Renal human organic anion transporter 3 increases the susceptibility of lymphoma cells to bendamustine uptake

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Hagos Y, Hundertmark P, Shnitsar V, Marada VV, Wulf G, Burckhardt G. Renal human organic anion transporter 3 increases the susceptibility of lymphoma cells to bendamustine uptake. Am J Physiol Renal Physiol 308: F330–F338, 2015. First published December 4, 2014; doi:10.1152/ajprenal.00467.2014.—Chronic lymphocytic leukemia (CLL) is often associated with nephritic syndrome. Effective treatment of CLL by chlorambucil and bendamustine leads to the restoration of renal function. In this contribution, we sought to elucidate the impact of organic anion transporters (OATs) on the uptake of bendamustine and chlorambucil as a probable reason for the superior efficacy of bendamustine over chlorambucil in the treatment of CLL. We examined the effects of structural analogs of p-aminohippurate (PAH), melphalan, chlorambucil, and bendamustine, on OAT1-mediated [3H]PAH uptake and OAT3- and OAT4-mediated [3H]estrone sulfate (ES) uptake in stably transfected human embryonic kidney-293 cells. Melphalan had no significant inhibitory effect on any OAT, whereas chlorambucil reduced OAT1-, OAT3-, and OAT4-mediated uptake of PAH or ES down to 14.6%, 16.3%, and 66.0% of control, respectively. Bendamustine inhibited only OAT3-mediated uptake of cytostatics for cell proliferation, we performed formed thymidine incorporation assays and measured apoptosis induced by the alkylating drugs. Surprisingly, lymphoma cells express the “renal” transporter OAT3, rendering them sensitive to bendamustine.

MATERIALS AND METHODS

Reagents. Cell culture materials, including FCS, PBS, RPMI-1640, and DMEM-LG, were purchased from Invitrogen (Groningen, The Netherlands). TRIzol Reagent was from Invitrogen, and murine leukemia virus (M-MLV) reverse transcriptase was purchased from Promega (Madison, WI). Cell culture media, including RPMI-1640, DMEM-LG, and DMEM-Ham’s F12, were purchased from Invitrogen (Groningen, The Netherlands).

Methods. For the measurement of OAT-mediated uptake of cytostatics for cell proliferation, we performed thymidine incorporation assays and measured apoptosis induced by the alkylating drugs. Surprisingly, lymphoma cells express the “renal” transporter OAT3, rendering them sensitive to bendamustine. The introduction of purine analogs almost 30 yr ago, especially of fludarabine, changed the frontline treatment paradigm for CLL. Compared with chlorambucil, fludarabine showed improved response rates and progression-free survival, but the overall survival was not enhanced (31). In parallel, bendamustine was developed with the intention of producing an anticancer agent that combined both alkylating and antimetabolite properties (13). Several studies have documented that bendamustine acts primarily as an alkylating drug, which induces the formation of intrastrand and interstrand cross-links between DNAs (15, 24, 36). In a patient with a B cell lymphoma treated with rituximab and bendamustine, markedly improved renal function was observed (3). In addition, urinary sediment became normal and proteinuria disappeared completely. These findings were in contrast to observations made during chemotherapy with cyclophosphamide, oncovin (vincristine), and prednisone (3).

Recently, bendamustine was compared with chlorambucil in a randomized first-line therapy with 319 previously untreated advanced CLL patients. Bendamustine showed significantly higher overall responses and progression-free survival compared with chlorambucil (20). Based on this vital clinical study, the United States Food and Drug Administration approved bendamustine for CLL treatment in the year 2008. Bendamustine shares similarities with chlorambucil and p-aminohippurate (PAH) with respect to the butyric acid side chain, as shown in Fig. 1. PAH is a well-known model substrate of renal organic anion transporters (OATs). OAT1 and OAT3 are expressed at the basolateral membrane and OAT4 at the apical membrane of proximal tubule cells, respectively, and are involved in the renal excretion of a large number of drugs (1, 5, 33, 37, 42).

In the present study, we examined the interactions of OATs (OAT1, OAT3, and OAT4) with the cytostatic agents melphalan, chlorambucil, and bendamustine. We then determined the expression of OATs in lymphoma cell lines as well as in samples from CLL patients. To evaluate the role of OAT-mediated uptake of cytostatics for cell proliferation, we performed thymidine incorporation assays and measured apoptosis induced by the alkylating drugs. Surprisingly, lymphoma cells express the “renal” transporter OAT3, rendering them sensitive to bendamustine.

CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) is the most common form of adult leukemia in the Western countries, accounting for nearly 40% of all leukemia (17). After the diagnosis of CLL, the survival period ranges from months to >10 yr and is often associated with nephritic syndrome (25). Histological examination of renal biopsies from CLL patients showed increased basement membrane thickness induced by interstitial CLL infiltration, causing a diffuse membranous glomerulonephritis (9). A retrospective analysis of clinical data confirmed the pathophysiological link between glomerulonephritis and B cell proliferation. Effective treatment of the malignancy by chlorambucil monotherapy showed, additionally, a chlorambucil-induced complete remission of the glomerulonephritis and substantial improvement of renal function (27).
Fig. 1. Chemical structures of bendamustine, chlorambucil, melphalan, and 
\( p \)-aminohippuric acid (PAH).

kocyte virus (MuLV) reverse transcriptase was obtained from Promega (Mannheim, Germany). Chemical reagents (analytical grade) were purchased from Sigma-Aldrich (Munich, Germany). Hygromycin was obtained from AppliChem (Darmstadt, Germany). [3H]thymidine was obtained from Amersham Bioscience (, Germany). [3H]estrone sulfate (ES) and [3H]PAH were from Perkin-Elmer (Rodgau, Germany). [3H]thymidine was obtained from Amersham Bioscience (, Freiburg, Germany). Real-time TaqMan primers and TaqMan buffer were purchased from Applied Biosystems (Darmstadt, Germany). Previously described B and T lymphoma cell lines (Karpas, SUDHL4, Raji, Hut78, and Jurkat cells) were obtained from a public depository (DSMZ, Braunschweig, Germany).

**Quantitative real-time PCR.** Suspension-cultured B and T lymphoma cell lines and patient samples were used to obtain total RNA using TRIzol Reagent following the manufacturer’s protocol. The concentration and quality of the purified RNA were quantified using a Gene Quant II spectrophotometer (Amersham Bioscience). cDNA was prepared from RNA using MuLV reverse transcriptase as per the protocol of the manufacturer. Briefly, 2 \( \mu \)g of total RNA were reverse transcribed into cDNA in a reaction mixture containing 200 units of MuLV enzyme, 500 \( \mu \)M of each dNTP, and 2.5 \( \mu \)g/\( \mu \)l of Ne-random primers in a 20-\( \mu \)l reaction volume for 1 h at 37°C and 10 min at 70°C. Diluted cDNA (5 \( \mu \)l) was used in the real-time PCR mixture containing 12.5 \( \mu \)l of 2\( \times \) reaction buffer, 1.25 \( \mu \)l of 25\( \times \) TaqMan-primer mixture, and 6.25 \( \mu \)l of nuclease-free water in a total volume of 25 \( \mu \)l. The mixture was transferred into a 96-well plate and covered with an optical adhesive film. The plate was then transferred to the ABI Prism 7000 real-time PCR cycler (Applied Biosystems). The PCR program consisted of the following steps: 2 min at a 52°C uridine glycosylase step, 10 min at a 96°C TaqMan polymerase activation step, and 40 cycles of 15 s at 96°C and 1 min at 60°C. Fluorescence reading was performed on the last step of each cycle. Data were analyzed by ABI Prism 7000 software.

**Real-time TaqMan primers.** Human GAPDH, Hs 9999995 m1; human hypoxanthine phosphoribosyltransferase, Hs 9999909 m1; human OAT1, solute carrier (SLC)22A6, Hs00537914_m1; human OAT2, SLC22A8, Hs0198527_m1; human OAT3, SLC22A8, Hs00185899_m1; and human OAT4, SLC22A11, Hs00945824_m1 and reagents were purchased from Applied Biosystems.

**Transport measurements.** T-REx human embryonic kidney (HEK)-293 cells stably expressing human OAT1, OAT3, or OAT4 were harvested and plated into 24-well plates (2 \( \times \) 10\(^4\) cells/well). After 72 h of incubation, cells were washed three times with mammalian Ringer solution containing 130 mM NaCl, 4 mM KCl, 1 mM CaCl\(_2\), 1 mM MgSO\(_4\), 1 mM NaH\(_2\)PO\(_4\), 20 mM HEPES, and 20 mM D-glucose with pH adjusted with 1 M NaOH to 7.4. After a wash, cells were incubated with 200 \( \mu \)l of transport solution containing 1 \( \mu \)M [\(^3\)H]PAH or 20 nM [\(^3\)H]ES with or without 100 \( \mu \)M of the specific cytostatic in mammalian Ringer solution. After 5 min of incubation at room temperature, cells were washed with ice-cold PBS and lysed with 500 \( \mu \)l of 1 M NaOH solution for 20 min. The lysed mixture was neutralized with 500 \( \mu \)l of 1 M HCl and transferred into scintillation vials. Incorporated radioactivity was counted by adding 2.5 ml Lumasafe scintillation solution (Perkin-Elmer) to each sample in a scintillation counter (TriCarb 1500 Packard, Meriden, CT). To determine the affinity of the transporter for the alkylating drugs, concentration-dependent cis-inhibition experiments were performed. The inhibitory effect of the drugs was calculated using four-parameter Hill equations with SigmaPlot 11.0 (SPSS Science, Chicago, IL) to determine the concentration that causes half-maximal inhibition of the uptake (IC\(_{50}\); \( \mu \)mol/l) as follows:

\[
I = I_o + \frac{I_{\text{max}} + c^n}{c^n + IC_{50}^{n}}
\]

where \( I_o \) is the inhibitory baseline effect (in %), \( I_{\text{max}} \) is the maximal inhibitory effect (in %), \( c \) is the inhibitor concentration (in \( \mu \)mol/l), and \( n \) is the Hill exponent (\( n = 1 \)).

**Proliferation assay.** Proliferation was estimated by [\(^3\)H]thymidine incorporation as previously described (32, 35). Human OAT1- or human OAT3-expressing HEK-293 cells were harvested and seeded onto 24-well plates at a concentration of 5 \( \times \) 10\(^4\) cells/well. After 24 h, cells were incubated with different concentrations of chlorambucil and bendamustine for 30 min and then washed three times with the medium. Cells were then incubated in a medium containing 4 \( \mu \)M [\(^3\)H]thymidine for 15 min. After this, cells were fixed with 5% trichloroacetic acid for 30 min on ice. Cells were washed twice with ice-cold PBS and once with 96% ethanol. Pellets were then dissolved in 1 M NaOH for 20 min and neutralized with 1 M HCl. Incorporated radioactivity was counted by a scintillation counter (TriCarb 1500 Packard).

**Bendamustine-dependent cytotoxicity.** Flow cytometric analysis of a B lymphoma cell line (Raji) and a T lymphoma cell line (Jurkat) was performed. Cells were washed and resuspended in PBS to a concentration of 5 \( \times \) 10\(^6\) cells/ml. Untreated cells were used as a control. After 15 min of incubation with cytostatics, cells were washed three times with PBS to remove the drugs and grown further for 12 h in culture medium without cytostatics. Cells were stained with annexin V-propidium iodide (PI). During this procedure, cells were pelleted and resuspended in staining buffer [10 mM HEPES-NaOH (pH 7.4), 140 mM NaCl, 5 mM CaCl\(_2\), and PI at a final concentration of 10 \( \mu \)M] containing 20 \( \mu \)l of annexin V-Fluos (Roche Diagnostics, Mannheim, Germany) for each milliliter of buffer and subsequently analyzed on a FACScalibur. All assays were performed in duplicate in three independent experiments.
We calculated the mean of the total apoptotic events from three independent experiments. Furthermore, we measured bendamustine-induced apoptosis as well as bendamustine plus probenecid-induced apoptosis in both lymphoma cells using a calculation system previously described by Friesen et al. (12). The formula used was 100 × [drug-induced apoptosis (in %) − spontaneous apoptosis in cell medium (in %)]/[100% − spontaneous apoptosis in cell medium (in %)].

RESULTS

Interaction of alkylating cytostatics with OAT1, OAT3, and OAT4. To elucidate the interactions of human OAT1, human OAT3, and human OAT4 with the alkylating cytostatics chlorambucil, melphalan, and bendamustine, we examined the inhibition of human OAT1-mediated [3H]PAH uptake and human OAT3- and human OAT4-mediated [3H]ES uptake. Transport activity of OAT1, OAT3, or OAT4 was demonstrated in stably transfected HEK-293 cells by measuring the uptake of the radiolabeled model substrates without cytostatic drugs in Ringer buffer compared with nontransfected cells (Fig. 2). Human OAT1-mediated PAH uptake and OAT3 and OAT4 significantly facilitated ES uptake in stably transfected cells with influx rates of $20.2 \pm 1.3$ pmol/5 min $^{-1}$·10$^6$ cells$^{-1}$ for PAH and $39.3 \pm 6.2$ and $45.2 \pm 4.8$ fmol/5 min $^{-1}$·10$^6$ cells$^{-1}$ for ES, respectively. Untransfected cells showed uptakes of $0.89 \pm 0.13$ pmol/5 min $^{-1}$·10$^6$ cells$^{-1}$ for PAH and $4.6 \pm 0.86$ and $3.9 \pm 0.6$ fmol/5 min $^{-1}$·10$^6$ cells$^{-1}$ for ES, respectively. To test for potential interactions of the alkylating cytostatic drugs with OATs, 100 μM of each drug were added as a competitor to PAH or ES into the assay. The inhibitory effects of these drugs on OAT1-, OAT3-, or OAT4-mediated uptake of PAH or ES are represented as percentages of uptake in the absence of these drugs (Fig. 3). OAT1-mediated PAH uptake was inhibited down to $90.4 \pm 5.7\%$, $80.9 \pm 7.2\%$, and $14.6 \pm 0.8\%$ of control by melphalan, bendamustine, and chlorambucil, respectively (Fig. 3A). Human OAT3 transport activity was reduced down to $16.3 \pm 1.3\%$ by chlorambucil and $14.3 \pm 2.7\%$ by bendamustine. Melphalan stimulated the uptake of ES by human OAT3 to $124.0 \pm 3.8\%$ (Fig. 3B). Chlorambucil was the only alkylating cytostatic drug that inhibited human OAT4-facilitated ES uptake to $66.0 \pm 4.0\%$ (P < 0.001). Bendamustine did not show any interaction with human OAT4. On the other hand, melphalan significantly stimulated human OAT4-mediated ES uptake (P < 0.001) by 86.4% (Fig. 3C).

Concentration-dependent inhibition of OAT1- and OAT3-mediated uptake. Next, we determined the affinity of OAT1 and OAT3 for chlorambucil as well as the affinity of OAT3 for bendamustine by performing concentration-dependent inhibition experiments. Figure 4 shows the inhibitory effects of the alkylating drugs in percentages of untreated samples. Calculated IC$_{50}$ values of OAT1 and OAT3 for chlorambucil were $44.3 \pm 2.6$ and $9.5 \pm 1.9$ μM, respectively. For bendamustine, the IC$_{50}$ value was $0.8 \pm 0.1$ μM.

Determination of cell proliferation. To elucidate whether cells expressing OAT1 and OAT3 exhibit an inhibition of proliferation upon treatment with alkylating drugs, we performed [3H]thymidine incorporation assays. Treatment of OAT3-transfected HEK-293 cells with increasing concentrations of chlorambucil induced a 20–25% decrease in thymidine incorporation similar to mock-transfected cells (Fig. 5A). However, OAT1-expressing cells demonstrated significantly higher reductions of thymidine incorporation compared with untreated or mock cells (Fig. 5A), suggesting that enhanced OAT1-mediated uptake of chlorambucil caused the increased inhibition of cell proliferation. Similarly, OAT3-expressing cells showed decreased proliferation in the presence of bendamustine (Fig. 5B); in contrast, chlorambucil and bendamustine were ineffective in mock-transfected cells (Fig. 5, A and B).

Expression of OAT3 in lymphoma cell lines and CLL patients. We examined the expression of OAT1, OAT2, OAT3, and OAT4 in six lymphoma cell lines (Karpas422, Raji, SudHL4, L428, Jurkat, and Hut78) by quantitative RT-PCR. Human OAT1, OAT2, and OAT4 were not expressed in any of the lymphoma cell lines tested. We discovered, however, high expression levels of human OAT3 in all lymphoma cell lines tested (Fig. 6A). Lowest expression was observed in Karpas422 and Raji cells at 6- to 8-fold higher ΔCt levels (where Ct is threshold cycle) than in normal lymphocytes, whereas all other

Fig. 2. Organic anion transporter (OAT)-mediated uptake of radiolabeled organic anions. Stable expression of human hOAT1, hOAT3, and hOAT4 was verified by measuring the uptake of 1.0 μM [3H]PAH (OAT1) or of 20 nM [3H]estrone sulfate (ES) (OAT3 and OAT4) (hatched columns). Solid columns represent the uptake of PAH and ES in vector-transfected mock human embryonic kidney (HEK)-293 cells. Values are means ± SE of three independent (n = 3) experiments with three repeats each.
cell lines showed 13- to 14-fold higher ΔCt levels of OAT3 expression compared with control lymphocytes (Fig. 6A). As OAT3 was highly expressed in lymphoma cell lines, we examined its expression in four samples of CLL patients. Expression of OAT3 in the four samples of patients showed 5- to 9-fold higher levels of OAT3 mRNA expression compared with control lymphocytes (Fig. 6B).

**Bendamustine-induced and OAT3-mediated specific apoptotic effects in lymphoma cells.** To examine OAT3-mediated, bendamustine-induced cytotoxicity in lymphoma cell lines, we selected Raji cells as a low-OAT3 expression system and Jurkat cells as a high-OAT3 expression system and then quantified apoptotic cell death by annexin V and PI staining. We incubated lymphoma cells with and without either bendamustine alone or bendamustine in combination with probenecid, an OAT3 inhibitor. After removal of the cytostatic drugs, cells were subsequently grown for 12 h, and apoptotic events were quantified by FACS analysis [Fig. 7A (a representative experiment)]. Treatment with bendamustine increased apoptotic events in both cell lines by threefold compared with low-level spontaneous apoptosis in untreated control cells. The simultaneous incubation of bendamustine with probenecid reduced the proportions of apoptotic cells significantly by 31–38% (Fig. 7B). Bendamustine-induced specific apoptosis for Raji and Jurkat cells was 6.0 ± 0.18% and 11.3 ± 0.4%, respectively, as calculated with the equation previously described by Friesen et al. (12). Simultaneous incubation with probenecid reduced bendamustine-induced specific apoptosis to 3.3 ± 0.8% and 5.2 ± 0.3% for Raji and Jurkat cells, respectively (Fig. 7C). These results demonstrate, for the first time, the impact of OAT3 on the cytostatic efficacy of bendamustine in lymphoma cells.

**DISCUSSION**

Renal syndromes caused by leukemic infiltration of the kidneys in CLL have been reported in several cases, and retrospective as well as autopsy studies have indicated a broad spectrum of nephropathy, including, e.g., membranoproliferative glomerulonephritis, mesangioproliferative glomerulonephritis, and tubular necrosis (9, 18, 25, 30). Treatment of CLL patients with severe kidney diseases by alkylating drugs like chlorambucil and bendamustine led to a significant improvement of renal function (3, 27).

Chlorambucil, bendamustine, and melphalan are antineoplastic agents containing an alkylating nitrogen mustard group. In lymphoid malignancies such as CLL, these drugs serve in efficient mono frontline therapy as well as in combination with other antineoplastic agents, e.g., fludarabine (11). Among other factors, the efficacy of chemotherapy depends on the intracellular drug concentration, which, in turn, is determined by an equilibrium of drug uptake into and drug release from tumor cells. Whereas drug release driven by ATP-dependent ABC transporters has been studied in detail in the past, relatively little is known on how antineoplastic agents are taken up into tumor cells. In this contribution, we...
considered renal OATs (OAT1, OAT3, and OAT4) as possible pathways for drug uptake for two reasons. First, OATs are known for their very broad substrate specificity, i.e., they can transport drugs of various chemical structures into cells. Second, melphalan, chlorambucil, and bendamustine share structural similarities with PAH, a model organic anion used to study renal plasma flow mediated by OAT function.

The alkylating agent melphalan carries a negatively charged carboxyl group and a positively charged amino group, resembling an amino acid. A previous study (26) demonstrated leucine-sensitive uptake of melphalan into murine leukemia cells, suggesting the involvement of an amino acid transporter.

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**Fig. 4.** Affinity of OAT1 and OAT3 for chlorambucil or bendamustine. Uptake experiments with 1.0 μM [3H]PAH for OAT1 and 20 nM [3H]ES for OAT3 were carried out in the absence or presence of different concentrations of chlorambucil or bendamustine. A and B: concentration-dependent inhibition of chlorambucil on OAT1 (A) or OAT3 (B). C: concentration-dependent inhibitory effect of bendamustine on OAT3-mediated ES uptake. IC₅₀ values were determined by sigmoidal calculation with the equation shown in MATERIALS AND METHODS. Data are means ± SE of three independent (n = 3) experiments with three repeats each.

**Fig. 5.** Effect of chlorambucil and bendamustine on cell proliferation. [3H]thymidine incorporation was measured after incubation of OAT1- or OAT3-expressing HEK-293 cells and untransfected mock cells with chlorambucil or bendamustine. Cells were treated for 30 min with various concentrations of chlorambucil or bendamustine. A: effect of chlorambucil on the proliferation of OAT1-expressing, OAT3-expressing, and vector-transfected HEK-293 cells. B: effect of bendamustine on OAT3-expressing cells and mock cells. All experiments were standardized by setting the control (without chlorambucil or bendamustine) of each experiment to 100%. Data are means ± SE of three independent experiments with two repeats. *Significant differences between untreated and treated cells; §significant difference between mock and OAT1-expressing cells; **significant chlorambucil-concentration dependent decrease of thymidine incorporation in OAT3-expressing cells similar to mock-transfected cells (*,§,SP < 0.05; **,§§,$$ P < 0.01; ***P < 0.001).
Indeed, L-type amino acid transporter 1 (LAT1; SLC7A5), expressed in *Xenopus laevis* oocytes, was inhibited by melphalan (40, 43), and downregulation of LAT1 expression in HeLa cells reduced uptake of melphalan (22). Besides its experimentally proven interaction with LAT1, melphalan inhibited organic cation transporter 3 (OCT3; SLC22A3), albeit with relatively low affinity (35). OCT3-expressing renal carcinoma cells were more sensitive to melphalan than other cells, suggesting that OCT3 increases intracellular melphalan accumulation (35). In the present study, we tested whether melphalan is a substrate of OAT1, OAT3, and OAT4. However, melphalan did not influence the activity of OAT1 and rather stimulated the function of OAT3 and OAT4. The reason for the stimulation of OAT3 and OAT4 is unclear and awaits further experimentation. At this point, we can conclude that OAT1, OAT3, and OAT4 are most probably not involved in melphalan uptake into cells.

Structurally, chlorambucil closely resembles melphalan but lacks the positively charged amino group. So far, transport of a chlorambucil-taurocholate conjugate by Na⁺-taurocholate cotransporting polypeptide (SLC10A1) and organic anion-transporting polypeptide 1A2 (SLCO1A2) has been reported (23). The ABC transporter multidrug resistance protein-1 translocated chlorambucil together with glutathione (2). Here, we show that chlorambucil inhibits OAT1, OAT3, and OAT4. This is the first report to demonstrate an interaction of chlorambucil itself with an SLC transporter. The IC₅₀ of OAT3 (9.5 μM) was about four times lower than that of OAT1 (44.3 μM). Therapeutic doses of chlorambucil are 0.1–0.2 mg/kg body wt, leading to a peak plasma concentration of ~0.5 μg/ml or 1.6 μM (molecular mass: 304.3 g/mol) (28). Although this concentration is below the IC₅₀ values determined here, OAT1 and OAT3 could well transport chlorambucil. As determined by thymidine incorporation, OAT3-expressing cells showed the same sensitivity to chlorambucil as nonexpressing control cells. Accordingly, there was no significant accumulation of chlorambucil mediated by OAT3. Thus, chlorambucil is a high-affinity inhibitor of OAT3 but not a substrate. On the other hand, chlorambucil may be taken up into cells by OAT3, but the transport-mediated substrate turnover was apparently not sufficient enough to influence cell proliferation, although the affinity of chlorambucil was higher for OAT3 compared with OAT1 and OAT4. In contrast, thymidine incorporation was clearly reduced by chlorambucil in OAT1-expressing cells compared with nonexpressing cells or OAT3-expressing cells. It appears that the expression of OAT1 renders cells more sensitive to chlorambucil, i.e., increases the cytostatic efficacy of this drug. Thus, the thymidine incorporation results indirectly demonstrate the uptake of chlorambucil in OAT1-expressing cells. An increased sensitivity to antineoplastic agents (irinotecan, paclitaxel, and vincristine) has been previously shown in lymphoma cell lines and in renal carcinoma cells expressing OCTs (OCT1 and OCT3) (14, 35). The present study adds OAT1 to the list of transporters that facilitate the intracellular accumulation of antineoplastic drugs. This makes a strong case for an indepth study of the role of these transporters in the uptake of chlorambucil into lymphomas that infiltrate the kidney, resulting in an improvement of renal function in leukemia patients treated with this alkylating drug. However, the cytostatic effect of chlorambucil in lymphoma cells is well documented. In light of our observations, OAT1 is not expressed in lymphoma cells; therefore, we suppose (or assume) that some other transporter from the SLC superfamily might be responsible for the uptake and cytotoxic effect of chlorambucil in lymphocytes.

Bendamustine mainly functions as an alkylating agent causing the formation of intrastrand and interstrand cross-links between the bases of DNA that, in turn, inhibit DNA replication and transcription (13). In preclinical studies, bendamustine acted on tumor cells otherwise resistant to alkylating or intercalating drugs, suggesting, among other reasons, a higher accumulation inside cells (16, 36). Several clinical studies with bendamustine have documented a higher overall response in
patients with non-Hodgkin’s lymphoma, multiple myeloma, and relapsed or refractory chronic lymphatic leukemia compared with chlorambucil (4, 19, 34). A comparison between chlorambucil and bendamustine in a phase III multicenter randomized study on 319 patients revealed a higher complete response, progression-free survival, and higher duration of remissions in bendamustine-treated patients (21). The molecular basis for the better outcomes in bendamustine-treated patients was hitherto unknown.

Bendamustine is extensively metabolized in the liver by oxidation, carboxylic acid formation, N-dealkylation, sulfation, as well as conjugation with cysteine and glutathione, leading to 7–25 different metabolites (8, 10, 38, 39). The metabolites and 30–50% of the parental drug are excreted into the bile and urine. Although the exact contribution of both pathways is controversial, reported data suggest that renal excretion of bendamustine amounts to 45% of the overall elimination (13) and is higher for the parental compound than for metabolites (29). The kidneys could, therefore, be equipped with transporters for bendamustine excretion.

Here, we tested a possible interaction of the anionic bendamustine with OAT1, OAT3, and OAT4, transporters involved in the renal excretion of a variety of anionic drugs (6, 41). OAT1 and OAT4 were slightly or not at all inhibited in the

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**Fig. 7.** Bendamustine-induced apoptosis in lymphoma cells. A: representative experiment of scattergrams of untreated and bendamustine or bendamustine plus probenecid treated lymphoma cells stained with annexin V antibodies and propidium iodide (PI). B: spontaneous apoptosis and apoptotic events resulting after 15 min of incubation with bendamustine alone or together with probenecid. Values are means ± SE of three independent experiments with each duplicate. C: bendamustine-induced specific apoptosis (in %) calculated from the whole gated cell number and spontaneous apoptotic events as previously published (12). §§Significantly induced apoptosis by bendamustine and **significant inhibition of bendamustine-induced apoptosis by probenecid (P < 0.01).
presence of 100 μM bendamustine, suggesting that both transporters hardly interact with this drug. In contrast, OAT3-mediated ES uptake was nearly abolished by bendamustine. The IC50 value was 0.8 μM, indicating a very high affinity of OAT3 for bendamustine. At a dose of 30 to 200 mg/m² surface area, peak plasma concentrations of bendamustine between 0.1 and 30 μg/ml were reported (7, 13). These values translate into concentrations between 0.2 and 8.3 μM (molecular mass: 357.8 g/mol), being in the range of the affinity of OAT3 for bendamustine. Hence, OAT3 could well be involved in renal bendamustine excretion.

A more intriguing question was whether OAT3-expressing cells are more sensitive to the cytostatic actions of bendamustine compared with nonexpressing cells. As tested by thymidine incorporation, OAT3-expressing HEK-293 cells showed clearly decreased proliferation compared with mock cells without OAT3. This finding strongly suggests that OAT3 is able to transport bendamustine into cells, leading to a higher intracellular concentration and efficacy of this antineoplastic drug. OAT3-expressing tumor cells should, therefore, be more sensitive to bendamustine treatment than cells not expressing OAT3.

We examined six established lymphoma cell lines and cells from four CLL patients for a possible expression of OAT3. Surprisingly, lymphoma cell lines and CLL patient cells, but not lymphocytes from a healthy donor, expressed mRNA for OAT3, as indicated by a 6- to 12-cycle difference in quantitative RT-PCR. Each cycle difference represents a twofold change (2ΔΔCt) in the relative mRNA expression level.

A 15-min treatment of Raji and Jurkat lymphoma cells with bendamustine and subsequent cultivation for 12 h resulted in a higher rate of apoptosis, as shown by FACS analysis. Considering total gated events and spontaneous apoptosis in a calculation previously described by Friesen et al. (12), bendamustine-induced specific apoptosis was 1.8- and 2.1-fold higher for Raji and Jurkat cells, respectively, compared with spontaneous apoptosis. Importantly, the OAT3 inhibitor probenecid reduced bendamustine-induced apoptosis significantly. Therefore, we conclude that the increase in apoptosis is due to the uptake of bendamustine by OAT3. Similarly, OAT3 might be responsible for the bendamustine-induced remission of a B cell CLL-associated membranoproliferative glomerulonephritis, as it would increase the cytotoxicity of bendamustine to CLL that infiltrated the kidney. However, we do not have a direct proof of this fact.

One of the basic problems of current chemotherapy is the poor accumulation of the antineoplastic agents within tumor cells. The application of cytostatic drugs taken up by transporters expressed in the individual tumor may help to ameliorate or even to overcome chemoresistance. The present study shows, for the first time, that OAT1 and OAT3 possess affinity for the anionic antineoplastic agents chlorambucil and bendamustine. OAT3 has an exceptionally high affinity for bendamustine and appears to transport this agent, because upon treatment, OAT3-expressing model cells showed reduced proliferation. Since OAT3 is expressed in lymphoma cells, the superior efficacy of bendamustine in treating CLL patients could now be partially explained by bendamustine uptake into tumor cells. From the results of the present study, it is evident that these transporters play a crucial role in the determination of cytotoxicity of chlorambucil and bendamustine; whether they play a direct role in the alleviation of CLL-induced nephropathy remains to be elucidated.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

Author contributions: Y.H., G.W., and G.B. conception and design of research; Y.H., P.H., V.S. analyzed data; Y.H., P.H., V.S., G.W., and G.B. interpreted results of experiments; Y.H. prepared figures; Y.H. and V.V.V.R.M. drafted manuscript; Y.H., P.H., V.S., V.V.V.R.M., G.W., and G.B. edited and revised manuscript; Y.H., P.H., V.S., V.V.V.R.M., G.W., and G.B. approved final version of manuscript; P.H. and V.S. performed experiments.

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