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Drug resistance: Still a daunting challenge to the successful treatment of AML

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Abstract

Resistance to chemotherapy remains a challenging issue for patients and their physicians. P-glycoprotein (Pgp, MDR1, ABCB1), as well as a family of structurally and functionally related proteins, are plasma membrane transporters able to efflux a variety of substrates from the cell cytoplasm, including chemotherapeutic agents. The discovery of ABCB1 made available a potential target for pharmacologic down-regulation of efflux-mediated chemotherapy resistance. In patients with acute myeloid leukemia (AML), a neoplasm characterized by proliferation of poorly differentiated myeloid progenitor cells, leukemic cells often express ABCB1 at high levels, which may lead to the development of resistance to chemotherapy. Thus, AML seemed to be a likely cancer for which the addition of drug efflux inhibitors to the chemotherapeutic regimen would improve outcomes in patients. Despite this rational hypothesis, the majority of clinical trials evaluating this strategy have failed to reach a positive endpoint, most recently the Eastern Cooperative Oncology Group E3999 trial. Here we review data suggesting the importance of ABCB1 in AML, address the failure of clinical trials to support a therapeutic strategy aimed at modulating ABCB1-mediated resistance, and consider the type of research that should be conducted in this field going forward.

Keywords

acute myeloid leukemia; multidrug resistance; ABC transporters; cancer stem cells; gene signatures

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Introduction

Acute myeloid leukemia (AML) is a hematologic malignancy characterized by proliferation of malignant precursors of the myeloid lineage, with impaired differentiation of normal hematopoietic progenitors coupled with proliferation of precursors of the myeloid lineage (Shipley and Butera, 2009). Despite advances in AML treatment, the projected survival for patients diagnosed from 2006 to 2010 was 21.4% at 5 years (Pulte et al., 2010). As these model-based projections are a minimal improvement from previous statistical data, they indicate the need for ongoing research on the treatment of AML. In particular, elderly patients, who make up the largest proportion of patients with AML and who have a <10% five year overall survival, are in dire need of novel therapeutic approaches.

Poor outcomes in patients with AML are related to the high proportion of patients with chemorefractory disease or relapse after an initial response. The discovery of ABCB1 and its association with chemotherapy drug efflux defined a therapeutic target associated with a state of multidrug resistance (MDR) in tumor cells. Over the following three decades, intense preclinical and clinical investigation led to the hypothesis that antagonism of ABCB1 could increase tumor chemosensitivity, which was tested in clinical trials evaluating ABCB1 inhibitors in over 2,000 patients with AML. As we review below, what has emerged from the study of ABCB1 in AML is that it is of strong adverse prognostic significance, mediates drug efflux, and can be fully overcome *in vitro* using competitive inhibitors. Despite these important features, clinical "MDR modulation" with competitive inhibitors has repeatedly failed, with few exceptions, to improve outcomes in patients with AML. Most recently ECOG 3999, a multi-center randomized trial, demonstrated no benefit from the addition of the third-generation MDR inhibitor zosuquidar to standard induction chemotherapy in patients with AML or high-risk myelodysplastic syndrome (MDS) (Cripe et al., 2010). Fundamentally, the strategy of modulating drug efflux transporters such as ABCB1 depends on the hypothesis that leukemic cells are either more dependent on drug efflux, overexpress the transporter relative to normal cells, or can be selectively targeted for inhibition of drug efflux as compared to normal tissues. However, the body of clinical trials reviewed here suggests that the current strategy of direct MDR/ABCB1 inhibition is in fact unlikely to generate a therapeutic "hand-hold" in unselected patients with AML based on these hypotheses. Indeed, an editorial that accompanied publication of the E3999 trial in the journal *Blood*, argued that the "MDR horse" should be dismounted. While the therapeutic strategy applied to date has fallen short, several features of ABCB1 outlined below suggest that its expression is an important contributor to drug resistance in AML, and that investigation surrounding its expression should not be completely abandoned. The purpose of this review is to summarize what has been learned in the development of drug resistance mediators for AML, specifically those targeting ABCB1, and to address what avenues within this effort are worthy of further pursuit in order to improve outcomes for patients with AML.

The Expression and Role of ABC transporters in AML

ABCB1/MDR1 (Juliano and Ling, 1976; Ueda et al., 1987), ABCC1/MRP1 (Cole et al., 1992), and ABCG2/BCRP (Doyle et al., 1998) are the most extensively studied genes in the ABC family, which includes 48 members divided into seven sub-families based on sequence similarities (Gillet et al., 2007). These three transporters have the ability to efflux a variety of structurally and mechanistically unrelated drugs out of the cell. In addition to the three broad spectrum ABC transporters noted above, extensive research has confirmed the ability of 12 other ABC transporters to confer resistance to chemotherapy drugs *in vitro*, and suggests that at least ten additional ABC transporters might confer resistance to chemotherapy (Gillet et al., 2007). Beyond this there is evidence of ABC expression,

particularly ABCB1, on normal tissues including hematopoietic progenitor cells (Chaudhary and Roninson, 1991; Cordon-Cardo et al., 1990). Therefore, any attempt to reverse drug resistance by inhibition of ABC transporters may present a challenge due to redundancy in substrates and function as well as normal tissue toxicity, the latter of which we discuss in more detail below.

Approximately 50% of AML patients have leukemic blasts that express ABCB1, with a slightly greater rate of expression and at higher levels in elderly patients (Leith, 1998). Detection of the *ABCB1* gene product is more frequent in elderly patients and has been more strongly linked to inferior outcomes (Table 2) in this population (Marie and Legrand, 1999; Pallis et al., 2002; Wuchter et al., 2000). This age-dependent prognostic relevance of ABCB1 expression could stem from the tendency of younger patients to receive more intensive chemotherapeutic regimens than older patients, a bias that could mask the relevance of ABCB1 expression as a prognostic factor in younger patients (Steinbach and Legrand, 2007).

While ABCG2 mRNA expression, protein expression, and function usually correlate with each other in leukemic cell lines, such a correlation is not always observed in clinical samples. This may be because ABCG2 is only expressed in a subset of AML samples, because of post-transcriptional regulation of ABCG2, or because other transporters not being measured influence the functional assays (Suvannasankha et al., 2004). Indeed, co-expression of ABCG2 and ABCB1 was found in a subset of AML samples from patients with poor outcomes, when assayed by cDNA array (Wilson et al., 2006). It is difficult to explain how ABCG2 would influence prognosis via drug efflux given that anthracyclines and cytarabine are not substrates of that transporter. Co-ordinate regulation of ABCG2 with ABCB1 (and a member of the ABCC family such as ABCC3) and possibly other transporters or resistance mechanisms, could explain why inhibition of a single transporter may not be an effective therapeutic strategy (Marzac et al., 2011; van den Heuvel-Eibrink et al., 2005). Along this same line, a recent report suggested that ABCG2 can be overexpressed in leukemias with a FLT3 internal tandem duplication, with an association between ABCG2 overexpression and reduced disease-free and overall survival but not with response rate (Tiribelli et al., 2011). This finding suggests an association of ABCG2 with the intrinsic biology of the leukemia rather than with drug efflux mediating resistance per se.

It is unknown how much ABC transporter expression is necessary to confer resistance in leukemic blasts. In one study, while 78% of patients with AML had elevated expression of ABCG2, only 7% had expression levels comparable to those of a resistant leukemia cell line (Abbott et al., 2002). It is not likely that expression levels similar to those seen with drug selection *in vitro* are relevant to those needed to confer resistance in patients. Functional assays have been relatively well established in AML samples and drug efflux can be confirmed *ex vivo*, with results that generally correlate with the gene expression data (Benderra et al., 2005; Laupeze et al., 2002; van der Pol et al., 2001).

Steinbach *et al.* conducted a low density cDNA microarray study involving 38 ABC transporters, and found 4 new ABC transporters, ABCA2, ABCA3, ABCB2, and ABCC10, that were overexpressed in the bone marrow of patients with pediatric AML compared to normal bone marrow; of these, ABCA3 correlated with inability to enter remission (Steinbach et al., 2006). The prognostic significance of ABCA3 was subsequently shown in a cohort of 81 pediatric and 38 adult patients with AML, where high ABCA3 expression was found to be associated with both reduced PFS ($p=0.0069$) and OS ($p=0.013$) (Chapuy et al., 2008). As opposed to ABCB1, which transports chemotherapeutic agents out of the cells, ABCA3 is thought to mediate drug sequestration in lysosomes. Taken together, these and other data suggest that multiple ABC transporters are likely coordinately expressed in

individuals with AML, a feature that complicates the single-target model of previous pharmacologic interventions, as we will discuss below.

Do single nucleotide polymorphisms of the ABCB1 gene influence its function?

A number of recent reports have addressed the impact of genetic polymorphisms on drug transporters (reviewed by Cascorbi and Haenisch (Cascorbi and Haenisch, 2010)). Among the 48 ABC transporters, ABCB1 is one of the most thoroughly studied and characterized, with more than 50 SNPs reported (Fung and Gottesman, 2009). Polymorphisms in ABCB1 or ABCG2 could alter clinical outcomes via a number of mechanisms. For example, the efficiency of transporter function could be increased via decreased binding to inhibitors or, on the other hand, dysfunctional transporters could lead to increased toxicity due to decreased export of chemotherapy drugs from normal tissues, particularly bone marrow cells. Kimchi-Sarfaty and colleagues analyzed the role of synonymous mutations in protein folding and function (Kimchi-Sarfaty et al., 2007). They reported that synonymous SNPs (3435C>T, 1236C>T and 2677G>T) in the ABCB1 gene sequence result in a protein with reduced drug and inhibitor interactions. One hypothesis to explain this result is that when frequent codons are changed to rare codons in a cluster of infrequently used codons, the timing of co-translational folding is affected and may result in altered function. Structural changes in mRNA caused by synonymous mutations could also alter the rate of co-translational folding.

The correlation of ABC transporter genetic variants to transporter expression or function in clinical samples and to treatment outcomes is under investigation. The United Kingdom National Cancer Research Network studied the effect of three SNPs (1199G>A, 2677G>T, and 3435C>T) in the ABCB1 gene, in a cohort of 817 patients with AML. Taking into account only the leukemias considered positive for ABCB1 expression (41% of the cohort), the polymorphisms 2677TT and 3435TT were found to be associated with lower ABCB1 protein expression when compared to the CT and CC genotypes (Seedhouse et al., 2007). In contrast, an association was found in a study of 101 Asian patients, where the presence of wild-type 3435CC significantly correlated with lower functional ABCB1 in a daunorubicin accumulation assay (Kim et al., 2006). Moreover, patients carrying 3435CC and 2677GG demonstrated higher complete remission and 3-year event-free survival, although there was no difference in the rate of overall survival. Lastly, a study conducted by van der Holt et al. on a cohort of 150 Caucasian patients over the age of 60 demonstrated no association with ABCB1 function, MDR1 expression or clinical outcome (Hampras et al., 2010; van der Holt et al., 2006). While the hypothesis remains plausible that ABCB1 variants could cause altered protein expression or function, thereby conferring differences in pharmacology or in drug resistance, and altered response to ABCB1 inhibitors, these three studies report divergent results that suggest that polymorphisms in ABCB1 may affect response to chemotherapy, but do not allow a determination of how these changes affect outcome. A recent study of 261 patients (mostly Caucasian) carried out at Roswell Park Cancer Institute (Buffalo, NY, USA) investigated eight non-synonymous SNPs not only in the ABCB1 gene, but also in ABCC1 and ABCG2 drug transporter genes (Hampras et al., 2010; van der Holt et al., 2006). Only the ABCG2 variants were associated with survival. Heterozygous (AG) or homozygous (AA) variant genotypes for SNP 34G>A in the ABCG2 gene, compared to the wild type (GG) genotype were associated with both significantly improved survival and increased odds for toxicity (Hampras et al., 2010). The implication of these studies is that impaired protein expression and function due to the presence of the ABCG2 SNP generated a less resistant AML.

In sum, evidence has emerged that genetic polymorphisms in the ABCB1 and ABCG2 genes may contribute to differential survival outcomes and toxicities in AML patients through a mechanism of decreased drug efflux in both AML blasts and normal progenitors. The goal

of these studies is a better understanding of inter-individual pharmacologic variation and therefore, the attractive possibility of tailored therapy based on an individual's ABC transporter genotype. The findings may also explain the repeated observation in the clinical trials of ABCB1 inhibitors that treatments including these inhibitors increased toxicity in some patients. If patients have an already limited ABCB1 expression and function, then addition of a modulator could increase toxicity to the normal bone marrow compartment while having little impact on the leukemic cell. Because of the likely effect of ABCB1 polymorphisms on sensitivity to inhibitors, it would be interesting to know what the results of the inhibitor trials would have been in terms of both efficacy and toxicity had patients been stratified to show the impact of the ABCB1 SNPs.

The role of epigenetic signals in ABC transporter expression

Methylation profiling studies have shown that aberrant DNA methylation is a dominant mechanism in the progression of MDS to AML (Gupta et al., 2009b). They have also demonstrated that methylation profiles allow the prediction of survival outcome (Hess et al., 2008). Although these studies did not focus on ABC transporters, they point to the important role of methylation in malignancy, and support future studies regarding epigenetics and ABC transporters. For example, it is known that methylation-controlled J protein (MCJ) is silenced by hypermethylation, which in turn prevents proper degradation of c-Jun, causing increased expression of *ABCB1* (Hatle et al., 2007). In contrast, both hypomethylation and hyperacetylation at the 5' promoter of *ABCB1* leads to increased expression of Pgp (Nakayama et al., 1998; Tabe et al., 2006). Similarly, hypomethylation of the ABCG2 promoter leads to increased expression in T-cell acute lymphoblastic leukemia cell lines (Bram et al., 2009). In a retrospective analysis using a small patient population, Nakayama and colleagues noted that 8 of 12 (66%) samples from patients who achieved first complete remission had hypermethylated *ABCB1* promoter versus none of 7 patients with chemorefractory leukemia (Nakayama et al., 1998). Additionally, two of six patients evaluated were found to have loss of methylation of the *ABCB1* promoter regions in leukemic cells at the time of relapse. Whether loss of *ABCB1* promoter methylation is causal versus circumstantial in initiating chemoresistance is unclear. Thus, while small, these studies suggest that ABC transporter expression or function should be monitored closely in clinical trials evaluating agents designed to modulate epigenetic control of gene expression.

Are cancer stem cells closely associated with multidrug resistance?

The existence of cancer stem cells (CSCs) has been proposed as a potential mechanism of drug resistance and a potential therapeutic target (for review, see Gupta et al. (2009a)). CSCs are a putative population of malignant cells within a tumor that possess the characteristics associated with normal stem cells. In this theory's most elaborate conception, CSCs can self-renew and divide by asymmetric division, reconstituting the heterogeneity of the original tumor. CSCs are also considered to be highly resistant to chemotherapeutic agents (for review, see Honoki (2010)). In this model, chemotherapy kills most of the cancer cells that are somewhat more differentiated and do not have long-term self-renewal capacity. When therapy is discontinued, the CSCs repopulate the cancer, thereby mediating disease relapse.

This model originated in the description of leukemic stem cells (LSCs), in individuals with AML, when Lapidot and colleagues showed that cells expressing CD34 but not CD38 (CD34⁺/CD38⁻) were capable of initiating human AML in non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice (Lapidot et al., 1994). This CD34⁺/CD38⁻ profile is identical to that of normal hematopoietic stem cells (HSCs), supporting the hypothesis that LSCs arise from HSCs (Bhatia et al., 1997).

Several studies have begun to unravel this aspect of the stem cell hypothesis. For example, it was demonstrated that LSCs can be generated from committed progenitors retrovirally transduced with either *MLL-ENL*, *MOZ-TIF2* or *MLL-AF9* oncogenes, leading to the reacquisition of self-renewal properties and the initiation of AML (Cuzzio et al., 2003; Huntly et al., 2004; Krivtsov et al., 2006). These reports are supported by recent studies on induced pluripotent stem cells (for review, see Yamanaka and Blau (2010)). Indeed, it was shown that either a loss of tumor suppressor function or a gain of oncogenic activity may cause the dedifferentiation of committed cells to cells possessing the stem cell property of self-renewal (Utikal et al., 2009; Yu et al., 2007). Furthermore, clonal evolution has been demonstrated in relapsed samples, suggesting that selection for fitness also occurs, prompting a more Darwinian view of drug resistance than accounted for in the stem cell model (Anderson et al., 2011; Bacher et al., 2010; van Delft et al., 2011).

Despite the accumulating evidence against the notion of a quiescent, long-lived, drug-resistant leukemic stem cell population characterized by long-term self-renewal and asymmetric cell division as the cell responsible for leukemic relapse, studies seeking such a cell type have identified “stemness” pathways of interest as therapeutic targets. In addition, normal hematopoietic stem cells have been functionally characterized by their ability to export Hoechst 33342 or Rhodamine123; the latter has been shown to be an ABCB1 substrate, whereas Hoechst 33342 is a substrate of both ABCB1 and ABCG2 (Lin and Goodell, 2006). These HSCs with the ability to efflux dye appear as a side population of cells that expands with enhancement of ABCB1 or ABCG2 function in murine or human bone marrow stem and progenitor cells (Apáti, et al., 2008; Bunting et al., 2000; McKenzie et al., 2007; Scharenberg et al., 2002). The normal regulation and timing of expression of the two transporters is still under study. *Mdr1a/1b*, *Bcrp1*, and *Mdr1a/Mdr1b/Bcrp1* mouse knockouts are viable and have hematopoietic stem cells, suggesting that these transporters are not essential for stem cell survival (Zhou et al., 2003).

It remains unclear if the prognostic value of drug transporters in patients with AML depends on their homogeneous expression by the entire population of blasts or on a subset of blasts with a stem cell phenotype. Nonetheless, to the extent that drug transporters may be expressed on cells bearing a stem cell phenotype, they may confer drug resistance, allowing selection and expansion of cells bearing that phenotype during treatment. ABCB1 and some members of the ABC C subfamily (ABCC1, C2, C3 and C5) were correlated with the CD34⁺ immature stem cell phenotype in AML (van der Kolk et al., 2001). Van den Heuvel-Eibrink and colleagues correlated the co-expression of ABCB1 and ABCG2 in a CD34⁺ immature cell population with poor prognosis in a group of elderly patients with AML (van den Heuvel-Eibrink et al., 2007).

Therapeutic Modulation of ABCB1

Competitive inhibitors of ABCB1 are categorized into three generations of drugs based on sequential refinements in the pharmacodynamic properties of these agents. First-generation ABCB1 inhibitors, including cyclosporin A (CSA) and quinine, among others, were agents with Food and Drug Administration (FDA) approval for other indications that were subsequently found to inhibit chemotherapeutic drug efflux. Second- and third-generation inhibitors, including valspodar and VX710 in the second generation and tariquidar and zosuquidar in the third generation, are agents specifically developed to elicit a high degree of ABCB1 inhibition *in vitro*. These agents were designed to have less off-target toxicity as compared to first-generation inhibitors. Selected phase III trials evaluating drug efflux inhibitors are summarized in Table 1.

The most positive findings with ABCB1 inhibitors were with CSA. A phase III trial conducted by the Southwest Oncology Group (SWOG) supported the combination of CSA with induction chemotherapy, demonstrating an improvement in 2-year overall survival (22% versus 12%, $P = 0.046$) (List et al., 2001). Approximately one-third of the patients had leukemia cells that expressed ABCB1 based on flow cytometric analysis using the MRK16 antibody. In this study, the survival benefit was derived from the subgroup with ABCB1+ leukemia cells, with an improvement in median overall survival from 4 to 12 months with the addition of CSA, whereas the median overall survival was only 6 months in both treatment arms for patients with ABCB1 negative leukemia cells. There was no correlation between treatment effect and the intensity of ABCB1 expression. Importantly, the use of CSA increased concentrations of daunorubicin and its principal metabolite, daunorubicinol, by 2- and 4-fold, respectively, presumably by inhibiting excretion of these compounds into bile and urine. Unfortunately, a subsequent phase III trial conducted by the United Kingdom Medical Research Council (MRC) did not support the use of CSA with induction chemotherapy for AML. The daily dose of CSA used in the UK MRC study, was significantly lower than that in the SWOG study (5 and later 10 mg/kg versus 16 mg/kg) (Liu Yin et al., 2001). A more recent, small study conducted by Matsouka and colleagues demonstrated a benefit in leukemia free survival from the addition of 5 mg/kg/day of CSA to idarubicin, cytarabine, and etoposide-based induction chemotherapy for 55 elderly patients with secondary AML (12 months versus 7 months; $P = 0.03$) (Matsouka et al., 2006). The variations in CSA dosing, along with the use of continuous infusion anthracycline in the SWOG study as opposed to the more standard bolus dosing used in the other two studies have made interpretation of the clinical utility of CSA difficult.

Similarly, the use of quinine with chemotherapy in high-risk MDS and MDS-AML was supported in a single phase III trial, demonstrating an improvement in patients with ABCB1+ AML in both CR (52% versus 18%, $P = 0.02$) and median OS (13 versus 8 months, $P = 0.01$) (Wattel et al., 1998). While survival benefit from quinine was not supported in two other phase III studies in AML sponsored by Groupe Ouest Est Leucémies Aiguës et Maladies du Sang (GEOLAMS) (Solary et al., 2003; Solary et al., 1996), in the more recent study there appeared to be some benefit in the subset of patients with detectable leukemic cell rhodamine efflux ($n = 54$) (Solary et al., 2003; Solary et al., 1996; Wattel et al., 1998). Here, the CR rate improved from 48% to 83% with the addition of quinine, although no difference in overall survival was observed.

Concerns over toxicity due to off-target effects with first generation inhibitors prompted the development of refined pharmacologic agents with increased ABCB1 specificity. The most extensively studied agents among this group are the cyclosporine D analog valspodar and the third-generation agent, zosuquidar. Initial trials incorporating valspodar with induction chemotherapy were complicated by high rates of toxicity, leading to dose reductions of induction chemotherapies, particularly anthracyclines (Kornblau et al., 1997). This was due in part to the off-target inhibition of CYP3A4-mediated drug metabolism. Despite using reduced doses of daunorubicin and etoposide with standard cytarabine in the induction regimen of the valspodar arm, the CALGB 9720 study was closed early due to a 42% rate of mortality during induction in the valspodar arm compared to 18% in the control arm (Baer et al., 2002). This trial only enrolled patients ≥ 60 years old. The CALGB 9621 study evaluated a similar regimen in 410 patients younger than 60 years old (Kolitz et al., 2004). In that study, the authors demonstrated an improvement in overall survival with the addition of valspodar in a subset of individuals ≥ 45 years old ($N = 220$, median OS 2.2 years versus 1.3 years, $P = 0.049$), although the trial comprised a series of dose escalations and was not a randomized trial designed to compare the two regimens. Unfortunately, a follow-up phase III study, CALGB 19808, demonstrated no improvement in clinical outcome, whether complete remission rate, disease-free survival or overall survival was considered (Kolitz et

al., 2010). Similarly, a European phase III trial in AML patients age 60 years and older performed by van der Holt and colleagues failed to demonstrate a benefit in the addition of valspodar to induction chemotherapy for patients over 60 years of age (van der Holt et al., 2005).

Zosuquidar, a third-generation ABCB1 inhibitor, also demonstrated promising results in early phase clinical trials. This agent was designed to have a high degree of ABCB1 specificity without interference with CYP3A4-mediated metabolism and without altering concentrations of other chemotherapeutic agents such as doxorubicin. Zosuquidar was found to completely inhibit ABCB1 function *in vitro* and demonstrated a favorable toxicity profile in early phase studies (Gerrard et al., 2004). Despite this, the recently reported ECOG 3999 study, a large, well conducted randomized trial, failed to demonstrate a benefit from the addition of zosuquidar to standard induction chemotherapy in either overall survival (zosuquidar, 9.4 versus placebo, 7.2 months, $P = 0.281$) or remission rates (51.9% versus 48.9%, $P = 0.583$) in older patients (Cripe et al., 2010). The rate of induction deaths was similar in both arms (22.2% with zosuquidar versus 16.3% without, $P = 0.158$). Individuals with high rates of rhodamine efflux from myeloblasts demonstrated worse OS compared to those without (median 6.8 versus 10.6 months, $P = 0.001$). There was no dose modification of daunorubicin planned, and pharmacokinetic studies showed no significant difference in the concentrations of daunorubicin between the treatment arms, but a generally higher level of its metabolite daunorubicinol with zosuquidar. It should be noted that the six-hour infusion of zosuquidar used in this trial may be insufficient to inhibit ABCB1. Lancet and colleagues demonstrated that a 12-hour continuous infusion of zosuquidar was required to reverse anthracycline resistance (Lancet et al., 2009). In summary, the addition of zosuquidar appeared to have little effect on the study population, with the caveat that there may have been insufficient dosing in this trial. However, further development of this drug has been suspended.

Taken in aggregate, clinical trials with ABC transporter inhibitors have demonstrated that despite increasing specificity for *ABCB1* as well as careful modifications to concurrent chemotherapy, toxicity with these agents remains a significant problem and likely obscures any potential benefit from these drugs. In fact, major dose reductions taken in the valspodar trials to prevent toxicity almost certainly undermined potential benefit. For example, in CALGB 19808, daunorubicin was reduced from 90 to 40 mg/m² and etoposide was reduced from 100 to 40 mg/m², more than a 50% dose reduction in both cases meant to mitigate the reduced clearance that would result from CYP3A4 inhibition (Kolitz et al., 2010). However, pharmacokinetic modeling of the area under the concentration curve (AUC) that occurs following a 50% reduction in drug dose associated with a 50% reduction in drug clearance suggests that peak (and likely therapeutic) drug levels are significantly reduced – followed by a long (and likely subtherapeutic) terminal phase (Bates et al., 2004). Since the goal of ABCB1 inhibition is to increase leukemic cell drug accumulation, this reduction in peak concentration would have negated any benefit accruing from efflux inhibition. Added to a 10 -15-fold interindividual variation in CYP3A4, it can be argued that the valspodar pharmacokinetic interaction and consequent dose reductions entirely undermine conclusions derived from those trials.

Beyond the drug interaction, there is still the question of whether these agents can *selectively* inhibit drug efflux in tumor cells and achieve a therapeutic window over normal tissues, in the absence of patient selection. As noted above, normal human tissue such as liver, endothelial, and hematopoietic progenitor cells express ABCB1 detected via functional and flow cytometric analysis, and that polymorphic differences may affect sensitivity (Chaudhary and Roninson, 1991; Cordon-Cardo et al., 1990). The data suggest that with the current repertoire of drugs the answer in AML is no; however, the impact of a

potent inhibitor without pharmacologic interactions in a population of patients selected for leukemic cell efflux prior to enrollment has not been tested. Additional problems include the possibility that other drug efflux mechanisms co-exist, providing redundancy in resistance mechanisms. The choice of mitoxantrone in several of the studies may have been unfortunate: as a substrate for ABCG2, the co-expression shown in several studies (Table 2) could more than compensate for the impact of an ABCB1-specific inhibitor. The most promising results from inhibitors of drug efflux were the early studies with agents with broad-spectrum transporter inhibition such as cyclosporine and quinine, suggesting that inhibition of a network of transporters may be worth exploring. This hypothesis is supported by Marzac et al (Marzac et al., 2011) as noted above, as well as by Qadir and colleagues, who demonstrated that cyclosporine modulates mitoxantrone toxicity in drug-resistant cell lines expressing a broad array of ABC transporters (Qadir et al., 2005).

Conclusion

The question of MDR1/ABCB1 modulation in AML would appear to be answered: There is no role for inhibition of ABCB1 in the clinic. So concluded the editorial accompanying the publication of the E3999 trial. Looking back at the development of ABCB1 as a therapeutic target indeed demonstrates a large number of failed clinical trials. Attempts to reverse resistance by inhibiting ABCB1-mediated efflux began almost immediately after the report by Takashi Tsuruo in 1981 that verapamil could inhibit drug efflux (Tsuruo et al., 1981). These early attempts to reverse resistance were flawed, as the inhibitors were insufficiently potent. Later attempts with valspodar were unsuccessful. Drug doses were compromised due to CYP3A4 interactions and more toxicity was observed in the experimental arm in randomized trials. By the time these trials were completed, enthusiasm for this line of investigation had disappeared.

In the wake of these disappointments, what evidence do we have that ABCB1 matters in acute myeloid leukemia? Three decades of investigation have repeatedly demonstrated the association of high ABCB1 expression in leukemia cells with poor outcomes. This strong association with an efflux transporter known to confer resistance to anthracyclines both *in vivo* and *in vitro* suggests that abandoning this therapeutic modality without fully understanding our failures is simplistic, scientifically unsatisfying, and premature. So then what do we not yet know? We do not know whether the inhibitors actually increase drug accumulation in leukemic cells in patients. We do not know whether the increased toxicity is due to a pharmacokinetic drug interaction, loss of a therapeutic window, or polymorphism-based interpatient variability. We do not know whether other drug transporters confound the inhibition of ABCB1 by a compensatory increase in drug efflux, thus requiring the inhibition of multiple transporters to increase drug accumulation. We do not know whether uptake transporters are dysregulated such that drug uptake is the limiting factor in drug accumulation. We do not know whether variant polymorphic transporters are associated with altered efflux, compensatory upregulation of other transporters, or reduced tolerability of anti-leukemic therapy, particularly with the addition of an ABCB1 inhibitor. We do not know whether the transporters are coordinately regulated with other biologic properties that confer drug resistance, regardless of drug efflux. Most of all, we do not know whether a patient population selected for leukemic cell ABCB1 overexpression would have fared differently in clinical trials of third-generation inhibitors. This latter question is critical in view of the studies showing that a specific subset of leukemia samples have ABC transporter upregulation.

Given the failures in AML clinical trials, it is tempting to ignore the link between ABCB1 expression and outcomes. But any investigator who has seen clear tumor cells floating in drug-stained media, reflecting the potent ability of an ABC transporter to efflux drugs

against a concentration gradient, will have difficulty thinking of ABCB1 as an innocent bystander. We argue that at this time, instead of more therapeutic clinical trials in AML, further efforts should be made to understand whether transporter-mediated drug efflux leading to reduced drug accumulation actually occurs in leukemic cells in patients. Clinical trials of targeted therapies over the last decade have clearly shown that targeted agents cannot be properly developed in the absence of the target. We know that *ex vivo* drug efflux is measurable by several assays. Drug concentrations need to be assayed in circulating leukemic cells. Imaging agents need to be developed that would allow leukemic cell drug accumulation to be quantitated *in vivo*. Further, we need to perform assays that examine all candidate drug uptake or efflux transporters that could confound clinical trial results. Until we confirm the hypothesis that underlies the clinical studies – ABCB1 expression confers a poor outcome due to drug efflux leading to reduced drug accumulation – the hypothesis that modulation of this pathway will be beneficial to patients cannot be properly tested.

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Table 1

Selected Trials Evaluating ABCB1 Inhibitors in Acute Myeloid Leukemia

Study/Group ^a	Chemotherapy + Modulator ^b	N	CR	OS	Comments
First Generation Inhibitors					
SWOG 9126 (List et al., 2001)	AD AD + CsA (16 mg/kg/d)	226	33% 39% (p=0.14)	12% (2 yr OS) 22% (p=0.046)	Effect of CsA greatest in patients with ABCB1 + AML
MRC(Liu Yin et al, 2001)	ADE ADE + CsA (5–10 mg/kg/d)	213	45% 41% (p=0.6)	8% (3 yr OS) 7% (p=0.5)	Worse survival in older patients with CsA
GOELAMS/GFM (Wattel et al., 1998)	AM AM + quinine (30 mg/kg/d)	131	41% 47% (p=NS)	11 m (median) 13 m (p=NS)	CR and OS improved in patients with Pgp+ AML
GOELAMS (Solary et al., 2003)	AI AI + quinine (30 mg/kg/d)	425	66% 68% (p=NS [§])	39% (4 yr OS) 44% (p=0.3)	CR improved with positive rhodamine efflux
Second Generation Inhibitors					
CALGB 9720 (Baer et al., 2002)	ADE ADE + valsopodar	120	46% 39% (p=0.008)	33% (1 yr OS) 33% (p=0.48)	Closed early due to high early death in PSC arm
CALGB 9621 (Koltz et al., 2004)	ADE ADE + valsopodar	394	75% 73% (p=NS)	1.5 y (median) 1.4 y (p=NS)	Improved survival in 220 patients 45 years old
ECOG 2995 (Greenberg et al., 2004)	AME AME + valsopodar	129	25% 17% (p=NS)	5.4 m (median) 4.6 m (p=NS)	Closed early due to lack of benefit
HOVON/MRC (van der Holt et al., 2005)	AD AD + valsopodar	419	48% 54% (p=0.22)	10% (5 yr OS) 10% (p=0.52)	
CALGB 19808 (Koltz et al., 2010)	ADE ADE + valsopodar	302	75% 75%	1.86 y (median) 1.69 y (p=0.82)	
Third Generation Inhibitors					
ECOG 3999 (Cripe et al., 2010)	AD AD + zosuquidar	433	49% 52% (p=.583)	23% (2 yr OS) 20% (p=.281)	ABCB1 positivity predicted worse outcome

^a Abbreviations: SWOG, Southwest Oncology Group; GOELAMS, Groupe Ouest Est d'Etude des Leucémies et Autres Maladies du Sang; GFM, Groupe Français des Myélodysplasies; HOVON, Dutch Hemato-Oncology Association; MRC, British Medical Research Council; CALGB, Cancer and Leukemia Group B; ECOG, Eastern Cooperative Oncology Group

^b Abbreviations: A, Ara-C; C, cytarabine; D, daunorubicin; CSA, cyclosporin A; E, etoposide; I, idarubicin; M, mitoxantrone; NS, not significant

Prognostic relevance of ABCB1 (P-gp), ABCC1 (MRP1), and ABCG2 (BRCP) in patients with acute myeloid leukemia

Table 2

Reference	Disease status	N	Assay	ABC transporter expression frequency			Association of transporter overexpression with outcomes (all P values < 0.05)
				ABCB1	ABCC1	ABCG2	
Pirker et al. (Pirker et al., 1991)	<i>de novo</i>	63	RT-PCR	71%			B1 negatively associated with CR
Michieli et al. (Michieli et al., 1999)	<i>de novo</i>	96	IFC	49%	8%		B1 negatively associated with CR, OS
Leith et al. (Leith et al., 1999)	<i>de novo</i>	352	FFC	35%	10%		B1 negatively associated with CR
Benderra et al. (Benderra et al., 2004)	<i>de novo</i>	149	FFC	CV		52%	G2 negatively associated with CR, DFS, OS
van der Kolk et al. (van der Kolk et al., 2000)	<i>de novo</i>	104	FFC	33%		31%	Rh123 efflux (B1) negatively associated with CR
Damiani et al. (Damiani et al., 2007)	<i>de novo</i> , normal karyotype	135	IFC	33%	23%		B1 negatively associated with CR, EFS
Damiani et al. (Damiani et al., 2006)	<i>de novo</i> , normal karyotype	73	IFC	33%	26%	33%	B1 negatively associated with CR, BRCP negatively associated with DFS
Zhou et al. (Zhou et al., 1995)	Mixed	91	RT-PCR		24%		C1 associated with resistant disease
Han et al. (Han et al., 2000)	Mixed	145	IFC	36%	8.3%		B1 negatively associated with CR
Shaich et al. (Shaich et al., 2005)	Mixed	331	RT-PCR	25%	45%		B1 negatively associated with CR, OS; MRP1 negatively associated with DFS
Walter et al. (Walter et al., 2007)	Relapsed/refractory	276	FFC	CV			B1 negatively associated with CR
Benderra et al. (Benderra et al., 2005)	Relapsed/refractory	85	FFC	50%		39%	B1 and G2 negatively associated with CR, DFS, OS

CV, continuous variable; FFC, functional flow cytometry; IFC, immune flow cytometry; RT-PCR, reverse transcription polymerase chain reaction; CR, complete response; OS, overall survival; EFS, event free survival; DFS, disease free survival