Multidrug Resistance Studies in Patients with Acute Myeloid Leukemia

Multidrug resistentie studies in patiënten met
acute myeloïde leukemie

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"We dance round in a ring and suppose, but the secret sits in the middle and knows".
(R. Frost, 1945)

Voor
GJ

Voor
mijn
ouders
en
mijn
kinderen

CONTENTS

Chapter 1		Introduction Paragraphs 1.2.2.1 and 1.2.3.1 are part of the review: The prognostic significance of membrane transport-associated multidrug resistance (MDR) proteins in leukemia *International J Pharm Ther 38: 94-110, 2000*	9
1.1		Acute myeloid leukemia	11
	1.1.1	Hematopoiesis and leukemia	11
	1.1.2	Characterization of AML	11
	1.1.3	Epidemiology of AML	12
	1.1.4	Prognostic factors in AML	12
	1.1.5	Treatment of AML	15
1.2		Multidrug resistance	18
	1.2.1	Resistance to chemotherapy	18
	1.1.2	Mechanisms of multidrug resistance	20
	1.2.2.1	(Membrane) transport associated drug resistance proteins	20
	1.2.2.2	Mediators of Ara-C response	26
	1.2.3	The clinical relevance of multidrug resistance in AML	27
	1.2.3.1	(Membrane) transport associated drug resistance proteins in AML	27
	1.2.3.2	Ara-C resistance in AML	33
	1.2.4	Reversal of multidrug resistance in AML	34
	1.2.4.1	In vitro	34
	1.2.4.2	In vivo	37
1.3		Aims of this Thesis	41
1.4		References	42
Chapter 2		*MDR*1 expression is an independent prognostic factor for response and survival in *de novo* acute myeloid leukemia *Br J Haematology 99:76-83, 1997*	83
Chapter 3		*MDR*1 gene related clonal selection and P-glycoprotein function. and expression in relapsed/refractory acute myeloid leukemia *Blood 97: 3605-3611, 2001*	103

Chapter 4 Increased expression of the breast cancer resistance protein BCRP in relapsed or refractory disease acute myeloid leukemia (AML) 125
Submitted

Chapter 5 *MDR*1 expression in poor-risk acute myeloid leukemia with partial or complete monosomy 7 145
Leukemia, 15:398-405, 2001

Chapter 6 Absence of mutations in the deoxycytidine kinase (*dCK*) gene in patients with relapsed and/or refractory acute myeloid leukemia (AML) 169
Leukemia, 2001, in press

Chapter 7 In vitro effect of GF 120918, a *novel* reversal agent of multidrug resistance, on acute leukemia and multiple myeloma cells 183
Leukemia 10:1930-1936, 1996

Chapter 8 General discussion 201

Summary 206
Samenvatting 209
List of abbreviations 212
List of publications 215
Curriculum vitae 217
Dankwoord/Acknowledgements 219

CHAPTER 1

Introduction

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1.1 Acute Myeloid Leukemia

1.1.1 Hematopoiesis and leukemia

In the bone marrow, a continuous, strictly organized process of blood cell production or hematopoiesis takes place. The human hematopoietic system is capable of replacing the normal daily turnover of blood cells and is capable of maintaining a balance between the blood cell formation and increased blood cell demands such as in bleeding or infection. The different types of cells that are normally present in the peripheral blood are all derived from committed progenitor cells (1-4). The compartment of these committed progenitor cells is maintained by a small number of pluripotent stem cells (2,5,6). The process of proliferation and differentiation is regulated by cellular interaction, the micro-environment of the bone marrow, several regulatory glycoproteins and hematopoietic growth factors (3,5,7,8).

Leukemia is the condition of malignant transformation of hematopoietic cells leading to the accumulation of immature abnormal cells in blood and bone marrow. According to the clinical presentation, the leukemias are divided in acute and chronic leukemias. Depending on the cell lineage involved, a further distinction can be made in myeloid and lymphoid leukemias. The focus of this thesis is on "acute myeloid leukemia" (AML).

AML is a clonal disease, characterized by a maturation arrest during the differentiation of the hematopoietic cells to mature blood cells (9-11), leading to accumulation of a population of immature abnormal myeloid cells, ultimately resulting in suppression of normal hematopoeisis. Clinically, the replacement of normal functional blood cells by leukemic blasts in bone marrow and peripheral blood will result in anemia, granulocytopenia and thrombocytopenia.

1.1.2 Characterization of AML

AML comprises a heterogeneous group of disorders of which the diversity is characterized by differences in morphologic, immunophenotypic and genotypic features. The currently used morphologic classification, is the French American British (FAB) classification described by Bennett *et al.* (12,13). It distinguishes FAB types M1-7 by morphologic and histochemical characteristics. A close correlation between morphologic and genotypic characterizations of AML has been described in the past decades (12-15). Recently, a new classification of myeloid hematological malignancies based on morphologic, immunophenotypic and cytogenetic features has been proposed by the

World Health Organization (WHO) (16). The immunophenotype represents the stage at which the maturation arrest has occurred during hematopoietic development. The karyotype, according to international guidelines (17), reveals the numerical and/or structural abnormalities of the chromosomes in the leukemic blast cells. The cytogenetic abnormalities play an important role in the leukemogenesis of AML. Some structural genetic abnormalities are specific for AML, like t(8;21), inv(16) and t(15;17), while others are also involved in other hematologic malignancies, such as t(9;22) and 11q23 with mixed lineage leukemia *(MLL)* gene rearrangements in acute lymphoblastic leukemia (ALL). Because of the heterogeneity of AML, it is important to classify every single case of AML using clinical characteristics (like age, white blood count, associated syndromes like Down's syndrome), morphology (FAB), immunophenotype and karyotype.

1.1.3 Epidemiology of AML

In adults, AML is the most frequently occurring form of acute leukemia. In childhood however, it is a rare disease, being diagnosed in only 15-20 pediatric patients per year in The Netherlands. The incidence increases with age from 1-3 per 10^5 each year in childhood and early adulthood to 15 per 10^5 persons each year at the age of 70 years, and to 35 per 10^5 at age 90 (18-20). The incidence rates are higher for males than for females (20-22). The median age of AML patients at diagnosis is over 60 years (23).

At present, the cause of AML is not known. Some environmental factors have consistently been linked to the origin of the disease, *i.e.* exposure to natural radiation, alkylating agents, chronic benzene exposure and cigarette smoking, and also exposure to radioactive irradiation as illustrated by long-term survival studies of exposants of the atomic bomb (24-34). Some hereditary diseases like Fanconi's anemia, Down's syndrome and Bloom's syndrome are associated with an increased risk for the development of AML (22,35,36). Infrequently, families with an unexplained high risk of AML have been described (37).

1.1.4 Prognostic factors in AML

A variety of clinical and biologic parameters has been examined for potential value in predicting treatment response and survival (Table 1) (38). Increasing age, higher peripheral white blood cell count, poor response to induction chemotherapy, short

Table 1. Prognostic factors in AML

Prognostic factors	Good risk	Poor risk	Reference
Clinical			(39,40-43)
Age	children	elderly	
WBC	low	high	
Response after induction	CR	PR/RD	
Time to relapse	long	short	
Performance scale	good	poor	
Morphology			(38,44-46)
FAB	M3, M4Eo Auer Rods	M0, M5, M6, M7	
Karyotype			(38,42,43,45-47)
Structural aberrations	inv(16), t(16;16), t(15;17), t(8;21)	7q-, 5q-, t(9;22), 11q23 with *MLL* rearrangements complex karyotypes	
Numerical aberrations		-5, -7 +8	
Immunophenotype			(38,48-56)
	panmyeloid	CD34+ CD13+,CD14+ CD11b, CD11c biphenotypic (>2 lymphoid markers)	
MDR			(54,56-64)
		MDR1, (LRP/MVP, MRP1) (co-)expression	
Other		Flt3/ITD	(65-68)

CR: complete remission after 1 or 2 cycles of chemotherapy; PR: partial remission after 2 cycles of chemotherapy; RD: refractory disease.

duration of first complete remission (CR), and a poor performance scale are important independent adverse clinical prognostic features (38-40).

Morphological features like the occurrence of dysmyelopoiesis, which is associated with a poor prognosis, have been reported to be of prognostic importance (39). Infants with AML with FAB types M4 and M5 have a relatively favorable prognosis (44). Also, both adult and pediatric patients with FAB M3 and M4Eo have a relatively good outcome (Table 1) (39), whereas adults with M1, M5, M6, and M7 fare worse (38). Several reports suggested a relationship between some antigens [CD11b, CD13, CD14, CD34, CD56 or terminal deoxynucleotidyl transferase (TdT)] and the poor prognosis of AML patients (38,48-56). Most studies confirm the correlation of the expression of the immature phenotypes with bad prognosis whereas the expression of panmyeloid markers was associated with a better outcome. However, subsequent studies have produced conflicting results (Table 1) (40,48-55).

Based on karyotyping, cytogenetic risk-groups have been recognized. In adult AML patients, monosomy 7, 7q-, t(9;22) and complex karyotypes are accepted as predictors for poor survival (45,46,69-71). The adverse prognostic value of 11q23 with *MLL* rearrangements, trisomy 8, monosomy 5, 5q- is more debatable (42). Good prognostic chromosomal aberrations are inv(16), t(8;21) and t(15;17) (45,46). Although in some studies abnormalities of chromosome 7 have been established as adverse prognostic factors, in childhood AML (42,47,72-74), monosomy 7 is not generally used as an unfavorable prognostic factor for stratification of therapy as yet (43,75,76). Until now, there is no explanation for the extremely poor outcome in adult AML patients with monosomy 7. However, monosomy 7 patients are more likely to have secondary leukemias and myelodysplastic syndrome related AML, which forms a group of more resistant leukemias.

The prognostic significance of the drug resistance proteins encoded by the breast cancer resistance protein (*BCRP*) gene, the multidrug resistance protein (*MDR*1) gene, the multidrug resistance related protein (*MRP*1) gene and the lung resistance related protein (*LRP*) gene, will be discussed in paragraph 1.2.3.1.

Recently, an internal tandem duplication (ITD) of the *Flt3* gene at chromosome 13, which encodes a tyrosinekinase receptor that regulates proliferation and differentiation of hematopoietic stem cells, has been associated with poor prognosis in adult (66,68) and childhood AML (65,67). This mutation seems to overrule other prognostic factors in

multivariate analyses and may be important for treatment stratification in the future, especially in AML patients with normal cytogenetics (65).

Based on the knowledge of prognostic factors in AML, several stratification scores have been proposed. In some of them the permeability-glycoprotein (P-gp) expression is involved, and combined with clinical, morphologic and cytogenetic risk factors. An example is a the recent study by Legrand *et al.*, who proposed a prognostic score combining the prognostic factors age, WHO performance status, P-gp activity, expression of panmyeloid markers, and the several cytogenetic risk groups for elderly AML patients (40). Another example of stratification in which MDR1 expression was involved has been proposed by Estey *et al.*, according to age, cytogenetics, performance status and MDR1 expression (77), and by Del Poeta *et al.* who identified an unfavorable risk group based on P-gp expression, TdT expression and unfavorable cytogenetic abnormalities, like complex karyotypes, numerical or structural deletions of chromosome 5 and 7, trisomy 8, t(9;22) and 11q23 with *MLL* rearrangements (78). Overall, until now, cytogenetic abnormalities are considered to be the most valuable prognostic determinants in AML (45,46).

1.1.5 Treatment of AML

The purpose of treatment of AML is to eradicate all malignant cells. Treatment strategies have been developed using combinations of cytostatic drugs, sometimes followed by bone marrow transplantation in order to restore normal hematopoiesis to obtain long term survival. Without treatment, the median survival of an AML patient is only 2 months (79).

Standard chemotherapy for AML consists of remission induction and post-remission chemotherapy (80). Complete remission (CR), is defined as a reduction in the marrow blast percentage to less than 5% as assessed by traditional light microscopic examination of the bone marrow. In adults, remission induction therapy usually includes one or two cycles of a combination chemotherapy of 7 days of cytosine-arabinoside (Ara-C) (81), and 3 days of an anthracycline (82), like doxorubicin, idarubicin (83-85), daunomycin or mitoxantrone (86). Amsacrine (87) and etoposide (88) have also been frequently included in induction regimens. In addition, others have combined high doses of Ara-C (HDAC) (1-3 g/m^2) with asparaginase or daunomycin (89). In children, comparable systemic induction chemotherapy is used in combination with central nervous system prophylaxis using intrathecal therapy. Acute promyelocytic leukemia (PML) with FAB type M3, is generally characterized by a t(15;17), in which the *PML* gene on chromosome 17 and the

retinoic acid receptor α (*RAR-α*) gene on chromosome 15 are involved. In this subtype of AML, induction therapy starts with all-trans retinoic acid (ATRA) as it induces maturation of the AML cells *in vitro* and *in vivo,* followed by induction chemotherapy (90).

Post-remission consolidation chemotherapy consists of intensive chemotherapy, that has proven to be more effective than the prolonged maintenance with low doses of chemotherapy strategies, used in the 1980s (91-94). However, in individual patients maintenance therapy might be of value by at least offering a longer relapse free survival, like for instance in elderly patients (95). Consolidation therapy as a curative regimen, usually consists of HDAC and anthracyclins, which in children often will be combined with L-asparaginase (96,97).

Recently, in adults, myeloid growth factors have been added to AML therapy with two distinct objectives: to recruit dormant malignant cells into the cell cycle for a more efficient cell killing by the chemotherapy given at the same time (41,98-103), and secondly, to reduce the duration of neutropenia and the toxic death rate when given after induction chemotherapy. Currently, clinical studies have shown that the application of these growth factors (104-106) did not decrease the number of documented infections or the number of days of hospitalization (106). An increase in the rate of initial responses (98), and survival using granulocyte-macrophage colony stimulating factor (GM-CSF) has been reported in adults (100), however a recent study in 652 young adolescents (< 21 years) and children, adding granulocyte colony-stimulating factor (G-CSF) did not influence induction CR rates post remission, outcome, nor overall survival (43). Although AML blast cells generally express functional G-CSF and GM-CSF receptors on their surface (107), thus far, the fear that treatment with G-CSF or GM-CSF could provoke the growth of leukemic cells in patients has not been substantiated (108).

Other contributions to the improvement of AML treatment in the past decade are the improvement of supportive care like treatment with antibiotic and anti-fungal drugs, the availability of anti-viral drugs, and improvement of feeding supplements.

Allogeneic bone marrow transplantation (BMT) in patients up to 55 years of age is generally recommended for patients in first CR (109). Recent protocols have omitted allogeneic BMT in AML patients with the karyotypes t(8;21), inv(16), t(16;16) and t(15;17), as the prognosis is good with chemotherapy only. For patients transplanted with bone marrow from a HLA identical sibling, the 5 years survival ranges from 40 to 60%. However, less than 10% of adult patients with AML are candidates for allogeneic BMT,

as the median age of AML patients in adults is over 60 years and the availability of HLA compatible donors is a major problem (109-111). Transplantation with stem cells from a matched unrelated HLA compatible donor (MUD) has been developed as an alternative donor source, but this approach is associated with a higher morbidity and mortality (109,110,112). In children and young adults T-cell depletion, variation of cell dosages (113) and CD34 concentration (114), and the availability of cord blood (115,116) as a powerful stem cell source, have pushed interest in unrelated donor transplants, especially in extremely poor prognostic groups (114). Apart from being effective for the restoration of normal hematopoiesis after engraftment, allogenic stem cell transplantation is also effective because of the 'graft-versus-leukemia' effect, a phenomenon that was first described in 1956 by Barnes and Loutit (117), confirmed after the excess of relapses after full T-cell depletion in the 1970s and 1980s (118) and currently consolidated by the use of donor lymphocyte infusions to reinduce remission in relapsed leukemia patients post-allogeneic BMT (119,120). In children, allogeneic BMT is recommended in first CR, in case of availability of an HLA identical sibling donor, with the exception of patients with Down's syndrome, and of AML patients with t(15;17), inv16 and t(8;21), which have an event free survival (EFS) of 60-90% with conventional chemotherapy (75,121-123).

Autologous bone marrow transplantation (ABMT) is less toxic than allogeneic BMT, however, it is less effective, most likely because of lack of the graft-versus-leukemia effect and the risk of reinfusion of minimal residual clonogenic disease. The benefit over conventional chemotherapy is disputable in adults (109,124,125) and not shown in children (43,123,126-129).

It is obvious that in subgroups of AML patients like for instance elderly, or very young infants, specific treatment choices have to be made depending on performance status, organ function and the presence of favorable or unfavorable prognostic factors (61,77,130-133).

With the current treatment protocols, a CR rate is achieved of 70-85% in younger adult AML patients, 50% in the elderly, and 75-92% in childhood AML. An event free survival of 20-30% is reached in younger adults and only 10-15% in the elderly patients. In general it could be stated that in AML outcome worsens continuously as age increases (93,97,108,123,127,128,134-143). It is not clear what accounts for the better prognosis of children with AML in comparison with adults. The different treatment protocols, heterogeneity of the disease, performance state and tolerance, combination with other diseases and the biological behavior, cellular resistance and pharmacokinetics and/or pharmacodynamics at different ages might play a role.

AML patients who relapse after chemotherapy only have a small chance to achieve a second remission after treatment with intensive chemotherapy (144-146). The probability of achieveing a second CR is mainly dependent on the duration of the first CR (147,148). After reaching a second CR, only 20 - 30% of younger adult patients can be cured by allogeneic stem cell transplantation, and only exceptionally by chemotherapy alone (108,149).

New treatment options are under investigation now. For instance, the efficacy of liposomal anthracyclins, alone or in combination with conventional doses of Ara-C or in combination with topoisomerase IIα (Topotecan) or angiogenesis inhibitors like thalidomide and SU5416 (77) is currently analyzed in AML (150-152). Another promising drug for the future treatment of AML is targeted immunochemotherapy with CMA 676 (Mylotarg), an anti-CD33 monoclonal antibody conjugated to the anthracyclin calicheamycin (153-157). Also, Phase I/II studies evaluating IL-2 after consolidation chemotherapy have been performed in AML. The results with respect to outcome are awaited (158). Moreover, Phase I/II trials are planned with hypomethylating agents like decitabine (77,159) and/or histone deacetylase inhibitors, like trichostatin A, butyrates, and depsipeptide (152,160-164). Also promising in AML treatment are protein kinase C (PKC) inhibitors, like bryostatin and UCN-01. There are a number of combination studies underway with bryostatin (152). Those that are involved in leukemia or myelodysplastic syndromes include phase I trials with Ara-C, fludarabine, and with 2-chlorodeoxyadenosine (2-CdA). There is also a phase II study in combination with ATRA for AML (152). Phase I trials in adults and children with novel retinoids, like fenretinide (165) that induce apoptosis in malignant cells, independent of the nuclear retinoid receptor pathway (166,167) in adults and children are being evaluated now (152).

1.2 Multidrug resistance (MDR)

1.2.1 Resistance to chemotherapy

The principal reason for treatment failure in patients with AML is the presence or development of resistance to chemotherapy. Treatment regimens in which different types of chemotherapy are combined, will not in all cases lead to CR. According to the Goldie and Coldman hypothesis, the multi-agent chemotherapy regimens can select drug resistant clones that develop by spontaneous mutation or tumor cell adaptation (168). From clinical settings it is widely accepted that patients with a high tumor burden, *i.e.*

organomegaly and high peripheral white blood cell counts are more susceptible to induction failure and relapse. Although most AML patients will reach CR after induction chemotherapy following a logarythmic decrease of malignant cells, eventually a large number of patients will relapse, probably because of the remaining minimal residual disease (MRD), which gives rise to the expansion of an AML clone with very often a more resistant phenotype in a later stage. At time of diagnosis most acute leukemic patients have a tumor burden that exceeds 10^{12} clonal cells. At time of complete remission, as much as 10^{10} leukemic cells might still be present in these patients. Currently, minimal residual disease (MRD) can be measured by very sensitive flowcytometric and polymerase chain reaction (PCR) techniques (169). In the future, these techniques might be helpful to identify patients that need more intensive or different forms of therapy, for instance before or after allogeneic BMT, to prevent relapses. Clinically, the probability of achieving a second CR and long term survival drops dramatically after relapse. As said, clinical resistance to anticancer agents can occur at relapse, but also at the time of presentation, which is called primary refractory disease. For these patients it is even more difficult to find a way to achieve a CR and subsequently long term survival.

Multidrug resistance (MDR) is the phenomenon that cancer cells are resistant to chemotherapy, even to drugs that have not previously been used (170,171). This concerns several of the most effective anticancer drugs, such as vinca alkaloids, bacterial anthracyclins and other semi-synthetic or synthetic analogues and organic compounds. These compounds differ in their mode of toxicity and cellular targets, which include the cell membrane (gramicidin D), microtubule assembly (vinca alkaloids), DNA replication (anthracyclins), transcription (actinomycin D) and protein synthesis. The drugs also differ in size, chemical composition and distribution of reactive groups. However, they do share a general hydrophobic and amphipathic character, being usually lipid soluble and possessing a positively charged nitrogen atom at neutral pH (172,173). In cell lines, the MDR phenotype is stably maintained during growth in the absence of the drug, while selection for higher levels of resistance results in a phenotype showing a decreased intracellular drug accumulation and an increased drug efflux (174,175). This phenomenon can be reversed by modifiers of the several multidrug resistance phenotypes (paragraph 1.2.4.1) (176,177). In the past decades, in AML, expression of several mechanisms of drug resistance have been reported. These will be discussed in the next paragraphs.

1.2.2 Mechanisms of multidrug resistance

1.2.2.1 (Membrane) transport associated drug resistance proteins

These proteins have in common that they act by reduction of the intracellular drug concentration by increased efflux, or by reduced accessibility due to drug sequestration.

Classical multidrug resistance *(MDR1)*. Classical multidrug resistance *(MDR1)* is characterized by the expression of the Permeability-glycoprotein *(P-gp)*, a 170-k*D*a membrane protein that has been believed for a long time to act as a 'classical' drug efflux pump. Indeed, upregulation of the *MDR1* gene, localized on chromosome 7q21.1, results in a decreased intracellular concentration of anthracyclins *(*doxorubicin, daunorubicin, idarubicin*)*, vinca alkaloids *(*vincristine, vinblastine*)* and epipodophyllotoxins *(*etoposide, tenoposide*)*, taxanes *(*taxol, taxotere*)* and amsacrine. These drugs have few structural and functional similarities except that they are small, hydrophobic molecules. Alkylating agents like cyclophosphamide, melphalan, chlorambucil, platinum derivates and antimetabolites do not share these characteristics. Hydrophobic compounds that are substrates for P-gp do not fully penetrate into the cytoplasma of cells that express P-gp *(*178*)*. Interaction of substrate with P-gp has been shown to take place within the membrane *(*179*)*.

The functional domains of P-gp have been studied by genetic analysis and biochemical studies in which labeling sites of photoaffinity analogues of drugs were identified *(*180*)*. P-gp is a transmembrane glycoprotein consisting of two similar, but not identical domains. Each domain has six transmembrane segments, and one intracellular adenosine triphosphate *(*ATP*)* binding site *(*181*)*. The presence of two ATP-binding sites defines the mammalian P-gp as a member of the ATP-binding cassette *(*ABC*)* superfamily of proteins *(*182*)*. *M*ost of these proteins are known to be transporters. For a long time it was hypothesized that the ability of P-gp to reduce drug accumulation was caused by an unidirectional energy-dependent drug-efflux pump mechanism with broad substrate specificity *(*183,184*)*. Currently, an alternative, so-called 'flippase' model *(*184,185*)*, is accepted which is based on the hypothesis that P-gp directly flips drugs from the inner leaflet of the lipid bilayer to the outer leaflet.

There is some evidence that P-gp is also involved in the cellular redistribution of drugs in the cytoplasm resulting in diminished accessibility of the drug to critical *(*nuclear*)* targets *(*186*)*. The physiological function of the mammalian *MDR1* gene remains unknown. The gene is differentially expressed in a variety of normal tissues, particularly along the apical

surface of secretory epithelium of the jejunum and colon, proximal tubular epithelium of the kidney, pancreatic small ductile epithelium, and the glandular epithelium of the pregnant uterus. In addition, P-gp is expressed in the adrenal gland, placenta, capillary endothelium of the brain and testis, as well as in hematopoietic precursors and lymphocytes (187,188). Based on this expression pattern, it is assumed that P-gp plays a role in the elimination of xenobiotic substances. The *MDR*1 gene, encoding P-glycoprotein has been cloned and sequenced (189-192), inserted into an expression vector, and transfected into drug-sensitive tumor cell lines. This has resulted in transfectants, that expressed the full MDR1 phenotype characterized by resistance to several classes of drugs comprising anthracyclins, vinca-alkaloids and epipodophyllotoxins (176,183,190,193,194). *In vitro* studies have also demonstrated that tumor cells are able to increase *MDR*1 gene expression in response to cytotoxic agents (195), as well as to physiologic stresses such as heat shock (196-198) and to agents which induce cell differentiation (199). The resistance can be partly overcome by competitive binding of P-gp modulators or modifiers (paragraph 1.2.4.1)(200-203).

Although MDR1 is extensively characterized as a mechanism of drug resistance, very little is known about its relationship to other cellular responses *e.g.* programmed cell death (apoptosis). Some authors revealed that loss of the *p53* tumor suppressor gene can result in a MDR phenotype in cells that normally require *p53* to undergo apoptosis following appropriate stress such as DNA damage (204-206). Other authors have reported point mutations in the promoter region of the *MDR*1 gene in correlation with a regulatory effect on *MDR*1 transcription and poor prognosis (207). Another epigenetic effect, *i.e.* methylation of the CpG islands has been investigated in AML. Until now no convincing data are available that suggest that the methylation status of the CpG-rich domain acts as a switch to regulate expression of the *MDR*1 gene in patient samples (208), although experiments with demethylating agents in P-gp positive and negative cell lines had suggested an important role for methylation of the *MDR*1 gene (209).

Recently, genetic polymorphisms of the *MDR*1 gene have been described at positions 2677 and 2995 (210). At position 2677, mutation is G to T, leading to an amino acid change from Alanine (Ala) to Serine (Ser), while at position 2995 the mutation G to A changses aminoacid Ala into Threonine (Thr) (210). Using this polymorphism, *MDR*1 gene related clonal selection was found during the development of disease towards resistant disease in Burkitt's lymphoma patients (211). Until now, it is not clear what the role of these polymorphisms is in AML.

It is difficult to compare MDR1 expression in cell lines and clinical samples from different studies because many different detection methods have been used such as immunocytochemistry, flow cytometry, RNase protection assays, quantitative PCR, and also because of the different thresholds for positivity, the use of different monoclonal antibodies, comparison of different expression levels (DNA/RNA *versus* protein), different internal controls, and differences in purification methods of the leukemic blasts. To deal with this problem, international workshops have been organized in order to define the objective methods to investigate MDR1 expression (212-216). For P-glycoprotein, functional assays (see paragraph 1.2.4.1) are available based on the MDR1 specific modifier effects in rhodamin 123 (Rho 123) or anthracyclin retention assays. For studying the expression of P-glycoprotein, a panel of monoclonal antibodies like MRK 16, UIC2, C219, JSB-1 is commercially available (217).

Multidrug resistance related protein (MRP1). MRP1 has been identified by Cole *et al.* (218) in cell lines that showed a typical MDR phenotype without elevated P-gp expression. Gene transfection studies have revealed that MRP1 expression resulted in a decrease of the intracellular concentrations of drugs, including anthracyclins, vinca-alkaloids and epipodophyllotoxins (219), and of toxic agents (220). The *MRP*1 gene, located on chromosome 16p13.1 encodes a 1531-aminoacid N-glycosylated integral membrane phosphoprotein, with a molecular weight of 190 kDa. Based on the characteristic structural motifs, MRP1 has been classified as a member of the ABC-transporter superfamily. The amino-acid homology between P-gp and MRP1 is 15%. Although MRP1 is a transmembrane protein, anti-MRP1 antibodies mainly stain intracellular epitopes.

The physiological role of MRP1 is unknown, but inside-out plasma membrane vesicles isolated from MRP1 overexpressing cells showed an increased ATP-dependent transport of glutathion S-conjugates and glucuronate and sulphate conjugates. Evidence that intact cells require glutathion (GSH) for extrusion of several drugs by MRP1 was obtained by depleting cells of GSH (221). MRP1 has been detected at high levels in all human tissues including blood. Only in erythrocytes and liver canaliculi, the levels of MRP1 were lower than in other tissues. Like P-gp, MRP1 is thought to be involved in altered drug distribution of intracellular compartments in the cytoplasm, leading to decreased concentrations of cytostatic drugs at their target sites (186). A major difference between P-gp and MRP1 is, that P-gp is less dependent on phosphorylation for function than MRP1 (222). Like P-gp, MRP1 can be investigated by functional assays using

carboxyfluorescein diacetate (CF) (223) or by measuring the expression levels using specific monoclonal antibodies like MRPr1, MRPm6, QRL-1 and QRL-3 (223).

Lung resistance-related protein/major vault protein (LRP/MVP). The lung resistance-related protein (LRP) was initially identified in an anthracyclin-resistant non-small cell lung cancer cell line characterized by the MDR phenotype, but lacking P-gp overexpression (224). The *LRP* gene is localized on chromosome 16p13.2 (225). LRP, also called the major vault protein (MVP) is the main constituent of a ribonucleoprotein particle: the vault complex. Vaults are evolutionarily conserved cytoplasmic organelles that may be present in the nucleus (226,227), and are found in most eukaryotic cells. Using electronmicroscopy (EM), purified vaults display a very distinct and complex morphology, resembling the multiple arches of a cathedral, the reason why they were named that way by Kedersha *et al.* (228). The vault components assemble into hollow barrel-like structures with an invaginated waist and two protruding caps which most likely consist of minor vault proteins (227). In normal tissue, LRP/MVP has been suggested to play a role in detoxification processes (186), but the physiological function of LRP/MVP is unknown. Until now, it is unclear which drugs are influenced by tumor expression of LRP/MVP. Transfection studies in cell lines have suggested that vaults may contribute to resistance to anthracyclins, vincristine and platinum derivates (229-231). There is circumstantial evidence for a role of vaults in clinical drug resistance (230-232). Most importantly, in a variety of drug-selected tumor cell lines, increased levels of LRP/MVP are consistently associated with increased levels of drug resistance (233,234). Furthermore, the expression of LRP/MVP closely reflects known chemoresistance characteristics in panels of unselected tumor cell lines and clinical cancers of different histogenetic origins. Some clinico-pathological studies, but not all, demonstrated that LRP/MVP expression at diagnosis is a strong and independent prognostic factor for poor response to chemotherapy in several malignancies (232,235-241). However, transfection of drug sensitive cells with only LRP cDNA (chromosomal DNA) did not confer drug resistance (231). This may either suggest that LRP/MVP is not the limiting component in vault assembly, or that vaults do not significantly influence MDR. Other vault components like the minor vault proteins and/or *vault* RNA are also necessary to obtain functional vault particles. Recently Kitazono *et al.* presented the first evidence that vaults are causally related to drug resistance (230). Reduction of LRP/MVP expression by the use of specific ribozymes in SW620 cells, that were induced to overexpress LRP/MVP by exposure to sodium butyrate, was sufficient to revert the drug resistant phenotype of these cells (230). The intriguing question that remains is, how vaults may confer the MDR

phenotype. It is suggested, that vaults may be involved in the sequestration of drugs in exocytotic vesicles and/or in the nucleo-cytoplasmic transport of various substrates. Future studies will have to corroborate the role of vaults in MDR and reveal the molecular mechanism by which they do so. As yet, there have been no reports of modulators of drug resistance mediated by vaults, that could be used for (pre-)clinical studies. For investigation of protein expression levels the specific monoclonal antibodies LRP-56, LMR-5 and MVP-37 are available.

Breast cancer resistance protein (BCRP). In 1990, Chen *et al.* reported on a 95-kDa MDR associated membrane protein (P-95), now called breast cancer resistance protein (BCRP), in a doxorubicin-resistant subline of the MCF-7 breast cancer cell line (242). The resistant subline, termed MCF-7/AdrVp, did not express P-gp, and was found to be highly resistant to anthracyclins, melphalan and teniposide (VM26), but not to vinblastine (243). P-95 has been noted to be expressed in the MDR small cell lung cancer lines NCI-H1688 and NCI-H660 and in clinical samples obtained from patients with solid tumors refractory to doxorubicin, (244). The murine gene is mapped on chromosome 6, 28-29 cM from the centromere, in humans on chromosome 4 at position q22, between the markers D4S2462 and D4S1557. An association of P-95 and the upregulation of the *H19* gene has been observed by Doyle in MDR cell lines (MCF-7/AdrVp and NCI-H1688) (245). The *H19* gene is an imprinted gene that has an important role in fetal differentiation, as well as a postulated role as a tumor suppressor gene (245). The new transporter P-95 was called 'breast cancer resistance protein' (BCRP) by Doyle *et al.*, because it caused drug resistance when transfected into drug-sensitive cells and because it was isolated from human breast cancer cells (243). The GenBank accession number for BCRP is AF098951. Other groups have termed BCRP 'mitoxantrone resistance protein' (MXR) (246) or 'placental ABC transporter' (ABCP) (247). The *BCRP/MXR/ABCP* gene is evolutionary distinct from the families that encode P-gp and MRP1, being on a completely separate limb of the phylogenetic tree (248). In contrast to the *MDR*1 and *MRP*1 gene it encodes a protein which is a half-transporter molecule requiring dimerization in order to function (248-250). Marked overexpression of BCRP has been observed in human cancer cell lines selected for daunorubicin, mitoxantrone or topotecan (251,252). Maliepaard *et al.* showed, that BCRP transported 70% of topotecan out of the T8 and MX3 cell lines in 30 seconds (252). In human normal tissue, the apparent localization of BCRP is quite distinct from that of P-gp and MRP1. BCRP levels are highest in the placenta, and in certain areas of the midbrain (putamen) (243,247). The

expression of BCRP in normal adult tissues and fetal tissues is relatively low, with the highest expressing adult organs being liver, small intestine, and colon. As yet, the monoclonal antibody (Moab BXP-34) is available for expression studies of BCRP (253). For functional assays Rho 123 in combination with the modulators GF 120918 or fumitremorgin C (FTC) can be used (251,254-257).

Other (membrane-) transport associated drug resistance proteins. Recently, several new members of the ABC transporter super family have been identified, such as the sister of P-glycoprotein (sP-gp), the transporter associated with antigen processing (TAP), the anthracyclin resistance-associated protein (ARA) and six new homologues of MRP (MRP2 or cMOAT, MRP3, MRP4, MRP5, MRP6 and MRP7).

sP-gp has a sequence homology of 61% with MDR1 (258), and is proposed to be the product of an earlier gene duplication and is only expressed in the liver of pigs. Whether it is expressed in human tumors is unknown. Overexpression of the peptide transporter involved in antigen-presentation (TAP) might also contribute to the multidrug resistant phenotype in many non-P-gp tumor cell lines. TAP is a heterodimer that is composed of TAP1 and TAP2 proteins. The heterodimer mediates the peptide translocation from the cytosol into the endoplasmatic reticulum (259). These peptides are then coupled to class I molecules of the major histocompatibility complex and the assembled complex is translocated to the plasma membrane for presentation to cytotoxic T lymphocytes. Besides its role in class I restricted antigen presentation, TAP has recently been associated with drug resistance, by transfection of *TAP1* and *TAP2* genes into TAP deficient lymphoblastic cells which resulted in resistance to MDR related-drugs including doxorubicin (DOX) (260). TAP might contribute to resistance by facilitating the sequestration of anthracyclins in the endoplasmic reticulum but this hypothesis awaits further investigation.

The *ARA* gene encodes a 49.5 kDa protein which resembles the C-terminal half of the MRP6 molecule (261). The 3' end of the *MRP6* gene corresponds with the 3' end of the *ARA* gene. The hypothesis is, that *ARA* is a splice variant of MRP6 (262). The relevance with respect to clinical resistance needs further investigation. Apart from MRP6, Kool *et al.* described MRP2, MRP3, MRP4 and MRP5 (262). The levels of *MRP2*-5 mRNA were analyzed in a panel of DOX and cisplatinum selected cell lines (262). Only the *MRP2* mRNA level was related to cisplatin resistance, whereas no relationship with DOX resistance was found. To date, MRP2 (also called canalicular multiorganic anion transporter, cMOAT) and MRP3 (also known as MOAT-D) have been cloned and transfected into drug-sensitive cells. Recently, an upregulation of *MRP2* and *MRP6* has

been reported in AML patients with inv(16) in patients especially in those with loss of one *MRP*1 homologue suggesting that there might be a compensatory role for these transporter proteins in AML (263). Enforced expression of MRP2 resulted in resistance to cisplatin, anthracyclins, etoposide, and methotrexate (264-266); enforced expression of MRP3 caused resistance to vinca alkaloids, etoposide, and methotrexate (267). Recently, MRP7 has been described as a ribosomal protein. The relevance of this protein for drug resistance has not been established as yet (268).

1.2.2.2 Mediators of Ara-C response

Cytosine-arabinoside (Ara-C) is a cytotoxic nucleoside analogue which acts by competing with the physiologic counterpart (cytidine) for incorporation into nucleic acids. As an inhibitor of DNA synthesis it has its greatest cytotoxic effects during the S-phase of the cell cycle (269,270). Ara-C enters the cell via a nucleoside carrier (271), although simple diffusion across the cell membrane has also been described (272).

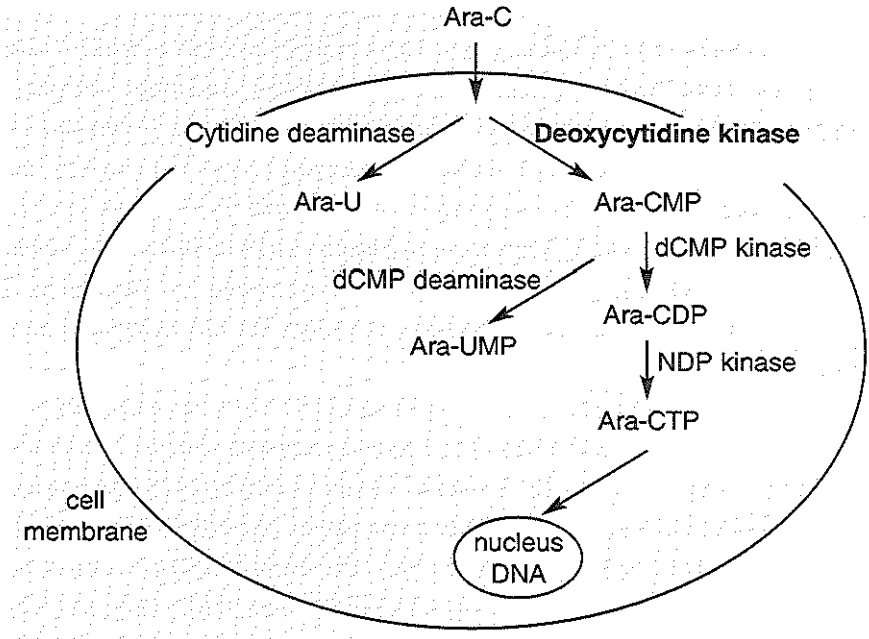

Figure 1. Metabolism of Ara-C by tumor cells

d: deoxyribose; MP: monophosphate; DP: diphosphate; TP: triphosphate; NDP: nucleoside diphosphate.

After entering the cell, Ara-C is phosphorylated into its mono-, di- and (the intracellulary) active triphosphate form Ara-CTP, by the action of three enzymes: deoxycytidine kinase (dCK), deoxycytidine monophosphate (dCMP) kinase and nucleoside diphosphate (NDP) kinase respectively (Figure 1). The first phophorylation step is catalyzed by deoxycytidine kinase (dCK), which has been appreciated as the rate-limiting enzyme in the metabolism of Ara-C. Ara-CTP inhibits DNA polymerase, and is competatively incorporated into DNA instead of the natural substrate deoxycytidine tripfosphate (dCTP) (273). Also, small amounts of Ara-CTP are incorporated into DNA, where the incorporated Ara-C residues act as relative chain terminators (274-277). Other mechanisms that have been suggested to be involved in cytotoxicity are effects on mitochondrial functions (278), direct induction of apoptosis (279,280), and inhibition of DNA ligase (281).

Several mechanisms of Ara-C resistance have been identified (Table 2) (282). In this thesis, we have focussed on the *dCK* gene (chapter 6), as this enzyme seems to play a major role in the mechanism of action of Ara-C (283). The gene has been assigned from a lymphoblast DNA library by Stegmann *et al.* (284). Murine neoplasm and human cell lines like HL-60 and AB 9228 confer resistance by decreased enzymatic activity (285-288).

Table 2. Mechanisms of Ara-C resistance

1. Kinetic resistance of Ara-C (non-cycling cells are less vulnerable)	
2. Pharmacologic resistance:	
A. Impaired Ara-CTP formation	
a. Failure of Ara-C to enter the cells (low concentration)	
b. Enzyme activity	- decrease of deoxycytidine kinase (dCK)
	- increase of cytidine deaminase (cDD)
B. Enhanced DNA repair mechanisms	
C. Reduced incorporation into the DNA (Mutations DNA polymerase)	
D. Direct influence on apoptosis	

1.2.3 The clinical relevance of multidrug resistance in AML

1.2.3.1 (Membrane) transport associated drug resistance proteins in AML

MDR1 and AML. In *de novo* AML, P-gp overexpression has been found in several studies of adult patients (56,59,62,64,289-291). The percentages of MDR1 positive samples in *de novo* AML vary from 17 to 71% (56,57,59,61,292-296). The quantitative

Table 3. Prognostic significance of MDR1 in *de novo* AML

Author	Year	No. of patients	a/c	Expression level	Correlation with outcome	
					CR	Survival
Sato	1990	36	a	RNA	S	S
Kuwarzuru	1990	17	a	protein	S	S
Pirker	1991	63	a	RNA	S	S
Marie	1991	23	a	RNA	S	-
Campos	1992	150	a	protein	S	S
Gruber	1992	34	a	RNA	NS	NS
Zhou	1992	51	a	RNA/protein	S	S
Marie	1993	42	a	RNA/protein	S	-
Te Boekhorst	1993	47	a	protein	S	S
Pirker	1994	63	a	RNA	S	-
Ino	1994	52	a	protein	NS	NS
Wood	1994	54	a	protein	S	NS
Hart	1994	36	a	RNA	S	S
Zöchbauer	1994	52	a	RNA/protein	S	S
Del Poeta	1994	117	a	protein	S	S
Guerci	1995	69	a	protein	S	S
Sievers	1995	130	c	protein	NS	NS
Zhou	1995	51	a	RNA	S	-
Schuurhuis	1995	17	a	RNA/protein	NS	NS
Del Poeta	1996	158	a	protein	S	S
Nussler	1996	102	a	protein	S	S
List	1996	21	a	protein	S	S
Samdani	1996	96	a	protein	S	S
Goasguen	1996	25	a+c	protein	NS	NS
V. d. Heuvel	1997	120	a+c	protein	S	S
Hunault	1997	110	a	RNA/ protein	S	S
Del Poeta	1997	223	a	protein	S	S
Martinez	1997	50	a	protein	S	S
Filipits	1997	80	a	protein	S	S
Hart	1997	67	a	RNA	NS	-
Lohri	1997	57	a	RNA	-	NS
Willman	1997	349	a*	protein	S	S
Willman	1997	203	a#	protein	S	S
Leith	1997	352	a	protein	S	S
Legrand	1998	53	a	protein	S	-
Borg	1998	91	a	protein	S	S
Kasimir	1998	40	a	protein	NS	-
Senent	1998	82	a	protein	S	NS
Pallis	1999	47	a	protein	S	S
Michieli	1999	96	a	protein	S	S
Del Poeta	1999	204	a	protein	S	S
Legrand	1999	80	a	protein	S	S
Broxterman	2000	98	a	protein	NS	NS

(N) S: (not) significant; -: not evaluated; CR: complete remission after induction chemotherapy; a: adults; c: children; *: < 55 year; :#: > 55 year

differences between these studies may result from the variety of analytical assays that were used for P-gp analysis. Moreover, several studies showed that MDR1 is expressed with high frequency (70%) in older patients, with the percentage of P-gp positive patients being similar in *de novo* and secondary AML (61,62). This is in striking contrast with the finding that MDR1 was expressed in 30% of younger adult (< 55 year) AML patients (62,297-299). MDR1 expression in AML is associated with the expression of the immature phenotype CD34 (56,295) and with CD7 expression (298). In contrast to the other AML subtypes, the promyelocyte subtype (FAB M3) is devoid of P-gp expression. (300-303). In most studies of relapsed AML, MDR1 expression does not exceed the level which was measured in groups of AML patients at diagnosis (212,304-313). However, studies of paired AML samples, investigating MDR1 expression during the development of refractory and/or relapsed disease in the same patients are scarce.

In adults, in *de novo* AML, MDR1 expression is a well-documented independent adverse prognostic factor for achieving CR and for survival (54,56-59,62,64,239,303,314)(Table 3). In children, the prognostic significance of MDR1 is less clear. The only study available on a respectable number of pediatric patients, *i.e.* a study of 30 infants and 100 children above the age of one, by Sievers *et al.* did not show a correlation of *MDR*1 upregulation with the achievement of CR, nor with long term survival (315). More prospective studies evaluating the prognostic significance of drug resistance proteins in large cohorts of children are needed.

MRP*1 *and AML. Several studies have investigated MRP1 expression in AML. The reported percentage of clinical samples with MRP1 expression ranges from 7 to 30% (60, 223,316-327). The incidence of MRP1 overexpression was obtained by RNA or protein techniques. As for the other resistance proteins, the different studies are difficult to compare because of heterogeneity of patient populations, techniques, and because of different criteria with regard to the definition of overexpression (60,316-327). No difference was found between *MRP*1 mRNA expression in normal bone marrow and in initial AML blasts. A relation of MRP1 expression and FAB subtype has been found by Lohri *et al.* (328). The *MRP*1 mRNA levels were clearly lower in the group of M4 and M5 as compared to the other FAB subgroups, and the lowest values were found in the 4 cases with a CBFB/MYH11 transcript caused by the chromosomal abnormality inv(16). In a small group of patients, Kuss *et al.* found a better clinical outcome in cases with FAB type M4Eo AML in which one allele of the *MRP*1 gene was deleted (329). However, Dohner *et al.* and Van der Kolk *et al.* did not find differences in MRP1

Table 4. Prognostic significance of MRP1, LRP and BCRP in *de novo* AML

Author	Year	No. of Patients	a/c	Expression level	Correlation with outcome	
					CR	Survival
MRP1						
Hart	1994	36	a	RNA	NS	-
Schuurhuis	1995	17a	a	RNA	NS	NS
Schneider	1995	29	a	RNA	NS	-
Zhou	1995	52	a	RNA	NS	-
Te Boekhorst	1995	35	a	RNA	NS	NS
Hunault	1997	110	a	RNA	S	NS
Filipits	1997	80	a	protein	NS	NS
Lohri	1997	57	a	RNA	NS	NS
Hart	1997	47	a	RNA	NS	-
Leith	1997	352	a	protein	NS	NS
Kasimir	1998	40	a	protein	NS	-
Legrand	1998	53	a	protein	NS(E)	-
					S(F)	-
Legrand	1999	50	a	protein	S	S
Borg	1998	91	a	protein	NS	NS
LRP /MVP						
List	1996	21	a	protein	S	S
Goasguen	1996	25	a+c	protein	NS	NS
Leith	1997	352	a	protein	NS	NS
Hart	1997	67	a	RNA	S	-
Pirker	1997	23	a	protein	S	S
Willman	1997	349	a*	protein	NS	NS
Willman	1997	203	a#	protein	NS	NS
Filipits	1998	86	a	protein	S	S
Borg	1998	91	a	protein	S	S
Legrand	1998	53	a	protein	NS	-
Pallis	1999	47	a	protein	NS	-
Michieli	1999	96	a	protein	NS	NS
BCRP						
Ross	2000	14	a	RNA	S?	-

(N)S: (not) significant; -: not evaluated; CR: complete remission after induction chemotherapy; a: adults; c: children; *: < 55 years; #: > 55 years; E: expression level; F: functional assay, S?: indicates a trend, very small group of patients.

expression in patients with and without deletion of an *MRP*1 allele in patients with inv(16) (330,331), suggesting that this deletion has no prognostic impact in these AML patients. In contrast to P-gp, MRP1 levels are higher in CD34- than in CD34+ leukemic cells (223,320). In several studies in relapsed AML, no or only minor differences have been found in *MRP*1 mRNA at relapse as compared to diagnosis (312,316-318). However, other studies found higher levels of *MRP* mRNA at time of relapse. Only one of these studies was performed in paired samples (316,317,323,332-334). MRP1 was found to be inversely related to the accumulated anthracyclin levels (318,335). It is not clear whether this is due to co-expression with P-gp in adult AML samples (60,323,336). Several studies on the prognostic value of MRP1 expression in AML patients have shown contradictory results (Table 4) (61,239,291,316,320). Overall, most studies conclude that MRP1 expression is not a prognostic factor for CR and survival in adult AML. Co-expression of both P-gp and MRP1 in a functional assay is likely to be of more prognostic value (223,291). The prognostic value of MRP1 in children with AML has not been studied as yet.

LRP/MVP and AML. LRP/MVP expression has been observed in 26-61% of the adult patients with AML (61,235-239,337,338). In some studies, LRP/MVP expression has been reported mainly in FAB M4 and M5 subtypes (60,238). The clinical impact of LRP/MVP in AML is contradictory (Table 4). A number of studies have reported a lower remission rate and/or a lower probability of long term survival in LRP-positive AML patients (236,238-240,335,340), while other studies have failed to demonstrate a prognostic value of LRP/MVP expression with regard to clinical outcome (60-63,339,341-343). Other studies failed to demonstrate a correlation between LRP/MVP expression and *in vitro* resistance to anthracyclins in adult AML (235,344). In adult AML, an association has been observed between LRP expression and anthracyclin accumulation, but not with rhodamin retention (335,345). Discrepancies in the clinical significance of LRP/MVP seem to be partly related to the methodology used, as the studies which do not show a prognostic value of LRP/MVP, use a flowcytometric technique to assess the expression in leukemic blasts (60,63,339,341,342). In contrast, LRP/MVP was a significant prognostic factor in adult AML in studies using immunocytochemical or mRNA LRP/MVP analysis by reverse transcriptase-polymerase chain reaction (RT-PCR) (235-240,337,338). In a paired analysis of 17 clinical AML samples, List *et al.* showed that LRP/MVP was higher at relapse than at diagnosis (235). However, in another study a paired analysis of 8 clinical AML samples revealed no

differences between diagnosis and relapse (236) as did non-paired studies (240). An association between LRP/MVP expression and older age, increased white blood cell counts and unfavorable karyotypes was reported (60,63,235,239,339). In childhood AML, no studies with respectable numbers of patients are available as yet.

BCRP and AML. Little is known about the expression of other ABC transporters and their relevance for clinical resistance in leukemia. Ross *et al.* investigated BCRP/P-95 in 14 samples of *de novo* adult AML patients, by means of a quantitative RT-PCR assay (323). He found a relatively high expression of *BCRP* in 30% of the *de novo* AML samples was found, as compared to the MCF7 cell line. The samples with a high expression of *BCRP* showed a higher remission induction failure than patients with a low expression, but the number of patients was small. (323). AML blasts positive for *BCRP* were more resistant in vitro to anthracyclins compared with the *BCRP* negative samples. Perhaps BCRP accounts for part of the recently described subset of AML patients whose blast cells had cyclosporin-resistant drug-efflux, not associated with the overexpression of P-gp, MRP1 or LRP/MVP (60,248). Ross *et al.* found a very weak correlation between the expression of *MDR*1 mRNA and *BCRP* expression (346). If BCRP expression indeed confers drug resistance in leukemia, it may be interesting to involve BCRP in current studies on the predictive value of MDR1, LRP/MVP and MRP1. Also, if BCRP and MDR1 are co-expressed in clinical AML samples, this might at least partly explain the disappointing results of MDR1 modifying agents in clinical studies so far (340). Currently, studies on the prognostic value of BCRP in AML are awaited. In children, no studies on BCRP expression are available as yet.

Co-expression of (membrane-) transport associated drug resistance proteins as a prognostic factor in AML. Several studies have shown that not the expression of one resistance protein, but a combination of two or even more may predict clinical drug resistance. Co-expression of P-gp and LRP/MVP occurs in a relatively small number (6-31%) of *de novo* AML patients (235,238,239,337,339). In these cases, both P-gp and LRP/MVP may potentially seem to contribute to the resistance phenotype. In one study, the longest survival was observed in AML patients with the lowest LRP and P-gp expression (239). This was also published by Broxterman *et al.*, who observed a strong association of AML with expression of both the LRP and P-gp phenotype with treatment failure (56 %, versus 8% in the double negative group) (347). Michieli *et al.* investigated 98 *de novo* AML patients and reported that co-expression of LRP/MVP and P-gp was associated with a low daunorubicin retention and a poor clinical outcome (63).

Co-expression of P-gp and MRP1 is associated with an extremely poor outcome in AML (236,317,339). Hunault *et al.* have pointed out that MRP1 and MDR1 expression were significantly correlated in AML (64). The MDR1/MRP1-double positive phenotype was expressed to a lesser extent in the 'good-risk' cytogenetic risk group. Recently, using a functional assay for MDR1 and MRP1 expression, patients with low accumulation of both Rho 123 and carboxyfluorescein (CF), had the lowest CR rate. However, no correlation was found with overall survival (348). A study of the co-expression of P-gp, MRP1, bcl-2, mutant p53 and heat-shock protein 27 in AML showed that co-expression of at least 2 proteins was predictive for CR (344). In this study no correlation was found between the expression of any of these proteins alone and treatment outcome (344).

1.2.3.2 Ara-C resistance in AML

Ara-C is used as a standard drug in the treatment of patients with AML. In combination with other chemotherapeutic agents it induces complete remission in 70-80% of adults and 80-90% of pediatric patients with *de novo* disease (43,75).

Ara-C is currently the most potent drug in the treatment of AML. As pointed out before, the enzyme dCK determines the rate limiting step in the phosphorylation process of Ara-C to the active component Ara-CTP. Cell lines from leukemic blasts of patients with ALL, who had become resistant to treatment with Ara-C showed markedly decreased Ara-CTP pools due to decreased dCK activity, suggesting that dCK deficiency is important in the clinical situation (349). Experiments on Ara-C resistant T-lymphoblast cell lines (Ara-C-8D and ddC50) revealed structural alterations, like point mutations and deletions within the coding region as well as decreased mRNA levels of *dCK* (287,350). In adult AML, low or altered dCK levels are associated with clinical cytarabine resistance (351,352).

Flasshove *et al.* studied 16 adult AML patients with relapsed and refractory AML (353). They found point mutations in the *dCK* gene in 7 patients, two silent mutations (codon 86 and 285), and five followed by amino-acid changes (codon 20, 93, 99, 98, 154). One of these, the point mutation in codon 99 (TAT→TGT) leading to an amino acid substitution from tyrosine to cysteine, was associated with absent dCK activity, whereas enzyme activity was normal in patients with a point mutation in codon 98 and 20. Recently, alternative splice variants of the *dCK* gene have been described in AML patients in which the resistant phenotype was not associated with *dCK* mutations. The alternatively spliced transcripts, in which one or more exons (354,355) were deleted, were shown to code for enzymatic inactive proteins *in vitro*.

1.2.4 Reversal of multidrug resistance in AML

1.2.4.1 *In vitro* reversal of multidrug resistance in AML

The first observations that drug resistance could be reversed were done by Tsuruo *et al.* (356), who showed that verapamil was able to enhance drug accumulation of vincristine and vinblastine in the P388/VCR drug-resistance cell line *in vitro* and *in vivo*. Since then, a number of miscellaneous compounds have been described to efficiently inhibit MDR (Table 5) in several cell lines expressing the MDR1 phenotype (177). Two possible approaches of MDR1 reversal can be distinguished. First, MDR1 specific anti-sense oligonucleotides (357) and protein C kinase inhibitors like staurosporine are capable to down-regulate MDR1 expression (195,358) which may result in decrease of resistance. The other way is to modify P-gp function by the (competitive) inhibition of P-gp by so-called reversing agents. It is currently accepted that such agents can restore drug accumulation by competing with cytostatic drugs for P-gp binding sites or by direct binding to P-gp. Agents that have this capacity include calcium channel blockers (*e.g.* verapamil), calmodulin inhibitors, indole alkaloids, detergents, steroids, anti-estrogens, cyclosporin analogues (*e.g.* cyclosporin A, PSC SDZ 833), pipecolinate derivatives (V-104), acridone carboxamide derivatives (GF120918 or GG918), polypropylene polymers (pluronic L61) and others (Table 5) (359-364). Several of these reversal agents share common chemical features, such as a planar aromatic domain and two amino groups, one of which has a cationic charge at physiologic pH and they all are highly lipophilic. A combination of different reversal agent, may result in a synergistic effect (365). This observation suggests that the exact mechanism of drug reversal may not be identical for different reversing agents. Recently, a study suggested that there are 4 drug binding sites which display complex allosteric interactions through which interaction of drug at one site switches other sites between high- or low-affinity conformations (366). As pointed out before, the function of the ABC transporters can be investigated using flow cytometric assays with fluorescent dyes using drug resistance modifiers. Various authors have reported theoretical and practical considerations in the choice of fluorescent dye, dye concentration, and choice of modulator in assays to determine functional activity of P-gp in leukemic samples (367-371). Recently, a new fluorescent probe (JC-1) to test P-gp activity has been reported, which might have a higher detection level for resistant cells in AML samples as compared to Rho 123 (372).

Table 5. Modulators of multidrug resistance

Mechanism of MDR	Mechanism of modulation	Examples
MDR1	P-gp binding inhibition	
	Calcium channel blockers	Verapamil, Dexniguldipine, PAK-200, AHC-52
	Calmoduline inhibitors	Trifluoperazine
	Immunosuppressive agents	Cyclosporine A, PSC 833 FK 506
	Quinolones	Quinidine
	Detergents	Cremophor EL
	Steroids	Progestesterone, Tamoxifen, Megestrol acetate
	Protein kinase C inhibition	Staurosporine
	Anti-sense oligodeoxy nucleotides	
	Acridone carboxamides	GF120918
	Propylene polymers	Pluronic L61
	Pipecolinate derivates	V-104
MRP1	Tyrosine kinase inhibiton	Genistein
	Direct binding to MRP1	Probenecid, MH-571 PAK-104P, VX-170
	Intracellular gluthathione depletion	BSO BCNU
	Inhibition glutathione S-transferase + direct binding to MRP1	Indomethacin, Ethacrynic acid
	Imidazothiazole derivates	
	ATP depletion	
LRP/MVP	Specific LRP/MVP ribozymes	
BCRP		Fumitremorgin C, GF120918

Currently, anthracycline or Rho 123 retention or efflux studies are commonly used in preclinical studies in cell lines or in clinical samples studying the functional level of P-glycoprotein using cyclosporin A or PSC 833 as modifiers (367,373,374).

In vitro reversal of drug resistance in fresh AML specimens has also been investigated. Verapamil, cyclosporin A, and other reversing agents increase the intracellular retention of daunorubicin in AML-blast cells which express P-gp, but not in drug-sensitive or P-gp-negative AML cells. This pharmacological effect is associated with increased cytotoxity of anthracyclins in *in vitro* clonogenic assays or in the MTT test, a cellular in vitro drug resistance assay using 3-4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide (MTT) (289,295,375-385). Because of potential side-effects of cyclosporin A, *i.e.* nephrotoxicity and immunosuppression, PSC 833, a cyclosporin D analogue was developed for clinical reversal of P-gp-mediated multidrug resistance. Te Boekhorst *et al.* showed that in AML patients the MDR1 phenotype was predominantly expressed in the CD34 positive cells. The level of daunomycin accumulation in the CD34 positive cells was restored to the level of CD34 negative population of cells using the modifier PSC 833 (361).

The difference in dependency on phosphorylation for function can be used for differentiating between the two efflux pumps MDR1 and MRP1 by the use of functional assays (222). The function of MRP1 can be blocked by many compounds with a variety of dissimilar structures (Table 5) (339,386). The best way to investigate functional MRP1 expression is to use carboxyfluorescein diacetate as a substrate, as it is specific for MRP1 (325). Other compounds like calcein AM, daunomycin and Rho 123 are substrates for both P-gp and MRP1. Another way to block transport by MRP1 is ATP depletion (387,388). In studies performed by Aszalos *et al.*, three prototype P-gp modifiers were used: verapamil, PSC 833 and cremophor EL (248,389,390). These "blockers" increase both the calcein uptake and the Rho 123 uptake in parental and VP16 selected cell lines (like UMCCO1 human lung cancer cells and MCF-7), in a flowcytometric assay. The fact that the substrate specificity for P-gp and MRP1 were overlapping but not identical was also found by Paul *et al.* (391).

Inhibition of BCRP mediated drug efflux has recently been described in cell lines using the compound GF120918 or Fumitremorgin C or its indolyl-diketopiperazinederivates as reversing agents (254,256,392), and mitoxantrone or daunomycin and Rho 123 as substrates. GF120918 was found to be an effective modifier in P-gp and BCRP expressing cell lines but was ineffective in MRP1 overexpressing cells (256,386,393). For LRP/MVP no functional assay is available.

1.2.4.2 *In vivo* reversal of multidrug resistance in AML

The discovery that the expression of MDR1 is an independent adverse prognostic factor for CR and survival in adults with AML, has led to several clinical studies with MDR1 modifiers in AML. At present, cyclosporin A and the cyclosporin D analogue PSC 833 (Valdospar) have been widely studied as reversal agents for resistance (394). These agents can be safely administered at sufficient doses to achieve effective serum levels for P-gp reversal and at the same time they can be combined with cytotoxic agents without an unacceptable increase of toxicity.

Administration of cyclosporin A and PSC 833 may result in increased toxicity of the anticancer drugs in patients for two reasons. First, the CD34+ hematopoietic progenitor cells are potentially harmed by a combination of a modulator and myelotoxic drugs because these cells express P-gp (395). Secondly, these modulators appear to alter pharmacokinetics of cytostatic drugs through modulation of ABC transporter proteins in the biliary canaliculi and renal tubuli, thereby blocking biliairy and renal drug elimination, leading to an increased plasma retention time and plasma area under the curve (AUC) of the anthracyclins and its metabolites (396).

Such an effect, recognized in mice has been observed in patients treated with verapamil and doxorubicin (397), verapamil with VAD (vincristine, doxorubicin and dexamethasone) (398,399), bipredil plus vinblastine (400), cyclosporin A plus daunorubicin and high dose Ara-C (340), cyclosporin A with vincristine, doxorubicin, and dexamethasone (401) and cyclosporin A plus etoposide (402). These studies showed that cyclosporin A, when present in the blood at effective levels, leads to an approximately two-fold increase of the plasma retention time of etoposide, daunorubicin, and doxorubicin. The first attempt to treat a refractory AML patient with daunomycin and cytarabine in combination with cyclosporin A was performed in 1990 by Sonneveld *et al.* (403). This patient was refractory to standard induction treatment and he subsequently achieved a short-lasting remission. Several phase I/II trials were initiated in AML patients who were either refractory to primary treatment or had relapsed after a previous response (Table 6). In general, combining modifiers and anthracyclines resulted in high areas under the curve (AUC) of the used cytostatic agents and metabolites (341,404,405), especially when the dose of the cytotoxic drug was not reduced. Also, considerable

Table 6. *Non-randomized* clinical trials with MDR1 modifiers in AML patients

Author, year	Phase	Reference	Number of patients	Modulator	Diagnosis	Age group	Chemotherapy
List, 1993	I/II	(340)	42	CsA	poor risk AML	3-75 year	Ara-C/ daunomycin
Berman, 1995	I	(417)	14	Tamoxifen	rel./refractory AML	22-67 year	daunomycin
Kornblau, 1997	I	(410)	10	PSC 833	rel./refractory AML/MDS	not mentioned	etoposide/ MXN
Advani, 1999	I/II	(408)	37	PSC 833	rel./refractory AML	27-70 year	etoposide/ MXN/Ara-C
Pea, 1999	I	(406)	27	CsA/Verapamil	*de novo* and rel. AML	adults	ida/Ara-C
Dahl, 2000	II	(411)	66	CsA	rel./refractory AML	children	etoposide/ MXN
Tidefelt, 2000	II	(404)	10	PSC 833	*de novo* AML	55-84 year	daunomycin
Sonneveld, 2000	II	(299)	39	PSC 833	*de novo* AML	elderly	daunomycin/Ara-C
Chauncey, 2000	II	(407)	31	PSC 833	*de novo* AML	≥ 56 year	etoposide/ MXN
Smeets, 2001	II	(396)	15	+/- CsA	poor risk AML	≤ 58 year	ida/ Ara-C
Damiani, 1998	III	(341)	46	+/- CsA	*de novo* AML	≤ 68 year	ida/ Ara-C
Tallman, 1999	III	(405)	38	+/- CsA	rel./refractory AML	≤ 65 year	etoposide/ MXN/Ara-C
Lee, 1999	III	(413)	110	+/-PSC 833	*de novo* AML	elderly	etoposide/ daunomycin/Ara-C

CsA: cyclosporin A; Ara-C: cytosine-arabinoside; MXN: mitoxantrone; Ida: idarubicin; rel.: relapse, =/-: comparing patients treated with or without the modulator, in a non-randomized way.

Table 7. ***Randomized*** **clinical trials with MDR1 modifiers in AML patients**

Author	Institute/ Group	Reference	Number of patients	Modulator	Diagnosis	Age group	Chemotherapy	Results
Solary	GOELAM	(414)	315	Quinine	poor risk	≤ 65 years	Ara-C/ MXN	no effect on CR no effect on DFS
Wattel	GOELAM/ GFM	(415)	131	Quinine	high risk MDS/ AML	≤ 60 years	Ara-C/MXN	higher CR rate P-gp+:higher DFS
Sonneveld	HOVON	completed	>80	CsA	Refractory AML	< 65 years	etoposide/MXN	analysis pending
Burnett	MRC	stopped	>100	CsA	AML	>60 years	daunorubicin/ Ara-C/ 6TG	high toxicity
Löwenberg	HOVON MRC	completed	450	PSC 833	AML	> 60 years	daunorubicin/ Ara-C	CR/DFS equal P-gp+:higher DFS
List	SWOG	(418)	220	CsA	poor risk AML	adults	daunorubicin/ HD-Ara-C	equal CR rate RFS and OS higher with CsA (p=0.04 and 0.05)
Ravindranath	POG	completed	650	CsA	AML	children	Ara-C/ anthracylins	analysis pending

CsA: cyclosporin A; Ara-C: cytosine-arabinoside; MXN: mitoxantrone; 6-TG: 6-thioguanine; CR: complete remission; DFS, disease-free survival; EFS, event-free survival; RFS: relapse-free survival. P-gp +: P-glycoprotein positive, POG: pediatric oncology group, SWOG: south west oncology group, MRC: Medical Research Council, HOVON: Dutch-Belgian Hemato-oncology collaborative group, GOELAM: Groupe quest est des leucémies aiguës myéloïdes, GFM: Groupe français des myélodysplasies.

toxicity, related to the dose of the modifier like severe marrow hypoplasia, nausea, hyperbilirubinemia and cardiovascular toxicity has been reported (340). This has resulted in dose adjustments for modifiers (299,406) and anthracyclins (340,407) in the clinical protocols.

In some studies, equal to higher CR rates than in historical controls have been reported in adults with relapsed and refractory AML (408-410). In children, thus far only one study has been performed in 37 patients with recurrent or refractory AML using cyclosporin A. The remission rate was 35%, while 12% achieved a partial remission and 9% died of infection. The use of cyclosporin A improved the response rate in the MDR1 positive patients, but not in the MDR1 negative AML patients (411).

In *de novo* AML patients, modifiers have been used in elderly (299,408,412,413) and in younger adults (341,404,407). Again, the addition of modulators was well tolerated but required adjustment of dosages of the cytostatic drugs (407). In the largest non-randomized in 110 elderly AML patients, Lee *et al.* did not find a difference in CR rate between the two groups of patients treated with or without PSC 833 (413).

A number of randomized studies have been conducted in AML. Solary *et al.* found that a slightly better response in patients was associated with higher toxicity, leading to an overall similar clinical benefit in a study of 315 poor risk leukemia patients treated with and without quinine as modifier (Table 7) (414). Wattel *et al.* studied the use of quinine in 131 high risk MDS and AML and found an increase in CR rate and survival in the P-gp positive patients (Table 7) (415). More recently, the South-Western Oncology Group (SWOG) performed a randomized study in relapsed or high-risk patients using standard induction treatment as compared to an attenuated dose of daunomycin with cytarabine plus cyclosporin A. Although the response rates were not different in both arms, the overall survival and the progression-free survival was significantly better in patients receiving cyclosporin A (416). The Dutch and British study groups have just completed a prospective randomized phase III trail to evaluate the clinical benefit of PSC 833 used in front line therapy in this group of 300 elderly AML patients.

Reversal of multidrug resistance in clinical setting warrants further investigation, preferably in randomized clinical trials. The results of more of these studies will become available soon. Clinical modifier studies of the other multidrug resistance proteins are not available as yet. For the future, emphasis should be part on the development of reversing agents that inhibit P-gp and preferably other drug resistance proteins in tumor cells only, and do not influence the pharmacokinetics of cytostatic agents.

1.3 Aims of this thesis

Resistance to chemotherapy is an important cause of treatment failure in AML. In case of relapsed AML, the probability of achieving complete remission is lower as compared to *de novo* disease. The aim of the studies described in this thesis is to evaluate the clinical relevance of different aspects of several multidrug resistance phenotypes in AML. We have investigated the prognostic significance of MDR1 expression in adults and children with *de novo* AML as compared to other prognostic factors in a multivariate analysis. Also, we have investigated several drug resistance mechanisms in paired analyses of adults and children with AML at relapse and/or refractory disease as compared to diagnosis.

In **chapter 2** we have shown the prognostic value of MDR1 expression as compared to other prognostic factors like age, WBC, karyotype and CD34 expression in *de novo* AML using uni- and multivariate analyses.

In **chapter 3** we investigated whether *MDR*1 gene related clonal selection plays a role in the development of the disease from diagnosis to relapsed/refractory disease by analyzing the genetic polymorphism of the *MDR*1 gene at position 2677 in AML.

In **chapter 4** we studied which of the drug resistance genes *MDR*1, *LRP/MVP*, *MRP*1 and *BCRP*, was overexpressed at relapse/refractory state compared to initial diagnosis using real time PCR (Taqman) in a paired analysis of AML patients.

In **chapter 5** the results are presented of a study in which we investigated the poor-risk AML patients with partial or complete monosomy 7, to determine the level of expression of MDR1 and the specificity of the allelic loss of the *MDR*1 gene, which is located on chromosome at position 7q21.1.

In **chapter 6** we have shown the results of a study in which we performed a mutation analysis of the *deoxycytidine kinase* (*dCK*) gene in AML patients at diagnosis and relapse, appreciating the fact that dCK is the rate limiting enzyme in the Ara-C metabolism.

In **chapter 7** the *in vitro* effect of one of the MDR1 specific modifier GF 120918 is reported in leukemia and myeloma cell lines.

In **chapter 8** the results and relevance of the presented experimental data will be discussed.

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CHAPTER 2

MDR1 expression is an independent prognostic factor for response and survival in *de novo* acute myeloid leukaemia

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Summary

The multidrug resistance gene (*MDR*1) is frequently expressed in acute myeloid leukaemia (AML). MDR1 is associated with resistance to chemotherapy *in vitro* and with a poor response rate in AML. We have investigated the prognostic value of MDR1 expression in relation to other patient characteristics with respect to response and survival.

One hundred and thirty patients aged 0-88 years were treated for *de novo* AML with standard induction and consolidation chemotherapy. MDR1 expression was determined by immunocytochemistry. Univariate and multivariate analyses were conducted to identify prognostic factors for reaching complete remission (CR) and for overall survival from diagnosis, in order to compare MDR1 with known prognostic factors. Univariate analysis showed that higher MDR1 was an adverse prognostic factor for CR ($P<0.001$), as was higher age ($P<0.001$) and unfavourable karyotype ($P<0.01$). These factors were also negative prognostic factors for overall survival (respectively $P<0.001$, <0.05 and <0.005, respectively). In the multivariate analysis MDR1 ($P<0.001$), higher age ($P<0.001$) and karyotype ($P<0.01$) were independent adverse prognostic factors for CR as well as for overall survival ($P<0.001$, $P<0.005$, $P<0.001$, respectively). Our data indicate that MDR1 expression is a disease-related unfavourable prognostic factor which has a significant impact on CR and overall survival in AML. Analysis of MDR1 may be used to determine prognosis in individual patients.

Introduction

Treatment of acute myeloid leukaemia (AML) with cytosine-arabinoside (Ara-C) and daunorubicin results in approximately 65% complete remissions (CR) in adults (1-4). However, with conventional post-remission chemotherapy only 25% of these patients remain relapse-free (1,5,6). Intensive consolidation chemotherapy or myeloablative therapy, followed by allogeneic or autologous stem cell transplantation may overcome minimal residual disease and prevent relapse, resulting in the accomplishment of long-term survival (3,7,8). Because of the significant toxicity associated with this myeloablative therapy, a substantial proportion of patients, such as those above the age of 60, do not benefit from intensification regimens. Other patients relapse with refractory disease, in spite of intensive consolidation therapy.

Expression of transmembrane transporter proteins in tumour cells has been identified as an important cause of chemotherapy resistance (9,10). Of these, the *MDR*1 gene encodes a membrane P-glycoprotein (P-gp), which is expressed in 19-75% of untreated AML and is

associated with a low probability to attain a CR (11-14).

At relapse, expression of MDR1 is more frequently observed, indicating that P-gp positive cells surviving induction therapy may form a reservoir of resistant leukaemia cells, ultimately resulting in treatment-refractory disease.

At present it remains to be determined if MDR1 expression of AML cells at diagnosis independently influences the outcome of remission induction therapy and long-term survival.

We have performed a prognostic study to identify the clinical role of MDR1 in *de novo* AML. For this purpose univariate and multivariate analyses were performed in protocol-treated patients to investigate the impact of MDR1 expression on response and survival in untreated AML in relation to other prognostic factors.

Patients and methods

Patients

After informed consent, bone marrow specimens from all newly diagnosed AML patients who were admitted during a period of 4 years (adults) and 7 years (children) respectively, were included in the study.

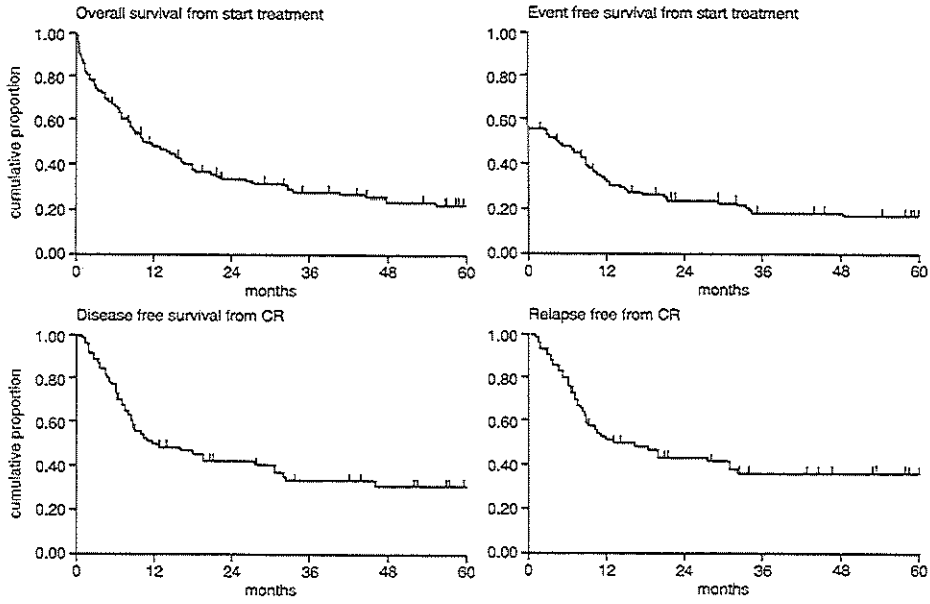

Figure 1. Survival of all AML patients from the start of treatment.

Patients who had received prior chemotherapy or radiotherapy for haematological disorders or solid tumours were excluded. BM aspirates were taken for evaluation of the AML phenotype, morphology, cytogenetic and other studies. Morphological classification was performed on May-Grünwald-Giemsa stained bone marrow smears according to the French-American-British (FAB) criteria (15).

Table 1. Clinical characteristics of the 130 AML patients

	Age group (years)			
	0-15	16-60	61-88	Total
Patients	23	61	46	130
Sex				
Male	13	35	26	74
Female	10	26	20	56
Age				
Median	8	44	67	51
Range	0-15	16-60	61-88	0-88
FAB classification				
M0	-	2	-	2
M1	5	10	10	25
M2	4	14	23	41
M3	-	5	1	6
M4	7	17	6	30
M5	6	8	6	20
M6	1	3	-	4
M7	-	2	-	2

Treatment protocols

The study included 61 adult patients (aged 16-60 years) who were treated according to the standard protocol of the Dutch Haematology-Oncology Group for AML (HOVON-4). Chemotherapy consisted of daunorubicin (DNR, 45 mg/m^2, i.v., days 1-3) and Ara-C (200 mg/m^2, i.v., days 1-7), followed by a second induction cycle of amsacrine (120 mg/m^2, i.v., days 4-6) plus Ara-C (2 g/m^2, i.v. , days 1-6) and a consolidation cycle of mitoxantrone (10 mg/m^2, i.v., days 1-5) plus etoposide (100 mg/m^2, i.v., days 1-5). Patients under 55 years with an HLA-identical sibling were eligible for allogeneic bone marrow transplantation. Patients in CR without a marrow donor were randomised to treatment with autologous, unpurged

bone marrow transplantation or no further therapy. Forty-six patients aged 61 years or more were treated according to an elderly AML protocol (HOVON/EORTC 9/11). Remission-induction treatment consisted of 2 cycles of DNR (30 mg/m^2, i.v., days 1-3) or mitoxantrone (8 mg/m^2, i.v., days 1-3), combined with Ara-C (200 mg/m^2, i.v., days 1-7), followed by consolidation with DNR (30 mg/m^2, i.v.) or mitoxantrone (8 mg/m^2, i.v., days 1-3), and Ara-C (100 mg/m^2, i.v., days 1-7). Twenty-three paediatric patients (0-15 years) were treated according to the protocols of the Dutch Childhood Leukaemia Study Group (DCLSG) with idarubicin or daunomycin (respectively 12 and 60 mg/m^2, i.v., days 3-5) plus Ara-C (100 mg/m^2, i.v., days 1 and 2; 200 mg/m^2, i.v., days 3-8), etoposide (150 mg/m^2, i.v., days 6-8), and intrathecal Ara-C on day 1, followed by consolidation with prednisolone (40 mg/m^2/d, orally for 28 days), 6-thioguanin (60 mg/m^2, orally for 43 days), vincristine (1.5 mg/m^2/week, i.v., four times), doxorubicin (30 mg/m^2/week, i.v., four times), Ara-C (75 mg/m^2, i.v., 24 times), cyclophosphamide (500 mg/m^2, i.v., twice), combined with intrathecal Ara-C (four times) and intensification with Ara-C (3000 mg/m^2, i.v., 10 times) plus mitoxantrone (10 mg/m^2, i.v., twice), followed by etoposide (125 mg/m^2, i.v., four times), or etoposide alone (125 mg/m^2/day, i.v., eight times) again with intrathecal Ara-C on day 1. Thereafter, patients were eligible for allogeneic bone marrow transplantation if an HLA-identical sibling was available.

CR status was defined according to the criteria of the CALGB, *i.e.*, normocellular marrow, < 5% blasts in a BM smear, or < 5% promyelocytes in case of AML-M3, and < 10% blast cells + promyelocytes (15), with normal peripheral blood cell counts. Both BM and blood cell counts should remain normal for at least one month. The clinical characteristics of the patients are listed in Table 1.

Cell samples

Bone marrow specimens were aspired from the posterior iliac crest at diagnosis and collected in heparinized tubes. Mononuclear cells (MNC) were isolated from the samples by Ficoll-Hypaque density gradient centrifugation (density 1.077 g/ml, Pharmacia, Uppsala, Sweden). Further purification of AML blast cells was performed by E-rosette and adherence depletion. After these steps > 95% of the cells were of AML blast cell morphology. Evaluation of CD34 membrane expression and MDR1 expression was performed on these purified blasts.

Analysis of MDR1 expression

MDR1 expression was evaluated by immunohistochemistry on cytospin slides using two

monoclonal antibodies (Moab) which are reactive with different epitopes of the P-gp molecule, *i.e.* C219 (cytoplasmatic epitope) and C494 (extracellular epitope) (Centocor, Malvern, PA, USA). Purified bone marrow specimens were washed twice in phosphate buffered saline (PBS) and cytocentrifuge slides were prepared. The slides were fixed in acetone/formalin phosphate buffer (50%/25%/25%), soaked in 50 mM Tris in 0.02% Tween 20 for 5 minutes and incubated with 10% normal rabbit + 1% normal goat serum for 30 min C219, C494, or isotype matched, non-reactive antibody controls (IgG2a)(Coulter Clone, Hialeah, Fla., USA) were added for 60 minutes at 37^{0}C. Anti-mouse immunoglobulin was added and after washing, the slides were incubated with alkaline phosphatase substrate (APAAP) for 60 minutes at 37^{0}C and washed three times after repeating these two steps. The cells were counterstained using Papanicolaou' solution1[a]/Harris' haematoxylin solution (Merck, Darmstadt, Germany). For each sample, four slides were prepared, which were stained with two antibodies and two controls, respectively. The whole staining procedure was performed twice on different days. The mean percentage of positive cells of four stained slides was counted by two independent investigators. The myeloma cell lines RPMI 8226S and its MDR1 expressing derivatives 8226D4 and 8226D40 were used as controls. Of these, 8226D4 expresses MDR1 at a low level of resistance, while 8226 D40 is highly positive. Using this technique of membrane staining of AML blast and control cells, the lower limit of detection in counterstained cytospin slides was 10% positive cells. Samples with < 20% positive cells (lower limit + twice standard error) were grouped together. All samples were scored in three prospectively defined arbitrary groups, *i.e.* with < 20%, 21-50 % and > 50% MDR1 positive AML cells.

Analysis of CD34 expression

Cells were washed in PBS supplemented with 2% bovine serum albumin (BSA). Next, the cells were incubated with phycoerythrin (PE) conjugated HPCA-2 [anti-CD34 PE; Becton & Dickinson (BD), San Jose, CA, USA] for 30 minutes at 0^{0}C. An irrelevant, isotype matched Moab was used as a negative control. After washing, 5000 events were counted using a FACScan flowcytometer (BD). The PE fluorescence signal was logarithmically amplified. The blast cell population was gated using scatter parameters. Data analysis was performed using PClysis software (BD), and the samples were divided in three groups: those with 0-10%, 11-50% and 51-100% CD34 positive AML cells.

Morphologic classification

Morphologic classification on May-Grünwald-Giemsa-stained bone marrow smears was performed according to the French-American-British (FAB) criteria (15).

Cytogenetics and immunologic marker analysis

Cytogenetic analysis of the AML blasts was performed by standard techniques (16). Chromosomes were identified by banding techniques according to the International System for Human Cytogenetic Nomenclature (ISCN) (17). For statistical analysis the patients were subdivided in three cytogenetic subgroups: *i.e.* favourable karyotype [t(15;17), t(8;21), inv(16)], unfavourable karyotype [-7/7q-, -5/5q-, t(9:22) or complex (more than two abnormalities) karyotype], or neutral [normal karyotype or karyotype not belonging to the above groups] (18). Immunologic surface membrane marker analysis was performed as described previously (19).

Statistical analysis

The data are based on 130 patients treated according to cooperative group protocols. CR was determined according to the treatment protocols, after 2 cycles of induction therapy. Overall survival was recorded from diagnosis to the date of death or last contact. Disease-free survival was recorded from the date CR was reached to the date of relapse, death or last contact, whichever occurred first. The actuarial method of Kaplan and Meier was used to calculate survival curves (20). The following variables were included in the analysis of prognostic factors: the percentage of CD34-positive bone marrow blasts, the percentage of MDR1 positive blasts, white blood cell count, age and, cytogenetics at diagnosis. Patients were divided in three arbitrary, *a priori* chosen groups for each variable. All reported P values are two-sided, and a significance level of ≤ 0.05 was used. Spearman's rank correlation test was used to determine the relation between the variables.

Logistic regression, univariate and multivariate, with the variables divided in three groups, was used to see whether there was a difference in CR rate between the subgroups. The variables that were significant in the univariate logistic regression were also used in a multivariate logistic regression (21). Univariate survival analysis was performed using the logrank test to see whether there was a difference in survival between the subgroups (22), and the univariate Cox regression was used to determine whether this relation was varied (23). The variables that appeared significant in the univariate Cox regression were also used in a multivariate Cox regression.

Table 2. Outcome of treatment according to age groups

	Age group (years)			
	0-15	16-60	61-88	Total
Total	23	61	46	130
Treatment				
Chemotherapy	14	47	46	107
Allogeneic BMT	5	8	-	13
Autologous BMT	4	6	-	10
Complete response	20 (87%)	33 (54%)	19 (41%)	72 (55%)
Relapse after CR	10 (50%)	20 (61%)	12 (63%)	42 (58%)
Death in CR	1	1	2	4
Death after relapse	7	17	10	34
Present clinical status				
Alive	12	15	7	34
Dead	11	46	39	96
Median follow-up of patients Still alive (months)	39.3	57.3	35.2	49.5
Median overall survival from diagnosis (months)	62.2	9.9	7.8	10.4

Results

One hundred and fifty-two patients with newly diagnosed *de novo* AML were treated in the participating centres, *i.e.* two haemato-oncology wards for adults (accrual from 1987 to 1990) and in the haemato-oncology departments of two children's hospitals (accrual from 1987 to 1994). All patients who were treated with at least one induction cycle of chemotherapy were included in the study. Of these, 22 patients were not included in the present analysis because of the following reasons: no protocol treatment applied (n=7), no evaluation or follow-up data available (n=5), no bone marrow available for immunocytochemistry (n=10). Thus, 130 patients (23 children, 107 adults) were included in the analysis. Of these, 23 patients received intensive consolidation treatment with autologous or allogeneic transplantation in first remission (Table 2).

Table 3. Distribution of risk factors of the 130 patients

	Age group (years)			
	0-15	16-60	61-88	Total
CD34 expression (%)				
< 10	8	22	15	45
11-50	4	20	13	37
> 50	11	19	17	47
Unknown	-	-	1	1
WBC($x10^9$/l)				
< 20	6	23	18	47
21-50	5	15	9	29
> 50	12	23	19	54
MDR1 expression (%)				
< 20	10	18	20	48
21-50	9	20	9	38
> 50	4	23	17	44
Karyotype*				
Favourable	2	12	-	14
Unfavourable	5	17	10	32
Neutral	16	32	36	84

*Favourable cytogenetics: t(15;17), t(8;21), inv(16). Unfavourable cytogenetics: -7/7q-, -5/5q-, t(9;22), complex karyotype (more than two abnormalities). Neutral: normal or abnormal karyotype.

Seventy-two patients (55%) attained a CR. Patients aged 15 or younger had a significant better CR rate (87%) as compared to adult patients (54%) or elderly patients (41%) (Table 2). The relapse rate was not different between age groups. Among these age groups, the distribution of risk factors for CR, *i.e* CD34 expression on AML blasts, leukaemia-associated cytogenetic abnormalities and a high white blood cell count (WBC) at presentation was investigated (Table 3). No significant difference of the presenting whiteblood cell counts was observed among paediatric, adult and older patients. MDR1 expression was observed in all age groups. Spearman's rank correlation test indicated that MDR1 was only correlated with CD34 ($P<0.01$) but not with WBC, age or cytogenetics. Univariate logistic regression analysis showed that MDR1 expression ($P<0.001$), higher age ($P<0.001$) and unfavourable cytogenetics ($P<0.01$) were negative prognostic factors for the probability to achieve a CR (Table 4).

Table 4. Univariate logistic regression analysis for reaching CR using the variables divided in the three subgroups

Variable	*n*	CR (%)	Odds ratio	95%CI	P- value
Sex					
Male	74	55	1		1.0
Female	56	55	0.998	0.50-2.01	
Age (years)					
0-15	23	87	1		<0.001
16-60	61	54	0.177	0.05-0.66	
61-88	46	41	0.106	0.03-0.41	
Karyotype					
Favourable	14	71	1		< 0.01
Unfavourable	32	31	0.182	0.05-0.72	
Neutral	84	62	0.650	0.19-2.25	
CD34 (%)					
< 10	45	76	1		<0.001
11-50	37	30	0.137	0.05-0.36	
> 50	47	57	0.437	0.18-1.07	
WBC ($x10^9/l$)					
0-20	47	60	1		<0.8
21-50	29	52	0.727	0.29-1.85	
> 50	54	54	0.787	0.36-1.74	
MDR1 (%)					
< 20	48	77	1		<0.001
21-50	38	50	0.297	0.12-0.75	
> 50	44	36	0.170	0.07-0.42	

When different thresholds for MDR1 positive cells were studied, MDR1 remained significant at levels from 2% to 50% positive cells. However, the optimum significance was reached at 20 % positive cells. CD34 expression was also a significant adverse factor for reaching a CR (P<0.001), but here the group with 11-50 % CD34+ cells did worse than the other groups. No significant correlation of the white blood cell count with CR was found from counts of 20 x 10^9/L up to 100 x 10^9/L.

Using univariate analysis, higher age (P<0.05), unfavourable karyotype (P<0.005) and MDR1 expression (P<0.005) but not CD34 were adverse prognostic factors for overall

survival (Table 5). Multivariate logistic regression revealed that increased MDR1 (P<0.001), age (P<0.001), and karyotype (P<0.01) were independent adverse prognostic factors for CR. By Cox multivariate regression analysis, increased MDR1 expression (P<0.001), higher age (P<0.005) and cytogenetics (P<0.001) were negative prognostic factors for overall survival (Table 6.). In Figure 1, Kaplan-Meier curves for the overall survival, event-free survival and disease-free survival of the whole group of patients are presented. Figure 2 shows the actuarial survival for different prognostic factors. These data indicate that MDR1 is an independent adverse prognostic factor for complete response and survival in *de novo* AML.

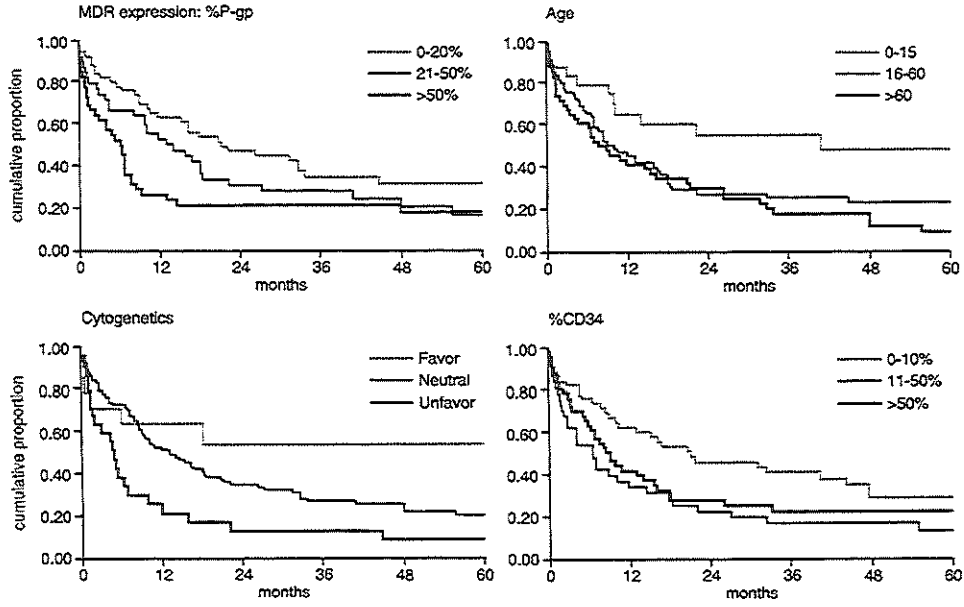

Figure 2. Survival according to MDR1 expression, age, cytogenetics and CD34 expression.

Table 5. Univariate analysis for overall survival using the logrank test to show a difference between the three subgroups

Variable	Hazard ratio	95% CI	P- value
Sex			
Male	1		< 0.9
Female	1.029	0.69-1.54	
Age (years)			
0-15	1		< 0.05
16-60	2.020	1.04-3.91	
61-88	2.559	1.30-5.03	
Karyotype			
Favourable	1		< 0.005
Unfavourable	3.06	1.26-7.47	
Neutral	1.90	0.82-4.40	
CD34 (%)			
< 10	1		< 0.06
11-50	1.865	1.12-3.12	
> 50	1.508	0.92-2.47	
WBC ($x10^9$/l)			
0-20	1		< 0.5
21-50	1.332	0.77-2.30	
51-100	1.317	0.83-2.09	
MDR1 (%)			
< 0-20	1		< 0.001
21-50	1.593	0.96-2.64	
50	2.205	1.34-3.62	

Table 6. Multivariate analysis for reaching complete remission (CR) and overall survival, using the risk factors that were significant in the univariate analyses

CR			
	Odds ratio	95% CI	P- value
Age	0.36	0.20-0.67	< 0.001
Cytogenetics	0.36	0.18-0.75	< 0.01
MDR1	0.39	0.23-0.64	< 0.001
Overall survival			
	Hazard ratio	95% CI	P- value
Age	1.54	1.15-2.07	< 0.005
Cytogenetics	1.99	1.36-2.92	< 0.001
MDR1	1.61	1.25-2.06	< 0.001

Discussion

Refractoriness to chemotherapy is the major cause of treatment failure in AML and several other haematological malignancies. Higher age, high WBC at diagnosis, and M_0 morphology have been recognised as clinically adverse prognostic factors in AML. Additional biological or disease-related prognostic variables which are associated with a poor survival include higher age, hyperleucocytosis, autonomous leukaemia growth *in vitro*, expression of the immature stem cell antigen CD34 and karyotypic abnormalities like monosomy 7, 5q-, 7q-, the Philadelphia chromosome [t(9;22)] and a complex karyotype (8,24,25). On the other hand, cytogenetic abnormalities like inv(16), t(8;21) and t(15;17) are associated with a good response to chemotherapy.

The presence of the multidrug resistance phenotype (MDR1) at diagnosis seems to be of biological and clinical importance in AML. There is strong evidence that the MDR1 encoded P-glycoprotein is associated with enhanced efflux of amsacrine, anthracyclins, vinca-alkaloids, etoposide and mitoxantrone from leukaemia cells, leading to lower intracellular concentrations of these drugs.

In untreated *de novo* AML patients MDR1 is expressed in 19 % to 75 % of the patients (11-14,26,27). In these patients, MDR1 is frequently associated with an immature phenotype (CD34) and autonomous AML growth *in vitro* (13). Univariate analysis of MDR1 and CD34 expression in our study indicates that both are unfavourable prognostic parameters in AML. A predominance of MDR1 expression may be present in AML cells with an immature

immunophenotype (CD34) (13). These characteristics emphasise that MDR1 is a biological marker which may offer an explanation for poor response to anthracyclins, mitoxantrone and etoposide in AML.

Several studies have shown that treatment of patients with leukaemia or myeloma with anthracyclins and vinca-alkaloids may even further increase MDR1 expression in (previously negative) patients or that the expression levels are higher in previously treated and/or refractory patients (10,28). MDR1 thus seems to be a potential important marker for refractory disease in AML. However, a major problem with studies of MDR1 in AML is caused by the use of different assays and different thresholds for MDR1 expression. In addition, all studies published to date, were retrospective studies. No study has performed an in depth analysis of MDR1 with other prognostic factors in uniformly treated patients. We have initialised a systematic analysis of MDR1 in AML patients 7 years ago, using an immunocytological method with which MDR1 could be identified in the actual tumour (blast) cells. Although this assay does not provide evidence of MDR1 functionality, it offers the possibility to study all accrued patients, even those from which not sufficient material can be obtained for functional or RNA assays (29). Moreover, we have found a good correlation with MRK16 staining using flow cytometry and the rhodamine 123 exclusion assay, as used in another prospective study (30). However, a substantial analysis of the role of MDR1 in relation to already known prognostic factors is needed to further define the relevance of MDR1 expression.

In the present study the value of age, white blood cell count, percentage CD34 expression, cytogenetics, and *MDR*1 expression at diagnosis on the outcome of induction treatment and on overall survival and disease-free survival were studied in 130 AML patients.

The non-selected groups of patients with representants of all age groups were all treated accordingly to their standard, age-restricted protocols. In this study *MDR*1 expression is an independent, unfavourable prognostic factor not only for complete response, but also for overall survival. It may therefore be an important marker of resistant disease, which provides insight in the cause of treatment failure. We now know that many antileukaemic agents are transported by P-gp, including anthracyclines and etoposide. MDR1 expression may be an explanation why dose-escalation of these agents does not improve the survival in AML. In contrast, the effect of Ara-C is not affected by MDR1 expression. High-dose Ara-C administered during induction or consolidation therapy significantly improves the disease-free and overall survival of AML patients (3,31). A significant improvement of survival may also be accomplished by intensification with alkylating agents or radiation therapy followed

by marrow transplantation. However, such intensive treatments cannot be tolerated by elderly patients, and consequently, the prognosis for these patients has remained poor (32). Previous studies have frequently failed to demonstrate an independent prognostic value of MDR1 after adjustment for both karyotype, CD34 and age, largely because these retrospective analyses did not determine all prognostic factors in the whole study population, or because of the small patient sample (33-35).

Recently, phase I/II clinical trials have evaluated the possibility to circumvent MDR1 efflux function by adding noncytotoxic agents such as cyclosporine A or verapamil (36-38). Second-generation reversal agents which lack major immunosuppressive or cardiovascular side-effects are now coming available. Patients with MDR1 positive AML may profit from reversal agents when these are combined with standard induction therapy. Future studies, however, should also take other resistance mechanisms into account which were identified after this prospective study had started, in order to further improve our knowledge about the prognostic value. Taken together, the results from the present study indicate that MDR1 expression is an independent adverse prognostic factor in AML, that allows us to identify patients who may benefit from agents that reverse resistance to chemotherapy.

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CHAPTER 3

*MDR*1 gene related clonal selection and P-glycoprotein function and expression in relapsed or refractory Acute Myeloid Leukemia (AML)

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Abstract

The expression of P-glycoprotein (P-gp), encoded by the *MDR*1 gene, is an independent adverse prognostic factor for response and survival in *de novo* acute myeloid leukemia (AML). Little is known about MDR1 expression during the development of disease. We investigated whether *MDR*1 gene related clonal selection occurs in the development from diagnosis to relapsed AML, using a genetic polymorphism of the *MDR*1 gene at position 2677. Expression and function of P-gp were studied using monoclonal antibodies MRK16 and UIC2 and the rhodamine 123 (Rho 123) retention assay with or without PSC 833.

No difference was found in the levels of P-gp function and expression between diagnosis and relapse in purified paired blast samples from 30 AML patients.

Thirteen patients were homozygous for the genetic polymorphism of *MDR*1 (n=7 for Guanine, n=6 for Thymidine), while 17 patients were heterozygous (GT). In the heterozygous patients no selective loss of one allele was observed at relapse. Homozygosity for the *MDR*1 gene (GG or TT) was associated with shorter relapse-free intervals (P=0.002) and poor survival rates (P=0.02), compared with heterozygous patients. No difference was found in P-gp expression or function in AML patients with either of the allelic variants of the *MDR*1 gene. We conclude that P-gp function or expression is not upregulated at relapse/refractory disease and expression of one of the allelic variants is not associated with altered P-gp expression or function in AML, consistent with the fact that *MDR1* gene related clonal selection does not occur when AML evolves to recurrent disease.

Introduction

Classical multidrug resistance (MDR) encoded by the *MDR*1 gene is characterized by expression of P-glycoprotein (P-gp), which acts as a drug efflux pump in the plasma membrane. Expression of MDR1 has been identified as an independent adverse prognostic factor for CR and survival in patients with acute myeloid leukemia (AML), especially in adults (1-11). Little is known about possible changes in *MDR*1 gene expression during the development to relapse or refractory disease, especially in paired analyses of clinical samples of AML patients. It is conceivable that MDR1 positive clones develop by clonal selection during chemotherapy or by *MDR*1 gene activation. This phenomenon has been described for Burkitt's lymphoma, in which single allelic MDR1 expression was found to be upregulated during the development of disease (12).

In the present study, we investigated whether clonal selection of one *MDR*1 allele contributes to drug resistance in AML, by studying the genetic polymorphism of the *MDR*1 gene at position 2677 (13). This study was performed in paired samples of AML patients at time of diagnosis and at first relapse or refractory disease. In addition, an analysis was performed of the expression and function of P-glycoprotein. To the best of our knowledge, no previous studies have been reported in which the P-glycoprotein levels were measured in the allelic variants of the *MDR*1 gene.

Patients

Bone marrow samples of 30 AML patients (9 children, 21 adults) were obtained from the posterior iliac crest at diagnosis and at time of first relapse (n=27) or refractory disease (n=3) (Table 1). From each patient and/or parents written informed consent was obtained to perform these studies. AML classification, according to the French-American-British (FAB) criteria (14) was M1 (n=8), M2 (n=11), M4 (n=2), M5 (n=7), M6 (n=2). Cytogenetic analysis was carried out by standard techniques, and the findings were described according to the international nomenclature (15). Patients with a deletion or loss of chromosome 7 were not included in the study, because of the (possible) loss of one *MDR*1 gene which is located on 7q21.1, which complicates the analysis of polymorphism in these patients. All patients were treated according to the Helsinki agreement and were included in treatment protocols of the Dutch-Belgian Hemato-Oncology Collaborative Group (protocol HOVON 4/4a resp. HOVON 29) for young adults (n=17), European Organization for Research and Treatment of Cancer (EORTC protocol LAM 9) (n=1) for patients ≥ 60 years, and the Dutch Childhood Leukemia Study Group (DCLSG: protocol ANLL 87 and 94)(n=9) for the children (age< 18 years). After relapse or in case of refractory disease after induction therapy, adults were treated according to the HOVON 30 protocol. The pediatric patients received treatment according to institutional protocols (Table 2).

For some patients, individual therapy choices were made (Table 1). Complete remission (CR) status was defined as normocellular marrow with < 5% blasts in a BM smear and normal peripheral blood cell counts.

Table 1. Clinical characteristics of the 30 AML patients

	Diagnosis				Relapse/Refractory disease		
	Expression of *MDR*1 gene poly-morphism (G/T variant)	Age (years)	FAB	Karyotype	Time to relapse (months)	Treatment at time of relapse/refractory disease	Response to reinduction
1	GT	2	M5	U	8	NT	
2	G	1	M6	N	4	2CdA/Ara-C/Ida	No CR
3	GT	47	M2	N	50	HOVON30	CR
4	T	55	M2	N	-	HOVON30	No CR*
5	T	50	M5	N	25	HOVON30	CR
6	GT	50	M2	N	7	HOVON30	CR
7	GT	62	M1	U	31	HOVON30	CR
8	GT	61	M1	N	29	HOVON30	No CR
9	GT	35	M1	F	12	HOVON30	CR
10	GT	9	M5	U	9	NT	
11	GT	12	M1	N	33	DCLSG ANLL94	CR
12	GT	37	M1	N	12	HOVON29	CR
13	GT	57	M4	N	4	NT	
14	G	46	M5a	N	6	Ara-C	TD
15	GT	67	M2	N	9	EORTC 9	No CR
16	T	16	M4eo	F	8	HOVON29	CR
17	GT	19	M5a	N	28	HOVON29	No CR
18	GT	42	M2	N	11	HOVON29	CR
19	GT	1	M1	N	14	DCLSG ANLL87	No CR
20	G	41	M6	N	4	HOVON30	CR
21	GT	10	M2	F	58	DCLSG ANLL94	CR
22	T	63	M2	N	8	NT	
23	G	1	M5	U	10	DCLSG ANLL87	No CR
24	GT	27	M2	N	14	HOVON30	CR
25	G	34	M5	N	-	HOVON30	No CR*
26	GT	5	M1	N	18	DCLSG ANLL87	CR
27	G	18	M2	N	8	Mitoxantrone	No CR
28	T	55	M1	N	-	HOVON30	No CR*
29	T	49	M2	N	6	NT	
30	G	67	M2	N	5	HOVON30	No CR

CR: complete remission after 1 or 2 courses of re-induction chemotherapy; No CR: refractory disease at time of relapse; No CR*: never CR after diagnosis; NT: not treated for relapse; Karyotype: U: unfavorable, t(9;22), 11q23 with *MLL* rearrangements, complex karyotype, 5q-; F: favorable, inv(16), t(15;17) and t(8;21), N: neutral, normal and other karyotypes; TD: toxic death..

Methods

Patient samples

Bone marrow aspirates were obtained in heparinized tubes. Mononuclear bone marrow cells (MNC) were collected by Ficoll Hypaque density gradient centrifugation (density 1.077g/m^3) (Pharmacia, Uppsala, Sweden). To obtain purified samples with more than 85% of blasts, T-cell depletion and adherence depletion was performed (16). Cells were cryopreserved in Iscove's Dulbecco's medium (IMDM; Gibco, Paisly, UK) supplemented with 10% dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany) and 20% fetal calf serum (FCS; Gibco) and stored in liquid nitrogen. On the day of the experiments bone marrow cells were thawed. For flowcytometry experiments, cells were washed and resuspended in IMDM supplemented with 10% FCS and gentamycin at a concentration of $4x10^6$ cells per ml. Total RNA was isolated using TRISOLV extraction (Biotecx, Houston, TX, USA).

Oligonucleotide hybridization and dotblot analysis

Both DNA and RNA were used as templates in the PCR. One microgram of genomic DNA was used as a template in the PCR for 40 cycles to investigate the genetic polymorphism at the DNA level. One microgram of total RNA was reversally transcribed and cDNA template was subjected to 40 cycles of PCR. The following primers were used as described by Mickley: 5' ^{2521} GCAAATCTTGGGACAGGAAT; 3' RNA, ^{2796} CTCCTTTCGTGTGTAGAAAC; 3',DNA,^{2681}CCTTC^{2687} CACTCAGTTTGATTT (12, 13). Reverse transcriptase treatment preceded amplification in order to evaluate RNA expression. All PCR experiments included controls without DNA or RNA. After amplification of 1 μg of template, 30% of the PCR product was loaded in each of two adjacent wells of a slot-blot apparatus. The Zeta Probe nylon filter was cut out into two halves and each halve was hybridized with a different oligonucleotide. Two 19-bp allele-specific oligonucleotide probes (HMO7 and HMO8) were 5'-phosphorylated with [γ^{32}P]-ATP and T_4 polynucleotide kinase. HMO7 and HMO8 cover residues 2667 to 2685 and were used for hybridizations. HMO7 possesses a G at position 2677 and HMO8 contains a T at this position. Internal controls for hybridization and specificity were included in all experiments. For this purpose two 30-bp oligonucleotides, designated HMC3 and HMC4, were used. These oligonucleotides cover residues 2656 to 2685 of the *MDR*1 gene with HMC3 possessing a G at position 2677 and HMC4 a T at this position. Equal amounts of each control were spotted on both sides of the filter. Because the hybridizations were performed under identical conditions, with probes labeled to similar specific activities,

Table 2. Cumulative drug doses (in mg/m^2) in the treatment protocols for acute myeloid leukemia

	Ara-C	DNR	Adria	Amsa	Ida	VP16	Mitox	Pred	6TG	VCR	CP	CsA
Induction												
HOVON 4/4A	13400	135		360		500	50					
HOVON 29*	13400			360	36	500	50					
DCLSG ANLL87**	22400	180	120			1050		1120	2580	6	1000	
DCLSG ANLL94**	33400		120		36	950	20	1120	2580	6	1000	
Reinduction												
EORTC-9	6000	90					50					
HOVON 30						500	50					±5 mg/kg

Ara-C: cytosine-arabinoside; Adria: adriamycine; DNR: daunorubicine; Amsa: amsacrine; Ida: idarubicine; VP16: etoposide; Mitox: mitoxantrone; Pred: prednisolone; 6TG: 6 thioguanine; VCR: vincristine; CP: cyclophosphamide; *: HOVON 29 patients were randomized to receive +/- G-CSF; **: + 5x intrathecal Ara-C; CsA: cyclosporine A.

the signals from the control oligonucleotides were similar. For quantification of the hybridization spots, the blots were exposed to a Phosfor Imager screen (Molecular Dynamics, Sunnyvale, CA, USA).

Expression of P-glycoprotein

For measurement of the expression of P-gp, cells were incubated at room temperature with the monoclonal anti-P-gp antibodies MRK 16 (17) (Kamiya Biomedical Company, Tukwila, WA, USA) at a concentration of 10 μg/ml and also, in separate tubes, with UIC2 (18) (Immunotech, Marseille, France) at a concentration of 12.5 μg/ml or with an isotype matched mIgG2a control antibody (Sigma, St. Louis, MO, USA) at a concentration of 10 μg/ml. Cell-bound antibodies were detected by fluorescein isothiocyanate (FITC)-labeled rabbit anti-mouse immunoglobulin antibodies (DAKO, Glostrup, Denmark). Results are given as the ratio of the mean fluorescence of cells incubated with the anti-P-gp antibody divided by the mean fluorescence of cells incubated with the control mIgG2a antibody. To measure the expression of P-gp in CD34-positive cells, cells were labeled with phyco-erythrin-Cy5-labelled CD34 antibody or a phycoerythrin-Cy5-labelled matched mIgG1 antibody (Immunotech, Marseille, France).

Function of P-glycoprotein

For measurement of the function of P-gp, the fluorescent molecule Rho 123 (Sigma, St. Louis, MO, USA) was used as a P-gp substrate (19,20). Cells were incubated for 1 hr at 37°C at 5% CO_2 in the absence or presence of 2 μM of the P-gp modulator PSC 833 (Novartis, Basel, Switserland). After this incubation, 200 ng/ml Rho 123 was added to the cells. A sample was taken at t=0 minutes (min) to correct for background fluorescence and at t=75 min to measure intracellular Rho 123 retention. Results were calculated as the PSC/Rho 123 retention ratio of the mean intracellular Rho 123 fluorescence of cells exposed to PSC 833 divided by the mean intracellular Rho 123 fluorescence of cells not exposed to PSC 833. As controls, the drug-sensitive human myeloma cell line 8226 S and the drug-resistant P-gp expressing variant 8226 D6 cells (21) were included in each experiment. Taken all experiments together, the mean ratio of P-gp function of the negative control cell line 8226 S was 0.91 ± 0.07 (mean ± SD). The mean ratio of P-gp function of the positive control cell line 8226 D6 was 7.03 ± 4.69 (mean ± SD).

For analysis of the function of P-gp in CD34-positive cells, cells were labeled with phyco-erythrin-Cy5-labelled CD34 antibody or as a control phycoerythrin-Cy5-labelled mIgG1 antibody (Immunotech). Fluorescence was measured using a FACScalibur

Table 3. Paired analysis of P-glycoprotein expression and function in AML patients at diagnosis and relapse/refractory disease

	At diagnosis	At relapse and/or refractory disease	P- value
MDR1			
MRK 16			
Median	2.16	1.83	0.14
Range	1.22- 7.65	1.02- 5.55	
n=	27		
MRK 16/CD34+			
Median	2.77	2.28	1.00
Range	1.52- 9.27	1.46- 9.6	
n=	11		
UIC2			
Median	2.37	1.74	0.22
Range	1.47- 12.3	0.99- 6.39	
n=	25		
UIC2/CD34+			
Median	3.5	2.42	0.07
Range	1.51- 26.6	0.86- 7.4	
n=	8		
PSC/Rho 123			
Median	1.13	1.10	0.26
Range	0.87- 2.11	0.81- 2.19	
n=	27		
PSC/Rho 123/CD34+			
Median	1.43	1.22	0.39
Range	0.98-2.7	0.82-3.26	
n=	12		

For P-gp expression the Moabs MRK 16 and UIC2 are used; for P-gp function the PSC/Rho 123 retention ratio. In samples with >10% CD34 expression the variables were also evaluated in the CD34 + subfraction of the blasts. The P-values indicate the differences between diagnosis and relapse/refractory disease.

flowcytometer (Becton-Dickinson, San José, CA, USA). Cells were incubated with 0.1 μM TO-PRO-3 (Molecular Probes, Eugene, Oregon, CA, USA) to exclude non-viable cells in the functional and expression studies.

Statistical analysis

Expression and functional levels of P-glycoprotein, either at diagnosis or at relapse or refractory disease, were compared between subgroups using the Mann-Whitney test in case of two subgroups, and the Kruskal-Wallis test in case of three subgroups. Moreover,

MDR1 expression at relapse or refractory disease was compared to that at diagnosis using the Wilcoxon matched-pairs signed-ranks test, which was restricted to patients with data available both at diagnosis and at relapse or refractory disease. All P-values are two-sided and a significance level α=0.05 was used.

Results

Thirty AML patients were studied at diagnosis and during the course of their disease. Twenty-seven patients developed a relapse after reaching complete remission with induction chemotherapy. Three patients were primary refractory to induction chemotherapy (Table 1).

Oligonucleotide hybridization and dotblot analysis

Oligonucleotide hybridization studies of position 2677 of the *MDR*1 gene revealed 7 patients with a G variant, 6 patients with a T variant, and 17 patients with a GT variant. The 17 patients with heterozygous expression at diagnosis also showed GT expression at relapse. In these patients, no upregulation of either allele was noticed during the development of disease at RNA level. Consequently, in this group of patients no evidence of a *MDR*1 gene associated selection of a resistant clone was found.

P-glycoprotein expression and function

MRK 16 expression (n=27) and UIC2 expression (n=25) revealed no differences in P-gp expression at relapse or refractory disease as compared to diagnosis (P=0.14 resp. 0.22) (Table 3). No difference of MRK 16 expression in the CD34-positive subpopulation, was found (n=11) (P=1.0) in the paired analysis. The analysis of UIC2/CD34 in matched pairs showed a trend to a lower expression level (P=0.07) in relapsed/refractory disease as compared to diagnosis, although the number of patients that could be analyzed for UIC2/CD34 was small (n=8) (Table 3, Figure 1C). The CD34 expression was not different at relapse as compared to diagnosis (P=0.31).

The PSC/Rho 123 retention ratio (n=27) was not significantly different between diagnosis and relapsed/refractory AML (P=0.26). When analyzed in the CD34-positive subpopulation of blasts (n=12), comparable result were found (P=0.39) (Table 3, Figure 1A). No difference was found in P-gp expression (P=0.67 for MRK 16 expression, P=0.82 for UIC2 expression) or PSC/Rho ratio (P=0.09) at diagnosis nor at relapse/refractory disease (P-values respectively 0.42, 0.67 and 0.11) between adults and children.

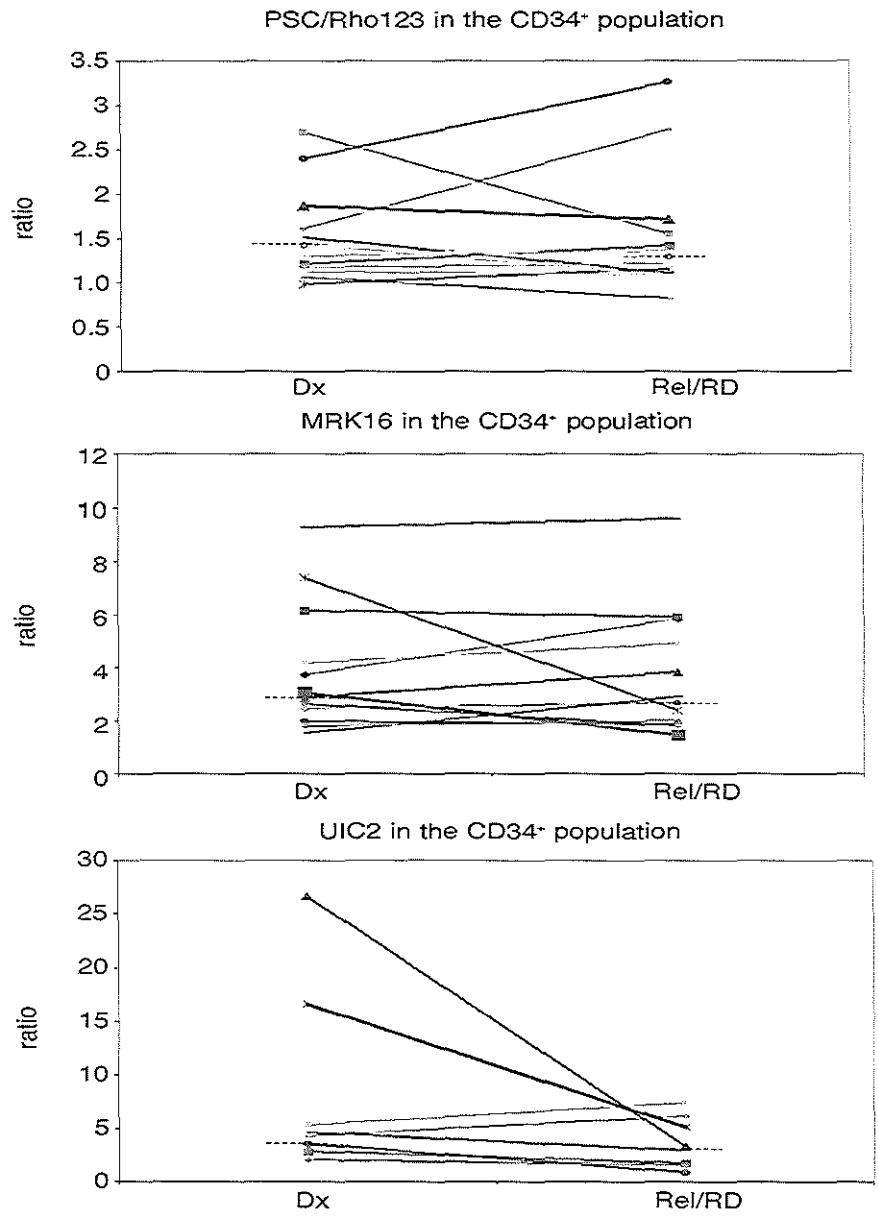


Figure 1. P-glycoprotein expression and function in the CD34-positive population of the paired AML samples. The UIC2 and MRK 16 ratio's represent the expression of P-glycoprotein. PSC 833/Rho 123 represents the function of P-glycoprotein. Dx: diagnosis, Rel/RD: relapsed/ refractory AML. The dotted lines indicate the median values.

P-glycoprotein versus MDR1 allelic expression

As the functional meaning of the genetic polymorphism of the *MDR*1 gene has not been established as yet, we analyzed P-glycoprotein in patients with expression of the G, T, and GT variants respectively. The median MRK 16 expression ratio was not significantly different in the various allelic variants (P=0.72 at diagnosis and P=0.34 at relapse). Also, no difference was found with Moab UIC2 (P=0.81 at diagnosis and P=0.25 at relapse) and the PSC/Rho 123 retention ratio (P=0.26 at diagnosis, P=0.11 at relapse). No difference was found in P-gp expression or function when homozygous patients were compared with heterozygous patients (Table 4). Similarly, in the CD34-positive fraction we did not find differences in P-gp expression and function between the different *MDR*1 allelic variants at diagnosis nor at relapse and/or refractory disease. The results show that there is no difference in P-gp expression and function in AML blast cells between the different specific allelic variants of the *MDR*1 gene. The therapeutic outcome of patients with the different allelic variants showed a significant difference, *i.e.* homozygosity was associated with a shorter time from diagnosis to relapse (P=0.002) and a shorter overall survival from relapse (P=0.02) (Figure 2A and 2B).

Discussion

Clinical resistance to chemotherapy is a major problem in relapsed and/or refractory AML. MDR1 expression in *de novo* AML is an adverse prognostic factor for CR and survival (3-7,11,22). It is conceivable that upregulation of the *MDR*1 gene is involved in the development of relapse and/or refractory disease, although this has not been investigated in paired analyses of respectable numbers of clinical samples of AML patients (12). In the present study we analyzed whether clonal selection associated with the *MDR*1 gene is involved in the development of relapsed AML.

This is the first study that examined the allelic expression of MDR1 in AML, using the genetic polymorphism of the *MDR*1 gene. Our data show, that there is no evidence of a *MDR*1 gene related clonal selection in the evolution of AML to relapse or refractory disease. This is consistent with our observation that P-glycoprotein expression and function did not increase from diagnosis to relapsed/refractory state. Several studies have reported a higher MDR1 expression at time of relapse as compared to diagnosis (23-29). However, most studies compared patients that were not matched and studies in paired patient samples are scarce and generally they were performed in small numbers of patients.

Table 4. Analysis of P-glycoprotein expression and function in the homozygous *vs* the heterozygous allelic variants of the *MDR*1 gene at time of relapse/refractory disease

	GG or TT	GT	P- value
MDR1			
MRK 16			
Median	1.54	2.14	0.15
Range	1.02- 5.45	1.18- 5.55	
n=	13	17	
MRK 16/CD34			
Median	1.88	2.68	0.22
Range	1.49- 4.90	1.46- 9.6	
n=	7	7	
UIC2			
Median	1.56	2.28	0.22
Range	1.12- 6.39	0.99- 5.24	
n=	13	17	
UIC2/CD34			
Median	1.67	2.72	0.10
Range	0.86- 7.34	1.39- 5.15	
n=	7	8	
PSC/Rho 123			
Median	1.08	1.14	0.30
Range	0.81- 1.85	0.84- 2.19	
n=	13	17	
PSC/Rho 123/CD34			
Median	1.21	1.42	0.40
Range	1.07- 1.72	0.82- 3.26	
n=	9	9	

For P-gp expression the Moabs MRK 16 and UIC2 are used, for P-gp function the PSC/Rho 123 retention ratio. In samples with more than 10% CD34 expression, the variables were also evaluated in the CD34 positive subfraction of the blasts. The P-values indicate the differences between the homozygous and heterozygous patients.

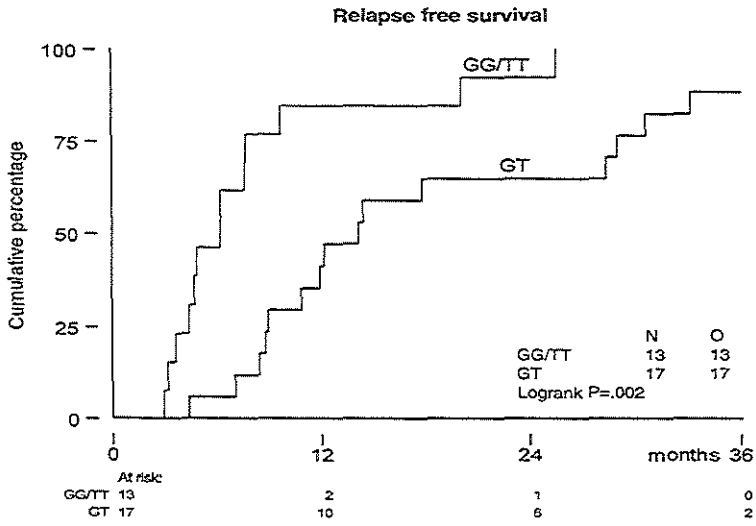
Most studies suggest an identical expression or even lower level of MDR1 in relapsed/refractory AML (29-32). Only the sequential analysis by Wood, who used immunocytochemistry techniques, showed a higher percentage of P-gp positive samples in 14 relapsed AML patients as compared to diagnosis (33). In pediatric patients only three case reports are available (25,34,35). Therefore, although many studies have suggested that MDR1 is upregulated in relapsed and/or refractory AML, sequential studies do not support this (Table 5 and 6).

Table 5. Review of analyses of MDR1 expression in paired samples of AML patients

Author	Reference	Number of patients	Expression level MDR1	Age category	MDR1 expression in relapsed versus *de novo* AML
Gekeler	(35)	1	RNA	children	higher
Beck	(46)	1	RNA	children	higher
Guerci	(26)	4	Protein	adults	higher/equal
Hart	(32)	9	RNA	adults	higher/lower
Ino	(31)	21	Protein	adults	equal/lower
Sato	(47)	6	RNA	adults	equal
Ito	(30)	10	DNA	adults	equal
		1	RNA	adults	equal
		10	Protein	adults	higher/lower
Kaczorowski	(34)	1	Protein	children	higher
List	(29)	17	Protein	adults	lower
Marie	(24)	4	RNA	adults	higher/equal
Marie	(48)	4	RNA	adults	higher/lower/ equal
Ma	(38)	2	Protein	adults	higher/equal
Musto	(23)	2	Protein	adults	higher
Maslak	(27)	5	Protein	adults	higher
Michieli	(28)	7	Protein	adults	higher/lower/ equal
Wood	(33)	14	Protein	adults	higher

The present analysis, which is the largest paired study in AML sofar, is an attempt to quantify MDR1 expression at genomic and protein level during the development towards resistant disease. In these 9 children and 21 adults we did not find evidence that MDR1, although being a strong prognostic factor at the time of diagnosis, is upregulated at time of relapse and/or refractory disease in AML. We suggest that similar sequenti al studies of other mechanisms of drug resistance should be performed in AML patients during the course of their disease in order to determine which drug resistance proteins are associated with clonal selection at relapse. In these studies it will be important to analyze children and younger adults separate from elderly patients with AML, since different mechanisms might be important in different age groups (6). Until now, the only study that analyzed P-gp expression in a large group of children with AML showed that in contrast with adult

A



B

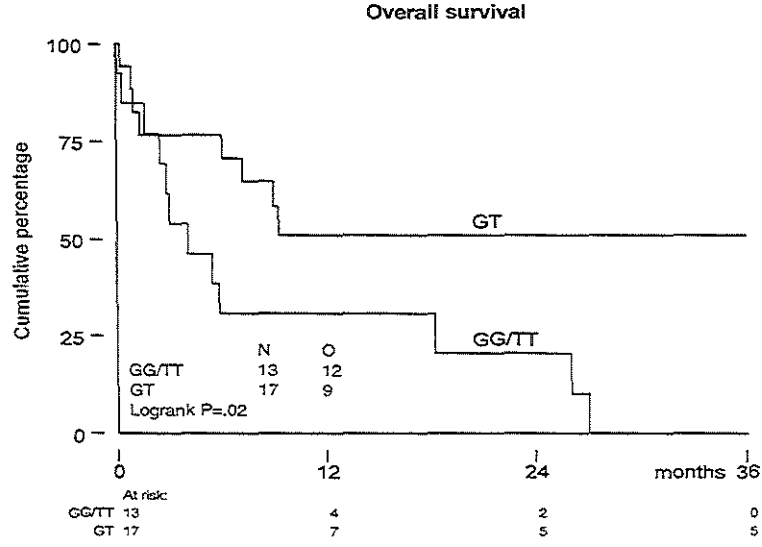


Figure 2. Survival of the AML patients, distinguishing patients that are homozygous (GG and TT) from patients that are heterozygous (GT) for the genetic polymorphism of position 2677 of the *MDR*1 gene.

A. Time from diagnosis until relapse/refractory disease. B. Overall survival from relapse/refractory disease. N: number of patients investigated; O: observed events.

Table 6. Review of MDR1 expression in AML in non-paired studies

Author	Ref.	No. of Patients		Expression level	Age category	MDR1 expression relapsed *vs* *de novo* AML
		Dx	Relapse			
Beck	(46)	14	23	RNA	children	higher
Guerci	(26)	69	10	Protein	adults	higher
List	(29)	21	29	Protein	adults	equal/ lower
Marie	(24)	21	6	RNA	adults	equal
Musto	(23)	8	7	Protein	adults	lower
Maslak	(27)	18	19	Protein	adults + children	higher
Michieli	(28)	38	21	Protein	adults	higher

Dx: at diagnosis, Ref: reference.

AML, MDR1 expression was not of prognostic significance (36). In the present study no difference was found in P-gp expression and function between adults and children.
This is consistent with our observation that P-glycoprotein expression and function did not increase from diagnosis to relapsed/refractory state. Several studies have reported a higher MDR1 expression at time of relapse as compared to diagnosis (23-29). However, most studies compared patients that were not matched. Studies in paired patient samples are scarce and generally they were performed in small numbers of patients. Most studies suggest an identical expression or even lower level of MDR1 in relapsed/refractory AML (29-32). Only the sequential analysis by Wood, who used immunocytochemistry techniques, showed a higher percentage of P-gp positive samples in 14 relapsed AML patients as compared to diagnosis (33). In pediatric patients only three case reports are available (25,34,35). Therefore, although many studies have suggested that MDR1 is upregulated in relapsed and/or refractory AML, sequential studies do not support this (Table 5 and 6). The present analysis, which is the largest paired study in AML sofar, is an attempt to quantify MDR1 expression at genomic and protein level during the development towards resistant disease. In these 9 children and 21 adults we did not find evidence that MDR1, although being a strong prognostic factor at the time of diagnosis, is upregulated at time of relapse and/or refractory disease in AML. We suggest that similar sequential studies of other mechanisms of drug resistance should be performed in AML patients during the course of their disease in order to determine which drug resistance proteins are associated with clonal selection at relapse. In these studies it will be important to analyze children and younger adults separate from elderly patients with

AML, since different mechanisms might be important in different age groups (6). Until now, the only study that analyzed P-gp expression in a large group of children with AML showed that in contrast with adult AML, MDR1 expression was not of prognostic significance (36). In the present study no difference was found in P-gp expression and function between adults and children.

Our study emphasizes that it is important to study MDR1 expression in clinical samples from AML patients. In many cell lines, including even AML cell lines, MDR expression may be upregulated as a direct response of cells to antineoplastic drugs. However, it seems apparent that this does not occur in AML patients (37-45).

This is the first analysis of the functional significance of the genetic polymorphism of *MDR*1 in highly purified samples of AML. P-glycoprotein function and expression were similar in either one of the specific allelic variants (G, T and GT). These findings suggest, that the genetic polymorphism of the *MDR*1 gene (at position 2677) lacks functional importance in AML. However, we found that patients with homozygous expression of the *MDR*1 gene (GG or TT) had a shorter time to relapse and overall survival from relapse/refractory disease than heterozygous patients. This finding warrants further studies on the role of genetic polymorphisms of *MDR*1 in AML.

MDR1 expression at diagnosis is a strong adverse prognostic factor in AML. However, our sequential analysis reveals that there is no higher function or expression of P-gp at relapse or refractory disease, and that specific allelic expression is not related with increased P-gp expression or function. Since no loss of a specific *MDR*1 allele has been observed in these AML patients, *MDR*1 gene related clonal selection plays no role in the development of resistant disease. These data suggest that other mechanisms than MDR1 might be responsible for the development of clinical resistance in these patients.

Acknowledgements

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Chapter 4

Increased expression of the breast cancer resistance protein (BCRP) in relapsed or refractory acute myeloid leukemia (AML)

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Abstract

Expression of the multidrug resistance proteins P-glycoprotein, encoded by the *MDR*1 gene, multidrug resistance associated protein (MRP1) and the lung resistance-related protein or major vault protein (LRP/MVP) is associated with clinical resistance to chemotherapy in acute myeloid leukemia (AML). Recently, the breast cancer resistant protein (BCRP), the equivalent of mitoxantrone resistant protein (MXR) or placental ABC transporter (ABCP), was described in AML. As no such simultaneous analysis has been reported in the past, we investigated *MDR*1, *MRP*1, *LRP/MVP* and *BCRP* mRNA expression in 20 paired clinical AML samples from diagnosis and relapse or refractory disease, using quantitative Taqman analysis. In addition, standard assays for P-glycoprotein expression and function were performed.

BCRP was the only resistance protein that was expressed at a significantly higher RNA level (median 1.7-fold, P=0.04) at relapsed/refractory state as compared to diagnosis. In contrast, *LRP/MVP* mRNA expression decreased as disease evolved (P=0.02), whereas *MDR*1 and *MRP*1 mRNA levels were not different at relapse as compared to diagnosis. Also, at the protein level no difference of MDR1 between diagnosis and relapse was found. A significant co-expression of *BCRP* and *MDR*1 was observed at diagnosis (r=0.47, P=0.04). The present results suggest that *BCRP*, but not *MDR*1, *MRP*1 or *LRP/MVP* is associated with clinical resistant disease in AML.

Introduction

Clinical resistance to chemotherapy in AML is often associated with expression of (membrane) transport associated multidrug resistance proteins (1). Expression of P-glycoprotein (P-gp), encoded by the *MDR*1 gene is an independent adverse prognostic factor for response and survival in *de novo* acute myeloid leukemia (AML) (2-11). More recently, it was observed that not only P-gp, but also the multidrug resistant associated protein (MRP1) and the lung resistance-related protein (LRP), also designated as the major vault protein (MVP), are expressed in AML. The precise prognostic significance of these latter resistance proteins is not yet known (8,10-20). Recently, a new drug resistant protein, *i.e.* the breast cancer resistance protein (BCRP) (21-23), which is the equivalent of the mitoxantrone resistant protein (MXR) (24,25) and of the placental ABC transporter (ABCP) (26), was found to be expressed in AML (27,28). BCRP maps to the human chromosome 4q22, between the markers *D4S2462* and *D4S1557* (26, 29).

Little is known about the expression of MDR1, LRP/MVP and MRP1 at presentation and at relapse in the same patients. Paired studies of the expression of these genes in clinical AML samples are limited to small numbers of patients (30-33). Consequently, it is still not clear if the expression of these resistance proteins increases from diagnosis to relapse, nor whether a resistant clone which is already present at diagnosis, regrows at relapse. At present, there is no information about the clinical relevance of *BCRP* gene expression in AML.

We investigated whether *MDR1*, *MRP1*, *LRP/MVP* and *BCRP* mRNA expression evolves from diagnosis to relapse/refractory disease in 20 paired AML samples, using a quantitative real-time polymerase chain reaction (PCR) assay. As upregulation of MDR1 has been claimed as a major event in resistant AML, we also investigated P-glycoprotein in these cells.

Table 1. Clinical characteristics of the 20 AML patients

Patient	Diagnosis				Relapse/refractory disease		
	Age (years)	FAB	Karyotype	Induction treatment (at diagnosis)	Time from CR to relapse (months)	Reinduction treatment (at time of relapse/refractory disease)	Response (to reinduction)
1	1	M6	Neutral	Ara-C/Adria	4	2CdA/Ara-C/Ida	No CR
2	50	M2	Neutral	Ara-C/DNR	7	Ida/VP16	CR
3	62	M1	Unfavorable	Ara-C/Mitoxantrone	31	Ida/VP16	CR
4	61	M1	Neutral	Ara-C/DNR	29	Ida/VP16	No CR
5	35	M1	Favorable	Ara-C/Ida	12	Ida/VP16	CR
6	12	M1	Neutral	Ara-C/Adria	33	Ara-C/Adria	CR
7	46	M5a	Neutral	Ara-C/Ida	6	Ara-C	TD
8	67	M2	Neutral	Ara-C/DNR	9	Ara-C/DNR	No CR
9	16	M4eo	Favorable	Ara-C/Ida	8	Ara-C/Ida	CR
10	19	M5a	Neutral	Ara-C/Ida	28	Ara-C/Ida	No CR
11	42	M2	Neutral	Ara-C/Ida	11	Ara-C/Ida	CR
12	41	M6	Neutral	Ara-C/Ida	4	Ida/VP16	CR
13	10	M2	Favorable	Ara-C/Adria	58	Ara-C/Adria	CR
14	63	M2	Neutral	Ara-C/DNR	8	NT	
15	27	M2	Neutral	Ara-C/Ida	14	Ida/VP16	CR
16	34	M5	Neutral	Ara-C/DNR	-	Ida/VP16	No CR*
17	5	M1	Neutral	Ara-C/Adria	18	Ara-C/Adria	CR
18	18	M2	Neutral	VP16/Mitoxantrone	8	Mitoxantrone	No CR
19	55	M1	Neutral	Ara-C/DNR	-	Ida/VP16	No CR*
20	67	M2	Neutral	Ara-C/DNR	5	Ida/VP16	No CR

CR: complete remission after 1 or 2 courses of re-induction chemotherapy; No CR: refractory disease at time of relapse; No CR*: never CR after diagnosis; NT: not treated for relapse; TD: toxic death; Karyotype: Unfavorable: t(9;22), 11q23 with *MLL* rearrangements, complex karyotype, 5q-; Favorable: inv(16) and t(8;21); Neutral: normal and other karyotypes. Ara-C: cytosine-arabinoside; DNR: daunorubicin;, Adria: adriamycin; Ida: idarubicin; VP16: etoposide.

Patients

Bone marrow samples of 20 AML patients (4 children aged <16 years and 16 adults), median age 38 years (range 1-66 years), were obtained from the iliac crest both at diagnosis and at time of first relapse (n=18) or primary refractory disease (n=2) (Table 1). AML classification, performed according to the French-American-British (FAB) criteria (34) was M1 (n=6) , M2 (n=8), M4 (n=1), M5 (n=3), M6 (n=2). Cytogenetic analysis was carried out by standard techniques, and the findings were described according to the international nomenclature (35). All patients and/or their parents had given informed consent, and they were treated according to the Helsinki agreement. Most of them were included in treatment protocols of the Dutch-Belgian Hemato-Oncology Collaborative Group (HOVON 4 resp. HOVON 29) for young adults, European Organization for Research and Treatment of Cancer (EORTC LAM 9) for elderly patients, and the Dutch Childhood Leukemia Study Group (DCLSG: ANLL 82, 87 and 94) for the children (30,36,37). At relapse or in case of refractory disease after repopulation following induction therapy, adult patients were treated according to the HOVON 30 relapse protocol. Treatment of the 4 pediatric patients was by the institutional protocol (Table 1). As shown in Table 1, for some adult patients individual treatment choices were made. One patient died before reinduction treatment started because of septicemia. The induction therapy used in HOVON 4, 29 and 30, the EORTC 9/11 and the DCLSG ANLL protocols all consisted of an anthracycline and Ara-C (30). Complete remission status was defined as normocellular marrow, with < 5% blasts in a BM smear, with normal peripheral blood counts.

Materials and methods

Patient samples

Bone marrow aspirates were obtained in heparinized tubes. Mononuclear bone marrow cells (MNC) were collected by Ficoll Hypaque density gradient centrifugation (density 1.077g/m^3) (Pharmacia, Uppsala, Sweden). To obtain purified samples with more than 85% of blasts, T-cell depletion and adherence depletion was performed as described (38). Cells were cryopreserved in Iscove's Dulbecco's medium (IMDM; Gibco, Paisly, UK) supplemented with 10% dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany) and 20% fetal calf serum (FCS; Gibco) and stored in liquid nitrogen. At the day of the experiments bone marrow cells were thawed. For flowcytometry experiments, cells were washed and resuspended in IMDM supplemented with 10% FCS and gentamycin at a concentration of 4x10^6 cells per ml. Before RNA and DNA isolation, cells were washed with PBS (Gibco).

*LRP/MVP, MRP*1, *MDR*1 and *BCRP* mRNA analysis

Isolation of RNA.. Total RNA was isolated using the TRISOLV extraction as described by the manufacturer (Biotecx, Houston, Tx, USA). RNA was aliquotted and stored at -80°C. RNA samples were analyzed for RNA integrity by gel electrophoresis.

cDNA synthesis. cDNA was synthesized by the use of the TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA). cDNA was diluted, aliquotted and stored at -80°C.

Quantitative Real-time PCR. The mRNA levels of *MDR*1, *MRP*1, *LRP/MVP*, *BCRP* and two endogenous reference genes, *i.e.* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and porphobilinogen deaminase (PBGD), were measured by quantitative real-time PCR based upon Taqman-chemistry on an ABI PRISM 7700 sequence detector (Applied Biosystems). PCR products were detected using a dual-fluorescent non-extendable probe containing a FAM reporter and TAMRA quencher for all reactions except for the GAPDH reaction in which FAM was replaced by VIC.

Table 2. Primer and probe combinations used in quantitative RT-PCR

LRP/MVP	Forward	5'-CAGCTGGCCATCGAGATCA
	Reverse	5'-TCCAGTCTCTGAGCCTCATGC
	Probe	5'-CAACTCCCAGGAAGCGGCGGC
*MRP*1	Forward	5'-CAATGCTGTGATGGCGATG
	Reverse	5'-GATCCGATTGTCTTTGCTCTTCA
	Probe	5'-AGACCAAGACGTATCAGGTGGCCCAC
*MDR*1	Forward	5'-GGAAGCCAATGCCTATGACTTTA
	Reverse	5'-GAACCACTGCTTCGCTTTCTG
	Probe	5'-TGAAACTGCCTCATAAATTTGACACCCTGG
BCRP	Forward	5'-TGGCTGTCATGGCTTCAGTA
	Reverse	5'-GCCACGTGATTCTTCCACAA
	Probe	5'-AGCAGGGCATCGATCTCTCACCCTG
PBGD	Forward	5'-CTGCACGATCCCGAGACTCT
	Reverse	5'-GCTGTATGCACGGCTACTGG
	Probe	5'-CTGAGGCACCTGGAAGGAGGCTG

All primers and probes, designed by the oligo 6.0 primer analysis software (Medprobe, Oslo, Norway), did not amplify contaminating genomic DNA (Table 2). The *GAPDH* mRNA levels were measured using the Pre-developed TaqMan Assay Reagents for human *GAPDH* (Applied Biosystems).

Forty nanograms of patient sample cDNA were used as a template in duplicate in the presence of 300 nM forward and reverse primers, 200 nM of probe, 200 μM dNTPs, 1.25 U of Ampli*Taq* DNA polymerase and 4 mM $MgCl_2$ in sample buffer A (Applied Biosystems) in a total volume of 50 μl. Samples were heated for 10 min at 95°C and amplified for 50 cycles of 15 sec at 95°C and 60 sec at 60°C. As a positive control, a serial dilution of cDNA from a cell line RNA pool (CEM, K562 and two EBV induced lymphoblastoid B-cell lines) in H_2O was used. All PCR reactions were performed with comparable efficiencies that exceeded $E = 0.95$. The relative expression levels of *MDR*1, *MRP*1, *LRP/MVP* and *BCRP*1 mRNA were calculated using the comparative cycle time (Ct) method (K. Livak, User bulletin #2) (39). Briefly, the target PCR Ct-values, *i.e.* the cycle number at which emitted fluorescence exceeds the 10 times standard deviation of base-line emissions as measured between cycles 3 to 15, is normalized to the average Ct-value of the reference PCRs (GAPDH and PBGD). From this ΔCt-value, the relative expression level for each target PCR was calculated using the equation: relative expression $= 2^{-[Cttarget-\bar{C}t(GAPDH/PGBD)]}$.

The average Ct-value of two reference PCRs was used instead of a single reference reaction to limit patient sample specific variation in housekeeping gene expression.

P-glycoprotein expression and function

Expression of P-glycoprotein

To measure the expression of P-gp, cells were incubated at room temperature using the monoclonal anti-P-gp antibodies (Moabs) MRK 16 (40) (Kamiya Biomedical Company, Tukwila, WA, USA) at a concentration of 10 μg/ml and also, in separate tubes, using Moab UIC2 (41) (Immunotech, Marseille, France) at a concentration of 12.5 μg/ml or with an isotype matched mIgG2a control antibody (Sigma, St. Louis, MO, USA) at a concentration of 10 μg/ml. Cell-bound antibodies were detected by fluorescein isothiocyanate (FITC)-labeled rabbit anti-mouse immunoglobulin antibodies (DAKO, Glostrup, Denmark). Results are given as the ratio of the mean fluorescence of cells incubated with the anti-P-gp antibody divided by the mean fluorescence of cells incubated with the control mIgG2a antibody. To measure the expression of P-gp in

CD34-positive (CD34-PECy5) cells, cells were labeled with phyco-erythrin-Cy5-labelled CD34 antibody or as a control phyco-erythrin-Cy5-labeled mIgG1 antibody (Immunotech).

Function of P-glycoprotein

For measurement of the function of P-gp, the fluorescent molecule rhodamine 123 (Rho 123) (Sigma) was used as a P-gp substrate (42,43). Cells were incubated for 1 hr at 37°C at 5% CO_2 in the absence or presence of 2 μM of the P-gp modulator PSC 833 (Novartis, Basel, Switserland). Next, 200 ng/ml Rho 123 was added to the cells. A sample was taken at t=0 min to correct for background fluorescence and at t=75 min to measure intracellular Rho 123 retention. Results were calculated as the PSC/Rho 123 retention ratio of the mean intracellular Rho 123 fluorescence of cells exposed to PSC 833 divided by the mean intracellular Rho 123 fluorescence of cells not exposed to PSC 833.

As controls in each experiment, the drug-sensitive human myeloma cell line 8226 S and the drug-resistant P-gp expressing variant 8226 D6 cells were included (44). Taken all experiments together, the mean ratio of P-gp function of the negative control cell line 8226 S was 0.91 ± 0.07 (mean ± SD). The mean ratio of P-gp function of the positive control cell line 8226 D6 was 7.03 ± 4.69 (mean ± SD). For analysis of the function of P-gp in CD34-positive cells, AML cells were labeled with the PE-erythrin-Cy5-labelled CD34 Moab or with mIgG1 PE-Cy5 (isotype matched control antibody)(Immunotech). Fluorescence was measured using a FACScalibur flowcytometer (Becton-Dickinson, San José, CA, USA). Cells were incubated with 0.1 μM TO-PRO-3 (Molecular Probes, Eugene, Oregon, CA, USA) to exclude non-viable cells in both the functional and expression studies.

Statistical analysis

The mRNA expression levels of the resistance proteins and of P-glycoprotein levels at time of relapse or refractory disease as compared to the levels at diagnosis were calculated using the univariate Wilcoxon matched-pairs signed-ranks test, which was thus restricted to patients with data available both at diagnosis and at relapse and/or refractory disease state. Also, *MRP*1, *LRP/MVP*, *BCRP* and *MDR*1 mRNA levels were compared with each other as well with patient characteristics at diagnosis and at relapse and/or refractory disease, by calculation of the Spearman rank correlation in order to test whether the two variables concerned are independent. All P-values are two-sided and a

significance level of $\alpha = 0.05$ was used.

Results

AML samples of 20 patients were investigated both at diagnosis and at relapse (n= 18) or refractory disease (n= 2). The patient characteristics are listed in Table 1. BCRP was the only resistance protein that was expressed at a significantly higher mRNA level at relapse or refractory disease state than at diagnosis (Figure 1, Table 3). No differences between the levels of *MDR*1 mRNA and *MRP*1 mRNA were observed at relapse compared with diagnosis, whereas *LRP/MVP* mRNA even decreased significantly at relapse (Table 3). *BCRP* mRNA expression was not associated with FAB classification, nor with CD34 expression or cytogenetic abnormalities at diagnosis. Interestingly, at presentation lower peripheral white blood cell counts (WBC) were associated with higher *BCRP* mRNA levels (r=-0.67, P=0.001).

At diagnosis, a correlation between the expression of *BCRP* mRNA and *MDR*1 mRNA expression was found (r=0.47, P=0.04). None of the other analyzed resistance proteins

Table 3. Paired analysis of the resistance genes in 20 AML patients

	Diagnosis Dx	Relapse/ RD	Ratio RD/Dx	P-value
BCRP mRNA				
Median	0.08	0.15	1.91	0.04*
Range	0.01- 2.54	0.00- 7.30		
LRP/MVP mRNA				
Median	11.6	4.75	0.62	0.02*
Range	1.55- 59,2	0.3- 18.2		
*MRP*1mRNA				
Median	16.6	10.5	0.72	0.37
Range	3.79- 100.2	0.19- 126.6		
*MDR*1 mRNA				
Median	0.11	0.09	0.69	0.17
Range	0.00- 5.26	0.0- 0.92		

The P-values indicate the differences between diagnosis and relapse or refractory disease using the Wilcoxon signed-ranks test. The 'n' indicates the number of patients involved in the paired analysis. The P-value indicates the difference between diagnosis and relapse/refractory disease, * indicates a statistically significant difference between relapse and diagnosis.

Table 4. Correlation between the quantitative mRNA expression of resistance proteins and the rhodamine 123 retention assay with/without PSC 833

		Correlation with PSC/Rho 123			
		Diagnosis		Relapse/refractory disease	
		(*r*)	P-value	(*r*)	P-value
BCRP	mRNA	0.48*	0.04*	0.04	0.88
*MDR*1	mRNA	0.59*	0.01**	0.88*	0.01**
LRP/MVP	mRNA	-0.01	0.97	0.12	0.61
*MRP*1	mRNA	0.06	0.80	0.22	0.34

(*r*): the Spearman rank-correlation coefficient; *: correlation is significant at the 0.05 level; **: correlation is significant at the 0.01 level.

were correlated with each other. Upregulation of *BCRP* mRNA at the time of relapse (Table 3) was not associated with upregulation of transcription of any of the other resistance genes. At relapse, only co-expression of *MRP*1 mRNA and *LRP/MVP* mRNA was found (r= 0.49, P=0.03).

We observed that *BCRP* mRNA expression was correlated with a positive effect of PSC 833 on Rho 123 retention in diagnostic samples, but not in relapsed/refractory AML samples (Table 4). This functional assay also showed a strong correlation between P-glycoprotein function and *MDR*1 mRNA levels at diagnosis (r=0.58, P=0.009) and at relapse (r=0.88, P=0.00). The *MDR*1 mRNA levels correlated well with the expression of P-glycoprotein (r=0.57, P=0.009 for MRK 16 and r=0.52, P=0.02 for UIC2) at relapse, but not at diagnosis (r=0.15, P=0.55 for MRK 16 and r=0.47, P=0.06 for UIC2).

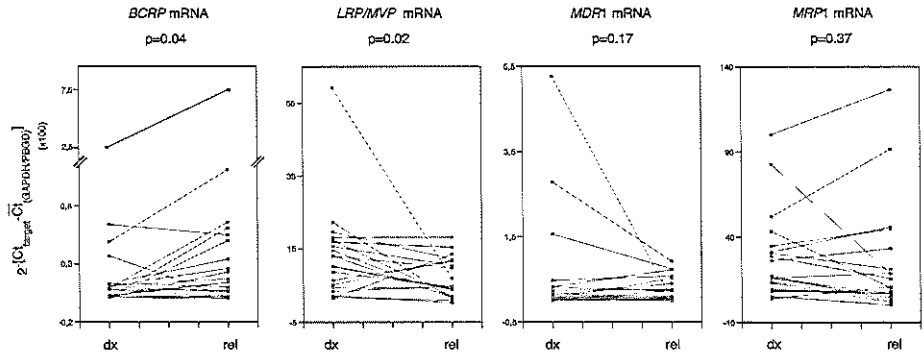

Figure 1. *BCRP, MDR1, LRP/MVP* mRNA expression in paired AML samples at diagnosis. The P-value indicates the difference between the two time points in a paired analysis.

Discussion

Clinical resistance to chemotherapy is a major problem in the treatment of AML. It is conceivable that functional expression of the resistance proteins P-gp, MRP1, LRP/MVP or BCRP is associated with the development of relapse and/or refractory disease. For instance, MDR1 and MRP1 have been reported as adverse prognostic factors for CR and survival (1,5,7,8,10,17,20). This is the first report on a cohesive analysis of the expression of these resistance proteins, in paired clinical samples of AML patients using quantitative PCR. Our data demonstrate, that *BCRP* is the single resistance gene which is expressed at a higher level at relapse as compared to diagnosis (P=0.04). In contrast to what has been suggested in the literature, no increase of *MRP*1 or *MDR*1 levels was found at relapse. *LRP/MVP* mRNA levels even showed a decrease at the time of relapse/refractory disease. Increased levels of *BCRP* mRNA were not correlated with an increase of expression of any of the other resistance genes at time of relapse/refractory disease, leaving only BCRP expression as a possible contributor to refractory/relapsed disease.

BCRP, also identified as mitoxantrone resistance protein (MXR) or ABCP (ABC transporter cloned from human placenta) is responsible for drug resistance phenotypes in MCF7 cell lines after transfection experiments. Resistance was found for mitoxantrone, daunomycin, doxorubicin and DNA topoisomerase I inhibitors like topothecan and SN-38, the active form of irinothecan, while sensitivity to platinum, paclitaxel and vincristine was retained (22,28,45,46). The *BCRP/MXR/ABCP* gene is evolutionary distinct from the other members of the ABC transporter family that encode P-gp and MRP1, being on a completely separate limb of the phylogenetic tree (28). In contrast to the *MDR*1 and *MRP*1 genes, it encodes a protein which is a half-transporter molecule requiring homo- or heterodimerization in order to function. This suggests a unique and probably complementary role for BCRP among the other resistance proteins (28,47,48). In normal tissues, BCRP is quite distinctly expressed from P-gp and MRP1. *BCRP* mRNA is highest in the placenta (22,26) and certain areas of the midbrain (putamen), and relatively low in other normal fetal and adult tissues (28).

Until now, only one study is available studying BCRP expression in clinical resistance in AML. In clinical samples studied by Ross *et al.*, a 1295-fold variation in expression levels in *de novo* AML patients was reported (27). Also, they described a weak association (r= 0.44) of *MDR*1 and *BCRP* mRNA expression in nine AML patients in which both drug resistance proteins were investigated. Based on this limited analysis, it was suggested that this co-expression of BCRP and MDR1 may explain

Table 5. Published studies of expression of the drug resistant proteins in paired samples of AML patients

Author	Reference	Number of patients	Expression level	Age	Expression in relapsed vs *de novo* AML
					MDR1
Guerci	(62)	4	protein	adults	higher/equal
Hart	(53)	9	RNA	adults	higher/lower
Ino	(31)	21	protein	adults	equal/lower
Sato	(66)	6	RNA	adults	equal
Ito	(32)	10	DNA	adults	equal
		10	protein		higher/lower
List	(33)	17	protein	adults	lower
Marie	(60)	4	RNA	adults	higher/equal
Marie	(67)	4	RNA	adults	higher/lower/ equal
Maslak	(63)	5	protein	adults	higher
Michieli	(64)	7	protein	adults	higher/lower/ equal
Wood	(65)	14	protein	adults	higher
Van den Heuvel	(30)	30	protein	adults + children	equal
					MRP1
Schneider	(54)	13	RNA	adults	higher
Zhou	(12)	not mentioned	RNA	adults	equal
Hart	(16)	8	protein	adults	equal
Van den Heuvel	(Ps)	20	RNA	adults + children	equal
					LRP/MVP
Hart	(16)	8	protein	adults	equal
List	(58)	17	protein	adults	higher
Van den Heuvel	(Ps)	20	RNA	adults + children	lower
					BCRP
Van den Heuvel	(Ps)	20	RNA	adults + children	higher

Ps: present study.

the disappointing results of MDR1 reversal agents in clinical studies in AML. Interestingly, Leith *et al.* described in a large SWOG study a distinct subgroup of AML patients of whom the blast cells exhibited cyclosporine resistant efflux of rhodamin 123, that was not correlated with MDR1, MRP1 or LRP/MVP protein expression (49). They suggested the existence of another as yet undefined efflux mechanism in adult AML blasts cells.

In our group of AML patients, the *BCRP* mRNA levels varied 240-fold at diagnosis. At diagnosis, we also found a weak, but significant correlation between the expression of *BCRP* and *MDR*1 mRNA (r=0.47, P=0.04), and not between any of the other resistance proteins. This association was confirmed by a correlation between *BCRP* mRNA expression and functional drug efflux which could be inhibited by PSC 833. This correlation disappeared at relapse, where no co-expression of BCRP and MDR1 was observed, leaving only BCRP expression as a possible contributor to refractory disease. If co-expression of *BCRP* and *MDR*1 would occur in AML, this could open a possibility for new clinical trials using reversal agents that block both P-gp and BCRP such as GF120918 (25,50,51). Moreover, recent studies have identified additional BCRP specific modulators such as fumitremorgin C (FTC) and analogues (46,51) which may be candidates for feasibility studies of BCRP blockers in a clinical setting.

Our study is the first that has investigated *BCRP* mRNA expression, simultaneously with *MDR*1, *MRP*1 and *LRP/MVP* mRNA expression in paired AML samples and shows that BCRP is the only drug resistance gene which is expressed at a higher level at relapse/refractory disease. Previous studies have shown conflicting results with regard to MDR1, MRP and LRP/MVP upregulation at relapse, probably because they were often performed in a heterogeneous pool of diagnostic, relapse and secondary AML samples (Table 5)(12, 16, 29, 52-58).

It has often been suggested that *MDR*1 expression at the time of relapse is higher as compared to diagnosis (33, 59-65). In the present study, we did not find a higher level of *MDR*1 mRNA, nor P-glycoprotein function and expression at relapse or in refractory disease in these AML patients.

We conclude that BCRP and not MDR1, LRP/MVP or MRP1 may play a relevant role in the development of relapsed or refractory AML. Studies in larger cohorts of AML patients are necessary to establish the prognostic role of expression and co-expression of these drug resistance proteins in relation to other already appreciated clinical prognostic factors.

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CHAPTER 5

MDR1 expression in poor risk acute myeloid leukemia (AML) with partial or complete monosomy 7

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Abstract

Expression of the multidrug resistance (MDR1) phenotype, encoded by the *MDR*1 gene, is an adverse prognostic factor for complete remisson (CR) and survival in acute myeloid leukemia (AML). Other prognostic factors, such as specific cytogenetic abnormalities, have been identified in AML. We have investigated the expression of the *MDR*1 gene in untreated AML patients with monosomy 7 (n=12), and partial deletions (n=7) of the long arm of chromosome 7 (resp. –7/7q-), because of the extremely bad prognosis associated with these cytogenetic abnormalities and because of the fact that the *MDR*1 gene is located on chromosome 7q21.1. The findings were compared with the level of MDR1 expression in a group of 42 other AML patients, matched for age with favorable, neutral or complex cytogenetic aberrations. *MDR*1 mRNA expression, as measured by the RNase protection assay was significantly higher in the -7/7q- group *vs* other AML patients (median 1.3 *vs* 0.1 AU, P=0.02). Protein expression of MDR1 in the –7/7q- group, as determined with the monoclonal antibody MRK 16, was found to be similar to the levels found in the control group. With a functional rhodamine retention assay using the modulator PSC833, increased MDR1 activity was observed in the –7/7q- group as compared to the control group of patients (P=0.05). Considering the higher *MDR*1 mRNA expression and equal or slightly elevated level of protein expression of MDR1, we studied the presence of *MDR*1 genes in this group of –7/7q- patients. Fluorescence *in situ* hybridization (FISH) studies, using a specific *MDR*1 probe revealed no loss of an *MDR*1 allele in any of the deleted q- arms of the 7 patients with 7q-, whereas all monosomy 7 patients lacked one *MDR*1 gene homologue. To determine whether there was selective loss of the *MDR*1 gene in the –7/7q- patients, the genetic polymorphism of the *MDR*1 gene was used. Both allelic variants (G and T) were represented in the -7/7q- and in the control group, showing a predominance for GT at position 2677 of the *MDR*1 gene in the control group. In the 12 monosomy 7 patients loss of the *MDR*1 allele was random. Methylation studies of the CpG island of the *MDR*1 gene revealed no hypermethylation in any of the –7/7q- patients. We conclude that MDR1 expression in –7/7q- AML patients is upregulated at transcriptional, but not at translational level, suggesting that other mechanisms than MDR1 are responsible for the poor prognosis in these patients.

Introduction

Intrinsic or acquired drug resistance is a major cause of treatment failure in acute myeloid

leukemia (AML). Resistance to anthracyclines, such as daunorubicin, doxorubicin, vinca alkaloids and epipodophyllotoxines, is associated with the classic multidrug resistance phenotype (MDR1). In cell lines that are cross-resistant to these drugs, the ATP-binding cassette (ABC) transporter P-glycoprotein (P-gp) is expressed (1-4). This acts as a transmembrane drug efflux pump and is encoded by the *MDR*1 gene, located on chromosome 7 at band q21.1 (5-7). In leukemic blast cells, P-gp expression is associated with a lower intracellular retention of cytostatic drugs and a relative resistance to these agents (8-11). In addition, other proteins are associated with multidrug resistance, including the *lung resistance protein* (*LRP*) gene, located on chromosome 16p13.2 and the 190 kD *multidrug resistance associated protein* (*MRP*1) gene, located on chromosome 16p13.1 (12-14).

In AML, MDR1 expression was identified as an independent adverse prognostic factor with respect to complete response to induction treatment and survival (15-22). Also, other prognostic factors such as specific cytogenetic abnormalities, age, CD34 expression and white blood count at diagnosis have been identified (15-20).

AML patients with –7/7q- have an extremely poor outcome, which is independent of the above mentioned clinical and immunological prognostic factors (15-22). It is not known why and how the (partial) loss of a chromosome 7 affects the sensitivity of these AML cells to chemotherapy (23-25). The breakpoint of partial chromosome 7 deletions is close to the 7q21.1 site of the *MDR*1 gene in many patients. Therefore, we have attempted to investigate whether the poor response to therapy of AML patients with –7/7q- is associated with an altered regulation of the *MDR*1 gene, and whether selective loss of one *MDR*1 allele is involved in this process.

Patients and methods

Patients

Routine cytogenetic studies on bone marrow samples of newly diagnosed AML patients revealed 19 patients to have a deletion of the long arm of chromosome 7 or monosomy 7 (Table 1). Cytogenetic analysis had been carried out by standard techniques, and the findings described according to the international nomenclature (26). Forty-two other karyotyped AML patients, matched for age, FAB, and WBC, were taken as controls (Table 2). Written informed consent was obtained to perform these studies, according to the Helsinki agreement. Morphologic classification was performed according to the French-American-British (FAB) criteria (27). In the –7/7q- group the FAB classifications were M0 (n=1), M1 (n=4), M2 (n=5), M4 (n=3), M5 (n=4), M7 (n=2).

Table 1. Karyotype and FAB type of the 19 newly diagnosed AML patients with complete or partial monosomy 7

Pat.no.	Age	WBC x 10^9/l	FAB	Karyotype (ISCN, 1995)
1	1	30	M4	45,XY,-7 [19] / 46,XY [3]
2	53	138	M5	45,XX,-7 [12] / 46,XX [4]
3	64	3	M4	45,XY,-7 [25] / 46,XY [6]
4	67	196	M5	45,XY,-7 [12] / 46,XY [16]
5	43	32	M1	45,XX,-7 [19] / 46,XX [1]
6	57	40	M7	45,XY,-7[27]
7	80	2	M7	46,XY,r(7) [8] / 45,XY,-7 [2]/ 46,XY [11]
8	35	206	M1	45,XX,inv(3)(q21q26),-7 [27]
9	16	130	M4	45,XY,inv(3)(q22q26),-7 [25]
10	22	51	M2	45,XX,inv(3)(q22q26),-7 [31] / 46,XX [1]
11	44	43	M2	44-48,X,-Y,add(3)(q27),dic(5;21)(q11;q22),-7,der(12)t(12;15)(p12;q15), -15, der(16)t(16;17)(p12;q11),17,18,idic(21)(q21),+idic(21)(q21)x1-2,+mar1,+mar2,+mar3 [32]*
12	51	256	M1	45,XX,ins(3;3)(q24;q25q21),del(5)(q11),-7,der(7)pseudic(7;5)(p13,cen) t(5;11)(q21;q11)add(7)(q35), der(11)t(7;11)(p15;p14)t(5;11)(q21;q11) [11] / 46,idem,+r(7) [7] / 46,XX [2]
13	54	49	M1	46,XX,?add(7)(q21) [41] / 46,XX [1]
14	66	23	M5	46,XX,del(7)(q21.3) [32] / 46,XX [5]
15	29	13	M2	46,XX,del(7)(q22) [33] / 46,XX [1]
16	76	10	M2	46,XX,del(7)(q22.2q36) [41]
17	76	119	M0	47,XX,del(7)(q22q36),+8 [12] / 46,XX [8]
18	57	2	M5	46,XY,del(7)(q2?q3?6),[20] / 47,XY,+13 [5]/46,XY [9]
19	73	1	M2	46,XX,del(7)(q31q34) [11] /46,XX [1]

WBC: white blood cell count, *add(3).ish der(3)t(3;6)(q2?7;?)(wcp3+,wcp6+) mar1.ish der(7)t(7;?)(wpc7+,cen7+,mde-) mar2.ish der(?)t(?;15)(wcp15+), mar3.ish der(?)t(?;17)(wcp17+)

The FAB classifications of the control group were M1 (n=9), M2 (n=15), M3 (n=2), M4 (n=9), M5 (n=7), and M7 (n=1). The –7/7q- group included 12 patients with a monosomy 7, and 7 with a 7q-karyotype. The cytogenetic abnormalities in the control group were favorable in 6 patients [t(8;21), inv (16), t(15;17)], unfavorable in 4 patients [t(9;22), 5q-, and 11q23 with mixed lineage leukemia (MLL) rearrangements], and normal or other karyotypes in 33 patients. The median age of the –7/7q- group of patients was 54 years, the median age of the control group was 55 years.

All patients were treated according the protocols of the Dutch-Belgian Hemato-Oncology Cooperative Group (HOVON 4/4a, respectively HOVON 29). Induction therapy consisted of daunorubicin (45 mg/m^2 for 3 days), cytosine arabinoside (200 mg/m^2 for 7 days followed by 2 g/m^2 for 6 days), and amsacrine (120 mg/m^2 for 3 days) (Hovon 4/4a). In Hovon 29 induction treatment consisted of cytosine-arabinoside (200 mg/m^2 for 7 days), idarubicine (12 mg/m^2 for 3 days), followed by amsacrine (120 mg/m^2 for 3 days and cytosine-arabinoside (2g/m^2) for 6 days. Sixteen of 19 patients with the -7/7q- karyotype received standard induction chemotherapy as compared to 36 out of the 42 in the control group. Three (19%) of sixteen -7/7q- AML patients, as compared to 22/36 (61%) of the controls, achieved complete remission (P=0.007). A group of a 104 normal healthy blood donors was, after informed consent, used as a pilot study to assess the genetic polymorphism of the *MDR*1 gene (peripheral blood mononuclear cells) in the normal population.

RNase protection assay

For RNA and protein studies in the AML patients, mononuclear cells from bone marrow or blood were freshly isolated and separated by Ficoll-Isopaque centrifugation (Nycomed, Oslo, Norway). All samples contained more than 85% of blasts. In normal subjects, blood nucleated cells were used as a control. Total RNA was isolated, using TRISOLV extraction (Biotecx, Houston, TX, USA) as is originally described by Chomczynski *et al.* (28). Quantitative detection of *MDR*1 and *MRP*1 gene transcripts was performed by the RNase protection assay. The assay was done with the RPA II kit (Ribonuclease Protection Assay Kit; Ambion, Austin, TX, USA), a modification of the method described by Zinn *et al.* (29). Ten μg of total RNA were hybridized with ^{32}P-CTP-RNA probes under standard conditions, followed by RNase-A/RNase T1 treatment. For RNase protection, an *MDR*1 specific mRNA antisense RNA probe was obtained by transcription of a 302 nucleotide cDNA fragment (nucleotide positions 3498 - 3801) with SP6 RNA polymerase (Ambion) (30).

Table 2. Control group of AML patients without partial or complete monosomy 7

Pat.no.	Age	WBC x 10^9/l	FAB	Karyotype
1	35	39	M4Eo	F
2	66	12	M2	N
3	74	42	M4	N
4	76	122	M2	N
5	76	51	M1	N
6	61	58	M2	N
7	58	194	M7	U
8	61	476	M4	N
9	73	200	M1	N
10	88	47	M2	N
11	51	31	M2	N
12	23	494	M4	N
13	24	3	M3	F
14	20	173	M4	N
15	50	70	M2	N
16	65	48	M1	N
17	61	14	M1	N
18	58	351	M1	N
19	54	227	M4	N
20	82	215	M5	U
21	55	5	M2	N
22	69	38	M1	N
23	44	173	M4	N
24	58	180	M2	N
25	61	164	M5	U
26	67	53	M2	N
27	37	108	M1	N
28	45	7	M2	N
29	41	32	M2	F
30	78	46	M4	N
31	30	28	M3	F
32	51	86	M5b	N
33	61	157	M5	N
34	69	103	M5	N
35	30	126	M4	U
36	53	2	M2	N
37	63	50	M5a	N
38	71	418	M2	N
39	17	13	M2	F
40	35	38	M1	F
41	71	72	M5	N
42	50	6	M2	N
43	44	25	M1	N

U: Unfavorable: 11q23 with *MLL* rearrangements, t(9;22), 5q- and multiple aberrations; F: t(15;17), t(8;21) and inv(16); N: normal or other cytogenetic abnormalities.

The *MRP*1-specific probe is complementary to sequences at the 5' end of the *MRP*1 mRNA (nucleotides 240-484) (7,13). A human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was included in all RNase protection assays as a control for RNA integrity and recovery. The mRNA levels of *MDR*1 and *MRP*1 were quantitated by scanning the films with the ultrascan XL-laser densitometer (LKB, Uppsala, Sweden) and data were analyzed with the gelscan XL software package (31,32). The colchicin-resistant KB 8-5 and KB 8 cell lines, and the drug-sensitive KB 3-1 parental cell line were used in each experiment as positive and negative controls (33). The signal obtained with a 10 μg total RNA sample of KB 8-5 cells was assigned an arbitrary expression level of 30 arbitrary units (AU), and the level in the KB8 cells was related to the level of the KB 8-5 cells, (3 AU). The cell lines GLC4 and GLC4/ADR were used as negative and positive controls for MRP1 expression levels in the different experiments (12). The *MRP*1 mRNA levels were expressed in units relative to the expression of MRP1 in GLC4/ADR which was arbitrarily set at 100 U. The calculated MRP1 expression of GLC4 was set to 4 U (34). The *MDR*1 and *MRP*1 mRNA levels were standardized according to the amount of *GAPDH* mRNA. All individual experiments included torulla yeast RNA as a control for specific hybridization of the probes to the mRNA samples.

Analysis of the expression of P-glycoprotein in bone marrow samples

For measurement of the expression of P-gp, cells were incubated (at room temperature) with monoclonal anti-P-gp antibody, MRK 16 Moab (Kamiya Biomedical Company, Tukwila, WA, USA) at a concentration of 12.5 μg/ml or an isotype matched control antibody mIgG2a (Sigma, St. Louis, MO, USA) at a concentration of 10 μg/ml. Cell-bound antibodies were detected by fluorescein isothiocyanate (FITC)-labeled rabbit anti-mouse immunoglobulin antibodies (DAKO, Glostrup, Denmark).

Results were given as the ratio of the mean of cell-associated fluorescence of cells incubated with the anti-P-gp antibody divided by the mean of cell-associated fluorescence of cells incubated with the control mIgG2a antibody.

As controls in each experiment, the drug-sensitive 8226 S and the drug-resistant 8226 D6 cells were included to measure expression of P-gp. The mean of the ratio of the MRK 16 expression of the negative control cell line 8226 S was 1.32 ± 0.29 (mean ± SD; n=59). The mean of the ratio of the MRK 16 expression of the positive control cell line 8226 D6 was 30.23 ± 5.01. The ratio of the expression was measured in the total population of blasts and also in the CD34-positive cells, when a subpopulation of more then 10% CD34 positive cells was present.

Analysis of the function of P-glycoprotein

For measurement of the function of P-glycoprotein, the fluorescent molecule rhodamine 123 (Rho 123) was used as a P-gp substrate. Therefore, cells were incubated for 1 h at 37°C at 5% CO_2 in the absence or presence of 2 μM PSC 833. After this incubation, 200 ng/ml Rho 123 (Sigma) was added to the cells. A sample was taken at t=0 min to correct for background fluorescence and at t=90 min to measure intracellular rhodamine accumulation.

Results are given as the ratio of the mean intracellular rhodamine fluorescence of cells exposed to PSC 833 divided by the mean intracellular rhodamine fluorescence of cells not exposed to PSC 833.

Fluorescence in situ hybridization (FISH)

Dual colored fluorescence *in situ* hybridization (FISH) was carried out, using standard techniques (35), on metaphases in cytogenetic preparations using a biotin labeled α-satellite probe for chromosome 7 (p7t1) detected with avidin FITC (green), and a digoxine-labeled cosmid probe CHMR6, specific for the *MDR*1 gene at 7q21.1, detected with Texas Red (red). The probes were labeled by standard nick translation using Biotin-16-dUTP according to the manufacturer's instructions (Gibco BRL, Gaithersburg State, MD, USA). Between 5 and 32 metaphases per patient were examined.

Detection of MDR1 polymorphism and allelic expression

The presence and allelic expression of the genetic polymorphism of *MDR*1 at position 2677 was detected using oligonucleotide hybridization as described by Mickley *et al.*, (36,37). The PCR products were dot-blotted to a Zeta Probe blotting membrane (Bio-Rad, Hercules, CA, USA) which was prehybridized for 30 min at 50^0 C in 5 x SSPE, 0.5% SDS, 5 x Denhardt's, 50 ug/ml denatured herring sperm DNA. Hybridization, using radiolabeled oligonucleotides as allele-specific probes, was performed for 2 hours at 50^0 C after which the blots were rinsed twice at room temperature in 2 x SSPE, 0.1% SDS and subsequently washed for 10 min at 55^0 C in 5 x SSPE, 0.1% SDS. Two 30-bp oligonucleotides, designated HMC3 and HMC4, were used. These oligonucleotides cover residues 2656 to 2685 of the *MDR*1 gene with HMC3 possessing a G at position 2677 and HMC4 a T at this position. Equal amounts of each control were spotted on both sides of

the filter, thus providing the means of an indicator of specificity. Because the hybridizations were performed under identical conditions, with probes labeled to similar specific activities, the signals from the control oligonucleotides were usually similar. Phosphor imager techniques (Image quant) were used to confirm G and T positivity.

PCR

One μg of genomic DNA was used as a template in PCR for 40 cycles to detect genetic polymorphism at the DNA level. To detect allelic expression, 1 μg of total RNA was reverse transcribed and cDNA template was subjected to 40 cycles of PCR. The primers used for DNA and RNA oligonucleotide hybridization are described above.

Methylation-specific PCR (MSP)

The MSP assay is a two step technique. In the first step DNA is pretreated with bisulfite. Bisulfite induces a chemical modification of the DNA sequence by altering cytosine to uracil (which subsequently is replaced by thymidine in the PCR reaction). In this reaction, all cytosines are converted to uracil, except methylated cytosines (5-methylcytosine), which are resistant to this modification. The second step is a PCR based amplification of the altered DNA. PCR primers are designed to distinguish methylated from unmethylated DNA, taking advantage of the sequence differences after bisulfite modification.

Bisulfite Modification: DNA (1 μg in a volume of 50 μl) is denatured by NaOH (final concentration 0.2 M) for 10 min at 37^{0}C. Thirty microliters of 10 mM hydroquinone (Sigma) and 250 μl sodium bisulfite (Sigma) at pH 5, both freshly prepared, are added and mixed. The samples are incubated under a layer of mineral oil at 50^{0}C for 16 h. Modified DNA is purified by using the Wizard DNA purification resin according to the manufacturer (Promega, Madison, WI, USA) and diluted in 50 μl of water. Modification is completed by NaOH (final concentration 0.3 M) treatment for 5 min at room temperature. Then follows an ethanol precipitation. DNA is resuspended in water and used immediately, or stored at –20^{0}C.

PCR Amplification: Primer pairs were designed in the 5'-UTR CpG island of the published *MDR*1 sequence (accession number AC002457, position 141267 sense and 141383 antisense). The primer sequences are: 5'-GGAGGGAGAATTGTATTGGTGGT-3' (unmethylated sense); 5'-GAGAATCGTATTGGCGGC-3' (methylated sense); 5'-

CATTAATACCCCAACTACTCTAACCACA-3' (unmethylated antisense); 5'-CCCCA-ACTACTCTAACCGCG-3' (methylated antisense).

The PCR mixture contains 1 x PCR buffer (16.6 mM ammonium sulfate, 67 mM Tris, pH 8.8, 6.7 mM MgCl2; 10 mM 2-mercaptoethanol), dNTPs (each at 1.25 mM), primers (300 ng each per reaction), and bisufite-modified DNA (50 ng) in a final volume of 50 µl. PCR reactions are hot started at 95^0C for 5 min before the addition of 1.25 units of Taq polymerase (Boehringer, Mannheim, Germany). Amplification is carried out for 35 cycles (30 sec at 95^0C, 30 sec at the annealing temperature of 60^0C, and 30 sec at 72^0C), followed by a final 4 min extension at 72^0C. Each PCR is loaded on a 6-8% nondenaturing polyacrylamide gel, stained with ethidium bromide and directly visualized under UV illumination (38,39).

Statistical analysis

The units of mRNA from *MDR*1 and *MRP*1, the MRK 16 and IgG ratios, as well as Rho 123 retention were compared between the two subgroups (-7/7q *vs.* control) using the Wilcoxon rank-sum test. The Hardy-Weinberg formula was used to evaluate the genetic polymorphism of *MDR*1 in the normal population (40,41). All reported P-values are two-sided and a significant level of $\alpha = 0.05$ was used.

Results

Patients characteristics

Of the 19 untreated AML patients with an abnormality of chromosome 7, 12 had a monosomy 7 (Table 1). In 6 instances (patients 1-6) this was the sole cytogenetic abnormality. Three patients (patients 8-10) also had an inv(3)(21q26), an association that has been previously reported (42). Patient 7 had two karyotypically independent clones, one with monosomy 7 and one with a ring chromosome 7. In patient 12, the main clone had monosomy 7 and a partial deletion of the remaining chromosome 7 was found. For this study all these patients were grouped together as monosomy 7 patients. Seven patients (patients 13-19) had deletions of the long arm of chromosome 7 (Table 1, Figure 1). Forty-two other AML patients without chromosome 7 abnormalities, were used as control group.

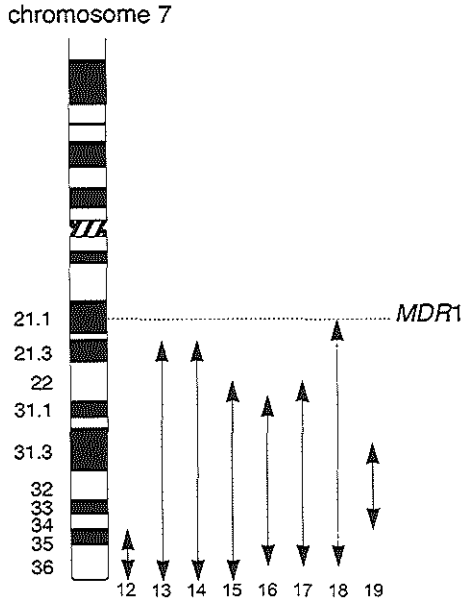

Figure 1. An ideogram of chromosome 7 showing the MDR1 gene at 7q21.1, and the deleted segments involved in the patients with 7q- (patients 12-19). The extent of the deleted region in patient 18 was unclear due to the quality of the metaphases.

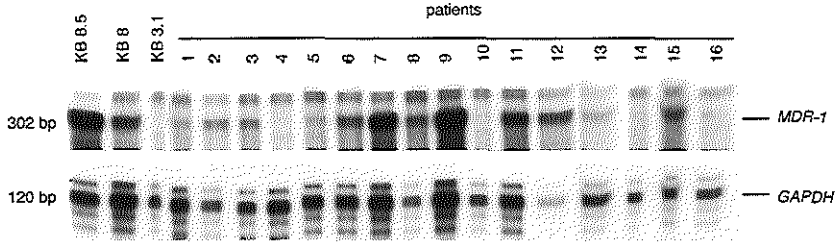

Figure 2. RNase protection assay. Analysis of MDR1 expression in control cell lines (KB 8.5, KB 8, KB 3.1) and BM mononuclear cells of 16 of the investigated 48 patients (13 -7/7q- and 35 controls).

mRNA expression

*MDR*1 mRNA expression was analyzed in 13/19 patients with a –7/7q- and in 35/42 control patients. In patients with the –7/7q- karyotype, the median *MDR*1 mRNA expression was 1.3 AU (range 0.05-107), as compared to 0.1 AU (range 0 -12.7) in the other combined karyotypes (P = 0.02) (Figure 2). The median *MRP*1 mRNA expression

Table 3. MDR1 and MRP1 expression in AML

	Karyotype		P-value*
	-7/7q-	Other AML	
Number of patients	19	42	
*MDR*1 mRNA (AU)			
Median	1.3	0.1	0.02
Range	0.05-1.07	0.0- 12.7	
Observations	13	35	
*MRP*1 mRNA (AU)			
Median	5.7	3.0	0.20
Range	1.1- 13.8	0.0- 31.1	
Observations	8	35	
MRK 16/IgG2a ratio			
Median	1.76	1.46	0.17
Range	0.82- 4.21	0.95- 3.04	
Observations	18	20	
PSC/Rho 123 retention			
Median	1.35	1.18	0.05
Range	0.96- 5.95	1.0- 1.9	
Observations	18	19	

*P-value belongs to the hypothesis that values in patients with karyotypic abnormalities –7 and 7q- are equal to those with other cytogenetic abnormalities or with normal cytogenetics.

was 5.7 AU (range 1.1-13.8) in 8/19 AML patients with a (partial) deleted chromosome 7 as compared to 3.0 AU (range 0-31.1) in the control group (P =0.20) (Table 3).

Protein expression

P-gp expression was analyzed by flowcytometry in 18 of 19 patients with –7/7q-, and the results were compared with 20/42 (for MRK 16 expression) and 19/42 (for PSC/Rho 123 modulation) control patients. The selection of the investigated subjects in the control group with flowcytometry was based on availability of viable cell samples. The median PSC/Rho 123 retention ratio's were 1.35 (range 1.01-2.34) in –7/7q- samples as compared to 1.18 (range 1.0-1.9) in the other AML patients (P = 0.05). The median value of the MRK 16/IgG2a staining ratio of all –7/7q- samples was 1.76 (range 0.82-4.21) as compared to 1.46 (range 0.95-3.04) in the controls (P = 0.17).

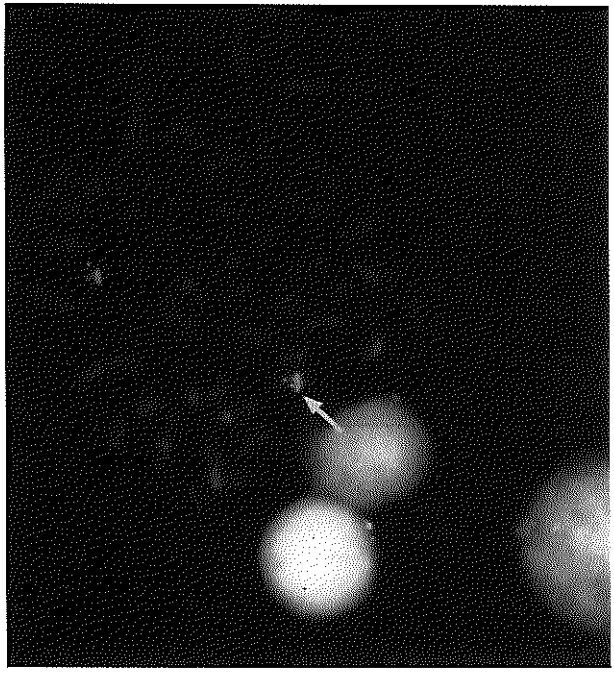

Figure 3. Results of the dual colored fluorescence in situ hybridization (FISH) study on one of the AML patients with a partial deletion of one chromosome 7, showing the biotin labeled α-satellite centromere probe (7pta) for detection of chromosome 7 (green) and the digoxinine-labeled (CHMR6) for the *MDR*1 gene (red). The arrows indicate the chromosomes 7. Two copies of the *MDR*1 gene are present in this metaphase.

Presence of the MDR1 gene

Dual colored FISH studies on metaphases from the monosomy 7 patients, showed that only one copy of the *MDR*1 gene on the remaining chromosome homologue was present. Patient 7 had two karyotypically independent clones, one with monosomy 7 and one copy of the *MDR*1 gene, and the other clone with two copies of the *MDR*1 gene due to a ring chromosome 7 and a normal chromosome 7 analogue. Two other monosomy 7 patients had complex variations (patient no. 11 and 12). For patient 11, additional FISH studies revealed that a marker chromosome was of chromosome 7 origin, which was negative for the *MDR*1 gene. In patient 12, the main clone had monosomy 7 and the remaining chromosome 7 had undergone structural changes but was shown by FISH to carry the *MDR*1 gene. A subclone of this patient had a ring 7 instead of monosomy 7. This ring also had retained the *MDR*1 gene. For the discussion all of these cases were grouped together as having monosomy 7 with one copy of the *MDR*1 gene.

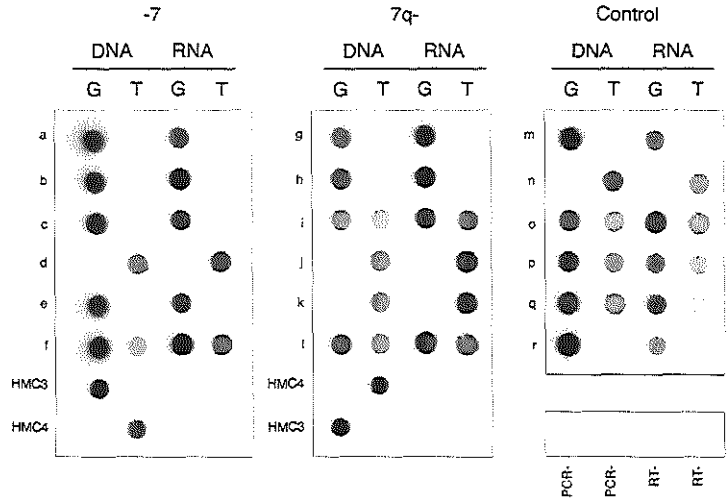

Figure 4. Detection of the genetic polymorphism of the MDR1 **gene in blasts of AML patients.** The DNA and RNA lanes indicate which alleles are expressed. The oligonucleotides HMC3 and HMC4 represent the hybridization controls for the G and T primer respectively. The box at the bottom side, right of the figure shows the results of initial controls on the procedures (PCR- and RT-).

MDR1 polymorphism and allelic expression.

Seven patients (pat.13-19) had deletions of the long arm of chromosome 7 (Table 1). In all instances dual colored FISH showed both chromosome 7 homologues to be positive for the *MDR*1 gene (Figure 1) allelic expression. Hybridization studies with the *MDR*1-specific primers for position 2677 at 7q21.1 were performed in 12 patients with monosomy 7. In eight cases, one primer hybridized (7x with G, 1x with T), while 4 of these patients hybridized with two primers (GT). Patients with 7q- expressed a G gene variant in 3 cases, a T variant in 2 cases, and a GT *MDR*1 variant in two cases. Five of these 7q- patients were homozygous for *MDR*1 since they had been shown to have 2 copies of the *MDR*1 gene by FISH (Figure 3). In the control group, 15 patients were examined with oligonucleotide hybridization. Twelve expressed the heterozygous variant, whereas the G variant was found twice and the T variant once (Figure 4). In the peripheral white blood samples of 104 healthy volunteers the G variant was found 43 times, the GT variant 45 times and the T variant 16 times.

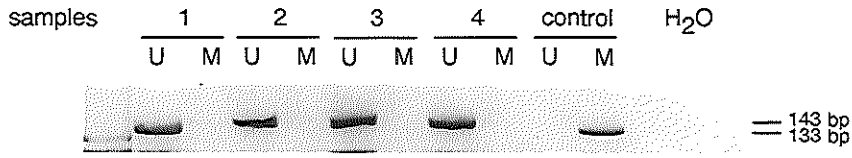

Figure 5. An example of the MDR1 DNA methylation assay in monosomy 7 (samples 1 and 2) and 7q- (samples 3 and 4) patients. All samples show unmethylated DNA in the CpG island of the *MDR*1 promoter. U, unmethylated; M, methylated.

Methylation studies of the MDR1 gene

Methylation analysis was performed in all monosomy 7 patients, 5/7 patients with 7q-, and 15/42 of the AML patients of the control group. In all samples the CpG island of the promoter of *MDR*1 gene was found to be unmethylated (Figure 5).

Discussion

AML patients with –7/7q- have a poor response rate to induction chemotherapy and a short overall survival (15-20). Expression of MDR1 in *de novo* AML patients is also associated with a poor outcome (16-19,21,22,43). The *MDR*1 gene is localized on the long arm of chromosome 7 at band 7q21.1 (5-7). The aim of our study was to investigate whether the extremely poor prognosis of –7/7q- AML patients could in part be due to a modified *MDR*1 gene expression.

Our data show that the *MDR*1 expression at the mRNA level in blasts of 19 –7/7q- AML patients, was 13-fold higher than in matched AML patients with other abnormal or normal cytogenetics. However, the increased mRNA level was not reflected in higher protein levels as measured with the monoclonal antibody MRK 16, *i.e.* levels were similar in –7/7q- patients as compared to the control group. Only a small increase of MDR1 activity was observed in the –7/7q- group (P=0.05). Dual colored FISH studies showed the presence of only one *MDR*1 gene in the monosomy 7 patients, whereas all 7q- patients revealed both *MDR*1 genes, even if the breakpoint was very close to band

7q21.1. Apparently, in the –7/7q- patients, P-glycoprotein levels are preserved, irrespective of the number of *MDR*1 alleles.

A high level of *MDR*1 mRNA expression in monosomy 7 patients may be explained by retention of the most active *MDR*1 gene. If the genetic polymorphism of *MDR*1 is functionally important, upregulation or activation of the remaining most resistant *MDR*1 allele would be expected. Evidence for this theory was found in a study of Mickley *et al.* who described this phenomenon in patients with Burkitt's lymphoma, who lost one of their *MDR*-1 alleles during development to resistant disease, suggesting selection of a drug-resistant clone (37). However, we observed, by analysis of the genetic polymorphism of the *MDR*1 gene at position 2677, that the loss of one *MDR*1 allele in patients with monosomy 7 was random, *i.e.* both G and T variants were found. The T variant was expressed less frequently, but this was concordant with the random distribution of the G and T variants, using the Hardy Weinberg formula for distribution of alleles in the normal population. A low incidence of the T variant was also reported in by Mickley *et al.* (37). He found 3x a T variant and 15x a G variant in cell lines, and 8x a T variant and 21x a G variant in normal tissue at position 2677 of the *MDR*1 gene in homozygous cases.

These findings indicate that, while the *MDR*1 gene is upregulated at the RNA level, the functional expression has not changed. Upregulation of the *MDR*1 gene as a result of (somatic) mutations was not the focus of our study. Methylation changes of the CpG islands in the promoter region of housekeeping genes is one of the mechanisms by which gene transcription is regulated (44). Especially in human cancers, *de novo* methylation of the islands usually has a significant (negative) effect on the transcription level of the gene involved. In our study the methylation analysis of the *MDR*1 promoter-associated CpG island in 17/19 of our –7/7q- patients and (15/42) control AML patients showed no abnormal methylation pattern in any case. Therefore, we conclude that the transcription of the *MDR*1 gene is not regulated through methylation changes of the promoter region. Fryxell *et al.* suggested in their detailed study that methylation changes upstream of the promoter, in an ALU repeat may be important for transcription regulation, but they did not show a correlation between the methylation level of the ALU repeat and transcription level of the *MDR*1 gene (45). The only study that showed a correlation between MDR1 expression and methylation of the *MDR*1 gene was performed by Kantharidis *et al.* They examined two AML cell lines, of which only one expressed MDR1. However, their

conclusions are mainly based on DNA digestion of HpaII sites, which are located just outside the CpG island of the *MDR*1 gene, and it is not known what the effect of the methylation status of this region will have on the expression of this gene (46).
We identified 4 patients with monosomy 7 and one copy of the *MDR*1 gene as shown by FISH, who had heterozygous MDR1 (G and T) expression. This phenomenon may be the result of contamination of the purified bone marrow samples with normal cells. An alternative explanation may be that these patients had a leukemia with disomy for chromosome 7, which had not been detected by karyotyping or FISH.
We have demonstrated, that MDR1 expression is upregulated in –7/7q- patients at a transcriptional level which is not translated to the protein level. The mechanism for higher expression level was found to be due neither to selective allelic loss of the *MDR*1 gene, nor to methylation changes of the promoter region of *MDR*1. As P-glycoprotein expression does not follow the upregulation at transcriptional level, we suggest that MDR1 is not a mechanism of drug resistance in poor prognostic AML with –7/7q- .

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CHAPTER 6

Absence of mutations in the deoxycytidine kinase (*dCK*) gene in patients with relapsed and/or refractory acute myeloid leukemia (AML)

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Leukemia, in press

Abstract

Resistance to chemotherapy is a major problem in the treatment of acute myeloid leukemia (AML). As cytosine-arabinoside (Ara-C) is an important agent in the treatment of AML it is conceivable that leukemic blasts become resistant to Ara-C during the development to relapse/resistant disease. Although several resistance mechanisms are involved in Ara-C metabolism, deoxycytidine kinase (dCK) is of particular interest because it is the rate-limiting enzyme in the phosphorylation process from Ara-C to Ara-CTP. Structural analysis of the *dCK* gene has revealed mutations which are associated with dCK deficiency and Ara-C resistance *in vitro* and *in vivo*. We searched for mutations in the *dCK* gene in a unique set of paired samples obtained from 31 AML patients at diagnosis and at relapse and/or refractory disease (10 children, 21 adults). Using a RT-PCR to amplify the *dCK* cDNA, followed by direct sequencing of the PCR product, we did not find any of the previously reported mutations in the *dCK* gene involving codons 20, 93, 98, 99 and 154. Also, we did not find new mutations at time of relapse, nor at diagnosis. These results show that mutations in the *dCK* gene are scarce and not of major importance for Ara-C resistance in AML patients.

Introduction

Cytosine-arabinoside (Ara-C) is a standard drug in the treatment of patients with acute myeloid leukemia (AML). In combination with other chemotherapeutic agents it induces complete remission (CR) in 70-80 % of adults and in 80-90 % of pediatric patients with *de novo* disease. Ara-C is a cytotoxic nucleoside analogue which is phosphorylated intracellular into its active form Ara-CTP, by the action of three enzymes; deoxycytidine kinase (dCK), dCMP kinase and nucleoside diphosphate (NDP) kinase. Ara-CTP inhibits DNA polymerase and acts by competing with its physiological counterpart for incorporation into nucleic acids, the natural substrate dCTP.

Clinical resistance resulting in relapse or refractory disease, contributes to a low survival rate in AML. *In vitro* drug resistance studies in AML have shown a strong correlation of Ara-C resistance with prognosis (1,2-6). Several mechanisms of Ara-C resistance have been identified (7). As dCK has been appreciated as the rate-limiting enzyme in the metabolism of Ara-C, we have focussed on the *dCK* gene in this study. The human *dCK* cDNA has been cloned by Chottiner *et al.* from a T lymphoblast cDNA library and the genomic structure has been established (8,9). Stegman *et al.* assigned the gene to 4q13.3-q21.1 in 1993 (10). Murine neoplasm and human cell lines like AB 9228 and HL-60 confer resistance by decreased dCK activity (11-14). Cell lines prepared from leukemic

blasts of a patient with acute lymphoblastic leukemia (ALL), showed markedly decreased Ara-CTP pools due to decreased dCK enzymatic activity after the patient had become resistant to treatment with Ara-C, suggesting an importance of dCK deficiency in the clinical situation (15). Experiments on Ara-C resistant T-lymphoblast cell lines (Ara-C-8D and ddC50) revealed structural alterations like point mutations and deletions within the coding region, as well as decreased mRNA levels (9,13). In adult AML, low or altered dCK activity has been associated with clinical Ara-C resistance (16,17).

Flasshove *et al.* (18) found different point mutations in the *dCK* cDNA in 7 out of 16 adult patients with relapsed and refractory AML. Two silent mutations (codon 86 and 42), and five mutations resulting in amino-acid changes (codon 20, 93, 99, 98, 154). One of them, a point mutation in codon 99 (TAT→TGT) leading to an amino acid substitution from tyrosine to cysteine, was associated with absent dCK activity, whereas the enzyme activity was normal in patients with a point mutation in codon 98 and 20.

The hypothesis of the present study was that point mutations in the coding region of the *dCK* gene might be responsible for changes in Ara-C sensitivity in the course of the disease towards relapse/refractory disease.

Patients

Bone marrow (BM) samples of 30 acute myeloid leukemia (AML) patients, and 1 chronic myeloid leukemia (CML) patient (10 children, 21 adults), were obtained from the iliac crest at diagnosis and at time of first relapse (n=27) or refractory disease (n=3), and in case of CML at the first and second blast crisis (n=1) (Table 1). From each patient and/or parents, written informed consent was obtained to perform these studies. AML classification performed according to the French-American-British (FAB) criteria (19) was M1 (n=8), M2 (n=11), M4 (n=2), M5 (n= 7), M6 (n=2). Cytogenetic analysis was carried out by standard techniques, and the findings were described according to the international nomenclature (20). All patients were treated according to the Helsinki agreement and were included in treatment protocols of the Dutch-Belgian Hemato-oncology Collaborative Group (HOVON 4/4a respectively HOVON 29) for young adults (n=17), European Organization for Research and Treatment of Cancer (EORTC LAM 9) (n=2) for patients ≥ 60 years, and the Dutch Childhood Leukemia Study Group (DCLSG: ANLL 87 and 94) (n=10) for the children (age <18 years). After relapse or in case of refractory disease after induction therapy, adults were treated according to the HOVON 30 relapse protocol (Table 2). The six pediatric patients received treatment according to institutional protocols. For

Table 1. Clinical characteristics of the 31 patients

Diagnosis				Relapse/Refractory disease		
	Age (years)	FAB	Karyotype	Time to relapse (months)	Treatment (at time of relapse/refractory disease)	Response (to reinduction)
1	2	M5	Unfavorable	8	NT	
2	1	M6	Neutral	4	2CdA/Ara-C/Ida	No CR
3	47	M2	Neutral	50	HOVON30	CR
4	55	M2	Neutral	-	HOVON30	No CR*
5	50	M5	Neutral	25	HOVON30	CR
6	50	M2	Neutral	7	HOVON30	CR
7	62	M1	Unfavorable	31	HOVON30	CR
8	61	M1	Neutral	29	HOVON30	No CR
9	35	M1	Favorable	12	HOVON30	CR
10	9	M5	Unfavorable	9	NT	
11	12	M1	Neutral	33	DCLSG ANLL94	CR
12	37	M1	Neutral	12	HOVON29	CR
13	57	M4	Neutral	4	NT	
14	46	M5a	Neutral	6	Ara-C	TD
15	67	M2	Neutral	9	EORTC 9	No CR
16	16	M4eo	Favorable	8	HOVON29	CR
17	19	M5a	Neutral	28	HOVON29	No CR
18	42	M2	Neutral	11	HOVON29	CR
19	1	M1	Neutral	14	DCLSG ANLL87	No CR
20	41	M6	Neutral	4	HOVON30	CR
21	10	M2	Favorable	58	DCLSG ANLL94	CR
22	63	M2	Neutral	8	NT	
23	1	M5	Unfavorable	10	DCLSG ANLL87	No CR
24	27	M2	Neutral	14	HOVON30	CR
25	34	M5	Neutral	-	HOVON30	No CR*
26	5	M1	Neutral	18	DCLSG ANLL87	CR
27	18	M2	Neutral	8	Mitoxantrone	No CR
28	55	M1	Neutral	-	HOVON30	No CR*
29	16	1st BC CML	Unclassified	3	EORTC 9	No CR
30	49	M2	Neutral	6	NT	
31	67	M2	Neutral	5	HOVON30	No CR

BC: blast crisis; CR: complete remission after 1 or 2 courses of re-induction chemotherapy; No CR: refractory disease at time of relapse; No CR*: never CR after diagnosis; NT: not treated for relapse; TD: toxic death; Uc: unclassified; Unfavorable karyotype: t(9;22), 11q23 with *MLL* rearrangements, Complex karyotype: 5q-; Favorable karyotype: inv(16), t(15;17) and t(8;21); Neutral karyotype: normal and other karyotypes.

some patients, after relapse, individual therapy choices were made (Table 1). Complete remission status was defined as normocellular marrow, with < 5% blasts in a BM smear, with normal peripheral blood (PB) counts.

Methods

Collection of bone marrow samples

Bone marrow aspirates were collected in heparinized tubes. Mononuclear BM cells (MNC) were isolated by Ficoll-Hypaque density gradient centrifugation (density 1.077 g/ml; Pharmacia, Uppsala, Sweden). To obtain purified samples with more than 85% of blasts, T-cell depletion and adherence depletion was performed (21). Cells were cryopreserved in Iscove's Dulbecco's medium (IMDM; Gibco, Paisly, UK) supplemented with 10% dimethylsulfoxide (DMSO; Merck, Darmstadt, Germany) and 20% fetal calf serum (FCS; Gibco) using a temperature controlled freezer (Kryo 10, Planer Biomed, UK) and were stored in liquid nitrogen. At the day of the experiments BM cells were thawed.

RNA Isolation

Total cellular RNA from BM or PB was isolated by using Trizol (a mono-phasic solution of phenol and guanidinium isothiocyanate) according to the recommendations of the manufacturer (Gibco BRL Life Technologies, Grand Island, NY, USA). Samples were stored at -80 °C.

cDNA synthesis

Following a denaturation step of 5 min at 70 °C, 500 ng RNA was used as a template in a reverse transcriptase reaction. The reaction contained 10 ng random hexamers (Gibco BRL Life Technologies), 0.5 mM of each dNTP, 200 U M-MLV Reverse Transcriptase (RT), 20 U RNAsin Ribonuclease Inhibitor and 4µl M-MLV-RT 5x first strand synthesis buffer (1 x buffer consists of 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$ and 10 mM DTT) in a total volume of 20 µl. After an incubation for 1 hour at 37°C the RT enzyme was heat-inactivated at 95°C for 5 min All products were purchased from Promega (Madison, Wisconsin, USA).

Amplification and direct sequencing of the dCK gene

The *dCK* cDNA was amplified by PCR using the Expand High Fidelity PCR System (Roche, Mannheim, Germany). Each PCR contained approximately 200 ng of cDNA, 200

μM of each dNTP, 3.5 U DNA polymerases (a mixture of *Taq* and *Pwo* DNA polymerase), 1.5 mM $MgCl_2$, 5 μl 10 x HF buffer and 25 pmol of each primer (forward primer 5' CTCCCAGCCCTCTTTGCCGGAC; reverse primer 5' ACAAAGCTGAA-GTATCTGGAACC) in a total volume of 50 μl. The PCR conditions were as follows: a 2 min 94°C denaturation step followed by 30 cycles of 1 min 94°C, 2 min 60°C, 3 min 72°C and finally 10 min 72°C. Part of the reaction products were analyzed on a 1.5% agarose gel in 0.5 x TAE buffer. Ethidium bromide was added to visualize the expected DNA fragment of 884 bp which was either isolated directly from the gel using the QIAquick gel extraction kit (QIAGEN, Hilden, Germany) or isolated from the PCR mix by the QIAquick PCR purification kit (QIAGEN). The complete nucleotide sequence of both strands of the purified fragment was determined by cycle sequencing using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin & Elmer Biosystems, Forster City, CA, USA) and analyzed in a ABI Prism 310 Genetic Analyzer (Perkin & Elmer Biosystems) using the following sequencing primers: 5'AGCTCTGGGCCGCCAC-AAGAC; 5'GGCTGCCTGTAGTCTTCAGCAAG, 5' AACGATCTGTGTATAGTGAC-AGG; 5'CCTGGGTCACTATTTACACAGGGATCGTTC.

Results

In order to investigate the BM samples of the AML patients for *dCK* mutations, we initially used the procedure as described by Flasshove *et al.* (18). Shortly, total RNA was isolated, converted to cDNA in a reverse transcriptase reaction and the *dCK* cDNA PCR amplified using *Taq* polymerase and two primers, flanking the *dCK* coding sequence as described in the Methods section. The single PCR product of 884 base pairs, that could be detected in all samples, was cloned into pCRII (TA cloning kit). From 21 diagnostic samples and 25 relapse and/or refractory disease AML patients BM samples, both strands of at least three clones of each sample were completely sequenced. In the diagnostic samples, 33 different point mutations were found (12 silent and 21 giving rise to amino acid substitutions) and in the relapse samples 61 mutations were found (15 silent and 46 giving rise to amino acid substitutions). Some *dCK* inserts contained no mutations at all whereas others, derived from the same patient sample displayed multiple mutations.

Also, mutations found in diagnostic samples were not present in relapse samples of the same patient, and *vice versa*. The large number of silent mutations that can not give rise to altered dCK enzymatic activity and as a consequence can not give rise to Ara-C resistance, and the abundant number of randomly found mutations was suggestive for experimental artifacts

Table 2. Cumulative drug doses in the treatment protocols for acute myeloid leukemia

	Ara-C	DNR	Adria	Amsa	Ida	VP16	Mitox	Pred	6-TG	VCR	CPH	CsA
Induction												
HOVON 4/4A	13400	135		360		500	50					
HOVON 29*	13400			360	36	500	50					
DCLSG ANLL87**	22400	180	120			1050	1120	2580		6	1000	
DCLSG ANLL94**	33400		120		36	950	20	1120	2520	6	1000	
Reinduction												
EORTC 9	6000	90					50					
HOVON 30						500	50					5mg/kg

(Total cumulative dose in mg/m^2). Ara-C: cytosine arabinoside; DNR: daunorubicine; Amsa: amsacrine; Ida: idarubicine; VP16: etoposide; Mitox: mitoxantrone; Pred: prednisolone; 6-TG: 6-tioguanine; VCR: vincristine; CP: cyclophosphamide; *: In HOVON 29 patients were randomized to receive +/- GCSF; **: + 5 x intrathecal Ara-C; BMT: bone marrow transplant (allo and/or autologous); CsA: cyclosporine A.

Therefore, to adjust our procedure, we used another thermostable DNA polymerase that, in contrast to *Taq* DNA polymerase, possesses 3' to 5' exonuclease proofreading activity enabling the polymerase to correct nucleotide incorporation errors. Secondly, we used a direct method to sequence the amplified PCR product. We attempted to use high-fidelity *Pfu* DNA polymerase but were unable to reliable amplify *dCK* cDNA. These problems were solved using the Expand High Fidelity PCR System (Roche), which uses a mixture of *Taq* and *Pwo* DNA polymerase and which displays proof-reading activity.
The modified procedure, i.e. the direct sequencing of *dCK* cDNAs obtained by RT-PCR from 31 paired patient samples, revealed no mutations in any of the 62 samples. So, none of the previously described mutations by Flasshove, involving the codons 20, 93, 98, 99 and 154 were found, nor the silent mutations in codon 42 and 86 (18). Also, other mutations that have been described in cell lines (13) were not found in our group of patients.

Discussion

In a group of 31 patients, in which the classical MDR1 phenotype was not upregulated at time of relapse/refractory disease as compared to diagnosis, we performed a sequence analysis of the *dCK* cDNA in order to screen for mutations including ones that were previously found to be associated with low or absent dCK activity (13,18). To our knowledge this is the largest group of AML patients that has been investigated for *dCK* mutations, and the first group with a respectable number of sequential samples. Using the method which has been described by Flasshove *et al.* (18), including a RT-PCR procedure using *Taq* polymerase, followed by cloning of the amplified fragment and sequencing of independent clones, we found a large number of random mutations in 21 diagnostic and 25 relapse/refractory AML samples. The large number of random 'mutations' prompted us to omit the cloning step from our procedure and to perform a direct sequence method on the PCR product. In addition, we used a thermostable DNA polymerase mixture, that is less prone to errors. Using this method, we did not find any of the previously described mutations (13,18) at diagnosis or at relapse. The abundant number of mutations we found in the method used by Flasshove is most likely due to the relatively low fidelity of the *Taq* polymerase. Our results do rise the question whether the 7 mutations found by Flasshove in 16 AML patients were true mutations, as the presence of the mutations are not confirmed at the genomic level and in only one of them a lowered dCK activity was found. The results of our present study indicate, that *dCK* mutations are not of major importance for Ara-C resistance in AML patients.

Several studies showed that a decreased dCK activity was not associated with mutations in the *dCK* gene. A study performed by Kobayashi *et al.* did not reveal mutations in the human Ara-C resistant cell line KY-RA (22) mutations in the cDNA coding region. Stammler *et al.* showed that pediatric ALL patients at diagnosis were more susceptible to relapse if dCK expression was low, but they did not find any mutations in the *dCK* gene (23). Kakihara *et al.* reported a great variability of *dCK* gene expression in a limited number of ALL and AML patients at diagnosis. In two patients that were investigated both at diagnosis and at relapse a lower *dCK* mRNA expression at relapse as compared to the diagnostic state was observed. No *dCK* point mutations were found in this study (24). Martincic *et al.* studied 7 leukemic samples, in which 5 showed Ara-C resistance *in vitro*. Four of the 5 leukemic samples showed decreased levels of *dCK* mRNA levels with semi-quantitative PCR. Using dideoxy fingerprinting (ddf) of the full-length *dCK* coding sequence, they did not find any mutations (25).

As dCK is the rate limiting step in the phosphorylation cascade towards Ara-CTP, it remains an interesting question, which biological mechanism is responsible for the lowered or absent dCK activity in several of the above mentioned studies in which no mutations were found in the *dCK* gene. There is some evidence that in cell lines not mutations, but hypermethylation of the cgcg boxes of the promoter region of the *dCK* gene might be responsible for downregulation of dCK. However, in a study of eight Ara-C resistant AML patients the *dCK* 5' CpG islands were largely unmethylated (25). As the dCK activity was not measured in these patients, the direct effect of a variegated methylated promoter region of the *dCK* gene is not established. Although other steps in the Ara-C metabolism like lowered deoxycytidine deaminase (cDD) activity, enhanced DNA repair mechanisms or an impaired apoptosis pathway have been suggested to be of importance in the mechanism of resistance to Ara-C, obviously, dCK plays a crucial role. Recently, Veuger *et al.* described AML patients in which the resistant phenotype not was associated with *dCK* mutations, but with the expression of alternative splice variants in addition to the wild type *dCK* (26). The alternatively spliced transcripts, in which one or more exons were deleted, were shown to code for enzymatic inactive proteins *in vitro* (27). In our study we did not amplify aberrantly spliced *dCK* variants.

Based on our own results in a relatively large panel of paired clinical samples and reports from the literature we conclude, that mutations in the *dCK* gene are scarce and not of major importance for Ara-C resistance in the development to resistant and/or refractory disease in AML.

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CHAPTER 7

In vitro effect of GF120918, a novel reversal agent of multidrug resistance, on acute leukemia and multiple myeloma cells

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Abstract

Resistance to chemotherapy in multiple myeloma (MM) and acute myeloid leukemia (AML) is frequently caused by multidrug resistance (MDR), characterized by a decreased intracellular drug accumulation. MDR is associated with expression of P-glycoprotein (P-gp). GF120918, an acridine derivative, enhances doxorubicin cell kill in resistant cell lines. In this study, the effect of GF120918 on MDR cell lines and fresh human leukemia and myeloma cells was investigated. The reduced net intracellular rhodamine 123 (Rho 123) accumulation in the MDR cell lines RPMI 8226/Dox1, /Dox4, /Dox6 and /Dox40 as compared with wild-type 8226/S was reversed by GF120918 (0.5 - 1.0 μM), and complete inhibition of rhodamine efflux was achieved at 1 - 2 μM. This effect could be maintained in drug-free medium for at least 5 hours (h). GF120918 reversal activity was significantly reduced with a maximum of 70 % in cells incubated with up to 100 % serum. GF120918 significantly augmented Rho 123 accumulation *in vitro* in CD34- positive AML blasts and CD38-positive multipel myeloma (MM) plasma cells obtained from 11/27 *de novo* AML and 2/12 refractory MM patients. A significant correlation was observed between a high P-gp expression and GF120918 induced Rho 123 reversal (P=0.0001). Using a MRK 16/IgG2a ratio ≥ 1.1, samples could be identified with a high probability of GF 120918 reversal of Rho 123 accumulation. In conclusion, GF120918 is a promising MDR reversal agent which is active at clinically achievable serum concentrations.

Introduction

Resistance to chemotherapy in multiple myeloma (MM) and acute myeloid leukemia (AML) is frequently caused by multidrug resistance (MDR), which is associated with expression of P-glycoprotein (P-gp), a 170 kDa ATP-dependent membrane protein encoded by the *MDR*1 gene (1-6) and the multidrug resistance-related protein (MRP1). Increased expression of MDR1 mRNA or P-gp has been observed in hematological malignancies, including untreated and refractory AML and refractory MM (1,2,4-9). Expression of P-gp by malignant cells is associated with a decreased intracellular accumulation of anthracyclines and vinca alkaloids, which is mediated through an enhanced transmembrane efflux (1,10-15). In AML, P-gp expression is associated with a lower probability to achieve a complete remission rate (8,11,15-19). Several structurally unrelated, noncytotoxic agents, such as verapamil and cyclosporine A (CsA) interfere with P-gp mediated efflux of cytostatic drugs *in vitro* (20). Some of these MDR reversal agents act by inhibition of the transmembrane drug transport through competitive binding to the active site of P-gp (21). *In vitro* studies have shown that

CsA and verapamil may completely restore the intracellular accumulation of daunorubicin and/or doxorubicin in resistant cell lines and in fresh AML and MM specimens (12,13,19,22-24). Recent clinical trials with verapamil and CsA added to standard chemotherapy suggest, that MDR reversal *in vivo* may represent a potential approach to treat refractory patients with MM and AML (25,26). Prospective phase III studies have now started in order to determine the therapeutic value of such an approach. GF120918 (N-{4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-iso-quinolinyl)-ethyl]-phenyl}-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide) is an acridine carboxamide analogue, which inhibits the transmembrane transport function of P-gp. In contrast to several other modulating agents, the *in vitro* concentrations of GF120918 that are required to block P-gp mediated efflux, can be achieved *in vivo* in animals (27). This property makes GF120918 a potential candidate for clinical trials to modulate multidrug resistant cancers. Based on these *in vitro* data, we undertook a study to clarify the conditions required for P-gp inhibition in cell lines. In addition, we examined the effect of GF120918 in isolated tumor cells obtained from patients with MM and AML, in order to establish a minimum effective concentration required for clinical modulation of P-glycoprotein with GF120918.

Materials and methods

Cell lines

For studies of MDR modulation, the sensitive multiple myeloma cell line, RPMI 8226/S (8226/S) and its multidrug resistant derivative lines 8226/Dox 1 (Dox1), 8226/Dox 4 (Dox4), 8226/Dox 6 (Dox6) and 8226/Dox 40 (Dox40) were used, which were kindly provided by WS Dalton (University of Arizona, Tucson, AZ, USA). 8226/Dox1, 8226/Dox4, 8226/Dox6 and 8226/Dox40 are cell lines of increasing resistance to doxorubicin and increasing levels of P-gp, which have been selected through doxorubicin (DOX) exposure. These lines are also cross-resistant to mitoxantrone, vincristine and etoposide (28). 8226/Dox1, 8226/Dox4, 8226/Dox6 and 8226/Dox40 were cultured in the presence of 10, 40, 60 and 400 nM DOX, respectively. DOX was dissolved in HBSS at 1 mg/ml, filtered sterile and stored as aliquots at -20°C. Dilutions were made immediately before use.

All cell lines were cultured in DMEM (Gibco, Paisley, UK) with 20 mM Hepes (Gibco), 10 % fetal calf serum (inactivated), 50 μg gentamycin/ml (Gibco) and 10 μg ciprofloxacin, a non-P-gp transported quinolone (Bayer, Mijdrecht, the Netherlands) in 175 cm^2 flasks (Falcon; Becton Dickinson, Mountain View, CA, USA) at 37°C in a fully humidified atmosphere comprising 5 % CO_2 in air. Medium was changed twice a week. One day before

the experiments, the cell medium was changed for medium without cytostatics.
MDR1/P-gp expression was examined by two different assays, *i.e.* firstly, P-gp expression was determined by MRK 16 monoclonal antibody (Moab) staining in fresh, unfixed cells by flow cytometry and secondly, the intracellular rhodamine 123 (Rho 123) retention ratio was determined by measuring the ratio of Rho 123 in the presence/absence of GF120918 (29,30). A RT-PCR of *MDR1* and *MRP*1 mRNA was performed in all cell lines, in order to exclude any interference of GF120918 with MRP1 (multidrug-resistance related protein), which is also an active membrane transporter of doxorubicin.

*MDR*1 *expression*
RNA was isolated by Trisolv extraction (Biotecx, Houston, TX, USA). Subsequently, RT-PCR was performed for analysis of *MDR*1 and *MRP* mRNA expression in all cell lines, in 17/27 AML and in 8/12 MM samples, using reverse transcriptase (Gibco/BRL) and Taq polymerase (Promega, Madison, WI, USA) for 30 cycles 1 min, 90 °C, 2 min, 60°C, 1 min 72°C in a Hybaid thermocycler (Omnigen/Biozym, Landgraaf, The Netherlands). The *MDR*1 and *MRP*1 primer sets were according to Futscher *et al.* (31). The presence of the PCR products was visualized by gel electrophoresis.

P-gp expression
Bone marrow specimens and cell lines were washed in buffer (PBS supplemented with 0.2% rabbit serum (Gibco), 0.02% goat serum (Gibco), 0.2% bovine serum albumin (BSA) and 2 mM sodium azide). Sera were inactivated for 30 min at 56°C. The samples were centrifuged for 5 min at 650 *g* and the pellet was resuspended with 25 µl (2.5 µg) of the monoclonal antibody MRK 16 (Hoechst, Amsterdam, The Netherlands). The cells were incubated at room temperature for 60 min. The same amount of irrelevant IgG2a was added to the control tubes. After washing, 50 µl 1:20 (vol/vol) diluted fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse Ig antiserum (Dakopatts, Glostrup, Denmark) was added and cells were incubated for 60 min at 0°C. The cells were washed with washing buffer and 10 µl of normal mouse serum (1:100) was added. In addition, in clinical specimens 10 µl of phycoerythrin (PE)-conjugated anti-CD34 (AML specimens) or CD38-PE (MM specimens) (Becton Dickinson) was added to the cells. To the control tubes 10 µl of IgG1-PE was added. The tubes were incubated at 0°C for 45 min, then washed with washing buffer and resuspended in 0.5 ml PBS containing sodium azide. Fluorescence was analyzed by flow cytometry using a FACScan (Becton Dickinson). The ratio between the MRK 16 and IgG2a

fluorescence was calculated, as this accounts for a measure of P-gp positivity (32).

Rhodamin 123 assay

To establish a dose-effect curve, GF120918 (Glaxo, Research Triangle Park, NC, USA) was prepared as a 2.5 mM solution in methanol/HCl 0.1 N (4:1) and stored as aliquots at -80°C. Dilutions were made in medium immediately before use. Rho 123 (Eastman Kodak, New York, USA) was kept as a stock solution of 2 mg/ml in ethanol 99 % at 4°C in the dark. Immediately before use, a fresh working solution was made by diluting the stock 1 : 100 in phosphate buffered saline (PBS). After suspending the cells at $1x10^6$/ml in DMEM containing 10 % FCS, GF120918 was added at various concentrations and Rho 123 at a final concentration of 200 ng/ml. The cell lines were incubated for 120 min, followed by washing, after which the suspensions were incubated for 120 min under the same conditions, yet omitting Rho 123. Samples were taken and immediately transferred to FACS tubes containing 2 ml PBS/sodium azide. The tubes were centrifuged for 5 min at 650 *g*, PBS was decanted and 0.5 ml fresh PBS containing 2 mM sodium azide was added. Intracellular 123 accumulation was measured using FACScan (Becton and Dickinson). The accumulation of Rho 123 was calculated as a function of GF120918 concentration. For comparison, the procedures described above were performed with cyclosporine A (Sandoz, Basel, Switzerland), which was kept as a 40 mM stock at 4°C.

The duration of the GF120918 effect was studied by adding Rho 123 at 200 ng/ml either with or without GF120918 (2 μM) to 8226/Dox40 or 8226/S in medium. Samples were taken at several times, starting at 0 min. After 120 min, the cells were washed with medium and Rho 123 was added. Then GF120918 was added at the same concentration and in control tubes medium only was added. Incubation continued for 5 h and samples were taken at several times. All samples were immediately added to FACS tubes containing 2 ml of PBS/azide. All samples were washed at 650 *g* for 5 min and resuspended in 0.5 ml PBS/azide. Intracellular Rho 123 accumulation was measured using a FACScan.

Effect of Protein binding

To determine the effect of protein binding on GF120918, the 8226/Dox40 cell line was incubated in PBS containing 4.5 g glucose/l, GF120918 (1.0 μM), Rho 123 (200 ng/ml) and 0, 0.10, 0.25, 0.50, 1.0, 5.0, 10.0, 25.0, 50.0, 75.0, or 100 % fetal calf serum (FCS). After incubation for one hour, the cells were washed with PBS, centrifuged at 650 *g* for 5 min and resuspended. The incubation continued in the above described suspension, omitting Rho 123.

After a 90 min incubation, 2 ml PBS/2 mM azide was added, cells were washed, centrifuged at 650 *g* for 10 min and resuspended in 0.5 ml PBS/azide. The intracellular Rho 123 accumulation was measured using the FACScan.

Patient tumor specimens

After informed consent, bone marrow aspirates from 27 patients with acute myeloid leukemia and 12 patients with multiple myeloma were obtained. Twentyfive of 27 AML patients had untreated, *de novo* AML. Two patients were sampled at the time of relapse after prior treatment with daunorubicin/cytarabine. Twelve MM patients were studied, *i.e.* 7 with VAD-refractory disease and 5 with untreated myeloma. Leukocytes were separated by density grade centrifugation using Lymphoprep (Nycomed Pharma, Oslo, Norway) and the remaining blasts or plasma cells were washed with Hank's Balanced Salt Solution (HBSS; Gibco). The cells were cryopreserved in freezing mixture (10% DMSO, 10% FCS in HBSS) using a temperature controlled freezer (Kryo 10; Planer Biomed, UK) and were subsequently stored in liquid nitrogen. Immediately before use, the material was thawed in water and then put on ice. The contents of the ampuls were transferred to a 50 ml Falcon tube and ice-cold HBSS was added every minute to 9 minutes in an increasing volume, till a dilution of 1:9 was reached. This suspension was centrifuged (5 min 650 *g*) and the pellet was resuspended in DMEM containing 10 % FCS. Viability was checked with Trypan Blue. No difference of P-gp expression or efflux was observed between fresh and cryopreserved AML/MM cells (12).

Accumulation studies in clinical specimens

The effect of GF120918 on P-gp blockade in human tumor cells was investigated in cryopreserved, purified tumor cells which had a viability >95 % as checked by Trypan Blue. Cells were washed at 650 *g* for 5 min and resuspended in DMEM containing 10% FCS to concentrations of $5x10^6$/ml. The suspension was transferred to 15 ml Falcon tubes and incubated at 37°C, 5% CO_2 for 1 hour, either with or without GF120918. In this experiment, concentrations were used that were derived from a concentration-effect curve of GF120918 determined in the 8226/Dox lines. After 1 hour, Rho 123 was added at a final concentration of 200 ng/ml and a 2 hour incubation followed. Samples were taken and immediately transferred to 12 x 75 mm FACS tubes containing 2 ml ice chilled phosphate buffered saline (PBS) containing 2 mM azide and 0.1% BSA. Intracellular Rho 123 was measured using the FACScan.

FACScan analysis

Analysis of P-gp expiation in clinical samples was performed by staining with MRK 16 in fresh cells using flow cytometry (FACScan; Becton Dickinson). All analyses were done in tumor cells by selecting those cells with a lineage specific epitope, *i.e.* CD34 in AML cases and CD38 in myeloma cases. Excitation was done at 488 nm and fluorescence was measured at 515-545 nm (green fluorescence: MRK 16-FITC and Rho 123) or 563-607 nm (red fluorescence: CD34-PE and CD38-PE). Both FITC and PE fluorescence signals were logarithmically amplified. Background autofluorescence was determined by measuring the fluorescence of cells not exposed to Rho 123 and subtracted from the signal (33). Each analysis was performed on 5000 cells. Rho 123 accumulation was determined by taking the mean channel fluorescence of the CD38+ plasma cells (MM) or CD34+ myeloblasts (AML) using the analysis program PCLysis (Becton Dickinson). In order to prevent background fluorescence of PE in the Rho 123 fluorescence, the settings of the signal amplifiers was adapted for each sample individually, as described before (12). Analysis of MRK 16 fluorescence and Rho 123 fluorescence in control cell lines was performed in unselected cells, with the same settings of the flow cytometer, such that 8226/S and 8226/Dox40 cells were used to calibrate the amplifier.

The data presented are based on three different experiments performed at different occasions, of which the median value is given.

Results

RT-PCR

*MDR*1 mRNA was present in the RPMI/8226 Dox40 cell line, but not in the parental cell line 8226/S (Figure 1). No *MRP*1 mRNA levels above background were found in the 8226/Dox40 as compared with the sensitive parental cell line, indicating that MRP expression was not increased in these resistant cells (results not shown).

Dose-effect

The effect of different concentrations of GF120918 on Rho 123 accumulation in cell lines is shown in Figure 2. The concentration of GF120918 required for restoration of intracellular Rho 123 fluorescence was different between the cell lines depending on the degree of resistance. In Table 1, the concentrations of GF120918 that induced a plateau of the intracellular Rho 123 retention are presented. The results are compared with equimolar

concentrations of cyclosporine A. In all resistant cell lines, the effect of GF120918 reaches a plateau at 2 μM, showing no additional increase even in highly resistant cells up to 10 μM of GF120198.

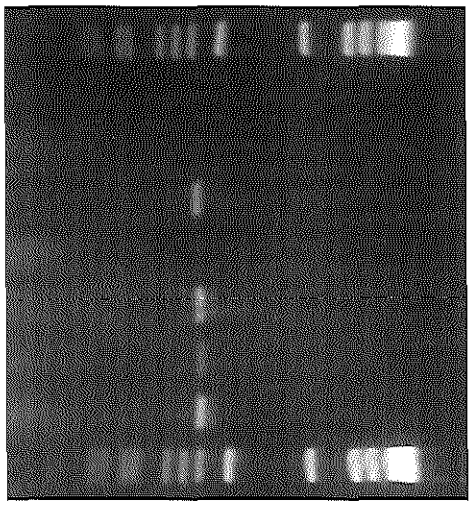

Figure 1. RT-PCR gel of MDR1 of MDR cell lines. The lanes were loaded with 10 μl of the reaction mix. RPMI8226/S, Dox40 and KB 3-1, 8, 8-5 and V-1 were loaded in lane 2 to 7 respectively. In lane 1 primer mix without template was loaded.

Table 1. Modifyer activity of GF 120918 compared with cyclosporine A

Cell line	Intracellular Rho 123 fluorescence (AU)	Concentration of GF 120918 (μm) for maximum modulation of Rho 123 retention	Concentration of cyclosporine A (μm) for maximum modulation of Rho 123 retention
8226/S	1108	no extra retention	no extra retention
8226/Dox40	16	1	> 5

AU, arbitrary units

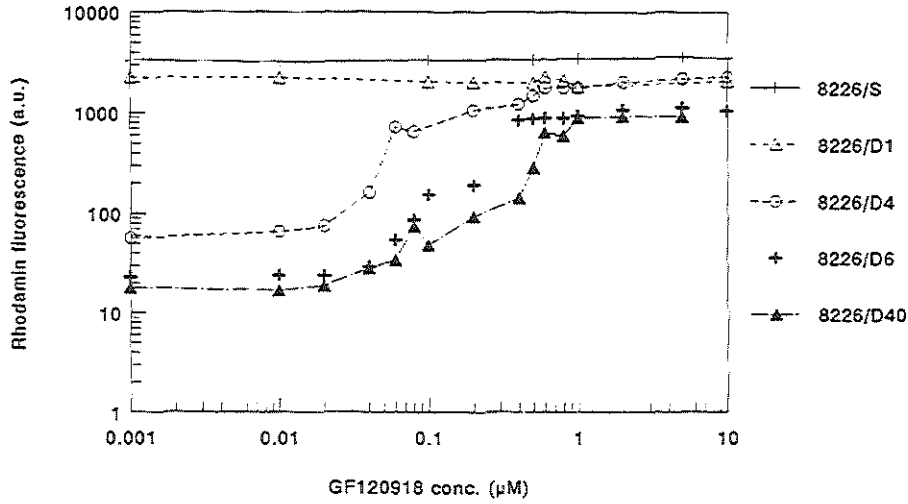

Figure 2. Dose-effect curve of GF120918 in 8226/S and Dox resistant cell lines. Rho 123 fluorescence after two hours of incubation and with different concentrations of GF120918 in the presence of 10 % FCS.

Accumulation/efflux curve

8226/Dox40 cells which were incubated with GF120918 during the entire period of 300 min accumulated Rho 123 in time. In separate samples, the cells were incubated with GF120918 for 120 min, after which it was omitted. In these cells, Rho 123 accumulation remained constant during 180 min after GF120918 was omitted. Cells incubated without GF120918 showed no significant accumulation of Rho 123. However, when GF120918 was added to these cells after 120 min, Rho 123 fluorescence increased, reaching the same plateau of cells incubated with GF120918 during the whole period of time (Figure 3).

Influence of protein binding on GF120918

After incubation of the 8226/Dox40 cell line in PBS containing 0 - 100% FCS with 2 µM GF120918, the effect of protein on the intracellular Rho 123 accumulation was analyzed. P - gp reversal by GF120918 was reduced by 42% in the presence of 1% FCS. Higher pro tein concentrations further reduced GF120918 activity by a maximum of 70% at 100% FCS (Figure 4).

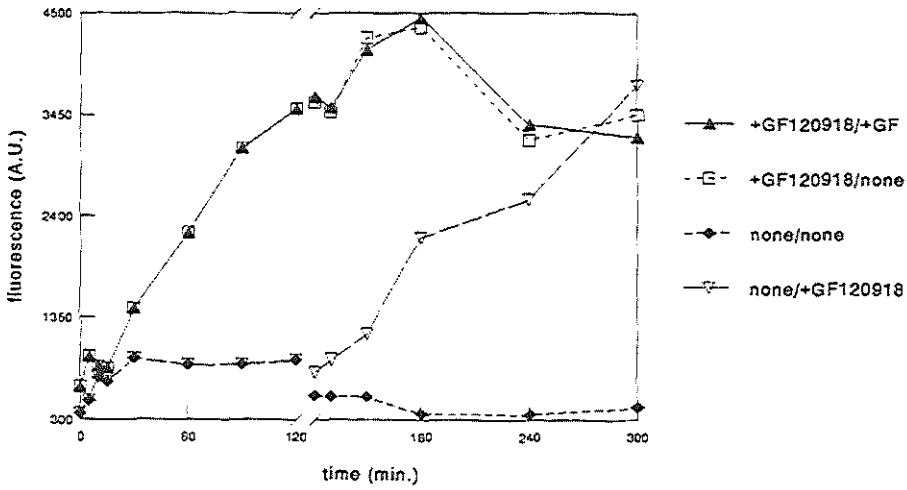

Figure 3. Accumulation/efflux curve of Rho 123 cell line 8226/Dox40 was incubated with 2 μM GF120918 and samples were taken at several times. Four different procedures were followed: incubation with GF120918 during the entire period (▲), incubation with GF120918 until t = 120 min, omission hereafter (□), incubation without GF120918 constantly (◆) and incubation without GF120918 until t = 120 min, hereafter with the drug (▽).

Patients

Twenty-seven cases of AML (25 *de novo*, 2 relapse AML) and 12 cases of MM (7 VAD refractory, 5 untreated) were analyzed. MRK 16 was determined in the live-gated sub-populations of tumor cells (plasma cells in MM samples; myelo blasts in AML samples) using the FACScan flowcytometer. Fluorescence was expressed as the mean peak fluorescence. In these tumor cells a significant relation between MRK 16 expression and the reversal of Rho 123 accumulation by GF120918 is observed (Figure 5). The level of P-gp expression, as quantified by the MRK 16 peak channel fluorescence correlated with the modulation effect by GF120918 in patient samples (Spearman correlation coefficient, 0.87, with a two-sided P value <0.0001). In 23/27 patients with AML and 6 of 12 with MM, the MRK 16/IgG2a ratio was greater than 1.1 which is consistent with increased P-gp expression as compared with values observed in normal blood cells. Seven VAD-refractory MM patients had a high MRK 16/IgG2a ratio, associated with P-gp expression on plasma cells.

MRP1 expression above background levels was detected in 11/17 tested AML and in 0/8 tested MM samples. Nine of 11 MRP1-positive AML samples were also MRK 16 positive

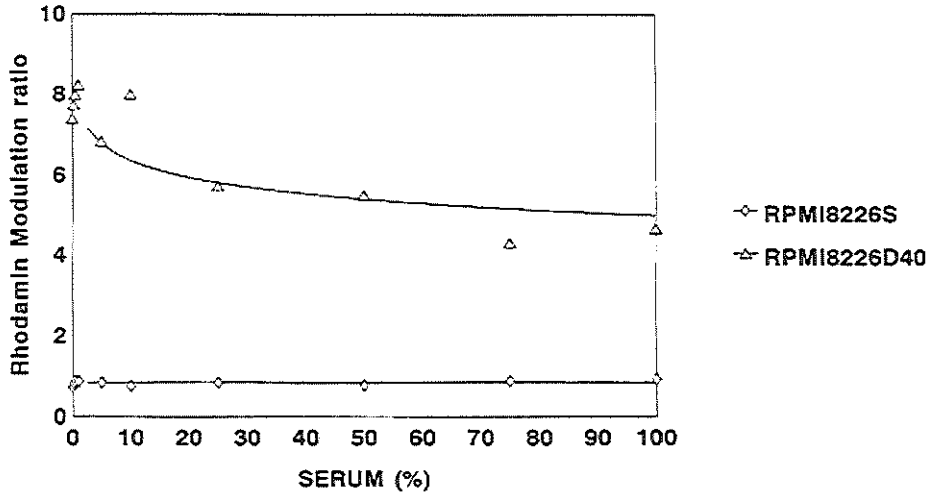

Figure 4. Effect of serum addition on GF120918 efficacy. The Rho 123 accumulation was determined after one hour incubation with 1.0 μM GF120918 in PBS/glucose containing Rho 123 and increasing final concentrations of fetal calf serum (FCS). The ratios of the fluorescence with/without GF120918 were calculated (modulation ratio).

(see below). Using the Spearman non-parametric test, no significant correlation was detected between MRP and MRK 16 positivity. These clinical samples were analyzed to determine the GF120918 reversal ratio in those which had a MRK 16/IgG2a ratio ≥ 1.1 (P-gp positive, mean ratio: 1.72, n=29) as compared with the P-gp negative samples (MRK 16/IgG2 ratio < 1.1, mean ratio: 0.94, n=8). These two groups had a significantly different reversal effect by GF120918, as determined by Fisher exact test (two-sided P=0.005; odds ratio 10.57, with 95 % confidence interval 1.9-58.5). Reversal of modulation of Rho 123 accumulation by GF120918 was significantly higher in the P-gp positive group as compared with the P-gp negative group (M ± s.e.m: 3.1 ± 0.7 *vs* 1.1 ± 0.1, P = 0.02, two-sided Student's t- test). However, in 12/27 AML cases, as opposed to 2 of 12 myeloma cases significant reversal was observed. This implies that reversal of the intracellular Rho 123 accumulation by GF120918 is restricted to MRK 16 positive tumor cells.

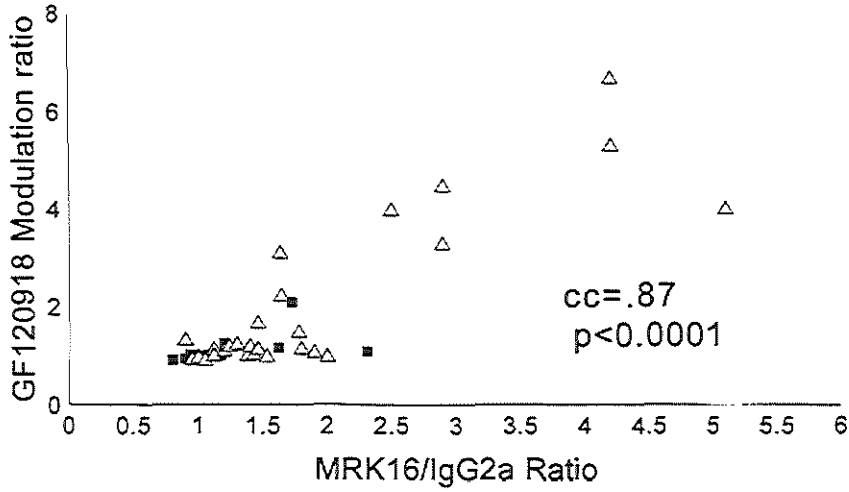

Figure 5. MRK 16/IgG2a ratio and Rho 123 modulation by GF120918: The MRK 16/IgG2a ratio in CD34+ blasts (AML, Δ) and CD38+ plasma cells (MM, ■) is compared with the rhodamine modulation ratio by GF120918 in these cells.

Discussion

MDR1 is a significant prognostic factor in untreated and relapse AML and in VAD-refractory MM (8,11,14,15,24-26). Phase II studies have shown that reversal of P-gp transmembrane transport function by so-called MDR modulating agents is feasible (24,26,34,35). *In vitro* studies indicate that a complete restoration of the intracellular accumulation of anthracyclines and vinca alkaloids is possible (9,12,13,19,23,24). Many agents that are capable of restoring sensitivity to MDR-type cytostatic drugs, lack specificity and therefore they cannot be used for clinical modulation of drug resistance.

GF120198 is a novel MDR modulating agent, which is active in several solid tumor cell lines (27). In this study, we evaluated its effect in two hematological malignancies, *i.e. de novo* AML and refractory MM, which frequently have P-gp over expression.

From experiments investigating the effect of protein binding on the GF120918 efficacy, it appears that increasing concentrations of protein in the incubation medium substantially reduce the reversal of rhodamin efflux with a maximum of 70% in full serum. In the present studies experiments were always performed in the presence of 10% fetal calf serum (FCS). Although at this serum concentration absolute inhibition of GF120918 activity is observed to

some extent, the ratio between GF120918 treated and control cells only diminishes from serum concentrations of 25% and over. However, these observations imply that the MDR1 reversal by GF120918 *in vivo* will be substantially affected by protein binding, and that dosing strategy of future clinical trials has to take into account the effect of protein binding of the drug.

In the sensitive cell line 8226/S and resistant Dox1, Dox4, Dox6 and Dox40, a dose-effect curve of GF120918 was established using Rho 123 as fluorescent probe for P-gp. The effect of GF120918 increased as the dose increased, until a plateau was reached at 1 μM. However, the level to which the intracellular Rho 123 accumulation could be restored, was different between cell lines of increasing resistance. Assuming a 50% reduction of the GF120918 effect *in vivo* by serum protein binding, it would therefore be appropriate to achieve a blood concentration greater than 1-2 μM GF120918 in order to attain an optimal reversal in clinical studies.

In this study we attempted to evaluate if GF120918 is capable of reversing MDR1 in tumor cells from patients with acute leukemia and multiple myeloma, which may also express alternative mechanisms of drug resistance besides MDR1. In these specimens, we attempted to analyze the effect of GF120918 in specific subsets of cells, *i.e.*, those with lineage specific markers such as CD34 (acute myeloid leukemia) and CD38 (multiple myeloma), thereby reducing the likelihood of analyzing non-malignant bone marrow cells. In both tumors, there was a correlation between the MRK 16/IgG2a ratio of the tumor cells with the Rho 123 modulation by GF120918. Based on the MRK 16/IgG2a ratio, two groups could be identified with a high *vs* low probability that GF120918 reverses Rho 123 accumulation. Thus, a MRK 16/IgG2a ratio greater than 1.1 could potentially be used to identify patients who could benefit from a GF120918 reversal effect. Recently, MRP1 has been designated as an alternative drug-efflux pump in refractory tumor cells. Also in our study, several clinical AML samples expressed MRP1 alone or both MRP1 and MDR1. No correlation between MRP1 and MDR1 expression was observed. However, the number of samples is limited, and does not allow conclusions about a possible effect of GF120918 on MRP1 mediated efflux.

From the experiments performed in cell lines we conclude that after saturation with 1-2 μM of GF120918, the inhibitory activity lasts for at least 5 hours in 8226/Dox40. These data also suggest that in clinical trials of GF120918 a dosing schedule of three to four times daily, resulting in trough blood level of 1-2 μM could be appropriate.

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CHAPTER 8

General discussion

General discussion

The major cause for treatment failure in acute myeloid leukemia (AML) is pre-existent or acquired resistance to chemotherapy. In the past two decades, clinical resistance to chemotherapy has been found to be associated with the expression of (membrane) transport associated drug resistance proteins like P-glycoprotein (P-gp), encoded by the multidrug resistance (*MDR*1) gene, multidrug resistance related protein (MRP1), lung resistance related protein (LRP) or major vault protein (MVP). Recently, the *novel* drug resistance protein 'breast cancer resistance protein' (BCRP) has been described. The results of our and several other studies that reported about the correlation between clinical resistance and the expression of these resistance proteins induce some arguments. First of all, this study underscores the value of studies in paired purified clinical samples of AML patients at diagnosis and relapse. One of our studies showed that comparing paired samples gave an insight in the clonal evolution of AML towards resistant or refractory disease, which was found not to be *MDR*1 gene related and revealed no P-glycoprotein upregulation at time of relapse as compared to diagnosis. In contrast, in previous reports, MDR1 expression was suggested to be upregulated at time of relapse or refractory disease in some studies of non-paired AML samples, and in cell lines studies, in which multidrug resistance phenotypes are upregulated after exposure to chemotherapeutic agents. The question remains what mechanisms are responsible for the higher clinical resistance levels of relapsed and refractory AML. Further studies described in this thesis showed that MRP1 and LRP/MVP were not upregulated at RNA level in relapsed AML as compared to diagnosis, again in paired analyses. Considering the fact that Ara-C in the most powerful drug in AML, we also investigated the *dCK* gene which encodes deoxycytidine kinase (dCK), the rate limiting enzyme in the metabolism of Ara-C. No mutations in this gene were found during the clonal expansion of AML cells to resistant disease, suggesting that *dCK* mutations do not play the important role in relapsed or refractory AML as has been suggested in previous reports.

However, we found that *BCRP* was upregulated in AML patients at time of relapse/refractory disease. Until now, it was not clear what role BCRP might play in clinical resistance in AML. Results of studies investigating the prognostic value of BCRP in AML are not available as yet. The upregulation of BCRP mRNA in our study was not correlated with an increase of any of the other resistance proteins at time of relapse/refractory disease. Interestingly, Leith *et al.* described a distinct subgroup which demonstrated cyclosporine resistant efflux that was not correlated with MDR1, MRP1 or

LRP/MVP expression, in a large study of adult AML patients. They suggested that the existence of another as yet undefined efflux mechanism might exist in adult AML. BCRP is a candidate protein, that might contribute to this resistance phenotype. The *BCRP* gene is evolutionary distinct from the families that encode P-gp and MRP1, being on a completely separate limb of the phylogenetic tree. In contrast to the *MDR*1 and *MRP*1 gene it encodes a protein which is a half-transporter molecule requiring homo-dimerization in order to function. These facts might suggest a unique and probably complementary role for BCRP among the other resistance proteins. The fact that BCRP in normal tissue is quite distinctly expressed from P-gp and MRP1 might underscore this.
Although P-gp expression in *de novo* AML is accepted as a poor prognostic factor for complete remission (CR) and survival in adults, it is still not clear whether this is also true for childhood AML. Studies of MDR1 but also MRP1, LRP/MVP and BCRP in large cohorts of children are not available as yet. Theoretically, differences in prognosis of childhood AML as compared to adults might be related to the differences in expression of (one of) the drug resistance proteins. Studies in the elderly age group have already pointed out the relationship between age and MDR1 expression. The increase in P-gp expression per decade of age is related to the decrease in prognosis, independently from other prognostic factors. At the moment the biologic mechanism which is responsible for the correlation between age and drug resistance is not fully understood. Comparing children and adults, the difference in incidence of certain cytogenetic and immunophenotypic features might partly explain the difference in outcome, in addition to the differences in treatment protocols, performance state, tolerance, combination with other diseases, cellular resistance and pharmacokinetics in the different age groups.
As the *MDR*1 gene is located on chromosome 7q21.1, not far from the breakpoints of the chromosome in several patients with 7q21, we hypothesized that MDR1 expression played a role in the poor prognostic subgroup of AML patients with partial or complete loss of chromosome 7. However, no increase in P-gp expression was found in this group of patients. On the other hand, interestingly, in monosomy 7 patients we did not find a downregulation of *MDR*1 if one allele was lost. This finding suggests an upregulation of the other remaining *MDR*1 homologue. This is consistent with the fact that the CpG islands of the promoter region of the *MDR*1 gene were all unmethylated. Until now, it is not known what mechanism is responsible for the poor prognosis in patients with a deletion of chromosome 7.

In *de novo* AML patients, our study confirmed the report of Ross *et al.* who suggested co-expression of MDR1 and BCRP. This finding needs confirmation in larger cohorts of AML patients. However, co-expression may well account for the rather disappointing results of clinical MDR1 specific modifier studies that have been reported so far, which follow the promising *in vitro* modifier studies performed in the past. Clinical studies with modifying agents like GF 120918 might be important for the future, because of the blocking capacity of both ABC transporter proteins. More studies are needed to identify the specificity, substrate identity, inhibitors, and (co-) expression of the several drug efflux pumps causing multidrug resistance in AML before we can fully explore the potential of transporter-specific modulators to improve clinical outcome. Also, it is important to point out that, apart from membrane transport related drug resistance proteins, other drug resistance mechanisms for cytostatic drugs like cytosine-arabinoside, thiopurines, asparaginase are important for the full understanding of clinical drug resistance in acute myeloid leukemia.

Summary

Acute myeloid leukemia (AML) is a clonal disease in which immature hematopoietic cells in the bone marrow do not mature to normal blood cells. Accumulation of these cells replaces normal hematopoiesis which causes anemia, thrombocytopenia and increased risk of infection. Without treatment AML is a fatal disorder.

Treatment of AML consists of cytostatic or chemotherapeutic agents, often followed by bone marrow transplantation. After treatment with chemotherapy, bone marrow evaluation in most patients does not reveal leukemic cells anymore. This is called complete remission (CR). Even though in most patients a recurrence or relapse of the disease occurs. Relapsed AML is difficult to cure with chemotherapy. In the last decades, AML has shown to be a heterogeneous disease. A number of subgroups of AML have been identified which reflect prognostic subgroups. Characterization of these subgroups is mainly determined by age and cytogenetic abnormalities of the leukemic blasts.

Treatment failure in AML is associated with the presence or development of resistance to several chemotherapeutic agents. This phenomenon is called multidrug resistance (MDR). Several mechanisms of multidrug resistance have been investigated in the past. This thesis describes studies involving the clinical relevance of multidrug resistance in patients with AML at diagnosis and relapse.

In chapter 1 the drug resistance proteins that have been investigated in this thesis are discussed. P-glycoprotein (P-gp) is the protein which is encoded by the *MDR*1 gene, which is localized on chromosome 7q21.1. This is usually called 'classical' MDR. P-gp is a transmembrane protein which transports drugs outside the cells, resulting in a diminished accumulation of chemotherapy in leukemic cells. Two other transmembrane multidrug resistance proteins are the multidrug resistance-related protein (MRP1), encoded by the *MRP*1 gene on chromosome 16p13.1 and the recently described breast cancer resistance protein (BCRP) encoded by the *BCRP* gene on chromosome 4q22. In contrast, the lung resistance related protein (LRP), which gene is localized on 16p13.2, is an intracellular protein of which the exact structure and function are not known as yet. In chapter 1 the prognostic value of these drug resistance proteins is discussed.

One of the most powerful drugs in the treatment of AML is cytosine-arabinoside (Ara-C). Ara-C is an effective drug which inhibits DNA replication during the S-phase of the cell cycle. Resistance to Ara-C is not regulated by multidrug resistance proteins. Mechanisms of Ara-C resistance in AML are discussed in chapter 1.

In chapter 2 a study of the prognostic value of the expression of P-glycoprotein (P-gp) in leukemic blasts in bone marrow smears in *de novo* AML is presented. Expression of P-gp

has shown to be an independent prognostic factor for complete remission (CR) and long term survival in a multivariate analysis.

In the next studies we investigated MDR1 and the other resistance proteins in paired analyses of AML patients as their disease evolved from diagnosis to refractory/relapsed disease. From cell line studies it is known that exposure of AML blasts to chemotherapy induces MDR1 expression. Translated to clinical settings it was suggested that at relapse a more resistant clone is selected, associated with a higher expression of multidrug resistance proteins. However, in a paired analysis of diagnostic and relapse samples of AML patients we did not find *MDR*1 gene related clonal selection (chapter 3). Expression studies, using specific antibodies for P-gp and functional assays, using a rhodamin retention assay with or without a P-gp specific modulator confirmed this. In consecutive studies we analyzed the role of the other drug resistance genes in relapsed AML. We showed that the expression of MRP1 and MDR1 is not different at relapse as compared to diagnosis. The expression of LRP even decreases at relapse. Only BCRP expression was expressed at a higher level at relapse as compared to diagnosis (chapter 4). At diagnosis a co-expression between MDR1 and BCRP was found, but not between any of the other genes involved. This co-expression may be important in the future for further studies of modifyers that do not only block P-gp but also BCRP, like the compound GF 120918, which is described in chapter 7.

The cytostatic drug Ara-C is being phosphorylated in the cell to Ara-CTP. This Ara-CTP competes with the incorporation of the natural counterparts, the nucleotides, in the DNA. Incorporation of Ara-CTP inhibits DNA replication. The rate limiting step in the formation of Ara-CTP is the phosphorylation of Ara-C to Ara-CMP which is catalyzed by deoxycytidine kinase (dCK). As in the past some reports have been made on the correlation between mutations in the *dCK* gene and decreasing dCK activity, we investigated the role of *dCK* mutations in relapsed AML. In chapter 6, we describe that this mutation was not found in our patient. We conclude that *dCK* gene mutations do not seem to play a major role in Ara-C resistance in AML.

One of the subgroups of AML which has a very poor prognosis is the group of patients with partial or complete monosomy 7. Because of the fact that the *MDR*1 gene is located on chromosome 7 we wondered whether the poor prognosis was associated with altered MDR1 expression in a group of –7/7q- patients as compared to a group of AML patients with normal chromosomes 7. In chapter 5 we report that the loss of chromosome 7 is random in monosomy 7 patients. For these studies we used a polymorphism of the *MDR*1

gene. P-gp expression was not elevated in the poor prognostic –7/7q- group of patients, which suggests that MDR1 expression does not play an important role in the resistance of patients with partial or complete monosomy 7.

In chapter 7 the results of this thesis are discussed. Emphasis is made on the fact that in contrast to adults AML, childhood studies on the clinical and prognostic value of drug resistance proteins are not available as yet. Also, we focussed on the potential importance of the *novel* drug resistance protein BCRP, which may contribute to clinical resistance in AML especially at time of relapse, and which may be an important target for future modulator studies in AML.

Samenvatting

Acute myeloide leukemie (AML) is een vorm van kanker waarbij onrijpe bloedvormende cellen in het beenmerg niet uitrijpen tot normaal functionerende bloedcellen en ongecontroleerd delen. Door ophoping van deze onrijpe leukemie cellen wordt de normale bloedcel-vorming verdrongen wat leidt tot bloedarmoede, verhoogde bloedingsneiging, en stoornissen in de afweer tegen infecties. Zonder behandeling is dit een fatale aandoening.

De behandeling van AML bestaat uit het toedienen van celgroei remmende middelen, ook wel cytostatica of chemotherapeutica genoemd, eventueel gevolgd door beenmerg transplantatie. Na chemotherapie worden bij het merendeel van de AML patienten geen leukemie cellen meer in het beenmerg of bloed aangetoond. Men spreekt dan van het bereiken van een complete remissie (CR). Desondanks treedt er bij het merendeel van deze patienten toch terugkeer, oftewel een recidief, van de ziekte op. Een recidief AML is met cytostatica minder goed te behandelen. Inmiddels is in de afgelopen jaren bekend geworden dat AML een heterogene ziekte is, dat wil zeggen dat er een aantal subgroepen van AML zijn te onderscheiden, die een al of niet grotere kans op overleving (betere prognose) hebben. De prognostische subgroepen worden met name bepaald door de leeftijd van de patient en de aanwezigheid van chromosomale afwijkingen in de leukemie cellen.

Het falen van chemotherapeutische behandeling kan veroorzaakt worden door het bestaan of ontwikkelen van ongevoeligheid voor verschillende vormen van cytostatica. Dit phenomeen noemen we multidrug resistentie (MDR). Verschillende mechanismen van multidrug resistentie zijn te onderscheiden. In dit proefschrift wordt beschreven wat de klinische relevantie is van multidrug resistentie in patienten met AML bij diagnose en recidief.

In hoofdstuk 1 worden de in dit proefschrift onderzochte multidrug resistentie eiwitten besproken. Het *MDR*1 gen, gelegen op chromosoom 7q21.1, codeert voor het eiwit P-glycoproteine (P-gp). Deze vorm van MDR wordt ook wel de klassieke MDR genoemd. P-gp is een eiwit in de celmembraan wat in staat is cytostatica uit de cel te transporteren, zodat deze hun werking niet optimaal kunnen doen. Twee andere transmembraan eiwitten die hier op lijken zijn het multidrug resistance-related protein (MRP1), gecodeerd door het *MRP*1 gen op chromosoom 16p13.1 en het recentelijk beschreven breast cancer resistance protein (BCRP), gecodeerd door het *BCRP* gen op chromosoom 4q22. Een ander resistentie eiwit wat zich in de cel bevindt, en waarvan de structuur en exacte

functie nog niet bekend zijn, is het lung resistance-related protein (LRP), gecodeerd door het gen op chromosoom 16p13.2. Naast de beschrijving van deze eiwitten wordt tevens de prognostische waarde van de expressie van deze eiwitten in AML patienten uiteengezet. Eén van de meest krachtige medicijnen tegen AML is cytosine arabinoside (Ara-C). De resistentie mechanismen voor Ara-C in relatie met AML worden besproken in hoofdstuk 1.

Uit ons onderzoek beschreven in hoofdstuk 2 blijkt dat de expressie van P-gp bij diagnose een onafhankelijke voorspellende waarde heeft voor het bereiken van complete remissie (CR) en lange termijn overleving bij AML patienten. We hebben ons afgevraagd wat er gebeurt met de expressie van MDR1 en de andere resistentie eiwitten op het moment dat de AML recidiveert. Vanuit laboratorium onderzoek met cellen die oorspronkelijk afkomstig zijn van kanker patienten die daarna zijn doorgekweekt (cellijnen) blijkt namelijk, dat er een verhoogde expressie van MDR1 is, naarmate er meer en/of langduriger chemotherapie aan toe gevoegd is. Vertaald naar de kliniek zou dit kunnen betekenen dat bij recidief AML patienten er een meer resistente groep cellen (kloon) uitgeselecteerd wordt die op basis van de verhoogde expressie van resistentie eiwitten minder gevoelig wordt voor chemotherapie. In een groep AML patienten die we bij diagnose én recidief (gepaarde analyse) hebben onderzocht, vonden we echter dat er geen sprake was van *MDR*1 gen gerelateerde klonale selectie (hoofdstuk 3). Onerzoek nar de expressie van het eiwit P-gp zelf en de functie hiervan bevestigden deze bevinding. Vervolgens werd onderzocht welke rol de andere resistentie genen spelen in het geval van een recidief. Hierbij werd gevonden dat *MRP*1 expressie, evenals *MDR*1 expressie niet verhoogd is bij recidief, *LRP* expressie zelfs afneemt en alleen *BCRP* expressie toeneemt bij recidief (hoofdstuk 4). Tevens werd bij diagnose een samenhang (co-expressie) in expressie gevonden tussen *MDR*1 en *BCRP* maar niet tussen de andere resistentie genen. Deze co-expressie is belangrijk omdat het de basis kan vormen voor verder onderzoek naar modulatoren die niet alleen P-gp maar ook BCRP blokkeren, zoals de stof GF120918 waarvan in hoofdstuk 7 de modulerende werking wordt beschreven.

Het cytostaticum Ara-C wordt, als het in de cel is opgenomen in een aantal fosforylatie stappen omgezet tot Ara-CTP. Dit Ara-CTP gaat een competitie aan met de natuurlijke inbouw van DNA bouwstenen, waardoor de DNA replicatie afgeremd wordt. De belangrijkste stap in de fosforylering van Ara-C tot Ara-CTP wordt geregeld door het enzym deoxycytidine kinase (dCK), wat Ara-C omzet in Ara-CMP. Omdat er in het verleden een relatie is gelegd tussen afwijkingen, mutaties in het *dCK* gen en verlaagde

dCK activiteit werd met behulp van een gepaarde analyse in beenmerg van diagnose en recidief van AML patienten, gezocht naar dergelijke mutaties. In hoofdstuk 6 wordt beschreven dat deze mutaties door ons echter niet werden aangetroffen bij diagnose en met name niet in de recidief monsters, op grond waarvan geconcludeerd wordt dat mutaties in het *dCK* gen geen belangrijke rol spelen voor Ara-C resistentie in AML.
Eén van de zeer moeilijk behandelbare subgroepen met AML is die met de chromosoom afwijkingen monosomie 7 waarbij één volledig chromosoom 7 verdwenen is, en 7q- waarbij een deel van een chromosoom 7 verdwenen is. Aangezien het *MDR*1 gen op chromosoom 7 gelegen is, was de vraag of de slechte prognose van deze patienten samenhangt met MDR1 expressie en wat er gebeurt met het *MDR*1 gen in deze patienten. In hoofdstuk 5 wordt beschreven dat het verlies van het betreffende *MDR*1 allel, gebruikmakend van een genetisch polymorphisme voor het *MDR*1 gen, willekeurig is. MDR1 expressie was niet verhoogd in de groep –7/7q- patienten in vergelijking met een controle groep AML patienten met twee chromosomen 7, hetgeen er op wijst dat MDR1 expressie in deze patienten geen belangrijke rol speelt, bij de slechte prognose.
In hoofdstuk 8 volgt een discussie van de resultaten van het onderzoek zoals dit beschreven is in dit proefschrift. Hierbij wordt met name vermeld dat in tegenstelling tot bij volwassenen met AML, over de prognostische waarde van deze resistentie eiwitten bij kinderen met AML nog bijna niets bekend is. Tevens wordt benadrukt dat het meest recent beschreven resistentie eiwit BCRP, een belangrijke bijdrage zou kunnen leveren aan klinische resistentie van AML en een belangrijk doelwit zou kunnen zijn bij interventie studies met modulatoren van multidrug resistentie.

List of frequently used abbreviations

ABC transporters	ATP-binding cassette protein.
ABCP	placental ABC transporter
ABMT	autologous bone marrow transplantation
ALL	acute lymphoid leukemia
Ala	alanine
AML	acute myeloid leukemia
ARA	Anthracycline resistance-associated protein
Ara-C	cytosine arabinoside
Ara-CMP	cytosine arabinoside monophosphate
Ara-CDP	cytosine arabinoside diphosphate
Ara-CTP	cytosine arabinoside triphosphate
ATP	adenoside triphosphate
BCRP	breast cancer resistance protein
BM	bone marrow
BMT	bone marrow transplantation
BSA	bovine serum albumin
C	cytosine
CD	cluster of differentiation/cluster of designation
cDNA	complementair DNA, copy DNA
CF	carboxyfluorescein
cMOAT	canalicular multiorganic anion transporter (=MRP2)
CR	complete remission
CsA	cyclosporin A
dCk	deoxycytidine kinase
dCTP	deoxycytidine triphosphate
DNA	deoxyribonucleine acid
DNR	daunomyscin/daunorubicin
DMSO	dimethylsulfoxide

EFS	event-free survival
FAB	French-American-British cytomorphological classification of acute leukemias
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FISH	fluorescent in situ hybridization
FTC	fumitremorgin C
G	guanine
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte macrophage colony-stimulating factor
G-v-H	graft – versus - host
G-v-L	graft – versus - leukemia
HLA	Human leucocyte antigen
Ida	idarubicin
IMDM	Iscove's modified Dubecco's medium
Il-2	interleukine-2
IV	intravenously
kDa	kilo Dalton
LRP	lung resistance-related protein
MVP	major vault protein
MDR	multidrug reisistance
MDS	myelodysplastic syndromes
MNC	mononuclear cells
Moab	monoclonal antibody
MOAT-D	MRP3
MP	monophosphate
MRD	minimal residual disease
mRNA	messenger RNA

MRP1-7	multidrug resistance related protein, subtype 1-7
MTT	methyl tetrazolium bromide
MVP	major vault protein
MXN	mitoxantrone
MXR	mitoxantrone resistance protein
NR	non-responder
P53	
P-170	P-glycoprotein
PB	peripheral blood
PBS	phosphate-buffered saline
PE	phycoerythrin
P-gp	P-glycoprotein
PkC	protein kinase C
PR	partial remission
RNA	ribonucleine acid
RT-PCR	reverse-transcriptase polymerase chain reaction
RD	refractory disease
Ser	serine
T	thymidine
TdT	terminal desoxynucleotidyl transferase
Thr	threonine
TP	triphosphate
VCR	vincristine
VP16	etoposide
U	uracil
WBC	white blood cell count

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Curriculum vitae

Marry van den Heuvel-Eibrink was born on the 22nd of April 1960 in 't Harde, a small village in the middle east of the Netherlands. She finished Atheneum B in Zwolle (Carolus Clusius College) in 1978. After she graduated medical school at the University of Utrecht (*cum laude*), she was resident internal medicine and cardiology in the Lorenz Hospital, Zeist for one year, before she started in the Sophia Children's Hospital in Rotterdam in April 1988. From September 1990 until April 1991, she had the opportunity to work in the Oncology/Hematology/BMT department in Great Ormond Street Children's Hospital in London, UK (Prof. dr. J.M Chessels), sponsored by the Erasmus exchange project. In 1993, she interrupted her clinical training for 12 months for a research project in the Laboratory of Experimental Hematology (Dr. P. Sonneveld, Prof. dr. B. Löwenberg) funded by a grant of the Dutch Cancer Society. Her pediatric training in Rotterdam (Prof. dr. H.K.A. Visser) was followed by a fellowship Oncology/Hematology in the Sophia Children's Hospital. In 1995, she was invited for a visiting fellowship in St. Jude Children's Research Hospital, Memphis, TN, USA. She was registered as Pediatric Oncologist/Hematologist in February 1998. From December 1996 until April 1999, she was *chef de clinique* in the department of pediatrics of the Sophia Children's Hospital (Prof. dr. H.A. Büller). In 1998 the project 'clonal selection in (childhood) leukemia', funded by the Sophia Foundation for Medical Research (SSWO, grant 247), the Foundation Pediatric Oncology Center Rotterdam (SKOR) and the Kröger Society, was started in the research group 'Multidrug resistance' in the laboratory of Experimental Hematology in the Erasmus University in Rotterdam (Prof. dr. P. Sonneveld). The study reported in chapter 3 was recently elected for a poster award on the Acute Leukemias meeting in München (February 2001). Since April 1999, she is staff member of the department of Oncology/Hematology in the Sophia Children's Hospital (Prof. dr. R. Pieters).

Marry van den Heuvel is an active member of the national Dutch Childhood Leukemia Study Group (DCLSG) ALL9 working group since 1994, the DCLSG/EWOG-MDS 97 study group, and the national DCLSG AML 2001 study group. She collaborates with the department of endocrinology (Dr. S.M.P.F. de Muinck Keizer-Schrama) and radiology in the local bone mineral density studies in patients with ALL (Ph.D. Thesis A.M. Boot, 1997; M. Lequin, 2000, I.M. van der Sluis, 2001) and endocrinologic late effects of therapy and disease in patients with M. Hodgkin (Ph.D. student R.D. van Beek, in collaboration with the pediatric Oncology/Hematology department in the Academic Medical Center in Amsterdam, Dr. H.M. van den Berg). She is chair of the national DCLSG study group investigating bone mineral density and avascular bone necrosis in

children with ALL. She is an active member of the American Society of Hematology (ASH), European Hematology Association (EHA) and for the Societé International Oncologique Pediatrique (SIOP) she participates in the Wilms tumor study group.
She married GJ van den Heuvel in 1982 and is the proud mother of the most wonderful children on earth: Floris (1994) and Simone (1996).

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