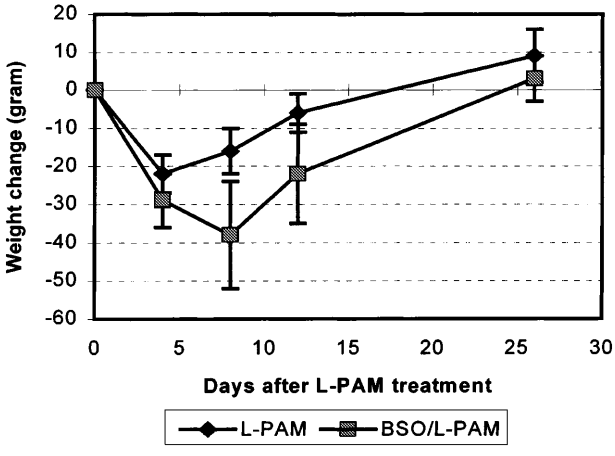

Fig. 1 Average changes in the body weight of rats after L-PAM treatment. The mean body weight on day 0 was 252 ± 7 g (range 243–261 g). Data represent mean values ± SD

Table 2 GSH levels determined in several tissues from rats treated with a 24-h infusion of 0.375 mmol/kg BSO via the hepatic artery ($n = 8$), portal vein ($n = 7$), or jugular vein ($n = 8$), respectively. Except for blood GSH, all other (tissue, bone marrow) GSH levels were significantly lower ($P < 0.05$) in BSO-treated rats. The P value denotes the significant differences observed in tissue GSH among the BSO-treated groups (LSD, procedure). Only the P value for tumor GSH reached statistical significance, indicating that hepatic artery infusion of BSO resulted in a further decrease in tumor GSH as compared with portal or jugular vein administration. Data represent mean values ± SD

	BSO infusion in			P value	Control
	Hepatic artery	Portal vein GSH level	Jugular vein		
Tumor (μmol/g)	0.47 ± 0.1*	0.61 ± 0.1	0.60 ± 0.1	0.02	1.2 ± 0.5
Liver (μmol/g)	2.3 ± 0.6	2.0 ± 0.3	2.4 ± 0.2	0.17	5.9 ± 0.6
Lung (μmol/g)	0.86 ± 0.2	0.85 ± 0.1	0.90 ± 0.1	0.75	1.4 ± 0.2
Heart (μmol/g)	0.99 ± 0.2	0.98 ± 0.1	0.99 ± 0.3	1.00	1.4 ± 0.1
Kidney (μmol/g)	0.59 ± 0.1	0.54 ± 0.2	0.50 ± 0.1	0.54	1.8 ± 0.4
Bone marrow (μmol/g protein)	7.7 ± 0.8	7.3 ± 0.2	7.1 ± 0.8	0.56	14.6 ± 1.0
Blood (mmol/mol Hb)	137 ± 30	136 ± 24	140 ± 34	0.97	139 ± 27

* $P = 0.02$ (LSD, one-way ANOVA)

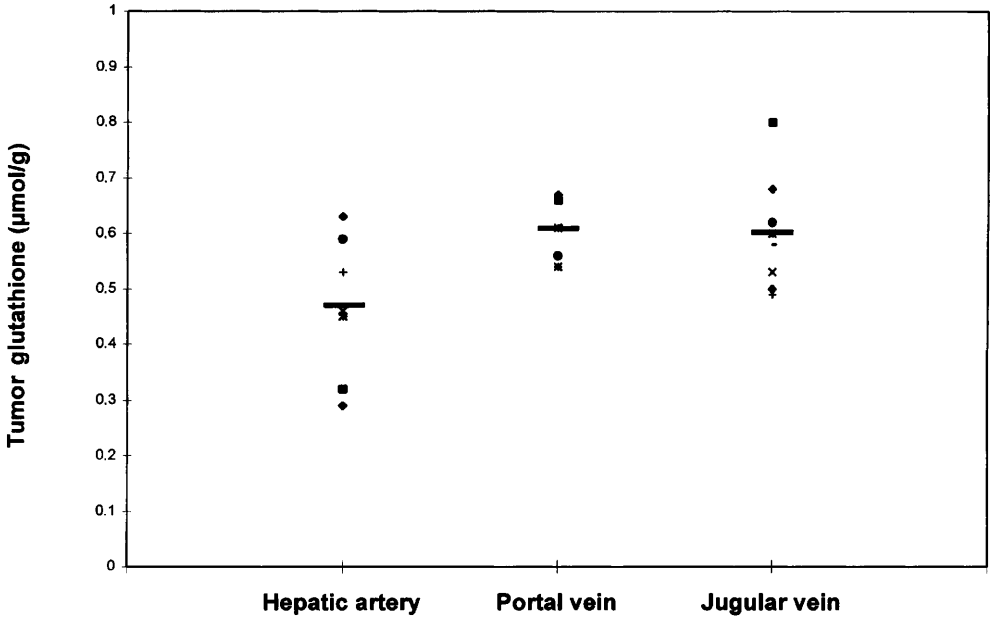

Fig. 2 Tumor GSH content determined after a 24-h continuous infusion of 0.375 mmol/kg BSO via the hepatic artery ($n = 8$), portal vein ($n = 7$), or jugular vein ($n = 8$), respectively (each *dot* represents one rat; the *bar* indicates the mean value). Hepatic artery infusion resulted in a statistically significant enhancement of tumor GSH depletion as compared with portal or jugular vein administration of BSO (LSD, one-way ANOVA, $P = 0.02$)

Discussion

GSH depletion resulted in the increased killing of CC531 cells by L-PAM in vitro (data not shown). We now confirm this finding in an in vivo model for CC531 colorectal cancer hepatic metastases; pretreatment of rats with a continuous infusion of BSO into the jugular vein increased the therapeutic efficacy of L-PAM 2-fold as determined at 14 days after treatment. Similar increases in the antitumor efficacy of L-PAM in mice in vivo by BSO pretreatment were reported for ovarian cancer, leukemia, melanoma, and medulloblastoma [8, 12, 21, 22, 24]. By day 28, however, this difference in the antitumor efficacy of L-PAM had virtually disappeared. Apparently this resulted from a very rapid growth of surviving CC531 tumor cells after BSO/L-PAM treatment. In a search for methods that would further increase the therapeutic efficacy of L-PAM by GSH depletion while circumventing a further increase in systemic toxicity (as reflected by body weight loss), we evaluated whether regional administration of BSO into the hepatic artery would increase tumor GSH depletion selectively.

Hepatic artery infusion of BSO statistically significantly enhanced GSH depletion only in the tumor as compared with portal or jugular vein administration of BSO. However, although statistically significant, this 10% additional decrease in tumor GSH would most likely be far too limited to increase the antitumor activity of L-PAM significantly. It is possible that an advantage of hepatic artery infusion of drugs can be obtained only when these drugs display a relatively high degree of regional extraction [5]. Interestingly, the cytostatic drug FUdR shows a 15.5-fold increase in tumor

concentration when infused into the hepatic artery as compared with portal vein administration [23]; indeed, FUdR is extremely efficiently extracted by the liver [5, 7]. Therefore, that there is so little difference between hepatic artery administration of BSO, on the one hand, and jugular or portal vein administration, on the other, might be due to a low degree of hepatic extraction of the only slightly lipid-soluble BSO. In humans, BSO is indeed a low-clearance drug [20] with a relatively low volume of distribution, suggesting that its hepatic extraction may be low.

The degree of GSH depletion observed after BSO infusion via either the hepatic artery, portal vein, or jugular vein in the various tissues was very similar to that reported by other investigators [13, 25]. However, whereas single high-dose injections of BSO seem to induce a strong depletion of bone marrow GSH [13, 22], this is observed to a lesser extent after continuous infusion (present study) and not at all after BSO administration via drinking water [22]. These results suggest that continuous administration of BSO by infusion at low dose rates in clinical trials of tumor GSH modulation may minimize the risk for increased bone marrow toxicity after L-PAM treatment, as seemed to be observed in the two aforementioned clinical phase I studies [2, 20]. In these clinical studies, BSO was given as a 30-min infusion every 12 h for 3 days. However, more recently, patients were treated by a 24- to 72-h continuous intravenous infusion of BSO followed by intravenous L-PAM, and this study [3] resulted in toxicities similar to those seen in the aforementioned studies on bolus BSO administration.

Whereas GSH levels in the tissues were significantly reduced after BSO infusion, no decrease was observed in blood GSH, indicating that blood GSH levels may not be appropriate monitors of the tissue or organ GSH availability in vivo.

Given these in vivo results, it is most likely that the administration of BSO via the hepatic artery in patients with colorectal cancer hepatic metastases prior to isolated hepatic perfusion with L-PAM would not translate into a therapeutically relevantly lower level of tumor GSH at the start of perfusion as compared with the systemic administration of BSO by either continuous infusion or multiple intravenous injections.

In conclusion, GSH depletion enhanced the therapeutic efficacy of L-PAM 2-fold. Locoregional administration of 0.375 mmol/kg BSO over 24 h directly into the hepatic artery in our CC531 rat model for colorectal cancer hepatic metastases led to considerable GSH depletion, especially in the tumor, liver, and kidney. However, hepatic artery infusion showed very little advantage over jugular or portal vein infusion as far as depletion of tumor GSH was concerned.

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