ATP binding cassette C1 (ABCC1/MRP1)-mediated drug efflux contributes to disease progression in T-lineage acute lymphoblastic leukemia*

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ABSTRACT

Purpose: In acute lymphoblastic leukemia (ALL), multidrug resistance is often mediated by ATPase Binding Cassette (ABC) proteins, which principally involve ABCC1 (multidrug resistance protein 1, MRP1) and ABCB1 (multidrug resistance 1, MDR1). However, direct comparisons between the differential effects of ABCC1 and ABCB1 have been difficult, since identical cell lines with differential expression of these transporters have not been developed. Experimental Design: In this study, we developed and compared the biological profiles of Jurkat cell lines that selectively over-expressed ABCC1 and ABCB1. Vincristine (VCR) plays an important role in the treatment of T-lineage ALL (T-ALL), and is often the first drug given to newly-diagnosed patients. Because of its importance in treatment, we provide descalating, sub-lethal doses of VCR to Jurkat cells, and extended our observations to expression profiling of newly diagnosed patients with T-ALL. Results: We found that VCR-resistant cells over-expressed ABCC1 nearly 30-fold. The calcein AM assay confirmed that VCR-resistant cells actively extruded VCR, and that ABCC1-mediated drug resistance conferred a different spectrum of multidrug resistance than other T-ALL induction agents. siRNA experiments that blocked ABCC1 export confirmed that VCR resistance could be reversed in vitro. Analyses of T-lymphoblasts obtained from 100 newly diagnosed T-ALL patients treated on Children’s Oncology Group Phase III studies 9404 and AALL0434 that induction failure could be could be partially explained by the over-expression of ABCC1 and ABCB1. Conclusions: Taken together, these results suggest that over-expression of ABC transporters plays a contributing role in mediating treatment failure in T-ALL, and underscore the need to employ alternate treatment approaches in patients for whom induction failed or for those with relapsed disease.

Keywords: ATP Binding Cassette Proteins C1 and B1; Multi-Drug Resistance; T-Lineage Acute Lymphoblastic Leukemia

1. INTRODUCTION

With modern, dose-intensive chemotherapeutic approaches, nearly 80% of children, adolescents and young adults with acute lymphoblastic leukemia are now being cured of their diseases [1,2]. Despite this steady progress towards improved outcome, leukemic relapse remains a common problem. Inherently involved in relapse are a super-family of ATP Binding Cassette (ABC) drug transport proteins that actively extrude many chemically unrelated compounds out of dividing cancer cells [3-5]. The ABC transport proteins have been widely implicated in disease relapse in a variety of hematopoietic malignancies, including T-lineage acute lymphoblastic leukemia.
(T-ALL) [6-8].

Malignant T-lymphoblast survival in the presence of chemotherapeutic agents is dependent on cellular and molecular alterations that are not present in chemother-apy-sensitive cells. The resistance of tumor cells to multiple, structurally or functionally dissimilar drugs is classified as multi-drug resistance (MDR) [9]. Experimentally-induced drug resistance in leukemic cell lines has shown that acquired MDR is commonly mediated by ATP-binding cassette (ABC) proteins ABCC1 (MRP1) and ABCB1 (MDR1) [7,8]. In T-ALL, over-expression of ABCB1 has not been uniformly associated with a poor outcome [10,11], but we found that intrinsic up-regulation of ABCB1 did correlate with induction failure within a subset of patients who were treated on Children’s Oncology Group Phase III studies 8704 and 9404 [8]. The role for ABCC1 in mediating therapy resistance for T-ALL has been even less clear. While the family of multidrug resistance-associated proteins has been implicated in causing chemotherapy resistance in T-ALL [6], others found that ABCC1 did not independently predict poor outcome [12]. The roles played by ABC transport proteins in T-ALL have yet to be fully understood, and may have differential effects on treatment within biologically-defined subsets of patients.

While treatment approaches have varied significantly over the preceding decades, induction therapy for T-ALL commonly begins with vincristine (VCR), which is typically given as 1.5 mg/m\(^2\) (maximum 2 mg) IVP once weekly [13-16]. Because of this consistent approach in VCR dosing, we developed a VCR-resistance cell line to investigate its resistance profile. We found that T-ALL cells acquire resistance to VCR through an up-regulation of ABCC1, and that the multi-drug resistance generated from ABCC1 expression is distinctly different from ABCB1 over-expression. We found that intrinsic over-expression of ABCC1 was associated with disease progression within the induction failure group, but not necessarily for individual patients, suggesting that additional resistance mechanism(s) remain to be defined. Taken together, these findings indicated that ABC proteins are important mediators of disease progression, and call for treatment approaches that bypass their resistance mechanisms.

2. PATIENTS MATERIALS AND METHODS

2.1. Cell Lines Chemotherapy Drugs
Chemicals and Reagents

Jurkat T-ALL cells was purchased from American Tissue Culture Corporation (Manassas, VA) and grown in RPMI 1640 medium (Gibco BRL, Grand Island, NY) with 100 units/ml penicillin-streptomycin (Invitrogen/ Gibco, Carlsbad, CA), 5 µg/l ciprofloxacin (Bayer Pharmaceuticals, Berkeley, CA) and 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT). Using a method adapted from others [7,8], Jurkat cells were exposed to incrementally increasing doses of VCR (Faulding Pharmaceutical Company, Paramus, NJ) to induce drug resistance while maintaining at least 80% viability. Daunorubicin (DNR) prednisone (PRED), and l-asparaginase (L-ASP) were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Quantification of Drug Resistance

As previously described [7,8], drug resistance was quantified first by simply measuring duration of survival in 300 nM VCR. Progenitor (parental) and VCR-resistant cells were maintained separately at a concentration of 2 × 10\(^5\) cells/ml with chemotherapy added at Day 0 and percent viability estimated with a hemacytometer (Haussers Scientific, Horsham, PA) and 0.4% trypan blue dye (Sigma). Secondly, the effective concentration resulting in 50% cell death (EC\(_{50}\)) was measured by culturing 1 × 10\(^5\) cells/ml with logarithmically increasing concentrations of VCR and assessing cell viability for parental and resistant cell lines after 7 days.

2.3. Microarray Analyses of Jurkat Cells

Total RNA was extracted from parental and resistant cell lines according to RNeasy Mini Kit manufacturer’s protocol (Qiagen, Baltimore, MD). Hybridization of RNA to the Human Genome U133 Plus 2.0 oligonucleotide microarray (Affymetrix, Santa Clara, CA) and data were normalized using the methods of the Robust Microarray Analyses, as previously described [17]. The differences in gene expression between parental and resistant cell lines were computed using GeneSpring 7.3.1 (Agilent Technologies, Inc., SantaClara, CA).

2.4. Flow Cytometry Analysis for Surface Expression and Functional Analysis of Transport Activity.

The ABC1 transporter pump was detected at the cell surface by flow cytometry using PE-labeled mouse anti-human ABC1 monoclonal antibody (clone QRCL-3, BD Biosciences, San Jose, CA). The antibody was incubated at 4°C for 30 minutes in the dark with 1 × 10\(^6\) parental and resistant cell lines and then were fixed and permeabilized according to the Cytofix/Cytoperm Kit (BD Biosciences) alternative protocol. In addition, to detect that the ABC1 pumps were functional, we employed a calceinacetoxymethyl ester (calcein AM) duplex assay [18]. In living cells, calcein AM is cell permeant and non-fluorescent until it is hydrolyzed by intracellular
esterase isoforms into a strongly green fluorescent anion, calcine. Calcine is retained within the cell cytoplasm, unless it is extruded by active protein transporters, such as ABCB1 or ABCC1. The Vybrant Multidrug Resistance Assay Kit (Invitrogen-Molecular Probes, Eugene, OR) was used to functionally test ABCC1 activity. Experiments were run according to manufacturer’s suggested protocol and at least 10,000 events were counted (FACscan cytometer) and analyzed with IDLeQuery software [19].

2.5. SiRNA-Mediated Suppression of Gene Expression

To investigate the effects of siRNA on ABCC1-mediated VCR resistance, 2 × 10^5 Jurkat cells were centrifuged and re-suspended in 75 μl siPORT electroporation buffer (Ambion, Austin, TX) and transferred into 1-mm gap electroporation cuvettes (Bio-Rad Laboratories, Hercules, CA). According to the manufacturer’s suggestion, 2.25 μg of siRNA was added and cells were then electroporated with a single square-wave pulse for 280 μs at 220 V delivered by a Gene PulserXCell (Bio-Rad). To preserve cell wall integrity, cells were incubated in the cuvette for 10 minutes at 37˚C before transfer. The siRNA sequence used to suppress ABCC1 transporter RNA was 5’-GGUGUUUAACAGACCAUUUt-3’ (sense) and 5’-AAAGGUCUUGUAACACCCtt-3” (antisense) at the optimal amount of 1 μg/sample (ABCC1-siRNA, #AM51334; Ambion). VCR resistant cell lines were used to establish experimental points, with and without GAPDH siRNA (Ambion) was used as a control. After 48 hours of maintaining viability, 500 nM VCR was added to each sample with percent viability and absolute cell concentration recorded on subsequent days. Total RNA was isolated with the RNeasy Mini Kit (Qiagen) and qRT-PCR performed using the SuperScript III Platinum Two-Step qR-PCR Kit with SYBR Green (Invitrogen, Carlsbad, CA). The relative rate of gene expression was compared using the delta-delta method [20].

2.6. Clinical Case Preparation and Microarray Analyses

Children’s Oncology Group (COG) Cell Bank newly-diagnostic samples from ALL Classification studies 9900/9904 and AALL03B1/AALL0434 were enriched to >90% T-ALL blasts with ficoll-hypaque density centrifugation prior to cryopreservation. RNA processing and hybridization to the Human Genome U133 Plus 2.0 oligonucleotide microarray (Affymetrix) were performed as previously described [17], and 100 cases were examined for their relative expression of ABC proteins based on their outcome into three groups: 1) induction failure (n = 11); 2) post-remission relapse (n = 20); and 3) complete continuous remission (CCR, n = 69). Patient subset compare-

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genes were differentially over-expressed. Interestingly, ABCB1 (MDR1) was not upregulated in response to VCR-dependent disease selection, as we previously described in response to DNR exposure [8].

**Up-regulated ABCC1 is an effective drug efflux protein.** In order to demonstrate that ABCC1 expression is responsible for VCR resistance, we examined the efflux capability of parental and resistant cells using a chemical VCR surrogate, calcein AM (Figure 3). Calcein AM did not accumulate in VCR-resistant cells, but did so in parental, drug sensitive cells. In addition, the efflux in ABC1-expressing, drug resistant cells could be blocked by verapamil, a known inhibitor of ABCC1 (Figure 3). [22]. These results show that VCR-induced chemoresistance in Jurkat is mediated by the specific up-regulation of ABCC1, which then results in increased surface expression of function ABCC1 efflux protein.

Silent interfering RNA inhibition of ABCC1 expression restores drug sensitivity. We performed siRNA experiments to show that the elimination of ABCC1 expression would return drug sensitivity. We exposed cells to siRNA directed against ABCC1 and quantified their growth over 6 days. Our measurements of ABC1 mRNA levels in resistant cells showed that ABCC1 expression remained high, allowing cells to maintain proliferative growth. When ABCC1 expression was markedly reduced, sensitivity to the cytotoxic effects of VCR was restored (Figure 4(a)). To confirm the knock-out effects of siRNA, we measured the expression level of the ABC1 mRNA over an 8-day period. Following a siRNA hiatus of 6 days, ABC1 mRNA expression increased to pre-siRNA levels (Figure 4(b)).

To confirm the knock-out effects of siRNA, we measured the expression level of ABC1 mRNA over an 8-day period. Following a siRNA hiatus of 6 days, ABC1 mRNA expression increased to pre-siRNA levels ([8]). In the resistant cells, L-asparaginase shows very minimal change, as it is not transported by ABC proteins [8]. Taken together, the results described for Figures 2-5 demonstrate two important findings: first, resistance to VCR was selectively mediated by ABCC1, and secondly, up-regulation of ABC1 resulted in multi-drug resistance that included DNR and PRED, but not L-ASP. Importantly, these drugs are commonly employed in the induction phases for newly-diagnosed and relapsed T-ALL patients.

**ABCC1 up-regulation is associated with treatment failure in patients treated for T-ALL.** To investigate the clinical significance of ABCC1 up-regulation in a cohort of patients with newly diagnosed T-ALL, we next investigated de novo ABC1 RNA levels in a cohort of 100 patients treated on COG T-ALL studies 9404 and AALL0434. The patient’s outcome status was characterized by those for whom induction failed, those who achieved a first remission, but later relapsed, and those who remained in complete continuous remission for at least four years from...
Figure 2. ABCC1 is up-regulated on the cell surface of vincristine resistant cells. (a1) Parental (therapy sensitive) cells do not express ABCC1 (grey peak: murine IgG2a PE isotypemAbs; white peak: PE-conjugated ABCC1 mAb). (a2) Cells resistant to VCR express increased levels of ABCB1, as demonstrated by a 2 log10 (100-fold) shift in peak fluorescence intensity (grey peak [41]; murine IgG2a PE isotypemAbs; white peak [right], PE-conjugated ABCC1 mAb). These data show that VCR induces the up-regulation of ABCC1 mRNA expression, resulting in increased cell surface expression of this efflux pump. (b) RMA-normalized gene profiling in parental (■) and VCR resistant (■) cells showed that ABCC1/MRP1 (202804_at) was approximately 16-fold up-regulated in the drug-resistant cell line. ABCB1/MDR1 (209993_at) was not up-regulated in response to VCR.

The one-way analysis of variance (ANOVA) test was performed and identified a significant difference of the ABCC1RNA levels among the three patient groups (P = 0.038). We also performed pairwise comparisons of the ABCC1 RNA levels using the Tukey-Kramer adjustment for multiple comparisons. As shown in Figure 5, a subset of 11 patients for whom induction failed had an RMA-normalized ABCC1 level of 9.695 ± 0.1646. This level was significantly higher than the 20 patients who relapsed, having a mean ABCC1 level of 8.883 ± 0.1573 (unpaired, two-tailed t test, P = 0.0025), or in comparison to 69 patients who remained in long-term remission, expressing a group mean level of 9.141 ± 0.0687 (unpaired, two-tailed t test, P = 0.0033).
ABCC1 is a functional drug efflux transporter. Left-centered white peak: VCR-resistant Jurkat cells maintained in calcein AM fluorescent dye without verapamil. Right-centered white peak: VCR-sensitive Jurkat cell maintained in calcein AM fluorescent dye without verapamil. Purple peak: VCR-resistant Jurkat cells maintained in calcein AM fluorescent dye with verapamil. These peaks demonstrate that verapamil can block the ABCC1-mediated efflux of the calcein AM fluorescent dye, demonstrating that ABCC1 retains functional transporter physiology in Jurkat cells.

Figure 4. siRNA restores sensitivity to vincristine resistant cells over-expressing ABCC1. (a) Cell growth of parental and “knock-out” cells. VCR-resistant cells were transfected with control (GAPDH) and experimental (ABCC1 siRNA) conditions. Cells electroporated cells with GAPDHsiRNA (solid grey) and 2.25 µg of ABCC1 siRNA (checkered grey) showed distinctly different growth rates. (b) Expression of ABCC1 in either mock (solid line) or siRNA (dotted line) transfectant cells. ABCC1 expression was normalized to Day 1 levels (n = 3 for all samples).

While over-expression of ABCC1 could not alone explain disease progression amongst individual patients, these data show that de novo drug resistance can be explained in part by a group mean over-expression of ABCC1 within these outcome subsets.

4. DISCUSSION

ABC transport proteins mediate drug resistance by exporting chemotherapeutic agents out of proliferating cancer cells, including malignant T-lymphoblasts [3,5,7-9]. We have previously reported that ABCB1 (MDR1) is selectively up regulated in T-ALL cells that have been exposed to sub-lethal concentrations of anthracyclines [8]. To further investigate the roles of structurally and functionally dissimilar ABC transporters in mediating drug resistance, we maintained cells in sublethal concentrations of VCR, a chemotherapeutic agent that is universally included during induction therapy for T-ALL and T non-Hodgkins Lymphoma [14,16,25]. Using molecular, cellular and functional assays, we identified that ABCC1 (MRP1) was specifically upregulated under VCR-induced cytotoxic pressure, but conferred resistance to other induction phase drugs, including PRED, and to a lesser extent, DNR. Our siRNA analyses showed that ABCC1 up-regulation in T-ALL cell lines resulted in profound resistance to VCR, suggesting that under conditions of that mimic sub-lethal drug exposure, ABCC1 may play a...
key role in mediating disease progression. In this study, our subsequent analyses of the patient samples indicated that ABCC1 was over-expressed in patients who suffered disease progression with induction therapy. Because second-line, non-ablative therapies for induction failure are often unsuccessful [12,26,27], our findings underscore the importance of early identification of such patients so that their treatment might be modified to utilize agents that bypass ABCB1 and ABCC1-mediated drug efflux.

For patients who do not achieve a first remission, de novo resistance is thought to be mediated by a number of biological events that control cell proliferation, including a disruption of cell division at the G0/G1 checkpoint, molecular aberrations that prevent therapy-induced apoptosis [28], or by extrusion of chemotherapeutic drugs by ABC transporters [8]. Over-expressed genes within our previously-described 116 member classifier and those over-expressed within the Jurkat VCR-resistant cell line included TMSB15A (205347_s_at), TJP2 (202085_at) and CDK1 (23153_at). We speculate that these genes maintain cell division in rapidly dividing leukemic blasts through their mediation of cytoskeletal integrity (TMSB15A), tight junction protein assembly (TJP2) and cell cycle progression (CDK1). Our experiments with sub-lethal VCR dosing resulted in highly VCR-resistant T-ALL cells, suggesting that in some patients for whom VCR dosing is not optimized, or for patients having primary VCR resistance, relapse or disease progression may be further complicated with the development of a multi-drug resistance phenotype early in treatment. Others have suggested that VCR under-dosing may be an important contributor to relapse, calling for further efforts to optimize VCR dosing in the treatment of ALL [29,30]. In contrast to the findings of Effert et al. [7], we found that under conditions of selective VCR exposure only ABC1, and not ABCB1, was significantly up-regulated in our T-ALL cell line model. We are the first to demonstrate that ABC1 contributes to de novo resistance against VCR in T-ALL, leading us to investigate its role in combination with other ABC transporters.

Multidrug resistance in T-ALL is conferred by a number of ABC transporters, which can mediate resistance to anthracyclines (ABCB1, ABCC1), methotrexate (ABCC1, ABCC2, and ABCG2) [31,32] and VCR (ABCC1). Compared to other ABC transporters, ABCC1 has several unique properties. While there is overlap between the xenobiotic substrates that are transported by ABCB1 and ABCC1, ABCC1 transports relatively broader ranges of compounds, including organic anions, heavy metals and vinca-alkaloids [31,33]. In a recent study of 72 T-ALL patients treated on DCOG ALL-7, -8 and -9, van Grotel and co-workers found CD34 expression was an important predictor of a poor 5 year disease free survival, but did not find an association between outcome and greater than median quantitative RT-PCR expression of ABCC1/
stem-cell like state, in which ABC transport molecules are highly expressed, resulting in de novo chemotherapy resistance. The collaborating roles between ABC transport proteins and other molecular features associated with induction failure or early relapse will require further study as potential biomarkers for high-risk disease.

Until recently, re-induction regimens containing VCR, DNR and other ABCC1 substrates produced only transient remissions in a fraction of patients, calling for the introduction of a novel set of agents that were not ABC substrates. Nelarabine, an RNA nucleoside analog that is not known to be a substrate of ABCC1, induced remissions that were achieved and maintained in up to 50% of first-time relapse patients, suggesting that relapse regimens must utilize cytotoxic drugs that might escape the transporting effects of ABC proteins [40]. Our findings suggest that patients who are at risk to not achieve a first remission should be identified early in the course of induction, as they may benefit from alternate treatment strategies that bypass ABC-mediated drug efflux.

5. ACKNOWLEDGEMENTS

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REFERENCES


Figure S1. VCR resistant cells with up-regulated ABCC1 express the multi-drug resistance phenotype. Resistance to prednisone (PRED), vincristine (VCR), and daunorubicin (DNR) are shown on the left axis, and resistance for L-asparagine (L-ASP) is shown on the right axis. (a) Parental cells demonstrate sensitivity to DNR (■), VCR (●), and PRED (♦), but not for L-ASP (○). (b) ABCC1 over-expressing cells maintained in VCR show greater resistance to DNR (■) and a significantly greater resistance to VCR (●) than the parental cells. Cells over-expressing ABCB1 maintained in DNR show relatively greater resistance to DNR (■) and VCR (●) [8].

Table S1. EC50 drug concentrations in ABCB1(8) and ABCC1-resistant Jurkat.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Parental Jurkat EC50</th>
<th>ABCB1 Jurkat EC50</th>
<th>ABCC Jurkat EC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vincristine</td>
<td>0.96 nM</td>
<td>275 nM</td>
<td>149 nM</td>
</tr>
<tr>
<td>Prednisone</td>
<td>196 μM</td>
<td>0.26 nM</td>
<td>545 μM</td>
</tr>
<tr>
<td>L-Asparaginase</td>
<td>0.13 IU</td>
<td>0.01 IU</td>
<td>0.22 IU</td>
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<tr>
<td>Daunorubicin</td>
<td>3.1 nM</td>
<td>1581 nM</td>
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