Renal Human Organic Anion Transporter 3 increases the Susceptibility of Lymphoma Cells to Bendamustine Uptake

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ABSTRACT

Chronic lymphatic leukaemia (CLL) is often associated with nephritic syndrome. Effective treatment of CLL by chlorambucil and bendamustine leads to restoration of renal function. In this contribution, we sought to elucidate the impact of the organic anion transporters on the uptake of bendamustine and chlorambucil as a probable reason for the superior efficacy of bendamustine over chlorambucil in treating CLL. We examined the effects of structural analogs of p-aminohippurate, melphalan, chlorambucil and bendamustine, on OAT1-mediated [3H]PAH uptake, and OAT3- and OAT4- mediated [3H]estrone sulfate uptake in stably-transfected HEK293 cells. Melphalan had no significant inhibitory effect on any OAT whereas chlorambucil reduced OAT1-, OAT3- and OAT4-mediated uptake of PAH or estrone sulfate down to 14.6%, 16.3% and 66.0% of control, respectively. Bendamustine inhibited only the OAT3-mediated estrone sulfate uptake, which was reduced down to 14.3% of control cells, suggesting that it interacts exclusively with OAT3. The IC_{50} value for OAT3 was calculated to be 0.8 µM. Real time PCR experiments demonstrated a high expression of OAT3 in lymphoma cell lines as well as primary CLL cells. OAT3-mediated accumulation of bendamustine was associated with reduced cell proliferation and an increased rate of apoptosis. We conclude that the high efficacy of bendamustine in treating CLL might be partly contributed to the expression of OAT3 in lymphoma cells and the high affinity of bendamustine to this transporter.
INTRODUCTION:

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 more than 10 years 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).

The introduction of purine analogs almost thirty years ago, especially of fludarabine, changed the front line 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 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 a 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, oncvin (vincristine) and prednisone (COP) (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 response and progression-free survival in comparison to chlorambucil (20). Based on this vital clinical study the U.S. Food and Drug Administration (FDA) 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 the renal organic anion transporters (OATs). OAT1 and OAT3 are expressed at the basolateral and OAT4 at the apical membrane of proximal tubules cells, respectively, and are involved in renal excretion of a large number of drugs (1; 5; 33; 37; 42).

In this study, we examined the interactions of OATs (OAT1, OAT3 and OAT4) with cytostatic agents, melphalan, chlorambucil and bendamustine. Then, we determined the expression of the OAT transporters 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.
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 Mouse Leukocyte Virus (MuLV) reverse transcriptase was obtained from Promega GmbH (Mannheim, Germany). Chemical reagents (analytical grade) were purchased from Sigma Aldrich (Munich, Germany). Hygromycin was obtained from AppliChem (Darmstadt, Germany). [\(^{3}\)H]ES and [\(^{3}\)H]PAH were from PerkinElmer (Rodgau, Germany). [\(^{3}\)H]Thymidine was obtained from Amersham Bioscience. Real-time TaqMan primers and TaqMan buffer were purchased from Applied Biosystems (Darmstadt, Germany). The previously described B- and T-lymphoma cell lines Karpas, SUDHL4, Raji, Hut78 and Jurkat 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 the TRIZOL® Reagent following the manufacturer’s protocol. Concentration and quality of the purified RNA were quantified using the Gene Quant II spectrophotometer (Amersham Bioscience, Freiburg, Germany). cDNA was prepared from RNA using MuLV reverse transcriptase as per the protocol of the manufacturer. Briefly, 2 µg total RNA was reverse transcribed into cDNA in a reaction mixture containing 200 units of MuLV enzyme, 500 µM of each dNTP, and 2.5 µg/µl N\(_6\)-random primer in a 20 µl reaction volume for 1 h at 37°C and 10 min at 70°C. Five µl of diluted cDNA was used in the real-time PCR reaction mixture containing 12.5 µl of 2X reaction buffer, 1.25 µl 25X TaqMan-primer mixture, 6.25 µl nuclease free water in a total volume of 25 µl. The mixture was transferred into a 96 well plate, and covered with an optical adhesive film. The plate was then transferred to the AbiPrism 7000 real time PCR cycler.
(Applied Biosystems). The PCR program consisted of the following steps: 2 min at 52°C uridine glycosylase step, 10 min at 96°C TaqMan polymerase activation step, 40 cycles of 15 sec at 96°C, 1 min at 60°C. Fluorescence reading was performed on the last step of each cycle. Data were analyzed by the AbiPrism 7000 software.

Real-time TaqMan primers (hGAPDH, Hs 99999905 m1; hHPRT, Hs 99999909 m1; hOAT1 (SLC22A6), Hs00537914_m1, hOAT2 (SLC22A8), Hs00198527_m1, hOAT3 (SLC22A8), Hs00188599 m1 and hOAT4, (SLC22A11), Hs00945824_m1) and reagents were purchased from Applied Biosystems.

Transport measurements: T-REx HEK 293 cells stably expressing human OAT1, OAT3 or OAT4 were harvested and plated into 24 well plates (2×10^5 cells per well). After 72h incubation, cells were washed 3 times with Mammalian Ringer solution containing: 130 mM NaCl, 4 mM KCl, 1 mM CaCl_2, 1 mM MgSO_4, 1 mM NaH_2PO_4, 20 mM HEPES, and 20 mM D-glucose, pH adjusted with 1 M NaOH to 7.4. After washing, cells were incubated with 200 µl of transport solution containing 1 µM [^3H]PAH, or 20 nM [^3H]ES with or without 100 µM of the specific cytostatic in Mammalian Ringer. After 5 min incubation at RT, the cells were washed with ice-cold PBS and lysed with 500 µl of 1 M NaOH solution for 20 min. The lysed mixture was neutralized with 500µl 1 M HCl and transferred into scintillation vials. Incorporated radioactivity was counted by adding 2.5 ml LumaSafe scintillation solution (PerkinElmer, Rodgau, Germany) 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 studies were performed. The inhibitory effect of the drugs was calculated using 4 parameter Hill equations with SigmaPlot 11.0 (SPSS Science, Chicago, IL) to determine the concentration (IC_{50}) that causes half-maximal inhibition of the uptake.
\[
I = I_o + \frac{I_{\text{max}} \cdot c^n}{c^n + IC_{50}^n}
\]

**Description of the equation.** \( I_o \): inhibitory baseline effect (%), \( I_{\text{max}} \): maximal inhibitory effect (%), \( n \): Hill exponent \( (n = 1) \), \( c \): inhibitor concentration (µmol/L), \( IC_{50} \): inhibitor concentration causing half-maximal inhibitory effect (µmol/L)

**Proliferation assay:** Proliferation was estimated by \([^{3}\text{H}]\text{thymidine incorporation as described previously by (32) (35). hOAT1 or hOAT3 expressing HEK293 cells were harvested and seeded onto 24 well plates at a concentration of 5}\times10^5 \text{ cells/well. After 24 h, cells were incubated with different concentrations of chlorambucil and bendamustine for 30 min and then washed 3 times with the medium. The cells were then incubated in a medium containing 4 µM of \([^{3}\text{H}]\text{thymidine for 15 min. Following this, the cells were fixed with 5% trichloracetic acid (TCA) for 30 min on ice. The cells were washed twice with ice-cold PBS and once with 96% ethanol. Then, the pellets were 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, Meriden, CT).**

**Bendamustine dependent cytotoxicity:** Flow cytometric analysis of a B-lymphoma cell line, Raji and a T-lymphoma cell line, Jurkat, was performed. The cells were washed and resuspended in PBS to a concentration of 2.5 \times10^6 \text{ cells. They were then incubated with either 100 µM bendamustine or simultaneously with bendamustine and 1 mM probenecid. Untreated cells were used as a control. After 15 min incubation with cytostatics, cells were washed thrice with PBS to remove the drugs and grown further for 12h in culture medium without cytostatics. The cells were stained with annexinV/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 CaCl₂ and PI at a final concentration of 10 µM, containing 20 µl of annexin V-Fluos (Roche Diagnostics, Mannheim, Germany) for each ml 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 calculated the bendamustine as well as bendamustine plus probenecid induced apoptosis in both lymphoma cells using a calculation system described by Friesen et al (12). The formula is 100x (Drug induced apoptosis (%) − spontaneous apoptosis in cell medium (%)) / (100% − spontaneous apoptosis in cell medium (%)).

RESULTS:

Interaction of alkylating cytostatics with the organic anion transporters OAT1, OAT3 and OAT4

To elucidate the interaction of hOAT1, hOAT3 and hOAT4 with the alkylating cytostatics chlorambucil, melphalan and bendamustine, we examined the inhibition of hOAT1- mediated [³H]PAH uptake and hOAT3- and hOAT4-mediated [³H]ES uptake. The transport activity of OAT1, OAT3 or OAT4 was demonstrated in stably transfected HEK293 cells by measuring the uptake of the radiolabeled model substrates without cytostatic drugs in Ringer buffer in comparison to non-transfected cells (Fig. 2). Human OAT1 mediated PAH uptake, and OAT3 and OAT4 significantly facilitated estrone sulfate uptake in the stably transfected cells with influx rates of
20.2 ± 1.3 pmol/5min/10^6 cells, 39.3 ± 6.2 fmol/5min/10^6 cells and 45.2 ± 4.8 fmol/5min/10^6 cells, respectively. Untransfected cells showed uptake of 0.89 ± 0.13 pmol/5min/10^6 cells PAH, 4.6 ± 0.86 fmol/5min/10^6 cells and 3.9 ± 0.6 fmol/5min/10^6 cells estrone sulfate, respectively. To test for potential interactions of the alkylating cytostatic drugs with OATs, 100 µM of each drug was 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% ± 5.7%, 80.9% ± 7.2%, and 14.6% ± 0.8% of control by melphalan, bendamustine and chlorambucil, respectively (Fig. 3A). The hOAT3 transport activity was reduced down to 16.3% ± 1.3% by chlorambucil and to 14.3% ± 2.7% by bendamustine. Melphalan stimulated the uptake of estrone sulfate by hOAT3 to 124.0% ± 3.8% (Fig. 3B). Chlorambucil was the only alkylating cytostatic drug which inhibited hOAT4 facilitated estrone sulfate uptake to 66.0% ± 4.0% (p<0.001). Bendamustine did not show any interaction with hOAT4. On the other hand, melphalan significantly stimulated hOAT4 mediated estrone sulfate 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 studies. Figure 4 illustrates the inhibitory effects of the alkylating drugs in percentages of the untreated samples. The calculated IC_{50} values of OAT1 and OAT3 for chlorambucil were 44.3 ± 2.6 µM and 9.5 ± 1.9 µM, respectively. For bendamustine, the IC_{50} value was 0.8 ± 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 \[^{3}H\]thymidine incorporation assays. The treatment of OAT3-transfected HEK293 cells with increasing concentrations of chlorambucil induced a 20 to 25% decrease in thymidine incorporation similar to mock-transfected cells (Fig. 5A). However, OAT1 expressing cells demonstrated significantly higher reductions of thymidine incorporation in comparison to 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 a decreased proliferation in the presence of bendamustine (Fig. 5B), in contrast, chlorambucil and bendamustine were ineffective in mock transfected cells (Fig. 5A - B).

Expression of OATs 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 levels 6 to 8 fold higher $\Delta$Ct than in normal lymphocytes whereas all other cell lines showed 13 to 14 fold higher $\Delta$Ct levels of OAT3 expression in comparison to control lymphocytes (Fig. 6A). As OAT3 was highly expressed in the lymphoma cell lines, we examined its expression in four samples of Chronic Lymphatic Leukemia patients. The expression of OAT3 in the four samples of
patients showed 5 to 9 fold higher levels of OAT3 mRNA expression compared to control lymphocytes (Fig. 6B).

**Bendamustine induced and OAT3 mediated specific apoptotic effect in lymphoma cells**

To examine the OAT3-mediated, bendamustine-induced cytotoxicity in the lymphoma cell lines, we selected Raji cells as a low OAT3 expression system and Jurkat cells as high OAT3 expression system, and quantified apoptotic cell death by Annexin V and PI staining. We incubated both lymphoma cells with and without either bendamustine alone or bendamustine in combination with probenecid, an OAT3 inhibitor. After removal of the cytostatic drugs, the cells were subsequently grown for 12 h and the apoptotic events were quantified by FACS analysis (Fig. 7A, a representative experiment). Treatment with bendamustine increased the apoptotic events in both cell lines by 3 fold compared to low-level spontaneous apoptosis in untreated control cells. The simultaneous incubation of bendamustine with probenecid reduced the proportions of apoptotic cells significantly by 31 to 38 % (Fig. 7B). The 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 deduced by Friesen et al 2002 (12). Simultaneous incubation with probenecid reduced the bendamustine-induced specific apoptosis to 3.3% ± 0.8 % and 5.2% ± 0.3% for Raji and Jurkat, respectively (Fig. 7C). These results demonstrate for the first time the impact of OAT3 on the cytostatic efficacy of bendamustine in lymphoma cells.
Renal syndromes caused by leukemic infiltration of the kidneys in Chronic Lymphatic Leukemia were reported in several cases, and retrospective as well as autopsy studies 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 the 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 front-line therapy as well as in combination with other antineoplastic agents, e.g. fludarabine (11). Among other factors, the efficacy of chemotherapy depends on intracellular drug concentration that, 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 the renal organic anion transporters 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 p-aminohippurate (PAH), a model organic anion used to study the renal plasma flow mediated by the OAT function.

The alkylating agent melphalan carries a negatively charged carboxyl group and a positively charged amino group, resembling an amino acid. Earlier studies (26)
demonstrated a leucine-sensitive uptake of melphalan into murine leukemia cells, suggesting the involvement of an amino acid transporter. Indeed, the L-type amino acid transporter, LAT1 (SLC7A5), expressed in Xenopus laevis oocytes, was inhibited by melphalan (40; 43), and down-regulation of LAT1 in HeLa cells expression reduced uptake of melphalan (22). Besides its experimentally proven interaction with LAT1, melphalan inhibited the 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 NTCP (SLC10A1) and OATP1A2 (SLCO1A2) has been reported (23). The ABC transporter MRP1 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 IC$_{50}$ of OAT3 (9.5 µM) was about four times lower than that of OAT1 (44.3 µM). The therapeutic doses of chlorambucil are 0.1 to 0.2 mg/kg body weight, leading to a peak plasma concentration of approximately 0.5 µg/ml or 1.6 µM (MW 304.3 g/mol) (28). Although this concentration is below the IC$_{50}$ 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 non-expressing 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 the cells by OAT3, but the transport mediated substrate turnover was apparently not sufficient enough to influence the cell proliferation, although the affinity of chlorambucil was higher for OAT3 compared to OAT1 and OAT4. In contrast, thymidine incorporation was clearly reduced by chlorambucil in OAT1-expressing cells as compared to non- 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 demonstrate indirectly the uptake of chlorambucil in OAT1 expressing cells. An increased sensitivity to the antineoplastic agents, irinotecan, paclitaxel, and vincristine, was shown earlier in lymphoma cell lines and in renal carcinoma cells expressing the organic cation transporters, 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 in depth study of the role of these transporters in the uptake of chlorambucil into the lymphomas which infiltrate the kidney, resulting in the 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 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 crosslinks between the bases of DNA that, in turn, inhibit
DNA replication, repair, 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 the cells (16; 36). Several clinical studies with bendamustine documented a higher overall response in patients with Non-Hodgkin’s lymphoma, multiple myeloma, and relapsed or refractory chronic lymphatic leukemia as compared to 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 the 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 renal excretion of a variety of anionic drugs (6; 41). OAT1 and OAT4 were slightly or not at all inhibited in the presence of 100 µM bendamustine, suggesting that both transporters hardly interact with this drug. In contrast, OAT3-mediated estrone sulfate uptake was nearly abolished by bendamustine. The IC$_{50}$ 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). This figure would translate into concentrations between 0.2 and 8.3 µM (MW 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 as compared to non-expressing cells. As again tested by thymidine incorporation, OAT3-expressing HEK cells showed a clearly decreased proliferation in comparison to mock cells without OAT3. This finding strongly suggests that OAT3 is able to transport bendamustine into the 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 6-12 cycles difference in quantitative RT-PCR. Each cycle difference represents a two-fold change (2ΔCt) in relative mRNA-expression level.

A fifteen minutes treatment of Raji and Jurkat lymphoma cells with bendamustine and subsequent cultivation for 12 hours resulted in a higher rate of apoptosis as shown by FACS analysis. Considering total gated events and spontaneous apoptosis in a calculation described by Friesen et al. (12), bendamustine-induced specific apoptosis was 1.8 and 2.1 fold higher for Raji and Jurkat cells, respectively, as compared to spontaneous apoptosis. Importantly, the OAT3 inhibitor probenecid reduced the
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 the 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 the 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 the organic anion transporters 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 show a 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 the tumor cells. From this study, it is evident that these transporters play a crucial role in determination of cytotoxicity of chlorambucil and bendamustine and whether they play a direct role in the alleviation of CLL induced nephropathy remains to be elucidated.
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STATEMENT OF COMPETING FINANCIAL INTEREST

No conflict of interest exists. Yohannes Hagos is a professor at the Institute for Vegetative Physiology and Pathophysiology of the University Goettingen and also the CEO and shareholder of PortaCellTec biosciences GmbH.

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LEGEND FOR FIGURES

Figure 1. Chemical structures of bendamustine, chlorambucil, melphalan and p-aminohippuric acid (PAH).

Figure 2. OATs mediated uptake of radiolabeled organic anions. Stable expression of OAT1, OAT3 and OAT4 was verified by measuring the uptake of 1.0 µM [³H]PAH (OAT1), or of 20 nM [³H]estrone sulfate (ES) (OAT3, OAT4) (hatched columns). The black columns in (A), (B), and (C) indicate the uptake of PAH and estrone sulfate in vector transfected mock HEK293 cells. The values represent the mean ± SEM of 3 independent (n=3) experiments with 3 repeats each.

Figure 3. cis-Inhibition of OAT-mediated PAH or ES uptake by alkylating drugs. The inhibition study was performed using 100 µM of either melphalan, chlorambucil
or bendamustine competing with hOAT1-mediated uptake of 1.0 µM [³H]PAH (A), or OAT3 and OAT4 mediated uptake of 20 nM [³H]ES (B, C) (hatched columns). The black columns represent the inhibition of the respective uptake in mock cells. All experiments were standardized by setting the uptake in control (Ringer without inhibitor) of each experiment to 100%. The values represent the mean ± SEM of 3 independent (n=3) experiments with 3 repeats each. * indicate significant inhibition of OATs mediated substrate uptake by alkylating drugs (***, P<0.001) and ns no significant inhibition.

**Figure 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. The concentration dependent inhibition of chlorambucil on OAT1 or OAT3 is presented in panels (A) and (B), respectively. The concentration dependent inhibitory effect of bendamustine on OAT3 mediated ES uptake is depicted in panel (C). The IC₅₀ values were determined by sigmoidal calculation with the equation showed in methods section. The data represent the mean ± SEM of 3 independent (n=3) experiments with 3 repeats each.

**Figure 5. Effect of chlorambucil and bendamustine on cell proliferation.** [³H]Thymidine incorporation was measured after incubation of the 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. The effect of chlorambucil on the proliferation of OAT1-expressing, OAT3-expressing and vector transfected HEK cells is presented in panel (A), and the effect of bendamustine on OAT3 expressing cells and mock cells
is depicted in panel (B). All experiments were standardized by setting the control
(without chlorambucil or bendamustine) of each experiment to 100%. Data are mean
± SEM of three independent experiments with 2 repeats. * indicates significant
differences between untreated and treated cells. § indicates 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. (*, §, $ P< 0.05; **, §§, $$ P<0.01; *** P<0.001).

**Figure 6. Quantification of OAT3 mRNA expression in lymphoma cells.** Total
RNA extracted from (A) six lymphoma cell lines and (B) peripheral blood from CLL
patients was used to perform a quantitative expression analysis by TaqMan real-time
PCR. The cDNA quantity of each cell preparation was normalized using the GAPDH
Ct (Cycle at threshold). After standardization, the OAT3 expression of control
lymphocytes of healthy donors (ΔCtc) was correlated to that of the lymphoma cell
lines (ΔCtL). The results are shown as ΔCt (ΔCtC /ΔCtL). Each cycle difference
represents a two-fold change (2ΔCt) in relative mRNA-expression level. Thereby,
larger columns indicate a higher expression in lymphoma cells as compared to
control lymphocytes. The control lymphocytes are set to unity (ΔCtC/ΔCtL). Data are
means ± SEM of three (A) and two (B) independent experiments with two repeats
each.

**Figure 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 annexinV antibodies and propidium
iodide (PI) is depicted in panel (A). The spontaneous apoptosis and apoptotic events
resulting after 15 min incubation with bendamustine alone or together with
probenecid, are as mean ± SEM of three independent experiments with each
duplicate (B). Panel (C) demonstrates bendamustine induced specific apoptosis in
(%) calculated from the whole gated cell number and the spontaneous apoptotic
events as published (12). §§ significantly induced apoptosis by bendamustine and **
significance inhibition of bendamustine-induced apoptosis by probenecid (p<0.01)

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Figure 2

- **Figure 2A**: hOAT1 and mock uptake comparison.
- **Figure 2B**: hOAT3 and mock uptake comparison.
- **Figure 2C**: hOAT4 and mock uptake comparison.
Figure 3A

Figure 3B

Figure 3C
Figure 4A

**Figure 4A**

**hOAT1**

IC$_{50}$ = 44.3 ± 2.6 µM

Figure 4B

**Figure 4B**

**hOAT3**

IC$_{50}$ = 9.5 ± 1.9 µM

Figure 4C

**Figure 4C**

**hOAT3**

IC$_{50}$ = 0.8 ± 0.1 µM
Figure 5A

![Graph showing the effect of chlorambucil on [%[^3]H]thymidine incorporation.](image)

Figure 5B

![Graph showing the effect of bendamustine on [%[^3]H]thymidine incorporation.](image)
Figure 6A

![Graph showing OAT3 expression in different cell lines.](image)

Figure 6B

![Graph showing OAT3 expression in different cell types.](image)