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Influx and efflux transport as determinants of melphalan cytotoxicity: Resistance to melphalan in *MDR1* overexpressing tumor cell lines

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ABSTRACT

There is a considerable variation in efficacy of melphalan therapy in multiple myeloma (MM) and other hematopoietic tumors. We hypothesized that this may be due to variations in the expression of influx and efflux transporters of melphalan. We measured the expression of the influx transporters *LAT1*, *LAT2*, and *TAT1* and the efflux transporters *MDR1*, *MRP1* and *BCRP* by quantitative RT-PCR and related their expression to the intracellular accumulation and cytotoxicity of melphalan in 7 MM and 21 non-MM hematopoietic tumor cell lines. Variation in the intracellular accumulation accounted for nearly half of the variation in the cytotoxicity of melphalan in MM cell lines ($r^2 = 0.47$, $P = 0.04$). High expression of the efflux transporter *MDR1* was associated with low intracellular accumulation and low cytotoxicity of melphalan ($r^2 = 0.56$, $P = 0.03$ and $r^2 = 0.62$, $P = 0.02$, respectively). The effect was reversed by the MDR1 inhibitor cyclosporine. In addition, the *MDR1* overexpressing HL-60 cell line showed 10-fold higher resistance to melphalan than the non-*MDR1* expressing one. Again, the resistance was reversed by cyclosporine and by MDR1-specific shRNA.

LAT1 was the major influx transporter in tumor cell lines with 4000-fold higher expression than *LAT2*. Down-regulation of *LAT1* by siRNA reduced the melphalan uptake by 58% and toxicity by 3.5-fold, but natural variation in expression between the tumor cell lines was not associated with accumulation or cytotoxicity of melphalan. In conclusion, tumor-specific variations in the expression of the efflux transporter *MDR1*, but not of the influx transporter *LAT1*, affect the intracellular accumulation of melphalan and thus determine its cytotoxicity.

1. Introduction

Melphalan (L-phenylalanine mustard) is an alkylating cytotoxic drug that is used in a high dose regimen followed by stem cell transplantation in therapy of multiple myeloma (MM; [1]). In addition, melphalan is used in combination with prednisolone in a palliative regime by patients not eligible for stem cell transplantation [2,3] and in combination with novel thalidomide or bortezomib based therapies [4,5]. Variations in the response and acquired resistance to melphalan are major problems in the treatment of MM [6,7]. Increasing the dose of melphalan may increase treatment efficacy [8], but at the cost of high toxicity, including leucopenia, mucositis, and diarrhea [9,10]. Therefore, predictors of melphalan efficacy are necessary to optimize the therapy.

Low influx or excessive efflux transport may be limiting factors for melphalan efficacy. Melphalan has been developed by coupling the bifunctional alkylating agent nitrogen mustard to the amino acid phenylalanine [11,12]. The conjugation with phenylalanine improved the bioavailability of the nitrogen mustard by increasing its transporter-mediated cellular uptake. Influx transporters from the SLC7 family, known as L-type amino acid transporters (LATs), were suggested to mediate the cellular uptake of melphalan [13]. Host-specific genetic polymorphisms in the two human LAT isoforms, *LAT1* and *LAT2*, do not affect melphalan pharmacokinetics or toxicity [14]. However, the effects of tumor-specific variation in *LAT1* and *LAT2* on melphalan cytotoxicity and clinical efficacy are unknown.

Abbreviations: MM, multiple myeloma; MDR1, multidrug resistance protein 1 (also known as P-glycoprotein, official symbol *ABCB1*); MRP1, multiple drug resistance-associated protein 1 (official symbol *ABCC1*); LAT, L-type amino acid transporter; TAT, T-type amino acid transporter; BCRP, breast cancer resistance protein (official symbol *ABCG2*).

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On the other hand, overexpression of the efflux transporter MRP1 is known to mediate resistance to melphalan [15,16]. The mechanism of resistance includes initial conjugation with glutathione by glutathione S-transferases alpha, mu and pi followed by excretion by the ATP-dependent efflux transporter MRP1. However, it is not clear whether MRP1 is the only efflux transporter that can cause resistance to melphalan. Overexpression of another efflux transporter *MDR1* (P-glycoprotein, *ABCB1*) causes resistance to anthracycline-based therapies in patients with MM [17,18]. However, an overexpression of *MDR1* has been less studied as a cause of resistance in melphalan-based therapies of MM.

In this study, we asked whether tumor-specific variations in the efflux and the influx transport of melphalan might cause variations in melphalan toxicity. To test this, we assessed the expression of the efflux transporters *MDR1*, *MRP1* and *BCRP*, and the influx transporters *LAT1*, *LAT2,* their heavy chain *4F2hc*, and *TAT1* in 28 tumor cell lines and related them to the intracellular accumulation and toxicity of melphalan. The observed correlations were validated by selective down-regulation using RNAi and with small-molecular inhibitors of drug transporters.

2. Materials and methods

2.1. Materials

[^{3}H]-melphalan was obtained from Jörg Kix Isotopes (Volxheim, Germany). Cyclosporine A was obtained from Roche (Mannheim, Germany). Chemically synthesized siRNAs and transfection reagents were obtained from Qiagen (Hilden, Germany) and all cell culture media and supplements were obtained from Gibco-Invitrogen (Karlsruhe, Germany). L-[4,5-^{3}H]-leucine, L-[2,3,4,5,6-^{3}H]-phenylalanine, unlabeled amino acids and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and the rest of the chemicals (except otherwise stated in the text) were obtained from Sigma (Sigma–Aldrich Chemie GmbH, Munich, Germany).

2.2. Cell lines and culturing conditions

The MM cell lines U266, SK-MM-2, RPMI-8226, OPM-2, NCI-H929, LP-1 and L363 and the AML cell line HL-60 were obtained from German cell depository (DSMZ, Braunschweig, Germany). The HL60/Dox cell line was kindly provided by Beran et al. [19]. The U266, HL60 and HL60/Dox cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and SK-MM-2, RPMI-8226, OPM-2, NCI-H929 and L363 in RPMI supplemented with 20% fetal bovine serum. The LP-1 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. All cells were cultured at 37 °C under 5% CO_2 in humidified atmosphere and in the presence of 100 U/ml penicillin and 100 μg/ml streptomycin. Details about source and culture conditions of the remaining 21 cell lines are available on request.

2.3. Cytotoxicity assay

The cytotoxicity of melphalan was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as follows: 2.5×10^4 cells per well were plated in 96-well plates (Sarstedt, Nümbrecht-Rommelsdorf, Germany). Following an overnight pre-incubation the cells were exposed to increasing concentrations of melphalan (0, 1, 2.5, 10, 25, 100 and 1000 μM). In the MDR1 inhibition experiments, 8 μM cyclosporine A was added 3 h prior to the melphalan treatment. After 24 h incubation with melphalan the cells were pelleted by centrifugation ($500 \times g$ for 5 min) and the medium was replaced by MTT solution (10 μl of MTT (5 mg/ml dissolved in PBS buffer) in 100 μl culture medium per well). The cells were incubated for 1 h under standard conditions and the reaction was stopped by adding an equal volume of 1 mM HCl and 20% SDS. On the next day, the amount of reduced MTT was assayed measuring the absorption of the samples at 570 nm using the TECAN Ultra plate reader (TECAN, Crailsheim, Germany). Each concentration point was measured in duplicates and the absorption means were used to calculate melphalan dose–response curves using SigmaPlot (Systat GmbH, Erkrath, Germany). The IC_{50} values were calculated for each cell line as the concentration of melphalan that reduced the number of viable cells to 50% of control.

2.4. Apoptosis assay

Apoptosis was assayed as an increasing caspase 3/7 activity using the EnzCheck® Caspase-3 assay kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. Briefly, the cells were plated and treated with melphalan as described for the cell vitality assay. After 16 h of incubation with melphalan, the cells were centrifuged at $500 \times g$ for 5 min and the medium was replaced by 40 μl lysis buffer (kit). For optimal cell lysis the plates were subjected to three cycles of freezing in liquid nitrogen and thawing. The caspase 3/7 activity was measured in the lysates by adding 40 μl of 50 μM solution of the caspase 3/7 substrate Z-DEVD-R110. The amounts of released R110 were assayed by fluorescence photometry (excitation = 485 nm and emission = 535 nm, at 37 °C over 3 h) using TECAN Ultra microplate reader. One unit caspase 3/7 activity was defined as 1 μmol R110 released per minute. Caspase 3/7 activity was dependent on melphalan concentrations with maximum observed after incubation with 25 μM melphalan, therefore the caspase 3/7 activity at this concentration was used in the analyses.

2.5. Measurements of intracellular accumulation of melphalan

The intracellular accumulation of melphalan was determined radioactively using [^{3}H]-labeled melphalan. The [^{3}H]-melphalan was repurified by reversed phase HPLC and then used as follows: 2×10^6 cells in suspension were washed three times in PBS and were finally resuspended in 100 μl Ringer solution. The incubation was started by the addition of 100 μl Ringer solution containing 160 pmol (0.044 μCi) [^{3}H]-melphalan (final concentration of 0.8 μM). The incubation was stopped after 10 min by adding 800 μl ice-cold PBS buffer. The entire incubation mixture was layered on top of 200 μl silicon oil (representing a combination of Dow Corning 500 and 200/1cs at a ratio of 84:14 (v/v)) in microcentrifuge tubes. The tubes were centrifuged at $12{,}000 \times g$ for 2 min at room temperature. Cell pellets were solubilized in 1 N NaOH overnight and neutralized with an equivalent volume of 1 N HCl. The radioactivity was measured by a liquid scintillation counter (TRI-CARB 2900TR Liquid Scintillations Analyzer, Perkin Elmer, MA, USA). The uptake of radiolabeled substrates in adherent cells (HeLa) was performed in 24-well plates. 2.5×10^5 cells treated with and without LAT1 siRNA were seeded into each well for 48 h before starting the uptake measurements. Before adding the substrate, cells were washed three times with PBS buffer and then the uptake experiments were started by the addition of transport medium (Ringer) containing 50 nM [^{3}H]-melphalan, 10 nM L-[4,5-^{3}H]-leucine (77 Ci/mmol) or 5 nM L-[2,3,4,5,6-^{3}H]-phenylalanine (120 Ci/mmol). The uptake was terminated after 10 min by removing the uptake solution followed by washing three times with ice-cold PBS buffer. The cell pellets were then solubilized and the radioactivity was measured following the procedure for the suspension cells.

2.6. Quantitative RT-PCR

Total RNA was isolated from each cell line three times using an RNeasy Mini kit (Qiagen, Hilden, Germany) and cDNA was

prepared as described previously [14]. The expression of *LAT2*, *4F2hc*, *MRP1,* and *TAT1* genes was quantified by real-time RT-PCR using TaqMan® based gene expression assays (Hs00794796_m1, Hs00374243_m1, Hs00219905_m1 and Hs00218593_m1, respectively; Applied Biosystems, Darmstadt, Germany) following the manufacturer's instruction. The expression of *LAT1* and *MDR1* was measured using SYBR® Green based QuantiTect Primer Assays (Hs_SLC7A5_QT00089145 and Hs_ABCB1_QT00081928, respectively; Qiagen, Hilden, Germany) and HotStart-IT™ SYBR® Green qPCR Master Mix (USB, Staufen, Germany). The expression of *BCRP* was measured using self-designed SYBR® Green based assay using primers 5′-ATG TCA CGT GGA ATA CCA GC-3′ and 5′-GAA GAC TGA ACT CCC TTC CT-3′. The reaction mixture was preheated at 95 °C for 2 min, followed by 40 cycles at 95 °C for 15 s, at 60 °C for 25 s, and 72 °C for 35 s. Each sample was analyzed in duplicate. All measurements were performed using Sequence Detection System 7900HT (Applied Biosystems).

The gene expression was normalized on the expression of TATA-box binding protein (*TBP*, Applied Biosystem TaqMan assay # 4326322E). Absolute numbers of *LAT1* and *LAT2* transcripts were calculated using plasmid based standard curves. The full cDNA plasmids were obtained from RZPD (Berlin, Germany; IRATp970E0255D for *LAT1* cDNA, and IRAUp969F09104 for *LAT2* cDNA). Relative *4F2hc*, *TAT1*, *BCRP, MRP1*, and *MDR1* expression was calculated using the $2^{-\Delta Ct}$ method with *TBP* as internal standard.

2.7. MDR1 gene silencing using shRNA

Plasmid expressing small hairpin RNA against MDR1 (sh*MDR1*), and a control plasmid expressing small hairpin RNA against GFP (sh*GFP*), were used to silence MDR1 expression in HL60/Dox cells. The plasmid construction and validation have been previously described [20]. The shRNA expressing plasmids were stably transfected in HL60Dox cells as follows: 2 μg of the plasmids were linearized with DrdI (Fermentas, St. Leon-Rot, Germany) enzyme and electrophorated in 2×10^6 cells using Cell Line Nucleofector® Kit V (Amaxa, Cologne, Germany) according to the manufacturer instructions. Stable transfected clones were selected in medium supplemented with 400 μg/ml Zeocin. Forty-three shMDR1 and 12 shGFP clones were isolated, expand and screened for MDR1 expression using quantitative RT-PCR. The two shMDR1 clones with the lowest MDR1 expression and two shGFP clones (none of the tested shGFP clones showed reduction in the MDR1 expression) were used in the further analyses.

2.8. LAT1 gene silencing using siRNA

A chemically synthesized 21 nt long siRNA was used (5′-AAG GAC ATC TTC TCC GTC ATC-3′) that was previously reported to be effective against human *LAT1* [21]. As negative control, we used a siRNA oligonucleotide against *GFP* (5′-CGG CAA GCT GAC CCT GAA GTT CAT-3′). 4×10^5 HeLa cells were plated in a single well of 6-well plates, and after overnight pre-incubation was transfected with 25 nM siRNA using the HiPerFect® transfection reagent (Qiagen). To achieve long term silencing effect, the siRNA transfection was repeated two additional times (at days 4 and 8 after initial plating). The silencing efficacy was monitored by measuring *LAT1* expression at days 3, 6, and 9, and the phenotypic characterization was performed at days 6 and 9 after initial plating.

2.9. DNA methylation analyses

The methylation pattern of *LAT1* 5′-region was determined by sequencing of bisulfite-treated DNA as follows: 1 μg genomic DNA was treated with sodium bisulfite for 5 h and purified using the EpiTect bisulfite kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The *LAT1* 5′-region was amplified using primers 5′-TTG GGA ATA GTT (T/G)GT TAG GTT GG-3′ and 5′-AC(A/C) AAC CTA CCT CCT TAA ACA C-3′, which were specific for bisulfite-treated DNA, but unspecific for DNA methylation. The amplification was carried out with the HotStarTaq master mix kit (Qiagen) under following conditions: 5 min at 95 °C, 45 cycles of 95 °C for 20 s, 55 °C for 30 s, and 70 °C for 1 min, and a terminal elongation for 5 min at 70 °C. The PCR products were cloned into the pCR-XL plasmid using the TOPO-XL PCR cloning kit (Invitrogen, Karlsruhe, Germany). Single clones were selected and sequenced using automated Sanger sequencing.

2.10. Statistical analyses

Correlations between metric data were analyzed by the Pearson's correlation analysis and one-tailed *P*-values for significance testing were used if there was a predefined direction for the correlation. Following directions of correlation were predefined: increased intracellular accumulation was expected to correlate with an increased toxicity of melphalan; increased expression of the influx transporters was expected to correlate with increased intracellular accumulation and increased toxicity of melphalan; and finally increased expression of the efflux transporters was expected to correlate with decreased intracellular accumulation and decreased toxicity of melphalan. The coefficient of determination (r^2) was calculated as a square of Pearson's correlation coefficient. Comparisons between two groups were analyzed by the Student's *t*-test for independent groups and comparisons between more than two groups were analyzed by one-way analysis of variance. Bonferroni adjustment was performed in case of multiple comparisons (e.g. cell vitality was compared between treated and non-treated cells under different melphalan concentrations). All statistical analyses were performed using SPSS version 12.0 (SPSS Inc., Chicago, USA).

3. Results

3.1. Relations between toxicity and intracellular accumulation of melphalan

A major question of this project was to what extent the variations in the intracellular accumulation of melphalan may affect its cytotoxicity. As initial step, we measured variation in the melphalan toxicity in 28 cell lines (Table 1). The 28 cell lines were selected to represent tumors clinically treated with melphalan either as a primary (MM cell lines) or as second-line therapy (the remaining cell lines). In the whole panel of 28 cell lines, the melphalan concentrations that caused 50% reduction in cell vitality (IC_{50}) varied 26.2-fold (IC_{50} from 3.7 to 96.9 μM). The highest median melphalan cytotoxicity was observed in the group of Mantel cell lymphoma (median IC_{50} 11.9 μM, range 5.7–24.4 μM, Fig. 1 and Table 1) and the lowest in the group of chronic myeloid leukemia (median IC_{50} 54.2 μM, range 3.7–79.0 μM). Although there were some differences between the tumor types, analysis of variance showed that variation between the different tumor types was not significantly bigger than variation within the tumor cell types (*F*-test). Special focus in our analyses was on the subgroup of MM cell lines. The MM is the most relevant group for the clinical application of melphalan and the MM cell lines showed the highest inter-group variability in the melphalan toxicity. In the group of MM cell lines, the cytotoxicity of melphalan, measured by MTT assay, varied 5.5-fold (IC_{50} between 17.5 and 96.9 μM) and caspase 3/7 activity 11-fold (Table 1). The measurements of cytotoxicity and caspase 3/7 activity correlated significantly ($r = 0.68$, $P = 0.04$). These results show high variability in the response to melphalan among all tumor cell lines with some variability between different

Table 1
Toxicity and intracellular accumulation of melphalan and expression of influx and efflux transporters in the analyzed 28 tumor cell lines.

	Cell lines	Melphalan cytotoxicity [IC_{50} in μM]	Caspase 3/7 activity [μU/10^6 cells]	Melphalan accumulation [pmol/10^6 cells]	Expression of influx and efflux transporters							
					LAT1	*LAT2*	*4F2hc*	*TAT1*	*MDR1*	*MRP1*	*BCRP*	*TBP*
Multiple myeloma	U-266	25.7	23.3	1.7	0.4	0.001	24.9	26.6	22.2	27.1	26.1	25.6
	SK-MM-2	96.9	2.2	1.0	1.4	0.003	23.2	37.3	21.2	29.8	30.0	26.4
	RPMI-8226	40.6	3.7	1.4	1.2	0.023	23.3	23.3	34.0	27.1	20.5	25.5
	OPM-2	23.8	7.6	2.5	0.8	0.004	23.6	26.4	38.0	28.3	23.3	25.4
	NCI-H929	17.5	15.3	2.3	6.6	0.053	23.9	26.0	25.7	28.1	24.6	27.7
	LP-1	58.0	2.9	2.3	1.3	<0.001	23.6	28.8	28.4	26.4	21.2	26.8
	L-363	26.8	15.8	2.7	1.8	<0.001	24.3	33.2	34.9	27.1	30.6	27.5
Mantel cell lymphoma	GRANTA-519	24.4	3.5	1.3	0.4	<0.001	24.4	29.0	26.7	26.8	29.3	25.3
	JEKO-1	7.2	10.2	0.7	0.3	0.001	25.9	31.4	34.3	28.9	33.4	26.8
	SP-53	16.5	4.8	2.2	0.5	0.001	25.0	31.9	27.7	28.4	33.9	26.3
	MINO	5.7	25.1	1.3	0.3	<0.001	24.1	32.4	35.0	27.2	31.3	25.5
Hodgkin lymphoma	L-1236	50.8	5.5	2.2	0.2	0.012	24.4	29.1	26.4	26.4	24.1	25.8
	KM-H2	21.2	4.6	2.2	0.2	<0.001	24.3	28.1	23.4	25.8	23.1	25.0
	L-428	23.3	1.0	1.8	0.5	0.014	23.4	26.3	22.0	25.5	20.6	25.5
	L-540	4.3	5.5	1.3	2.7	0.002	24.3	38.9	24.0	26.7	23.5	26.3
	L-591	10.4	0.4	1.4	0.8	0.002	24.3	38.0	23.2	26.6	27.1	26.7
Burkitt lymphoma	BL-2	11.8	8.4	1.4	1.5	0.004	24.2	27.4	34.8	27.1	34.4	27.0
	BL-41	53.3	4.7	0.9	0.5	<0.001	24.1	27.6	38.0	27.6	>40	25.4
	RAMOS	26.0	2.5	1.0	1.7	0.037	24.2	27.6	19.2	27.2	>40	26.3
	RAJI	72.9	0.6	1.7	0.3	<0.001	23.8	38.2	29.2	27.1	34.8	25.4
Diffuse B-cell lymphoma	BALM3	30.3	1.0	2.0	0.5	<0.001	23.8	38.0	32.2	27.0	36.4	24.8
	KARPAS-422	41.3	0.9	1.6	1.0	0.001	23.8	31.0	31.2	27.4	34.8	25.2
	SU-DHL-4	31.6	1.6	2.6	0.9	0.001	24.9	31.0	36.3	26.9	35.3	26.6
CML	BV-173	3.7	2.2	1.5	0.5	<0.001	23.4	>40	36.3	27.0	26.3	25.1
	K-562	79.0	0.9	2.8	0.7	0.011	22.7	35.5	28.4	27.1	26.8	24.7
	LAMA-84	54.2	5.6	3.3	0.1	0.013	23.7	25.3	24.1	27.7	25.6	24.6
AML	HL-60	4.8	33.7	1.2	0.7	<0.001	23.5	26.3	35.5	27.5	31.6	25.3
	U-937	45.2	5.0	1.2	0.5	0.068	23.0	24.8	21.3	27.3	30.8	24.8

Caspase 3/7 activity was assayed after treatment with 25 μM melphalan for 16 h. The intracellular accumulation was assayed after exposing the cells to 0.8 μM melphalan for 10 min. The expressions of *LAT1* and *LAT2* are shown in number of transcripts per TBP transcript and of the remaining genes in Ct values. *Abbreviations*: CML, chronic myeloid leukemia; AML, acute myeloid leukemia.

hematopoietic tumors and high variability within the subgroup of MM cell lines.

To explore the role of membrane transport in the variable cytotoxicity of melphalan, we tested to what extent the variations in the cytotoxicity correlate with variations in the intracellular accumulation of melphalan. In the whole panel of 28 cell lines, there was no statistically significant correlation between intracellular accumulation and cytotoxicity. However, in the subset of MM cell lines, intracellular accumulation of melphalan correlated significantly with melphalan toxicity. Increased intracellular accumulation corelated with increased toxicity ($r^2 = 0.47$, $P = 0.04$; Fig. 2). Thus, variations in the membrane transport of melphalan and therefore of its intracellular accumulation may account for up to the half of the variation in its toxicity in MM cells. But it remained to be resolved whether the variations in the intracellular accumulation are due to variation in the efflux or in the influx transport of melphalan.

3.2. Efflux transport by MDR1, MRP1 and BCRP and the intracellular accumulation and toxicity of melphalan

To assess the role of the efflux transport in the intracellular accumulation of melphalan we measured expression of the efflux transporters *MRP1*, *BCRP* and *MDR1* in all tumor cell lines (Table 1). Among all the 28 tumor cell lines (Table 1), there was no statistically significant correlation between efflux transporter expression and melphalan accumulation or cytotoxicity. Within the MM cell lines, the *MRP1* expression varied 10-fold (Ct values from 26.4 to 29.8) and the *BCRP* expression 1000-fold (Ct from 20.5 to 30.6). However, neither *MRP1* nor *BCRP* expression correlated with the intracellular accumulation or with the toxicity of melphalan. On the other hand, the *MDR1* expression was highly variable (more than 200,000-fold). Increased *MDR1* expression correlated with decreased intracellular accumulation ($r^2 = 0.56$, $P = 0.03$; Fig. 3A) and increased resistance to

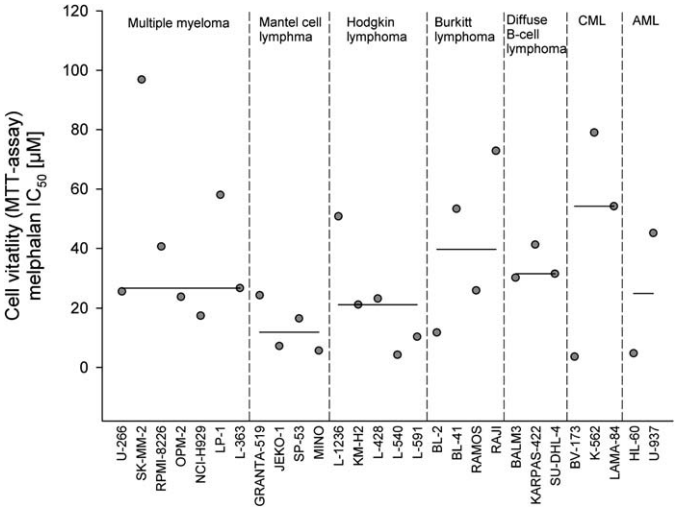

Fig. 1. Variations in the sensitivity to melphalan among the 28 cell lines originating from seven different types of tumors. Sensitivity to melphalan was measured using the MTT cell vitality assay. IC_{50} values were calculated from cell survival curves for each cell line (shown as grey circles) and median IC_{50} values (shown as lines) were calculated for the seven groups of tumors.

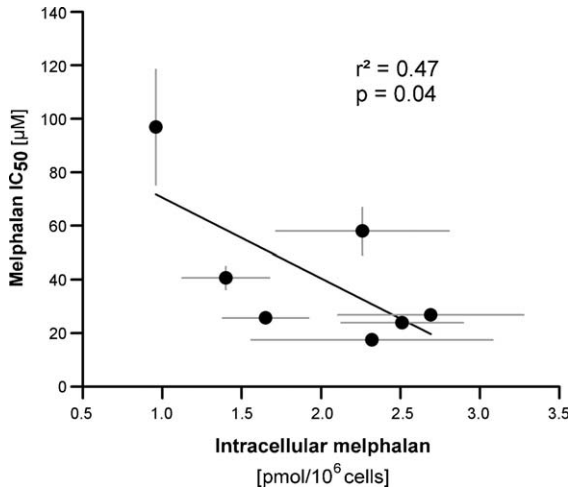

Fig. 2. Correlation between intracellular accumulation and toxicity of melphalan in multiple myeloma cell lines. Cytotoxicity of melphalan was determined using the MTT assay and are given as melphalan concentrations causing a 50% reduction in cell vitality (IC_{50}). The intracellular accumulation was assessed in cells treated with 0.8 μM [^{3}H]-melphalan for 10 min and was correlated with melphalan cytotoxicity. Means and their standard errors of three independent experiments are shown. The coefficient of determination (r^2) was calculated as a square of the Pearson's correlation coefficient. One-tailed significance of the correlation was calculated accounting for an a priori expected increase of cytotoxicity by increased intracellular accumulation of melphalan.

melphalan ($r^2 = 0.62$, $P = 0.02$; Fig. 3B) within the MM cell lines. Furthermore, when MDR1 activity was inhibited by cyclosporine A, the melphalan toxicity in SK-MM-2 cells, the cells with the highest *MDR1* expression, increased from IC_{50} of 93 μM to IC_{50} of 18 μM ($P = 0.025$, Fig. 3C). After this cyclosporine treatment, the melphalan cytotoxicity in SK-MM-2 cells treated was even higher than the median cytotoxicity (IC_{50} of 26.8) observed in the panel of all MM cell lines. Thus, MDR1 expression, but not MRP1 and BCRP expression were predictive for melphalan cytotoxicity in MM cell lines.

In addition, the impact of MDR1 on melphalan cytotoxicity was validated in an independent cell model. We compared the melphalan toxicity in the doxorubicin resistant cell line HL-60/Dox [19] with the melphalan toxicity in the parental cell line HL-60. The doxorubicin resistance in the HL-60/Dox cells is related with overexpression of MDR1 [19]. Indeed, also in our hands the HL-60/Dox cells showed high *MDR1* expression (Ct of 17.3), whereas in the maternal cell line HL-60 no *MDR1* expression was detected (Ct > 40; Fig. 4A). In contrast, there was constantly low background of *MRP1* expression in both cell lines (Ct of 27.8 and 27.6, respectively).

The melphalan toxicity was 10.1-fold lower in the HL-60/Dox cells compared to HL-60 cells (IC_{50} of 41.8 μM and 4.12 μM, respectively; $P = 0.0008$) and treatment of HL-60/Dox cells with cyclosporine A significantly reduced the melphalan IC_{50} by 58% ($P = 0.012$, Fig. 4B). However, cyclosporine A could affect melphalan toxicity also in some MDR1-independent mechanisms. Therefore, we validated the role of MDR1 in causing resistance to melphalan by silencing the *MDR1* expression using small hairpin RNA (shRNA). Using stable transfection, we generated shRNA expressing HL60/Dox cell lines that showed more than 70% reduction in their *MDR1* expression (Fig. 4A). The melphalan toxicity in shRNA expressing cells was also significantly increased by more than 3-fold compared to those of the paternal HL60/Dox cells (IC_{50} of 12.7 and 41.8 μM, respectively, $P < 0.0001$; Fig. 4B). On the other hand, no effects on the *MDR1* expression and melphalan toxicity were observed when a control shRNA against GFP was expressed (Fig. 4). In summary, melphalan resistance in HL60/Dox cells was reversed both by inhibition of MDR1 with cyclosporine A and by silencing the MDR1 expression with RNAi.

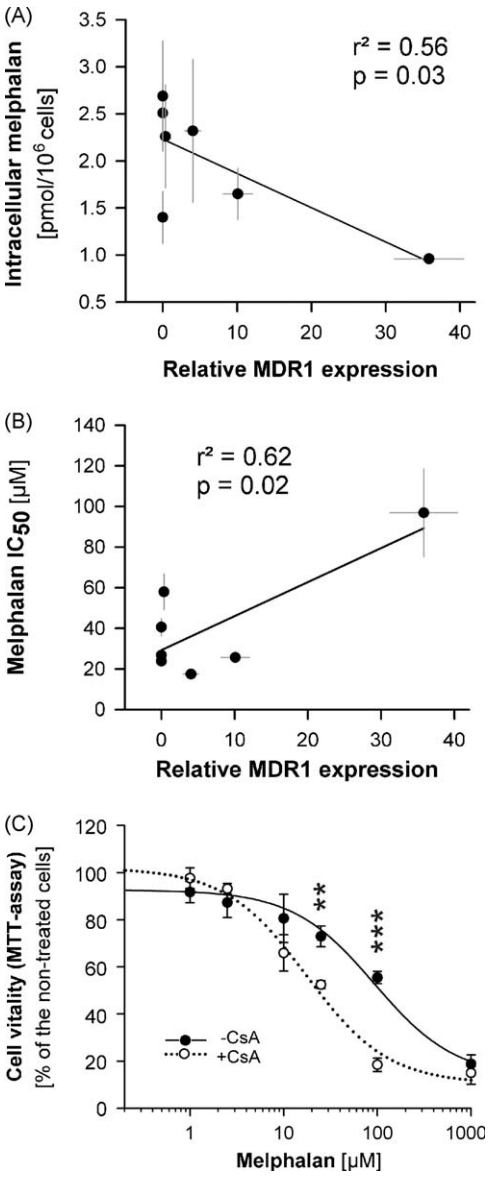

Fig. 3. Effect of MDR1 expression on intracellular accumulation and toxicity of melphalan. MDR1 expression was correlated with the intracellular accumulation of melphalan (A) and melphalan toxicity (B). The means of three independent experiments and their standard errors are shown. The coefficient of determination (r^2) and the one-tailed significance of the correlation are also represented. (C) Treatment with the MDR1 inhibitor cyclosporine A (CsA) increased sensitivity of SK-MM-2 cells to melphalan. The SK-MM-2 cell line showed the highest MDR1 expression among the MM cell lines. The means of at least three independent experiments and their standard errors are depicted ($^{**}P < 0.01$; $^{***}P < 0.001$ according to the Student's *t*-test after Bonferroni adjustment for six multiple tests).

Thus, a *MDR1* overexpression was confirmed to cause melphalan resistance using two independent approaches.

3.3. Influx transport by LAT1, 4F2hc and TAT1 and the uptake and toxicity of melphalan

After the efflux transport, we assessed the role of the influx transport on the intracellular accumulation and toxicity of melphalan. We first asked whether *LAT1* or *LAT2* might be the relevant influx transporter in hematological tumor cell lines. The expression of *LAT1* was on average more than 4000-fold higher than the expression of *LAT2* in all the 28 cell lines, and in the subgroup of MM cell lines in particular ($P < 10^{-6}$ and <0.01, respectively, paired samples *t*-test; Table 1). This suggests the *LAT1*

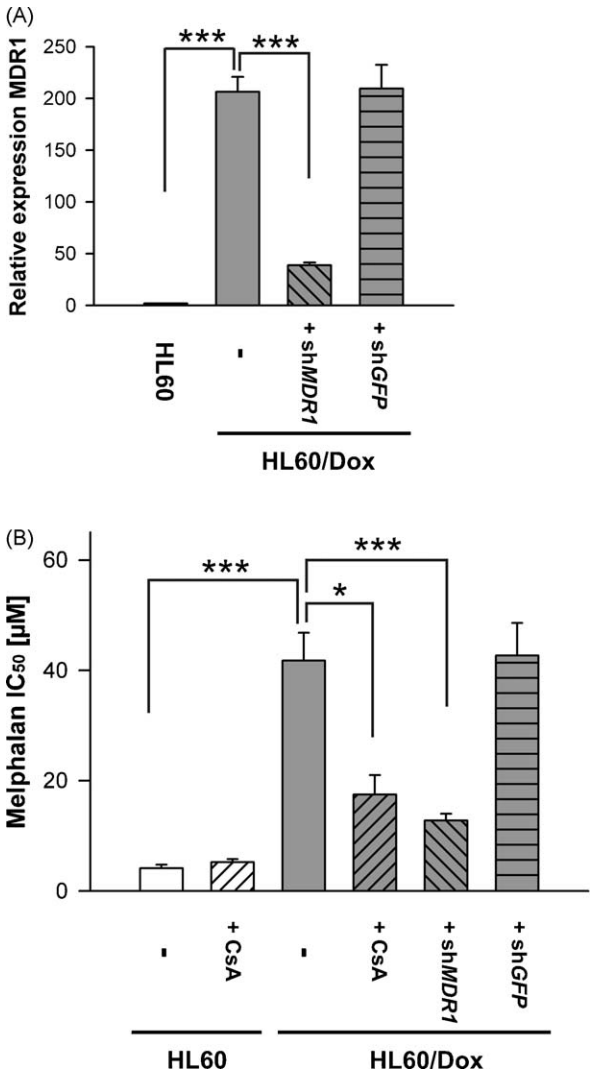

Fig. 4. Dependence of melphalan toxicity on MDR1 overexpression in HL-60/Dox cells. HL60/Dox cell line was originally obtained by selecting for HL60 mutants with high resistance to antracyclines [19]. (A) This figure shows the relative MDR1 expression in the parental HL60 cells, HL60/Dox cells and HL60/Dox cells stably transfected with plasmids expressing short hairpin RNA against MDR1 (sh*MDR1*) and controls expressing short hairpin RNA against green florescence protein (sh*GFP*) (***$P < 0.001$ according to the Student's *t*-test). (B) This figure shows the reversal of melphalan resistance in HL60/Dox cells by cyclosporine A (CsA) or by silencing of MDR1 using small hairpin RNA (sh*MDR1*). Mean IC_{50} values from three independent experiments and their standard errors are shown and the experiments were performed using two independent shMDR1 and shGFP expressing clones (*$P < 0.05$; ***$P < 0.001$ according to the Student's *t*-test).

rather than the *LAT2* isoform plays a role in uptake of melphalan in tumor cell lines.

To confirm the impact of LAT1 on the influx transport and toxicity of melphalan, we knocked down *LAT1* in HeLa cells using siRNA. HeLa cell lines expressed 2.9 *LAT1* transcripts/TBP and only 0.007 *LAT2* transcripts/TBP, which closely resembled the expression pattern observed in the MM cell lines (Table 1). Treatment with siRNA results in a rapid down-regulation of *LAT1* mRNA (Fig. 5A) and in a graduate decrease of transport activity over 9 days (Fig. 5B). After 9 days of treatment the influx transport of typical LAT1 substrates leucine and phenylalanine was significantly decreased by 69.5% ($P < 0.0001$) and 39.2% ($P < 0.0001$), respectively. Importantly, the influx of radioactive melphalan was also significantly decreased by 58.2% ($P < 0.0001$, Fig. 5B) and the toxicity of melphalan was significantly decreased by 3.5-fold (from IC_{50} of 84 of 292 μM, $P = 0.001$; Fig. 5C). This confirms LAT1 as a functional influx transporter of melphalan.

In the next step, we asked whether the variation in *LAT1* expression correlated with intracellular accumulation and toxicity of melphalan in the hematological tumor cell lines. The *LAT1* expression varied 66-fold among all the 28 cell lines and 16.5-fold among the subgroup of MM cell lines (Table 1). However, the *LAT1* expression correlated neither with the intracellular accumulation nor with the toxicity of melphalan in the whole panel of 28 cell lines ($r^2 < 0.01$, $P > 0.34$). There were no correlations within the subgroup of MM cell lines either ($r^2 < 0.03$, $P > 0.36$). Thus, in spite that LAT1 can mediate the influx transport of melphalan, expression of LAT1 in the individual tumor cell line was not predictive for intracellular accumulation or toxicity of melphalan neither in hematological tumor cell lines in general nor in MM tumor cell lines.

Furthermore, we analyzed the expression of *4F2hc*, the heavy chain of the LAT complex, and the T-type amino acid influx transporter *TAT1*. The MM cell line with the highest *LAT1* expression, NCI-H929, showed also the highest *4F2hc* and *TAT1* expression and was with an IC_{50} of 17.5 μM the most melphalan sensitive cell line among the MM cell lines tested. However, no correlation was observed between *4F2hc* or *TAT1* expression and intracellular accumulation or toxicity of melphalan neither in the whole group of 28 cell lines nor in the subgroup of MM cell lines alone ($r^2 < 0.09$, $P > 0.23$). Interestingly, the expression of *4F2hc* correlated significantly with the expression of *LAT1* ($r^2 = 0.78$, $P = 0.004$) suggesting co-regulation of both genes in tumor cells. These results suggested that variations in the expression of the known melphalan influx transporter are not limiting for melphalan intracellular accumulation and are not determining melphalan toxicity.

Since our earlier studies could not explain differences in *LAT1* expression by genomic variation [14], we hypothesized that expression might be modulated by epigenetic factors. Thus, we asked whether tumor-specific epigenetic effects like methylation of the *LAT1* promoter may be predictive for *LAT1* expression or for melphalan cytotoxicity. Using the strict criteria of Takai and Jones [22] we identified a CpG island overlapping with the 5′-region and exon 1 of *LAT1* (GC contents of 73.6% and observed vs. expected ratio of 0.94). We analyzed the methylation status of this island in the seven MM cell lines and in non-malignant blood and liver tissues. Only scarce methylation was observed in all samples analyzed, with slightly higher methylation rates in the liver samples where *LAT1* is not expressed (supplementary Fig. 1). The methylation status correlated neither with *LAT1* expression nor with melphalan toxicity in the MM cells. Furthermore, treatment of MM cells for 72 h with 1 μM of the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine did not increase their response to melphalan (data not shown). Thus, methylation of the *LAT1* promoter does not differ between tumor cell lines and non-malignant tissues and the methylation pattern is not predictive for melphalan toxicity.

4. Discussion

Our results indicate that the transmembrane transport is an important determinant of melphalan toxicity and that variation in the efflux, rather than variation in the influx transport, may determine tumor-specific variation in melphalan efficacy. We observed a strong variation in melphalan toxicity within the different tumor cell lines. In an unselected panel of MM cell lines, nearly the half of the observed variation in melphalan cytotoxicity was associated with the variations in membrane transport (Fig. 2). Alternative mechanisms, including differences in the DNA-repair capacity or apoptosis signaling, may account for the rest of the

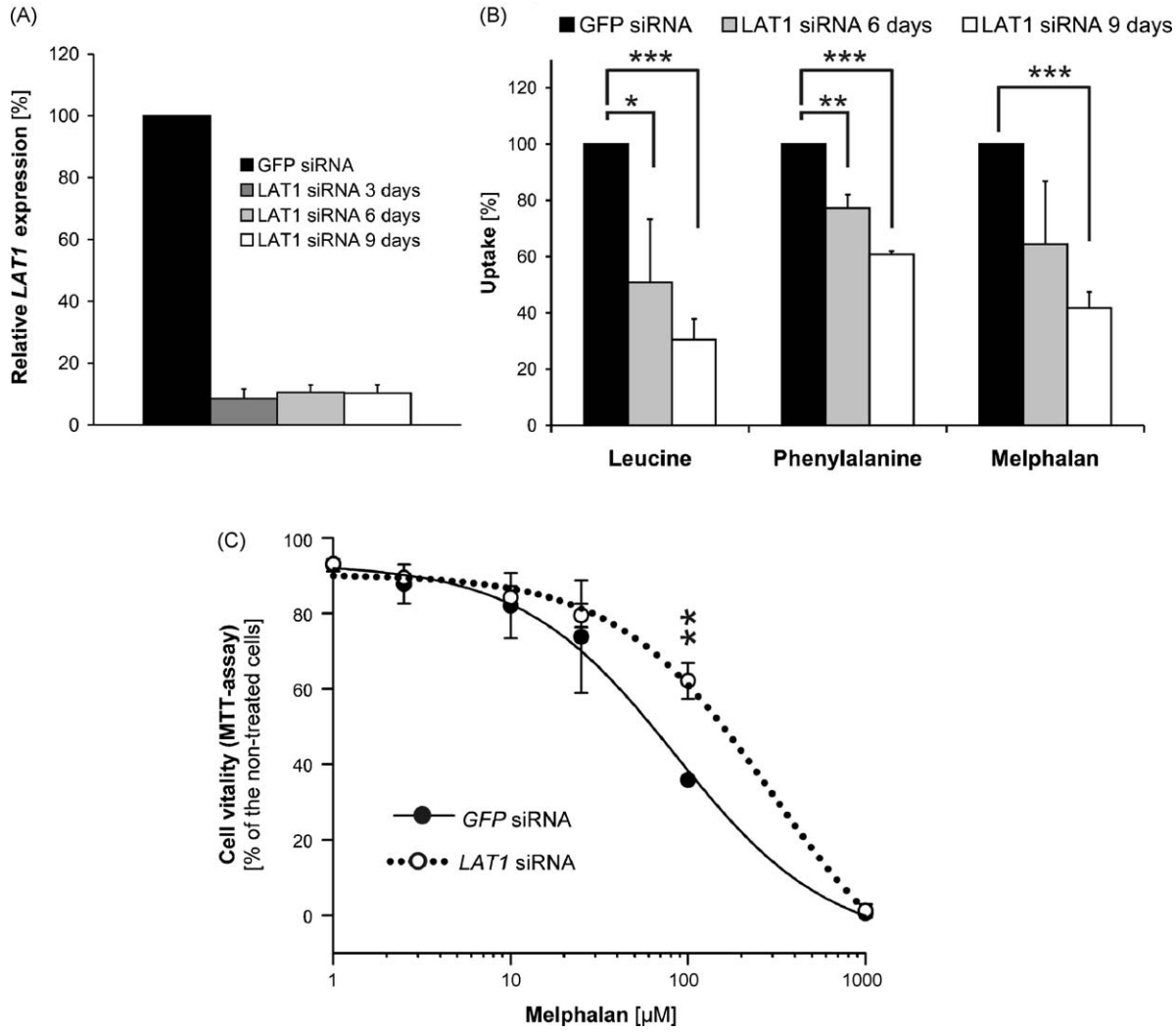

Fig. 5. Effects of *LAT1* silencing on intracellular accumulation and toxicity of melphalan. The expression of *LAT1* in HeLa cells was down-regulated using siRNA in comparison to a control siRNA against GFP. (A) Addition of LAT1 siRNA resulted in a significant and rapid decrease of *LAT1* mRNA. The means of three independent experiments and their standard errors are depicted. Significance was tested with one-way analysis of variance (*F*-test $P < 0.0001$). (B) *LAT1* knock-down resulted in decreased influx of leucine, phenylalanine and melphalan. The means of at least two independent experiments and their standard errors are depicted (*F*-test $P < 0.0001$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ according to the Student's *t*-test). (C) *LAT1* knock-down resulted in decrease of melphalan cytotoxicity. The means of at least three independent experiments and their standard errors are depicted ($^{**}P < 0.01$ according to the Student's *t*-test after Bonferroni adjustment for six multiple tests).

observed variation in melphalan cytotoxicity [6,23–26]. Overexpression of the efflux pump *MDR1* resulted in decreased intracellular accumulation and toxicity of melphalan both in MM and in HL-60 cell line (Figs. 3 and 4). On the other hand, although our data confirmed that the influx transporter *LAT1* can mediate the uptake of melphalan in tumor cells (Fig. 5), variations in LAT1 expression did not explain the variations in melphalan intracellular accumulation and toxicity.

Here we suggest MDR1 as an important efflux transporter for melphalan. The efflux pump MRP1, but not MDR1, has been previously known to mediate melphalan resistance [15,16]. However, in our study we did not observe a correlation between *MRP1* mRNA expression and the intracellular accumulation or activity of melphalan. One explanation may be that only moderate variability in the *MRP1* expression was present in our samples (less than 11-fold among the MM cells, which corresponds to difference of 3.4 in the Ct values; Table 1). Alternative explanation may be based on the requirement of conjugation with glutathione by glutathione S-transferases (GSTs) before melphalan is exported by the efflux transporters from the MRP family. Thus, the activity of the GST enzymes or availability of intracellular glutathione may be limiting for the efflux transport of melphalan and the variability of the GST activities was not measured in our tumor cell lines.

On the other hand, in our experiments the MDR1 overexpression was associated with resistance to melphalan in MM cells. The *MDR1* overexpressing cell line SK-MM-2 (the *MDR1* expression was 100-fold higher in SK-MM-2 cells than the median expression in the MM panel) showed high resistance to melphalan. The initially observed association between MDR1 overexpression and reduced melphalan cytotoxicity was based mostly on one cell line (Figs. 2 and 3). However, the assumption that MDR1 overexpression causes melphalan resistance was then strongly supported by the results of two additional experiments. First, the MDR1 inhibitor cyclosporine A reversed the resistance of the SK-MM-2 cells to melphalan (Fig. 4). Second, we tested an independent cell line model consisting of a parental line with no MDR1 expression, HL-60, and a daughter line with high MDR1 expression, HL-60/Dox. The *MDR1* overexpressing cell line showed increased resistance to melphalan compared to the parental line, and this resistance was again reversed by cyclosporine A. Even more importantly, this resistance was reversed using a small hairpin RNA specific against MDR1 (Fig. 4). Both the SK-MM-2 cells

and the HL60 and HL60/Dox cell lines had only marginal *MRP1* expression. Therefore, we claim that in the absence of strong *MRP1* overexpression also *MDR1* overexpression causes resistance to melphalan.

The finding that *MDR1* overexpression causes resistance to melphalan may be of clinical relevance. There is no evidence that melphalan treatment can directly induce *MDR1* overexpression [27,28]. Melphalan, however, is commonly used as a second-line treatment for tumors previously treated with anthracyclines, which are known to induce *MDR1* overexpression [18]. Furthermore, in HL60/Dox cells, the *MDR1* overexpression was also caused by previous treatment with doxorubicin [19]. Therefore, it may be also that in clinical settings the tumors that developed resistance to anthracyclines by overexpressing *MDR1* will not respond to melphalan either. In addition, the effects of a combination therapy of melphalan and bortezomib may be modulated by interactions at MDR1 and there are several other strong inducers of MDR1 like rifampin or carbamazepine which thus may compromise melphalan treatment response.

Surprisingly, we were not able to find a correlation between the expression of any of the putative melphalan influx transporters (LAT1, LAT2, TAT1) and melphalan toxicity. It has been shown earlier that the *LAT1/4F2hc* complex catalyzes the uptake of melphalan when overexpressed in *Xenopus* oocytes [13]. Using the knock-down technique we confirmed that also endogenously expressed *LAT1* mediates (at least part of the) intracellular uptake of melphalan, and this down-regulation of *LAT1* results in resistance to melphalan (Fig. 3). Furthermore, we observed substantial variation in the expression of *LAT1* (and *4F2hc*) among the tumor cell lines, which may be explained by the role of oncogenes like MYC in the transcriptional regulation of the both genes [29]. Indeed, also in our hands the lowest *LAT1* expression was observed in the MYC-independent MM cell line U266 (Table 1). However, in our study the expression of *LAT1* correlated neither with the intracellular accumulation nor with the toxicity of melphalan. Possible explanations for this lack of correlation are first that LAT activity is not predominantly regulated by LAT1 expression but by other factors, or second, the melphalan concentrations used in our experiments were below the transport maximum even in those cell lines with relatively small LAT expression, or third, that there may be additional, LAT-independent, influx transporters of melphalan in the MM cells. In addition, we cannot exclude that there is some extend of influx transport not mediated by transport proteins but just by passive transmembrane diffusion even with the relatively hydrophilic substance melphalan (octanol–water partition coefficient of about 0.3).

Factors different from LAT1 expression may regulate LAT1 activity. The LAT complexes consist of a light chain, represented by *LAT1* in tumor cells [30] and our results, and a heavy chain glycoprotein encoded by *4F2hc*. Although not catalytically active, 4F2hc is essential for functional cell surface expression [31] and its down-regulation in myeloma cells was associated with reduced uptake and sensitivity to melphalan [32]. The expression of *4F2hc* in our model, however, was strongly correlated with the expression of *LAT1*, but not with the intracellular accumulation or toxicity of melphalan. In addition, the LAT system represents obligatory exchangers, which require an export of equimolar amounts of small amino acids, like methionine, for the uptake of melphalan [30,33]. We cannot exclude that the availability of intracellular substrates rather than the expression may limit the activity of the exchanger LAT system in tumor cells [33].

Alternatively, amino acid transporters different from the LAT system may also be involved in the uptake of melphalan in tumors. This is supported by the observation that knocking down of *LAT1* resulted in almost complete abolishment of *LAT1* expression (more than 90% on RNA levels, Fig. 2A) but in only 58% reduction in the uptake of melphalan (Fig. 3B). However, we analyzed the expression of *TAT1* in the MM cell lines, but did not see a correlation between *TAT1* gene expression and intracellular accumulation or toxicity of melphalan. Thus, the influx system of melphalan seems to be more complex and variations in the expression of a single influx gene are not predictive for the intracellular accumulation or the toxicity of the drug.

In conclusion, we have found that variations in a membrane transport of melphalan contribute to variations in its toxicity. The LAT1 uptake system is only one component mediating intracellular uptake of melphalan and variation in the expression of *LAT1* and *4F2hc* genes was not predictive for melphalan toxicity. However, overexpression of the efflux transporter *MDR1* may cause resistance to melphalan in tumors. Our data suggest that *MDR1* expression should be considered in analysis of melphalan resistance and in sequential therapies or in combination therapies with drugs like bortezomib or anthracyclines.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2009.03.026.

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