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Expression of ABCC-Type Nucleotide Exporters in Blasts of Adult Acute Myeloid Leukemia: Relation to Long-term Survival

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Abstract

Purpose—Successful treatment of acute myeloid leukemia (AML) remains a therapeutic challenge, with a high percentage of patients suffering from persistent or relapsed disease. Resistance to drug therapy can develop from increased drug export and/or altered intracellular signaling. Both mechanisms are mediated by the efflux transporters ABCC4 (MRP4), ABCC5 (MRP5), and ABCC11 (MRP8), which are involved in cellular efflux of endogenous signaling molecules (e.g., cyclic adenosine 3′, 5′-monophosphate and cyclic guanosine 3′,5′-monophosphate) and nucleoside analogues. The nucleoside analogue cytosine arabinoside (AraC) is administered to all patients with AML.

Experimental Design—Expression of ABCC transporters MRP4, MRP5, and MRP8 in blast samples from 50 AML patients was investigated by real-time reverse transcription-PCR analysis and correlated with clinical outcome measures. Accumulation of radiolabeled AraC, transport of AraC metabolites, and AraC cytotoxicity were analyzed in MRP8-transfected LLC-PK1 cells.

Results—Regression analysis revealed that high expression of MRP8 is associated with a low probability of overall survival assessed over 4 years ($P < 0.03$). MRP8-transfected LLC-PK1 cells accumulated reduced intracellular levels of AraC (63% of the parental vector-transfected LLC-PK1 control cells) as well as AraC metabolites. Furthermore, AraC monophosphate was transported by MRP8-enriched membrane vesicles (116 ± 6 versus 65 ± 13 pmol/mg/10 minutes by control vesicles), and MRP8-transfected cells were resistant to AraC.

Conclusion—These data suggest that MRP8 is differentially expressed in AML blasts, that expression of MRP8 serves as a predictive marker for treatment outcome in AML, and that efflux of AraC metabolites by MRP8 is a mechanism that contributes to resistance of AML blasts.

A major obstacle to the successful treatment of acute leukemia is the development of resistance. Approximately 50% to 75% of adults with acute myeloid leukemia (AML) achieve complete remission (CR), whereas only 20% to 30% of patients are long-term relapse-free survivors. The majority of patients suffer from persistent or relapsed disease (1). Resistance can occur by various mechanisms, among them active drug export from tumor cells or alterations in intracellular signal transduction. Both mechanisms can be affected by blast expression of members of the C-family of ABC drug transporters, particularly ABCC4 (MRP4), ABCC5 (MRP5), and ABCC11 (MRP8). These transport proteins are unique in their capability to enhance cellular efflux of cyclic nucleotides (2–4), which are important signaling molecules in the regulation of cell proliferation, differentiation, and apoptosis, particularly in hematopoietic development (5–8). In addition, recent experiments suggest that intracellular levels of cyclic nucleotides can modulate the sensitivity of leukemia cells toward certain antineoplastic agents (9, 10) and other proapoptotic stimuli (11). Furthermore, MRP4, MRP5, and MRP8 are able to confer resistance to nucleoside analogues, which are important cytostatic drugs in the treatment of acute leukemia. MRP4 confers resistance to 6-mercaptopurine, 6-thioguanine (2, 12), and methotrexate (13, 14) in cells in which the protein is ectopically expressed. For MRP5, ectopic expression is associated with resistance to 6-mercaptopurine (15), 6-thioguanine (15), and methotrexate (16) as well as to cladribine (12, 17) and 5-fluorouracil (5-FU; ref. 18). In addition, MRP8, a more recently identified ABCC family member (19), is reported to confer resistance to 5-

FU, and possibly methotrexate (4). Thus, MRP4, MRP5, and MRP8 by virtue of their ability to confer resistance to certain nucleoside-type drugs and to efflux cyclic nucleotides have the potential to affect the treatment of AML. We therefore investigated the expression of ABCC transport proteins in blast cells from AML patients. Results presented here indicate that expression of MRP8 in blasts predicts decreased long-term survival in these patients. In addition, experiments showing that MRP8-transfected cells are resistant to and accumulate lower levels of cytosine arabinoside (cytarabine, AraC), a key agent used in the treatment of AML, indicate that reduced survival may be attributable to MRP8-mediated efflux of this agent.

Materials and Methods

Patients and sample collection

Bone marrow samples of 50 patients taken at diagnosis were selected randomly from a study pool of blast samples and examined. The patient population consisted of 26 males and 24 females with a median age of 60 y (range, 2078). Five of the patients were diagnosed with secondary disease, the resulting 45 with *de novo* AML. Patients were subdivided into the following French-American-British (FAB) subtypes: M0 ($n = 4$), M1 ($n = 12$), M2 ($n = 14$), M4 ($n = 7$), and M5 ($n = 13$). Patients diagnosed with the FAB subtype M3 were not included and treated within other European trials. Median white blood count was 50.7×10^9 cells/L (range, 0.7-303; $n = 48$), median rate of bone marrow blasts was 78% (range, 51-95), and the median amount of CD34-positive blasts was 28% (range, 0-97; $n = 48$). All of the patients included here were treated within a multicenter treatment trial of the German Study Initiative Leukemia for *de novo* and secondary AML (DSIL AML96, NCT 00180115) as published previously (20). Briefly, double induction therapy was stratified according to age. Patients ≤60 y ($n = 27$) received one course of MAV (10 mg/m^2 mitoxantrone, days 4-8; 100 mg/m^2 AraC, days 18; 100 mg/m^2 etoposide, days 4-8) and a second course of MAMAC (2 × 1,000 mg/m^2 AraC, days 1-5; 100 mg/m^2 m-amsacrine, days 1-5). Patients older than 60 y ($n = 23$) were treated with two courses of DA (45 mg/m^2 daunorubicin, days 3-5; 100 mg/m^2 AraC, days 1-7). CR rate was defined in accordance to the previously published consensus criteria (21) with <5% of blast cells in a standardized bone marrow puncture after the second course of induction therapy. Only patients with fully regenerated peripheral blood count were considered to be in CR. Postremission therapy for individuals ≤60 y was priority based and adapted for cytogenetic risk (20). Patients with intermediate cytogenetic risk were referred to allogeneic hematopoietic stem cell transplantation (HSCT) from an HLA-identical sibling donor. Patients with intermediate cytogenetic risk without a sibling and low-risk patients were randomized to receive intermediate (2 × 1,000 mg/m^2 every 12 h, days 1-6; I-MAC) or high-dose (2 × 3,000 mg/m^2 every 12 h, days 1-6; H-MAC) AraC + mitoxantrone (10 mg/m^2, days 4-6), which was followed by autologous HSCT (intermediate cytogenetic risk) or MAMAC (low cytogenetic risk). Patients with cytogenetic high risk were referred to allogeneic HSCT, including the option of unrelated HSCT. Patients without a donor were treated with either I-MAC or H-MAC and referred to autologous HSCT. Patients over 60 y of age received one postremission therapy consisting of 2 × 1,000 mg/m^2 AraC (days 1-5) and 100 mg/m^2 m-AMSA (days 1-5; MAMAC).

The study was approved by the local ethics committee and each patient gave written informed consent. Leukemic blast cells from patients as well as peripheral blood monocytes from human volunteers were isolated by Ficoll-Hypaque density gradient separation and frozen in liquid nitrogen.

Cytogenetics

Chromosome analyses were done in all 50 AML patients studied and were done on metaphases from direct preparations, as well as from 24- and 48-h cultures of bone marrow and/or peripheral blood samples as described previously (22). The cytogenetic preparation and G-banding were done according to routine laboratory procedures. Cytogenetic risk groups were defined as follows: unfavorable: −5/del(5q), −7/del(7q), hypodiploid karyotypes (besides 45,X,-Y or -X), inv(3q), abnl12p, abnl11q, +11, + 13, +21, +22, t(6;9); t(9;22); t(9;11); t(3;3), multiple (i.e., three or more independent aberrations; $n = 13$); intermediate: patients without low-risk or high-risk constellation ($n = 37$); favorable: t(8;21) and t(8;21) combined with other aberrations ($n = 0$).

Real-time PCR

RNA was prepared using the Qiagen RNA isolation kit according to the manufacturer's instruction and complementary cDNA was synthesized using the reverse transcription kit (Invitrogen) with random hexamer primers. Real-time PCR for MRP5 was done as described previously (23), and for MRP4, the following primer and probes were used: 5′-GTCTTCATTTTCCTTATTCTCCTAAACAC (forward), 5′-CCATTTACAGTGACATTTAGCATACTTTGT (reverse), and 5′-(FAM)-CCAGTATGAAAGCCACCAATCTTGAAGCA (probe). MRP8 expression was determined using the Assay-on-Demand (Applied Biosystems) system Hs00261567_m1 (ABCC11). Measurements were done in duplicate and normalized to 18S rRNA expression.

Cellular accumulation of [^{3}H]AraC-associated radioactivity

Previously described MRP8-transfected (LLC-PK1-MRP8-1) and control (LLC-PK1-pcDNA) LLC-PK1 cells were used for cellular studies (4). For accumulation experiments, cells were plated in triplicate at a density of 2×10^5 per well in six-well plates. The next day, the cells were incubated with 1 μmol/L [^{3}H]AraC (56 mCi/mmol; Moravek) for 1.5 h in complete growth medium. At various times, the medium was removed, and the cells were put on ice and washed thrice with 2 mL of ice-cold PBS. Trypsin solution (0.2 mL) was applied to the plates and the cell suspension was added to 4 mL of scintillation fluid.

Analysis of intracellular AraC and AraC metabolites

Cells were incubated for 3 h in medium containing 10 μmol/L [^{3}H]AraC and 50 nmol/L tetrahydrouridine. At the end of incubation, cells were washed with Dulbecco's PBS and processed for nucleotide extraction. Nucleotides were extracted using perchloric acid and the extracts were neutralized with KOH and stored at −20°C until analyzed. The intracellular concentration of normal nucleotides and [^{3}H]AraC metabolites was quantitated using high-performance liquid chromatography as described previously (24). The lower limit of

sensitivity of this assay using radioactive AraC was less than 1 pmol in an extract of 2×10^6 cells corresponding to a cellular concentration of 0.2 to 0.3 μmol/L.

Preparation of membrane vesicles and transport experiments

Membrane vesicles were prepared by the nitrogen cavitation method (25), and transport experiments were done using the rapid filtration method, essentially as described previously (26). Transport experiments were carried out in medium containing membrane vesicles (10 μg), 0.25 mol/L sucrose, 10 mmol/L Tris-HCl (pH 7.4), 10 mmol/L $MgCl_2$, 4 mmol/L ATP, 10 mmol/L phosphocreatine, 100 μg/mL creatine phosphokinase, and 40 μmol/L [^{3}H]AraC monophosphate (AraCMP; 24 Ci/mmol, Moravek), in a total volume of 50 μL. Reactions were carried out at 37°C and stopped by the addition of 3 mL of ice-cold stop solution [0.25 mol/L sucrose, 100 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 7.4)]. Samples were passed through 0.22-μm GVWP filters (Millipore) under vacuum. The filters were washed thrice with 3 mL of ice-cold stop solution and dried at room temperature for 30 min. Rates of net ATP-dependent transport were determined by subtracting the values obtained in the presence of 4 mmol/L AMP from those obtained in the presence of 4 mmol/L ATP.

Drug sensitivity analysis

Drug sensitivity of parental vectortransfected LLC-PK1 cells (LLC-PK1-pcDNA) and two clones of MRP8-transfected LLC-PK1 cells (LLC-PK1-MRP8-1 and LLC-PK1-MRP8-2) toward AraC was analyzed by the use of a tetrazolium salt microtiter plate assay (CellTiter 96 Cell Proliferation Assay, Promega). Cells were seeded at a density of 5,000 per well in 96-well dishes in complete medium, and drugs were added at various concentrations the following day. Growth assays were done after 72 h of growth in the presence of drugs.

Statistical methods

The Kaplan-Meier method was used to estimate the distribution of relapse-free and overall survival. End points were set according to Cheson et al. (21). Confidence interval (CI) estimation for the survival curves was based on the cumulative hazard function using Greenwood's formula for the SE estimation. Survival distributions were compared using the log-rank test. Data analysis of overall and disease-free survival was based on uncensored data (i.e., outcome was measured irrespective of whether patients were transplanted or not). This accounts for the fact that treatment outcome might be influenced by the blast phenotype at diagnosis despite allogeneic transplantation and subsequent cytogenetic switch of the bone marrow progenitor cells. The effects of ABCC expression on induction outcome, as defined by CR or not CR, were examined with Fisher's exact test. The correlation of the MRP4, MRP5, and MRP8 mRNA expression with categorical clinical findings, such as status at diagnosis and cytogenetic risk group, was calculated by the two-tailed Mann-Whitney U test. For correlation of MRP4, MRP5, and MRP8 mRNA expression with continuous variables such as age, leukocyte count, bone marrow blasts, or CD34 expression, Spearman's correlation was used. The differences of MRP expression between different FAB subtypes were evaluated by ANOVA. For statistical analysis of AraC metabolites, the one-tailed paired Wilcoxon test was used. For statistical analysis of AraC cytotoxicity, the two-tailed Wilcoxon test was used. Statistical analyses were done with the software package of Statistical Package for the Social Sciences version 12.0.1.

Results

Expression of MRP4, MRP5, and MRP8 mRNA in blast cells of adult AML patients

MRP4, MRP5, and MRP8 mRNA were expressed in all blast cells of adult patients with AML, albeit with considerable variability. By comparison with MRP4 and MRP5, MRP8 mRNA was expressed at a markedly lower level (Table 1). All investigated transporters showed a log normal distribution as assessed by normality testing of logarithmic mRNA expression values (data not shown). Expression of MRP4, MRP5, and MRP8 in peripheral blood monocytes of human volunteers was in the range of the mRNA expression of blast cells from AML patients. However, MRP8 could not be detected in 7 of 14 volunteer samples. Higher levels of MRP4 and MRP8 transcripts were observed in the less differentiated FAB subtypes M0 and M1 compared with the M2 subtype ($P = 0.038$ and 0.025, respectively), whereas expression of MRP5 mRNA followed the same trend but did not reach significance ($P = 0.088$). Transporter expression in subtypes M4 and M5 did not differ from the M2 subtype (Table 1).

Correlation of MRP4, MRP5, and MRP8 transporter expression with diagnostic and clinical features of AML

The relationship between MRP4, MRP5, and MRP8 mRNA levels and available laboratory and clinical data were explored. No correlation was observed with age, sex, initial WBC count, blast count, cytogenetic risk, and CD34 expression. To analyze the effect of MRP4, MRP5, and MRP8 on treatment outcome, the patients were divided into two groups using the median of the respective ABCC mRNA expression as a cut-off point for a low- and high-expression group. CR was achieved in 28 of 50 patients (56%). The median follow-up for survival was 4.3 years. Analysis of the effect of ABCC expression on treatment response did not reveal a significant correlation with remission rate (data not shown). Notably, although expression of MRP4 and MRP5 did not relate to overall and relapse-free survival (Table 2), a statistically significant difference was found in the probability of 4-year overall survival between patients with high and low MRP8-expressing blasts [8% (95% CI, 0-19%) versus 28% (95% CI, 10-46%), respectively; $P = 0.03$; Fig. 1A ; Table 2]. A similar difference could be shown for relapse-free survival [15% (95% CI, 0-35%) versus 33% (95% CI, 9-57%), respectively], which was, however, not statistically significant ($P = 0.41$; Fig. 1B; Table 2). This influence of MRP8 expression on survival was also found when patients were analyzed separately according to age groups. For patients ≤60 years ($n = 27$), 4-year overall survival and relapse-free survival rates were 43% and 46% for low MRP8-expressing patients compared with 15% and 20% for high MRP8-expressing patients, respectively. These survival rates were generally lower in patients >60 years ($n = 23$) with 9% and 25% compared with 0% and 0%, respectively. Due to low patient numbers within the groups, these differences did not reach statistical significance.

MRP8 confers resistance to AraC by extrusion of AraC metabolites

Anthracyclines and AraC are mainstays in the treatment of adult AML. Previously, it was shown that MRP8 does not affect anthracycline cytotoxicity but that it is able to reduce cytotoxic effects of nucleoside-based agents such as 5-FU (4), suggesting the possibility that MRP8 could modify outcome of AML by functioning as a resistance factor for AraC.

Therefore, the effect of MRP8 on the cellular kinetics of AraC was analyzed. First, the ability of MRP8-transfected LLC-PK1 cells (LLC-PK1-MRP8-1) to accumulate radiolabeled AraC was compared with parental vector-transfected LLC-PK1 cells (LLC-PK1-pcDNA). After 30 and 90 minutes in growth medium containing 1 μmol/L [^{3}H]AraC, accumulation of radioactivity in LLC-PK1-MRP8-1 cells was 72% and 63% of the control cells, respectively (Fig. 2).

Inside the cell, AraC is converted to the phosphorylated anabolites AraCMP, AraC diphosphate (AraCDP), and AraC triphosphate (AraCTP), which are the cytotoxic drug species (Fig. 3A). The influence of MRP8 on the accumulation and distribution of phoshorylated AraC was analyzed. LLC-PK1-pcDNA and LLC-PK1-MRP8-1 cells were incubated with [^{3}H]AraC and accumulation of AraCMP, AraCDP, and AraCTP was measured. As expected, the major intracellular metabolite was AraCTP with lower levels of AraCMP (Fig. 3B). The levels of each of these species were depressed as a result of ectopic expression of MRP8. In LLC-PK1-MRP8-1 cells, the levels of AraCTP and AraCMP were 21.9 ± 2.9 μmol/L and 7.0 ± 0.8 μmol/L, respectively, whereas the corresponding values in the control cells were 35.8 ± 3.1 μmol/L and 10.6 ± 2.1 μmol/L. Thus, the levels of AraCTP and AraCMP in the MRP8-transfected cells were 61% and 66% of the control cells, respectively ($P < 0.05$ for AraCTP). Levels of free AraC were very low (less than the diphosphate; data not shown) because intracellular AraC is rapidly phosphorylated.

MRP8 is a lipophilic anion transporter (27), and in previous studies, it was shown that the pump confers resistance to 5-FU by transporting the monophosphate metabolite of this agent, as opposed to unmetabolized 5-FU, which is uncharged (4). Reduced cellular accumulation of AraC metabolites in MRP8-transfected cells suggests that MRP8 similarly transports AraC metabolites. Transport of AraC metabolites by MRP8 was evaluated by analyzing uptake of [^{3}H]AraCMP into inside-out membrane vesicles prepared from control and MRP8-transfected cells. Although membrane vesicles prepared from control cells were able to catalyze the ATP-dependent uptake of [^{3}H]AraCMP (65 ± 13 pmol/mg/10 minutes), an increment attributable to MRP8 was readily detected in membrane vesicles prepared from the LLC-PK1-MRP8-1 and LLC-PK1-MRP8-2 cells (116 ± 6 and 97 ± 13 pmol/mg/10 minutes, respectively; Fig. 3C). Negligible uptake of [^{3}H]AraC was observed for control or MRP8-enriched membrane vesicles (data not shown).

Finally, to analyze the ability of MRP8 to affect the cytotoxic effects of AraC, the sensitivities of LLC-PK1-MRP8-1 and LLC-PK1-MRP8-2 were compared with the parental vector-transfected LLC-PK1-pcDNA cells. Representative AraC toxicity curves are shown in Fig. 4. The IC_{50} for LLC-PK1-pcDNA cells was 0.23 μmol/L, by comparison with IC_{50} values of 1.18 and 1.38 μmol/L for LLC-PK1-MRP8-1 and LLC-PK1-MRP8-2 cells (P = 0.032 and 0.016), respectively. This corresponded to resistance levels of 5.1- and 5.9-fold for LLC-PK1-MRP8-1 and LLC-PK1-MRP8-2, respectively.

Discussion

Resistance to drug therapy remains a major challenge in the treatment of AML, which underscores the need to identify key mechanisms of drug resistance. Cellular drug efflux

mediated by drug transport proteins is thought to play an important role. Here, we show that expression of ABCC11 (MRP8) mRNA in blast progenitor cells of patients diagnosed with AML predicted long-term survival. Furthermore, MRP8 conferred resistance to cytarabine (AraC) and was able to extrude AraC metabolites when ectopically expressed in a cellular model system. Because other ABC transport proteins have not been described to be involved in AraC efflux thus far, extrusion of AraC from AML blast cells by MRP8 may represent a unique situation, where modulation of MRP8 activity might be highly beneficial for treatment outcome.

The most extensively investigated drug transporter in acute leukemia is multidrug resistance 1 (MDR1; ABCB1, P-glycoprotein; refs. 28–30). Several studies suggest that MDR1 is an independent adverse prognostic factor for response and survival in *de novo* AML (28, 31, 32). MDR1 has also been analyzed in acute lymphoblastic leukemia (ALL). Whereas in childhood ALL the relationship between MDR1 status and CR rate as well as survival is unsettled (33), high MDR1 expression and activity have been reported to be associated with poor clinical outcome in adult ALL (34). Based on these findings, a rationale was developed for modulation of MDR1 activity as a therapeutic approach (35). Randomized trials with first-generation MDR1 inhibitors such as cyclosporin A, quinidine, or verapamil were largely inconclusive because of unexpected interactions affecting pharmacokinetics of the cytostatic agents and major adverse side effects (36). Subsequently, administration of the second-generation inhibitor valspodar (PSC-833) with a more favorable pharmacologic profile failed to achieve clinical benefits (37, 38). One reason that was considered to explain this failure was the fact that cytostatic drugs were included in the treatment regimens that were not substrates of MDR1, such as AraC, which is respected as one of the most potent antileukemic drugs available today. This in turn led to the consideration that other drug transport mechanisms may contribute to clinical resistance.

In fact, MRP1 emerged as a relevant player in predicting treatment outcome in *de novo* AML patients, and the predictive value was even stronger if activities of MRP1 and MDR1 were combined (28, 32). In contrast, MRP1 does not seem to play any role in childhood or adult ALL (33, 39). Among the other members of the ABCC drug transporter family, high expression of MRP3 was reported to correlate with a poor prognosis in childhood AML and childhood as well as adult ALL (40–42), whereas expression of MRP2 and MRP6 predicted treatment outcome in adult ALL (41). The predictive roles of MRP4 and MRP5 have been addressed in childhood and adult ALL as well as in childhood AML (40–42). Whereas for MRP4 no influence on treatment outcome was found, MRP5 expression was related to a higher relapse rate as well as a shorter relapse-free and overall survival in childhood ALL (41). In our own investigation, we found MRP4 and MRP5 mRNA expressed in blast cells of adult AML patients with considerable variability. However, their expression did not predict therapy response and clinical outcome.

Recently, a new member of the ABCC family, ABCC11 (MRP8), was identified (43). Similar to MRP4 and MRP5, MRP8 is able to confer resistance to nucleoside-based agents such as 5-FU and PMEA (4). Because AraC is a nucleoside analogue and is an important drug used in the treatment of AML, we hypothesized that MRP8 may affect AraC metabolite levels in leukemia blast cells. Consistent with this notion, we found that ectopic expression

of MRP8 resulted in decreased uptake of radiolabeled AraC, decreased intracellular accumulation of AraC metabolites, and increased resistance to AraC. AraC exerts its cytotoxicity by inhibiting the DNA polymerase as well as being incorporated into the DNA molecule (44). However, to do this, AraC has to be converted intracellularly into its triphosphate form AraCTP via a series of phosphorylation steps (45). These steps comprise conversion of AraC to AraCMP, which is further converted to AraCDP before finally AraCTP is formed. The interference with one of these steps will diminish the formation of the active compound AraCTP and hence confer resistance to AraC. Here, we show that AraCMP is effluxed by MRP8 to a high extent, thus removed from being converted to AraCTP. This most likely represents the mechanism of resistance to AraC induced by MRP8 in our model system. Interestingly, preliminary data exist that support the conclusion that neither MRP4 (12, 46) nor MRP5 (12), despite their otherwise similar substrate spectrum compared with that of MRP8, is able to affect the cytotoxicity of AraC, which implies that AraC is a specific substrate of MRP8.

The possibility that expression of MRP8 influences treatment outcome of AML patients treated with AraC is supported by our findings. Our analysis shows that expression of MRP8 mRNA correlated with treatment outcome. High levels of MRP8 expression were significantly associated with an unfavorable prognosis of long-term overall survival over 4 years and correlation with relapse-free survival followed a similar trend but did not reach significance. As a similar trend for survival differences of patients with low and high MRP8-expressing blasts was also found when patients ≤60 years and patients >60 years were analyzed separately, the influence of MRP8 expression on survival seems to be independent of the dosage of AraC delivered during antileukemic treatment. Overall survival and relapse-free survival are in turn mainly influenced by postremission treatment, which predominantly consists of AraC. In contrast, remission rate was not correlated with MRP8 expression, which suggests that factors other than AraC efflux are involved. These factors might be related to anticancer drugs additionally included into the induction treatment, such as mitoxantrone, etoposide, and daunorubicin, which are not transported by MRP8 but are in part substrates of other drug transport proteins. The level of MRP8 mRNA expression varied extensively among the investigated blast samples. With regard to the MRP8-transfected cell model, the MRP8 mRNA expression was 2- to 100-fold lower in AML blasts with over-the-median expression. By translating our results from the transfected cell line to the blast cells, we might overestimate the role of the efflux of the AraC metabolites for predicting treatment outcome. Hence, we cannot rule out that alternative mechanisms exist or that MRP8 serves as surrogate parameter unless substances are available that selectively inhibit MRP8 transport activity and can be used to modulate the efflux of AraC metabolites in the patient setting.

One such alternative mechanism could be that MRP8 contributes to cellular resistance by virtue of its ability to affect cyclic nucleotide levels (4). Cyclic AMP (cAMP) signaling has been shown to regulate vital cellular functions and is thought to be abnormal in transformed and cancer cells (47). Activation of cAMP signaling led to growth inhibition and cell cycle —specific apoptosis in a variety of cancer cell lines but not in untransformed cells (5) and differentiation was induced in a promyelocytic leukemia model (7). Moreover, self-renewal capacity of blast progenitors from AML patients was effectively suppressed on activation of

cAMP signaling (48) and patients whose blast progenitors displayed a high self-renewal capacity *in vitro* showed a low survival rate (49). One has to keep in mind, however, that intracellular levels of cyclic nucleotides are only modestly decreased by ectopic MRP8 expression (4). Whether alteration of intracellular cAMP concentrations by MRP8 might contribute to therapy resistance and treatment outcome needs further investigation.

In conclusion, the present study suggests that expression of MRP8 mRNA in blasts of adult AML patients predicts overall long-term survival and *in vitro* data support the hypothesis that the influence of MRP8 on survival is mediated by increased cellular efflux of phosphorylated metabolites of AraC.

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Translational Relevance

Successful treatment of acute myeloid leukemia remains a therapeutic challenge, with a high percentage of patients suffering from persistent or relapsed disease. One of the main backbones of antileukemic treatment is the nucleoside analogue cytarabine, and mechanisms of cytarabine resistance are therefore important to delineate. Here, we present evidence that expression of the ABC drug transport protein MRP8 is an important factor in acute myeloid leukemia patient survival and that the cause of treatment failure in those patients with high expression of MRP8 is very likely an increased extrusion of cytarabine from blast cells mediated by MRP8. Two possible consequences may result from these findings. Firstly, expression of MRP8 mRNA might serve as a predictive marker for treatment outcome and should therefore be investigated for stratification of patients with high MRP8 expression to treatments that rely to a lesser extent on cytarabine. Secondly, patients with high expression of MRP8 might benefit from adjuvant therapies that modulate MRP8 transport activity, which underscores the need for clinical development of specific MRP8 inhibitors.

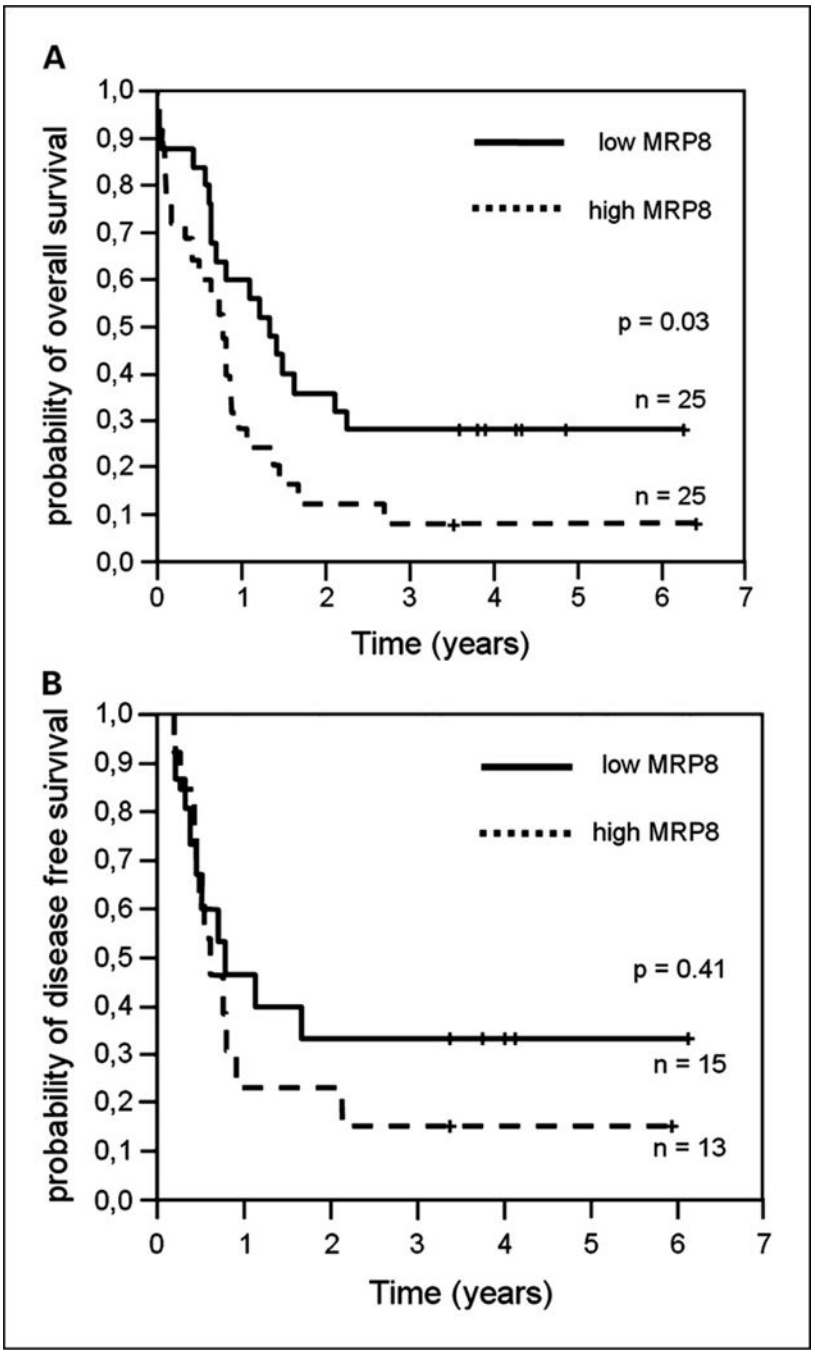

Fig. 1.

MRP8 mRNA expression in blasts of AML patients predicts long-term survival. Overall survival (*A*) and relapse-free survival (*B*) in adults diagnosed with AML subdivided into two groups of low and high MRP8 expression. mRNA of blast samples of at least 85% purity was prepared and MRP8 mRNA expression was quantified as described in Materials and Methods. The median of MRP8 mRNA expression was used as a cutoff for low and high expression. The Kaplan-Meier method was used to estimate the distribution of overall survival, and CI estimation for the survival curves was based on the cumulative hazard

function using Greenwood's formula for the SE estimation. Survival distributions were compared using the log-rank test.

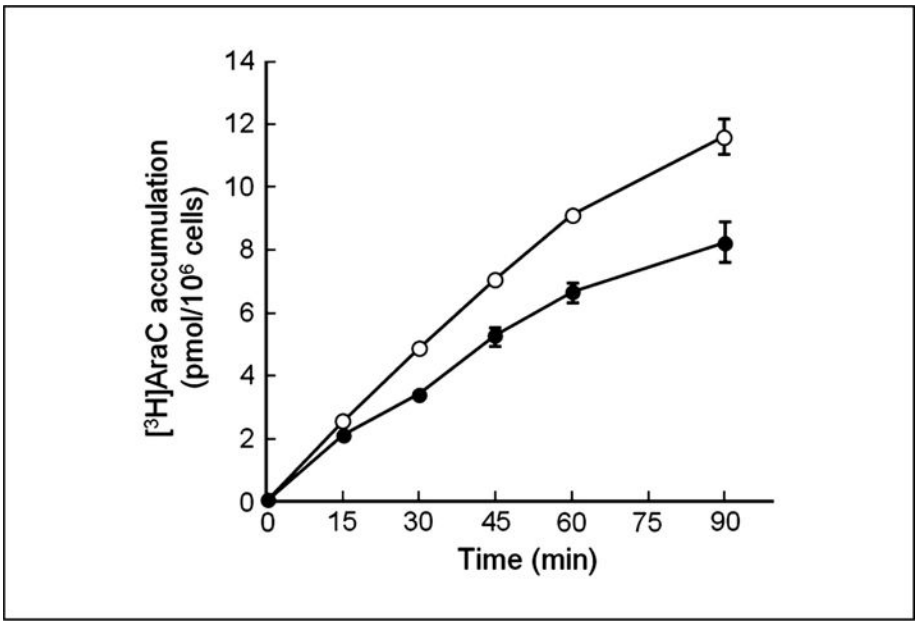

Fig. 2.

Cellular accumulation of AraC. Time course of accumulation of [^{3}H]AraC in parental vector-transfected (LLC-PK1-pcDNA, ○) and MRP8-transfected (LLC-PK1-MRP8-1, •) LLC-PK1 cells. Cells were incubated in 1 μmol/L [^{3}H]AraC and intracellular radioactivity was measured at various time points. Points, mean of a representative experiment done in triplicate; bars, SE.

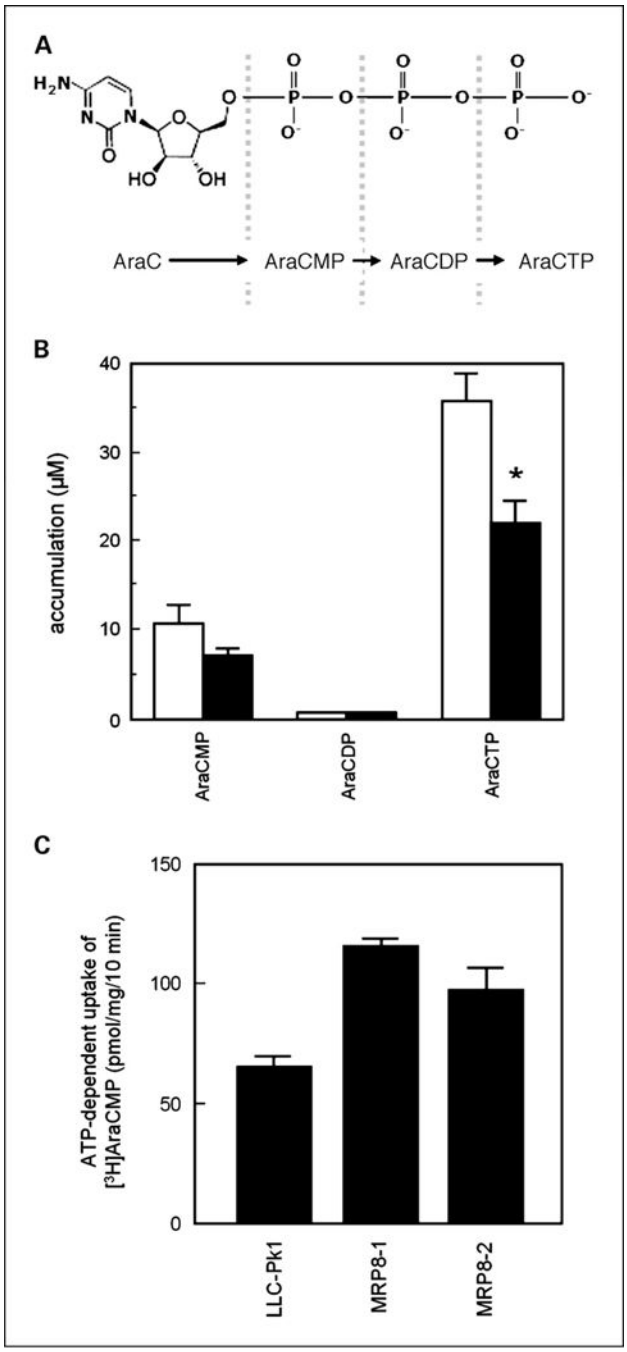

Fig. 3.

Analysis of AraC metabolites in MRP8-transfected cells. *A*, chemical structure and metabolic pathway of intracellular AraC phosphorylation. *B*, parental vector-transfected (*white columns*, LLC-PK1-pcDNA) and MRP8-transfected (*black columns*, LLC-PK1-MRP8-1) cells were incubated with 10 μmol/L [^{3}H]AraC and 50 nmol/L tetrahydrouridine for 3 h. Intracellular AraC metabolites were extracted by the perchloric acid method, and AraCMP, AraCDP, and AraCTP were separated and quantitated using high-performance liquid chromatography as described in Materials and Methods. Columns, mean of three or

more separate experiments; bars, SD. *, $P < 0.05$. *C*, membrane vesicles (10 μg) prepared from parental vectortransfected (LLC-PK1-pcDNA) or MRP8-transfected (LLC-PK1-MRp8-1 and LLC-PK1-MRP8-2) cells were incubated for 10 min at 37°C in uptake medium containing 4 mmol/L ATP or 4 mmol/L AMP and 40 μmol/L [^{3}H]AraCMP. MgATP-dependent uptake was calculated by subtracting the values obtained in transport medium containing AMP from the values obtained in medium containing MgATP. Columns, mean of a representative experiment; bars, SE.

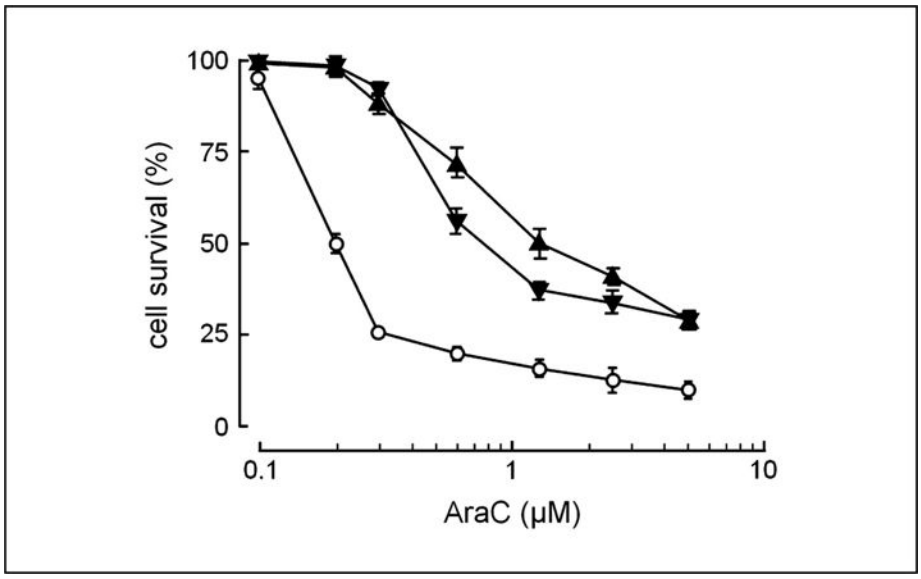

Fig. 4.

Sensitivity of MRP8-transfected and parental vector-transfected LLC-PK1 cells to AraC. The sensitivity of parental vector-transfected (○, LLC-PK1-pcDNA) and MRP8-transfected LLC-PK1 cells (▲, LLC-PK1-MRP8-1; ▼, LLC-PK1-MRP8) to AraC was analyzed using the tetrazolium salt microtiter plate assay as described in Materials and Methods. Points, mean of a representative experiment done in triplicate; bars, SE.

Table 1

Expression levels of ABCC drug transporter mRNA in the total AML population and in blasts of different FAB subtypes as well as in peripheral leukocytes of normal individuals

Population	n	MRP4			MRP5			MRP
				Median; mean (95% CI)*	P†	Median; mean (95% CI)*	P†	Mediar mean (95% CI)*
Total	50	27.0; 35.4 (25.7-45.1)	—	63.7; 105.6 (64.6-146.7)	—	0.3; 16.7 (1.3-32.1)	—	
M0/M1	16	53.8; 55.6 (29.7-81.6)	0.038	91.8; 164.3 (40.2-288.4)	0.088	2.6; 45.8 (–2.3 to 93.8)	0.025	
M2	14	32.8; 30.3 (21.6-39.0)	—	57.8; 73.5 (36.4-110.7)	—	0.2; 1.9 (–0.7 to 4.5)	—	
M4	7	11.0; 21.3 (–2.7 to 45.2)	0.549	61.9; 104.0 (14.6-193.5)	0.646	0.5; 1.3 (–0.3 to 3.0)	0.981	
M5	13	19.8; 28.0 (14.9-41.1)	0.853	61.4; 68.7 (50.6-86.8)	0.931	0.4; 5.1 (–1.6 to 11.7)	0.875	
Peripheral leukocytes‡	14	50.0; 52.8 (40.4-65.1)	—	33.0; 35.8 (29.2-42.5)	—	0.3; 0.3 (0.1-0.5) §	—	

*Drug transporter mRNA expression/18S rRNA × 10^{-7}.

†ANOVA with M2 as control group.

‡Derived from normal individuals.

§$n = 7$ (expression of MRP8 mRNA was not detectable in 7 of 14 samples).

Table 2

Four-year survival rates of 50 AML patients in dependence of mRNA expression of the respective MRP protein

	Expression level	4-y OS in % (95% CI)	Logrank OS	4-y DFS in % (95% CI)	Logrank DFS	
MRP4	High	12 (0-25)	P = 020	21 (0-43)	P = 0.45	
		Low		24 (7–41)		29 (5-52)
MRP5	High	16 (0-33)	P = 0.64	25 (2–49)	P = 0.99	
		Low		20 (4–36)		25 (4–46)
MRP8	High	8 (0-19)	P = 0.03	15 (0-35)	P = 0.41	
		Low		28 (10–46)		33 (9-57)

Abbreviations: OS, overall survival; DFS, disease-free survival.