Title Page

SYNTHESIS AND CHARACTERIZATION OF SOME NEW PHASE II METABOLITES OF THE ALKYLATOR BENDAMUSTINE AND THEIR IDENTIFICATION IN HUMAN BILE, URINE, AND PLASMA FROM PATIENTS WITH CHOLANGIOCARCINOMA

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Running Title Page

Phase II metabolites of bendamustine

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Abbreviations used are:

CID, collision induced dissociation; CLL, chronic lymphocytic leukemia; COSY, correlation spectroscopy; GST, glutathione S-transferase; HMBC, heteronuclear multiple bond connectivity; HMQC, heteronuclear multiple-quantum coherence; NHL, non-Hodgkin’s lymphoma; TFA, trifluoroacetic acid.
Abstract

The alkylating agent bendamustine is currently in phase III clinical trials for the treatment of hematological malignancies and breast, lung, and gastrointestinal tumors. Renal elimination mainly as parent compound is thought to be the primary route of excretion. Because polar biliary conjugates were expected metabolites of bendamustine, three cysteine S-conjugates were synthesized, purified by quantitative high-performance liquid chromatography (HPLC) and characterized by nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS). HPLC assays with MS as well as fluorescence detection of bile, urine, and plasma after single-dose intravenous infusion of bendamustine 140 mg/m² in five subjects with cholangiocarcinoma indicated the existence of these phase II metabolites, which were identified as cysteine S-conjugates by comparison with the previously characterized synthetic reference standards. The sum of the three cysteine S-conjugates of bendamustine determined in human bile and urine was 95.8 and 26.0 %, respectively, expressed as mean percentage of the sum of parent compound and identified metabolites. The percentage of administered dose recovered in urine as cysteine S-conjugates ranged from 0.9 to 4.1 %, whereas the total percentage of administered dose excreted in urine as parent drug and seven metabolites ranged from 3.8 –16.3 %. The identification of cysteine S-conjugates provide evidence that a major route of bendamustine metabolism in humans involves conjugation with glutathione (GSH). Results indicate the importance of phase II conjugation in the elimination of bendamustine beside phase I metabolism and hydrolytic degradation and require further investigation.
Bendamustine (4-{5-[bis-(2-chloro-ethyl)-amino]-1-methyl-1H-benzoimidazol-2-yl}-butyric acid, 1, Figure 1), is a promising bifunctional alkylating agent containing a heterocyclic nucleus that induces more long-lasting DNA double-strand breaks than other alkylating drugs (Strumberg et al. 1996). The compound was first synthesized in 1963 with anticipated purine based antimetabolic activity and has been shown preferentially cytotoxic activity in the treatment of hematological malignancies, including NHL, CLL, and multiple myeloma, as well as breast cancer (Höffken et al., 1998; Heider and Niederle, 2001; Kath et al., 2001; Bremer, 2002; Konstantinov et al., 2002; Weidmann et al., 2002; Aivado et al., 2002; Pönisch and Niederwieser, 2002). Moreover, anticancer activity was reported in advanced small cell lung cancer and head or neck tumors (Reck et al., 1998; Rahn et al. 2001). 1 has been approved and used in Germany for about 30 years, but has not been approved outside of Germany. Currently, it is being studied as an investigational drug in the United States.

There is limited information on the metabolism and disposition of 1. To date, only one metabolite resulting from biotransformation in humans has been identified as N-demethyl bendamustine (4-{5-[bis-(2-chloro-ethyl)-amino]-1H-benzoimidazol-2-yl}-butyric acid, 7, Figure 2) (Matthias et al., 1995; Preiss et al., 1998). An additional metabolite was thought to be formed via beta-oxidation of the butyric acid moiety as demonstrated by MS data but has not yet been structurally characterized. Own structural investigation did not confirm formation of the assumed 2-hydroxy bendamustine. Hence, this metabolite was tentatively named oxidized bendamustine (8, Figure 2). Similarly to other mustards containing the bis-chloroethyl moiety, two products of chemical hydrolysis namely monohydroxy and dihydroxy bendamustine (4-{5-[bis-(2-chloro-ethyl)-(2-hydroxy-ethyl)-amino]-1-methyl-1H-benzoimidazol-2-yl}butyric acid, 5 and 4-{5-[bis-(2-hydroxy-ethyl)-amino]-1-methyl-1H-benzoimidazol-2-yl}-butyric acid, 6, Figure 2) have been detected (Preiss et al., 1985). The mean percentage of the administered dose excreted in urine as the sum of 1, 5, 6, 7, and 8 was 20% as shown in 12 cancer patients with normal renal function (Teichert et al., 2003). This finding provides strong
evidence that a considerable part of the administered dose is eliminated by nonrenal mechanism and is compatible with earlier results obtained from animal experiments that revealed a biliary excretion of nearly 42% for 1 in rats within 2 h after i.v. administration of radioactively labeled 1 (Bezek et al., 1991). On the other side, 30% of the administered radioactivity was excreted via urine.

To understand the metabolic fate of 1, we conducted the studies on the metabolism and disposition of 1 in five patients with cholangiocarcinoma. In particular, we sought to determine whether the GSH detoxification mechanism plays a role in the metabolism of this alkylating agent. In vitro spontaneous and GST mediated reaction of melphalan and chlorambucil both structurally related to 1 (Figure 1) with GSH has been investigated as reported in numerous articles (Dulik et al., 1986, 1990; Ciaccio et al., 1990, 1991; Meyer et al., 1992; Horton et al., 1999; Paumi et al., 2001; Zhang et al., 2003; Zhang and Lou, 2003). However, no papers have been published describing the qualitative or quantitative determination of these conjugates in humans. In this article, we present the chemical syntheses of the major biliary metabolites, their characterization and identification in human bile, urine, and plasma.
Materials and Methods

Chemicals

L-Cysteine was purchased from Sigma-Aldrich Fine Chemicals Co. (Taufkirchen, Germany). Acetonitrile, water for HPLC, ammonium acetate, perchloric acid, and acetic acid were obtained from J. T. Baker (Deventer, The Netherlands). D$_6$-DMSO was obtained from Chemotrade Chemiehandelsgesellschaft mbh, (Leipzig, Germany). All other chemicals were obtained from Merck KGaA (Darmstadt, Germany). All reagents were of analytical grade and the solvents were of HPLC grade. 1 (as hydrochloride), 5, 6, and 7 (as hydrochloride) were generous gifts from ribosepharm GmbH (Munich, Germany).

Instrumentation and Analytical Conditions

LC-MS

A ConstaMetric 4100 MS Series pump with a SCM 1000 Vacuum Membrane Degasser, an autosampler AS 3000, and a model spectromonitor 3200 programmable wavelength detector (Thermo Separation Products, Riviera Beach, Florida, USA) was interfaced to a Finnigan (Finnigan MAT, Bremen, Germany, now Thermo Electron Corporation) SSQ-7000 single quadrupole mass spectrometer equipped with an electrospray ionization (ESI) /APCI interface and coupled to a Digital Personal DEC 5000/25 workstation. The LC was carried out on a narrow bore column (125 x 2.0 mm i.d.) packed with Ultrasep ES PHARM RP18 5µm (Separation Service, Berlin, Germany). The mobile phase consisted of two components, namely solvent A (water with 5 mM ammonium acetate and 0.1% acetic acid, v/v) and solvent B (acetonitrile with 5 mM ammonium acetate and 0.1% acetic acid, v/v). Samples were separated using a slow gradient from 5 to 80% B in 60 min at a flow rate of 0.3 ml/min. The flow was split 5:1 into the mass spectrometer. Positive-ion electrospray-mass spectrometric analysis was carried out with a capillary temperature of 220°C and a capillary voltage of 4.5 kV. The value of the CID offset voltage was 10.0 V. The sheath gas and auxiliary gas both of
nitrogen 4.6 (Messer Griesheim GmbH, now Air Liquide Deutschland GmbH, Krefeld, Germany) were set to 60 psi and 10 psi, respectively. Mass spectra were recorded at an electron multiplier voltage of 1300V. Peaks were detected either by single ion recording or by scanning over an appropriate mass range. Data acquisition, reduction, selected ion monitoring and peak area calculations are performed under software control by Alpha AXP DEC 3000 Data System (Digital Equipment Corp, Maynard, MA, USA).

NMR

$^1$H and $^{13}$C NMR spectra were obtained on Varian Gemini 200 (Palo Alto, CA, USA) and Bruker DRX-600 (Bruker BioSpin GmbH, Rheinstetten, Germany) spectrometers at 26° C, with DMSO-$d_6$ as the solvent. Residual solvent signals were used as internal chemical shift references for proton ($\delta_{\text{DMSO}} = 2.49$ ppm) and carbon ($\delta_{\text{DMSO}} = 39.52$ ppm) spectra. J values are given in Hz. Signals were assigned by means of 2D proton-proton (COSY) and proton-carbon (HMQC, HMBC) shift-correlation spectra.

Preparative HPLC

All conjugates were purified by gradient HPLC procedure using two Shimadzu LC 8 pumps (Kyoto, Japan) and a Vydas 218 TPB 1520 column (Grace Vydas, Hesperia, CA, USA) 300 x 40 mm i.d. Flow rates were set at 70 ml/min. Mobile phase A consisted of 1 ml 12M HCl in 1 L water and mobile phase B of acetonitrile/water/12M HCl 1000:200:1 (v/v/v). The column was equilibrated with mobile phase A. From 5 to 50 min, a linear gradient ran from 100% A / 0% B to 100% B. Purity and identity of each conjugate was verified by analytical HPLC and LC-MS.

Analytical HPLC

Instruments used in this study were: Alliance 2695 and fluorescence detector 2475 (Germany Waters GmbH, Eschborn), HP 1090 gradient HPLC system with photo diode array (PDA) and
HP 1046A fluorescence detector (Hewlett Packard, predecessor of Agilent Technologies, Palo Alto, CA, USA). A column SYNERGI 4 µ MAX-RP 80A, 250 x 2 mm i.d. equipped with a guard cartridge 4 x 2 mm (Phenomenex, Torrance, CA, USA) was used for identification of metabolites. The mobile phase consisted of 0.1 ml 12M HCl in 1 L water (A) and acetonitrile/water/12M HCl 800:200:0.02 (v/v/v) (B). The gradient was 5 - 40% B in 70 min at a flow rate of 0.3 ml/min. For analysis by PDA detection, UV absorption was recorded at 233 nm. The excitation wavelength of the fluorescence detector was set to 328 nm and the emission wavelength to 420 nm to monitor eluted components. For quantitative analysis of 1 as well as its metabolites, a six-point calibration curve was constructed for each compound except 8 according to an internal standard method. Metabolite 8 was tentatively quantitated by means of the calibration curve of 1. (5-{5-[bis(2-chloro-ethyl)-amino]-1-methyl-1H-benzoimidazol-2-yl}-pentanoic acid was used as internal standard.

Synthesis of Reference Compounds

Cysteine S-Conjugate of 1

4-{5-[(2-Amino-2-carboxy-ethy)sulfanyl]-ethyl}-(2´-chloro-ethyl)]-amino]-1-methyl-1H-benzoimidazol-2-yl]-butyric acid, 2, Figure 1, was synthesized following the method reported by Paumi (16). Accordingly, 0.4 mg/ml L-cysteine and 0.6 mg/ml 1 hydrochloride was dissolved in 100 ml of a solution of 0.1 M sodium bicarbonate and 0.2 M NaCl. The reaction mixture was incubated at 25 °C for 1 h and terminated by addition of 2 ml 12M HCl to the reaction mixture. The product was purified by preparative HPLC followed by lyophilisation. LC-MS: C_{19}H_{27}ClN_{4}O_{5}S requires [MH]^+ 443; found 443.04.
Cysteine S-Conjugate of 5

4-{5-[[2-Amino-2-carboxy-ethylsulfanyl]-ethyl]-[2´-hydroxy-ethyl]-amino]-1-methyl-1H-benzoimidazol-2-yl}-butyric acid, 3, Figure 1, was synthesized and purified as described for 2 using 5 instead of 1. LC-MS: C_{19}H_{28}N_{4}O_{5}S requires [MH]^+ 425; found 425.16.

Dicysteine S-Conjugate Trifluoroacetate of 1

4-(5-{Bis-[2-(2-amino-2-carboxy-ethylsulfanyl)-ethyl]-amino}-1-methyl-1H-benzoimidazol-2-yl)-butyric acid, 4, Figure 1, was accomplished as described for synthesis of 2. The reaction was terminated by addition of 2 ml TFA to the reaction mixture. The product was purified as described for 2. LC-MS: C_{22}H_{33}ClN_{5}O_{6}S_{2} requires [MH]^+ 528; found 528.10.

Sample Collection

Samples were obtained from five patients (2 m, 3 f) with cholangiocarcinoma who have been on treatment with 1 hydrochloride. The intravenous dose received by each patient on day 1 of the first of overall 4 cycles was 140 mg/m^2, the average age of the patients was 69.0 ± 3.4 years. 20 blood samples (4.5 ml) including a pre-dose blank sample were drawn from the cubital vein of the arm contra lateral to that used for the administration of 1 hydrochloride up to 8 hours after starting the i.v. infusion. After centrifugation, the supernatant plasma was withdrawn and immediately frozen at -70°C. 10 ml aliquots of the urine samples collected prior dosing and during 6 sampling intervals up to 24 hours postdose were stored in polypropylene tubes at -70°C. The tubes were pre-treated with HCl and NaCl to prevent chemical hydrolysis. For bile sampling, two temporary external nasobiliary drainages (7F, 290 cm, 8 holes; Endo-Flex®, Voerde, Germany) were placed into the right and left hepatic duct each via endoscopic retrograde cholangiography and left in place for the entire collection period. After placement the complete biliary secretion was collected without any loss during
the collection period. After collecting the bile samples, the nasobiliary drainages were removed and permanent endoscopic stenting was performed. Bile aliquots were stored as described for plasma and urine samples before dosing and during 16 intervals up to 24 hours after starting the 30-minute infusion. The Ethics Committee of the Faculty of Medicine of the University of Leipzig issued approval for this study.

**Sample Preparation for the Identification and Quantitation of the Metabolites**

For the identification and characterization experiments, chromatographic and mass spectral characteristics of the conjugates from biological fluids were compared with those of the previously synthesized reference compounds. During shaking on a rotational shaker at 400 min\(^{-1}\), 100 µL 20% perchloric acid were added to 0.2 ml of the plasma samples. The precipitated proteins were removed by centrifugation at 4°C and 15,000 g for 5 min. The supernatant was used for chromatographic analysis. For LC-MS experiments, each urine and bile sample (1 ml) was extracted by SPE procedure after dilution with 2.5 ml water pH 3.0 (adjusted with HCl). For the determination of polar conjugates, urine and bile samples (0.1 ml each) were diluted with 0.9 ml of the respective mobile phase, centrifuged at 15,000 g for 5 min and filtered through a centrifugal filter device (0.22 µm, Amicon ULTRAFREE\textsuperscript{®}-MC) before an aliquot was injected into the HPLC system.
Results

Synthesis and Characterization of Synthetic L-Cysteine S-Conjugates of 1 and 5

The cysteine S-conjugates were synthesized by adaptation of a method for the preparation of GSH conjugates described for the structurally similar compounds chlorambucil and melphalan. Sodium phosphate was replaced by sodium bicarbonate to prevent formation of phosphate adducts. Results of recent pharmacokinetic studies have been indicated that 1 and 5 are the major urinary components following i.v. administration of 1 hydrochloride. Therefore, we decided to prepare the cysteine S-conjugates of 1 and 5 as well. The reference compound was purified by preparative HPLC from the reaction mixture of 5 with L-cysteine and characterized by \(^{1}\text{H-NMR},\ ^{13}\text{C-NMR},\ \text{and}\ \text{LC-MS}\) analysis. Beside the parent compound 1 (12-20%) and its hydrolysis product 5 (1%), the reaction of 1 with L-cysteine yielded three peaks of unknown identity separable by reverse-phase HPLC. The minor peak was identified as 3 by comparison with the reaction product of 5 with L-cysteine. In addition, 3 was obtained by hydrolysis of 2 with aqueous NaOH. Addition of chloride to the reaction mixture of 1 and L-cysteine reduces formation of 3 by preventing both, hydrolysis of 1 and 2. Under the chosen experimental conditions we achieved product yields of 35-40% for 2 and 35-48% for 4. Hence, two major peaks were obtained from this reaction mixture under the above described experimental conditions, which were individually collected with preparative HPLC and characterized by \(^{1}\text{H-NMR},\ ^{13}\text{C-NMR},\ \text{and}\ \text{LC-MS}\). LC-MS and HPLC analyses with UV as well as fluorescence detection revealed a purity of 96.4% for 2, 94.8% for 3, and 92.8% for 4, respectively. The presence of the cysteine moiety was shown by the detection of characteristic aliphatic protons and carbon atoms in the NMR spectra. COSY, HMQC, and HMBC spectra supported this conclusion. The HMBC spectrum of 3 is depicted in Figure 3 showing the characteristic chemical shifts as well as long-range couplings attributable to the assigned structure of all cysteine S-conjugates. The NMR data for the synthesized reference standards
are given in Table 1, and were used along with the MS and HPLC data as basic data for identification of metabolites.

**Identification of 1 and its metabolites in bile, urine, and plasma from cancer patients**

An HPLC-method was developed by which both phase I and phase II metabolites of 1 could be analyzed simultaneously in bile, urine, and plasma samples. The separation of 1, 5, 6, 7, and 8 and the three cysteine S-conjugates was achieved by gradient elution followed by either MS or fluorescence detection. Upon HPLC analysis with fluorescence detection of bile from patients treated with 1 hydrochloride, the 3 peaks at 10.3, 11.5 and 27.3 min were prominently visible (Figure 4). Further, no background peaks were observed in this elution region of the blank bile samples collected before drug administration indicating little potential for interference from background at low concentrations (Figure 4). The cysteine S-conjugates 1, 2, and 3 present in human bile were characterized by LC-MS analyses (Figure 5). In addition, the expected molecular ions (m/z 425), (m/z 443), and (m/z 528) were detected by MS in urine as well as bile samples from preparative HPLC (Figure 6). Identical mass spectra were obtained from the authentic reference compounds previously characterized (not shown). Quantitative assessment was carried out by HPLC/fluorescence detection. The six-point calibration curve of each compound showed excellent linearity in the respective concentration range, which was chosen according to peak intensity of the respective component determined in preliminary experiments. The lower limits of quantitation for 2, 3, and 4 were 0.1, 0.15, and 0.2 µg/ml, respectively. The concentration-time profiles indicated that the cysteine derived adducts were the major drug-related components in all bile samples from the five subjects. The percentage related to the sum of the parent compound and its metabolites determined in this study ranged from 90.3 to 98.0 %, with a mean value of 95.8 %. The total percentage of administered dose recovered in bile ranged from 1.5 to 4.8 %, with a mean
value of 2.6%. The sum of the cysteine S-conjugates recovered from urine accounted for 26.0% of all metabolites including the parent ranging from 14.1 to 41.2%. On the other side, the mean percentage of 1 and 5 related to all components determined in urine was 39.0% and 21.4%, respectively. We measured a mean total urinary recovery of 8.3%, including 0.9, 0.5, and 1.0% of the dose as 2, 3, and 4, respectively. We measured a mean total urinary recovery of 8.3%, including 0.9, 0.5, and 1.0% of the dose as 2, 3, and 4, respectively. Figure 7 shows a characteristic chromatogram of a urine sample as well as a plasma sample from a cancer patient after administration of 1. Maximum plasma concentrations of 1 ranged from 4.54 to 24.25 µg/ml with a mean of 16.81 µg/ml. Those for 5, 6, 7, and 8 were in the range 0.12 to 0.77 µg/ml (mean values). Maximum plasma concentrations of the cysteine S-conjugates were in the range 0.82 to 2.89 µg/ml (mean values). The mean terminal elimination half-life was 45 min for 2 and comparable to those for 1 (39 min), whereas prolonged values of 85 and 163 min were calculated for 3 and 4, respectively. Neither glucuronide and sulfate conjugates nor GSH conjugates of 1 were detected.
Discussion

Although 1 is being increasingly used for cancer management, the metabolic profile of this alkylating agent has been poorly investigated. In this paper, we report the synthesis and characterization of the monocysteine S-conjugates of 1 as well as 5 and the dicysteine S-conjugate of 1. The conjugates that were synthesized according to a procedure previously published for chlorambucil and melphalan yield a characteristic NMR pattern for a cysteine S-conjugated bendamustine heterocyclus. All NMR spectra were similar to each other except for the cysteine proton signals of 4, showing twofold intensity indicating two identical cysteine groups. Broadened signals appearing in the 1H-NMR spectra of 4 indicated the presence of the trifluoroacetate ion in the molecule, whereas both 2 and 3 occurred in the non-ionic form. The data described in this manuscript provide the first conclusive characterization of these cysteine S-conjugates of 1. In the present study, the cysteine S-conjugates were identified in bile, urine, and plasma samples from cancer patients after intravenous administration of 1 hydrochloride using LC-MS as well as HPLC with fluorescence detection by comparison with the synthetic standards previously synthesized and characterized. Quantitative determination of 1 and its metabolites was performed using HPLC with fluorescence detection.

The mean amount of bile collected from the subjects in 24 h was 638 ml. This is in good accordance with the normal bile production of 600–800 ml per day. The cysteine S-conjugates presumably formed via the prominent GSH conjugation pathways are excreted into bile in high concentrations. The cysteine S-conjugate concentrations in the bile sampled directly from the hepatic bile duct were much higher than the simultaneous plasma concentrations. This finding provides evidence for efficient biliary excretion of 1 in man, as was found earlier in an animal experiment (Bezek et al., 1991).

A large variation in total urinary excretion of 1 was seen between patients, with recovery percentages ranging from 0.7 to 9.5 % of the administered dose. As expected, in all patients the highest concentrations of 1, 5, 6, 7, and 8 were observed in the first urine samples. For the
cysteine S-conjugates we observed a mean cumulative urinary excretion of 1.3–4.1 % of the administered dose within 24 h. The major part of 2 was excreted within the first 3 h, whereas 4 was predominantly excreted within 10–24 h after administration. 3 was recovered throughout the whole sampling period. The individual maximum amount excreted within 24 hours into urine was 16 % of the administered dose including the parent drug as well as seven metabolites. Plasma kinetics of 1, 5, 6, 7, and 8 were similar to those reported previously.

There are some unidentified peaks in the chromatograms indicating the presence of unknown derivatives of 1 in the present study. Therefore, it must be concluded that 1 is excreted into urine and/or bile in the form of as yet unknown metabolites (e.g. mercapturic acids). This may be the reason for not being able to account for all of administered parent drug. In general, glutathione and cysteine conjugates are subjected to further metabolism e.g. N-acetylation to give mercapturic acid conjugates prior to excretion in mammals. Theoretically, 2 can still form DNA cross-links via formation of the aziridinium intermediate. Therefore, further investigations should be targeted to the metabolic pathways of 1.

In this study, three cysteine S-conjugates exemplified by 2, 3, and 4 were detected. The existence of these S-containing metabolites demonstrates that 1 is conjugated with glutathione and further metabolized. The results indicate that the detoxifying pathways of 1 in man primarily involves phase I (and hydrolytic degradation) as well as phase II metabolism followed by urinary excretion of polar metabolites. Structures of metabolites that have been identified are shown in Figure 2. However, the major amount of the administered dose was recovered as parent drug. Further investigations should be address fecal components to assess the extent that biliary elimination contributes to the overall elimination of 1 in humans.
Acknowledgments

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References


Legends for Figures

Figure 1  Structures of the synthesized cysteine $S$-conjugates used as reference standards in this study as well as those of the alkylators chlorambucil and melphalan structurally related to 1.

Figure 2  Proposed metabolic pathways of 1 in humans.

Figure 3  Chemical structure of the cysteine $S$-conjugates including numbering of carbon atoms and aromatic protons as well as an example of characteristic long-range couplings observed in the two-dimensional HMBC spectrum of 2 after chemical synthesis and purification by preparative HPLC. Numbering of carbon skeleton was according to Table 1 in comparison to NMR data and does not correspond to the rules of chemical nomenclature.

Figure 4  HPLC chromatograms of a human (upper panel) bile sample 90-120 min after administration of 140 mg/m$^2$ 1 as hydrochloride and (lower panel) blank bile sample before drug administration spiked with internal standard.

Figure 5  HPLC-ESI-MS chromatogram (selected mass tracks, m/z = 425, 443, 528; and reconstructed, or total, ion chromatogram, RIC) of a human bile sample (1.5-2 h) after start of the 1 hydrochloride infusion 140 mg/m$^2$.

Figure 6  Mass spectra of the cysteine $S$-conjugates 2, 3, and 4 obtained by preparative HPLC from the human bile sample described in Figure 5.

Figure 7  HPLC chromatograms of a human (upper panel) urine sample 3 – 6 h and (lower panel) plasma sample 60 min after drug administration.
Table 1  
NMR spectral data of the synthetic reference standards 2, 3, and 4.

(A) $^1$H NMR Data (200 MHz, 600 MHz, $^1$H, $^1$H COSY)$^a$

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(B) $^{13}$C NMR Data (50 MHz, 150 MHz, APT, HMQC, HMBC)

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*a s, singlet; d, doublet; dd, doublet-doublet; t, triplet; m, multiplet; *, not observed due to broadened signals caused by the ionic structure;*
FIG. 1

Chlorambucil

Melphalan

1

2

3

4
FIG. 3

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HOOC  

R  

H₃C

NH₂
FIG. 5
FIG. 7