ABC TRANSPORTERS AS PREDICTIVE FACTORS FOR CHEMOTHERAPEUTIC RESPONSE IN ACUTE MYELOID LEUKEMIA

by

Maria Ming Chee Ho

B.Sc., The University of British Columbia, 2001

A THESIS SUBMITTED IN PARTIAL FULFILLMENT

OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Biochemistry and Molecular Biology)

The University of British Columbia

April 2007

ABSTRACT

Multidrug Resistance (MDR), resistance to multiple chemotherapeutic drugs, is a major problem in the treatment of acute myeloid leukemia (AML). Overexpression of members of the ATP-Binding-Cassette (ABC) transporter superfamily has been associated with clinical MDR and failure of conventional chemotherapy. The work in this thesis was the first in investigating expression of ABC transporters and functional effects of their modulation in AML subpopulations along the leukemic stem cell hierarchy: CD34+CD38- (primitive and disease maintaining), CD34+CD38+ (differentiating progenitors), and CD34- (depleted of progenitors). An initial profiling of mRNA expression of the 47 human ABC transporters in total *de novo* blasts by RT Real-Time PCR showed no consistent differences between patients who subsequently achieved complete remission following conventional remission induction chemotherapy (responders) and patients who remained refractory (non-responders). Subsequent profiling of isolated subpopulations, however, revealed elevated expression of *MDR1* and/or *BCRP1*, two main drug-resistance ABC transporters, in the primitive CD34+CD38- fraction of 7/10 non-responders compared to 0/7 responders. To test their functional activity *ex vivo*, daunorubicin sensitivity with or without ABC modulators was determined in AML subpopulations by the apoptotic assay. I found high ABC-dependent drug resistance, correlated to high *MDR1/BCRP1* expression level and reversible by ABC inhibition, in the CD34+CD38- fraction of non-responders compared to responders. This suggests an active functional role of ABC transporters in the primitive, disease-maintaining fraction. Taken as a whole, my studies suggest a prognostic significance of ABC transporters in the primitive CD34+CD38- leukemic subpopulation, and support a modified approach in investigating the value of ABC modulating agents in AML. It may be possible to pre-screen and identify patients for whom ABC transporters is a major factor for MDR before initial treatment, who are most likely to benefit from the combination of conventional chemotherapy and ABC inhibitors. This will be invaluable especially to patients with a normal karyotype (50% of patients), since cytogenetic aberrations currently remain the most useful prognostic marker for AML.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
ABCR	ATP-binding cassette transporter-retina
AE	amplification efficiency
AML	acute myeloid leukemia
APL	acute promyelocytic leukemia
Ara-C	arabinoside
BAALC	Brain and Acute Leukemia Cytoplasmic
BAC	bacterial artificial chromosome
BCRP1	Breast cancer resistance protein 1
bp	base pair(s)
BSEP	Bile-salt export pump also known as SPGP
CBF	Core-binding Factor
CD	Cluster of Differentiation molecule
CEBPA	CCAAT enhancer binding protein
CFTR	Cystic Fibrosis Transmembrane conductance Regulator
CGH	comparative genomic hybridization
CR	complete remission
CSC	cancer stem cells
Ct	threshold cycle
Cy	cyanine
DMSO	dimethylsulfoxide
DNA	deoxy ribonucleic acid
FAB	French-American-British
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FCS	fetal calf serum
FISH	fluorescence *in situ* hybridization
Flt3	FMS-like tyrosine 3
GAPDH	Glyseraldehyde-3-phosphate dehydrogenase
G-CSF	granulocytic-colony stimulating factor
GSH	glutathione
HSC	hematopoietic stem cell
IMDM	Iscove's modified Dulbecco's medium
ITD	internal tandem duplication
LSC	leukemic stem cell
MDR	multidrug resistance
MDR1	Multidrug Resistance 1
MEM	minimal essential medium
MLL	Mixed Lineage Leukemia
MRP1	Multidrug Resistance protein 1
MTS	tetrazolium salt
NBD	nucleotide-binding domain
NOD-SCID	non-obese diabetic severe-combined immunodeficiency
NR	non-responsive

PB	peripheral blood
PCR	polymerase chain reaction
Pgp	Permeability-glycoprotein
PI	propidium iodide
PML	promyelocytic leukemia
PS	phosphatidylserine
RARα	Retinoic Acid Receptor α
RNA	ribonucleic acid
RT	reverse transcription
Shh	Sonic Hedgehog
SKY	spectral karyotyping
SL-IC	severe-combined immunodeficiency leukemia initiating cell
SP	side population
SPGP	Sister Permeability-glycoprotein also known as BSEP
VLB	vinblastine

ACKNOWLEDGEMENTS

I thank my supervisor, Dr. Victor Ling, for giving me the opportunity to work in his laboratory. The guidance, challenge, resources and autonomy he provides are all pivotal in shaping me into an independent thinker.

To Dr. Donna Hogge, my collaborator: her support and expert advice are critical to the successful completion of this project.

Special thanks to Dr. Jaclyn Hung for constant encouragement and fruitful intellectual discussions.

To my fellow lab members and friends, it was a pleasure and privilege working with them. I shall remember this special period of time in my life.

I thank my committee members, Dr. Connie Eaves, Dr. Donna Hogge, and Dr. Ross MacGillivray, for their help and suggestions on my thesis work.

I also thank Gitte Gerhard and Leman Yalcintepe for their technical assistance.

Finally, I thank my parents, my sister and brother for their unfailing love and support throughout the years.

To my father, who spurred me on the pursuit of knowledge.

To my Father from Above: without Him I am nothing.

I Introduction

1.1 – Challenges in cancer therapy

Cancer has become the current leading cause of death for people under age 85 in North America[8]. It is a genetic disease, arising from the transformation of a normal cell, with a derangement in normal regulation of cell proliferation, differentiation and death. One major difficulty in the treatment of cancer stems from its heterogeneity: individual cancer patients typically show different combinations of genetic and/or epigenetic aberrations in specific cellular pathways. This is thought to explain, at least in part, why there is no form of therapy that is equally successful in all patients presented with the same type of cancer. Recognition of this heterogeneity has resulted in increasing support for the concept of "personalized therapy" or "individual therapy" – tailoring the treatment according to individual patient condition, in particular, therapeutically targeting specific genetic abnormalities for the patient in question – as emphasized in the 2006 annual report from American Society of Clinical Oncology[9].

There are three broadly used types of treatment for cancer: surgery, radiation therapy and chemotherapy. While the former two are very effective on local tumors, chemotherapy remains the main form of systemic treatment for inoperable, metastasized or more advanced cancer. Adverse side effects often ensue, however, due to drug toxicity on normal cells in the body, particularly those that must divide to maintain organ integrity. It would therefore be invaluable if an individual patient's response to a given regimen could be predicted before deciding on the best form of therapy available. Recent years has seen the development of hypothesis-driven, mechanism-based drug discovery in support of personalized therapy – designing drugs specifically targeting the molecular pathology underlying individual cancers (reviewed by

Collins and Workman, 2006[10]). This type of molecular-targeted therapy is best combined with characterization and pre-screening of patients with the relevant genetic alteration. One prominent example is the use of Gleevac (imatinib, STI571), a specific inhibitor of BCR-ABL tyrosine kinase, in chronic myeloid leukemia (CML)[11,12]. BCR-ABL is the product of the aberrant Philadelphia chromosome and is present in virtually all cases of the disease. This prototypical specific targeting of a molecular product in cancer achieves high success in newly diagnosed CML patients, although cases of resistance undermines its effectiveness in more advanced cases (reviewed by Kantarjian, 2006[13]). In Acute Myeloid Leukemia (AML), the internal tandem duplication (ITD) of the *FLT3* gene is the most common mutation and hence an attractive target for therapy. Flt3 is a tyrosine kinase receptor important in regulation of proliferation, differentiation and apoptosis of hematopoietic progenitors[14]. Several studies reported that karyotypically normal patients with FLT3 activation have a poorer prognosis[15-17], and a number of agents against the *FLT3* mutation were being tested on patients bearing the mutation[18,19].

This thesis sought to evaluate the possible prognostic value of ABC transporter expression in anticipating the chemotherapeutic response of the malignant population in patients with AML. This type of cancer offers a number of unique features relevant to such a study. Technically, it is possible to select patients with over 90% leukemic cell counts in their blood system, so that results are not skewed by a substantial or undefined normal cell contamination, a common problem in solid tumors. Since chemotherapy is the main form of treatment for leukemia, almost all patients will undergo a standard initial drug regimen, thereby allowing direct comparisons with treatment outcome. Furthermore, critical cancer biology models are well established for AML, notably the origin and perpetuation of the leukemia by a rare subset of "leukemic stem cells" of known phenotype[2,20] (Discussed in Section 1.7). This forms the basis of

my main hypothesis that it is the rare leukemic stem cells, not the mature or differentiated cells comprising the majority of leukemia, which dictate clinical outcome. This is supported by results of this study, which highlights the value of determining ABC transporter levels in a leukemic stem cell-enriched population to predict treatment response. I then went on to illustrate the effects of ABC inhibitors on this small cell fraction, proposing their use as drug-sensitizing agents in combination with chemotherapy to improve initial treatment outcome for otherwise non-responding patients. This is especially crucial for AML, in which its fast progression augments the importance of choosing the best treatment at diagnosis.

1.2 – Acute Myeloid Leukemia: an overview

Leukemias make up ~2% of adult cancers [21] but comprise a heterogeneous group of diseases. The lymphoid leukemias affect the lymphoid lineages (notably B- and T-cells) and the myeloid leukemias affect primarily the myeloid lineages including the granulocytes and monocytes/macrophages, red blood cells, and megakaryocytes. Leukemia can be classified as either acute or chronic. AML is a malignancy of the myeloid elements, the hallmark being a block in normal differentiation, leading to the massive accumulation of immature leukemic "blast cells". This usually results in rapid and severe disruption of normal bone marrow function, which can take the clinical form of anemia (decrease in hemoglobin), fever and infection (decrease in white cells), and bleeding and bruising (decrease in platelets). Accumulation of leukemic cells in other tissues is also common, such as the lymph nodes, spleen and skin. AML requires urgent diagnosis and treatment. If left untreated, the disease results in death within weeks or days. In contrast, the chronic leukemias are characterized by unregulated proliferation

and overexpansion of a range of differentiated cells, are slow-growing and usually progress over a period of several years[21].

The incidence of AML increases sharply with age, from less than 1/100,000 under age 35 to 15/100,000 over 75. Recent Canadian statistics report an incidence of 816 new cases in 2001 and a mortality rate of 690 deaths in 2003[8]. While the etiology of AML remains largely unknown, a number of risk factors have been identified, including radiation and/or chemical exposure, tobacco use, prior cancer-related chemotherapy or radiation therapy, old age, genetic syndromes, and a history of prior blood disorders. Certain recurring cytogenetic abnormalities have been found to be closely associated with AML and form a major area of investigation (see Section 1.3). Diagnosis is based on morphological data which further classify AML into eight subgroups (M0-7) under the French-American-British (FAB) scheme established in 1976 (Table 1.1). An example of distinct morphological and histochemical differences between AML subtypes is shown in Figure 1.1. More recently the World Health Organization (WHO) has proposed a new classification which includes immunophenotyping, cytogenetics and clinical features to allow a more prognostically relevant description of AML (Table 1.2).

Given that AML is a rapidly progressing disease with a fatal outcome if not adequately controlled, initial treatment is often targeted at eradicating leukemic blasts and re-establishing normal bone marrow function. This is usually achieved through high-dose chemotherapy with general supportive measures such as blood cell transfusions, antibiotics administration, and leukapheresis to temporarily clear patient blood of blasts. The standard remission-induction chemotherapeutic regimen for AML is a combination of cytosine arabinoside (Ara-C) and an anthracycline such as daunorubicin. Clinical MDR (Multidrug Resistance), cross resistance arising in cancer cells to a wide range of chemically unrelated drugs, is commonly observed and

presents a major problem in AML therapy. While ~70% of patients achieve remission with initial therapy, approximately 75% of these will relapse within 2 years of diagnosis in spite of additional consolidation chemotherapy[21]. In addition, 20-30% of patients are unresponsive even to initial chemotherapy. Overall, long-term remissions are obtained in only 25% of patients[21]. A small number of patients under 60 years of age and who have a suitable histocompatible donor are eligible for curative treatment supported by an allogeneic bone marrow or mobilized peripheral blood transplant. However, chemotherapy necessarily remains the main form of post-remission treatment. Given that chemotherapy is a highly invasive treatment with both long term and short term side effects, it would be valuable to be able to predict which patients are more likely to benefit from current chemotherapy treatments. In addition, the poor prognostic rate raises the need for more effective curative therapies.

1.3 – Current prognostic factors for predicting chemotherapeutic response in AML

Older AML patients (over 65 years) have a poor prognosis compared to young patients due to their lower drug tolerance and higher toxicity during high-dose chemotherapy[21]. They are also considered clinically ineligible for allogeneic transplant therapy. Other than age, the current most important prognostic variable for patients with AML is the detection of cytogenetic abnormalities in a diagnostic bone marrow sample[22-24]. Almost 200 different recurring acquired cytogenetic aberrations have been identified in ~50% of AML patients[24]. The most common ones are listed in Table 1.3. These abnormalities, in the forms of translocations, inversions, deletions monosomies and trisomies, play an important role in determining the biological basis of AML. Intense molecular studies of specific genes at the sites of aberration revealed that they are usually

involved in normal blood cell development and homeostasis. Most inversions and translocations in AML result in gene fusion products that can dysregulate proliferation, differentiation or apoptosis of blood cell precursors[25]. One of the best known translocations (8;21) (Figure 1.2) is frequently associated with AML subtype M2. It places the gene *AML1* on 21q22 beside the gene ETO (a transcription factor) on 8q22. AML1 is the α subunit of the heterodimeric transcription factor Core Binding Factor (CBF) critical for normal hematopoiesis[26]. The fusion protein retains the ability to bind to AML1 consensus regions and acts as a competitive inhibitor for the normal *AML1* product, resulting in defective myeloid differentiation. Another frequent abnormality, inversion (16), is molecularly related to t(8;21) because it disrupts the gene encoding the β subunit of CBF. AML subtype M3, also known as acute promyelocytic leukemia (APL), is characterized by translocation (15;17) (Figure 1.3). This fuses the retinoic acid receptor α gene (RARα) on 17q12-21 with the promyelocytic leukemia gene (PML) on 15q22, which has a dominant effect on normal RARα, antagonizing its differentiation function[27].

Large cooperative studies have documented a significant relationship between detection of non-random chromosomal abnormalities and disease outcomes, including complete remission, disease-free survival and overall survival[6,28-30].. The prognostic significance of common abnormalities is summarized in Table 1.4. The presence of certain aberrations including inversion (16) and translocations (8;21) and (15;17) is associated with significantly better overall survival compared to normal cytogenetics[6] (Figure 1.4A). While use of the novel targeted therapeutic drugs all-trans retinoic acid (ATRA)[31,32] and arsenic trioxide[33-36] have drastically improved outcome for patients with t(15;17), the molecular basis of higher sensitivity to chemotherapy for t(8;21) or inv(16) AML remains to be elucidated. On the other hand, other chromosomal changes such as -5, -7 and del(5) are frequently related to poor prognosis

compared to patients whose blasts are cytogenetically normal[6] (Figure 1.4B), although the molecular mechanisms responsible remain unclear.

Despite the prognostic value of the many cytogenetic abnormalities identified almost 50% of patients present with an apparent normal karyotype. A patient's karyotype is routinely determined by traditional chromosome-banding and less frequently by newer methodologies such as spectral karyotyping (SKY) or fluorescence in situ hybridization (FISH). Studies have confirmed the validity and consistency of these karyotyping techniques in detecting translocations and large-scale gains or losses of genomic DNA[37-40]. It will be useful, as suggested by large-scale microarray gene expression studies[41,42], to try to subcategorize patients with a normal karyotype into groups with varying prognosis. Indeed, ongoing studies have suggested a few genes that may act as possible prognostic markers for AML patients with a normal karyotype[43], including FMS-like tyrosine 3 (*FLT3*) (see Section 1.1), mixed lineage leukemia (*MLL*)[44], CCAAT enhancer binding protein (*CEBPA*)[45], nucleophosmin (*NPM1*)[46] and brain and acute leukemia cytoplasmic (*BAALC*)[47]. Studies on more biomarkers should facilitate predicting treatment outcomes within the cytogenetically normal group. The objective of this thesis was to investigate the prognostic value of expression of the multidrug resistance-related ATP-binding cassette transporters (ABC transporters).

1.4 – ABC transporters: an Overview

ATP-binding cassette (ABC) transporters represent the largest transmembrane protein superfamily in eukaryotes and prokaryotes and are important factors in drug resistance. They are ATP-dependent protein transporters that actively pump a wide range of substrates across biological membranes. To date, 48 ABC transporters have been identified in humans[1,48,49]. Figure 1.5 shows

the structural organization of the ABC transporter protein and gene. ABC proteins share a highly conserved ATP-binding cassette (ABC), also known as nucleotide-binding domain (NBD), which consists of the characteristic motifs Walker A (G-X_2-G-X-G-K-S/T-T/S-X_4-hydrophobic) and Walker B (R-X-hydrophobic$_2$-X_2-P/T/S/A-X-hydrophobic$_4$-D-E-A/P/C-T-S/T/A-A/G-hydrophobic-D)[50]. The ABC gene also contains motif C, or the "signature motif" bearing the sequence (hydrophobic-S-X-G-Q-R/K-Q-R-hydrophobic-X-hydrophobic-A) organized between motifs A and B[51]. A fully functional unit of ABC transporters consists of two similar halves, each containing a transmembrane domain and an ATP-binding domain. Members of the superfamily can either be "full transporters" with both halves present or "half transporters" requiring dimerization for function[1].

The crystal structure of MsbA, an ABC transporter homolog in *Escherichia coli*, has been described by Chang *et al*[5]. This provides insights into the molecular structure and transport mechanism of ABC transporters. As seen in Figure 1.6, MsbA consists of two similar halves embedded in the lipid bilayer, organized much like a clamp hinging on extracellular connecting loops. The twelve transmembrane domains collectively serve as the substrate binding site. Based on this structure, the same group also proposed a general model for lipid transport by ABC transporters (Figure 1.7). In this "flippase" model, the transmembrane chamber first interacts with and intercepts the substrate, causing conformational changes that result in ATP hydrolysis by the NBD. This in turn causes a conformational shift that brings the two NBDs together. The resulting change in the chamber now produces an energetically unfavorable environment for the hydrophobic substrate and it "flips" from the inner to the outer membrane layer. After the flip, the NBDs separate leading to the expulsion of the substrate to the extracellular environment. Recently, Dawson and Locher proposed an alternate "access and release" model based on their crystal structure on the bacterial

ABC transporter Sav1866, that postulates conformational changes to reflect the hydrolysis state of ATP[52].

The human ABC transporters are further categorized into subfamilies A to G based on sequence homology and structural similarity. Table 1.5 lists the current subfamily members, nomenclature and function. Human ABCs perform a variety of physiological functions by facilitating unidirectional shuttling of compounds within the cell as part of a metabolic process or outside the cell to other organs[1,49]. Unlike other types of transport proteins, one unique characteristic of the members of the ABC superfamily are their wide substrate specificity. The first[53] and best characterized ABC transporter, *MDR1* (encoding the P-glycoprotein, PGP), is a promiscuous transporter of hydrophobic substrates. Physiologically, PGP is important in removing toxic metabolites from cells, especially in the brain[54,55]. Other ABCs perform functions ranging from liver bile salt excretion (SPGP) to vitamin A transport in photoreceptors (ABCR) to cholesterol transport (ABCA subfamily). A number of ABC transporters have been linked to genetic disorders, for example CFTR (mutations of which causes cystic fibrosis) and SPGP (mutations of which result in progressive familial intrahepatic cholestasis 2[56]). The promiscuity of ABC transporters, however, becomes a major clinical problem when cancer occurs: some ABCs are able to transport multiple chemotherapeutic drugs out of cancer cells, resulting in Multidrug Resistance.

1.5 – ABC transporters and multidrug resistance in cancer

Overexpression of members of the ATP-Binding-Cassette (ABC) transporter family is found to be a main factor for MDR in cancer. There are three main ABC transporters involved in multidrug resistance: the classical PGP/*MDR1*, the multidrug resistance associated protein (*MRP1*), and the breast cancer resistance protein (*BCRP1*, *ABCG2*). *MDR1*, the prototype ABC

transporter, was first cloned from a drug-selected cell line displaying multidrug resistance[53]. Knockout studies showed that *mdr1a/mdr1b*(-/-) (homologs of human *MDR1*) mice had a high accumulation of drug levels in many tissues, especially the brain, confirming that PGP confers a general detoxification function against xenotoxins[57]. The observation that not all MDR cell lines overexpress *MDR1* led to the discovery of another multidrug resistance transporter, multidrug resistance protein 1 (*MRP1*). Cloned from a small-cell lung cancer cell line, *MRP1* transports drugs that are conjugated to glutathione (GSH) via the GSH reductase pathway[58]. Knockout studies showed that *MRP1* is important in inflammation as well as for detoxification in the brain[59]. The third multidrug resistance transporter, *BCRP1*, was identified in cell lines selected for mitoxantrone resistance[60,61]. Unlike PGP and MRP1 which are full ABC transporters, BCRP1 is a half ABC and is thought to function as a homodimer. Knockout studies have suggested an in vivo role for this transporter in tissue detoxification and heme metabolite transport under hypoxic conditions[62,63]. Hence all three MDR-related ABC transporters are physiologically important in protecting normal cells from a broad array of xenobiotics. This versatile defense mechanism, however, can be utilized by cancer cells for protection against a wide variety of chemotherapeutic drugs. Early clinical studies found high expression of PGP in many types of cancer, such as the leukemias, colon, kidney, adrenocortical and hepatocellular cancers[64,65]. Amplification of *MDR1* is frequently reported in many drug-selected MDR cell lines, although this is not a commonly observed mechanism for overexpression in clinical cases[66]. *MRP1* is overexpressed in leukemia, esophageal cancer and non-small cell lung cancers[67]. Expression of *BCRP1* has been detected in the leukemias[68], gastric cancer, hepatocellular carcinoma, endometrial cancer, colon cancer, melanoma and lung cancer[69,70]. Figure 1.8 illustrates the mechanism of drug resistance via these transporters: the incoming

chemotherapeutic drug is intercepted by the transporter in the plasma membrane of the cancer cell and expelled, thereby failing to reach the intracellular site of action, resulting in drug resistance and failure of therapy.

Other ABC transporters have been implicated in drug resistance. For instance, ABCA2 can confer resistance to estramustine, a nitrogen mustard derivative of oestradiol[71]. SPGP, an ABC that shares high homology to PGP, is reported to confer resistance to paclitaxel[72]. Members of the ABCC subfamily, commonly known as MRP2 to MRP9, bear similarities to MRP1 and have the potential to confer drug resistance. Given that many members of the ABC superfamily have not been well characterized, they may also have the capacity to efflux substrates of clinical interest that has not yet been identified.

Table 1.6 lists the common chemotherapeutic drugs that are known substrates of these transporters. PGP and BCRP1 preferentially extrude large hydrophobic, positively charged molecules[60,73-75], while MRP1 can extrude both hydrophobic uncharged molecules and water-soluble anionic compounds[58,76-79]. All three display capacity to transport daunorubicin[80], the common drug used for AML, although daunorubicin efflux by MRP1 is strictly dependent on GSH levels[81]. Many agents have been investigated in an effort to reverse PGP-mediated clinical MDR. These are generally competitive inhibitors that are substrates for the ABC transporters. Verapamil is a commonly used inhibitor that can modulate activity of a number of ABC transporters (most effective against PGP). More specific inhibitors have been developed for each transporter, for example PSC-833 for PGP[82-84]. Clinical trials using ABC inhibitors yielded mixed results in AML, with complications including high bone marrow and neurological toxicity, confounding interpretations on the usefulness of these agents. Nonetheless, successful therapy has been reported for other types of cancer. In highly drug-resistant retinoblastoma for

example, combination of PGP inhibitor cyclosporin A with chemotherapy resulted in marked increase in relapse-free rate, demonstrating the value of ABC modulation in improving clinical outcome[85].

1.6 – ABC transporters in AML

Expression of *MDR1* in leukemic cells likely contributes to chemotherapy resistance in AML patients. Treatment failure can be observed either as intrinsic chemotherapy resistance at diagnosis or at relapse. Overexpression of PGP is the most extensively studied mechanism of MDR in AML. The presence of PGP detected by antibody staining as measured by flow cytometry has been demonstrated in 20% to 75% of *de novo* AML patients according to different studies[87-89]. Expression of PGP in patient peripheral blood or bone marrow is associated with lower remission rates[87,88], shorter overall survival and lower disease-free survival (Figure 1.9)[4,7,90,91]. In addition, a number of studies have also reported an increase in PGP during relapsed disease[92-94]. It is suggested that PGP-positive cells might escape initial chemotherapy and remerge at relapse, or alternately, *MDR1* expression may be induced during chemotherapy. *Ex vivo* studies have shown that PGP expression decreases intracellular accumulation of daunorubicin in leukemic cells, and is reversed by PGP inhibitors[82,95,96]. An additional anti-apoptotic role has also been attributed to PGP in that it appears to protect leukemic cells from caspase-dependent programmed cell death[97-99].

Several investigations on the MRP1 transporter suggested a role in AML drug resistance[100-102], but larger studies failed to identify a consistent relationship between MRP1 and prognosis[89,103]. Studies on the expression of BCRP1 in AML have also yielded mixed results. While some showed that up-regulation of *BCRP1* is associated with a poor prognosis[104,105] and is

common during relapse[106], others reported a lack of consistent overexpression in AML patients[107-109]. Several studies have reported the combination of different drug-resistant transporters, or their co-expression with cell survival factors, important in drug resistance[89,100,109]. Almost all past studies, however, have been limited to the three MDR-related transporters.

Another important feature of *MDR1* and *BCRP1* are their changes in expression during the early differentiation of very primitive hematopoietic cells. As described in the following sections, this feature has important clinical implications in the treatment of AML.

1.7 – The leukemic stem cell model and its implications in drug response

The concept of "cancer stem cells" has emerged as an important theme for cancer research in the past few years. Normal stem cells are defined by their dual capacity to regenerate themselves through self-renewal mechanism and to produce mature cells through differentiation[110]. The "cancer stem cell model" proposes that a tumor is similarly sustained by a biologically distinct subpopulation of "cancer stem cells" (CSC), with the same ability to perpetuate the production of progeny with limited proliferative ability. Three observations support the existence of CSCs in human cancers. First, only a small fraction of a tumor has the capacity to regenerate a new tumor, operationally demonstrable upon transplantation into immunodeficient mice. Second, these tumor-initiating cancer cells can be identified and prospectively isolated by a distinct phenotype, usually based on flow cytometric or immuno-magnetic detection of differentially expressed surface antigens. Third, secondary tumors regenerated by these cells contain an array of mixed tumorigenic and non-tumorigenic cells, recapitulating the heterogeneity of the original tumor[111]. Hence irrespective of the origin of CSC,

this population bears the hallmarks of stem cells – self-renewal and "differentiation" into a functional hierarchy of "primitive" (tumorigenic) and "mature" (non-tumorigenic) cells.

The CSC model was first and best developed in leukemia[2,112]. In 1979, Minden and colleagues first described colony forming cells, termed "blast progenitor cells", from AML patient peripheral blood[113], suggesting that a subpopulation of leukemic cells was clonogenic. It was, however, John Dick and colleagues who provided direct evidence for the leukemic stem cell (LSC) model[20]. They described a primitive leukemic cell they termed SL-IC (SCID leukemia initiating cell) that can initiate AML in mice. The SL-ICs from most patients tested are CD34++/CD38-, a surface phenotype similar to that of primitive normal cells that can regenerate normal hematopoiesis in immunodeficient mice (Figure 1.10). In addition to its tumorigenic potential, the SL-IC also shows the capacity to differentiate into the non-dividing leukemic cells similar to those which constitute the majority population in the malignant clone in the AML patient from which it was isolated. The authors conclude that it is possible to isolate a small fraction enriched with LSC activity (0.2-100 stem cells in 10^6 blast cells), and the common surface properties and hierarchical organization support the hypothesis that the LSC originates by transformation of an initially normal hematopoietic stem cell (Figure 1.11). Nevertheless, it has also been shown that leukemic stem cells can be generated from more mature progenitors that reacquire stem cell properties[114-116].

CSCs appear to share many common properties with normal stem cells[3]. For example, overlap in their regulation by the Wnt, Notch and Sonic hedgehog (Shh) pathways, are associated both with oncogenesis and normal stem cell renewal[117]. As well, CSC can share normal stem cell properties that allow a long life-span, such as protection against cytotoxins via expression of ABC transporters (see section 1.8 below).

Validation and adoption of the leukemic stem cell model calls for a paradigm shift in the treatment of the disease. Figure 1.12 gives a schematic representation of new treatment strategies that are likely more effective. Because the leukemia is sustained by the rare LSCs, this small population must be included as a target for effective therapies rather than just the majority of blast cells that have very limited proliferative ability. Existing drug therapies, however, are commonly targeted against the bulk leukemic population or their immediate precursors. Although a dramatic initial response can often be achieved, if the LSCs are not also effectively eliminated they can eventually regenerate the disease. Hence to achieve more durable responses or even cure, there is a need for novel treatment methods more specifically directed against this primitive subpopulation.

1.8 – ABC transporters in normal and leukemic stem cells

In recent years, MDR-related ABC transporters, in particular *MDR1* and *BCRP1*, have been associated with stem cells of the hematopoietic system[118]. Early investigation by Chaudhary and Roninson first showed elevated expression of *MDR1* in primitive (CD34+) normal hematopoietic cells[119]. As discussed above, CD34, a cell surface phosphoprotein, is a marker for both normal hematopoietic stem cells (HSC) and LSCs. Engraftment studies in humans[120], baboons[121] and mice[122] demonstrated the CD34+ population to be highly enriched with stem cell repopulation activity. It has also been suggested that CD34 expression may be in part regulated by the activation state of stem cells[123]. Further studies demonstrated a marked decline in *MDR1* expression during differentiation[119,124]. Like *MDR1*, expression of *BCRP1* is high in normal human HSC, drops dramatically in more committed progenitors, and remains low in mature hematopoietic cells[125]. In addition, *BCRP1* is reported to be a molecular determinant of the side

population (SP) in hematopoietic cells, a small distinct cell fraction with enriched HSC repopulating activity in adult mouse bone marrow[126] or human fetal liver[127] but not vice versa. The SP, defined by its ability to efflux the fluorescent dye Hoechst 33342, was first identified in murine bone marrow cells[128] and later also found in many different human tissues[126,129-131], in spite of its failure to be detected on normal adult human HSC. One important property of the SP is its ability to exclude a number of drugs, reflective of the transport activity of ABC transporters. Interestingly, Uchida and colleagues reported that the dye (Rhodamine 123 and Hoechst 33342) efflux ability of PGP and Bcrp1 are unstable in murine HSCs[132]. This fluctuation appears to parallel changes in other HSC markers such as CD34 and CD38 and relate to the activation state of HSCs, suggesting a common control mechanism operated by a cell cycle checkpoint.

One likely function of ABC transporters in stem cells is to protect this population from toxic substances over their long life-span. Another possible function is that these transporters can efflux regulatory molecules that can alter stem cell fate. For example, studies conducted by our lab revealed that PGP is a functional bile salt transporter in SPGP knockout mice, compensating for the lack of SPGP. The recently elucidated connection between ABC transporters and normal hematopoietic stem cells sheds a new light on the drug resistance property of CSCs. It has been suggested that CSCs can employ the same protective mechanisms operating in normal stem cells to defend themselves from chemotherapeutic drugs. Indeed, an increasing number of studies are associating ABC transporters to CSCs. For example, PGP expression has been correlated to CD34 positivity in AML[133,134]. As well, the SP has been identified in neuroblastoma, breast cancer, ovarian cancer, glioblastoma and gastrointestinal cancer cell lines[135-139]. These studies demonstrated that this small subpopulation has both enriched tumorigenicity and high drug

extrusion capacity. Wulf and colleagues found the SP to be detectable in most AML patients, displays significantly increased drug efflux ability, and is able to regenerate the disease in NOD-SCID mice[140]. Studies by Feuring-Buske and Hogge confirmed the prevalence of SP in AML, although SP+CD34+CD38- cells appeared to represent normal rather than leukemic primitive cells in AML patients[141].

The emerging paradigm on cancer biology posits functional heterogeneity within a cancer and the existence of a small distinct group of CSC reminiscent of normal stem cells. It remains to be investigated whether higher expression and activity of MDR-related ABC transporters contributes to higher drug tolerance in LSC, the presumptive subpopulation responsible for perpetuation of AML.

1.9 – Thesis objectives

The overall objective of this thesis was to investigate ABC transporter expression as a possible predictive factor for initial drug response in AML patients. My original hypothesis was that upregulation of ABC transporters is responsible for the lack of response to initial chemotherapy in AML patients. In that context, I investigated the expression level of ABC transporters in total AML blast cells and in different subpopulations along the leukemic hierarchy – $CD34^{+}CD38^{-}$ (most primitive), $CD34^{+}CD38^{+}$, and $CD34^{-}$ (most mature). Hence the three goals of this thesis were:

1. To profile mRNA expression patterns of the ABC transporter superfamily in bulk AML patient materials, and compare profiles between patients that responded or failed to respond to initial chemotherapy.

2. To examine expression patterns of key MDR-related ABC transporters in primitive and mature AML subpopulations of responders and non-responders.

3. To determine and compare the *ex vivo* drug sensitivity of CD34+CD38-, CD34+CD38+, and CD34- AML subpopulations of responders and non-responders.

The first and second goals are addressed in Chapters 2 and 3 where the RT-Real-Time-PCR technique was utilized to profile mRNA levels of ABC transporters in unsorted and sorted populations of AML patient samples. In Chapter 4, I investigated the *ex vivo* drug sensitivity of sorted leukemic subpopulations using the fluorescence-based Annexin V-PI assay for apoptosis.

Table 1.1. FAB classification of AML.

FAB	Morphology
M0	Minimally differentiated
M1	Myeloblastic leukemia without maturation
M2	Myeloblastic leukemia with maturation
M3	Hypergranular promyelocytic leukemia
M4	Myelomonocytic blasts
M4Eo	Variant, increase in marrow eosinophils
M5	Monocytic leukemia
M6	Erythroleukemia
M7	Megakaryoblastic leukemia

Table 1.2. WHO classification of AML.

AML with recurrent cytogenetic translocations AML with t(8;21)(q22;q22) AML1/CBFalpha/ETO Acute promyelocytic leukemia: AML with t(15;17)(q22;q12) and variants PML/RARalpha AML with abnormal bone marrow eosinophils inv(16)(p13;q22) vagy t(16;16)(p13;q22) CBFbeta/MYH1 AML with 11q23 MLL abnormalities
AML with multilineage dysplasia With prior MDS Without prior MDS
AML with myelodysplastic syndrome, therapy related Alkylating agent related Epipodophyllotoxin related Other types
AML not otherwise categorized AML minimally differentiated AML without maturation AML with maturation Acute myelomonocytic leukemia Acute monocytic leukemia Acute erythroid leukemia Acute megakaryocytic leukemia Acute basophilic leukemia Acute panmyelosis with myelofibrosis Myeloid sarcoma
Acute Leukemias of ambiguous lineage

Table 1.3. Frequencies of common recurring cytogenetic aberrations in adult AML.

Cytogenetic abnormality	Cooperative Group Study (No. of patients)			Adults total (n = 4257) No. (%)
	CALGB (n = 1311) No. (%)	MRC (n= 2337) No. (%)	SWOG/ECOG (n = 609) No. (%)	
None (normal karyotype)	582 (44)	1096 (47)	244 (40)	1922 (45)
+8	123 (9)	211 (9)	53 (9)	387 (9)
-7/7q-	95 (7)	209 (9)	52 (9)	356 (8)
t(15;17)(q22;q21)	88 (7)	210 (9)	27 (4)	325 (8)
-5/5q-	86 (7)	183 (8)	36 (6)	305 (7)
t(8;21)(q22;q22)	81 (6)	104 (4)	50 (8)	235 (6)
inv(16)	96 (7)	53 (2)	53 (9)	202 (5)
-Y	58 (4)	NA	20 (3)	78 (4)
t/inv(11q23)	54 (4)	45 (2)	42 (7)	141 (3)
+21	28 (2)	51 (2)	NA	79 (2)
abn(17p)	30 (3)	NA	12 (2)	42 (2)
del(9q)	33 (3)	37 (2)	17 (3)	87 (2)
inv(3)	12 (1)	61 (3)	12 (2)	85 (2)
Complex with ≥ 3 abn	135 (10)	NA	71 (12)	206 (11)
Complex with ≥ 5 abn	99 (8)	222 (9)	53 (9)	374 (9)

Abbreviations: CALGB, Cancer and Leukemia Group B[28]; MRC, United Kingdom Medical Research Council[6,29]; SWOG/ECOG, Southwest Oncology Group/Eastern Cooperation Oncology Group[30]; abn, abnormality; NA, not available. Modified from Mrozek et al, *Blood Reviews*, 2004[24].

Table 1.4. Prognostic significance of frequent chromosomal abnormalities in AML.

Good	Standard	Poor
inv(16)	Normal	-5, del(5q)
t(8:21)	+8	-7
t(15;17)	+21	Abnormal 3q
	+22	Complex
	del(7q)	
	del(9q)	
	Abnormal 11q23	
	All other structural abnormalities	

Modified from Grimwade et al, *Blood*, 1998[6].

Table 1.5. List of human ABC transporters, location and physiological function.

Gene (subfamily)	Common name	Location	Expression	Function
ABCA1	ABC1	9q31.1	Ubiquitous	Cholesterol efflux
ABCA2	ABC2	9q34	Brain	
ABCA3	ABC3, ABCC	16p13.3	Lung	
ABCA4	ABCR	1p22.1-p21	Photoreceptors	N-retinylidene-PE efflux
ABCA5		17q24	Muscle, heart, testes	
ABCA6		17q24	Liver	
ABCA7		19p13.3	Spleen, thymus	
ABCA8		17q24	Ovary	
ABCA9		17q24	Heart	
ABCA10		17q24	Muscle, heart	
ABCA12		2q34	Stomach	
ABCA13		7p11-q11	Low in all tissues	
ABCB1	MDR1/PGP	7p21	Adrenal, kidney, brain	Multidrug resistance
ABCB2	TAP1	6p21	All cells	Peptide transport
ABCB3	TAP2	6p21	All cells	Peptide transport
ABCB4	PGY3, MDR3	7q21.1	Liver	PC transport
ABCB5		7p14	Ubiquitous	
ABCB6		2q36	Mitochondria	Iron transport
ABCB7		Xq12-q13	Mitochondria	Fe/S cluster transport
ABCB8		7q36	Mitochondria	
ABCB9	TAPL	12q24	Brain, testis, spinal cord	Peptide transport
ABCB10		1q42	Mitochondria	
ABCB11	SPGP, BSEP	2q24	Liver	Bile salt transport
ABCC1	MRP1	16p13.1	Lung, testes, PBMC	Drug resistance
ABCC2	MRP2	10q24	Liver	Organic anion efflux
ABCC3	MRP3	17q21.3	Lung, intestines, liver	
ABCC4	MRP4	13q32	Prostate	Nucleoside transport
ABCC5	MRP5	3q27	Ubiquitous	Nucleoside transport
ABCC6	MRP6	16p13.1	Kidney, liver	
ABCC7	CFTR	7q31.2	Exocrine tissue	Chloride ion channel
ABCC8	SUR1	11p15.1	Pancreas	Sulfonylurea receptor
ABCC9	SUR2	12p12.1	Heart, muscle	
ABCC10	MRP7	6p21	Low in all tissues	
ABCC11	MRP8	16q11-q12	Low in all tissues	
ABCC12	MRP9	16q11-q12	Low in all tissues	
ABCD1	ALDP	Xq28	Peroxisomes	VLCFA transport regulation
ABCD2	ALDR	12q11-q12	Peroxisomes	
ABCD3	PMP70	1p22-p21	Peroxisomes	
ABCD4	PMP69	14q24.3	Peroxisomes	
ABCE1	OABP, RNASELI	4q31	Ovary, testes, spleen	Oligoadenylate binding
ABCF1	ABC50	6p21.33	Ubiquitous	
ABCF2		7q36	Ubiquitous	
ABCF3		3q25	Ubiquitous	
ABCG1	WHITE1	21q22.3	Ubiquitous	Cholesterol transport
ABCG2	BCRP1, ABCP	4q22	Placenta, intestines	Toxin efflux, drug resistance
ABCG4	WHITE2	11q23	Liver	
ABCG5	WHITE3	2p21	Liver, intestines	Sterol transport
ABCG8	WHITE4	2p21	Liver, intestines	Sterol transport

Modified from Dean et al, *Genome Research*, 2001[1].

Table 1.6. Substrate specificity of PGP, MRP1 and BCRP1.

Gene	Protein	Non-chemotherapy substrates	Chemotherapy substrates
ABCB1	PGP	Neutral and cationic organic compounds, many commonly used drugs	Doxorubicin, daunorubicin, vincristine, vinblastine, actinomycin-D, paclitaxel, docetaxel, etoposide, teniposide, bisantrene, STI-571[86]
ABCC1	MRP1	Glutathione and other conjugates, organic anions, leukotriene C4	Doxorubicin, daunorubicin, epirubicin, etoposide, vincristine, methotrexate[58,76-79]
ABCG2	BCRP1, ABCP	Prazosin	Doxorubicin, daunorubicin, mitoxantrone, topotecan, SN-38[60,73-75]

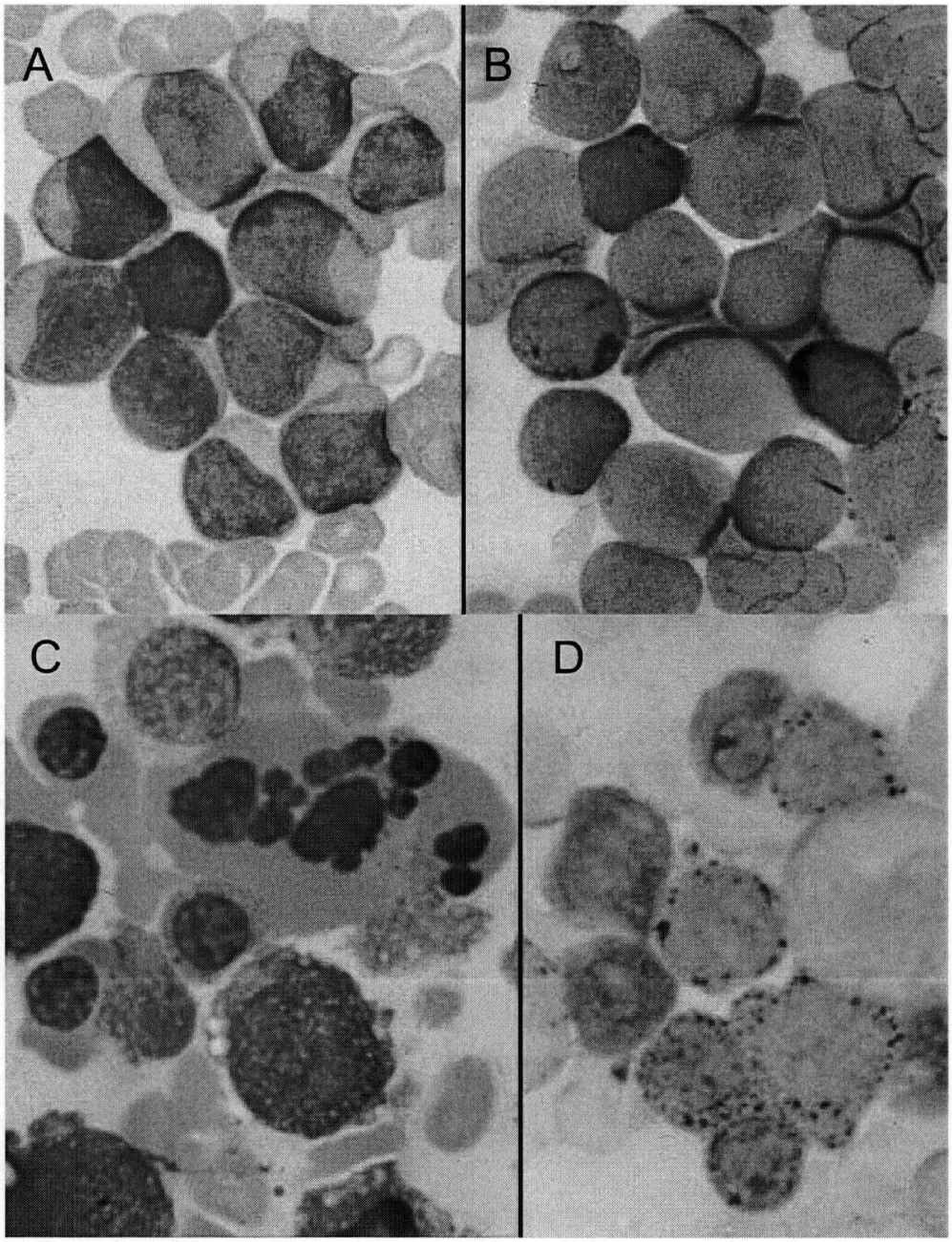


Figure 1.1. Morphological differences between different AML subtypes. AML subtypes M1 (A & B) and M6 (C & D) are shown as examples. A, M1 myeloblasts with eccentric nuclei. B, Sudan Black stain of M1 blasts. C, M6 erythroblasts. D, coarse PAS stain of M6 blasts. Courtesy of Haematological Malignancy Diagnostic Service http://www.hmds.org.uk.

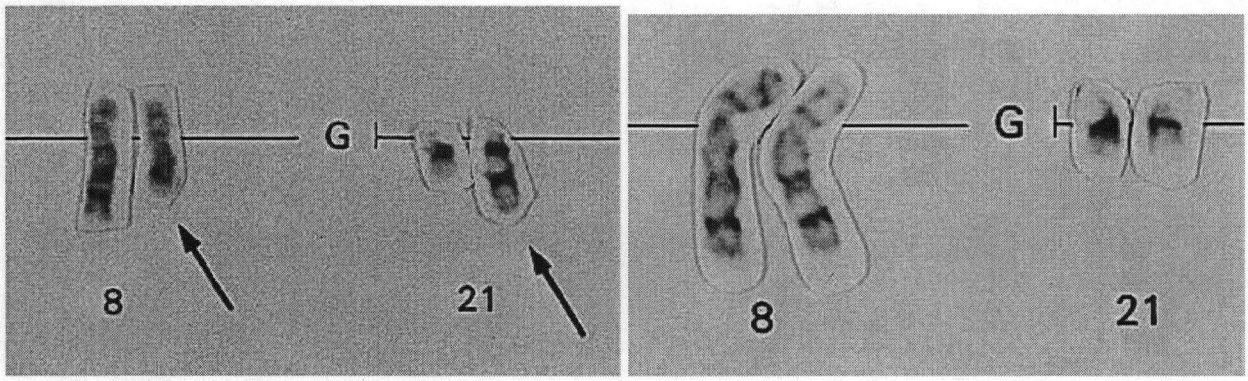


Figure 1.2. AML translocation (8;21) (left) compared to normal chromosomes 8 and 21 (right). Courtesy of Guide for Detection of MRD in AML **www.meds.com/leukemia/guide**.

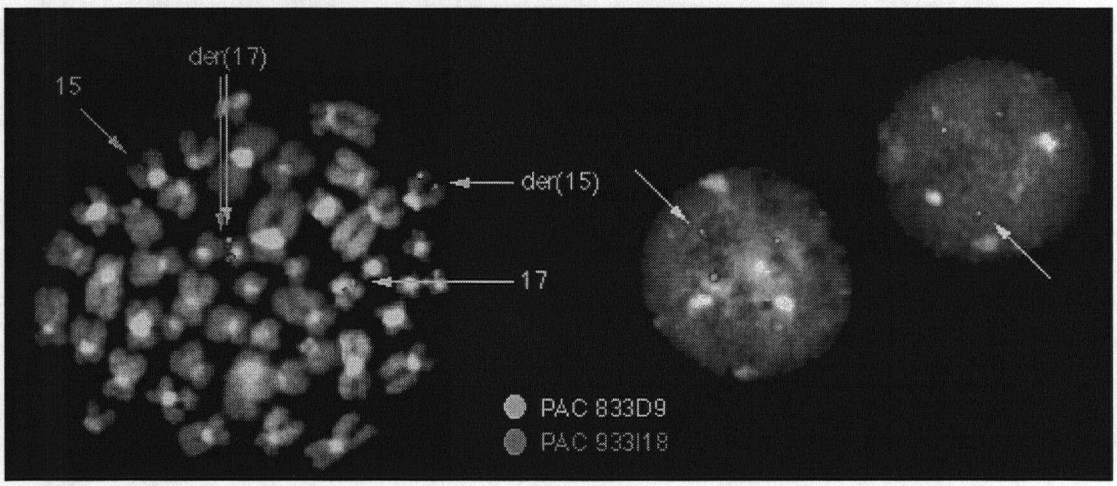


Figure 1.3. AML translocation (15;17) detected in APL. Two probes (red: chromosome 15, green: chromosome 17) are utilized to visualize the translocation (yellow arrows). Courtesy of Atlas of Genetics and Cytogenetics in Oncology and Hematology.

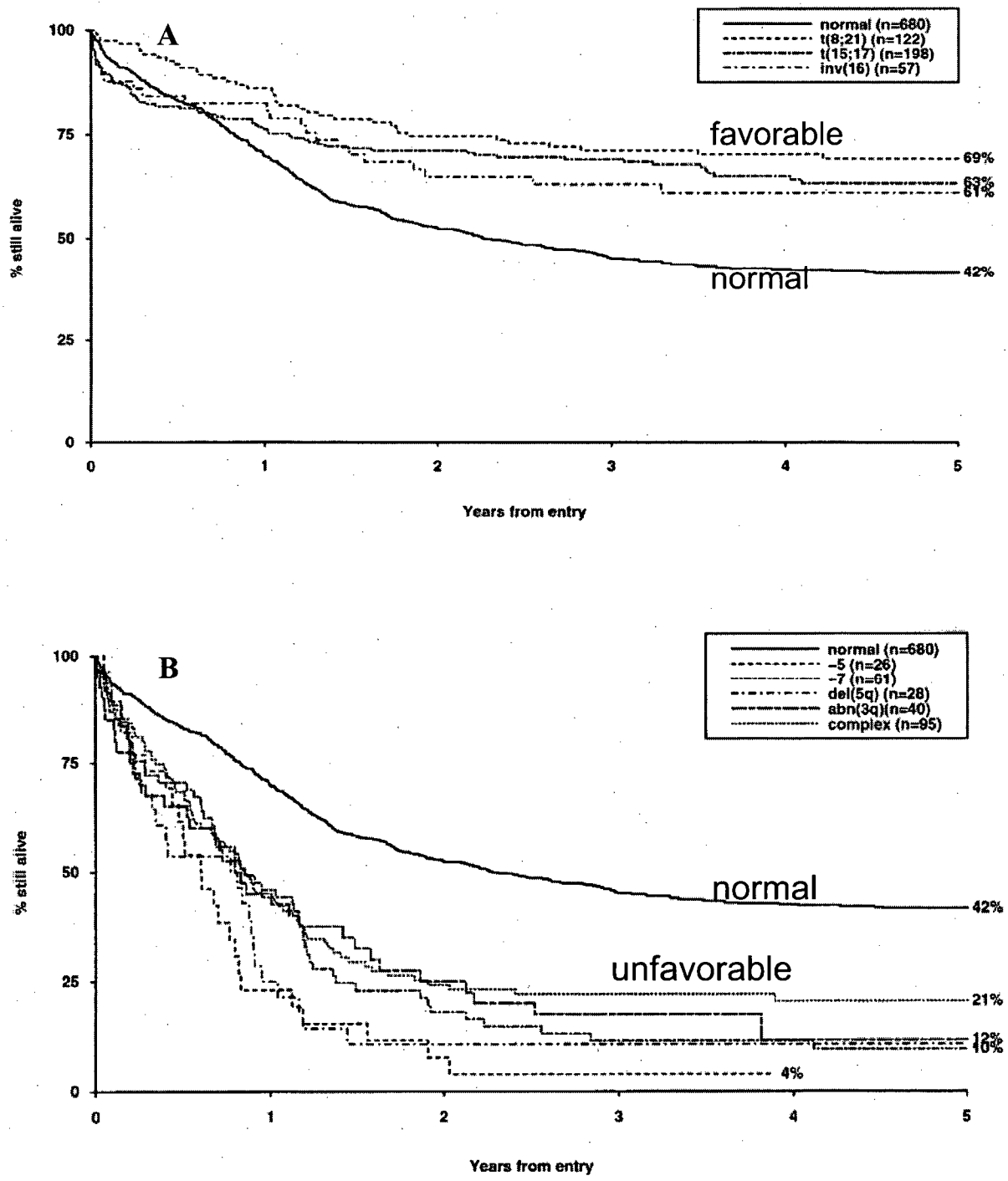


Figure 1.4. Overall survival of AML patients with favorable (A) or adverse (B) cytogenetic abnormalities compared to the group with normal karyotype. Reproduced from Grimwade et al, *Blood*, 1998[6].

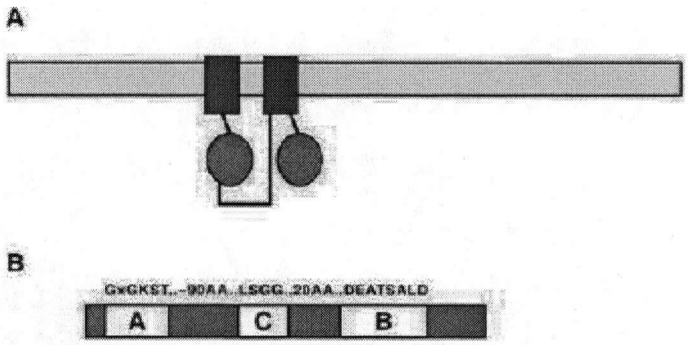


Figure 1.5. Schematic diagram of a typical ABC transporter. A, an ABC protein is embedded in the lipid bilayer of a cellular membrane (yellow). The transmembrane domain is depicted as blue rectangles and the NBD as red circles. B, Common sequence organization of the ATP-binding cassette of an ABC gene, with Walker motifs in the order A-C-B. Reproduced from Dean *et al*, *Genome Research*, 2001[1].

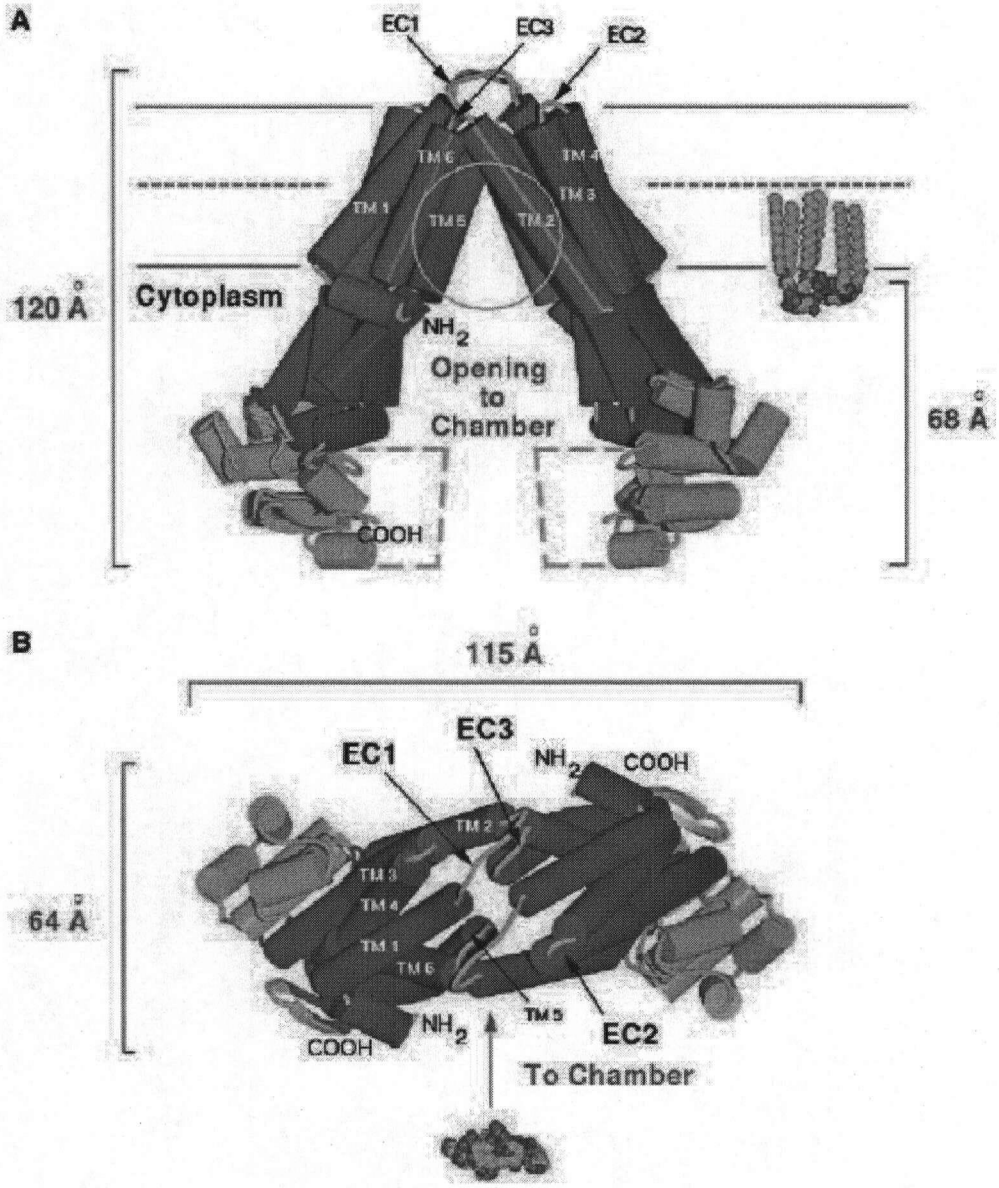


Figure 1.6. X-ray crystallography structure of MsbA. A, View of dimer looking into the chamber opening. The transmembrane domain, NBD, intracellular domain, and connecting loops are in red, cyan, dark blue and green, respectively. A potential substrate lipid A is shown to the right of the structure. Solid and dotted green lines represent the boundaries of the membrane bilayer leaflets. B, View of MsbA from the extracellular side, perpendicular to the membrane with lipid A. Reproduced from Chang et al, *Science*, 2001[5].

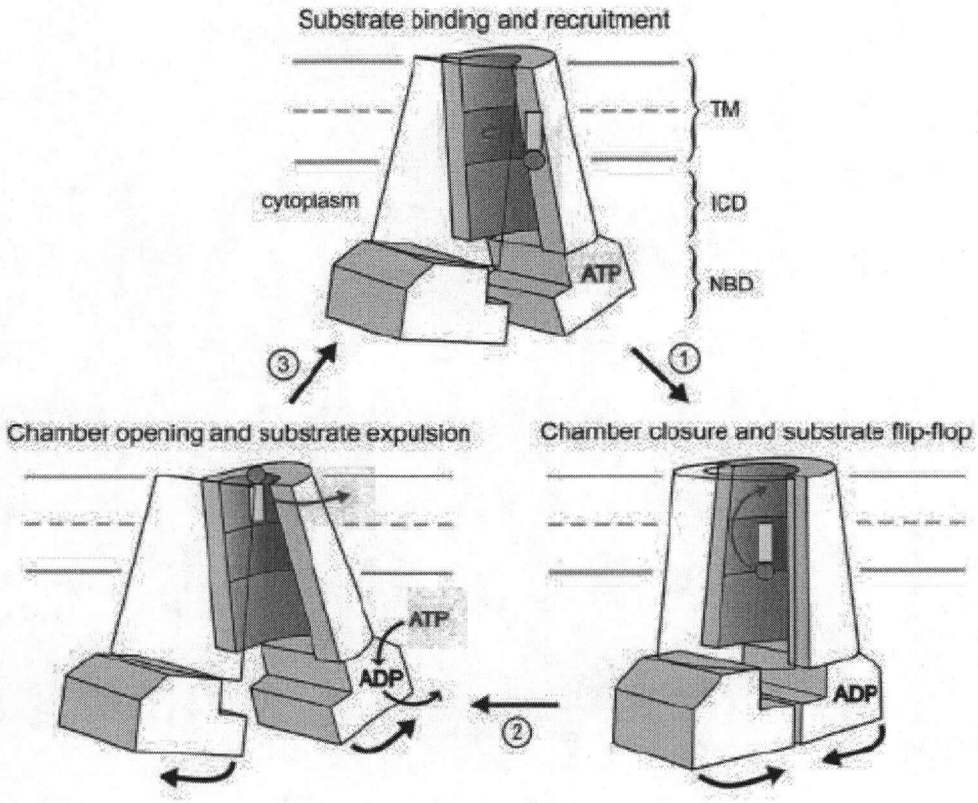


Figure 1.7. Proposed model for lipid A transport by MsbA. Stages 1 to 3 begin at top and proceeds clockwise. See text for details. (1) Lipid A binding, triggering of ATP hydrolysis, and recruitment of substrate to chamber. (2) Closure of the chamber and translocation of lipid A. Interaction between the two NBDs is possible. (3) Opening of the chamber, movement of TM2/5, release of lipid A to the outer bilayer leaflet, and nucleotide exchange. A small yellow rectangle and a green circle denote the hydrophobic tails and sugar head groups of lipid A, respectively. The transmembrane domain (TM), intracellular domain (ICD), and nucleotide-binding domain (NBD) are labeled. Blue regions indicate positive charge lining the chamber, and purple regions represent the intracellular domain. The gray region on the outer membrane side of the chamber is hydrophobic. Red and black arrows show the movement of substrate and structural changes of MsbA, respectively. Reproduced from Chang et al, *Science*, 2001[5].

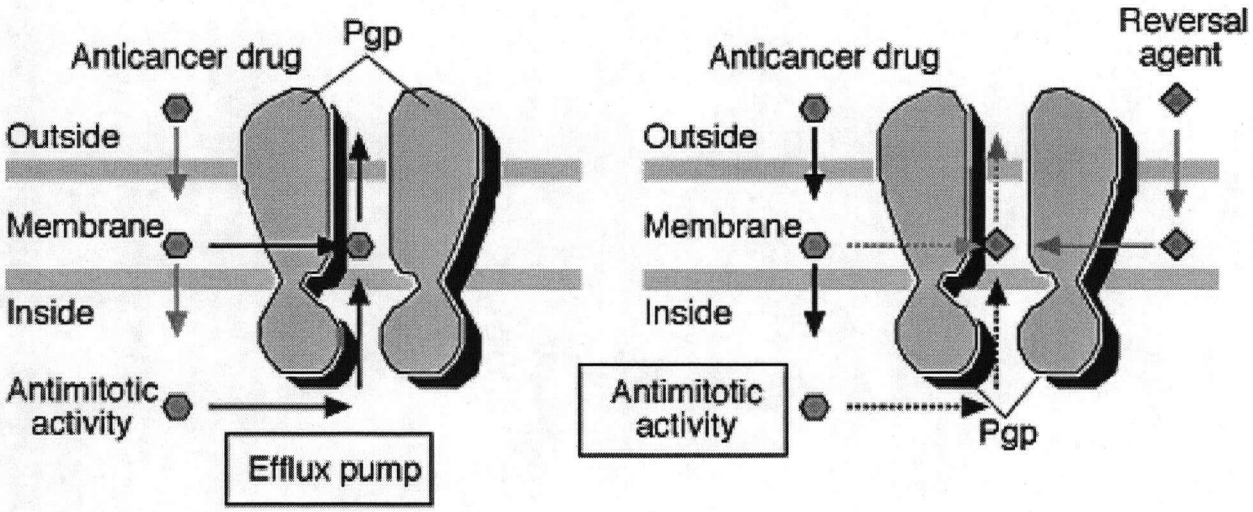


Figure 1.8. PGP as a classic drug pump in cancer cells. Left, PGP is located in the plasma membrane. A drug molecule going from the outside to the inside of the cell is intercepted by PGP in the membrane and subsequently transported out of the cell, resulting in drug resistance. Right, Addition of a reversal agent such as PSC-833 inhibits PGP transport activity by binding to its substrate binding site. Reproduced from *Chemtech* 1998, 28 (6), 31-36.

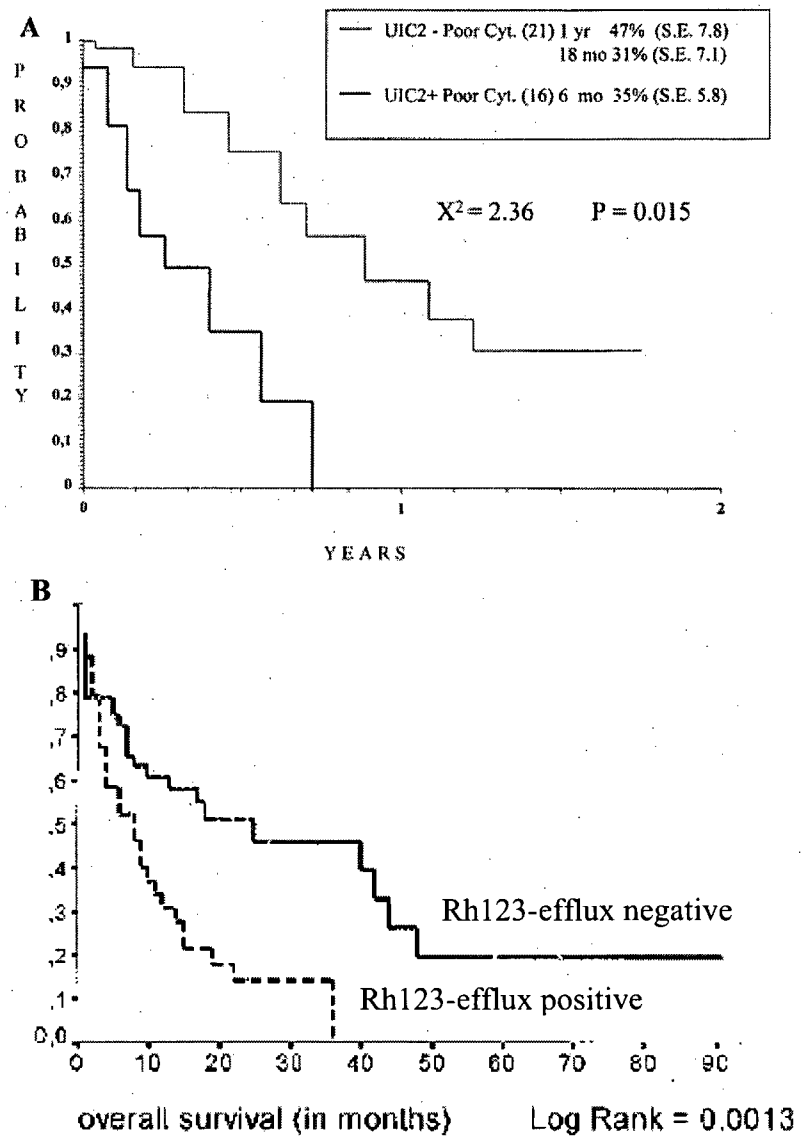


Figure 1.9. Prognostic significance of PGP in AML. A, PGP protein positivity as measured by UIC2 antibody staining is associated with reduced complete remission duration in the poor risk cytogenetic group. B, PGP-related functional activity as measured by dye Rhodamine 123 efflux correlates with lower survival in *de novo* AML. Reproduced from Del Poeta et al, *Leukemia Research*, 1999[4] (A) and Wuchter et al, *Haematologica*, 2000[7] (B).

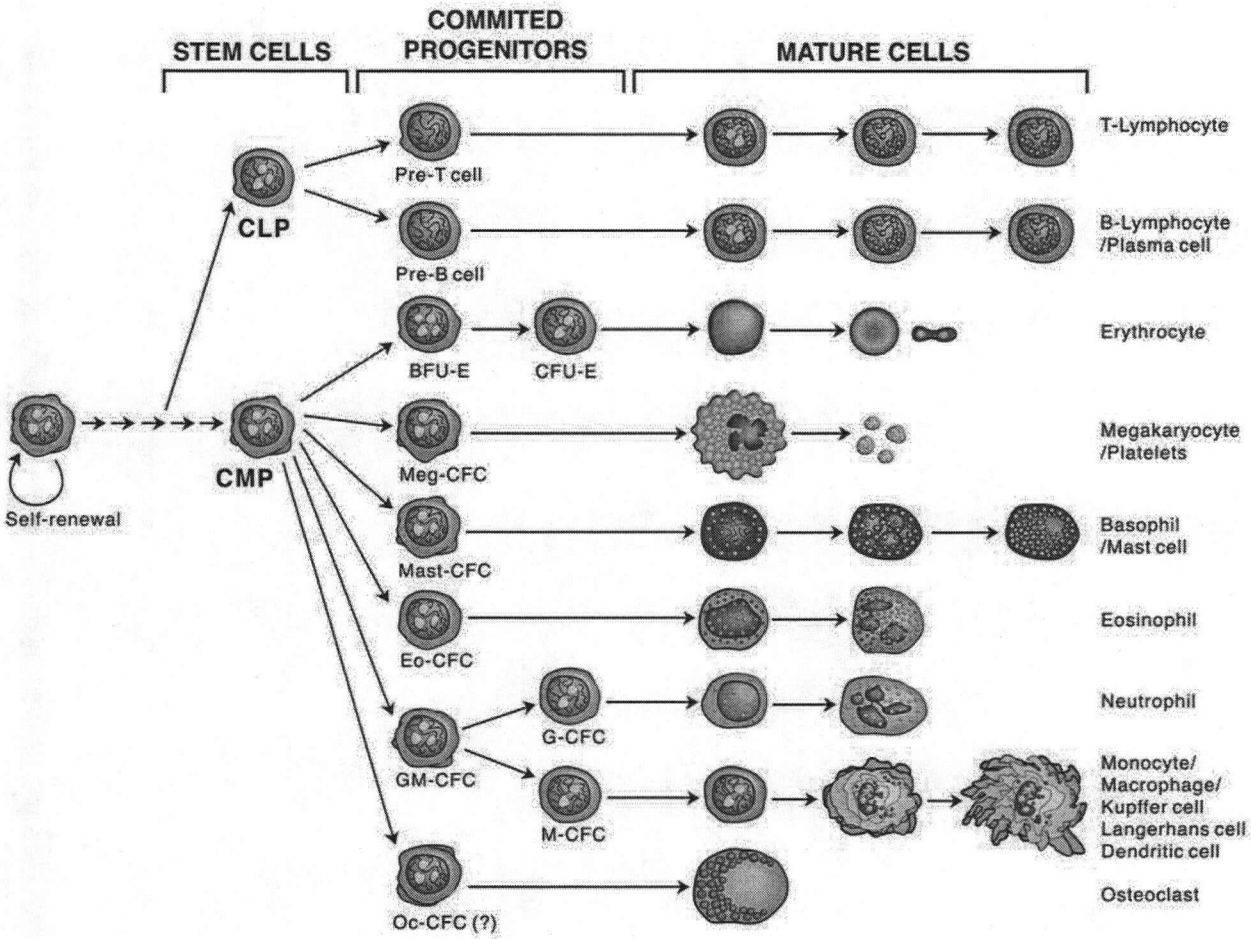


Figure 1.10. Normal human hematopoiesis. Hematopoietic stem cells give rise to all the types of blood cells of the lymphoid lineage (B lymphocytes, T lymphocytes, natural killer cells) and the myeloid lineage (red blood cells neutrophils, basophils, eosinophils, monocytes, macrophages, and platelets) via more commited progenitors. Reproduced from Metcalf D, *Blood Lines*, 2005. **www.bloodlines.stemcells.com**.

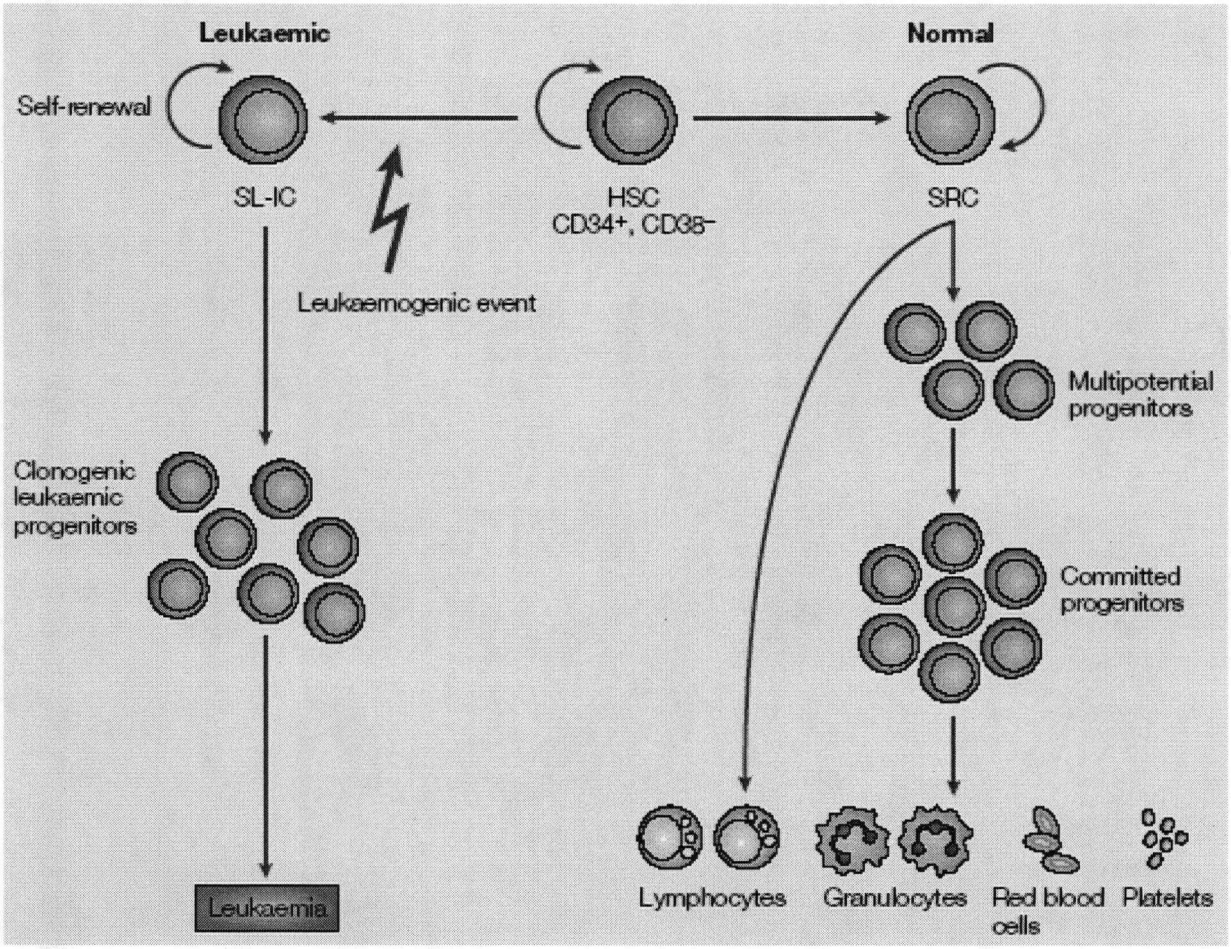


Figure 1.11. AML forms a stem cell hierarchy. Leukemia cells are believed to be mainly derived from transformed CD34++/CD38- hematopoietic stem cells (HSC) and share common surface markers with the HSC. The HSC is capable of self-renewal and production of the normal myeloid and lymphoid lineages through a series of progenitors (right). Similarly, the leukemic stem cell (LSC) is responsible for producing the leukemic progenitors and non-clonogenic blast cells which form the bulk of the leukemia (left). Reproduced from Huntly & Gilliland et al., *Nature Reviews/Cancer*, 2005[2].

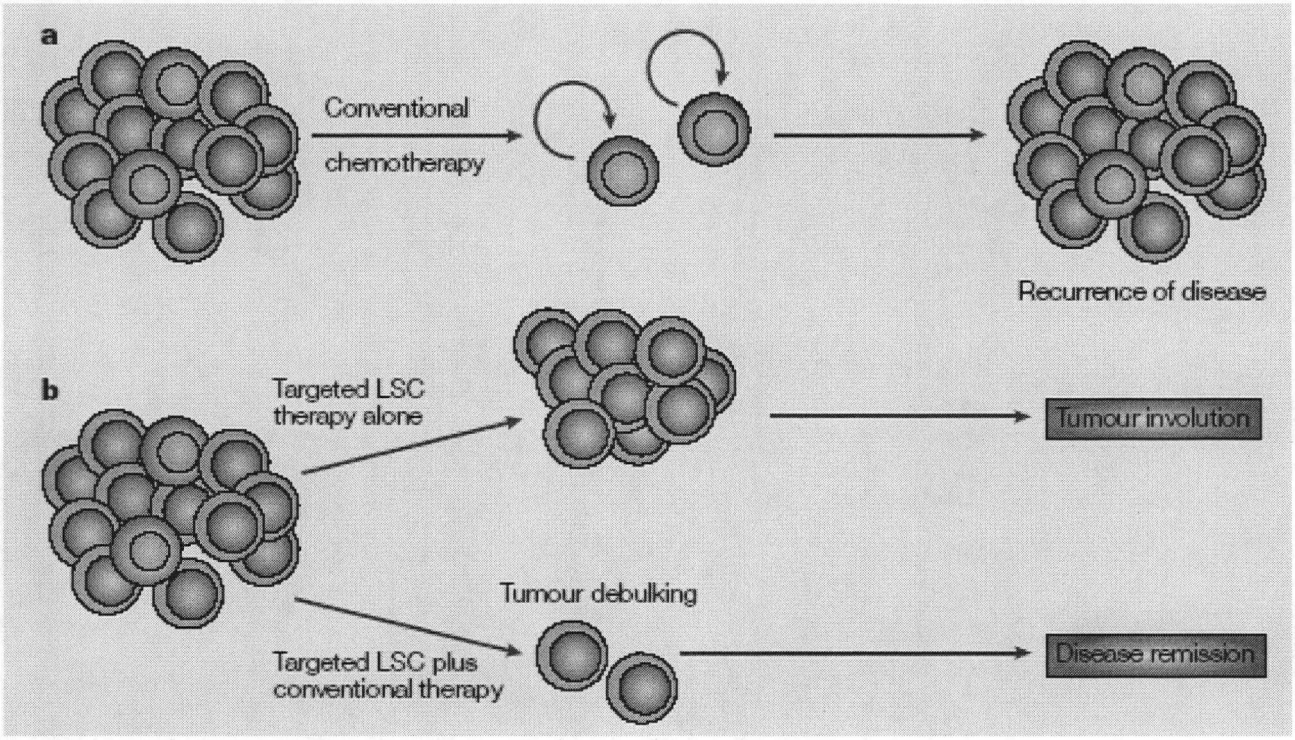


Figure 1.12. Targeting leukemic stem cells as a curative therapy. a, Current treatment focuses on the eradication of all leukemia cells (grey) and alleviation of symptoms, but is ineffective for long-term remission since remaining leukemic stem cells (LSCs, green) are capable of repopulating the leukemia. b, Specific targeting of these LSCs, with or without combination of conventional chemotherapy, can allow effective cure of the disease. Reproduced from Huntly & Gilliland, *Nature Reviews/Cancer*, 2005[2].

II mRNA expression profiling of the ABC transporter superfamily in unfractionated AML patient samples

2.1 – Introduction

ABC transporters are an important factor in cancer MDR (see Section 1.5), a major problem in AML treatment. Because neither radiation therapy nor surgery is applicable to leukemia, and few patients are eligible for allogeneic transplants, most AML patients rely on high-dose chemotherapy to overcome their disease. Due to presentation of MDR during initial treatment or at relapse; however, only 20-30% patients will ultimately achieve long-term remission. There is an urgent need to investigate the role of drug resistance factors in AML in order to circumvent MDR. Previous studies of ABC transporters on AML have largely focused on the three known MDR-related transporters – PGP, MRP1, and BCRP1, with mixed results. With the identification of more novel, uncharacterized ABC transporters (up to 48), it is possible that some of these promiscuous transporters also play a role in drug resistance. A systematic study of the expression of the whole ABC superfamily is required to determine how many of these transporters might contribute to MDR in AML patients.

Classic detection of MDR-related ABCs has mainly relied on use of monoclonal antibodies to measure protein levels by immunocytochemistry or flow cytometry, typically defining ABC "positivity" by arbitrary thresholds. But because antibodies are not available for many of the more recently identified ABC transporters, protein measurement was not suitable for a systematic study. As well, an international, multi-centered workshop organized by William Beck's group in 1996[142] identified the variability in measurements by these methods as a major

impediment to reaching consensual conclusions on the role of PGP in AML. This inconsistency was most apparent when measuring low levels of ABC transporters in clinical samples, calling for an improvement in the methods used for their detection.

Since then, advances in technology have allowed simultaneous, sensitive detection of many genes with known sequences at the mRNA level. Data generated by this RT-Real Time PCR assay gives a profile that is semi-quantitative and represents expression as a continuous variable. In this chapter, I tailored and utilized the RT-Real Time PCR assay for semi-quantification of mRNA expression of the ABC superfamily in AML samples. Because ABC transporters are expressed at relatively low levels, past results by old methods were likely obscured by the limitation of low sensitivity. Since 20%-30% patients fail to respond to initial therapy, intrinsic resistance mechanisms such as ABC transporter overexpression may already be in place at diagnosis in these patients. I therefore hypothesized that intrinsic mRNA expression of ABC transporters might be predictive of chemotherapeutic response. To test this, expression profiles were generated on samples of leukemic cells taken from AML patients at diagnosis and the results between patients that responded or not to treatment were then compared retrospectively.

2.2 – Materials and Methods

2.2.1 – Patient samples, cell lines and culture

CCRF-CEM and CEM/VLB human acute lymphoblastic leukemia cell lines established in our laboratory[143,144] were grown in Alpha MEM with 10% FBS (GIBCO Invitrogen, Burlington, Ontario, Canada). An additional 1.0 μg/ml vinblastine (Sigma-Aldrich, Oakville, Ontario, Canada) was supplemented to CEM/VLB to maintain drug resistance.

Peripheral blood (PB) cells were obtained from 31 patients with newly diagnosed AML, after an informed consent and with the approval of the Clinical Research Ethics Board of the University of British Columbia. Diagnosis and classification of AML were based on the criteria of the FAB group. Cytogenetic analysis was performed on the bone marrow at initial diagnosis. Mononuclear cells from AML blood samples were isolated by Ficoll Hypaque density gradient centrifugation (Pharmacia, Uppsala Sweden) and cryopreserved in Iscove's modified Dulbecco's medium (IMDM) with 50% FBS (both from StemCell Technologies, Vancouver, Canada) and 10% dimethylsulfoxide. More than 90% of the cells in AML samples were leukemic blasts. Thawed cells were washed twice in IMDM containing 10% FBS before use in the experiments described below. RNA was extracted immediately from steady-state PB samples.

All patients selected for this study received remission induction therapy consisting of daunorubicin (45 mg/m^2 daily for 3 days) and cytarabine (100 mg/m^2 IVq12h x 7 days for patients $\geq$ 60 years old or 1.5 g/m^{2}IVq12h x 6 days for patients < 60 years old). Patients who entered a complete morphological remission with this therapy constitute the complete remission group (CR; responders) in this study. Patients who failed to achieve remission with this initial chemotherapy plus one additional cycle of treatment (either a second course of the same regimen

or an alternate regimen usually containing cyclophosphamide and etoposide[145]) constitute the non-responding group (NR; non-responders). Complete remission (CR) was defined as less than 5% blasts in a normocellular bone marrow with a neutrophil count >1.0 x 10^9/L, and an unsupported hemoglobin of >100 gm/L and platelet count >100 x 10^9/L. CR patients received consolidation therapy consisting of either two cycles of additional chemotherapy the same as induction treatment or allogeneic transplantation (for patients <50-60 years of age with a suitable sibling donor and intermediate risk or a suitable sibling or unrelated donor and high risk cytogenetics as defined by the MRC (UK) criteria[6]).

2.2.2 – RT-Real Time PCR assay

2.2.2.1. Overview. A reverse transcription (RT) -Real Time polymerase chain reaction (PCR) assay was developed and utilized to detect the relative expression levels of ABC transporters in AML patient samples. This methodology was preferred over expression array chips for its high sensitivity. This proved crucial since many ABC transporters were expressed at low levels. A flow-chart of the assay was shown in Figure 2.1. Total RNA was first extracted from frozen patient samples and DNase was used to eliminate contaminating genomic DNA. RNA was then reverse-transcribed into cDNA and an aliquot of the same RT reaction was used for individual Real Time PCR reactions for each gene. The resulting Ct value (see below) from each PCR was used to calculate the relative expression of each gene of interest.

The amount of PCR product was detected and measured by the fluorescence intensity caused by the binding of the fluorescent dye SYBR Green to DNA. The threshold cycle, Ct, was defined as the cycle when fluorescence first becomes detectable above the threshold value. The Ct is inversely proportional to the amount of starting RNA transcript and is used to calculate the

relative expression level of the gene. To correct for differences in amplification efficiency (AE) between primer pairs, a standard curve was constructed for each gene. A housekeeping gene (*GAPDH*) was amplified from the same sample as an endogenous control to account for variability in concentration and quality of total RNA, and in the RT reaction efficiency.

2.2.2.2. – RNA isolation, DNase treatment and Reverse Transcription. Total RNA was isolated using Trizol for AML PB samples (Invitrogen) and quantified by measuring its absorbance at 260/280 nm on a U-2000 spectrophotometer (Hitachi, Tokyo, Japan). Total RNA was treated with DNase I (Invitrogen) following manufacturer's instructions and subsequently reverse-transcribed using random hexamers and the Superscript II RT enzyme (Invitrogen) at a concentration of 1 μg total RNA per 20 μl reaction.

2.2.2.3. Primer design and optimization. Primers for Real-Time PCR for all 47 ABC transporter genes were designed using PrimerExpress software, Version 2.0 (Applied Biosystems, Streetsville, Ontario, Canada). All primers were designed to yield a unique gene-specific product that does not overlap with consensus ABC walker sequences. Parameters used for design included 100 bp amplicon size, 19-22 bp primer size, 40-60 % GC content and 80-90 °C melting temperature. The validity of the primers was tested by conventional PCR and products were analyzed by agarose gel electrophoresis to ensure they gave a single product of the correct size.

2.2.2.4. Real-Time PCR. Real-Time PCR was performed with SYBR Green Real-Time Core Reagents (Applied Biosystems) according to manufacturer's instructions on the ABI Prism 7900 Sequence Detection System (Applied Biosystems). Each 15 μl PCR reaction contained 1.5 μl diluted cDNA (24 ng starting total RNA). Thermal cycling conditions were 50°C for 2 min and 95°C for 5 min, followed by 40 cycles of 15 sec at 95°C, 30 sec at 58°C and 30 sec at 72°C. An

additional cycle – 15 sec at 95°C, 15 sec at 60°C and 15 sec at 95°C - was performed at the end of the reaction to generate the dissociation curve of the amplicon to ensure a single, specific product with the corresponding melting temperature was produced (Figure 2.2). A negative RT control without the RT enzyme was included for each sample total RNA to ensure no reminiscent genomic DNA was amplified in the PCR reaction, and a negative PCR water control without cDNA was included per PCR reaction plate to check for reagent contamination.

2.2.2.5. Generation of standard curves. To determine the AE, a standard curve was constructed for each gene on a 2x cDNA dilution series equivalent from 30 ng to 0.47 ng of starting total RNA (Figure 2.3). The AE was calculated from the formula: $10^{1/M} - 1$, where M = the slope of the standard curve.

2.2.2.6. Data analysis. The Sequence Detector Software SDS 2.0 (Applied Biosystems) was used for data analysis. The threshold cycle value (Ct), defined as the cycle at which a statistically significant increase in SYBR fluorescence (normalized to a passive reference Rox) is first detected, is automatically calculated and reported by SDS 2.0 for each reaction. Ct is inversely proportional to the log of cDNA. To determine the fold expression of a gene relative to the housekeeping gene, we used the formula: $(1 + AE)^{-dCt}$ where AE is the amplification efficiency of the specific gene and dCt = (Ct of the gene) – (Ct of the housekeeping gene).

2.2.2.7. Statistical analysis. Two statistical tests, the student's t test and the permutation test, were performed to assess expression differences between the CR and NR patient groups. A two tail-distribution, homoscedastic (assume two sample groups with equal variance) t test was utilized to compare the means of the patient groups. The permutation test is a randomization test which requires no assumptions about statistical distributions (random-sampling, equal variance). Statistical significance was set at $p \leq 0.05$ for the t test and $Z \geq 2$ for the permutation test.

2.3 – Results

2.3.1 – Profiling of ABC transporters in the drug-sensitive leukemic cell line CEM and its vinblastine-selected, drug-resistant subline CV1.0

I first profiled all 47 human ABC transporters in the lymphoblastic leukemic cell lines CCRF-CEM and CEM/VLB (although 48 human ABCs were predicted, sequences were available for only 47). These were chosen for comparison since the CCRF-CEM parental line is drug-sensitive while the vinblastine-selected CEM/VLB is multidrug resistant (at least 500-fold more resistant to vinblastine and 150-fold more resistant to doxorubicin than CCRF-CEM)[143,144]. As described in the Materials and Methods Section, Ct values generated from the amplification plots were used to calculate the level of expression of each test gene relative to that of *GAPDH* (set at 10^6). Bustin and colleagues reported the transcript copy number per cell of *GAPDH* to be in the order of 2×10^3 to 5×10^3 in PB[146,147]. In addition, I found Ct values above 35 to be generally unreliable because it approaches the machine detection limit (40 = undetectable). Under the conditions used, this translates to approximately 5×10^3-fold fewer transcripts than *GAPDH*. I therefore set the tentative "biologically relevant" expression level at $1/10^3$ of *GAPDH* (discussed in Section 2.4), which I estimated to correspond to ~2-5 transcripts per cell. Similar to previous reports, *MDR1* mRNA was upregulated by at least 2×10^3-fold in CEM/VLB cells due to gene amplification as compared to CEM (Figure 2.4)[148]. The drug-sensitive parental CCRF-CEM cells expressed very low level of *MDR1* (almost 10-fold lower than the ~2 copy per cell reference line). There is no significant difference in *MRP1* expression and *BCRP1* levels are below the level of detection. A number of genes in the *ABCA* subfamily, notably *ABCA5, 6, 9, 10*, appear to be also upregulated in the drug-selected cell line. As these are all clustered on

17q24, this chromosomal segment is likely to be amplified independently of *MDR1* located on 7q21 during drug selection. Amplification of this cluster of genes in drug resistance warrants further investigation as the function of these ABC transporters are not well known.

2.3.2 – Lack of consistent differences was observed in ABC transporter expression between responsive and non-responsive patients

Table 2.1 lists the characteristics of 31 AML patients selected for expression profiling. Samples were chosen to represent patients differing in clinical response to induction chemotherapy with the combination of cytosine arabinoside and daunorubicin. Of the 31 samples, 18 were from patients who achieved CR after initial therapy and 13 were from the NR group (patients who did not achieve remission). 25 of the 31 patients had only normal karyotypes seen in the diagnostic bone marrow sample.

In an initial set of experiments, amplification profiles were generated for all 47 ABC transporters from PB samples of 12 of the CR patients, and 6 NR patients. Figure 2.5 shows representative expression profiles (CR, Patient #7 and NR, Patient #9). Transcripts for over 40 ABC transporters were detectable in these AML samples. These included the MDR-related transporters *MDR1*, *MRP1* and *BCRP1*, as well as ABC transporters that have been previously reported to be restricted to other tissues, such as *MDR3* (*ABCB4*) that transports phosphatidylcholine in the liver, *SPGP*(*ABCB11*) that exports bile salts in the liver, *CFTR* (*ABCC7*) the chloride ion channel in the lung, *WHITE3* (*ABCG5*) and *WHITE4* (*ABCG8*) which transport sterols in the liver and the intestine. However, expression levels of ABC transporters were generally low (at least 10-fold lower than *GAPDH*), with many below the reference line (2 copies per cell). This may represent differential expression among subpopulations of cells. As

well, there was a significant variation (10 to 100-fold) among these 18 AML samples in the mRNA levels of each ABC transporter detected. *MDR1* expression in patients generally fell between that of CCRF-CEM and CEM/VLB.

To evaluate the difference in expression between the CR and NR patients for every gene, the data set was tested independently using both a t test and a permutation test. Both statistical tests indicated no significant difference ($p>0.05$ for the t test and $Z<2$ for the permutation test) in any ABC transporter between the CR and NR groups, implying that mRNA levels of ABC transporters in the bulk population prior to treatment is not predictive of drug response. We also compared expression levels between AML samples from the NR group and a CR subgroup of 6 patients (#24, #30 to #34) who achieved long-term remission for over 3 years - CR(L). But, again, no statistically significant difference ($p>0.05$ for the t test and $Z<2$ for the permutation test) was found for any of the genes examined.

Expression of a selected subset of 9 ABC transporters including the *MDR*-related ABC transporters *MDR1, MRP1* and *BCRP1*, as well as *ABCA2, ABCA3, ABCB9, SPGP, MRP4*, and *WHITE1* was evaluated in an expanded population of 31 AML samples which included 18 CR and 13 NR patients. These genes were selected because they were either MDR-related or closely related to the MDR-transporter genes. Expression profiles of these genes are shown in Figure 2.6. Again, for each ABC transporter, both groups covered a wide expression range that overlapped with each other. Statistical analysis also confirmed that there was no consistent difference in mRNA expression between the two groups for these ABC transporters. As apparent in the overlapping expression ranges, some CR patients actually show the highest expression of MDR-related transporters among all patients, while some NR patients have very low expression of these transporters.

2.4 – Discussion

In this section, I used the sensitive RT-Real Time PCR technique to quantify and compare expression of the ABC transporter superfamily in various leukemic cell populations of defined drug responsiveness. Testing on the parental CEM cell line and its drug-selected sub-line validated the ability of this approach to detect differences in ABC expression levels. Consistent with prior reports, dramatic upregulation of the *MDR1* gene was observed in the drug-resistant CV1.0 cell line. Interestingly, a number of other ABC transporters were also elevated (the *ABCA* subfamily), most of which are poorly characterized. Whether or not these are inducible by drugs or related to drug resistance are not known and warrant further investigation.

In this initial study I constructed the mRNA expression profiles of the ABC transporter superfamily in patient blast cells of 90% purity. Because ABC transporters are a major contributor to MDR in other cancers, I asked whether this would also be the case in AML. However, I observed no consistent, statistically significant difference between the CR and NR patients' cells in all ABC transporters examined. Hence my results suggest that expression levels of ABC transporters in the total blast population are not a useful predictor of response to initial chemotherapy.

There are several interpretations for this apparent discordance with older studies relating ABC expression to a poor prognosis)[4,7,90,91,100-102,104,105]. The first may be purely technical since differences in laboratory techniques and experimental conditions are known to produce variable results that confounding comparisons. In particular, old studies commonly measured the protein levels of ABC transporters, setting somewhat arbitrary levels of "positivity". Levels of ABCs, however, are likely a continuous variable. Thus my data likely give a more accurate depiction of

the expression profiles. On the other hand, mRNA expression levels may not translate to corresponding protein levels and drug efflux activity of the ABC transporters. Third, ABC transporters may simply not contribute significantly towards resistance to initial chemotherapy via high intrinsic levels, as suggested by the very low levels of expression detected. Instead, ABC transporters may play a role in AML-induced resistance via rapid and dramatic upregulation of expression under the stress of drug exposure. In support of this, acute induction of *MDR1* expression after exposure to doxorubicin[149] and carcinogens[150] has been reported.

Another plausible explanation, in line with the existing paradigm of cancer heterogeneity, is that the difference in expression lies not in the heterogeneous bulk sample, but in subpopulations of cells. This is supported by the very low expression levels (below reference line - less than one copy per cell) observed in a significant number of transporters in many patients. Possibly, a small subpopulation expressed a much higher, biologically-relevant level that is "diluted" in the average profile for the total population. This could also explain why some CR patients express relatively high levels of known MDR-related transporters and some NR patients express relatively low levels. Perhaps it is expression in the "relevant" cell fraction that accounts for differences in drug response. In order to obtain a more comprehensive picture on the subject, an examination of sorted subpopulations could be useful.

Table 2.1. Patient characteristics.

Patient no.	Gender	Age at diagnosis	FAB	Cytogenetics	Treatment Response
1	M	36	M4eo	Inv(16)(p13;q22),+22	CR
2	M	39	M5B	Normal	CR
4	F	17	M5A	+8,del(5)(q31;q33)	NR
7	M	54	M5A	Normal	CR
8	M	36	M2	Inv(3)(q21;q26)	NR
9	M	18	M5A	Normal	NR
10	F	63	M4	Normal	NR
11	M	68	M1	+11	NR
12	F	25	M1	Normal	NR
13	M	69	M1	Normal	NR
14	F	68	M4	Normal	NR
15	F	46	M2	Normal	NR
16	F	25	M2	Normal	NR
17	F	49	M5	Normal	NR
18	M	57	M2	Normal	NR
19	F	28	M4	Normal	NR
20	M	51	M1	Normal	CR
21	M	41	M5B	Normal	CR
22	M	46	M4	Normal	CR
23	M	36	M2	Normal	CR
24	M	69	M6	Normal	CR(L)
25	F	39	M1	Normal	CR
26	M	69	M3	Normal	CR
27	F	66	M4	Normal	CR
28	M	27	M4	Normal	CR
29	F	47	M4	Normal	CR
30	M	22	M4	Normal	CR(L)
31	M	25	M2	+4, t(8;21)(q22;q22)	CR(L)
32	M	64	M2	Normal	CR(L)
33	F	62	M2	Normal	CR(L)
34	M	50	M4eo	inv(16)(p13;q22), +22	CR(L)

CR indicates complete remission achieved after induction chemotherapy; CR(L), continuous remission >3years; NR, no response to induction therapy.

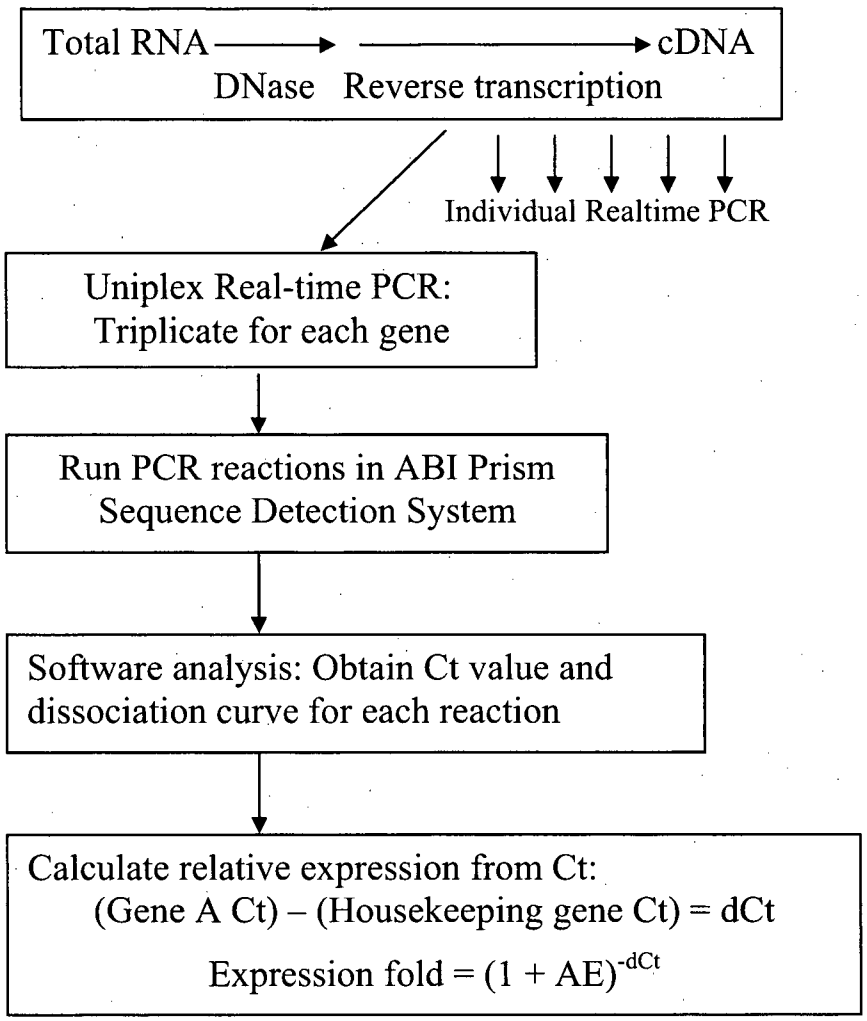


Figure 2.1. Flow-chart of RT-Real Time-PCR. Total RNA isolated from patient peripheral blood was first DNased and reverse-transcribed. The cDNA was subsequently used for uniplex Real Time PCR for each gene. PCR reactions were prepared and aliquoted into a 384-well plate and run in the ABI Prism detection system, generating a Ct value per reaction. Expression of gene A was normalized to the housekeeping gene (*GAPDH*) by subtracting the housekeeping Ct value from Ct of gene A. The resulting dCt value and the amplification efficiency (AE, see below) were used to calculate the relative expression, expressed as a fold difference to the housekeeping gene.

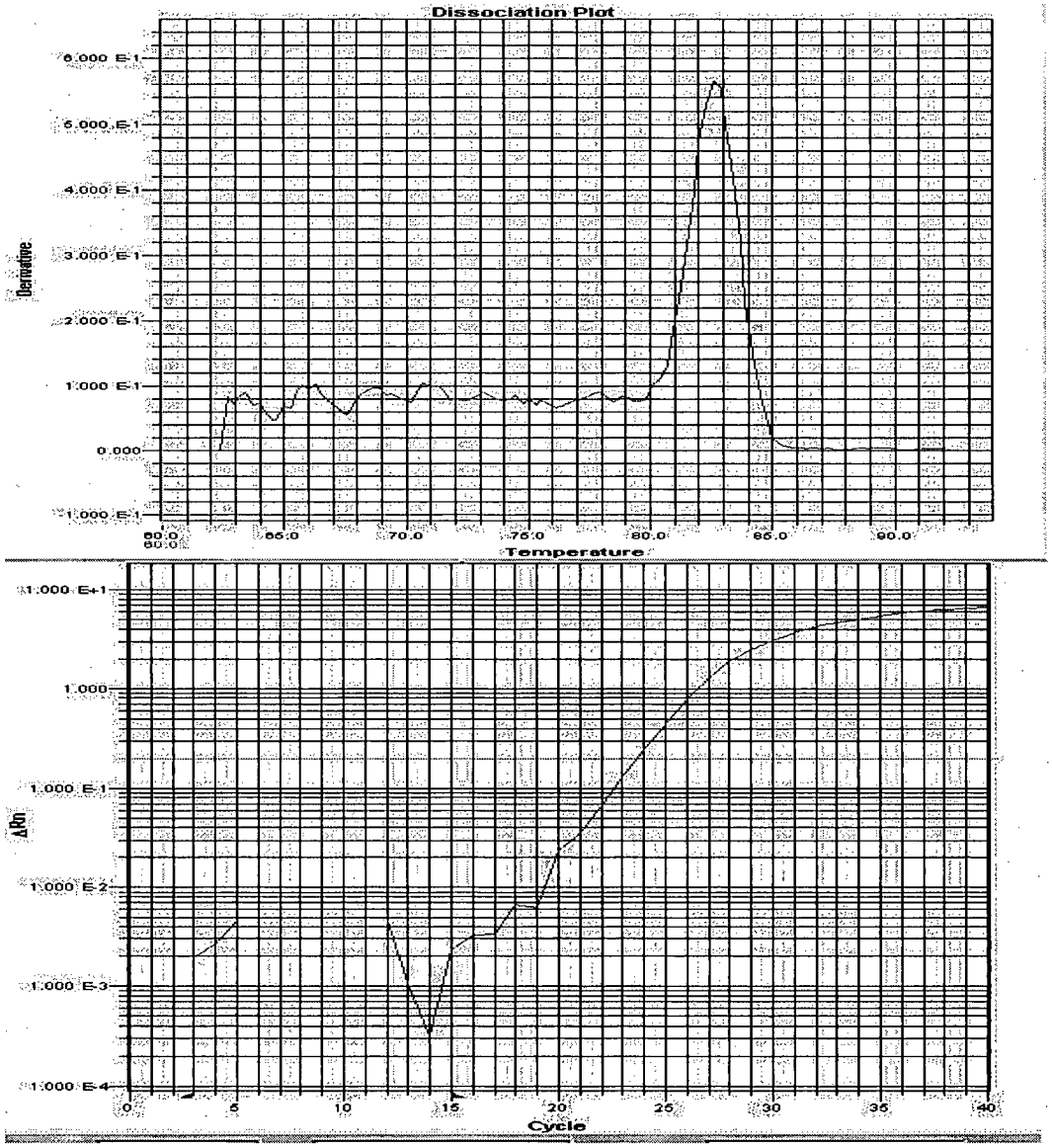


Figure 2.2. Typical dissociation curve and amplification plot of a Real Time PCR product. A dissociation curve (top) was generated for the amplified product of the housekeeping gene *GapDH* in an AML sample at the end of a Real Time PCR cycle. The melting temperature (Tm), 83 °C, corresponds to that of the expected *GapDH* amplicon from the primer design. The threshold cycle (Ct) of the product is 24 as observed in the amplification plot (bottom), with the red horizontal line indicating the threshold intensity.

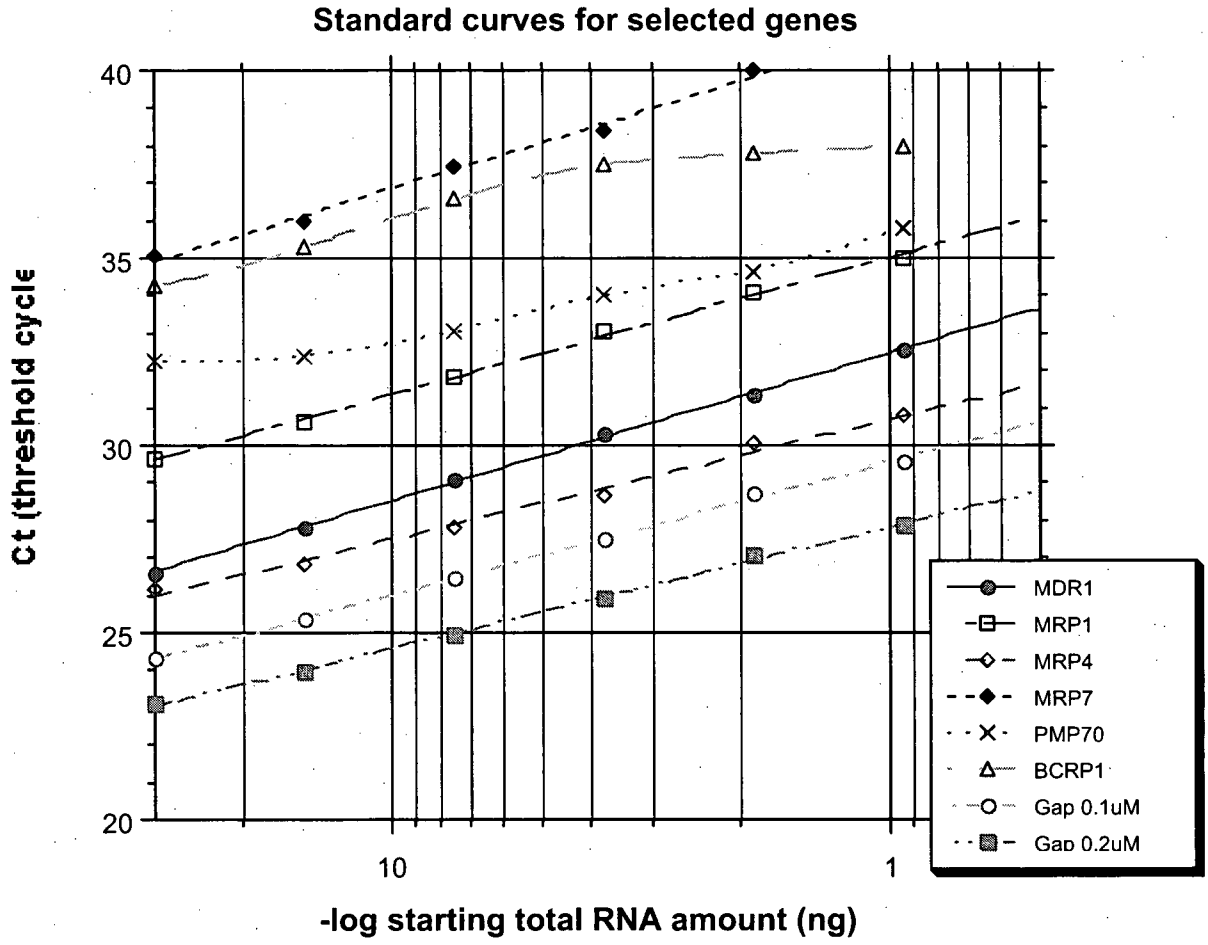


Figure 2.3. Standard curves for selected genes. Ct values of real time PCR reactions were plotted against the amount of starting total RNA. For details, see above.

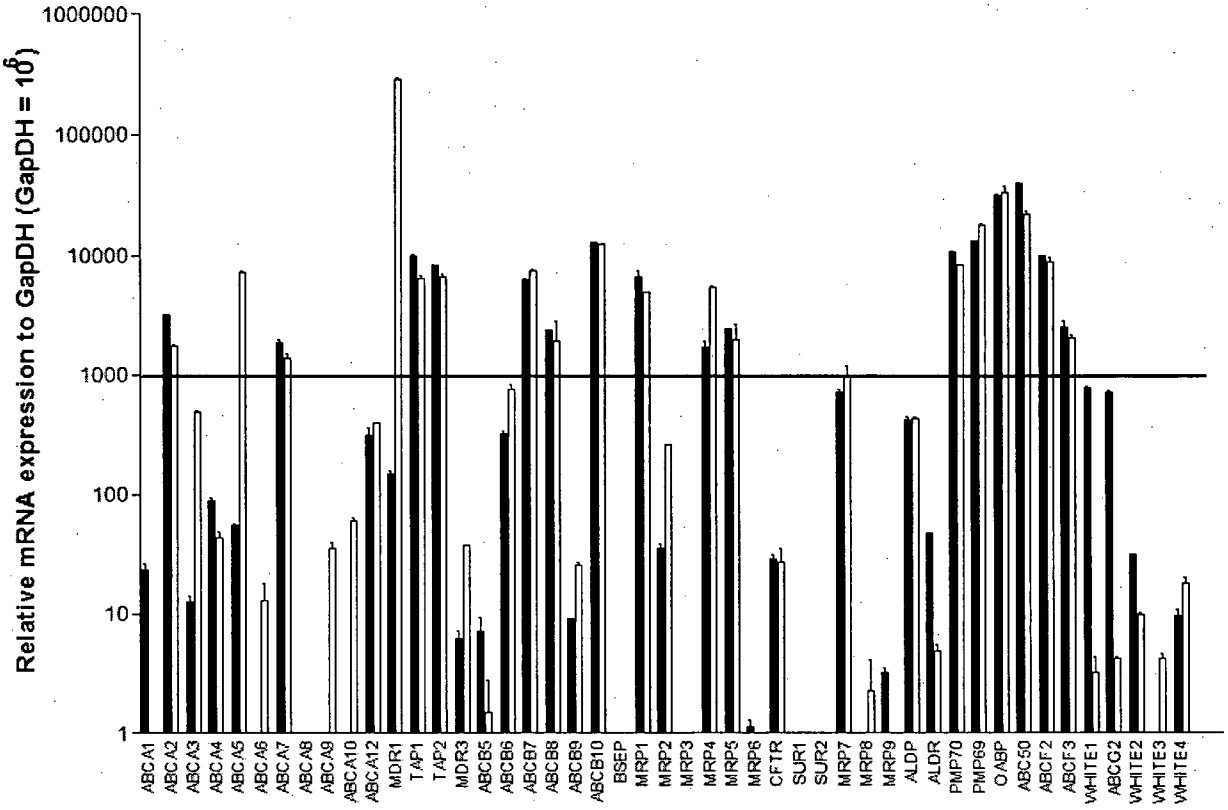


Figure 2.4. Profiling of the ABC transporter superfamily in CEM and CV1.0. mRNA expression levels normalized to *GapDH* (set at 10^6) of 47 known ABC transporters in cell lines CEM (black bar) and CV1.0 (white bar). Solid horizontal line indicates the biologically-relevant reference line.

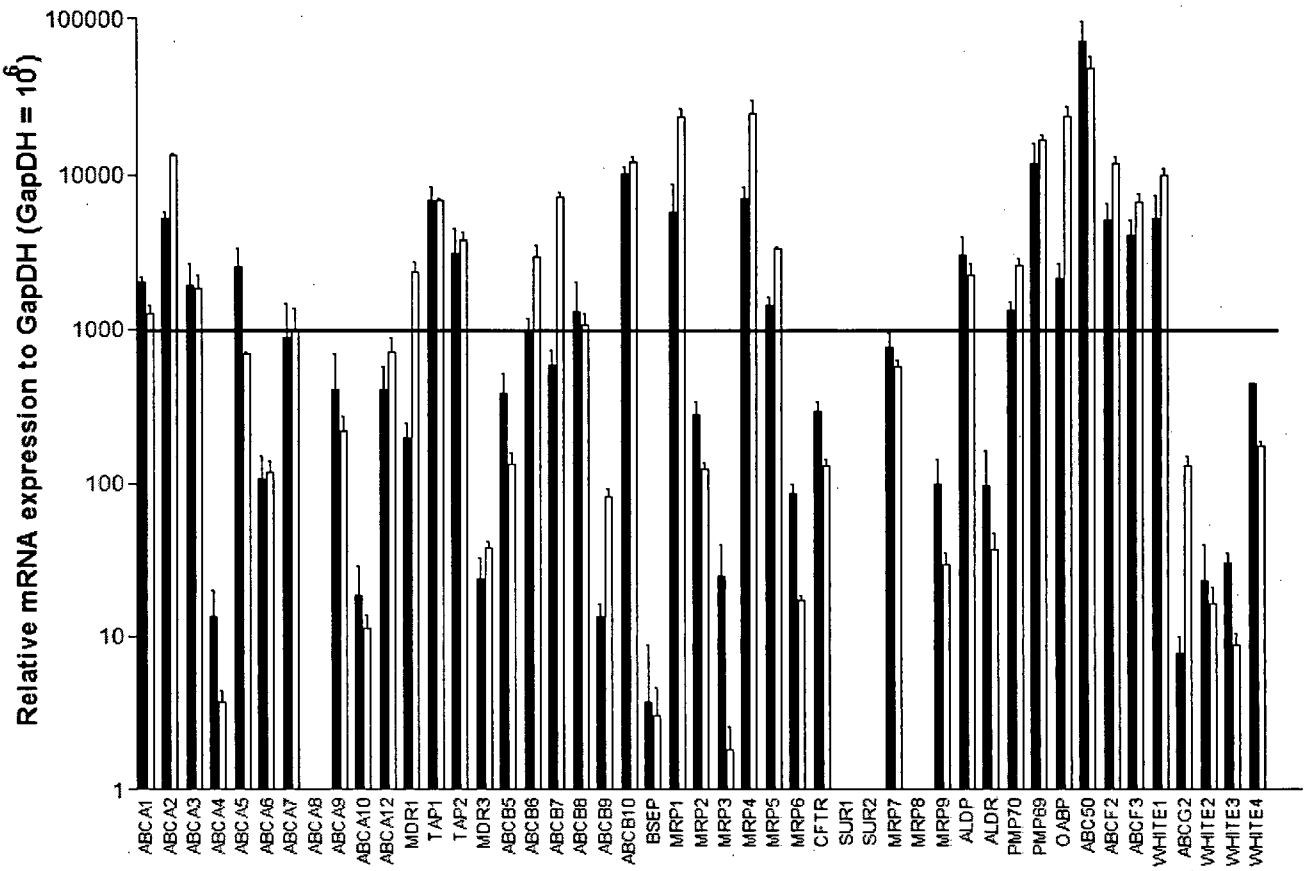


Figure 2.5. Profiling of the ABC transporter superfamily in patients CR#7 and NR#9. mRNA expression levels normalized to *GapDH* (set at 10^6) of 47 known ABC transporters in cell lines CR#7 (black bar) and NR#9 (white bar). Solid horizontal line indicates the biologically-relevant reference line.

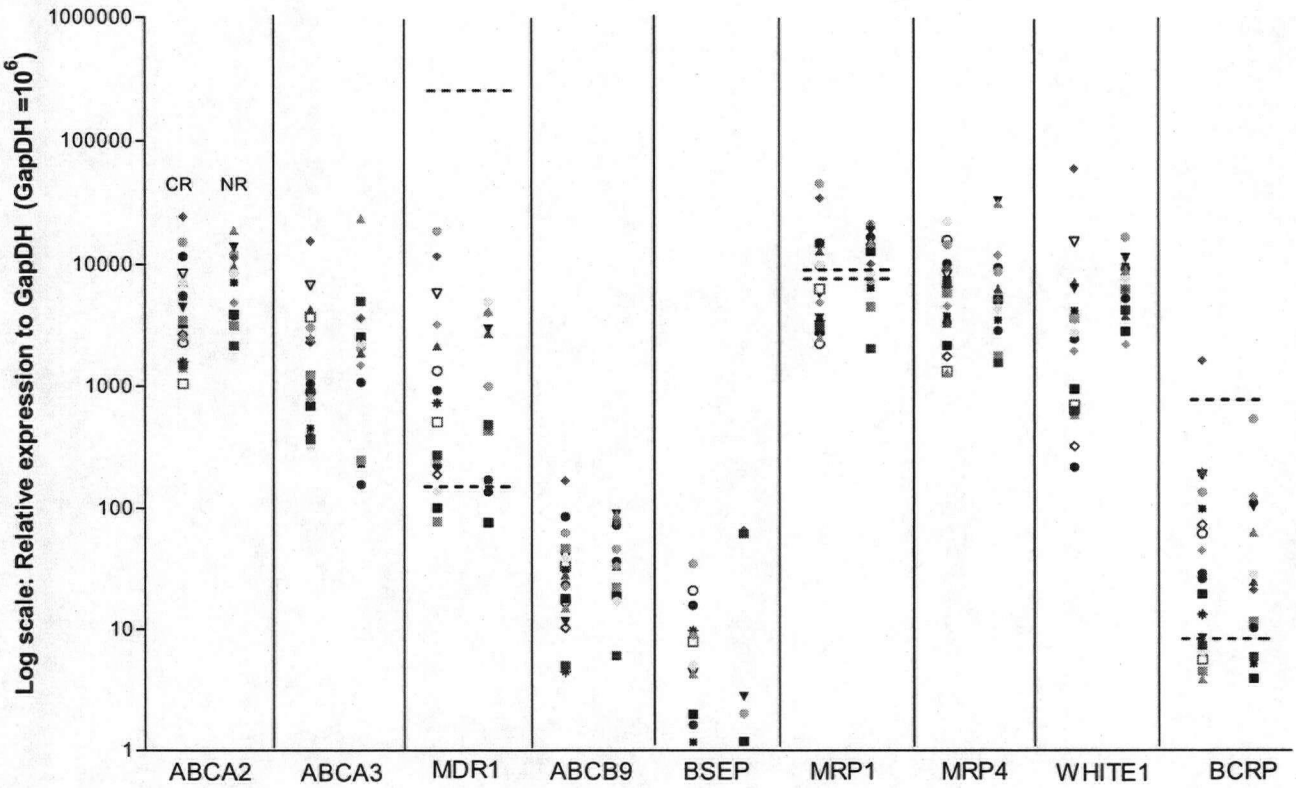


Figure 2.6. mRNA levels of selected ABCs in unfractionated AML patient samples. ABC transporters were profiled in cells from 18 CR patients, and 13 NR patients. For each column (gene), each point represents a single patient from 3 groups (left: remission, right: refractory). Dotted lines indicate expression levels in cell lines (Blue: CEM, Red: CV1.0)

III Expression profiling of drug resistance-related transporters in FACS-sorted AML subpopulations

3.1 – Introduction

Results from Chapter 2 raised the question of whether ABC transporters are differentially expressed in different subpopulations of leukemic cells. Based on the LSC model, high ABC expression in the fraction enriched in leukemia-initiating $CD34^{+}CD38^{-}$ cells, would be expected to be the most critical to explain initial AML treatment failure. As discussed in Chapters 1.6 and 1.7, this fraction is chiefly responsible for initiation and maintenance of the whole leukemic population, and its frequency has been implicated to be an independent prognostic marker in AML. Many studies have been conducted to examine the prognostic value of CD34 expression in AML (typically categorizing patients into CD34-positive and CD34-negative for comparison of clinical outcome), albeit with mixed results (reviewed by Kanda et al in 2000[151]). Although older studies frequently report an association between CD34 "positivity" and lower remission rates[152-157], more recent studies found no such correlation[158-161].

CD34+CD38- leukemic cells display self-renewal and differentiating properties that are reminiscent of the function of normal HSC. It is, therefore, also likely that these cells would show higher drug tolerance, another characteristic of normal HSC. Recent studies by de Grouw et al[162] and Peeters et al[163] have demonstrated preferential expression of ABC transporters in both normal and leukemic CD34+CD38- cells. Since CD34+CD38- cells that express *MDR*-related ABCs will have a survival advantage under cytotoxic stress, the presence of these transporters in this fraction of cells may predict chemotherapeutic failure. This may in part explain the mixed results on the prognostic value of CD34 expression[151] – it may be ABC

"positivity" of the CD34 positive leukemic cells, rather than CD34 "positivity" among total AML blasts, that is prognostically significant.

In this section, I profiled the key MDR-related ABC transporters – *MDR1, MRP1*, and *BCRP1* - in different AML subpopulations. My working hypothesis was that intrinsic ABC expression in the "relevant" subpopulation, specifically the CD34+CD38- fraction, might be more predictive of initial treatment response than the bulk cells, and might possibly be an even more useful prognostic factor than the number of these primitive leukemic cells present.

3.2 – Materials and Methods

3.2.1 – Flow cytometric sorting of AML subpopulations

Frozen AML patient cells were thawed at 37 °C and counted in 1:1 trypan blue for viability. A small aliquot was reserved as the unsorted population and the remaining was centrifuged for 9 min at 1000 rpm in 10 ml Iscove's medium (Invitrogen) + 20 % FBS (Invitrogen). Cells were resuspended in HFN (Hank's Balanced Salt Solution + 2% FBS + 0.05% NaN_3) + 5% human serum at a concentration of 7 x 10^4 cells per µl. For each 2 x 10^7 cells, 12 µl of each of the following antibodies were added: CD3-FITC, CD19-FITC, CD38-PE, CD34-Cy5 (APC-A). After incubation in the dark for 30 min on ice, 1 ml HFN was added to each tube and centrifuged for 7 min 1000 rpm. Cells were resuspended in 1 ml HFN with 2µg/ml PI, re-centrifuged and resuspended in 1.5 ml HFN. FACS-sorting was performed on a FACSAria flow cytometer (Becton Dickinson). PI negative cells were first gated as the viable fraction. CD34+CD38-, CD34+CD38+, and CD34- cells were gated within the viable CD3-, CD19- fraction. The following controls were included: IGG1-FITC only, IGG1-PE only (non-specific staining), CD3-FITC only, CD19-FITC only, CD38-PE only, CD34-Cy5 only (for compensation). A sample FACS sort is shown in Figure 3.1.

3.2.2 – RNA isolation, DNase treatment and RT-Real Time-PCR

Sorted AML subpopulation cells were centrifuged at 1200 rpm for 5 min. Lysis buffer was added to the cell pellet immediately after centrifugation and stored at -80 °C until RNA isolation. Total RNA was isolated using the RNeasy Micro spin column kit (Invitrogen) according to manufacturer's instructions. No quantification was performed due to limitation of

material, and all total RNA isolated was directly treated with DNase I (Invitrogen), reverse-transcribed and subsequently used for Real Time PCR using a similar protocol as in Chapter 2.2.2.4 scaled down for smaller amounts of RNA. A two-tailed, homoscedastic student's t test was utilized to evaluate statistical difference between the NR and CR groups. Statistical significance was set at $p \leq 0.05$.

3.3 – Results

3.3.1 – Profiling of selected drug resistance-related transporters in FACS-sorted subpopulations of AML patient samples

Three fractions from each of 7 CR and 10 NR patients were sorted by FACS: CD34+CD38- (most primitive), CD34+CD38+ (differentiating progenitors), CD34- (depleted of progenitors). CD3-CD19- cells were first gated to exclude contaminating normal T and B lymphocytes. The three major MDR-related ABC transporters were profiled for each subpopulation: *MDR1* (Figure 3.2), *MRP1* (Figure 3.3), *BCRP1* (Figure 3.4). Variation in levels of each gene among subpopulations of the same patient is apparent, indicating heterogeneity within the cancer. Expression levels of *MDR1* and *MRP1* were frequently detected above the reference line. Detectable *BCRP1* levels were less frequent and fall below the reference. Significant expression for *MDR1* and *BCRP1* appeared to be restricted to the CD34- fraction for CR patients, while NR patients showed high levels also in the primitive subpopulations (see below). *MRP1* expression, on the other hand, was observed across all fractions.

3.3.2 – Higher expression of *MDR1* and *BCRP1* in the CD34+CD38- cells from non-responders

Figure 3.5 shows a scatter plot of expression levels in the CD34+CD38- fraction. For *MRP1*, there was significant overlap between the CR and NR groups and no significant difference was observed. *MDR1* and *BCRP1*, however, showed interesting patterns in the primitive fraction. All 7 CR patients expressed uniformly low levels of *MDR1* and *BCRP1* in the CD34+CD38- cells. In contrast, 5/10 NR patients (NR6, NR8, NR10, NR14, NR15, 50%) show significantly higher expression of *MDR1* (all of which are above the median), and 5/10 NR patients (NR6, NR8, NR9, NR15, NR18, 50%) have high expression of *BCRP1* (most of which are significantly

above the median). Differences for both genes reached statistical significance ($p<0.05$). In combination, 7/10 NR patients (70%) show high expression of *MDR1* and/or *BCRP1*. Interestingly, *MDR1/BCRP1* expression profiles of the NR group do not resemble a continuum. Rather, there seem to be a distinct segregation within the group, with patients' cells expressing high *MDR1/BCRP1* being well separated from the rest of the NR patients clustered with the CR group (especially for *BCRP1*). Of note, *MDR1/BCRP1* levels in CD34+CD38- cells are very low, especially *BCRP1* ($\geq 10^3$-fold lower than the housekeeping gene *GAPDH*). To test if the proportion of CD34+CD38- cells among AML blasts could predict treatment response, the CD34+CD38- fraction size was also compared between NR and CR patients (Figure 3.6). Contrary to old studies reporting the prognostic significance of CD34 expression[152-157], however, comparison of the two groups revealed no statistically significant difference (means of % CD34+CD38- for CR and NR = 2.9 and 12 respectively, $p>0.05$).

3.4 – Discussion

In this chapter, I profiled the MDR-related transporters in FACS-sorted subpopulations of CR and NR patients. Results demonstrate that, consistent with the concept of cancer heterogeneity, ABC transporters were differentially expressed across subpopulations along the leukemic hierarchy. This argues against the concept that expression levels are homogeneous across all cells in the leukemic clone. Indeed, profiles on subpopulations proved far more revealing on the potential underlying mechanisms of AML MDR.

No difference in *MRP1* expression between the two patient groups was observed, showing that *MRP1* is not a useful predictive factor of initial treatment outcome and likely not an important contributor to drug resistance in AML. On the other hand, expression of *MDR1* and/or *BCRP1* was elevated in CD34+CD38- cells of 7/10 NR patients compared to uniformly low levels in the CR group. Hence intrinsic high expression of either transporter in this primitive fraction enriched for leukemia-initiating cells appears predictive of poor response. Furthermore, *MDR1/BCRP1* mRNA levels in CD34+CD38- cells were more predictive of response than the size of the CD34+CD38- fraction itself, which showed no significant difference between the CR and NR groups.

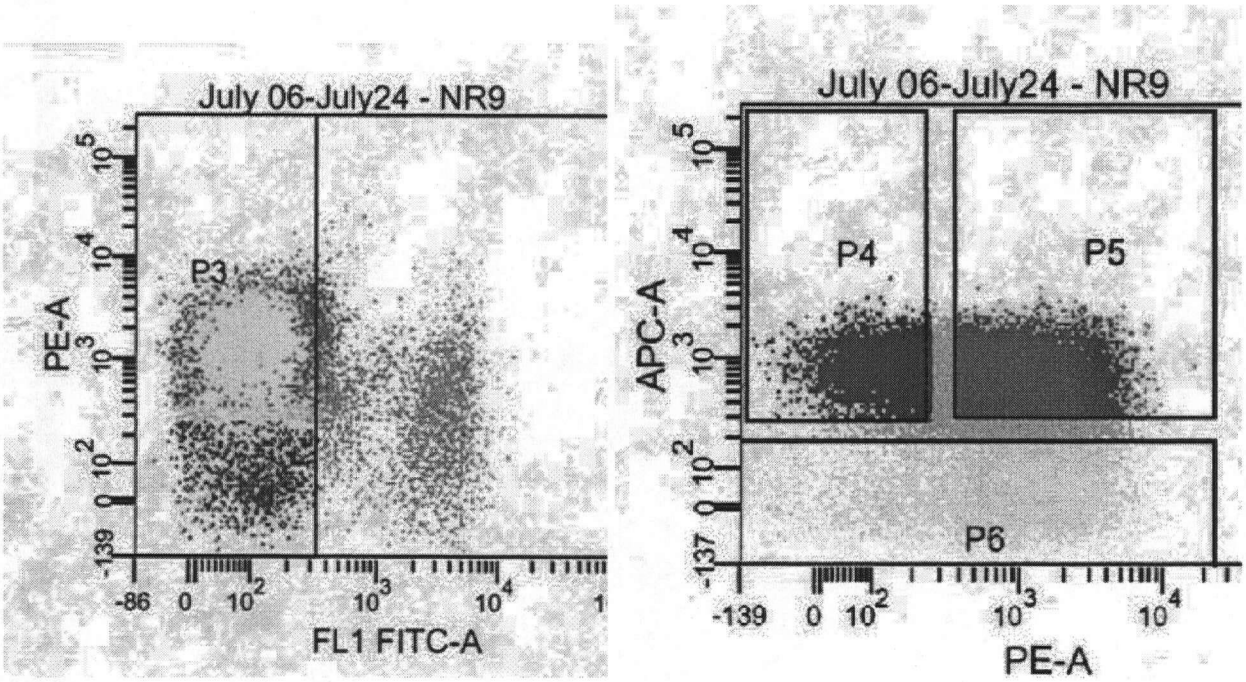
Both *MDR1* and *BCRP1* can efflux daunorubicin, an important chemotherapeutic drug used for most AML patients. According to the LSC model, CD34+CD38- cells are responsible for maintenance of the whole leukemic population, thus high expression of these transporters will give them a critical survival advantage under drug exposure, allowing them to regenerate a leukemic population resulting in refractory disease. Although high levels of ABC transporters were also observed in the CD34- fraction of some responders, this mature subpopulation is

incapable of propagating the disease on its own and thus will not contribute to treatment outcome. Comparing differences between the CR and NR groups, both *MDR1* and *BCRP1* reached statistical significance. More notably however, it is observed that within the NR group, expression levels are either significantly elevated (above the mean) or very low like the CR group, making these "outliers" clearly identifiable. Hence it may be possible to distinguish the potential ABC-dependent (high expression) responders within the NR group for more specifically targeted therapy (see Chapters 4 and 5).

Higher relative expression of ABC transporters in the primitive leukemic fraction is not an entirely surprising finding. In fact, based on the current existing paradigm that LSC originate from transformed HSC, the leukemic CD34+CD38- cells would be expected to be found to express the highest ABC levels among the three subpopulations examined, as previously reported for their normal counterparts[119,125]. Indeed, profiling of a limited number of CD34+ normal PB samples (with HSC mobilization by G-CSF injection) showed *MDR1* transcript levels to be even higher than those of the high-expressing NR patients (data not shown). Hence these patients appear to be retaining existing normal stem cell protection mechanisms, while the low-expression patients may have actually "lost" this defense system during leukemogenesis. Even more intriguing is the observation that some CD34- fractions displayed high ABC expression. Since normal CD34- cells do not express high levels of these transporters[119,124,125], their leukemic counterpart may have abnormally turned on the expression of these genes. Whether this is a random product of aberrant epigenetics in AML or part of a more systematic mechanism remains to be elucidated.

Results of this study suggest the utility of evaluating the leukemic CD34+CD38- subpopulation for both *MDR1/BCRP1* expression in predicting a NR outcome. Despite the

apparent difference between the CR and NR groups; however, the low *MDR1/BCRP1* expression levels detected in NR patients raises the possibility of their biologically relevance. In particular, *BCRP1* levels were so low that they fell below the biological reference line. Further studies are required to discern what expression level translates to functionally significant activity.



Tube: July24 - NR9

Population	#Events	%Parent	%Total
All Events	61,200		100.0
P1	44,374	72.5	72.5
P2	41,521	93.6	67.8
P3	38,745	93.3	63.3
P4	6,350	16.4	10.4
P5	19,824	51.2	32.4
P6	7,073	18.3	11.6

Figure 3.1. FACS analysis of AML patient sample NR#9. Left: Staining of cells with CD3-FITC and CD19-FITC to gate the CD3-CD19- (P3) fraction. Right: Staining and sorting of CD34+CD38- (P4), CD34+CD38+ (P5), and CD34- (P6) cells from the P3 gate. Bottom: Size of each gate is shown as a percentage of the parental gate or percentage of total population.

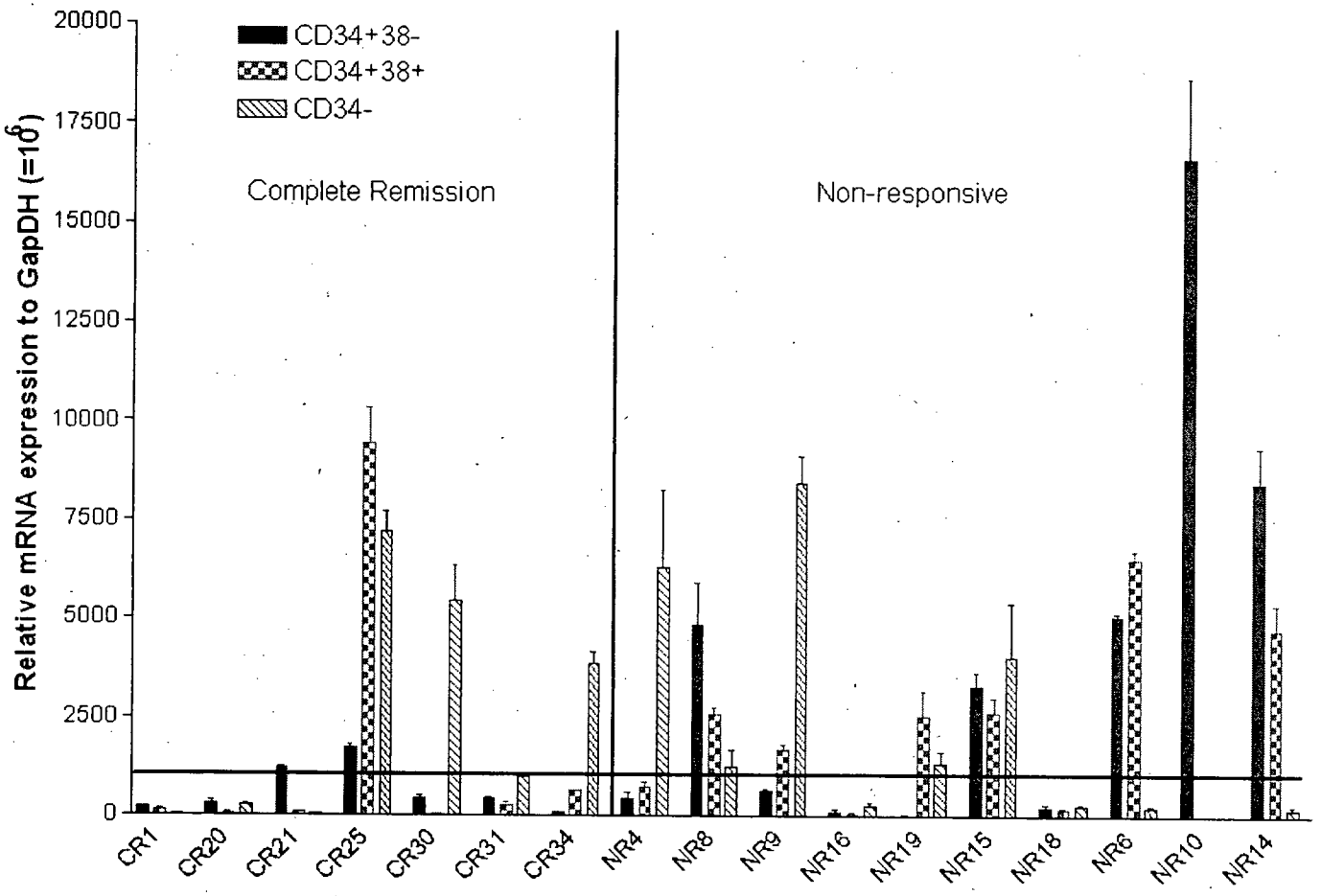


Figure 3.2. mRNA expression levels of *MDR1* in FACS-sorted AML subpopulations. CD34+CD38- (red), CD34+CD38+ (green) and CD34- (blue) cell fractions of AML patient samples were sorted by FACS analysis for Real Time-PCR. Expression levels were expressed relative to *GAPDH* (set at 10^6). Solid horizontal line indicates the biologically-relevant reference line.

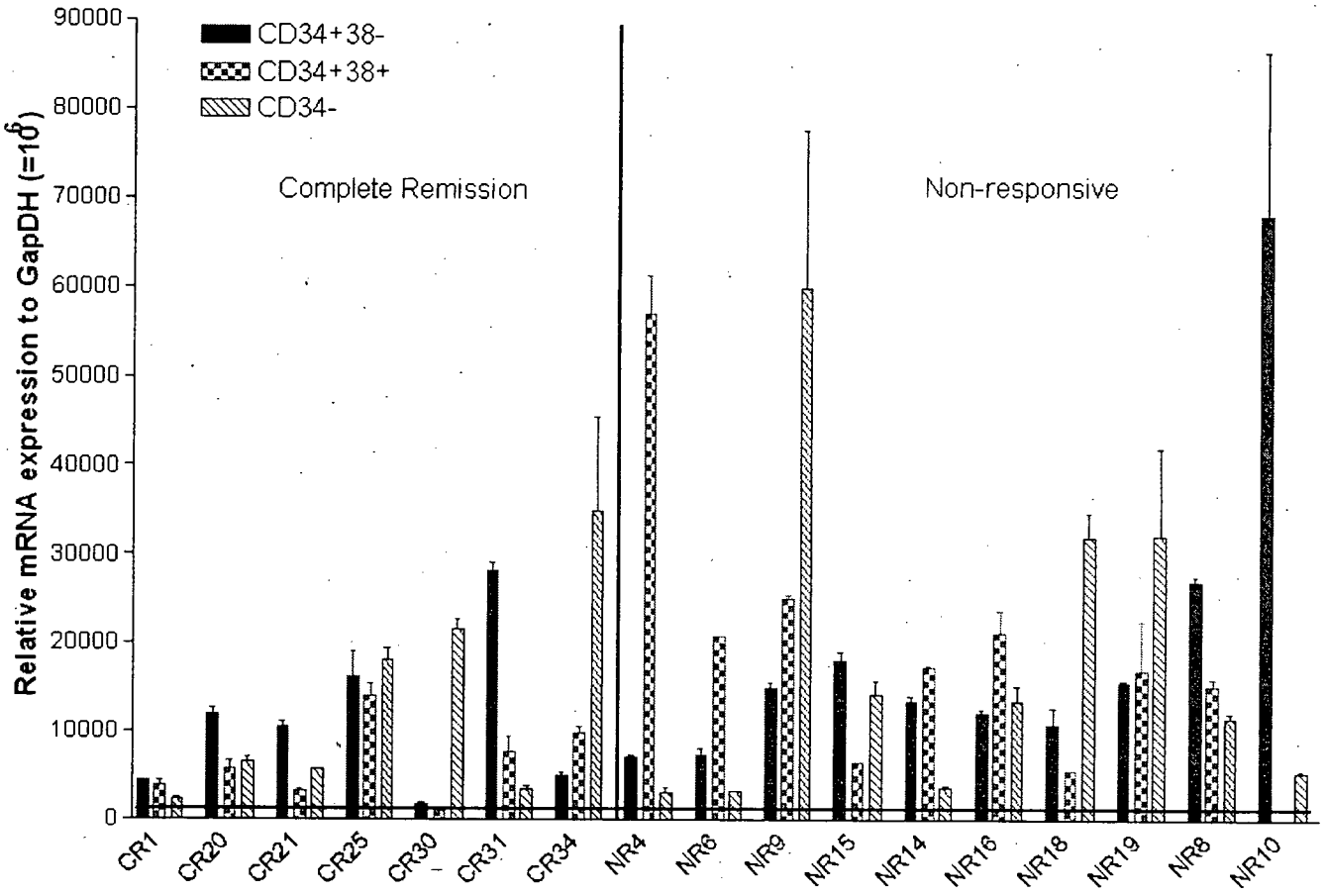


Figure 3.3. mRNA expression levels of *MRP1* in FACS-sorted AML subpopulations. CD34+CD38- (red), CD34+CD38+ (green) and CD34- (blue) cell fractions of AML patient samples were sorted by FACS analysis for Real Time-PCR. Expression levels were expressed relative to *GAPDH* (set at 10^6). Solid horizontal line indicates the biologically-relevant reference line.

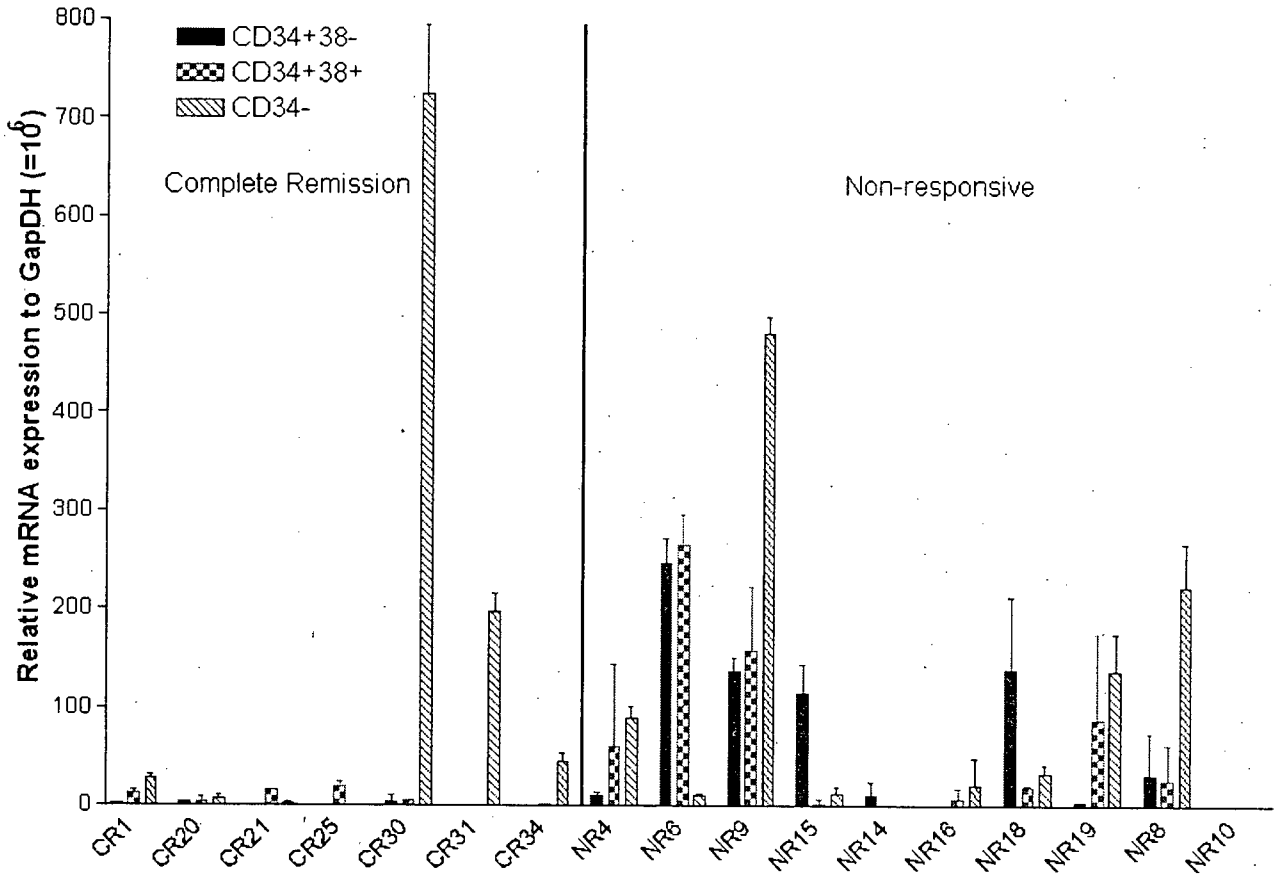


Figure 3.4. mRNA expression levels of *BCRP1* in FACS-sorted AML subpopulations. CD34+CD38- (red), CD34+CD38+ (green) and CD34- (blue) cell fractions of AML patient samples were sorted by FACS analysis for Real Time-PCR. Expression levels were expressed relative to *GAPDH* (set at 10^6). Expression levels fall significantly below the biologically-relevant reference line at 1000 (not shown).

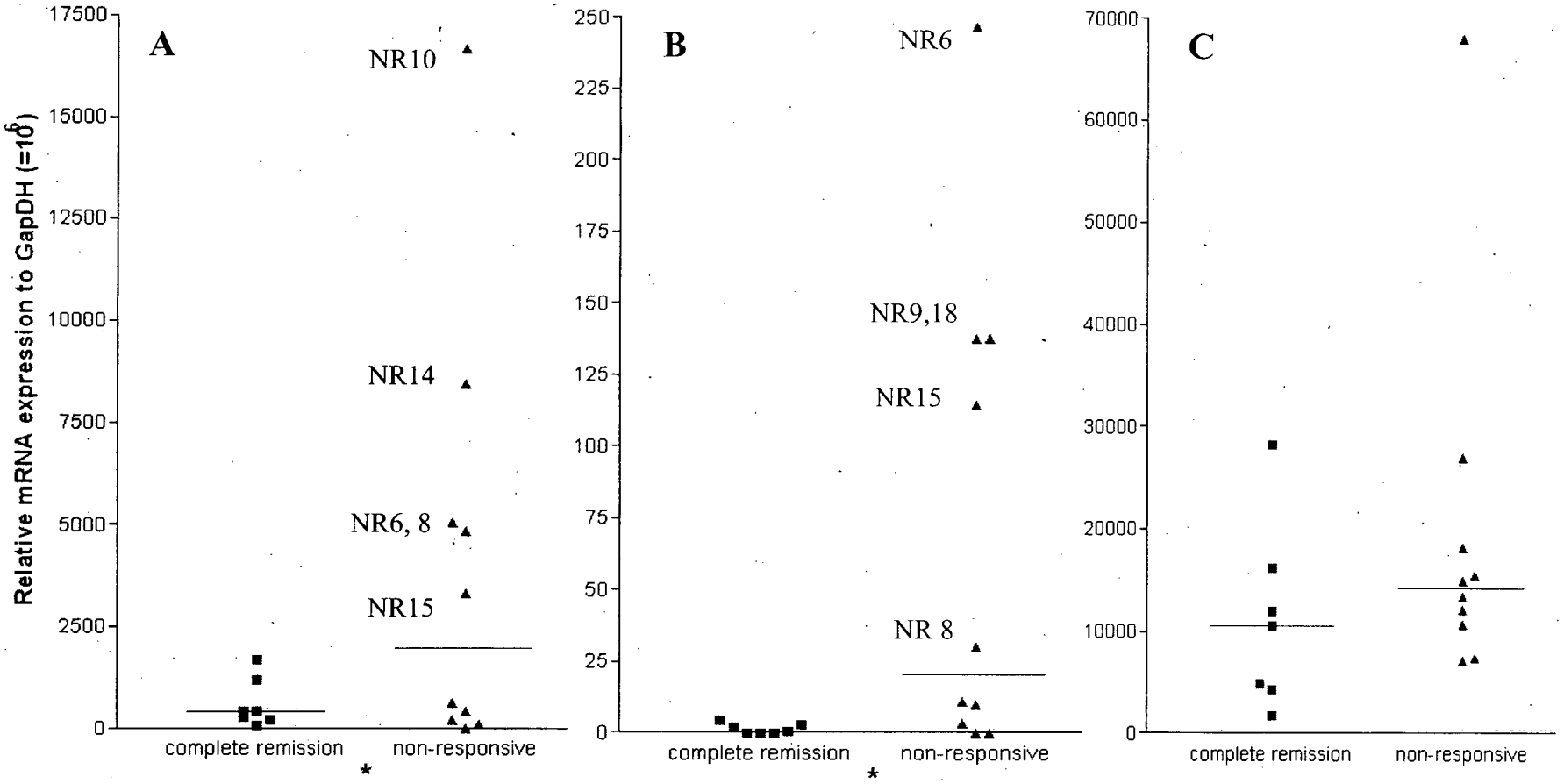


Figure 3.5. Comparison of expression of *MDR1*, *BCRP1* and *MRP1* between the CR and NR groups in the CD34+CD38- fraction. Expression levels of CR patients (square) and NR patients (triangle) were plotted for *MDR1* (A), *BCRP1* (B) and *MRP1* (C). Red and blue horizontal lines represent median levels of CR and NR, respectively. Patients with high *MDR1/BCRP1* levels are identified beside their symbols. Expression levels relative to *GAPDH* (set at 10^6). Asterisk indicates statistical significance.

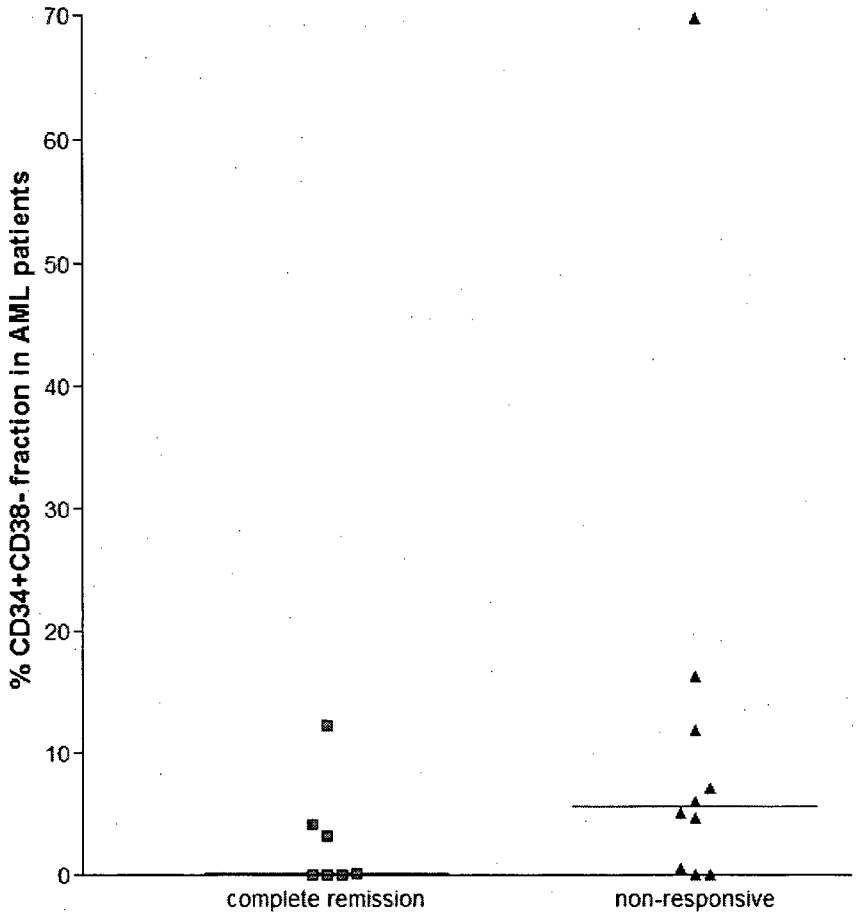


Figure 3.6. CD34+CD38- fraction size in CR and NR AML patients. Percentages of the FACS-sorted CD34+CD38- subpopulation is plotted for CR (square) and NR (triangle) patients. Horizontal lines represent the median of each group.

IV *Ex vivo* drug sensitivity of primitive and mature subpopulations of Acute Myeloid Leukemia and effects of ABC transporter modulation

4.1 – Introduction

Expression profiling as described in the previous chapter allowed rapid examination of a high number of genes in patient samples. However, high gene expression does not necessarily correlate to high protein expression or functional activity. To evaluate the biological relevance of expression data in drug response, a functional assay is necessary. In this chapter I investigated the *ex vivo* drug sensitivity of AML patient cells to determine the functional relevance of *MDR1/BCRP1* gene expression, particularly in the primitive CD34+CD38- subpopulation. I first adapted and validated two functional assays, the Annexin V-PI apoptotic assay and the MTS proliferation assay, on the CEM and CV1.0 leukemic cell lines (see section 2.3.1). These two assays were used to test the sensitivity of CEM and CV1.0 to exposure of daunorubicin, a major chemotherapeutic drug used for AML treatment and a known substrate for both PGP and BCRP1. The CEM cell line with low ABC transporter expression should display higher sensitivity to the drug than the CV1.0 cell line with *MDR1* amplification. Addition of ABC modulators PSC-833 (highly specific inhibitor of PGP) and verapamil (inhibitor of ABC transporters including PGP and BCRP1) is expected to result in re-sensitization of CV1.0 to daunorubicin.

The objectives of this chapter were two-fold. The first was to examine possible differences in drug sensitivity among FACS-sorted subpopulations from AML patients. As discussed earlier, the LSC fraction exhibits properties reminiscent of the normal HSC. Hence I

hypothesized that the primitive CD34+CD38- fraction would be associated with drug resistance, mirroring protection of HSC from cytotoxins. Specifically, I hypothesized that the CD34+CD38- fraction of NR patients would exhibit the highest tolerance to daunorubicin. My second objective was to investigate the functional significance of ABC transporters in AML subpopulations. Based on my observations of higher ABC gene expression in CD34+CD38- cells from AML NR as compared to CR patients (Chapter 3), I hypothesized that *ex vivo* drug resistance would be associated with high expression levels of *MDR1* and/or *BCRP1*, and that addition of ABC inhibitors would have the largest drug re-sensitization effects on the primitive fraction of NR patients, while the low expresser CR fractions would remain unaffected.

4.2 – Materials and Methods

4.2.1 – Exposure of AML cells to drugs

Unsorted or FACS-sorted AML patient cells were centrifuged and re-suspended in Iscove's medium (Invitrogen) + 10% FBS at a concentration of 250 cells per µl. To each well, 100 µl cells, 50 µl daunorubicin at varying dilutions (0.001 to 0.5 µg/ml) and 50 µl PSC-833 (1 µM or 3 µM) or verapamil (5 µg/ml or 20 µg/ml) were added. Cells were incubated at 37 °C for 24 hours before subjected to apoptosis or proliferation assay (see below).

4.2.2 – Annexin V-Propidium Iodide assay

The Annexin V-PI assay utilizes two cell-death markers Annexin V (conjugated to FITC fluorochrome) and propidium iodide (PI) to stain for cells which are undergoing or have undergone apoptosis, respectively. In the early stages of apoptosis, membrane rearrangement causes the translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane. Thus the binding of PS by Annexin V-FITC allows detection of cells in the early stages of apoptosis. This is coupled with the vital dye PI that stains for later-stage apoptotic cells with a loss of membrane integrity. Cells that are both Annexin V-FITC and PI negative are defined as viable. Figure 4.1 shows the detection of viable cells as double negatives under exposure of a low and high concentration of daunorubicin.

Apoptosis was measured using the Annexin V-FITC + PI detection kit I (BD Biosciences) with a modified manufacturer's protocol. After incubation with daunorubicin for 24 hours, AML patient cells were centrifuged for 5 min at 1200 rpm. Medium (170 µl) was removed from the top of each well with caution before addition of 100 µl of 1x Binding Buffer

and 20 µl of 1/8 diluted Annexin-V and PI mix. IGG1-FITC Antibody (BD Biosciences) was added to the mock-treatment well as negative control. Additional staining controls were carried out with addition of Annexin V-FITC only and PI only. Cells were incubated in the dark at room temperature for 15 min. Reactions were terminated by addition of 100 µl 1x Binding Buffer. Fluorescence was measured using a FACSCalibur flow cytometer (High Throughput Sampler, BD) and analyzed by Flow Jo v.2.0 software.

4.2.3 – MTS assay

The MTS assay is a colorimetric, proliferation assay that utilizes the soluble chemical tetrazolium salt (MTS). MTS is reduced to formazan by metabolically active cells (Figure 4.2). Production of formazan can be detected by development of a brown colour and is proportional to the number of viable cells. Proliferation was measured using the CellTiter 96 Aqueous Non-Radioactive Cell proliferation Assay (Promega). After 24-hour incubation with daunorubicin, 20 µl of Aqueous Solution 1 was added to cells and incubated for 2 hours at 37 °C. Color development was quantified as absorbance at 490 nm by the MRX Microplate Reader (Dynex Technology).

4.2.4 – Analysis

For the MTS assay, absorbance was plotted against daunorubicin concentration to generate a drug sensitivity curve for each concentration of ABC inhibition (no inhibition, 1µM PSC-833, 3 µM PSC-833, 5 µg/ml verapamil, 20 µg/ml verapamil). For the Annexin V-PI assay, % viability was defined as % of cells in the Annexin V-FITC and PI double-negative quadrant (in reference to the non-specific IGG1 control). Using this value, % kill was calculated

for each daunorubicin concentration with respect to the viability control (no daunorubicin) using the formula (% viable control-% viable sample)/% viable control x 100%. The drug sensitivity curve was then generated by plotting % kill against daunorubicin concentration for each concentration of ABC inhibition. IC_{50} for each curve was obtained as the daunorubicin concentration at 50% kill. Fold change in IC_{50} by ABC modulation was calculated using the formula IC_{50} unmodulated/ IC_{50} highest inhibition dose. A two-tailed, heteroscedastic student's t test was utilized to evaluate statistical difference between the NR and CR groups. Statistical significance was set at $p \leq 0.05$.

4.3 – Results

4.3.1 – Comparison of the Annexin V-PI apoptotic assay to the MTS proliferation assay on CEM and CV1.0 cell lines.

The Annexin V-PI assay and the MTS assay were performed to measure and compare drug tolerance of CEM and CV1.0 cells. Daunorubicin sensitivity was first determined by the apoptosis assay. Cells were stained with Annexin V-FITC and PI after exposure to different concentrations of daunorubicin with or without PSC-833 or verapamil. As expected, Figure 4.3 shows that CV1.0 cells without ABC modulation is much more resistant to daunorubicin (only 20% kill at highest dose of 0.5 μg/ml) than parental CEM cells (IC_{50} = 0.025 μg/ml). Neither PSC-833 nor verapamil had a significant effect on CEM while both had a dose-dependent re-sensitizing effect on CV1.0 cells, which is consistent with *MDR1* amplification being a main cause of the drug-resistance mechanism in this cell line. Results from the MTS assay on a similar drug study were represented in Figure 4.4. Again, CV1.0 cells were more resistant, as indicated by their higher proliferation activity (proportional to higher absorbance) after daunorubicin exposure, and ABC modulation had a dose-dependent effect in decreasing their proliferation/survival. Similar results from the two assays gave results consistent with expectations showing that both assays provided valid measures of *ex vivo* drug responses of leukemic cells. Because of its single-cell analysis, the apoptotic assay was more sensitive and reliable compared to the colorimetric MTS assay, and was thus used for subsequent studies on AML patient cells.

4.3.2. Adaptation of the apoptotic assay to AML patient cells.

Due to the limitation of sorted cells, I next tested assay conditions on unsorted AML patient cells to adapt the apoptotic assay further to clinical material. To determine the maximum dose of ABC modulators PSC-833 (dissolved in DMSO) and verapamil that can be used, bulk cells from AML patient #18 were subjected to different concentrations of the ABC modulators in culture and % kill was determined using the Annexin V-PI apoptotic assay. Because the concentrated PSC-833 stock solution was dissolved in DMSO, the matching volume equivalent of DMSO was also tested for each PSC-833 concentration. As seen in Figure 4.5, the maximum dose of PSC-833 and verapamil without significant toxicity on AML primary cells was 3 μM and 20 μg/ml, respectively. DMSO had no independent toxic effect in the assay conditions used.

Next, a range of daunorubicin concentrations with or without addition of the modulator PSC-833 was tested on two unsorted patient samples. As expected, ABC modulation has no significant effect on patient #18 (Figure 4.6), whose expression of *MDR1* was among the lowest of all patients (Figure 4.6, small panel). On the other hand, Figure 4.7 shows that patient #25, who had the highest *MDR1* expression (Figure 4.7, small panel), displayed a more resistant profile (% kill plateaued at about 60%) than patient #18 (% kill reached about 100% at 0.1 μg/ml). Moreover, PSC-833 had a dose-dependent effect, increasing the sensitivity of patient #25 cells to the PGP substrate daunorubicin. This validated the application of the apoptotic assay on clinical samples and demonstrated ABC inhibition as a useful measure of ABC transporter activity.

4.3.3 – Subpopulation size and patient material availability as a source of limitation.

For study coherence and to allow direct comparison between expression data and functional results, I performed the *ex vivo* drug sensitivity assay on the same patient samples used in Chapters 2 and 3. Unfortunately, there were limitations in the selection of patient samples for functional studies. The main limitation was the size of subpopulations obtainable. Taking into account the length of sort time and number of patient cells per frozen aliquot, a size of at least 2% of each subpopulation was typically needed from a single patient. Table 4.1 lists the fraction sizes of the same patients that have been profiled for ABC expression in their subpopulations, with an asterisk marking those with high expression of *MDR1* and/or *BCRP1* in the CD34+CD38- cells (Chapter 2). Another limitation was the availability of patient samples (for example, no more cells were available for patient CR#30). Taken together, two CR patients (CR#1 and #34) and six NR patients (NR#8, #9, #14, #15, #16, #19) matched the criteria of availability and ample fraction size. Four of the six NR patients (NR #8, #9, #14, #15) had high *MDR1/BCRP1* expression in CD34+CD38- cells. These eight patients were used for the functional assay.

4.3.4. Higher daunorubicin resistance and larger effect of ABC modulation in the CD34+CD38- fraction of non-responders.

CD34+CD38-, CD34+CD38+, and CD34- fractions were sorted by FACS from the eight selected AML patients. These as well as unsorted cells were subjected to 24-hour daunorubicin exposure in the presence or absence of PSC-833 or verapamil followed by the apoptotic assay.

As illustrated by examples of drug sensitivity curves from the CR and NR groups in Figures 4.8 and 4.9 (other profiles were included in the Appendix), striking differences were observed between the two. For CR patient #1, unsorted (total), CD34+CD38-, CD34+CD38+ and CD34- fractions showed high sensitivity to daunorubicin, reaching over 85% kill at 0.1 μg/ml (IC_{50} = 0.03 to 0.04 μg/ml) with or without ABC modulation, and addition of either PSC-833 or verapamil only had a slight effect of increasing its sensitivity. In contrast, NR patient #9 exhibited significant differences among subpopulations. As shown in Figure 4.9, unmodulated CD34+CD38- and CD34+CD38+ (35% - 40% kill at 0.1 μg/ml, IC_{50} > 0.1 μg/ml) of NR#9 were significantly more resistant than CD34- and unsorted (55% and 70% at 0.1 μg/ml respectively). ABC modulation had a dose-dependent re-sensitizing effect on all subpopulations, with a magnitude following the order CD34+CD38- > CD34+CD38+ > CD34-, suggesting that ABC transporter activity was a significant contributor to drug resistance in the non-responder, especially in the primitive subpopulation.

Figure 4.10 shows the IC_{50} of daunorubicin for each subpopulation of CR and NR patients. Although only two CR patients were tested, they gave similar results. Both showed very high drug sensitivity (IC_{50} <0.04 μg/ml) in the primitive CD34+CD38- fraction, while the mature CD34- fractions were slightly more resistant to daunorubicin. On the other hand, the NR patients showed a range of resistance in terms of higher IC_{50} values across all fractions. As a group, NR patients were much more resistant than the CR patients, particularly in the CD34+CD38- fraction in which there was no overlap between the two groups and difference reached statistical significance ($p \leq 0.05$). Drug sensitivity corresponded with ABC expression, in that the four patients (NR #8, #9, #14, #15) with high *MDR1/BCRP1* expression displayed the highest resistance in this primitive fraction.

Figure 4.11 shows the effect of ABC modulation on drug sensitivity for each subpopulation. Both CR patients showed little change in the presence of ABC inhibitors, especially in the CD34+CD38- fraction, and only a minor increase in sensitivity in the CD34- fraction. Contrastingly, ABC inhibition markedly decreased IC_{50} in the primitive CD34+CD38- and CD34+CD38+ fractions of NR patients. There is a statistically significant difference between the CR and NR groups in CD34+CD38- ($p \leq 0.05$, student's t test) but not in CD34+CD38+, CD34- and total fractions. As with drug resistance, observed effects of ABC modulation is consistent with expression data, with the *MDR1*/BCRP1-expressing NR patients being most affected by ABC inhibition, demonstrating the MDR activity of these transporters.

4.4 – Discussion

In this section, I followed up on the expression results from Chapter 3 with *ex vivo* functional studies to test the drug sensitivity of sorted patient subpopulations and the activity of ABC transporters in these fractions. As with levels of ABC expression, differences in functional characteristics were apparent among subpopulations along the leukemic hierarchy. Sensitivity curves of unsorted cells, as well as the effects of ABC modulation on these, were generally similar to that of the predominant fraction of CD34- cells rather than the small primitive CD34+CD38- fraction, again showing that studies on total population may not be representative of the tumorigenic cells of importance for prognosis. Although the population size is small, results of this study revealed that ABC-dependent daunorubicin resistance in the CD34+CD38- fraction was common among NR patients who are mostly karyotypically normal (not observed in the CR group) and might thus be predictive of poor clinical response to initial therapy. This difference between the CR and NR patients was not seen in either the mature CD34- fraction or the bulk population. For CR patients, the CD34- fraction displayed slightly higher resistance than the primitive cells which can be overcome by ABC modulation, showing some level of ABC transporter activity in this fraction. Nevertheless, this did not correlate with clinical outcome (CR patients), consistent with the hypothesis that the features of the non-tumorigenic fraction may not be prognostic.

Modulation of ABC activity increased daunorubicin sensitivity in a dose-dependent fashion in the CD34+CD38- cells of most NR patients studied. Such an effect was most dramatic for NR#8, #9 and #15, patients with high *MDR1/BCRP1* expression (3/6 tested). ABC transporter activity was consistent with the expression data in that high *MDR1/BCRP1*

expression corresponded with high drug resistance, reversible by ABC-specific inhibition. This can have important clinical implications. Drug studies, especially on subpopulations, is time-consuming and laborious, hence may not be feasible for routine testing in a clinical laboratory, especially for an acute disease such as AML that requires rapid intervention. On the other hand, because expression studies are faster, require less clinical material, and may be adapted to high through-put protocols, it is potentially more valuable for identification of the NR cases where *MDR1/BCRP1* is a significant contributor to drug resistance.

Interestingly, although CD34- cells of NR patients exhibited a range of drug resistance like that of CD34+CD38- cells, effects of ABC modulation on CD34- is much smaller (except NR16). Therefore, while *MDR1/BCRP1* expression may significantly contribute to drug resistance in primitive leukemic cells, it is not the dominant resistance mechanism in mature cells. Based on the existing paradigm that the LSC originate from HSC, the LSC will likely retain many of the normal stem cell properties, including its protective mechanism against cytotoxins. As evident from my studies, LSC from a number of patients did appear to have high ABC expression like HSCs, which became advantageous under chemotherapy resulting in treatment failure. What additional mechanisms drive drug resistance in the CD34- fraction remains to be elucidated.

The LSC model is in congruence with older principles of chemotherapy, in particular the classification of cell cycle-specific and non-cell cycle-specific drugs. Cycle-specific drugs, such as Ara-C, have a dramatic but exclusive effect on proliferating cells in the S-phase of the cell cycle, while non-cycle-specific drugs such as daunorubicin are active throughout the cell cycle, killing both proliferating cells and quiescent (G_0) cells. The latter, however, tends to associate with much higher tissue toxicity compared to cycle-specific drugs. In light of the LSC model, an

updated interpretation of this is that cycle-specific drugs are effective in killing the more differentiated, actively proliferating cancer cells that usually comprise of the majority of the tumor. On the other hand, non-cycle-specific drugs are needed to eradicate the rare CSC that may be in G_0. Hence in AML, although Ara-C is useful in tumor de-bulking and alleviating symptoms, it is daunorubicin (the substrate for *MDR1/BCRP1*) that targets the LSC fraction and is necessary for long-term remission. Following this premise, it makes sense how patients with high *MDR1/BCRP1* in CD34+CD38- should fail to achieve complete remission in my study, since daunorubicin is ineffective against the disease-maintaining compartment.

Table 4.1. % of CD34+CD38-, CD34+CD38+ & CD34- fractions in AML patients.

Patient	% CD34+38-	% CD34+38+	% CD34-
CR1	12.3	41.2	27.9
CR20	0.1	63.5	11.8
CR21	0.1	0.8	78
CR25	0.1	10.4	60.6
CR30	3.3	39.9	38
CR31	0.2	75.3	1.2
CR34	4.2	21.6	36.5
NR4	0.6	1.4	79.3
*NR6	0.05	2.7	89.3
*NR8	69.8	13.8	12.1
*NR9	16.4	51.2	18.3
*NR10	5.2	1.1	80.7
*NR14	6.1	15.2	70.7
*NR15	4.8	63.9	17.3
NR16	11.9	18.6	52.5
*NR18	0.1	69.8	11.7
NR19	7.1	48.7	36.4

* - NR patients with high expression of *MDR1*/BCRP1 in CD34+CD38-.

Highlighted – patient samples suitable AND available for functional studies.

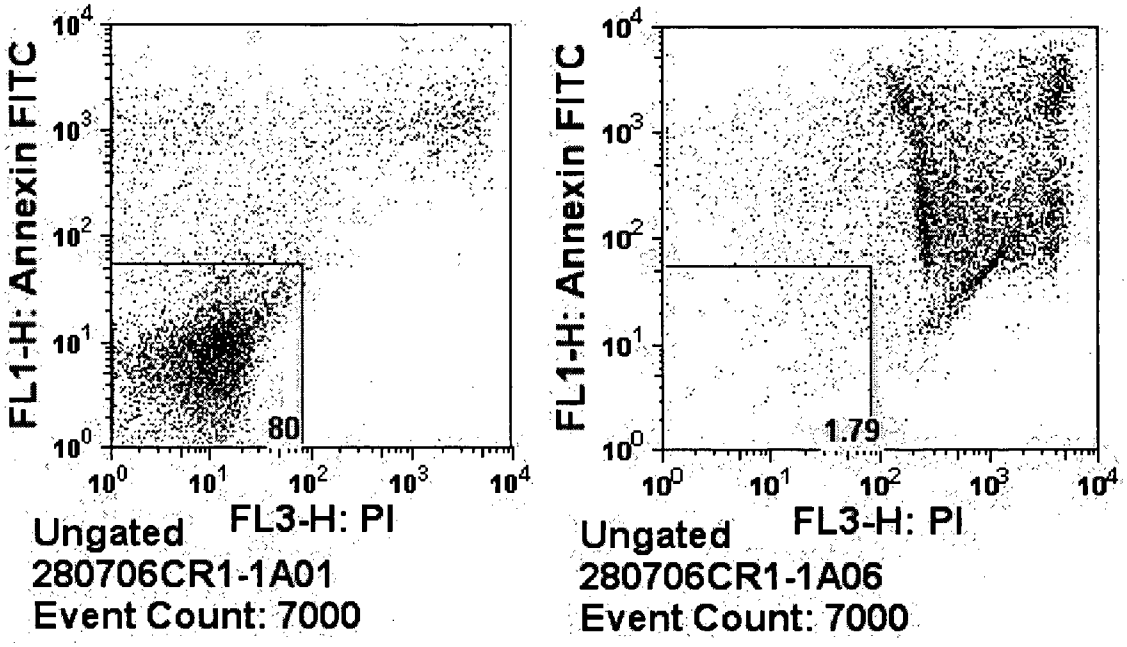


Figure 4.1. Apoptosis as detected by the Annexin V-PI assay. AML patient cells were exposed to 0.001 μg/ml (left) and 0.5 μg/ml (right) daunorubicin for 24 hours before stained by Annexin V and PI and analyzed. The blue gate indicates the quadrant containing viable cells. Cells were significantly more viable when exposed to low drug concentrations (80% viable) than high drug concentrations (1.8% viable).

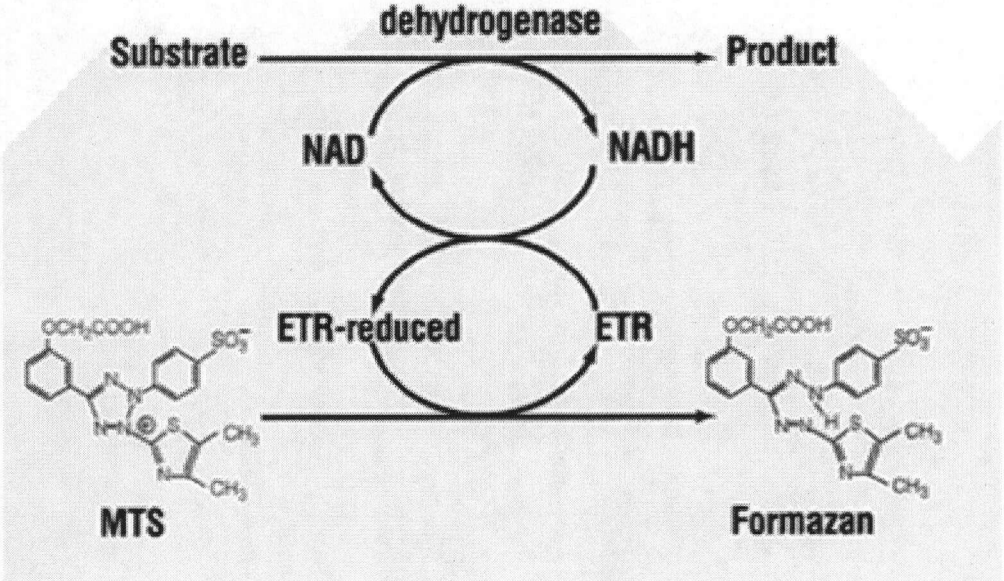


Figure 4.2. Schematic representation of conversion of MTS to formazan. Production of NADH or NADPH by metabolically-active cells is the main cause for conversion of MTS to formazan. Electron transfer occurs from NADH to an electron transfer reagent (ETR) such as PMS, which subsequently causes the production of brown formazan that can be detected by a colorimetric method. Reproduced from www.ebiotrade.com.

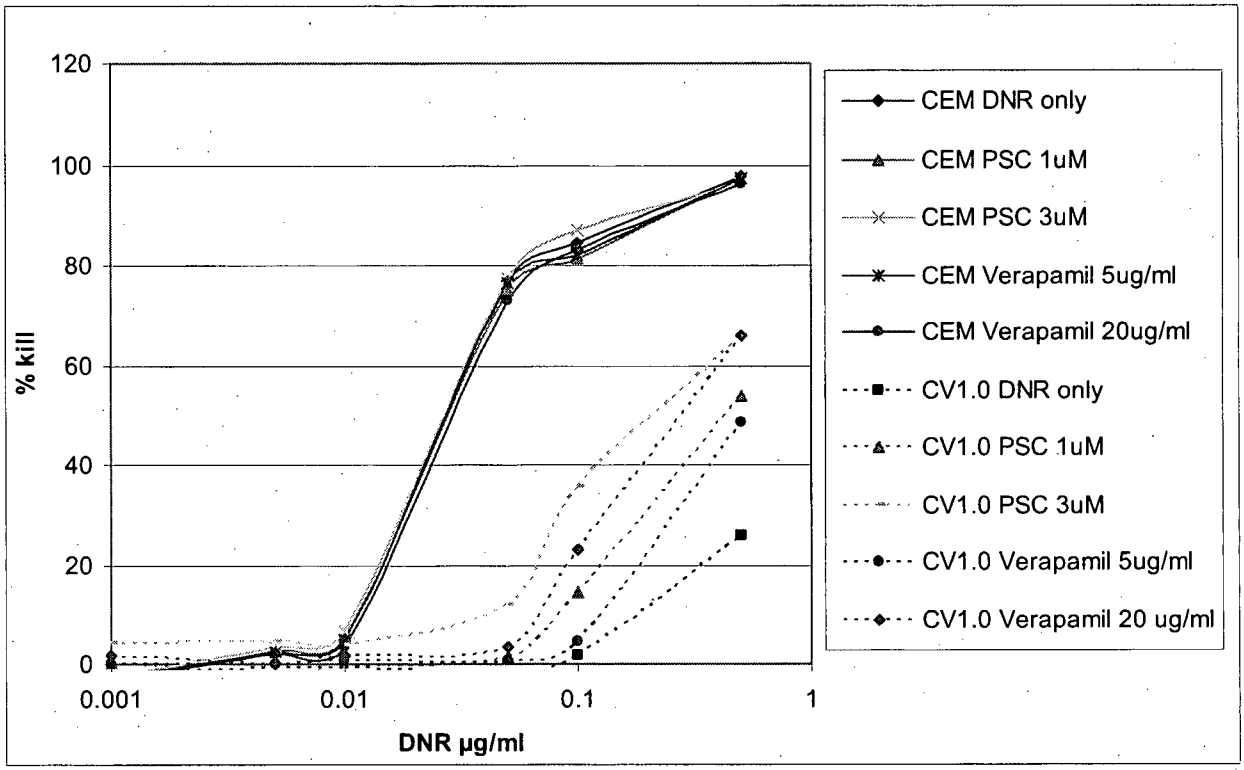


Figure 4.3. Effects of PSC-833 and verapamil on daunorubicin sensitivity in CEM and CV1.0 as measured by the Annexin V-PI assay. CEM and CV1.0 cells were exposed to daunorubicin at concentrations ranging from 0.001 to 0.5 µg/ml for 24 hours, with or without PSC-833 (1 µM, 3 µM) or verapamil (5 µg/ml, 20 µg/ml). Annexin V and PI were subsequently added to the cells and incubated at room temperature for 15 min in the dark. FACS analysis was performed to determine the viability (defined as both Annexin V and PI negative).

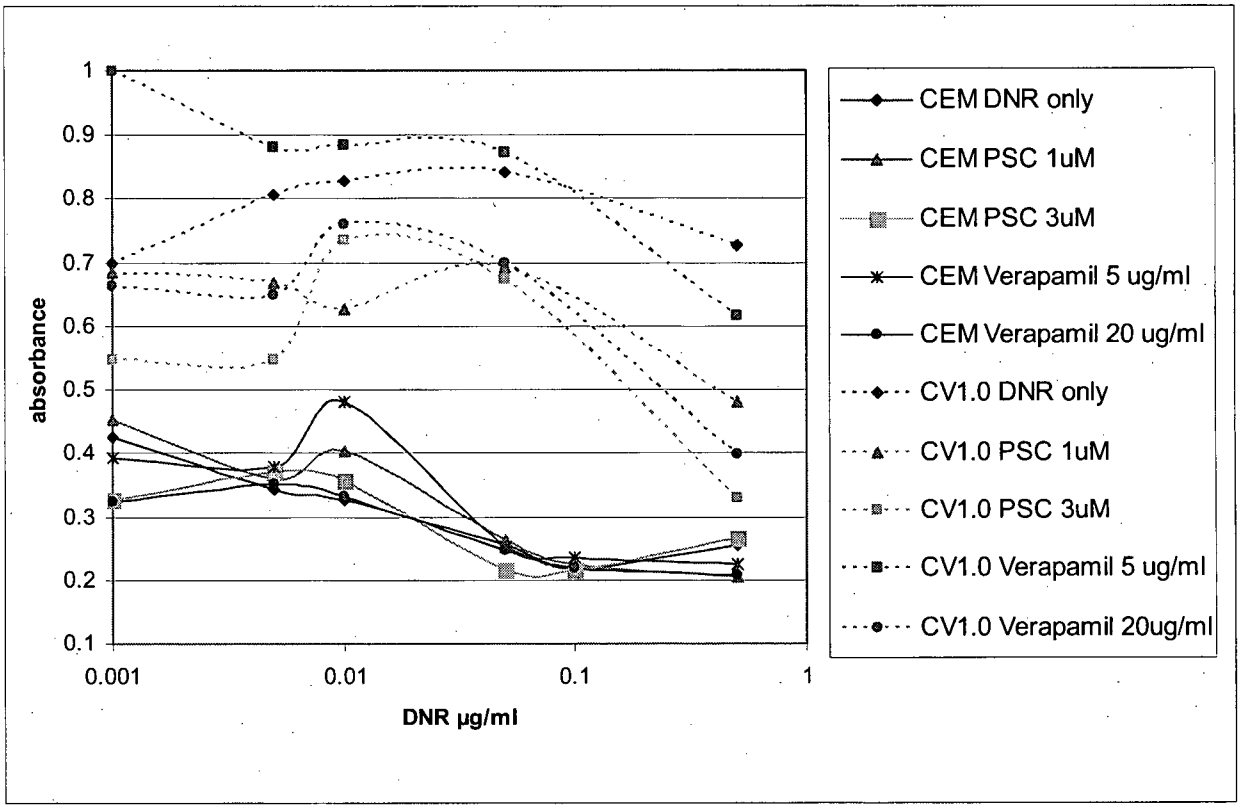


Figure 4.4. Effects of PSC-833 and verapamil on daunorubicin sensitivity in CEM and CV1.0 as measured by the MTS assay. CEM and CV1.0 cells were exposed to daunorubicin at concentrations ranging from 0.001 to 0.5 µg/ml for 24 hours, with or without PSC-833 (1 µM, 3 µM) or verapamil (5 µg/ml, 20 µg/ml). MTS was subsequently added to the cells and incubated for 2 hours at 37 °C. Color development was measured as absorbance at 490 nm.

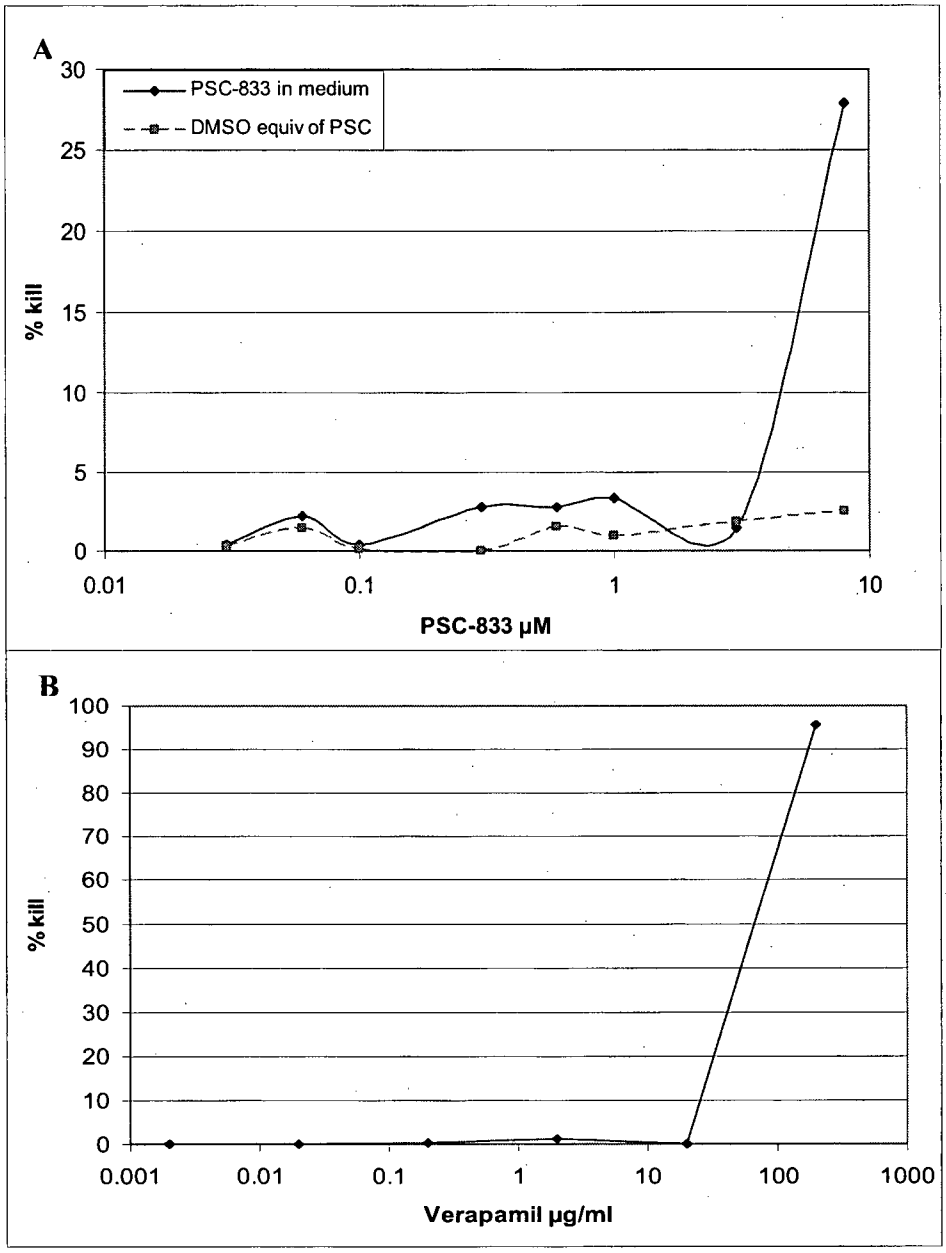


Figure 4.5. Toxicity assay of PSC-833 and verapamil on AML patient cells. Primary AML patient cells were exposed to PSC-833 at concentrations ranging from 0.03 µM to 8 µM and the DMSO volume equivalent (A) or verapamil at concentrations ranging from 0.002 to 200 µg/ml (B) for 24 hours. Viability and % kill were determined by the Annexin V-PI assay.

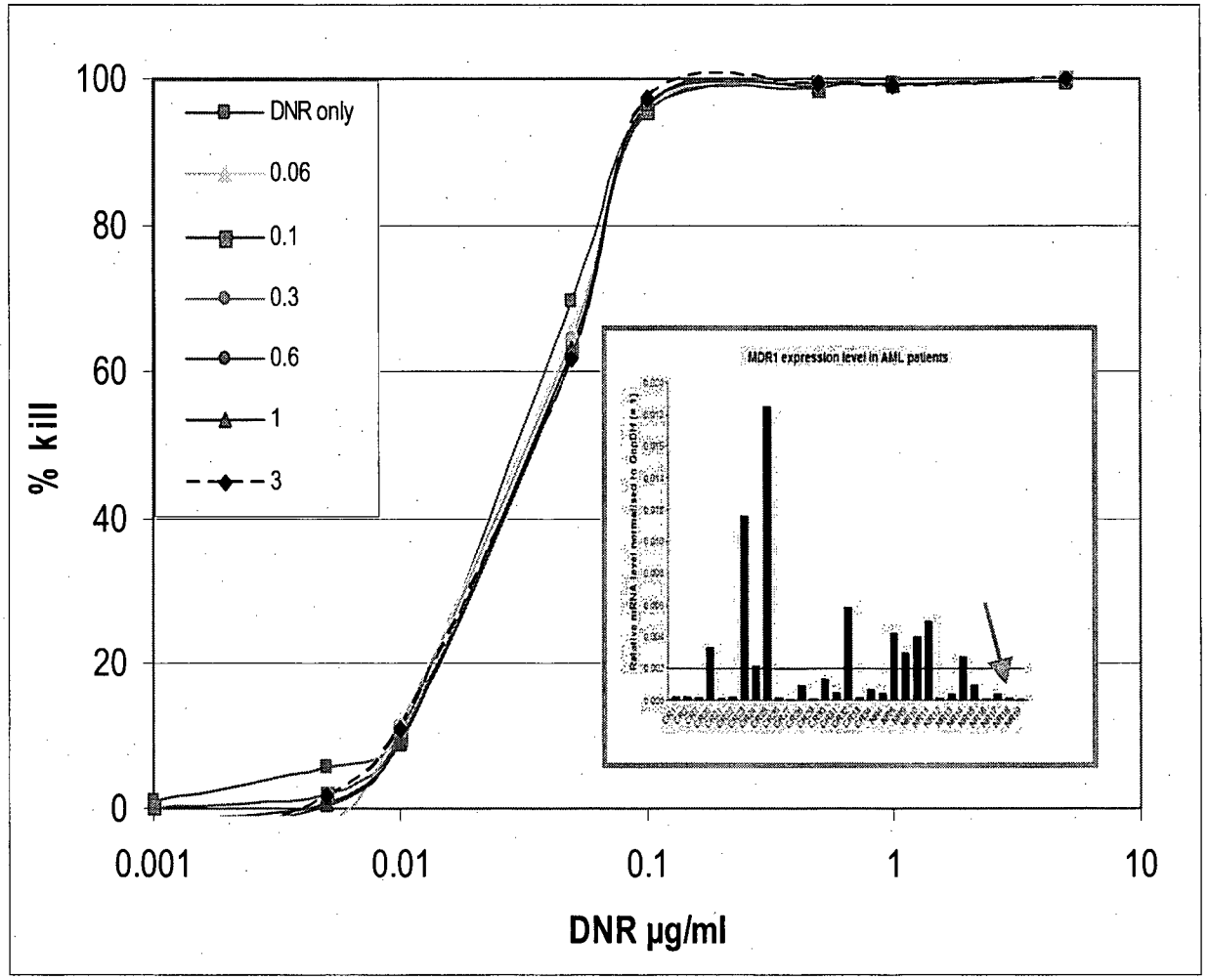


Figure 4.6. Effect of PGP inhibition on daunorubicin sensitivity of AML patient #18. Unsorted cells from patient #18, was subjected to daunorubicin exposure with or without PSC-833 for 24 hours before the Annexin V-PI assay. Small panel: *MDR1* expression in total population of #18 (arrow) in comparison to other patients.

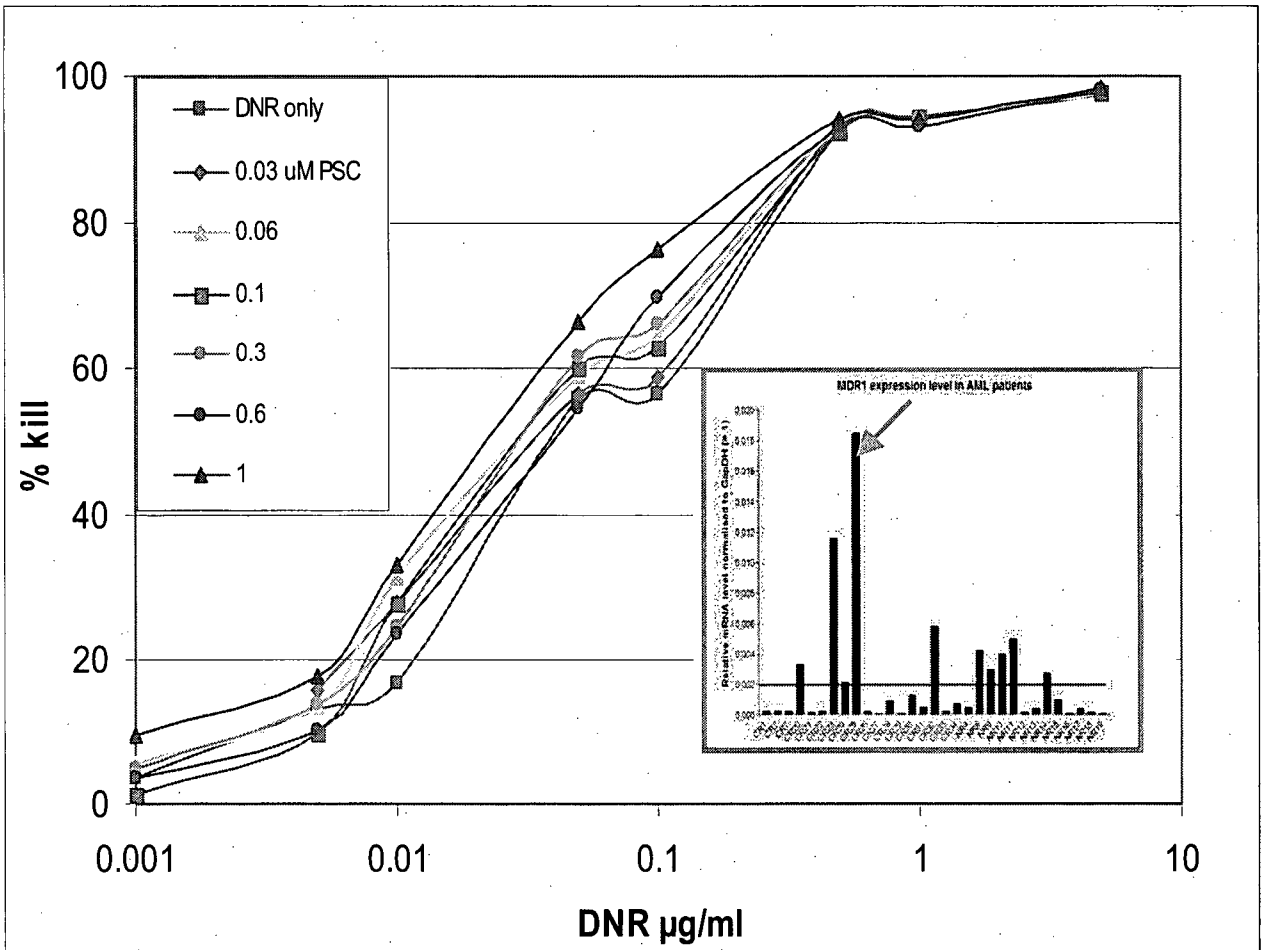


Figure 4.7. Effect of PGP inhibition on daunorubicin sensitivity of AML patient #25. Unsorted cells from patient #25, was subjected to daunorubicin exposure with or without PSC-833 for 24 hours before the Annexin V-PI assay. Small panel: *MDR1* expression in total population of #25 (arrow) in comparison to other patients.

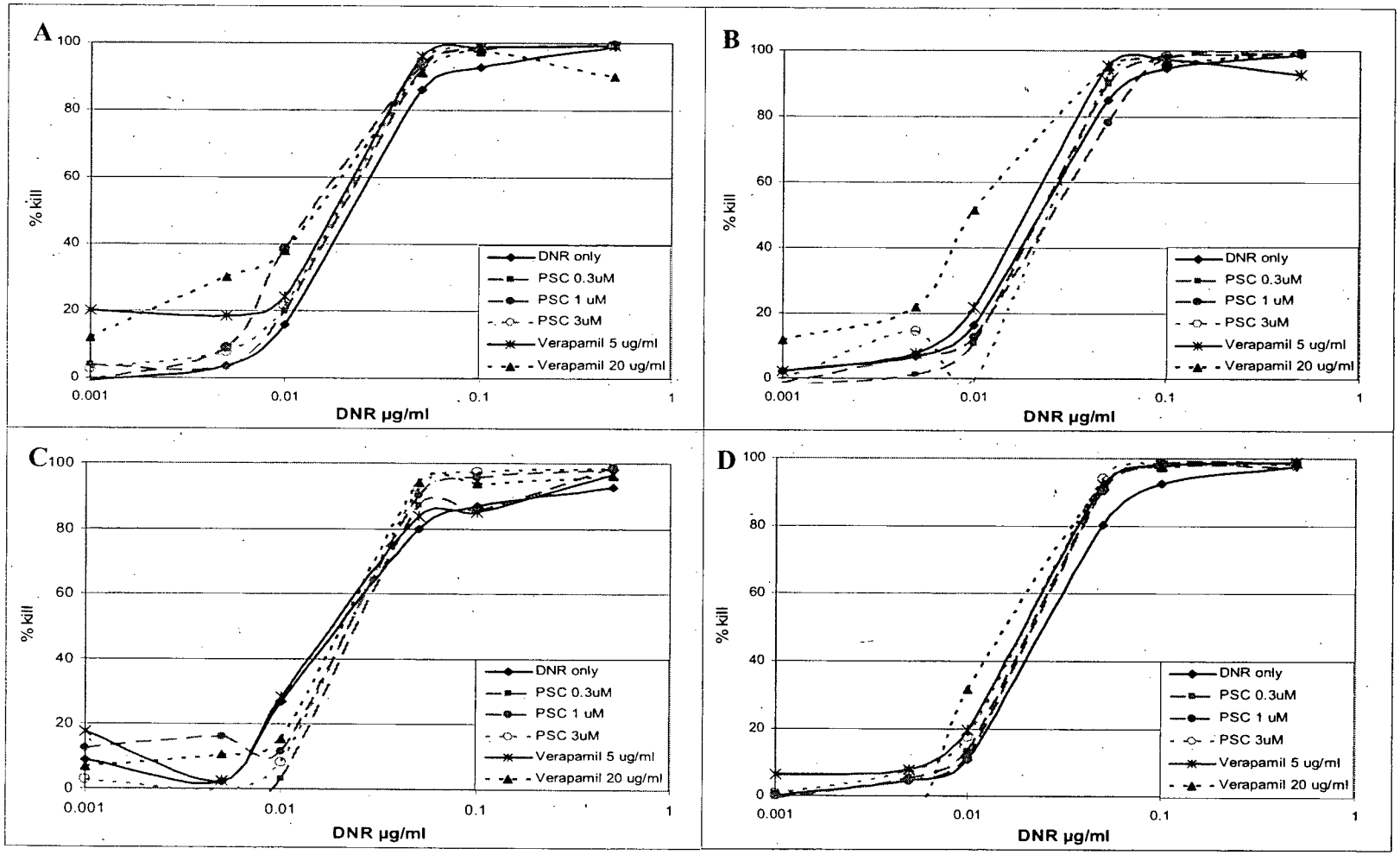


Figure 4.8. Drug sensitivity and effects of ABC modulation on CR patient #1 subpopulations. FACS-sorted CD34+38- (A), CD34+38+ (B) and CD34- (C) and unsorted cells (D) were exposed to daunorubicin at a range of concentrations +/- ABC inhibitors verapamil and PSC-833 for 24 hours. Cell viability was measured by the AV-PI assay and expressed as % kill.

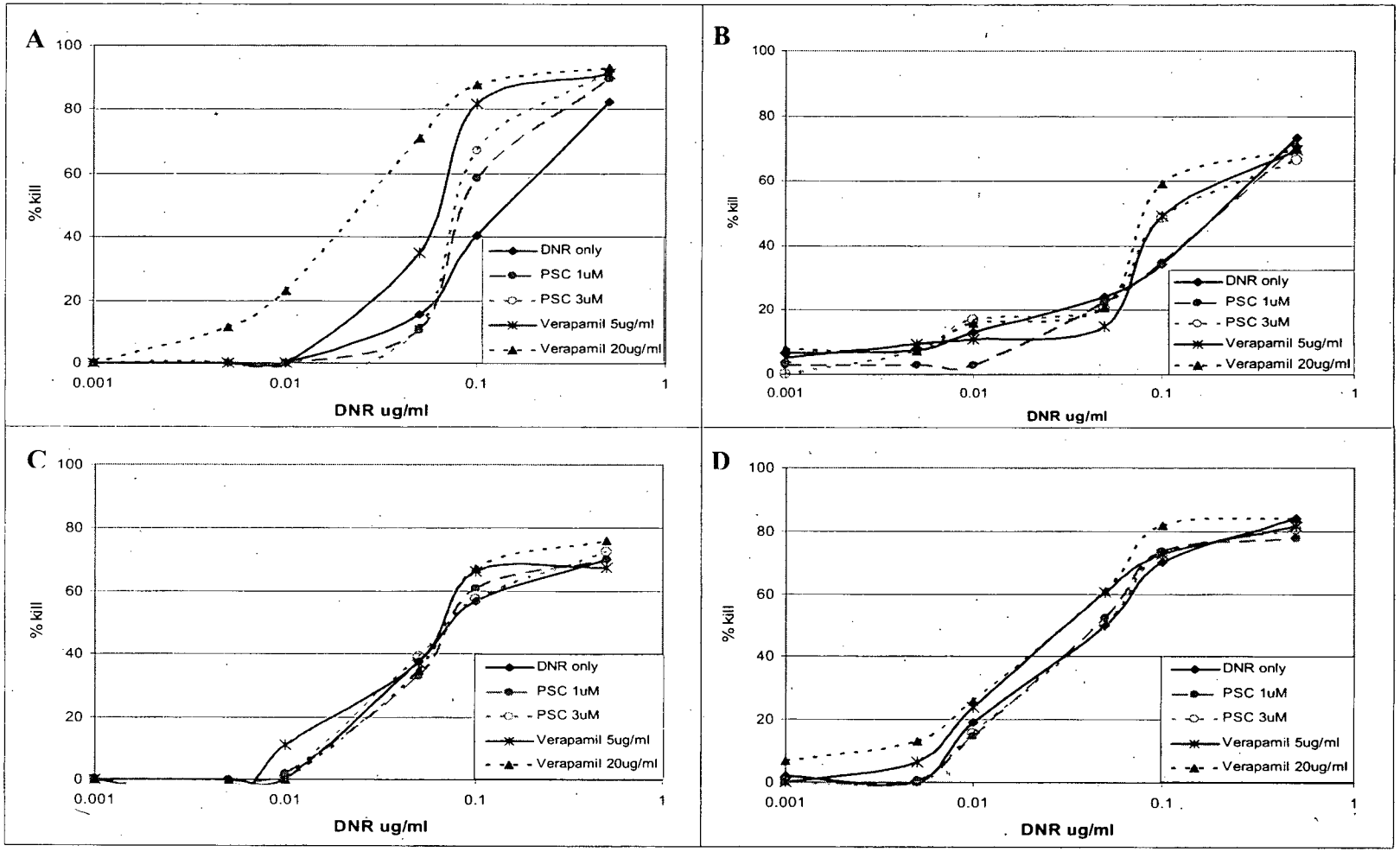


Figure 4.9. Drug sensitivity and effects of ABC modulation on NR patient #9 subpopulations. FACS-sorted CD34+38- (A), CD34+38+ (B) and CD34- (C) and unsorted cells (D) were exposed to daunorubicin at a range of concentrations +/- ABC inhibitors verapamil and PSC-833 for 24 hours. Cell viability was measured by the AV-PI assay and expressed as % kill.

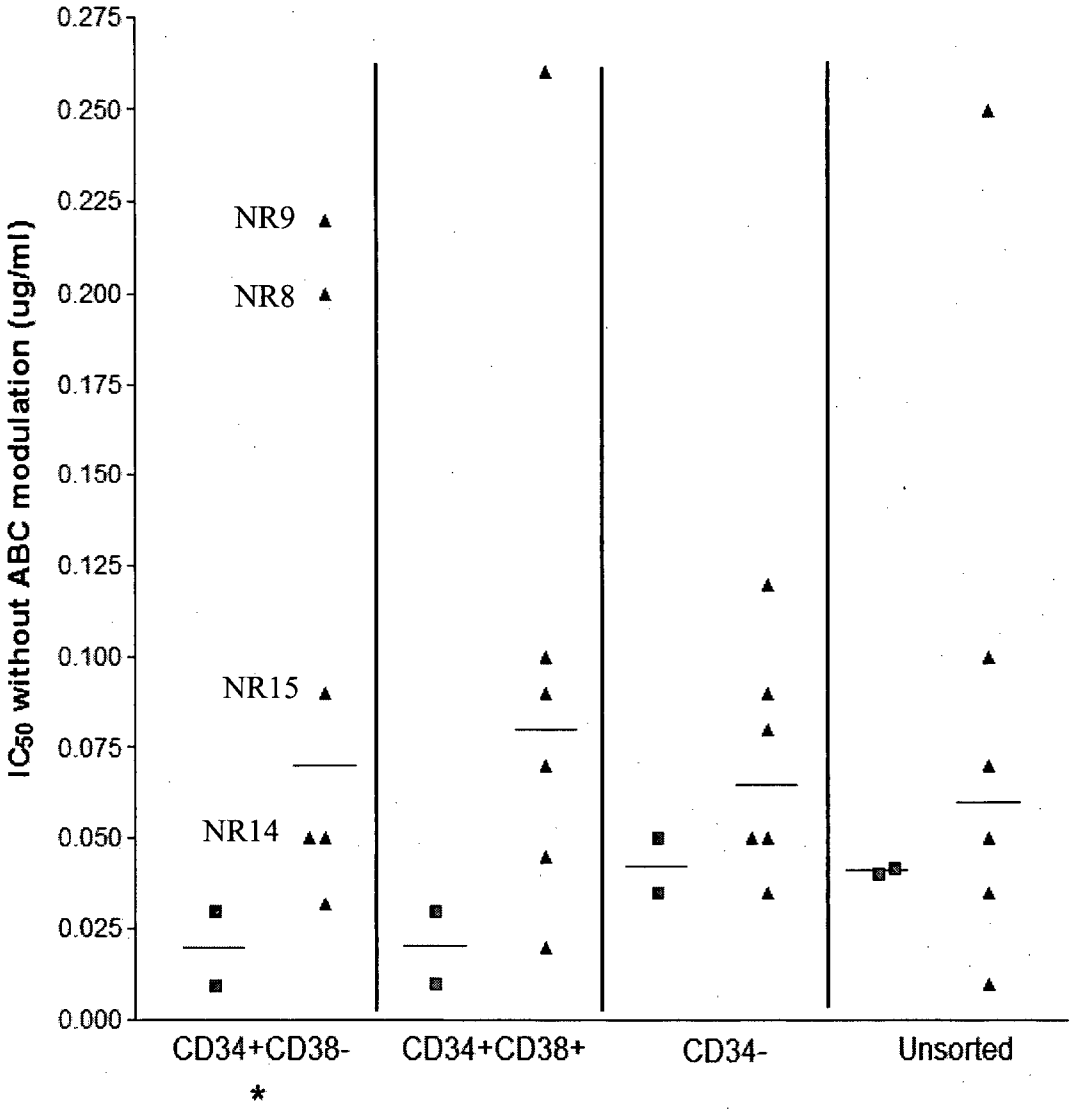


Figure 4.10. Daunorubicin sensitivity of different AML subpopulations in CR and NR patients. IC_{50} of daunorubicin on AML cells without ABC modulation was plotted for unsorted cells and each subpopulation. Square, CR patients. Triangle, NR patients. Patients with high *MDR1/BCRP1* expression are identified beside their symbols. Horizontal line represents median of each group. Statistical significance in difference between CR and NR indicated by an asterisk *.

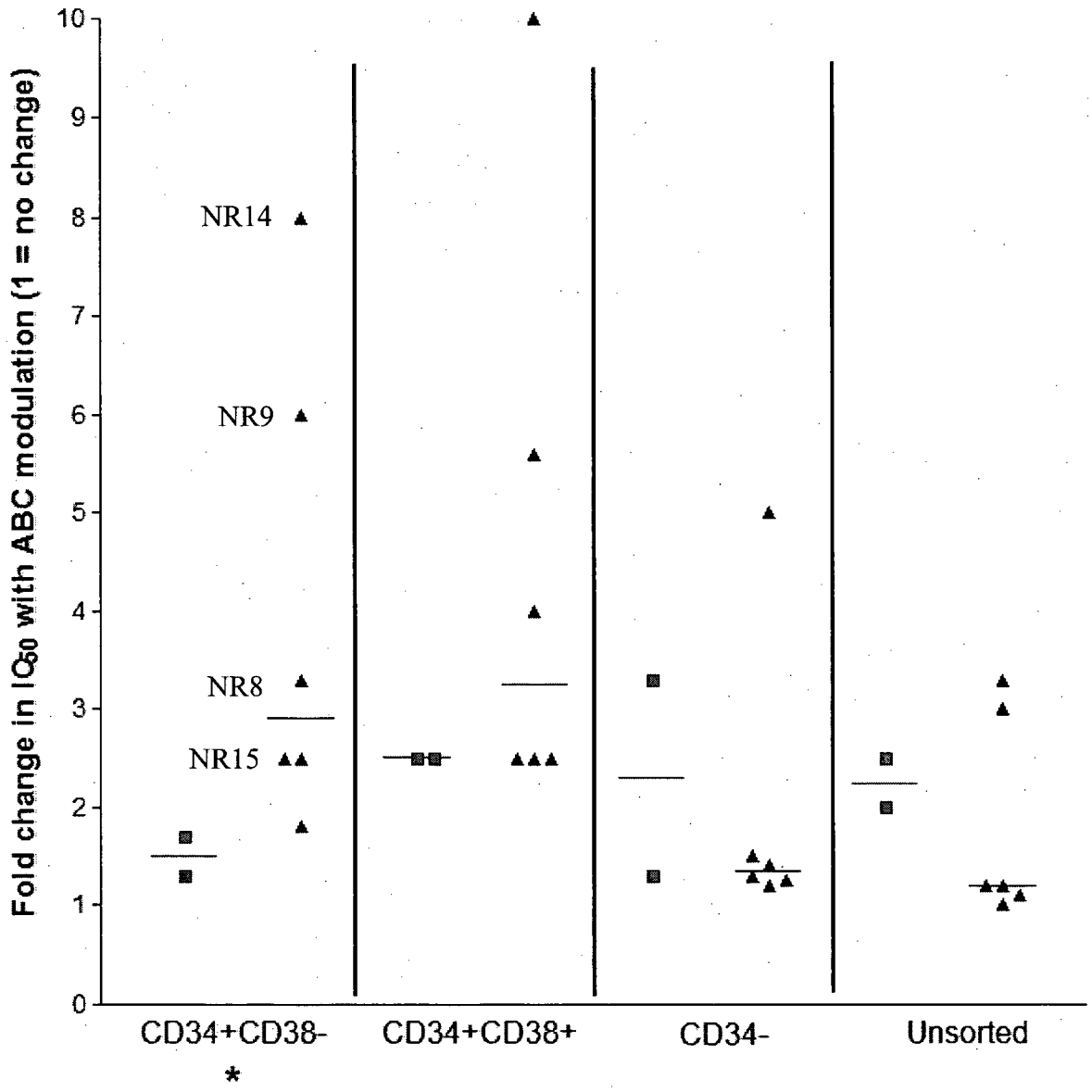


Figure 4.11. Effects of ABC modulation on drug sensitivity in different AML subpopulations. Fold-change in daunorubicin IC_{50} by the highest dose of ABC inhibitor (IC_{50} unmodulated / IC_{50} inhibited) was plotted for unsorted cells and each subpopulation. Square, CR patients. Triangle, NR patients. Patients with high *MDR1/BCRP1* expression are identified beside their symbols. Horizontal line represents median of each group. Statistical significance in difference between CR and NR indicated by an asterisk *.

V Conclusion and future prospects

5.1 – Overall discussion and conclusion

Drug resistance has been a major obstacle in cancer treatment. Some forms of resistance, which result from alteration of a specific drug target or loss of the surface receptor for a given drug, are specific to a small number of related drugs and can be overcome by combination therapy[164]. The emergence of MDR, however, which involves resistance to multiple unrelated drugs, poses a far more difficult problem for which combination therapy is not a solution. To effectively reverse MDR, an understanding of the underlying mechanisms is necessary. This thesis sought to address the problem of MDR in AML by investigating the role of ABC transporters, a classic contributor to MDR in cell lines.

In Chapter 2, I applied the RT-Real Time PCR assay to profile and compare the expression levels of the 47 known human ABC transporters between the AML responders and non-responders to initial chemotherapy. This is the first systematic study on the prognostic significance of the full ABC transporter superfamily in AML. I first asked whether some of these transporters, especially the MDR-related transporters, might be present in high levels in the bulk leukemic cells from NR patients. However, I found no consistent difference in the expression of any ABC gene between the bulk samples of the two patient groups. Based on the LSC model, I then hypothesized that expression differences might be hidden within the most primitive subpopulations. This was addressed in Chapter 3, where subpopulations along the leukemic functional hierarchy were isolated and profiled for expression of MDR-related ABC transporters. High *MDR1* and/or *BCRP1* expression in the primitive CD34+CD38- fraction was found to be consistently associated with NR outcomes. Neither the more committed CD34+CD38+

progenitors nor the mature CD34- cells provided such an association. This is the first indication of a possible prognostic value of ABC transporter expression in the CD34+CD38- cells, the small subpopulation that is thought to be responsible for maintenance of the leukemia in patients and for relapses.

In Chapter 4, I further investigated the *ex vivo* drug sensitivity of patient subpopulations and functional relevance of ABC transporters. Using the Annexin V-PI apoptotic assay, I confirmed that ABC-dependent resistance, corresponding to high *MDR1/BCRP1* expression and reversibility by ABC-specific inhibitors, is common among non-responsive patients, particularly those with a normal karyotype. This suggested that the expressed PGP/BCRP1 are actively extruding drugs from the LSC and thus may make a significant contributor to intrinsic drug resistance *in vivo*.

My study demonstrated that the properties of primitive subpopulations may facilitate better understanding of how a cancer operates than examination of the properties of the bulk cells. In particular, the drug response of the primitive CD34+CD38- AML subpopulation seems a more accurate predictor of treatment outcome than the bulk leukemic population. This is in line with the LSC model and calls for a continued research focus on this small fraction of cells. Of note, the very low mRNA levels observed (especially for *BCRP1* which was consistently below the biological reference line) even in the relative "high" expressers hints towards a further subset within the CD34+CD38- fraction that expresses much higher levels. Hence although CD34+CD38- marks a subpopulation enriched in tumorigenicity that is important for prognostic and therapeutic purposes, it is likely a heterogeneous group of cells in itself.

Figure 5.1 shows the different proposed models of drug resistance in cancer[3]. The conventional model of cancer drug resistance (Figure 5.1A) conceives that a number of cells

acquired mutations that confer drug resistance. These cells outgrow the others to form a new resistant tumor population following chemotherapy. In the CSC model (Figure 5.1B), the CSC (CD34+CD38- in AML) is inherently drug-resistant. At least some of these survive chemotherapy to regenerate a tumor similar to the original disease. A variation of the CSC model, the acquired-resistance CSC model (Figure 5.1C), posits that additional mutations in the surviving CSC generate a drug-resistant tumor. In the last intrinsic resistance model (Figure 5.1D), both the CSC and its descendants are intrinsically drug-resistant. Therapies are ineffective, resulting in uncontrolled tumor growth. The results of my study are consistent with the CSC model and the acquired-resistance model in that CD34+CD38- cells with intrinsic high *MDR1/BCRP1* expression and activity can survive chemotherapy. These can drive tumor regeneration that is rapid enough to result in persistent disease, hence failure to achieve complete remission (no detectable leukemic cells). In this respect, non-responsiveness may be viewed as a very fast relapse within the chemotherapeutic regimen. During or following initial therapy, the primitive fraction may acquire further mutations that confer MDR to its descendants as well (acquired resistance model). ABC transporter expression may also be induced in the general population upon drug exposure, producing a drug resistant disease often seen in AML relapse.

The presence of drug resistance mechanisms such as ABC transporters in the LSC is a more accurate predictor of response than the *de novo* size of this fraction. Thus not all LSC are resistant enough to withstand the initial high-dose chemotherapy given to AML patients. Those patients without high *MDR1/BCRP1* expression in CD34+CD38- cells responded well to initial therapy, while those that apparently retained this normal HSC defense system did not respond. Clearly, AML is a heterogeneous disease and the LSC that originates it possesses varying properties in each patient.

It is interesting that non-responders with high ABC expression (*MDR1/BCRP1*) appear well separated from the other non-responders. Practically, this may represent a convenient means to distinguish the ABC-dependent (high expression) from the ABC-independent (low expression) NR patients. Conceptually, this segregation also demonstrates the value in identifying outliers within a pre-defined group, an approach that has recently been successfully applied by Tomlins and colleagues to discover the TMPRSS2-ETS fusion in prostate cancer[165]. Cancer as a dynamic disease can display heterogeneity within both itself (as exemplified by expression differences observed among subpopulations) and among its assigned "type" and "group", such as responders and non-responders in this study. In fact, it is important to bear in mind that these "subcategories" merely serve as working definitions and hence is subjected to redefinition or further division as more information became known. In this case, patients within the NR group appear to fall into the ABC-dependent and ABC-independent subcategories that may require different strategies in treatment (Section 5.2).

Overall, my work fulfilled all three objectives: to profile expression levels of the ABC transporter superfamily in total AML patient samples, to compare expression of MDR-related ABC transporters in subpopulations along the leukemic hierarchy, and to investigate the drug resistance of subpopulations and functional activity of ABC transporters. Based on my studies, the following conclusions are reached. First, expression of ABC transporters in *de novo* unfractionated patient samples is not predictive of response. Second, high intrinsic levels of *MDR1* and/or *BCRP1* in the primitive CD34+CD38- fraction are associated with poor response to initial chemotherapy. Third, high *ex vivo* functional activity correlates with high expression levels of *MDR1/BCRP1* in CD34+CD38- cells. Fourth, ABC-dependent drug resistance in CD34+CD38- is common among non-responders, especially those with normal cytogenetics, and

is reversible via ABC-inhibition. Taken as a whole, my studies suggest a prognostic significance of ABC transporters in the primitive CD34+CD38- leukemic subpopulation, and support a modified approach in investigating the value of ABC modulating agents in AML, as discussed in the next section.

5.2 – Future Prospects

Previous clinical trials of PGP inhibition, such as second-generation inhibitor PSC-833, yielded largely negative results in AML, with high toxicity and lack of significant improvement in outcome[83,84]. My findings suggest a functional role of ABC transporters in the primitive, disease-maintaining fraction of some NR AML patients. Based on this initial study, disappointing results from clinical ABC inhibition can be viewed in a new light. *MDR1*/PGP expression and activity was low in all CR samples and half of the NR samples, to whom PGP inhibition is unlikely to have a significant effect. Because past studies did not distinguish these patients from the small subset of ABC-expressing non-responders, clinical benefits of ABC modulation was probably diluted and underestimated.

My work raises the importance and feasibility of pre-screening patients for targeted therapy in AML. While ABC activity may not be the major mechanism of MDR in all non-responders, it may be possible to identify those where ABC transporter expression is a major factor for drug resistance and apply appropriate therapeutic intervention. Figure 5.2 outlines a proposed scheme for predicting treatment response and overcoming MDR. Before a patient is subjected to chemotherapy, levels of *MDR1/BCRP1* in the CD34+CD38- fraction can be quickly determined. Patients with high expression of either ABC transporter are predicted to be ABC-dependent non-responders (to chemotherapy alone). These are likely to significantly benefit from the combination of conventional chemotherapy and ABC inhibitors, outweighing the high toxicity effects. The low-expressers are predicted to be either responsive to chemotherapy alone or ABC-independent non-responders and may be spared of the toxicity of ABC inhibition. As

more MDR-related factors (factor X) are characterized, it may be possible to incorporate additional testing and targeted-therapeutic options to the treatment scheme.

As discussed in the introduction, cytogenetic aberrations are the major prognostic factor in AML. But for patients who are karyotypically normal (half of all patients), only a few molecular biomarkers have been investigated (discussed in Section 1.3). Most of the patients (25/31) profiled for ABC transporter expression in my study belonged to this category. In search for other prognostic markers especially for this cytogenetically normal group, I have identified high expression and activity of *MDR1/BCRP1* in CD34+CD38- cells as one significant predictive factor of poor response. Although this was a common MDR mechanism among the patients tested, however, about half of the non-responders appear relatively independent of ABC activity. Evidently, ABC transporters are not the only determinants for treatment outcome.

Technical advancements in recent years have opened up new possibilities to study the molecular genetics of a disease. One such technique, comparative genomic hybridization (CGH), can be used to detect micro amplifications and deletions in DNA. Recent construction by Lam and colleagues of a high-resolution array with over 30,000 BAC clones that cover the entire human genome allows analysis of copy-number changes in the global genome, revealing down to gene-size alterations (Figure 5.3)[166]. While half of the patients are cytogenetically normal, there may be micro-scale alterations in their cancer genome not detected by karyotyping. It is possible that these micro-amplifications and deletions recur at the loci of novel genes that account for the lack of response to chemotherapy.

As a further extension of my thesis, I would seek to explore genomic changes occurring in AML samples using the novel array-CGH technique. I hypothesize that micro genomic differences can be detected between drug sensitive and resistant patients. The objective of this

study would be two-fold: first, to determine whether high expression of *MDR1* and *BCRP1* in NR cells is due to micro-amplifications; second, to see if additional amplified markers can be identified in a genomic signature predictive of clinical response. Eight CR patients and nine NR patients (17 in total) from previous studies were selected for arrayCGH, 12 of which have normal cytogenetics. Microalterations not reported by cytogenetics have been found in each sample. As shown in Figure 5.4, micro-gains and losses were observed in the different chromosomes of patient NR#3 (A). For example, there was a fragment loss on the p arm of chromosome 9 (B) not detected by cytogenetics, and even smaller but clear micro-amplifications and micro-deletions on chromosome 5q. Some of these gains and losses were recurrent in the samples tested, which may indicate genes critical to leukemogenesis. Furthermore, some differences are seen between the CR and NR samples. Further work on these may provide insight into finding possible prognostic patterns in the *de novo* leukemic genome to predict treatment response, and identifying novel genes that contribute to the pathogenesis and/or drug response of AML.

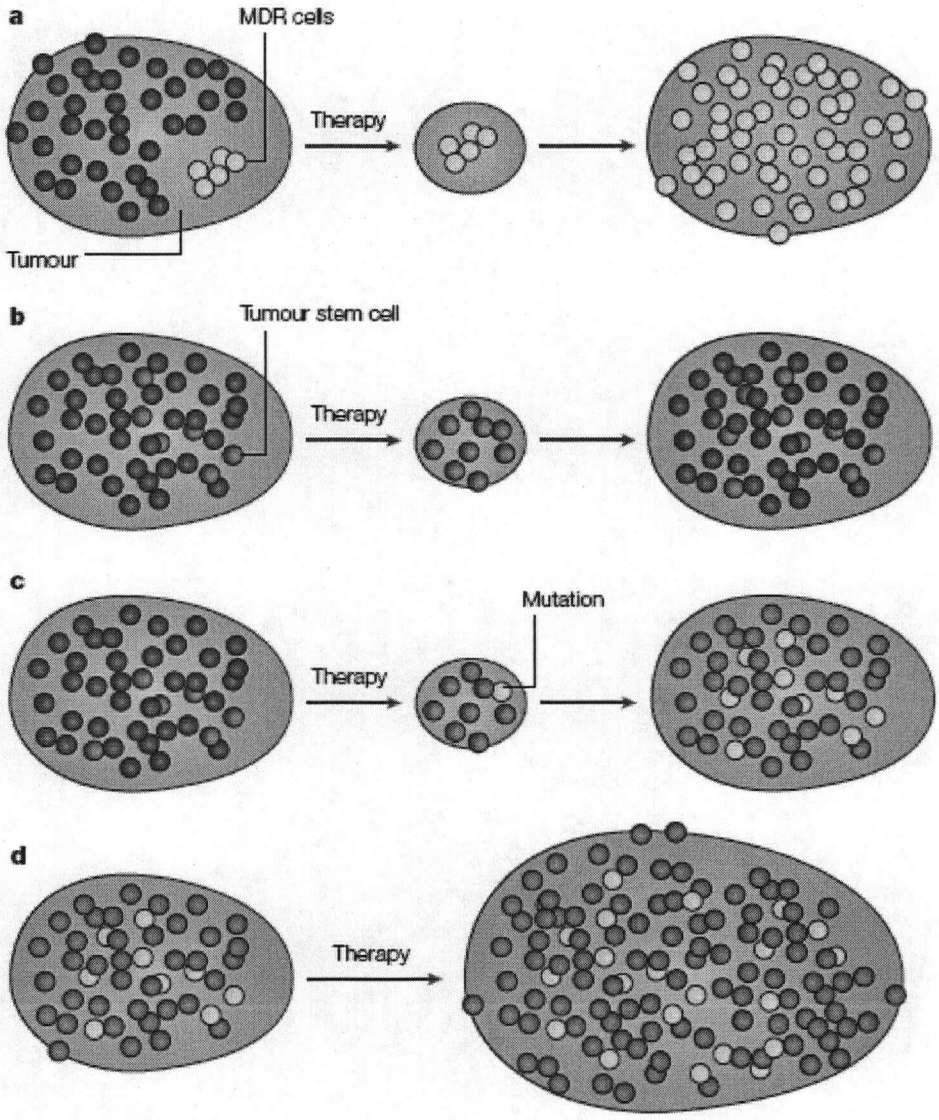


Figure 5.1. Models of tumor drug resistance. A, in the conventional model of cancer drug resistance, rare cells with genetic alterations that confer MDR form a resistance clone (yellow). These cells survive chemotherapy and proliferate, giving rise to relapsed disease with offspring of the resistant clone. B, in the CSC model, the CSC (red) that has protective mechanisms survive chemotherapy while the committed cells (blue) are killed. The CSC repopulates a functional tumor hierarchy. C, in the acquired resistance CSC model, CSC surviving chemotherapy accumulates mutations (yellow) conferring a resistant phenotype in also the committed descendants (purple). D, in the intrinsic resistance model, both the stem cells and the committed cells are inherently resistant. Therapy has no effect, resulting in tumor expansion. Reproduced from Dean et al, *Nature Reviews/Cancer*, 2005[3].

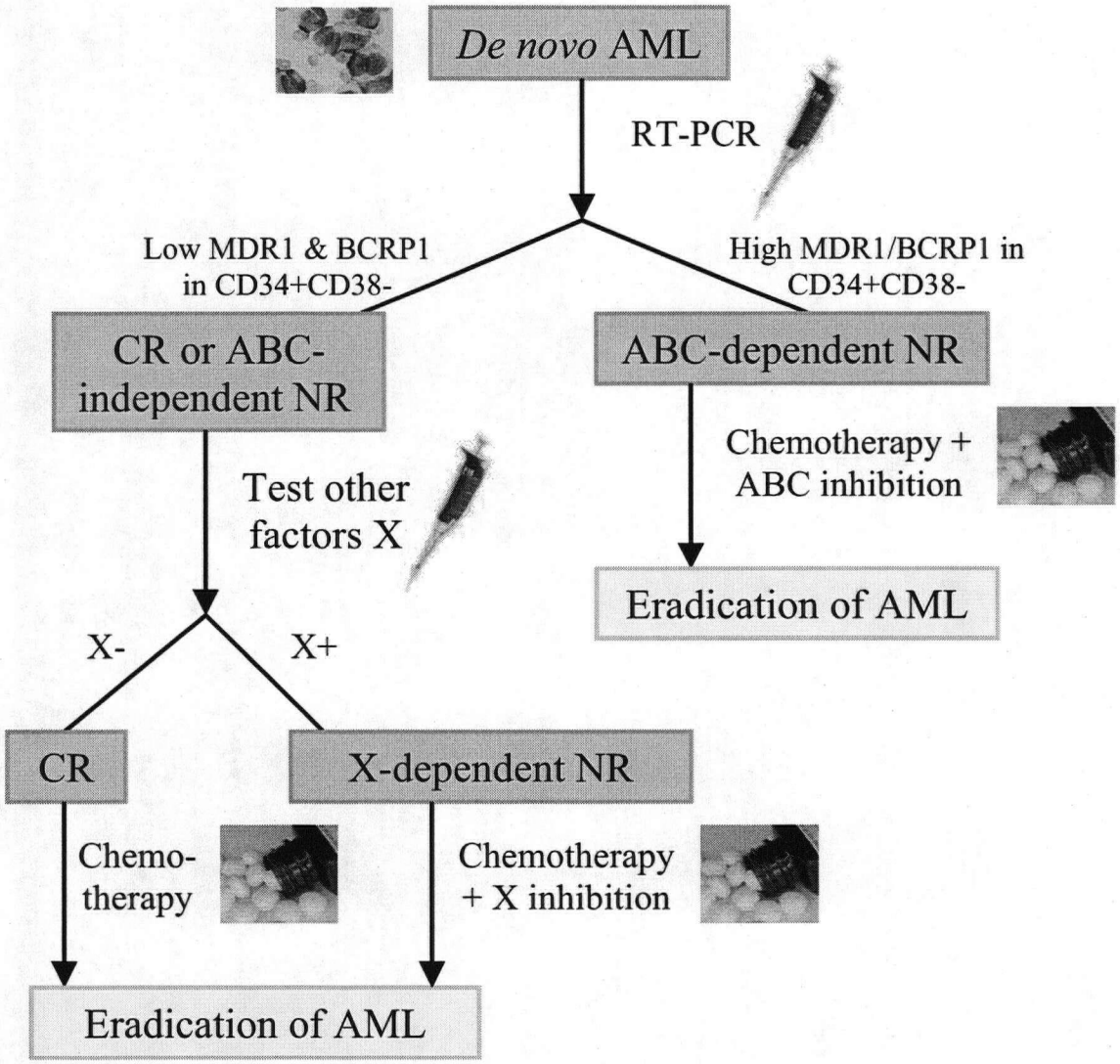


Figure 5.2. Predicting response and overcoming MDR. At diagnosis, AML patients can be tested for *MDR1* and *BCRP1* expression in the CD34+CD38- cells. Those with high expression are likely ABC-dependent non-responders (NR) to chemotherapy alone and will benefit from the combination of chemotherapy and ABC inhibitors. Patients with low ABC expression are either responders who will achieve complete remission with chemotherapy alone (CR) or ABC-independent non-responders who has alternate drug resistance mechanisms. As more knowledge becomes available, patients may be tested for other MDR-related factors (X) and appropriate therapeutic intervention may be applied for eradication of the disease.

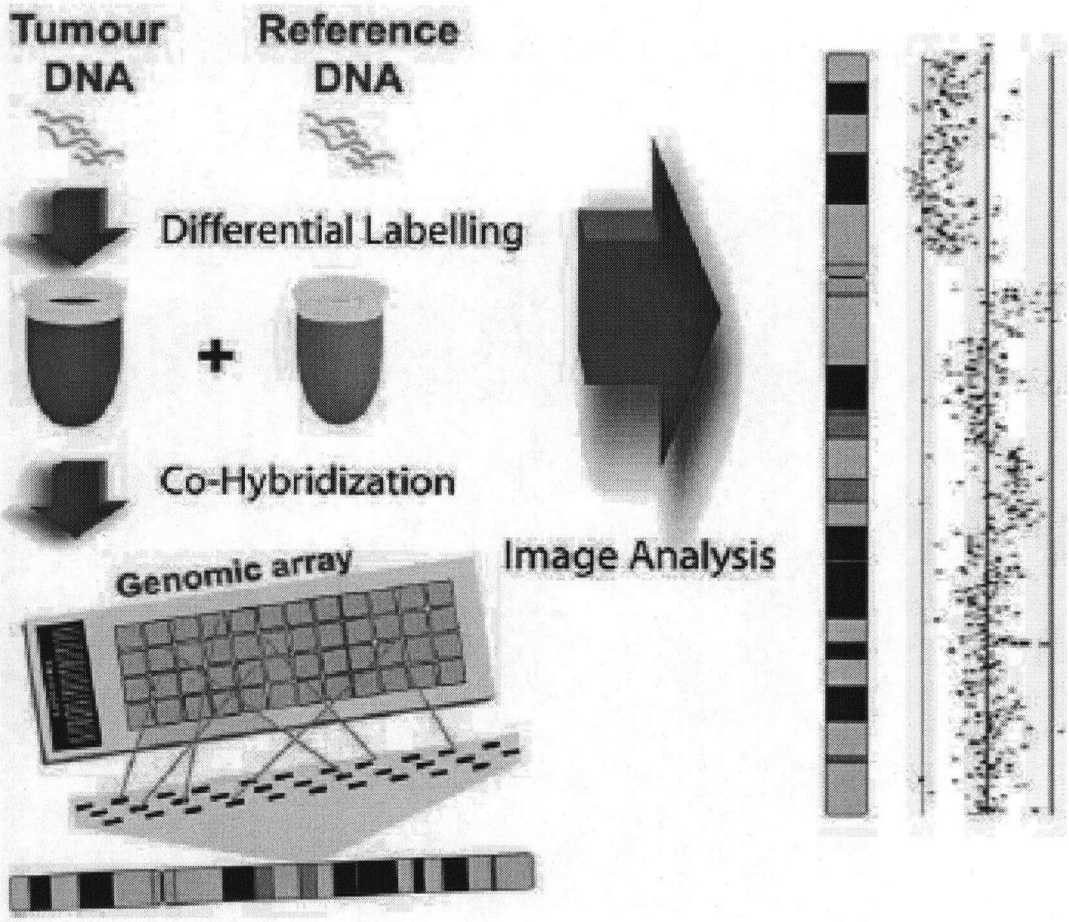


Figure 5.3. Principles of array comparative genomic hybridization. A, normal and tumor DNA samples are isolated and used to create fluorescently labeled probes, commonly with cyanine 3 (Cy3, green) and cyanine 5 (Cy5, red) dyes. The probes are pooled and competitively cohybridized to a glass slide spotted with a known array of mapped genomic clones. The arrays are analyzed with a microarray scanner, producing an image that is used to assess the log2 ratios of the Cy5 to Cy3 intensities for each clone. B, A log2 ratio profile is assembled to determine relative copy number changes between the cancer and the normal samples. Each dot on the graph represents a clone. Values to the left of "0" indicate a loss of a genomic region, while values to the right indicate a gain or amplification. Values at "0" indicate no change. Reproduced from Davies et al, *Chromosome Research*, 2005.

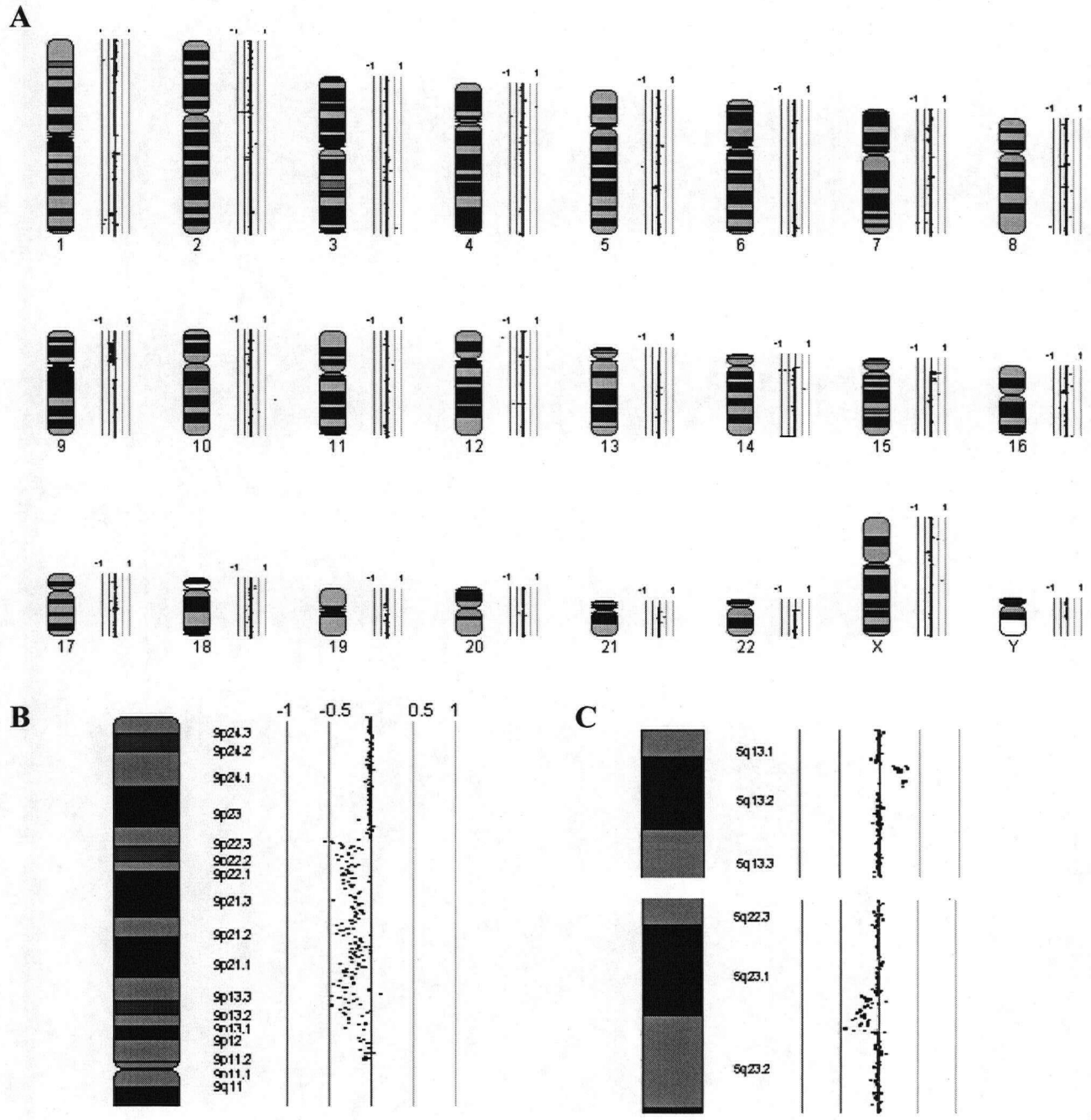


Figure 5.4. CGH karyogram pf patient NR#3. DNA from patient NR#3 was isolated and subjected to arrayCGH. The raw data was analyzed using the SeeGH program to generate a karygram for every chromosome. Each dot is a BAC clone representing a small section of DNA. Values to the left of the centre line ("0") indicate a loss, and values to the right indicate a gain. A, microalterations on the 23 chromosome pairs. B, Fragment loss on chromosome 9p. C, micro-amplifications and micro-deletions on chromosome 5q.

Bibliography

1. Dean M, Rzhetsky A, Allikmets R. The human ATP-binding cassette (ABC) transporter superfamily. Genome Res. 2001;11:1156-1166.
2. Huntly BJ, Gilliland DG. Leukaemia stem cells and the evolution of cancer-stem-cell research. Nat Rev Cancer. 2005;5:311-321.
3. Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. Nat Rev Cancer. 2005;5:275-284.
4. Del Poeta G, Venditti A, Stasi R, et al. P-glycoprotein and terminal transferase expression identify prognostic subsets within cytogenetic risk classes in acute myeloid leukemia. Leuk Res. 1999;23:451-465.
5. Chang G, Roth CB. Structure of MsbA from E. coli: a homolog of the multidrug resistance ATP binding cassette (ABC) transporters. Science. 2001;293:1793-1800.
6. Grimwade D, Walker H, Oliver F, et al. The importance of diagnostic cytogenetics on outcome in AML: analysis of 1,612 patients entered into the MRC AML 10 trial. The Medical Research Council Adult and Children's Leukaemia Working Parties. Blood. 1998;92:2322-2333.
7. Wuchter C, Leonid K, Ruppert V, et al. Clinical significance of P-glycoprotein expression and function for response to induction chemotherapy, relapse rate and overall survival in acute leukemia. Haematologica. 2000;85:711-721.
8. Canadian Cancer Statistics. 2006.
9. Ozols RF, Herbst RS, Colson YL, et al. Clinical cancer advances 2006: major research advances in cancer treatment, prevention, and screening--a report from the American Society of Clinical Oncology. J Clin Oncol. 2007;25:146-162.
10. Collins I, Workman P. New approaches to molecular cancer therapeutics. Nat Chem Biol. 2006;2:689-700.
11. Druker BJ, Sawyers CL, Kantarjian H, et al. Activity of a specific inhibitor of the BCR-ABL tyrosine kinase in the blast crisis of chronic myeloid leukemia and acute lymphoblastic leukemia with the Philadelphia chromosome. N Engl J Med. 2001;344:1038-1042.
12. Druker BJ, Talpaz M, Resta DJ, et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. N Engl J Med. 2001;344:1031-1037.
13. Kantarjian HM, Talpaz M, Giles F, O'Brien S, Cortes J. New insights into the pathophysiology of chronic myeloid leukemia and imatinib resistance. Ann Intern Med. 2006;145:913-923.
14. Stirewalt DL, Radich JP. The role of FLT3 in haematopoietic malignancies. Nat Rev Cancer. 2003;3:650-665.
15. Whitman SP, Archer KJ, Feng L, et al. Absence of the wild-type allele predicts poor prognosis in adult de novo acute myeloid leukemia with normal cytogenetics and the internal tandem duplication of FLT3: a cancer and leukemia group B study. Cancer Res. 2001;61:7233-7239.
16. Frohling S, Schlenk RF, Breitruck J, et al. Prognostic significance of activating FLT3 mutations in younger adults (16 to 60 years) with acute myeloid leukemia and normal cytogenetics: a study of the AML Study Group Ulm. Blood. 2002;100:4372-4380.
17. Kainz B, Heintel D, Marculescu R, et al. Variable prognostic value of FLT3 internal tandem duplications in patients with de novo AML and a normal karyotype, t(15;17), t(8;21) or inv(16). Hematol J. 2002;3:283-289.

18. Smith BD, Levis M, Beran M, et al. Single-agent CEP-701, a novel FLT3 inhibitor, shows biologic and clinical activity in patients with relapsed or refractory acute myeloid leukemia. Blood. 2004;103:3669-3676.
19. Stone RM, DeAngelo DJ, Klimek V, et al. Patients with acute myeloid leukemia and an activating mutation in FLT3 respond to a small-molecule FLT3 tyrosine kinase inhibitor, PKC412. Blood. 2005;105:54-60.
20. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med. 1997;3:730-737.
21. Mitus A, Rosenthal D. The Adult Leukemias. Clinical Oncology. 1999.
22. Schoch C, Haferlach T. Cytogenetics in acute myeloid leukemia. Curr Oncol Rep. 2002;4:390-397.
23. Mrozek K, Heinonen K, Bloomfield CD. Prognostic value of cytogenetic findings in adults with acute myeloid leukemia. Int J Hematol. 2000;72:261-271.
24. Mrozek K, Heerema NA, Bloomfield CD. Cytogenetics in acute leukemia. Blood Rev. 2004;18:115-136.
25. DeVita JV, Hellman S, Rosenberg S. Cancer Principles and practice of oncology. 2001;2nd edition.
26. Miyoshi H, Shimizu K, Kozu T, Maseki N, Kaneko Y, Ohki M. t(8;21) breakpoints on chromosome 21 in acute myeloid leukemia are clustered within a limited region of a single gene, AML1. Proc Natl Acad Sci U S A. 1991;88:10431-10434.
27. Warrell RP, Jr., de The H, Wang ZY, Degos L. Acute promyelocytic leukemia. N Engl J Med. 1993;329:177-189.
28. Byrd JC, Mrozek K, Dodge RK, et al. Pretreatment cytogenetic abnormalities are predictive of induction success, cumulative incidence of relapse, and overall survival in adult patients with de novo acute myeloid leukemia: results from Cancer and Leukemia Group B (CALGB 8461). Blood. 2002;100:4325-4336.
29. Grimwade D, Walker H, Harrison G, et al. The predictive value of hierarchical cytogenetic classification in older adults with acute myeloid leukemia (AML): analysis of 1065 patients entered into the United Kingdom Medical Research Council AML11 trial. Blood. 2001;98:1312-1320.
30. Slovak ML, Kopecky KJ, Cassileth PA, et al. Karyotypic analysis predicts outcome of preremission and postremission therapy in adult acute myeloid leukemia: a Southwest Oncology Group/Eastern Cooperative Oncology Group Study. Blood. 2000;96:4075-4083.
31. Fenaux P, Chevret S, Guerci A, et al. Long-term follow-up confirms the benefit of all-trans retinoic acid in acute promyelocytic leukemia. European APL group. Leukemia. 2000;14:1371-1377.
32. Tallman MS, Andersen JW, Schiffer CA, et al. All-trans retinoic acid in acute promyelocytic leukemia: long-term outcome and prognostic factor analysis from the North American Intergroup protocol. Blood. 2002;100:4298-4302.
33. Shen ZX, Chen GQ, Ni JH, et al. Use of arsenic trioxide (As2O3) in the treatment of acute promyelocytic leukemia (APL): II. Clinical efficacy and pharmacokinetics in relapsed patients. Blood. 1997;89:3354-3360.
34. Soignet SL, Frankel SR, Douer D, et al. United States multicenter study of arsenic trioxide in relapsed acute promyelocytic leukemia. J Clin Oncol. 2001;19:3852-3860.

35. Raffoux E, Rousselot P, Poupon J, et al. Combined treatment with arsenic trioxide and all-trans-retinoic acid in patients with relapsed acute promyelocytic leukemia. J Clin Oncol. 2003;21:2326-2334.
36. Shen ZX, Shi ZZ, Fang J, et al. All-trans retinoic acid/As2O3 combination yields a high quality remission and survival in newly diagnosed acute promyelocytic leukemia. Proc Natl Acad Sci U S A. 2004;101:5328-5335.
37. Garson OM, Hagemeijer A, Sakurai M, et al. Cytogenetic studies of 103 patients with acute myelogenous leukemia in relapse. Cancer Genet Cytogenet. 1989;40:187-202.
38. Frohling S, Skelin S, Liebisch C, et al. Comparison of cytogenetic and molecular cytogenetic detection of chromosome abnormalities in 240 consecutive adult patients with acute myeloid leukemia. J Clin Oncol. 2002;20:2480-2485.
39. Trost D, Hildebrandt B, Muller N, Germing U, Royer-Pokora B. Hidden chromosomal aberrations are rare in primary myelodysplastic syndromes with evolution to acute myeloid leukaemia and normal cytogenetics. Leuk Res. 2004;28:171-177.
40. Casas S, Aventin A, Fuentes F, et al. Genetic diagnosis by comparative genomic hybridization in adult de novo acute myelocytic leukemia. Cancer Genet Cytogenet. 2004;153:16-25.
41. Bullinger L, Dohner K, Bair E, et al. Use of gene-expression profiling to identify prognostic subclasses in adult acute myeloid leukemia. N Engl J Med. 2004;350:1605-1616.
42. Valk PJ, Verhaak RG, Beijen MA, et al. Prognostically useful gene-expression profiles in acute myeloid leukemia. N Engl J Med. 2004;350:1617-1628.
43. Marcucci G, Mrozek K, Bloomfield CD. Molecular heterogeneity and prognostic biomarkers in adults with acute myeloid leukemia and normal cytogenetics. Curr Opin Hematol. 2005;12:68-75.
44. Hess JL. MLL: a histone methyltransferase disrupted in leukemia. Trends Mol Med. 2004;10:500-507.
45. Frohling S, Schlenk RF, Stolze I, et al. CEBPA mutations in younger adults with acute myeloid leukemia and normal cytogenetics: prognostic relevance and analysis of cooperating mutations. J Clin Oncol. 2004;22:624-633.
46. Thiede C, Koch S, Creutzig E, et al. Prevalence and prognostic impact of NPM1 mutations in 1485 adult patients with acute myeloid leukemia (AML). Blood. 2006;107:4011-4020.
47. Baldus CD, Tanner SM, Ruppert AS, et al. BAALC expression predicts clinical outcome of de novo acute myeloid leukemia patients with normal cytogenetics: a Cancer and Leukemia Group B Study. Blood. 2003;102:1613-1618.
48. Childs S, Ling V. The MDR superfamily of genes and its biological implications. Important Adv Oncol. 1994:21-36.
49. Higgins CF. ABC transporters: from microorganisms to man. Annu Rev Cell Biol. 1992;8:67-113.
50. Walker JE, Saraste M, Runswick MJ, Gay NJ. Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. Embo J. 1982;1:945-951.
51. Hyde SC, Emsley P, Hartshorn MJ, et al. Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. Nature. 1990;346:362-365.

52. Dawson RJ, Locher KP. Structure of a bacterial multidrug ABC transporter. Nature. 2006;443:180-185.
53. Juliano RL, Ling V. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochim Biophys Acta. 1976;455:152-162.
54. Rao VV, Dahlheimer JL, Bardgett ME, et al. Choroid plexus epithelial expression of MDR1 P glycoprotein and multidrug resistance-associated protein contribute to the blood-cerebrospinal-fluid drug-permeability barrier. Proc Natl Acad Sci U S A. 1999;96:3900-3905.
55. Borst P, Evers R, Kool M, Wijnholds J. The multidrug resistance protein family. Biochim Biophys Acta. 1999;1461:347-357.
56. Gottesman MM, Ambudkar SV. Overview: ABC transporters and human disease. J Bioenerg Biomembr. 2001;33:453-458.
57. Schinkel AH, Smit JJ, van Tellingen O, et al. Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. Cell. 1994;77:491-502.
58. Borst P, Evers R, Kool M, Wijnholds J. A family of drug transporters: the multidrug resistance-associated proteins. J Natl Cancer Inst. 2000;92:1295-1302.
59. Wijnholds J, deLange EC, Scheffer GL, et al. Multidrug resistance protein 1 protects the choroid plexus epithelium and contributes to the blood-cerebrospinal fluid barrier. J Clin Invest. 2000;105:279-285.
60. Doyle LA, Yang W, Abruzzo LV, et al. A multidrug resistance transporter from human MCF-7 breast cancer cells. Proc Natl Acad Sci U S A. 1998;95:15665-15670.
61. Miyake K, Mickley L, Litman T, et al. Molecular cloning of cDNAs which are highly overexpressed in mitoxantrone-resistant cells: demonstration of homology to ABC transport genes. Cancer Res. 1999;59:8-13.
62. Jonker JW, Buitelaar M, Wagenaar E, et al. The breast cancer resistance protein protects against a major chlorophyll-derived dietary phototoxin and protoporphyria. Proc Natl Acad Sci U S A. 2002;99:15649-15654.
63. Krishnamurthy P, Ross DD, Nakanishi T, et al. The stem cell marker Bcrp/ABCG2 enhances hypoxic cell survival through interactions with heme. J Biol Chem. 2004;279:24218-24225.
64. Fojo AT, Ueda K, Slamon DJ, Poplack DG, Gottesman MM, Pastan I. Expression of a multidrug-resistance gene in human tumors and tissues. Proc Natl Acad Sci U S A. 1987;84:265-269.
65. Goldstein LJ, Galski H, Fojo A, et al. Expression of a multidrug resistance gene in human cancers. J Natl Cancer Inst. 1989;81:116-124.
66. Roninson IB. From amplification to function: the case of the MDR1 gene. Mutat Res. 1992;276:151-161.
67. Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. Nat Rev Cancer. 2002;2:48-58.
68. Ross DD, Karp JE, Chen TT, Doyle LA. Expression of breast cancer resistance protein in blast cells from patients with acute leukemia. Blood. 2000;96:365-368.
69. Diestra JE, Scheffer GL, Catala I, et al. Frequent expression of the multi-drug resistance-associated protein BCRP/MXR/ABCP/ABCG2 in human tumours detected by the BXP-21 monoclonal antibody in paraffin-embedded material. J Pathol. 2002;198:213-219.
70. Kawabata S, Oka M, Soda H, et al. Expression and functional analyses of breast cancer resistance protein in lung cancer. Clin Cancer Res. 2003;9:3052-3057.

71. Mack JT, Beljanski V, Tew KD, Townsend DM. The ATP-binding cassette transporter ABCA2 as a mediator of intracellular trafficking. Biomed Pharmacother. 2006;60:587-592.
72. Childs S, Yeh RL, Hui D, Ling V. Taxol resistance mediated by transfection of the liver-specific sister gene of P-glycoprotein. Cancer Res. 1998;58:4160-4167.
73. Allikmets R, Schriml LM, Hutchinson A, Romano-Spica V, Dean M. A human placenta-specific ATP-binding cassette gene (ABCP) on chromosome 4q22 that is involved in multidrug resistance. Cancer Res. 1998;58:5337-5339.
74. Komatani H, Kotani H, Hara Y, et al. Identification of breast cancer resistant protein/mitoxantrone resistance/placenta-specific, ATP-binding cassette transporter as a transporter of NB-506 and J-107088, topoisomerase I inhibitors with an indolocarbazole structure. Cancer Res. 2001;61:2827-2832.
75. Maliepaard M, Scheffer GL, Faneyte IF, et al. Subcellular localization and distribution of the breast cancer resistance protein transporter in normal human tissues. Cancer Res. 2001;61:3458-3464.
76. Cole SP, Bhardwaj G, Gerlach JH, et al. Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. Science. 1992;258:1650-1654.
77. Loe DW, Deeley RG, Cole SP. Characterization of vincristine transport by the M(r) 190,000 multidrug resistance protein (MRP): evidence for cotransport with reduced glutathione. Cancer Res. 1998;58:5130-5136.
78. Jedlitschky G, Leier I, Buchholz U, Barnouin K, Kurz G, Keppler D. Transport of glutathione, glucuronate, and sulfate conjugates by the MRP gene-encoded conjugate export pump. Cancer Res. 1996;56:988-994.
79. Muller M, Meijer C, Zaman GJ, et al. Overexpression of the gene encoding the multidrug resistance-associated protein results in increased ATP-dependent glutathione S-conjugate transport. Proc Natl Acad Sci U S A. 1994;91:13033-13037.
80. Marbeuf-Gueye C, Broxterman HJ, Dubru F, Priebe W, Garnier-Suillerot A. Kinetics of anthracycline efflux from multidrug resistance protein-expressing cancer cells compared with P-glycoprotein-expressing cancer cells. Mol Pharmacol. 1998;53:141-147.
81. Renes J, de Vries EG, Nienhuis EF, Jansen PL, Muller M. ATP- and glutathione-dependent transport of chemotherapeutic drugs by the multidrug resistance protein MRP1. Br J Pharmacol. 1999;126:681-688.
82. Tidefelt U, Liliemark J, Gruber A, et al. P-Glycoprotein inhibitor valspodar (PSC 833) increases the intracellular concentrations of daunorubicin in vivo in patients with P-glycoprotein-positive acute myeloid leukemia. J Clin Oncol. 2000;18:1837-1844.
83. Greenberg PL, Lee SJ, Advani R, et al. Mitoxantrone, etoposide, and cytarabine with or without valspodar in patients with relapsed or refractory acute myeloid leukemia and high-risk myelodysplastic syndrome: a phase III trial (E2995). J Clin Oncol. 2004;22:1078-1086.
84. Baer MR, George SL, Dodge RK, et al. Phase 3 study of the multidrug resistance modulator PSC-833 in previously untreated patients 60 years of age and older with acute myeloid leukemia: Cancer and Leukemia Group B Study 9720. Blood. 2002;100:1224-1232.
85. Chan HS, DeBoer G, Thiessen JJ, et al. Combining cyclosporin with chemotherapy controls intraocular retinoblastoma without requiring radiation. Clin Cancer Res. 1996;2:1499-1508.
86. Ambudkar SV, Dey S, Hrycyna CA, Ramachandra M, Pastan I, Gottesman MM. Biochemical, cellular, and pharmacological aspects of the multidrug transporter. Annu Rev Pharmacol Toxicol. 1999;39:361-398.

87. Marie JP, Zittoun R, Sikic BI. Multidrug resistance (mdr1) gene expression in adult acute leukemias: correlations with treatment outcome and in vitro drug sensitivity. Blood. 1991;78:586-592.
88. Pirker R, Wallner J, Geissler K, et al. MDR1 gene expression and treatment outcome in acute myeloid leukemia. J Natl Cancer Inst. 1991;83:708-712.
89. Leith CP, Kopecky KJ, Chen IM, et al. Frequency and clinical significance of the expression of the multidrug resistance proteins MDR1/P-glycoprotein, MRP1, and LRP in acute myeloid leukemia: a Southwest Oncology Group Study. Blood. 1999;94:1086-1099.
90. Zochbauer S, Gsur A, Brunner R, Kyrle PA, Lechner K, Pirker R. P-glycoprotein expression as unfavorable prognostic factor in acute myeloid leukemia. Leukemia. 1994;8:974-977.
91. Borg AG, Burgess R, Green LM, Scheper RJ, Yin JA. Overexpression of lung-resistance protein and increased P-glycoprotein function in acute myeloid leukaemia cells predict a poor response to chemotherapy and reduced patient survival. Br J Haematol. 1998;103:1083-1091.
92. Lyttelton MP, Hart S, Ganeshaguru K, Hoffbrand AV, Mehta AB. Quantitation of multidrug resistant MDR1 transcript in acute myeloid leukaemia by non-isotopic quantitative cDNA-polymerase chain reaction. Br J Haematol. 1994;86:540-546.
93. Wood P, Burgess R, MacGregor A, Yin JA. P-glycoprotein expression on acute myeloid leukaemia blast cells at diagnosis predicts response to chemotherapy and survival. Br J Haematol. 1994;87:509-514.
94. Zhou DC, Zittoun R, Marie JP. Expression of multidrug resistance-associated protein (MRP) and multidrug resistance (MDR1) genes in acute myeloid leukemia. Leukemia. 1995;9:1661-1666.
95. Michieli M, Damiani D, Ermacora A, et al. P-glycoprotein, lung resistance-related protein and multidrug resistance associated protein in de novo acute non-lymphocytic leukaemias: biological and clinical implications. Br J Haematol. 1999;104:328-335.
96. Broxterman HJ, Sonneveld P, Pieters R, et al. Do P-glycoprotein and major vault protein (MVP/LRP) expression correlate with in vitro daunorubicin resistance in acute myeloid leukemia? Leukemia. 1999;13:258-265.
97. Smyth MJ, Krasovskis E, Sutton VR, Johnstone RW. The drug efflux protein, P-glycoprotein, additionally protects drug-resistant tumor cells from multiple forms of caspase-dependent apoptosis. Proc Natl Acad Sci U S A. 1998;95:7024-7029.
98. Johnstone RW, Cretney E, Smyth MJ. P-glycoprotein protects leukemia cells against caspase-dependent, but not caspase-independent, cell death. Blood. 1999;93:1075-1085.
99. Robinson LJ, Roberts WK, Ling TT, Lamming D, Sternberg SS, Roepe PD. Human MDR 1 protein overexpression delays the apoptotic cascade in Chinese hamster ovary fibroblasts. Biochemistry. 1997;36:11169-11178.
100. van der Kolk DM, de Vries EG, van Putten WJ, et al. P-glycoprotein and multidrug resistance protein activities in relation to treatment outcome in acute myeloid leukemia. Clin Cancer Res. 2000;6:3205-3214.
101. Legrand O, Simonin G, Beauchamp-Nicoud A, Zittoun R, Marie JP. Simultaneous activity of MRP1 and Pgp is correlated with in vitro resistance to daunorubicin and with in vivo resistance in adult acute myeloid leukemia. Blood. 1999;94:1046-1056.
102. Legrand O, Simonin G, Perrot JY, Zittoun R, Marie JP. Pgp and MRP activities using calcein-AM are prognostic factors in adult acute myeloid leukemia patients. Blood. 1998;91:4480-4488.

103. Filipits M, Suchomel RW, Zochbauer S, Brunner R, Lechner K, Pirker R. Multidrug resistance-associated protein in acute myeloid leukemia: No impact on treatment outcome. Clin Cancer Res. 1997;3:1419-1425.
104. Damiani D, Tiribelli M, Calistri E, et al. The prognostic value of P-glycoprotein (ABCB) and breast cancer resistance protein (ABCG2) in adults with de novo acute myeloid leukemia with normal karyotype. Haematologica. 2006;91:825-828.
105. Steinbach D, Sell W, Voigt A, Hermann J, Zintl F, Sauerbrey A. BCRP gene expression is associated with a poor response to remission induction therapy in childhood acute myeloid leukemia. Leukemia. 2002;16:1443-1447.
106. van den Heuvel-Eibrink MM, Wiemer EA, Prins A, et al. Increased expression of the breast cancer resistance protein (BCRP) in relapsed or refractory acute myeloid leukemia (AML). Leukemia. 2002;16:833-839.
107. Abbott BL, Colapietro AM, Barnes Y, Marini F, Andreeff M, Sorrentino BP. Low levels of ABCG2 expression in adult AML blast samples. Blood. 2002;100:4594-4601.
108. van der Kolk DM, Vellenga E, Scheffer GL, et al. Expression and activity of breast cancer resistance protein (BCRP) in de novo and relapsed acute myeloid leukemia. Blood. 2002;99:3763-3770.
109. van der Pol MA, Broxterman HJ, Pater JM, et al. Function of the ABC transporters, P-glycoprotein, multidrug resistance protein and breast cancer resistance protein, in minimal residual disease in acute myeloid leukemia. Haematologica. 2003;88:134-147.
110. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. Nature. 2001;414:105-111.
111. Dalerba P, Cho RW, Clarke MF. Cancer Stem Cells: Models and Concepts. Annu Rev Med. 2006.
112. Huntly BJ, Gilliland DG. Cancer biology: summing up cancer stem cells. Nature. 2005;435:1169-1170.
113. Buick RN, Minden MD, McCulloch EA. Self-renewal in culture of proliferative blast progenitor cells in acute myeloblastic leukemia. Blood. 1979;54:95-104.
114. Turhan AG, Lemoine FM, Debert C, et al. Highly purified primitive hematopoietic stem cells are PML-RARA negative and generate nonclonal progenitors in acute promyelocytic leukemia. Blood. 1995;85:2154-2161.
115. Castor A, Nilsson L, Astrand-Grundstrom I, et al. Distinct patterns of hematopoietic stem cell involvement in acute lymphoblastic leukemia. Nat Med. 2005;11:630-637.
116. Brown D, Kogan S, Lagasse E, et al. A PMLRARalpha transgene initiates murine acute promyelocytic leukemia. Proc Natl Acad Sci U S A. 1997;94:2551-2556.
117. Taipale J, Beachy PA. The Hedgehog and Wnt signalling pathways in cancer. Nature. 2001;411:349-354.
118. Bunting KD. ABC transporters as phenotypic markers and functional regulators of stem cells. Stem Cells. 2002;20:11-20.
119. Chaudhary PM, Roninson IB. Expression and activity of P-glycoprotein, a multidrug efflux pump, in human hematopoietic stem cells. Cell. 1991;66:85-94.
120. Berenson RJ, Bensinger WI, Hill RS, et al. Engraftment after infusion of CD34+ marrow cells in patients with breast cancer or neuroblastoma. Blood. 1991;77:1717-1722.
121. Berenson RJ, Andrews RG, Bensinger WI, et al. Antigen CD34+ marrow cells engraft lethally irradiated baboons. J Clin Invest. 1988;81:951-955.

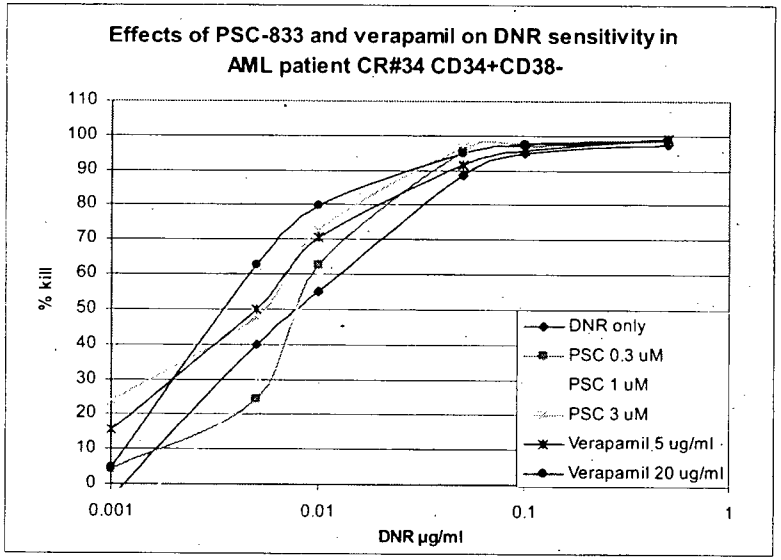
122. Krause DS, Ito T, Fackler MJ, et al. Characterization of murine CD34, a marker for hematopoietic progenitor and stem cells. Blood. 1994;84:691-701.
123. Sato T, Laver JH, Ogawa M. Reversible expression of CD34 by murine hematopoietic stem cells. Blood. 1999;94:2548-2554.
124. Zinovyeva MV, Zijlmans JM, Fibbe WE, Visser JW, Belyavsky AV. Analysis of gene expression in subpopulations of murine hematopoietic stem and progenitor cells. Exp Hematol. 2000;28:318-334.
125. Scharenberg CW, Harkey MA, Torok-Storb B. The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. Blood. 2002;99:507-512.
126. Zhou S, Schuetz JD, Bunting KD, et al. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nat Med. 2001;7:1028-1034.
127. Uchida N, Fujisaki T, Eaves AC, Eaves CJ. Transplantable hematopoietic stem cells in human fetal liver have a CD34(+) side population (SP)phenotype. J Clin Invest. 2001;108:1071-1077.
128. Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med. 1996;183:1797-1806.
129. Alvi AJ, Clayton H, Joshi C, et al. Functional and molecular characterisation of mammary side population cells. Breast Cancer Res. 2003;5:R1-8.
130. Asakura A, Rudnicki MA. Side population cells from diverse adult tissues are capable of in vitro hematopoietic differentiation. Exp Hematol. 2002;30:1339-1345.
131. Lechner A, Leech CA, Abraham EJ, Nolan AL, Habener JF. Nestin-positive progenitor cells derived from adult human pancreatic islets of Langerhans contain side population (SP) cells defined by expression of the ABCG2 (BCRP1) ATP-binding cassette transporter. Biochem Biophys Res Commun. 2002;293:670-674.
132. Uchida N, Dykstra B, Lyons K, Leung F, Kristiansen M, Eaves C. ABC transporter activities of murine hematopoietic stem cells vary according to their developmental and activation status. Blood. 2004;103:4487-4495.
133. te Boekhorst PA, de Leeuw K, Schoester M, et al. Predominance of functional multidrug resistance (MDR-1) phenotype in CD34+ acute myeloid leukemia cells. Blood. 1993;82:3157-3162.
134. Drenou B, Fardel O, Amiot L, Fauchet R. Detection of P glycoprotein activity on normal and leukemic CD34+ cells. Leuk Res. 1993;17:1031-1035.
135. Haraguchi N, Utsunomiya T, Inoue H, et al. Characterization of a side population of cancer cells from human gastrointestinal system. Stem Cells. 2006;24:506-513.
136. Hirschmann-Jax C, Foster AE, Wulf GG, et al. A distinct "side population" of cells with high drug efflux capacity in human tumor cells. Proc Natl Acad Sci U S A. 2004;101:14228-14233.
137. Kondo T, Setoguchi T, Taga T. Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line. Proc Natl Acad Sci U S A. 2004;101:781-786.
138. Smalley MJ, Clarke RB. The mammary gland "side population": a putative stem/progenitor cell marker? J Mammary Gland Biol Neoplasia. 2005;10:37-47.

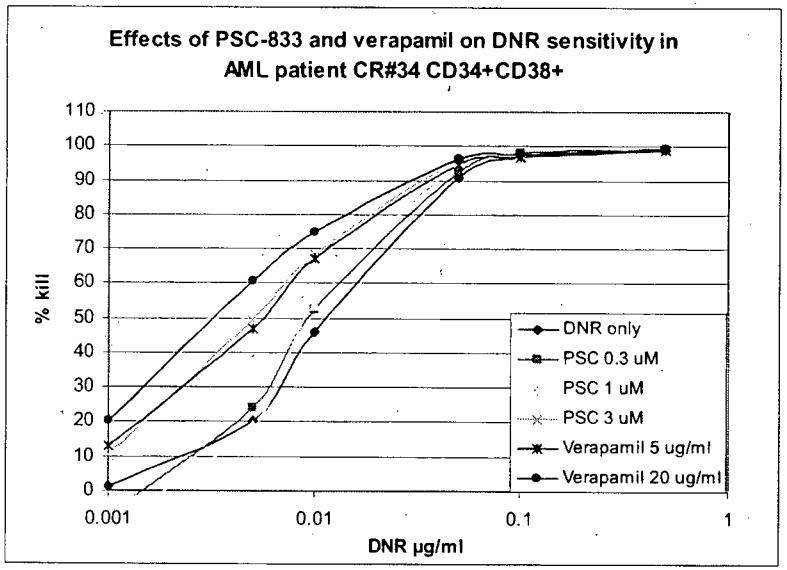
139. Szotek PP, Pieretti-Vanmarcke R, Masiakos PT, et al. Ovarian cancer side population defines cells with stem cell-like characteristics and Mullerian Inhibiting Substance responsiveness. Proc Natl Acad Sci U S A. 2006;103:11154-11159.
140. Wulf GG, Wang RY, Kuehnle I, et al. A leukemic stem cell with intrinsic drug efflux capacity in acute myeloid leukemia. Blood. 2001;98:1166-1173.
141. Feuring-Buske M, Hogge DE. Hoechst 33342 efflux identifies a subpopulation of cytogenetically normal CD34(+)CD38(-) progenitor cells from patients with acute myeloid leukemia. Blood. 2001;97:3882-3889.
142. Beck WT, Grogan TM, Willman CL, et al. Methods to detect P-glycoprotein-associated multidrug resistance in patients' tumors: consensus recommendations. Cancer Res. 1996;56:3010-3020.
143. Bradley G, Naik M, Ling V. P-glycoprotein expression in multidrug-resistant human ovarian carcinoma cell lines. Cancer Res. 1989;49:2790-2796.
144. Efferth T, Davey M, Olbrich A, Rucker G, Gebhart E, Davey R. Activity of drugs from traditional Chinese medicine toward sensitive and MDR1- or MRP1-overexpressing multidrug-resistant human CCRF-CEM leukemia cells. Blood Cells Mol Dis. 2002;28:160-168.
145. Johny A, Song KW, Nantel SH, et al. Early stem cell transplantation for refractory acute leukemia after salvage therapy with high-dose etoposide and cyclophosphamide. Biol Blood Marrow Transplant. 2006;12:480-489.
146. Bustin SA, Gyselman VG, Williams NS, Dorudi S. Detection of cytokeratins 19/20 and guanylyl cyclase C in peripheral blood of colorectal cancer patients. Br J Cancer. 1999;79:1813-1820.
147. Bustin SA, Siddiqi S, Ahmed S, Hands R, Dorudi S. Quantification of cytokeratin 20, carcinoembryonic antigen and guanylyl cyclase C mRNA levels in lymph nodes may not predict treatment failure in colorectal cancer patients. Int J Cancer. 2004;108:412-417.
148. Hu XF, Slater A, Wall DM, et al. Rapid up-regulation of mdr1 expression by anthracyclines in a classical multidrug-resistant cell line. Br J Cancer. 1995;71:931-936.
149. Abolhoda A, Wilson AE, Ross H, Danenberg PV, Burt M, Scotto KW. Rapid activation of MDR1 gene expression in human metastatic sarcoma after in vivo exposure to doxorubicin. Clin Cancer Res. 1999;5:3352-3356.
150. Thorgeirsson SS, Huber BE, Sorrell S, Fojo A, Pastan I, Gottesman MM. Expression of the multidrug-resistant gene in hepatocarcinogenesis and regenerating rat liver. Science. 1987;236:1120-1122.
151. Kanda Y, Hamaki T, Yamamoto R, et al. The clinical significance of CD34 expression in response to therapy of patients with acute myeloid leukemia: an overview of 2483 patients from 22 studies. Cancer. 2000;88:2529-2533.
152. Campos L, Guyotat D, Archimbaud E, et al. Surface marker expression in adult acute myeloid leukaemia: correlations with initial characteristics, morphology and response to therapy. Br J Haematol. 1989;72:161-166.
153. Borowitz MJ, Gockerman JP, Moore JO, et al. Clinicopathologic and cytogenic features of CD34 (My 10)-positive acute nonlymphocytic leukemia. Am J Clin Pathol. 1989;91:265-270.
154. Geller RB, Zahurak M, Hurwitz CA, et al. Prognostic importance of immunophenotyping in adults with acute myelocytic leukaemia: the significance of the stem-cell glycoprotein CD34 (My10). Br J Haematol. 1990;76:340-347.

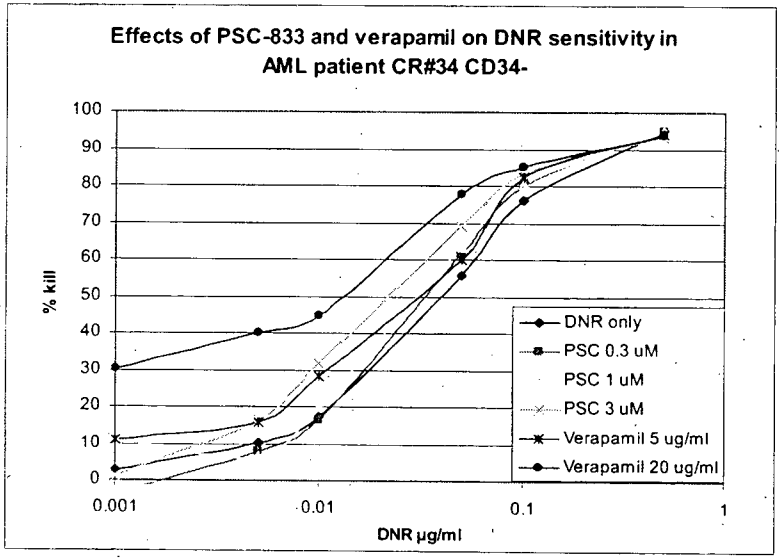
155. Campos L, Guyotat D, Archimbaud E, et al. Clinical significance of multidrug resistance P-glycoprotein expression on acute nonlymphoblastic leukemia cells at diagnosis. Blood. 1992;79:473-476.
156. Solary E, Casasnovas RO, Campos L, et al. Surface markers in adult acute myeloblastic leukemia: correlation of CD19+, CD34+ and CD14+/DR--phenotypes with shorter survival. Groupe d'Etude Immunologique des Leucemies (GEIL). Leukemia. 1992;6:393-399.
157. Lee EJ, Yang J, Leavitt RD, et al. The significance of CD34 and TdT determinations in patients with untreated de novo acute myeloid leukemia. Leukemia. 1992;6:1203-1209.
158. Lamy T, Goasguen JE, Mordelet E, et al. P-glycoprotein (P-170) and CD34 expression in adult acute myeloid leukemia (AML). Leukemia. 1994;8:1879-1883.
159. Bradstock K, Matthews J, Benson E, Page F, Bishop J. Prognostic value of immunophenotyping in acute myeloid leukemia. Australian Leukaemia Study Group. Blood. 1994;84:1220-1225.
160. Fruchart C, Lenormand B, Bastard C, et al. Correlation between CD34 expression and chromosomal abnormalities but not clinical outcome in acute myeloid leukemia. Am J Hematol. 1996;53:175-180.
161. Arslan O, Akan H, Beksac M, et al. Lack of prognostic value of CD34 in adult AML. Leuk Lymphoma. 1996;23:185-186.
162. de Grouw EP, Raaijmakers MH, Boezeman JB, et al. Preferential expression of a high number of ATP binding cassette transporters in both normal and leukemic CD34+CD38- cells. Leukemia. 2006;20:750-754.
163. Peeters SD, van der Kolk DM, de Haan G, et al. Selective expression of cholesterol metabolism genes in normal CD34+CD38- cells with a heterogeneous expression pattern in AML cells. Exp Hematol. 2006;34:622-630.
164. Gottesman MM. Mechanisms of cancer drug resistance. Annu Rev Med. 2002;53:615-627.
165. Tomlins SA, Rhodes DR, Perner S, et al. Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science. 2005;310:644-648.
166. Ishkanian AS, Malloff CA, Watson SK, et al. A tiling resolution DNA microarray with complete coverage of the human genome. Nat Genet. 2004;36:299-303.

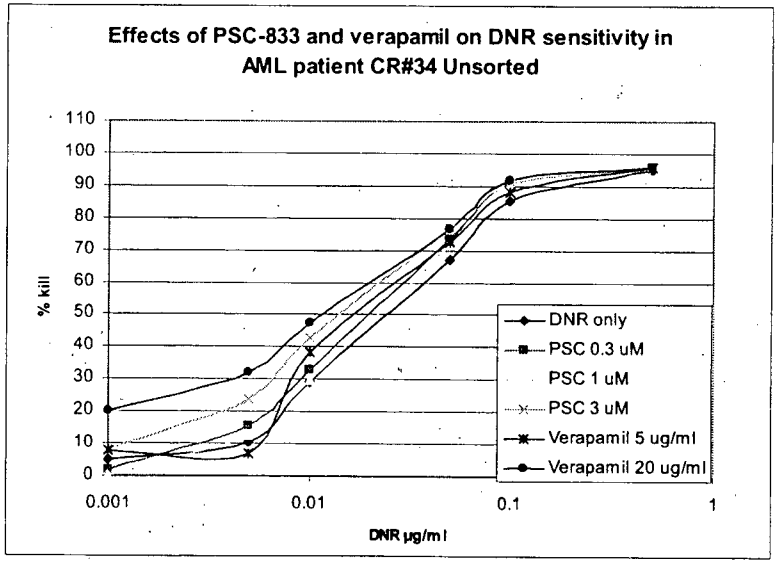
Appendix: Drug sensitivity curves of CR and NR patients.

CR34

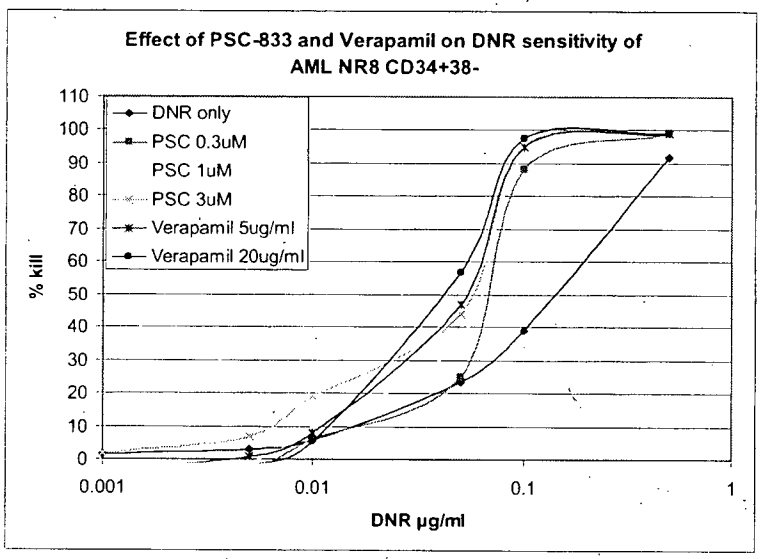


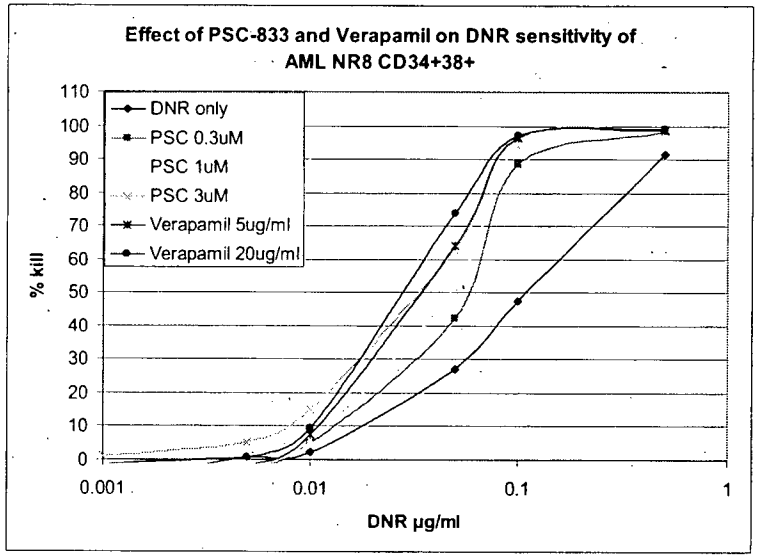


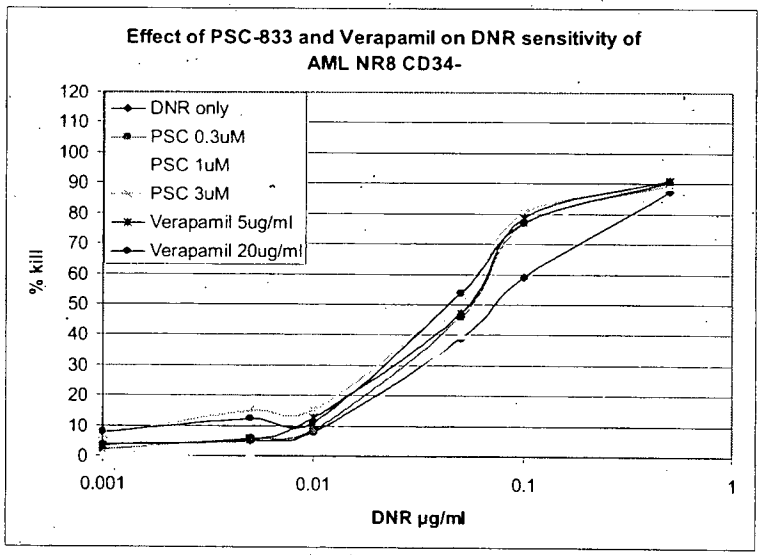


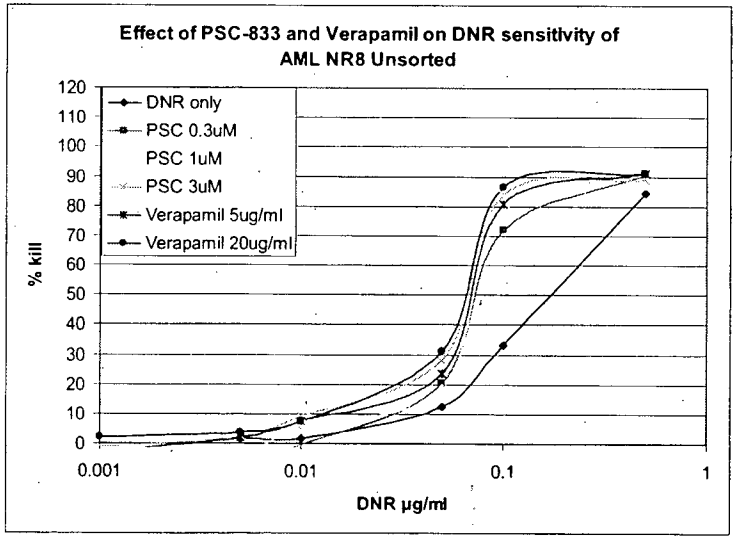


NR8

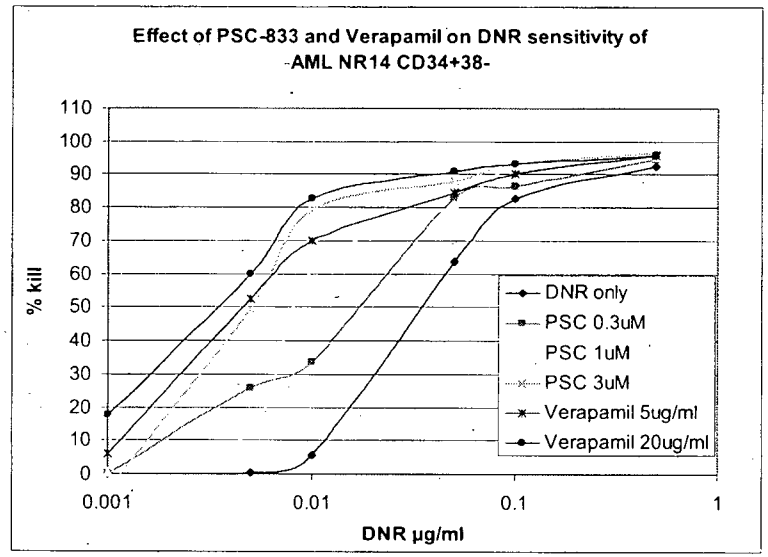

Effect of PSC-833 and Verapamil on DNR sensitivity of AML NR8 CD34+38-
DNR only
PSC 0.3uM
PSC 1uM
PSC 3uM
Verapamil 5ug/ml
Verapamil 20ug/ml
% kill
DNR µg/ml

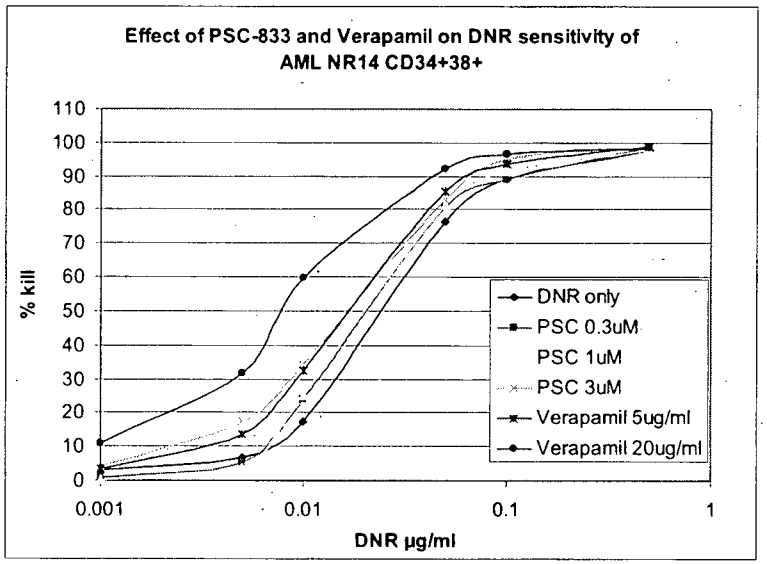

Effect of PSC-833 and Verapamil on DNR sensitivity of AML NR8 CD34+38+
DNR only
PSC 0.3uM
PSC 1uM
PSC 3uM
Verapamil 5ug/ml
Verapamil 20ug/ml
% kill
DNR µg/ml

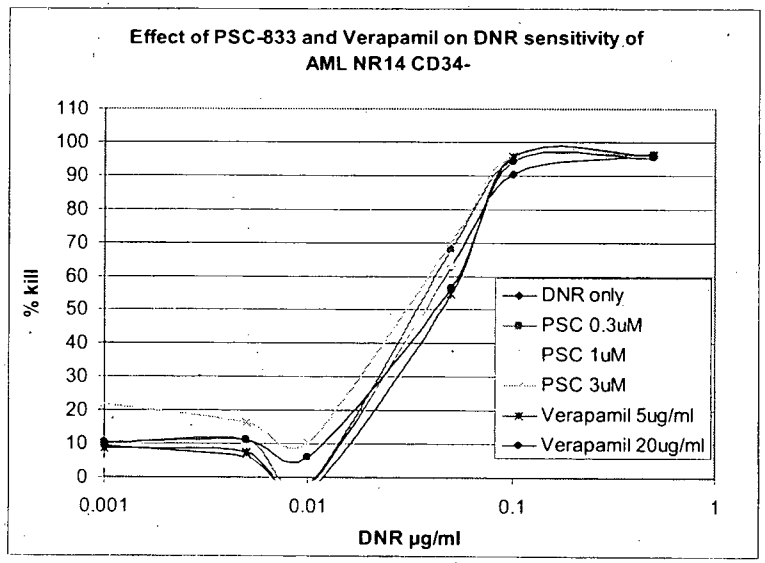

Effect of PSC-833 and Verapamil on DNR sensitivity of AML NR8 CD34-
DNR only
PSC 0.3uM
PSC 1uM
PSC 3uM
Verapamil 5ug/ml
Verapamil 20ug/ml
% kill
DNR µg/ml

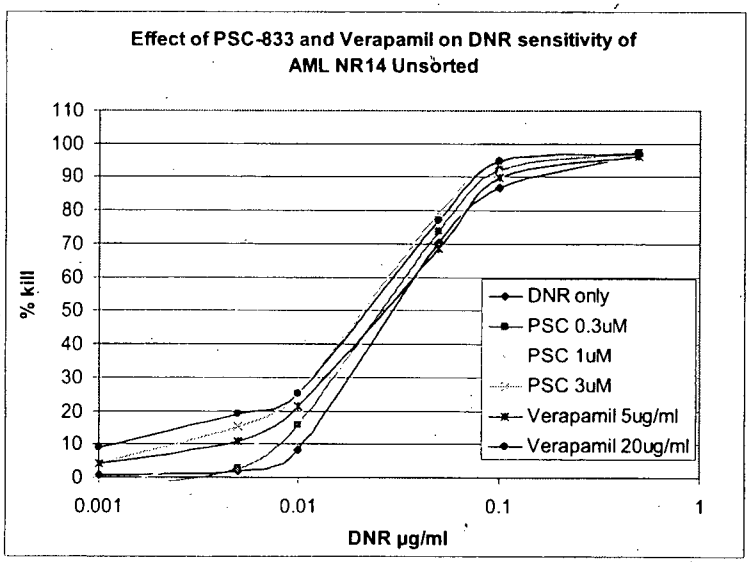

Effect of PSC-833 and Verapamil on DNR sensitivity of AML NR8 Unsorted
DNR only
PSC 0.3uM
PSC 1uM
PSC 3uM
Verapamil 5ug/ml
Verapamil 20ug/ml
% kill
DNR µg/ml

NR14

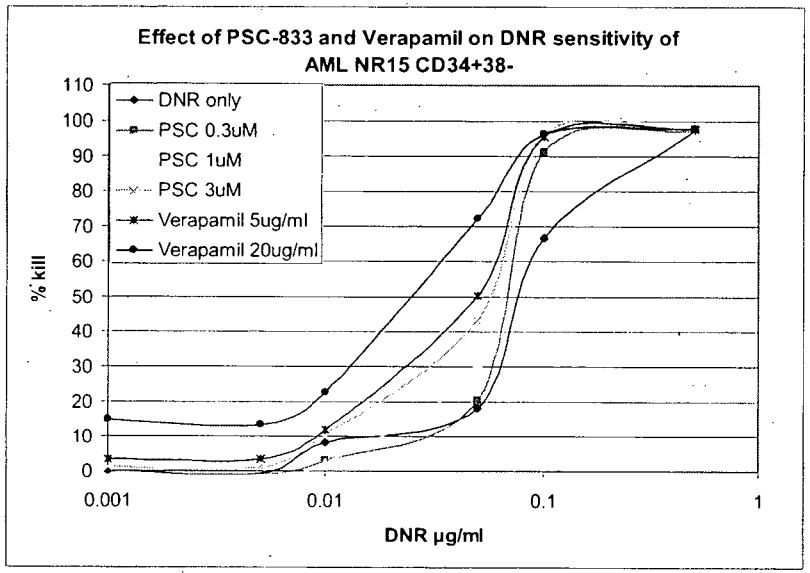

Effect of PSC-833 and Verapamil on DNR sensitivity of
AML NR14 CD34+38-
% kill
DNR µg/ml
DNR only
PSC 0.3uM
PSC 1uM
PSC 3uM
Verapamil 5ug/ml
Verapamil 20ug/ml

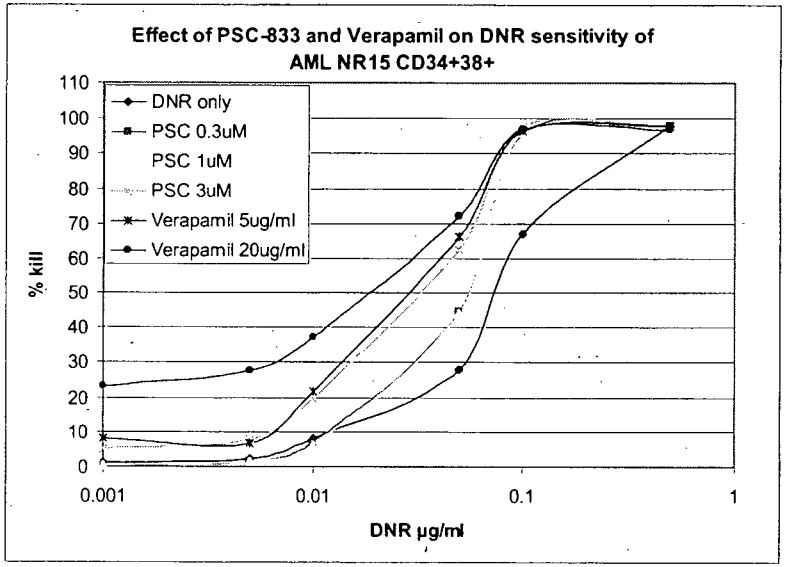

Effect of PSC-833 and Verapamil on DNR sensitivity of
AML NR14 CD34+38+
% kill
DNR µg/ml
DNR only
PSC 0.3uM
PSC 1uM
PSC 3uM
Verapamil 5ug/ml
Verapamil 20ug/ml

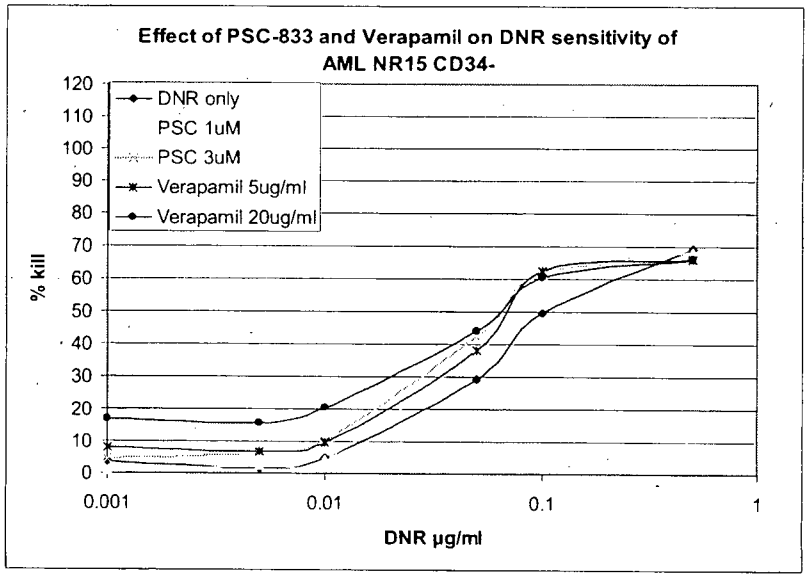

Effect of PSC-833 and Verapamil on DNR sensitivity of
AML NR14 CD34-
% kill
DNR µg/ml
DNR only
PSC 0.3uM
PSC 1uM
PSC 3uM
Verapamil 5ug/ml
Verapamil 20ug/ml

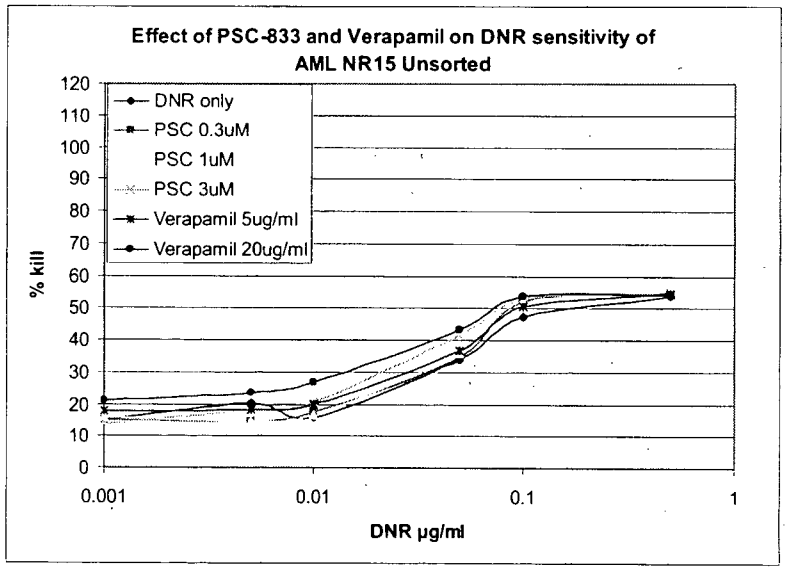

Effect of PSC-833 and Verapamil on DNR sensitivity of
AML NR14 Unsorted
% kill
DNR µg/ml
DNR only
PSC 0.3uM
PSC 1uM
PSC 3uM
Verapamil 5ug/ml
Verapamil 20ug/ml

NR15

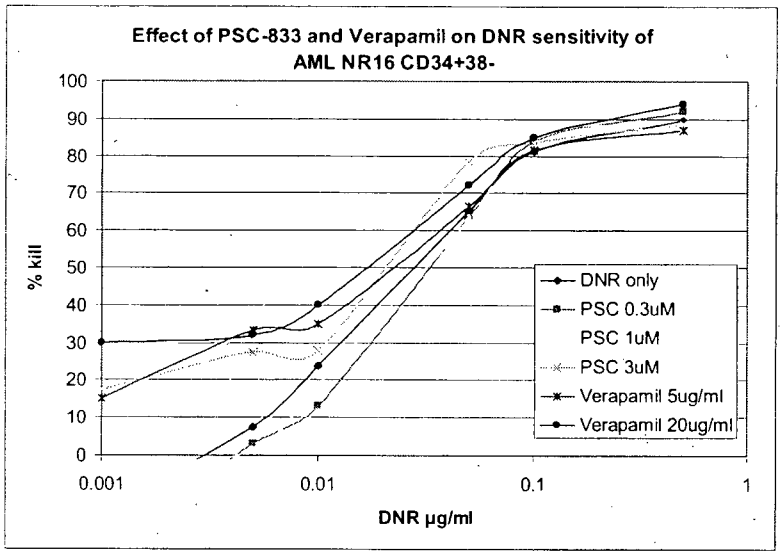

Effect of PSC-833 and Verapamil on DNR sensitivity of AML NR15 CD34+38-
DNR only
PSC 0.3uM
PSC 1uM
PSC 3uM
Verapamil 5ug/ml
Verapamil 20ug/ml
% kill
DNR µg/ml

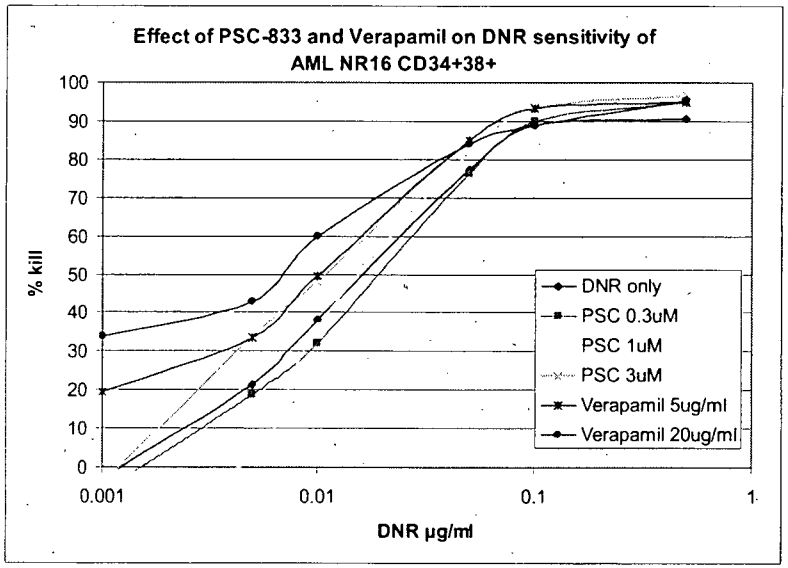

Effect of PSC-833 and Verapamil on DNR sensitivity of AML NR15 CD34+38+
DNR only
PSC 0.3uM
PSC 1uM
PSC 3uM
Verapamil 5ug/ml
Verapamil 20ug/ml
% kill
DNR µg/ml

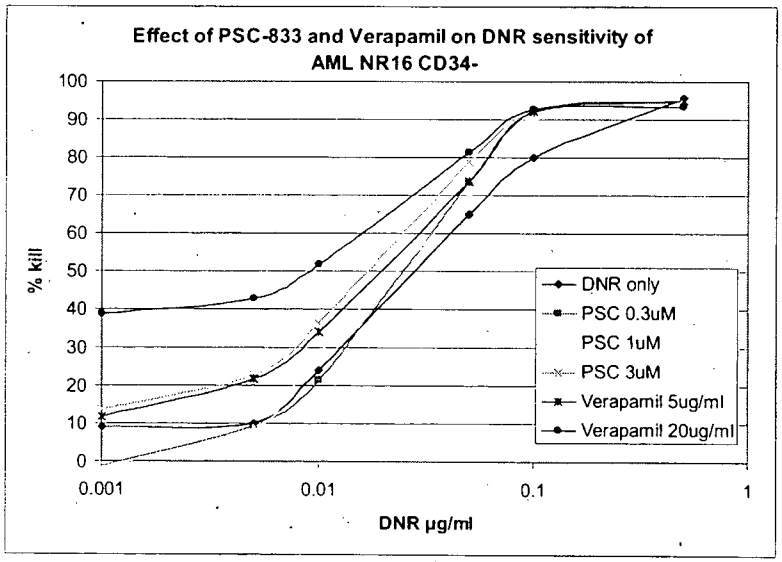

Effect of PSC-833 and Verapamil on DNR sensitivity of AML NR15 CD34-
DNR only
PSC 1uM
PSC 3uM
Verapamil 5ug/ml
Verapamil 20ug/ml
% kill
DNR µg/ml

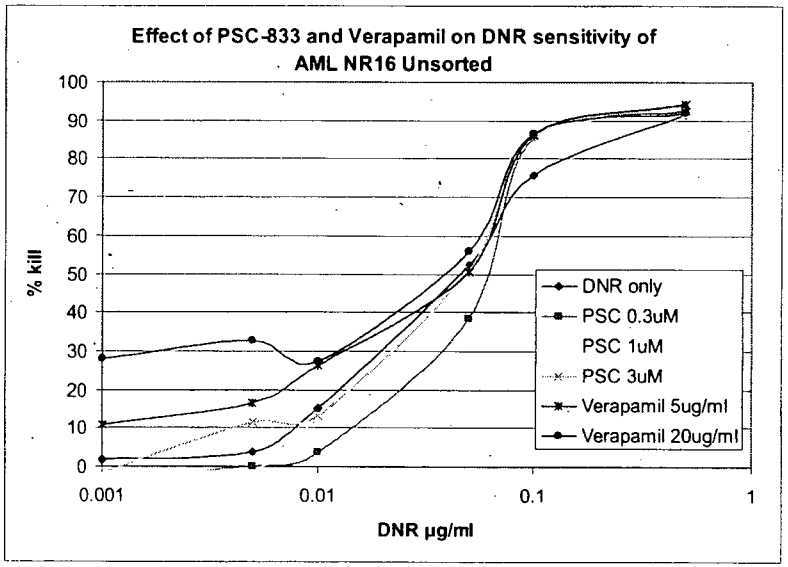

Effect of PSC-833 and Verapamil on DNR sensitivity of AML NR15 Unsorted
DNR only
PSC 0.3uM
PSC 1uM
PSC 3uM
Verapamil 5ug/ml
Verapamil 20ug/ml
% kill
DNR µg/ml

NR16

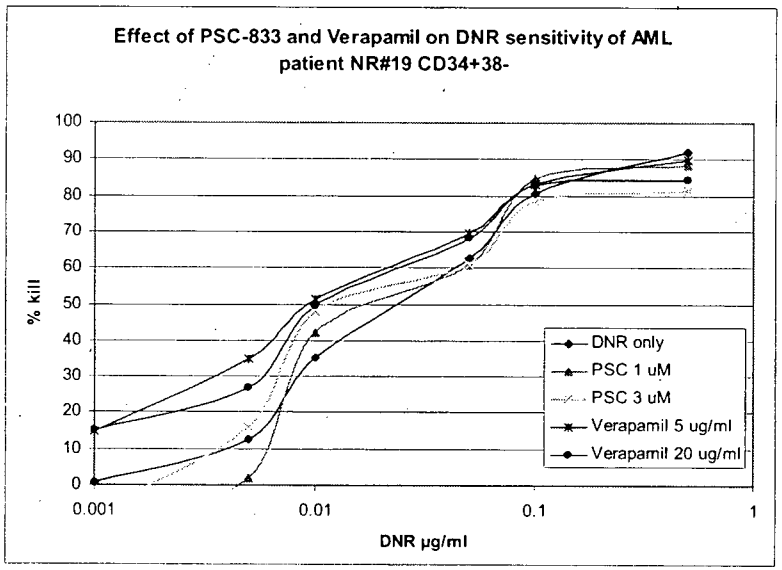

Effect of PSC-833 and Verapamil on DNR sensitivity of AML NR16 CD34+38-
% kill
DNR µg/ml
DNR only
PSC 0.3uM
PSC 1uM
PSC 3uM
Verapamil 5ug/ml
Verapamil 20ug/ml

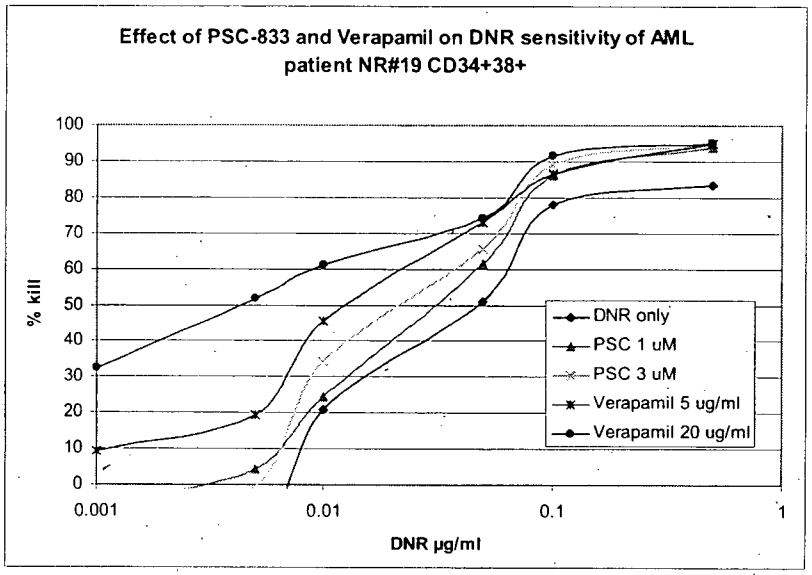

Effect of PSC-833 and Verapamil on DNR sensitivity of AML NR16 CD34+38+
% kill
DNR µg/ml
DNR only
PSC 0.3uM
PSC 1uM
PSC 3uM
Verapamil 5ug/ml
Verapamil 20ug/ml

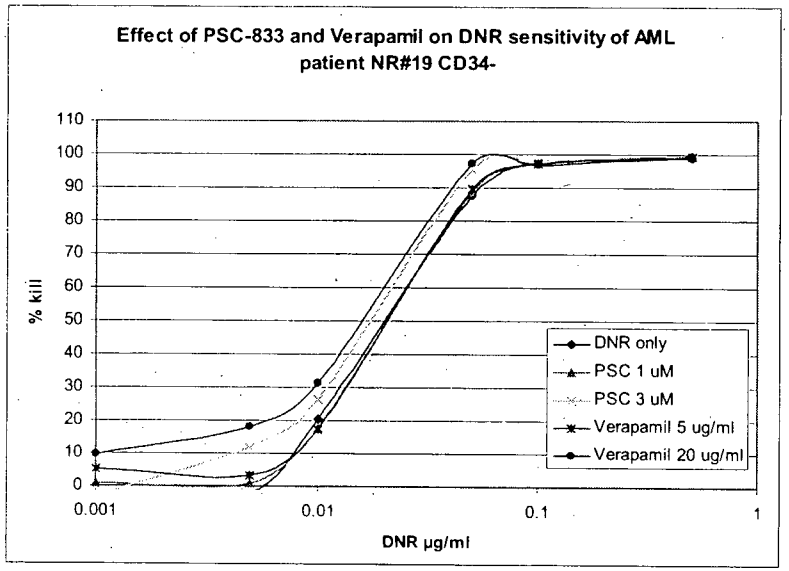

Effect of PSC-833 and Verapamil on DNR sensitivity of AML NR16 CD34-
% kill
DNR µg/ml
DNR only
PSC 0.3uM
PSC 1uM
PSC 3uM
Verapamil 5ug/ml
Verapamil 20ug/ml

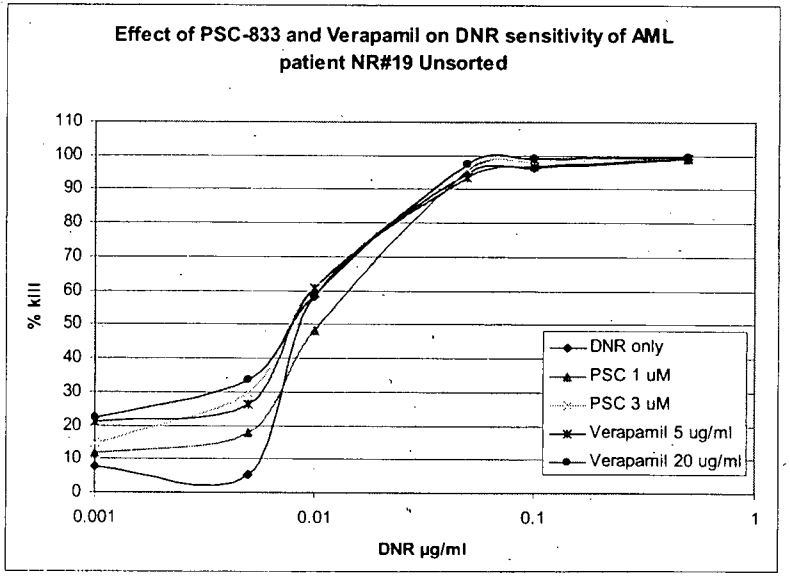

Effect of PSC-833 and Verapamil on DNR sensitivity of AML NR16 Unsorted
% kill
DNR µg/ml
DNR only
PSC 0.3uM
PSC 1uM
PSC 3uM
Verapamil 5ug/ml
Verapamil 20ug/ml

NR19


Effect of PSC-833 and Verapamil on DNR sensitivity of AML patient NR#19 CD34+38-
% kill
DNR µg/ml
DNR only
PSC 1 uM
PSC 3 uM
Verapamil 5 ug/ml
Verapamil 20 ug/ml


Effect of PSC-833 and Verapamil on DNR sensitivity of AML patient NR#19 CD34+38+
% kill
DNR µg/ml
DNR only
PSC 1 uM
PSC 3 uM
Verapamil 5 ug/ml
Verapamil 20 ug/ml


Effect of PSC-833 and Verapamil on DNR sensitivity of AML patient NR#19 CD34-
% kill
DNR µg/ml
DNR only
PSC 1 uM
PSC 3 uM
Verapamil 5 ug/ml
Verapamil 20 ug/ml


Effect of PSC-833 and Verapamil on DNR sensitivity of AML patient NR#19 Unsorted
% kill
DNR µg/ml
DNR only
PSC 1 uM
PSC 3 uM
Verapamil 5 ug/ml
Verapamil 20 ug/ml