Thesis for doctoral degree (Ph.D.)
2008

DRUG RESISTANCE IN ACUTE MYELOID LEUKEMIA

CLINICAL AND EXPERIMENTAL STUDIES

Bertil Uggla

Karolinska Institutet

Thesis for doctoral degree (Ph.D.) 2008

DRUG RESISTANCE IN ACUTE MYELOID LEUKEMIA

Bertil Uggla

Karolinska Institutet

From THE DEPARTMENT OF MEDICINE, DIVISION OF HEMATOLOGY, HUDDINGE
Karolinska Institutet, Stockholm, Sweden

DRUG RESISTANCE IN ACUTE MYELOID LEUKEMIA

CLINICAL AND EXPERIMENTAL STUDIES

Bertil Uggla

Karolinska Institutet

Stockholm 2008

Published by Karolinska Institutet.

ISBN 978-91-7409-201-1

Printed by

REPROPRINT AB
Stockholm 2008
www.reproprint.se
Gårdsvägen 4, 169 70 Solna

For Ylva, Fredrika
and Gustaf

ABSTRACT

The long-term result of chemotherapy in acute myeloid leukemia (AML) is poor, even though a majority of AML patients initially respond to chemotherapy and achieve complete remission (CR). Initially resistant disease as well as relapse is a result of resistance to chemotherapy – drug resistance. The aim of this thesis was to further explore the relevance of three putative mechanisms of drug resistance in AML - regarding drug transport, drug target and apoptosis - as well as the immediate effect of drug exposure on the expression of three markers of drug resistance in leukemic cells *in vitro*.

Topoisomerase IIα (topo IIα) is the target of topoisomerase poisons, which are widely used in AML treatment. We investigated topo IIα at the protein level, in relation to cell cycle phases, as well as the mRNA level, in samples from patients with acute leukemia. We found that, in contrast to the situation with normal cells, topo IIα was expressed not only in the S/G2/M cell cycle phases (mean 76% topo IIα positive cells) but also in G0/G1 (mean 39% positive cells). Despite an association between low topo IIα protein expression and drug resistance *in vitro*, we found no association between topo IIα mRNA or protein expression and clinical outcome.

The recently described efflux pump breast cancer resistance protein (BCRP) was determined in a material of 40 AML patients. The majority of samples showed lower BCRP mRNA expression than that seen in a drug sensitive reference cell line, MCF-7. Overall we found no significant association between BCRP mRNA and clinical outcome; however we did find an association between BCRP mRNA levels and survival in a subgroup of patients responding to intial chemotherapy (n=28, median survival 18 months in the high-BCRP group vs 52 months in the low- BCRP group, p=0.047), suggesting a predictive value for BCRP mRNA in AML.

The initial effect of drug exposure on the expression of the efflux pumps P-glycoprotein (Pgp) and BCRP as well as on the cytosolic enzyme glutathion-S-transferase π (GSTπ) was investigated *in vitro* using HL-60 leukemic cell lines with different levels of drug resistance. After 10 min exposure to cytarabine, Pgp mRNA had increased (1.7-3.1-fold); and Pgp protein was detectable in the initially Pgp-negative HL-60 S cell line after 8 hours exposure. Since cytarabine is administered simultaneously with the Pgp-substrate daunorubicin in standard treatment of AML, this finding may be of clinical importance but needs to be further studied *in vivo*.

P53 is a key protein regarding apoptotic response to cytostatic treatment. The protein $p14^{ARF}$ stabilizes p53 via the ARF-MDM2-p53 pathway and, in theory, high levels of $p14^{ARF}$ would make the cell more prone to apoptosis. High levels of $p14^{ARF}$ mRNA have been associated with longer survival in AML. We investigated $p14^{ARF}$ mRNA expression in 57 AML patients with normal karyotype and found an association with survival. In a multivariate Cox regression analysis, the hazard ratio for death for patients with low $p14^{ARF}$ mRNA expression was 3.83 (95% CI 1.13-9.44). *In vitro*, patient samples with low expression of $p14^{ARF}$ mRNA tended to be more sensitive to the recently described compound PRIMA-1, suggesting a role for this compound for patients with altered regulation of the ARF-MDM2-p53 pathway.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to by their Roman numerals.

I. **B Uggla**, L Möllgård, E Ståhl, LL Mossberg, MG Karlsson, C Paul, U Tidefelt. Expression of topoisomerase IIalpha in the G0/G1 cell cycle phase of fresh leukemic cells. *Leukemia Research 2001;25:961-966.*

II. **B Uggla**, E Tina, H Nahi, C Paul, M Höglund, A Sirsjö, U Tidefelt. Topoisomerase IIα mRNA and protein expression vs. in vitro drug resistance and clinical outcome in acute leukaemia. *International Journal of Oncology 2007;31:153-160*

III. **B Uggla**, E Ståhl, D Wågsäter, C Paul, MG Karlsson, A Sirsjö U Tidefelt. BCRP mRNA expression v. clinical outcome in 40 adult AML patients. *Leukemia Research 2005;29:141-146.*

IV. M Prenkert, **B Uggla**, E Tina, U Tidefelt, H Strid. Rapid induction of MDR1 mRNA and protein expression by cytarabine in HL-60 cells. *Submitted*.

V. E Paul*, **B Uggla***, S Deneberg, S Bengtzen, M Hermansson, I Dahlman, KG Wiman, R Rosenquist, H Nahi. Expression of $p14^{ARF}$ in de novo AML with normal karyotype – implications on drug resistance and survival. *Submitted*.

*These authors contributed equally to the manuscript

CONTENTS

LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
ABCG2	gene encoding for BCRP
ALL	acute lymphocytic leukemia
AML	acute myeloid leukemia
ARF	alternative reading frame
ATP	adenosine 5´-triphosphate
BCRP	breast cancer resistance protein
CI	confidence interval
CML	chronic myeloid leukaemia
CpG	cytosine and guanine separated by a phosphate
CR	complete remission
dCK	deoxycytidine kinase
DFS	disease-free survival
EFS	event-free survival
ET	essential thrombocytemia
FAB	French-American-British
FISH	fluorescence *in situ* hybridization
FITC	fluorescein isothiocyanate
FLT3-ITD	fms-like tyrosine kinase 3 – internal tandem duplication
GSTπ	glutathione-s-transferase π
HDM-2	human mdm-2
IC50	50% inhibitory concentration
JNK1	c-Jun N-terminal kinase 1
mdm-2	murine double minute 2
MDR1	gene encoding for Pgp
MDS	myelodysplastic syndrome
MRC	Medical Research Council
MRP3	multidrug resistance-associated protein 3
NOD/SCID	nonobese diabetes/severe combined immunodeficiency
NPM1	nucleophosmin 1
OS	overall survival
Pgp	P-glycoprotein
PRIMA-1	p53-dependent reactivation and induction of massive apoptosis 1
RITA	reactivation of p53 and induction of tumor cell apoptosis
RPMI	Roswell Park Memorial Institute medium
RT-PCR	reverse transcriptase-polymerase chain reaction
SD	standard deviation
SEM	standard error of mean
7AAD	7-amino-actinomycin D
topo IIα	topoisomerase IIα
topo IIβ	topoisomerase IIβ
TP53	gene encoding for tumor protein 53
WBC	white blood cell count
WHO	World Health Organization

INTRODUCTION

INCIDENCE

Acute myeloid leukemia (AML) is a life-threatening malignant disease which affects both children and adults. The annual incidence is usually reported to be 3-4/100,000[1], but in truth is probably slightly higher due to undernotification in cancer registries[2]. The incidence increases with age. In the United States, the incidence rate has been reported as 1.8/100,000 in people <65 years and 17/100,000 in people ≥65 years[1]. Even if AML is a rare disease, due to its poor prognosis it has a great impact on cancer death among young adults[3].

ETIOLOGY

Several risk factors for developing AML have been identified, such as constitutional genetic defects, ionizing radiation and exposure to chemical compounds (e.g. benzene, or cytotoxic therapy)[1]. However, for most individual cases of AML the etiology is unknown, the exception being the 10-20 % of AML which arise in patients earlier treated with cytotoxic agents or radiotherapy[1].

PATHOPHYSIOLOGY

AML is characterized by a rapid proliferation of myeloid progenitor cells, blasts, in the bone marrow, leading to suppression of normal hematopoiesis. The symptoms are the result of deficiency of normal blood cells; that is, they are due to anemia, neutropenia and thrombocytopenia, which causes fatigue, infections and bleeding[4]. Without treatment, patients with AML deteriorate rapidly and usually die within weeks or months after diagnosis, often due to infections.

DIAGNOSIS

Diagnosis of AML is based primarily on morphology, by demonstration of an accumulation of blast cells in the bone marrow. The FAB (French-American-British) classification[5,6], originally proposed in 1976, requires ≥30% blasts in the bone marrow. The recently adopted WHO (World Health Organization) classification[7], proposed in 1999, requires ≥20% blast cells. Hence, patients who according to FAB would have been diagnosed with refractory anemia with excess blasts in transformation (RAEB-t), an entity with similar prognosis as patients with ≥30% blast cells, will be identified as AML cases. The AML diagnosis also requires that the blasts be of myeloid origin, demonstrated with cytochemical staining or immunophenotyping showing positivity for myeloid surface antigens such as CD13 or CD33.

CLASSIFICATION

AML is not a homogeneous entity but a heterogeneous group of myeloid malignancies. This has long been the clinical experience and now the rapidly growing

knowledge of genetic aberrations in AML has had a great impact on understanding and classification of different clinical entities within the disease.

The FAB classification, which is based mainly on morphology and cytochemical stainings, supported by immunophenotyping[8], recognizes eight classes of AML with subgroups (AML M0-M7, Table I). The WHO classification incorporates cytogenetic data and, to some extent, etiology (Table II). With this approach, however, a large proportion of AML cases must be classified according to the FAB classification, since they do not display any distinct cytogenetic or etiologic features.

Furthermore, the blast cell population from an individual patient is heterogeneous. Within the malignant clone there are cells of different phenotype, such as stem cell like cells and more mature cells[9-11].

Table I. FAB classification of AML

Myeloblastic leukemia minimally differentiated	M0
Myeloblastic leukemia without maturation	M1
Myeloblastic leukemia with maturation	M2
Promyelocytic leukemia	M3
Myelomonocytic leukemia	M4
Monocytic leukemia	M5
Erythroleukemia	M6
Megakaryoblastic leukemia	M7

Table II. WHO classification of AML

AML with recurrent cytogenetic translocations
- t(8;21)(q22;q22)
- t(15;17)(q22;q11-12) and variants
- inv(16)(p13q22) or t(16;16)(p13;q11)
- 11q23 abnormalities

AML with multilineage dysplasia
- with prior myelodysplastic syndrome
- without prior myelodysplastic syndrome

AML and myelodysplastic syndrome, therapy related
- Alkylating agent related
- Topoisomerase II inhibitor related
- Other types

AML not otherwise categorized
- Classification basically according to FAB classification

PROGNOSIS

The overall prognosis in AML is poor. For example, the 5-year overall survival (OS) in AML in the United States was reported to be 34.4% in patients <65 years and 4.3% in patients ≥65 years[1]. In a Swedish population-based study, the median survival time was 24.8 months for patients <60 years and 3.1 months for patients ≥60 years[12].

There are several prognostic factors among which age is a well-known and important one, as illustrated above. Other previously established prognostic factors are high white cell count and prior hematological malignancy[13]. However, cytogenetic and molecular genetic features have been shown to be of very strong prognostic importance, and this field is currently under rapid development[14]. It has been possible to differentiate prognostic groups based on cytogenetics (for example[15], see Table III), and these groups also have an impact on the therapeutic strategy for individual patients. Regarding molecular genetics, studies have identified an internal tandem duplication in the *FLT3* gene, which has been associated with poor prognosis in the heterogeneous group of patients with normal karyotype[16]. In the absence of *FLT3* mutation, a mutated *NPM1* gene is associated with relatively favourable prognosis in the same group of patients with normal karyotype[17]. In addition, gene expression profiling has also recently been used to identify different prognostic groups[18,19].

Table III. Cytogenetic risk groups according to the MRC AML 10 trial[15]

Risk group	Abnormality	Comment
Favorable	t(8;21) t(15;17) inv(16)	Whether alone or in conjunction with other abnormalities
Intermediate	All other abnormalities	Normal karyotype or abnormalities not classified as favorable or adverse
Adverse	-5 -7 del(5q) Abnormal 3q Complex	Whether alone or in conjunction with intermediate-risk or other adverse-risk abnormalities

TREATMENT

AML treatment with curative potential consists of intensive chemotherapy, given as induction therapy and post-remission therapy. The goal of induction therapy is to achieve complete remission (CR), which is basically defined by a bone marrow smear with less than 5% blasts at the time of hematological recovery after induction

therapy[20]. CR is a prerequisite for cure, but not equivalent to it, since relapse after achieving CR is very common. The risk of relapse is most pronounced during the first years after achievement of CR; after three years the risk of relapse has declined to below 10%[21]. Post-remission therapy is given to reduce the risk of relapse, and consists of further cytostatic courses, sometimes followed by allogeneic stem cell transplantation.

The possibility of curing AML with cytostatic drugs is based on the difference in susceptibility to drug toxicity between malignant and normal cells. Without this difference, which has no obvious explanation, normal bone marrow cells would not recover but would instead deteriorate along with the malignant clone. On the other hand, if the malignant clone proves to be resistant to conventional drug doses, increased dosage is limited by toxicity to normal tissues including bone marrow.

Induction therapy

Although there have been numerous attempts to develop better treatment by different scheduling and combinations of cytostatic drugs, no induction regimen has consistently proved better than the combination of an anthracycline, basically daunorubicin (45-60 mg/m^2 days 1-3), and the antimetabolite cytarabine (100 mg /m^2 per day, given as continuous infusion days 1-7)[22,23]. Attempts to give more dose-intensive regimens, often by adding a third drug, have usually resulted in worse toxicity without gain in terms of survival. Patients aged 18-60 years treated with induction therapy will achieve CR at a rate of 65-75%[4]. However, a majority of these patients will relapse and eventually die of their disease[24].

Post-remission therapy

Post-remission therapy traditionally consists of repeated cytostatic courses including anthracyclines or anthracycline analogues, cytarabine and sometimes a third drug such as etoposide. Dose intensification has generally failed to improve survival, but data indicate that high-dose cytarabine (3 g/m^2 per dose) instead of conventional doses is beneficial for younger patients with core-binding-factor AML (AML with t(8;21), inv(16) or t(16;16)[25,26]. Further consolidation therapy using allogeneic stem cell transplantation may also be given, with the decision being based on individual risk factors, mainly the cytogenetic and molecular genetic features mentioned above.

Stem cell transplantation

Allogeneic stem cell transplantation reduces the risk of relapse. This is believed to be due to the graft-versus-leukemia effect rather than to intensive conditioning regimens[27]. However, the lower relapse rate has not consistently improved survival since treatment related mortality due to infections and graft-versus-host disease is a major problem. There are on-going attempts to minimize treatment-related mortality by reduced-intensity-conditioning regimens, taking advantage of the graft-versus-leukemia effect; this may make allogeneic transplantation a feasible and effective

option for elderly patients, too[28]. As for other treatment options, results of allogeneic transplantation have been evaluated in different cytogenetic and molecular genetic risk groups in the hope of identifying groups that will benefit from this treatment.

However, the role of allogeneic stem cell transplantation in AML treatment is controversial[22,29]. It is an established post-remission treatment for patients with a karyotype indicating intermediate risk and with an available sibling donor, but no truly randomized comparison to standard treatment has been performed. Patients with adverse cytogenetic profile have a very poor prognosis if treated with chemotherapy alone. Allogeneic stem cell transplantation is currently used as an option to improve outcome for these patients, and there are data supporting this strategy[30]. On the other hand, patients with favourable karyotype do not benefit from allogeneic stem cell transplantation in first CR[29].

Autologous stem cell transplantation has not proved better than cytostatic treatment or allogeneic transplantation in terms of survival and is currently not widely used in AML[29].

Elderly patients

Most data on treatment of AML are derived from younger patients (in this context <60-65 years), while a majority of AML patients are elderly. For example, Swedish adult patients diagnosed with AML (AML M3 excluded) during 1997-2001 had a median age of 71 years[31]. The therapeutic principles are also applicable to these patients, but the possibility of giving intensive treatment decreases with age due to higher treatment morbidity and mortality, which also strongly limit the use of allogeneic stem cell transplantation. Cytogenetic aberrations have a major prognostic importance in the elderly, among whom there is a higher proportion of patients with unfavourable karyotype as compared to younger patients[32-36]. It is often possible to perform standard induction therapy in patients aged up to 75-80, but the result in terms of survival is generally poor[37]. Patients not eligible for standard induction therapy receive investigational therapy or palliative therapy, which can include low-dose chemotherapy.

In practice, a decision on treatment intensity must be based on several factors which have an impact on total mortality rate[37]. Adverse outcome in AML can be due to either treatment-related death or resistance to therapy. Theoretically, treatment related death is predicted mainly by poor performance status and comorbidity, while the above-mentioned adverse cytogenetic and molecular genetic prognostic factors indicate resistance to therapy; that is drug resistance.

CYTOSTATIC DRUGS

Topoisomerase poisons

Anthracyclines are cytostatic drugs with various possible mechanisms of action, such as intercalation into DNA, free radical formation, DNA cross-linking and interaction with chromatin[38,39]. However, they are mainly considered as topoisomerase II

poisons, that is they are believed to exert their effect primarily by interfering with topoisomerase II, thereby causing damage to DNA and subsequent apoptosis[38,40,41]. The drugs most widely used in AML treatment are daunorubicin and idarubicin. Daunorubicin is given in doses of 45-60 mg/m^2 for three days but the optimal dosage is not known. In addition, uncertainty about optimal dosing has made comparisons between daunorubicin and its analogue idarubicin difficult to interpret, although randomized trials have shown a tendency toward better results for idarubicin[42]. Apart from immediate toxicity, an important cumulative dose-limiting factor regarding anthracyclines is cardiotoxicity[43].

The anthracene mitoxantrone also interacts with topoisomerase II and has been shown to be effective in induction treatment and as salvage therapy[44,45], but has not proved to be superior to standard regimens[22,23].

Amecrine is another topoisomerase II poison. It is used as an alternative to anthracylines for patients at increased risk for cardiotoxicity or as part of salvage regimens in case of primarily refractory disease or at relapse[46,47].

Etoposide is a derivative of epipodophyllotoxins and acts as a topoisomerase II poison. It has been used as a third drug in combination with anthracyclines and cytarabine in induction therapy, but these regimens have not proved to be beneficial in comparison to standard therapy[22,23]. It is also frequently a part of salvage regimens[48].

Nucleoside analogues

The pyrimidine analogue arabinosylcytosine (cytarabine, Ara-C) is an antimetabolite. Cytarabine is intracellularly phosphorylated to cytarabine triphosphate (Ara-CTP), which is incorporated into DNA in competition with deoxycytidine triphosphate (dCTP) thereby causing strand termination and subsequent cell death[49,50]. Ara-CTP is also an inhibitor of DNA polymerase[51]. The phosphorylation of cytarabine to Ara-CTP is catalyzed by deoxycytidine kinase (dCK) and decreased activity of this enzyme is considered one of several likely mechanisms of resistance to cytarabine[52]. Another such mechanism might be increased metabolism to inactive uracil arabinoside (ara-U) catalyzed by cytidine deaminase[53].

Cytarabine has been included in every induction and post remission therapy protocol for more than 30 years. Standard dosage is 100-200 mg/m^2 as continuous infusion for seven days[23]. The dose-limiting factor is bone marrow toxicity. High-dose cytarabine, 3g/m^2 intravenously twice daily, has as previously mentioned been indicated to be beneficial in post-remission therapy of younger patients[25], especially for patients with favorable cytogenetics[26]. With higher doses, there is an increased risk of cerebellar toxicity[54].

Fludarabine and cladribine are both purine analogues resistant to deamination. Intracellularly, like cytarabine, they are phosphorylated by dCK and incorporated into DNA. In the context of AML, they are used in salvage regimens in combination with cytarabine with or without topoisomerase II poisons[55]. Promising effects of

combinations of fludarabine or cladribine with cytarabine and anthracyclines in primary induction chemotherapy of AML have been demonstrated, although no randomized controlled studies have been performed to show its superiority over standard treatment[56,57].

The more recently investigated compound clofarabine has shown promising results in AML[58-60], but its clinical importance needs to be validated in controlled trials[61].

DRUG RESISTANCE

A majority (65-75%) of younger AML patients who receive induction treatment will achieve CR[4], while the CR rate among the elderly is lower (40-50%)[31,37]. Most patients who achieve CR will relapse, usually within three years[21]. Patients who do not respond to induction treatment display resistance to chemotherapy, and resistance is also common at relapse[62]. Relapse itself could be interpreted as a result of resistance to treatment in a subgroup of leukemic cells, which survive initial induction treatment despite CR. Thus, drug resistance is a major problem in treatment of patients with AML.

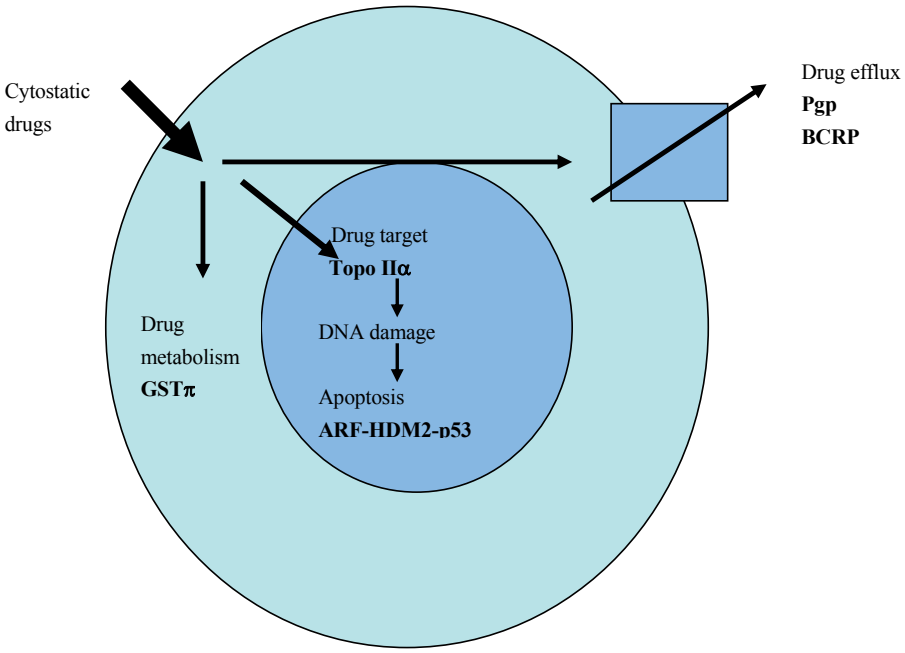

Figure 1. Overview of the putative mechanisms of drug resistance discussed in this thesis. Once the cytostatic drug has entered the cytoplasm (light blue), enhanced drug efflux or drug metabolism can prevent the drug from reaching the target in the cell nucleus (dark blue). In the nucleus, alteration of drug target could interfere with drug action, thereby limiting DNA damage and subsequent apoptosis. Disposition to apoptosis could also be altered *per se*. The specific proteins that are discussed in this thesis are indicated by bold letters.

Drug resistance in AML can be caused by several mechanisms, often in combination. The mechanisms may be located in different parts of the leukemic cell and include for example altered drug transport, drug metabolism, drug target or, less specifically, disposition to apoptosis[63,64]. An overview of the putative mechanisms discussed in this thesis is shown in Figure 1.

Drug resistance can be investigated *in vitro*, using cell lines or patient samples or *in vivo*, by investigating whether the patient enters CR after one or more induction courses and whether relapse occurs. Since drug resistance is a major cause of treatment failure in AML, survival may also be used as a surrogate endpoint of drug resistance.

DRUG TRANSPORT

ATP-binding cassette (ABC-) proteins

ABC transporter proteins are a large family of evolutionary highly preserved transmembrane proteins[65]. They are present in virtually every cell type and are located in the plasma membrane or in intracellular membranes, exerting transmembrane transport of drugs, peptides, ions and xenobiotics. Energy for this efflux is generated by hydrolysis of ATP (adenosine triphosphate)[66]. The ABC transporters can be subdivided into seven subfamilies, ABCA-ABCG. They have distinct substrate profiles, but several are able to extrude substrates of very heterogeneous structures. In the context of AML, the most extensively-studied ABC-transporters are ABCB1 (originally designated P-glycoprotein, Pgp), ABCC1 (multi-drug resistance associated protein, MRP1) and ABCG2 (breast cancer resistance protein, BCRP)[67]. MRP1 has been associated with drug resistance in AML[67] and so has MRP3 (ABCC3), as recently reported[68,69]. In terms of drug transport, this thesis focuses on Pgp and BCRP.

P-glycoprotein

In cell lines selected for resistance to colchicin and vinblastin, a 170-kDa cell membrane glycoprotein was found to be overexpressed in comparison to drug-sensitive parental cell lines. Since this glycoprotein was believed to alter cell membrane permeability, it was designated P-glycoprotein, Pgp[70-72]. Pgp, which is encoded by the *MDR1* (multidrug resistance) gene, located on chromosome 7q21[73], was later shown to exert ATP-dependent drug transport[74]. Although Pgp is expressed in many tissues, its physiological role, besides protection against toxic compounds, is not fully understood. In a study of *mdr1*-deficient mice, no biological defects could be demonstrated[75]. A wide range of cytotoxic compounds are substrates of Pgp; in this context, the most important to mention are anthracyclines, anthracenes and epipodophyllotoxins[76]. However, in terms of cellular uptake and retention, Pgp might have lesser impact on the the anthracycline idarubicin than on daunorubicin[77]. Expression of Pgp has been associated with drug resistance in cell lines[71], and

transfection of the *MDR1* gene results in a multidrug-resistant phenotype in transfected cells[78].

Expression of *MDR1* or Pgp has also been associated with clinical drug resistance and prognosis in AML[34,79-83]. There exist a number of compounds with inhibitory effect on Pgp, such as verapamil, cyclosporine and the cyclosporine analogue PSC-833, and attempts to improve cytostatic effect by modulating Pgp activity have been made in several studies; however, these have yielded conflicting but mainly disappointing results[84-91]. One problem has been adequate dosage of cytostatic drugs, since these drugs are also pharmocokinetically influenced by the modulators, aside from the Pgp effect[92]. At relapse, AML patients often display increased drug resistance[62], probably as a result of selection of resistant AML cell subpopulations and/or clonal development during chemotherapy. However, Pgp has not been proven to be up-regulated at relapse[93]. *In vitro*, long-term drug exposure of leukemic cell lines can induce a drug-resistant phenotype with increased Pgp expression[94].

The short-term effect of exposure to cytostatic drugs on Pgp expression has been studied in different malignant cells including leukemic cells. It has been shown that Pgp substrates as well as non-Pgp substrates can induce Pgp mRNA and Pgp protein expression in human leukemic cell lines[95], and that Pgp mRNA expression can be induced within only four hours of exposure to anthracyclines[96-98]. In samples from patients with myeloid leukemia, Hu *et al.* demonstrated that Pgp protein expression and function was induced *in vitro* 16 hours after the start of exposure to daunorubicin or cytarabine[99]; the latter is not considered to be a substrate of Pgp[78,100]. Hu *et al.*[99] also found that concomitant treatment of one patient with daunorubicin and cytarabine *in vivo* was followed by Pgp expression after four hours.

Regulation of *MDR1* gene in AML has been associated with the methylation status of CpG islands in the promoter region, insofar as hypomethylation has been associated with increased Pgp mRNA expression[101]. A recent study using leukemic cell lines indicated that induction of Pgp mRNA by cytostatic drugs requires a hypomethylated status of the promoter region and is associated with increased histone acetylation[98].

Breast cancer resistance protein

Another member of the ABC superfamily is ABCG2, or breast cancer resistance protein (BCRP). BCRP is a 655-amino acid transmembrane protein coded on chromosome 4q21-22[102]. It was originally isolated from a breast cancer cell line, MCF-7AdrVp, which was developed by culturing the drug-sensitive cell line MCF-7 with doxorubicin in the presence of verapamil in order to identify mechanisms of drug resistance other than Pgp. A 95 kDa protein (P-95) was found to be increased in the resistant cell line compared to the parental cell line[103]. Later, P-95 was found in AML blasts with reduced daunorubicin accumulation. These cells were Pgp-negative according to Western blot and cyclosporine did not reverse the decreased accumulation of daunorubicin[104]. MCF-7 cells transfected with *BCRP* cDNA acquired a drug-resistant phenotype resembling that of MCF-7AdrVp. The amino acid sequence displayed a high degree of homology to members of the ABC super family

and it was also demonstrated that drug efflux due to BCRP could be attained by ATP depletion[105].

BCRP probably functions as a homodimer or a homotetramer[106]. It is highly expressed by normal stem cells[107], including hematopoietic cells[108], but its physiological role is not fully understood. Its abundant expression in human placenta[109] implies a role in fetal protection and its importance in protection against dietary compounds has been demonstrated in *bcrp*-deficient mice[110]. In normal hematopoietic cells, the expression of BCRP decreases during differentiation. BCRP is also expressed by the so-called "leukemic stem cells" in AML, resulting in lower drug accumulation in these cells compared to more differentiated leukemic cells[111].

Mitoxantrone, like doxorubicin and daunorubicin[105,112], was initially shown to be a substrate of BCRP in cell lines, while idarubicin was not[77,113]. However, the *BCRP* gene in the MCF-7AdrVp cell line (and also in the colon carcinoma cell line S1-M1-80) was later shown to be mutated in comparison to wild-type *BCRP*, which is found for example in human placenta[114]. The gene from the drug-selected cell lines contains a single-nucleotide mutation at position 482 (coding for arginine in wild type cells, threonine in MCF-7AdrVp cells and glycine in S1-M1-80 cells). The mutated forms of BCRP differ from the wild type regarding substrates. Most important in this context is the ability to extrude anthracyclines and mitoxantrone. Doxorubicin, and possibly other anthracyclines, is believed not to be a substrate of wild-type BCRP but of the mutated forms, while mitoxantrone is a substrate of both wild-type BCRP and the mutated forms[115].

Inhibitors of BCRP have been identified. The first to be described was fumitremorgin C, a compound extracted from *Aspergillus fumigatus*[116,117]. However, this fungal toxin has proved to be neurotoxic. More recently described is KO143, an analogue to fumitremorgin C, which is highly potent and specific[118]. Despite the above mentioned findings of Doyle *et al.*[104], there are data indicating that cyclosporine could also be an inhibitor of BCRP, although only a weak one at clinical concentrations[119], while PSC-833 probably is not[120].

The expression and clinical importance of BCRP in AML have been studied in recent years. Ross *et al.* reported that 20 out of 21 acute leukemia samples expressed less BCRP mRNA than the drug-sensitive cell line MCF-7[121]. In another study of adult AML, the BCRP mRNA expression in patient samples was of the same level as the BCRP mRNA expression in a drug sensitive cell line and well below that of a resistant cell line[113]. In the same study, flow cytometry data suggested that the BCRP expression was heterogeneous, with only a small minority of BCRP-positive cells. A study of 31 AML patients demonstrated small subpopulations of leukemic cells expressing BCRP at mRNA and protein level, while another study of 25 AML patients found no evidence for subpopulations with different BCRP protein expression or function[122]. Two additional studies showed that BCRP mRNA was solely of wild type sequence at codon 482 in 17 and 31 cases of AML, respectively[123,124].

BCRP mRNA has been shown to be up-regulated at relapse in comparison to diagnosis[125,126]. In contrast, in two studies on BCRP protein in AML, there was no evidence of increased expression at relapse/refractory disease as compared to diagnosis[122,127].

In clinical studies, the BCRP mRNA and protein expression have been associated with both drug resistance (resistance to induction treatment) and prognosis. The available data are summarized in Table IV.

Table IV. Studies on BCRP expression and clinical outcome in AML

Authors	**Number of patients**	**Method**	**Age**	**CR rate (%)**	***De novo*/ secondary (%)**	**Results**
Van den Heuvel-Eibrink[128] (2007)	154	RT-PCR	67 (mean)	52	80/20	BCRP/MDR1 mRNA coexpression associated with low CR rate
Benderra[129] (2004)	149	RT-PCR	52 (mean)	66	100/0	BCRP mRNA positivity associated with RD and shorter 4-year EFS and OS
Damiani[130] (2006)	72	Flow cytometry	53 (median)	75	100/0	BCRP positivity associated with shorter DSF in AML with normal karyotype
Benderra[68] (2005)	85	Flow cytometry	<55 53% ≥55 47%	66	100/0	High BCRP activity associated with RD and shorter EFS/OS
Wilson[19] (2006)	170	Gene expression profiling	65 (median)	43	61/19*	Overexpression of *ABCG2* and *MDR1* associated with RD and short OS

*not all cases were categorized

DRUG METABOLISM

Glutathione S-transferase π

Glutathione S-transferases are a family of enzymes with the ability to catalyze the conjugation of glutathione to a variety of compounds, including cytostatic drugs; this conjugation is the first step in elimination of toxines[131]. High levels of glutathione S-transferase π (GSTπ), a subclass of cytosolic glutathion S-transferases, have been associated with unfavorable clinical outcome in AML[132].

DRUG TARGET

Topoisomerase IIα

Topoisomerases are a group of nuclear enzymes with the ability to induce transient breakage of single (topoisomerase I) or double (topoisomerase II) DNA strands, followed by religation. Single-strand breaks allow relaxation of supercoiled double

helices, while double-strand breaks allow separation of intertwined double helices. Relaxation and disentangling of DNA is essential for important cellular functions such as replication, transcription and mitosis[133]. The transient cleavage of double-stranded DNA by topoisomerase II is illustrated in Figure 2.

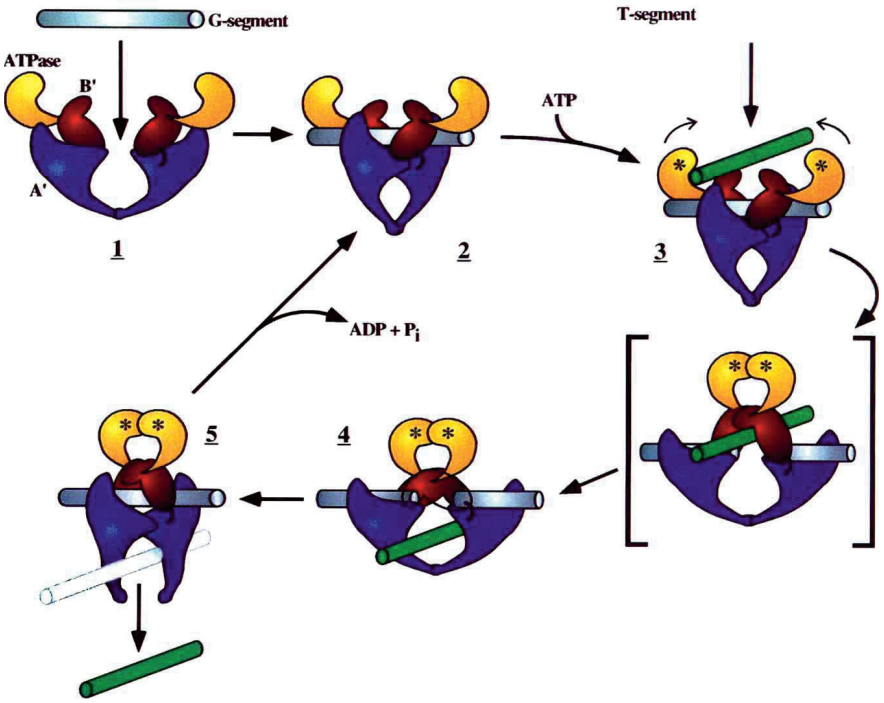

Figure 2. Proposed structure and catalytic cycle of topoisomerase II. One DNA double helix (grey) is cleaved, allowing the passage of another intact DNA double helix (green). The enzyme is a homodimer consisting of three functional segments: an ATPase (top), a cleavage (B´), and a C-terminal (A´) part. The topoisomerase II poisons act by stabilizing stage 3 or 4 (cleavable complex), where the DNA double helix is cleaved. Figure reprinted with permission from Berger et al, Nature 379 (1996), pp. 225–232.

Topoisomerase II has two isozymes; topoisomerase IIα (topo IIα) and topoisomerase IIβ (topo IIβ)[134]. Topo IIα is a 170 kDa protein[135] coded on chromosome 17q21-22[136]. The expression of topo IIα is cell cycle dependent in normal cells with expression predominantly occurring in the S/G2/M phases, while topo IIβ expression is not cell cycle dependent[137]. Data indicate that topo IIα expression in malignant cells could be less dependent on the cell cycle and that the enzyme may also be significantly expressed in the G0/G1 phases[138-140].

Topo IIα is believed to be the main target for anthracyclines and epipodophyllotoxins[40]. Anthracyclines and epipodophyllotoxins are designated as topoisomerase poisons, since they do not inhibit but instead interact with the enzyme. The drugs stabilize the reversible double-strand break, the "cleavable complex" (Figure 2)[41], and this persistent DNA damage, if extensive enough, is believed to be the main mechanism inducing apoptosis[38]. The double-strand breaks also make the cells vulnerable to mutations by unsuccessful DNA repair, which could cause secondary leukemias in patients treated with topoisomerase poisons. Data from mice suggest that topo IIβ is primarily responsible for the carcinogenic drug effect, while the tumor cell toxicity is primarily dependent on topo IIα[141].

The effect of topoisomerase poisons is thus dependent on the presence of topo IIα; and down-regulation of topo IIα is considered a possible mechanism of drug resistance[142]. Low topo IIα mRNA, protein or activity has been associated with drug resistance in a number of different tumor cell lines, including leukemic cells[143-148]. Previous reports on topo IIα expression and clinical outcome in AML have been negative regarding both mRNA[149-151] and protein levels[152], with one exception[153]. There are data indicating that the effect of topoisomerase poisons - DNA damage or cell killing - is predominantly evident in, but not limited to, the S/G2/M cell cycle phases[154-156]. In addition to down-regulation or mutation of the topo IIα gene, post-translational modification of topo IIα may also be of importance for its activity and possibly for the effect of topoisomerase poisons[157-160].

DNA DAMAGE AND APOPTOSIS

Cytostatic drugs are believed to exert their effect mainly by inducing programmed cell death, or apoptosis. The complicated chain of events that eventually leads to phagocytosis of an apoptotic cell is triggered by DNA damage caused by the drugs. A key protein in this process is p53.

P53

The p53 protein is encoded by the *TP53* gene located on chromosome 17p13[161]. It is a transcription factor which is activated by cellular stress, for example DNA damage[162]. Its activation is followed by transcription of cell cycle inhibitors and pro-apoptotic proteins, leading to differentiation, cell cycle arrest or apoptosis[163,164]. During normal cellular conditions, p53 is constitutionally expressed but its activity is strictly controlled by a complicated regulatory network[165]. An important modulator of p53 is HDM-2, a protein which promotes ubiquitination and degradation of p53[166]. Mutations of *TP53* are a common feature of many tumours and are associated with loss of apoptotic function and hence with drug resistance[167]. In AML, p53 mutations are found in about 10% of newly diagnosed cases[168-170]. Inactivation of p53 is usually caused by loss of one p53 allele and a point mutation in the remaining allele[168,171]. *TP53* mutations and abnormalities of chromosome 17 have been associated with poor prognosis in AML[170,172]. The function of mutated p53 can be restored by a recently identified compound known as PRIMA-1 (p53-dependent reactivation and induction

of massive apoptosis)[173,174]. Leukemic cells with mutated p53 and drug-resistant phenotype have been shown to be sensitive to PRIMA-1 *in vitro*[175].

P14^{ARF}

The protein p14^{ARF} is encoded as an alternative reading frame with p16^{INK4a} by the INK4a/ARF gene locus on chromosome 9p[176]. It is involved in regulation of p53 by binding and inhibiting HDM-2, thereby stabilizing p53[177]. There are data indicating that both HDM-2 and p14^{ARF} genes are overexpressed in AML blasts and that low levels of p14^{ARF} mRNA are associated with poor prognosis in AML[178].

LEUKEMIC STEM CELLS

Hematopoietic stem cells constitute a pool of rare immature cells with the capacity for self-renewal and differentiation; this is displayed by assymmetric cell division giving rise to both an identical stem cell and a more mature cell[179]. Similarly, human leukemic cells with the ability to initiate AML in NOD/SCID mice and with self–renewal, proliferative and differentiative capacities have been identified as CD34+CD38- cells[9]. These leukemic stem cells are believed to arise either from normal hematopoietic stem cells or from lineage-committed progenitors which have acquired self-renewal properties[179]. Hematopoietic cells with self-renewal properties have also been identified by their ability to efflux the fluorescent dye Hoechst 33342, forming a "side population" (SP) by dual wave-length flow cytometry[180,181]. Normal SP cells are CD34- and may represent even more immature cells than CD34+CD38- cells. SP cells have also been identified in AML samples, but seem less phenotypically distinct, and may be both CD34+ and positive for CD13 and CD33, both markers of further myeloid differentiation[182]. The ability to efflux Hoechst 33342 is probably mediated by BCRP, which is highly expressed in the SP and CD34+CD38- cells but down-regulated in more mature progenitors[108,183]. The expression of Pgp in leukemic stem cells seems more complex. Data indicate that Pgp levels are higher in leukemic CD34+CD38- cells than in more mature CD34+ progenitors[184], but that they may also be up-regulated again in mature CD34- leukemic cells[183]. It has recently been demonstrated that both BCRP and Pgp mRNA expression in CD34+CD38- cells are associated with resistance to chemotherapy in AML[185]. On the other hand, despite similar levels of Pgp protein, Pgp function *in vitro* was found to be impaired in leukemic CD34+CD38- cells in comparison to their normal counterparts[184]. Thus, the leukemic clone includes small populations of cells which display distinctive patterns of drug resistance markers. If the cells with self renewal properties are able to escape apoptosis induced by cytostatic drugs, they may well be responsible for treatment failure.

AIMS OF THE STUDY

The main objective of this thesis was to contribute to a better understanding of drug resistance in AML by exploring the clinical relevance of three putative mechanisms of drug resistance, involving topo IIα, BCRP or $p14^{ARF}$, and by investigating the immediate effects of drug exposure on Pgp, BCRP and GSTπ expression in leukemic cells. The specific aims were to investigate

- the topo IIα protein expression in different parts of the cell cycle in leukemic cells (paper I)
- the expression of topo IIα mRNA and protein in leukemic cells in relation to drug sensitivity *in vitro* and to clinical outcome in AML (paper II)
- the expression of BCRP mRNA in AML in relation to clinical outcome (paper III)
- the initial response to drug exposure *in vitro* in leukemic cells regarding expression of Pgp, BCRP and GSTπ (paper IV)
- the expression of $p14^{ARF}$ mRNA expression in relation to prognosis in AML with normal karyotype (paper V)
- the expression of $p14^{ARF}$ mRNA in AML samples in relation to drug sensitivity *in vitro* with special reference to PRIMA-1 (paper V)

MATERIALS AND METHODS

ETHICS

The four studies involving patients and patient samples (papers I-III and V) were conducted with the approval of the ethics committees and after informed consent had been obtained.

PATIENTS AND PATIENT SAMPLES (PAPERS I-III AND V)

Blood or bone marrow samples were obtained from patients with acute leukemia, as a portion of the routine samples taken for diagnostic procedures. The samples were used in the different studies as indicated below (papers I-III and V). Separated mononuclear cells were used for flow cytometry and *in vitro* cytotoxicity assays or vitally frozen and stored at -150ºC for real-time RT-PCR.

Paper I used samples from 25 consecutive patients (22 AML and 3 ALL). Fifteen of these patients had *de novo* AML, while the remaining seven had secondary or MDS-AML. The patients were aged between 19 and 84 years, with a median age of 67 years. Thirteen patients were evaluable for clinical response; that is they received remission-induction therapy and could be evaluated for response. Sixteen of the samples were from bone marrow aspirations and 9 were blood samples. Blood samples were also obtained from 10 voluntary blood donors.

Paper II included samples from an additional 35 patients (32 AML and 3 ALL), giving a total of 60 samples for flow cytometry. Frozen samples were available from 24 of these patients, and were supplemented with frozen samples from another 34 patients (all AML), giving a total of 58 samples for real-time RT-PCR. Additional frozen material was available from 40 of these 58 patients, and these samples were used for real time RT-PCR in paper III. Hence, samples for flow cytometry were obtained from consecutive patients while inclusion of samples for real-time RT-PCR was based on availability of frozen samples. Of the 94 patients included in total in paper II, 76 patients had *de novo* AML and 12 had AML secondary to myelodysplastic syndrome (n=4), CML (n=3), Hodgkin's lymphoma (n=1), essential thrombocythemia (n=1), or cytostatic treatment for other reasons (n=3). Three patients had pre-B-ALL and three patients had T-ALL, all *de novo*. Mean age was 58 years (range 18-84); 61 were women and 33 were men. Cytogenetic analyses were routinely performed for 85 patients.

Paper III used samples from 40 patients as described above; 37 of these had *de novo* AML while three had AML secondary to myelodysplastic syndrome (MDS), Hodgkin's lymphoma or essential thrombocythemia (ET). Their mean age was 57 years (range 22–79).

Among the patients with AML included in paper I-III, those who were evaluable for clinical response to induction treatment received at least one course of induction chemotherapy containing an anthracycline or mitoxantrone (a few patients received amsacrine instead of an anthracycline or mitoxantrone in the first or second induction

course) in combination with cytarabine. Patients with ALL received treatment according to the then-current Swedish Adult ALL Group protocol, which included daunorubicin in the first induction course and amsacrine in the second induction course. Three patients underwent allogeneic stem cell transplantation.

Paper V used frozen bone marrow samples collected at diagnosis from 57 adult patients (six of whom were also included in papers II-III) with *de novo* AML and normal karyotype. Mean age of the patients was 63 years (range 20–84). All patients were treated according to a standard protocol, including cytarabine and an anthracycline, mainly daunorubicin, in a 7+3 treatment scheme with three or four consolidation courses. Four patients underwent allogeneic stem cell transplantation.

Cytotoxocity testing *in vitro* was performed using fresh blood or bone marrow samples from consecutive patients. The results were available to a different extent for different drugs, partly because the number of drugs varied over the years and partly due to technical failure.

CELL LINES, CULTURING AND DRUG INCUBATIONS (PAPERS II, IV AND V)

The lymphoblastic cell line CEM-CCRF (paper II), the breast cancer cell line MCF-7 (paper III) and the cervical cancer cell line HeLA (paper V) were used for construction of standard curves. MCF-7 was also used as a reference for BCRP mRNA expression (paper III).

In paper IV, the human leukemic HL-60 cell line (HL-60 S), which is highly sensitive to doxorubicin, and the sublines HL-60 R0.5 and HL-60 R5, which are resistant to 0.5 μM and 5 μM doxorubicin respectively[186], were grown in a culture medium consisting of RPMI 1640 (GIBCO, Paisley Scotland, UK) with 10% fetal calf serum and 1% L-glutamine. Resistant cells were maintained in the absence of the drug for two weeks before the experiments were performed. The sublines HL-60 R0.5 and R5 have previously been shown to express high levels of Pgp, while HL-60 S has been shown to be Pgp-negative. Compared to HL-60 S, the degree of resistance to daunorubicin was measured at 80-fold in HL-60 R0.5 and 858-fold in HL-60 R5[186].

Cells were suspended in pre-heated culture medium (37°C) in culturing tubes, at a concentration of 0.5 x 10^6 cells/ml. Each cell line was exposed to a final concentration of 0.2 μM daunorubicin or 0.5 μM cytarabine. These concentrations were chosen to achieve clinically relevant exposure[187]. Cells cultured in RPMI 1640 without drugs were used as negative controls. From each of the three series, samples were collected before exposure and at 0, 10 and 30 minutes and 1, 2, 4, 8, 12, 16, 24 and 36 hours, for real-time RT-PCR and Western blot analyses. The reaction was terminated with 10 ml of ice-cold RPMI 1640; samples were then pelleted at 400 *g* for 5 min at 4°C and the supernatant was discarded. For flow cytometry, samples were collected from each series before exposure and after 4, 8, 12 and 24 hours of exposure to daunorubicin or cytarabine. The samples were immediately pelleted at 400 *g* for 5 min at room temperature and the supernatant was discarded.

In paper II, separated mononuclear cells were washed and then incubated in culture medium, as described above, with the cytostatic drugs: amecrine 1 μM continuously, daunorubicin 0.2 μM for 1 hour, etoposide 20 μM for 1 hour, idarubicin 0.05 μM for 1 hour, mitoxantrone 0.1 μM for 1 hour and cytarabine 0.5 μM, cladribine 50 μM and fludarabine 2 μM, all three continuously. In paper V, cells were incubated with daunorubicin, etoposide, mitoxantrone and cytarabine, in the same concentrations as in paper II, and also with PRIMA-1 (5, 10 and 20 μM) continuously. After interruption of drug exposure in the short time cases, all samples were cultured for 4 days. All incubations were performed in duplicate and with a drug-free control.

TOPO IIα PROTEIN EXPRESSION (PAPERS I-II)

The samples were prepared within 24 hours. Separated mononuclear cells were washed and diluted to a concentration of 10 x 10^6/ml. One million cells were added to each of two tubes and incubated with lysing solution. After fixation and washing, the nuclei in one of the two tubes were incubated with a monoclonal antibody to topo IIα (SWT3D1 IgG1κ, DAKO, Glostrup, Denmark, 1:100). After incubation, a FITC-conjugated rabbit anti-mouse antibody was added to both tubes. After a second incubation, propidium iodide with RNAse was added, followed by a third incubation.

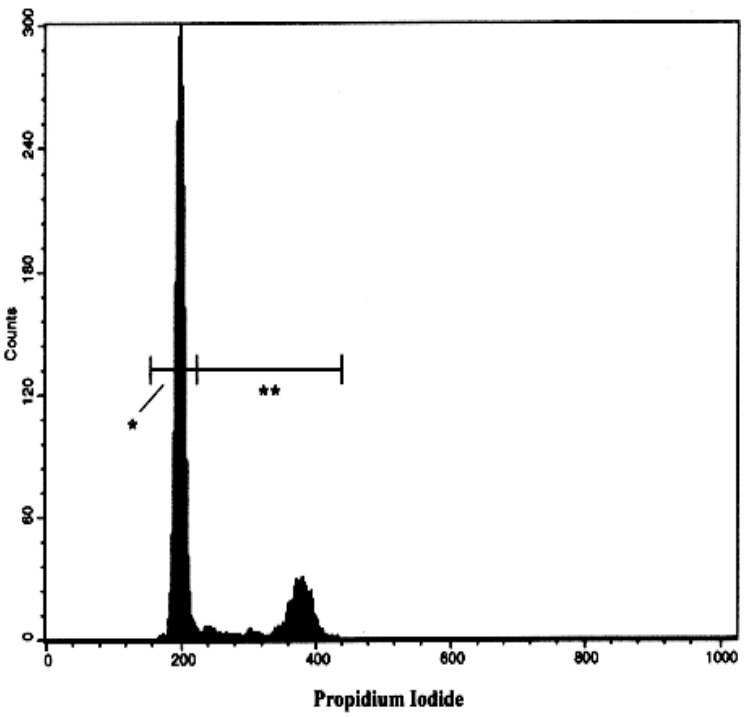

Figure 3. Example of DNA histogram used for separation of G0/G1 cells (*) from S/G2/M cells (**).

The stained nuclei were analyzed in a FACScan equipped with an argon laser (488 nm) (Becton Dickinson, Immunocytometry Systems, CA, USA). CellQuest software (Becton Dickinson) was used to analyze 5000 events; non-cellular events as determined by the propidium iodide fluorescence were excluded by gating. The DNA histogram was used to separate G0/G1 cells from cells in S/G2/M (Figure 3). The cut-off limit for topo IIα-positivity was set for each population based on the negative controls, that is the cells incubated with the secondary antibody only (Figure 4).

Granulocytes from healthy donors were used as negative control[188]. Separation was performed on Histopaque (Sigma) and the supernatant was removed. The pellet, containing granulocytes and red cells, was washed once in 0.05 M PBS pH 7.4, and then incubated with 8 ml Ortho-mune Lysing Reagent (Ortho Diagnostic Systems, Raritan, NJ, USA) for 10 minutes, in order to lyse the red cells; next it was centrifuged at 300 *g* for 5 minutes and washed once in 0.05M PBS. The remaining granulocytes were diluted to a concentration of 10 x 10^6 /ml in 0.05M PBS and further analyzed as described above for the mononuclear cells from patient samples. A microscopic control of these samples showed >99% granulocytes.

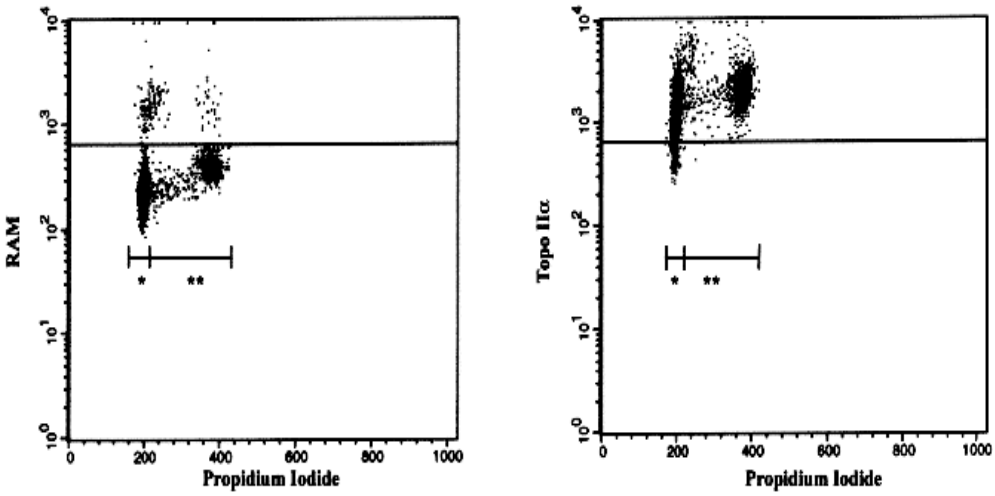

Figure 4. Example of scattergrams: negative control with FITC-labeled rabbit anti-mouse antibody (RAM) alone (left) and test sample with the addition of topo II α antibody (right). G0/G1 phase is indicated by * and S/G2/M by **.

TOPO IIα AND BCRP mRNA EXPRESSION (PAPERS II-III)

RNA from thawed cells from patient samples and cultured cells was isolated using QIAamp RNA Blood Mini Kit (Qiagen) following the manufacturer's instructions and then stored at -80ºC. The quality and quantity of RNA was determined using RNA chips with RNA 6000 Nano Reagents & Supplies (Agilent Technologies) in a Bioanalyzer (Agilent Technologies).

cDNA was synthesized from 400 ng RNA from each patient sample and from cell line samples to a volume of 40 µL, which was stored at –20°C. Omniscript RT Kit (Qiagen) with OligodT primer (Qiagen) was used for cDNA synthesis. To perform a real-time PCR, a mix containing Taqman Universal PCR Master Mix (Applied Biosystems), primer (1 µM), probe (0.25 µM), 2 µL cDNA and water to a total volume of 25 µL was prepared and the samples were amplified in duplicate using ABI Prism 7700 (Applied Biosystems, Weiterstadt, Germany).

Standard curves were constructed for β-actin, topo IIα and BCRP by serial dilutions of the purified (QIAquick Gel Extraction Kit, Qiagen) amplification products using cDNA from CEM and MCF-7, respectively. Patient samples were related to the standard curves, giving a relative quantification of the gene product. The mRNA expression (i.e. the mean value of the duplicates) of topo IIα or BCRP was normalized by means of division by the β-actin expression. This quotient was used for statistics and (for BCRP) for relating patient samples to the MCF-7 cell line.

CYTOTOXICITY IN VITRO (PAPERS II AND V)

The bioluminescence assay utilizes an ATP monitoring reagent (AMR, luciferin-luciferase reagent from the firefly), which emits light proportionally to the ATP content of a cell. Since the amount of ATP in a certain cell type is relatively constant and since ATP is rapidly degraded if the respiratory cycle is disturbed in aerobic cells, the amount of ATP can be used as an indirect method of measuring cell growth and cell death[189,190].

After incubation with drugs as described above, ATP was extracted from the cells by mixing equal volumes (100 µL) of cell suspension and 2.5% trichloracetic acid. The bioluminescence assay was performed in a Bio Orbit photometer (Turku, Finland). Twenty µL of a sample was added to 900 µL Tris-EDTA buffer in a cuvette which was placed in the photometer. AMR 100 µL was added and the resulting light emission was measured. The ATP standard (10 µM) was then added and light emission measured again. In this way, the concentration of ATP in each sample could be determined and a percentage of ATP in every drug-exposed sample compared to the drug-free control was calculated. Thus, a relatively high percentage would indicate a relatively drug-resistant cell population.

PGP, BCRP AND GSTΠ mRNA EXPRESSION (PAPER IV)

RNA was isolated with an RNeasy® mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol and stored at -80º C. The quality and quantity of RNA was determined using RNA chips with RNA 6000 Nano Reagents & Supplies (Agilent Technologies, Waldbronn, Germany) in a Bioanalyzer (Agilent Technologies).

First-strand cDNA synthesis was achieved using Omniscript RT kit (Qiagen) according to the manufacturer's instructions. Briefly, 1µg of total RNA was used to

produce first-strand cDNA with OligodT to a final volume of 40 µl in RNase free water and stored at -20º C.

Real-time PCR was performed on a thermal cycler, the TaqMan 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with 7500 Fast Sequence Detection software, Relative Quantification. Combined primers and probes were purchased from Applied Biosystems (Sweden); Hs00184491_m1 was used to detect Pgp, Hs00184979_m1 to detect BCRP and Hs02512067_s1 to detect GSTπ, while 18S (Hs99999901_s1) was used as house-keeping gene. Standard curves were obtained by serial dilutions ranging from 1:1 to 1:100. PCR reactions were performed in 20 µl using the TaqMan Universal PCR Master Mix (Applied Biosystems) primer and probe and 2 µl cDNA. Experiments were carried out in duplicate and the mean value was used as the result.

PGP PROTEIN EXPRESSION (PAPER IV)

Western blot

Pelleted samples were lysed in 10 mM TRIS/HCl lysis buffer with 150 mM NaCl, 1 % Nonidet P-40, 1 mM EDTA, 50 mM Sodium fluoride, 40 mM β-glycerophosphate, 1 mM sodium orthovanadate and protease inhibitors such as phenylmetylsulphonyl fluoride, leupeptin, pepstatin and aprotinin (all diluted to a final concentration of 1µg/ml), at 4°C for 10 minutes and stored at -80°C in cryo-preservation tubes (Sarstedt AG & Co, Nümbrecht, Germany). Protein concentrations were determined with the DC Protein Assay Kit (Bio-Rad Laboratories, Hercules CA, USA) as absorbance at 750 nm on a Multiscan Ascent (Thermo Labsystems, Stockholm, Sweden). Protein lysate, 20 µg in urea buffer (8 M urea, 170 mM SDS, 0.5 mM bromphenol blue, 455 mM dithiotretiol and 50 mM Tris (pH 6.8 adjusted with HCl)) with 30 mM DTT (Sigma-Aldrich), in a lysate to buffer ratio of 2:1, as well as a molecular weight marker (Precision Plus Protein Standards Dual Color, Bio-Rad), was separated on 7 % SDS gel at 140V for 1 hour. After electrophoresis, proteins were transferred to a PVDF membrane at 30V over night. Nonspecific binding was blocked by 2 % bovine serum albumine (Roche Diagnostics GmbH, Indianapolis IN, USA) in TBS buffer. Membranes were incubated with P-glycoprotein specific primary mouse monoclonal antibody C219 (Abcam, Cambridge, UK), diluted 1:500 in TBS containing 0.1% Tween (TBS-T, Sigma-Aldrich), at 4°C over-night. Blots were washed thoroughly in TBS-T and incubated in secondary horseradish peroxidase (HRP)-conjugated goat-anti-mouse IgG antibody C1607, dilution 1: 1000 (Santa Cruz Biotechnology, Santa Cruz CA, USA), at room temperature for 1 hour. After a final wash in TBS-T, horseradish peroxidase was detected using the Immun-Star HRP Substrate kit from Bio-Rad and Molecular Imager ® ChemiDoc™ XRS, as described in the manufacturer's instructions.

Flow cytometry

Pelleted cells were incubated with FITC-conjugated anti-Pgp (clone 17F9) and 7AAD (BD Biosciences, Stockholm, Sweden) for 10 minutes at room temperature in the dark. Cells were then resuspended in 400 µl of PBS, transferred to 5 ml Falcon test tubes (VWR) and analyzed immediately using an EPICS® *ALTRA*™ (Beckman Coulter, Fullerton CA, USA) flow cytometer equipped with an Argon laser (488 nm) and Expo32 software (Beckman Coulter). Pgp expression on drug-exposed cells was determined as mean fluorescence intensity (MFI) in relation to Pgp expression in cells cultured in RPMI only, at each point of time. Flow cytometric analyses were performed in duplicate for each sample and the mean value of the duplicates was used as the result.

$P14^{ARF}$ mRNA EXPRESSION (PAPER V)

RNA was isolated using a QuickGene RNA cultured cell kit with Nucleic Acid Isolation System QuickGene-810 (Science Imaging Scandinavia AB, Nacka, Sweden) and RNeasy plus mini kit (Qiagen AB, Solna, Sweden) according to the manufacturer's instructions. 12 µl of total RNA were used for cDNA synthesis with the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics Scandinavia AB, Bromma, Sweden) in a total volume of 20 µl, using 1 µl of Anchored-oligo primer, 4 µl of Transcriptor RT Reagent Buffer, 0.5 µl of Protector RNase Inhibitor, 2 µl of Deoxynucleotide Mix and 0.5 µl of Transcriptor reversed Transcriptase. 25 µL reaction volumes were prepared for real time PCR. They contained 12.5 µL of Sybr®Green PCR Master Mix (Applied Biosystems, Stockholm, Sweden), 1 µL of each primer at a final concentration of 300 nmol/L, 5.5 µL H_2O and 5 µL of cDNA product, diluted 1:2 in H_2O. The reactions were run on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Stockholm, Sweden). Thermal cycling conditions were as follows: 50°C for 2 minutes, 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The primers used for $p14^{ARF}$ amplification were: forward 5´-CCC TCG TGC TGA TGC TAC TGA-3´ and reverse 5´-ACC ACC AGC GTG TCC AGG AA-3´. Amplification of the house-keeping gene abl was performed using the primers forward 5´-CGA AGG GAG GGT GTA CCA TTA C-3´ and reverse 5´-CGT TGA ATG ATG ATG AAC CAA CTC-3´. All primers were purchased from DNA Technology A/S, Aarhus, Denmark. Standard curves were constructed by serial dilution of the amplification product from HeLa cells. Experiments were performed in triplicate and the mean values were used for further calculations.

MUTATIONS OF *NPM1*, *FLT3* AND *TP53* (PAPER V)

Detection of insertion mutations in exon 12 of the *NPM1* gene was performed by PCR as described previously[191], using 10 ng of genomic DNA and *NPM1* forward primer 5´-TTA ACT CTC TGG TGG TAG AAT GAA-3´ and reverse primer 5´-ACC ATT TCC ATG TCT GAG CACC-3´. The PCR products were separated by capillary electrophoresis in an ABI 3130 XL genetic analyzer (Applied Biosystems, Foster City,

CA, USA) and fragment sizing was performed using GeneMapper 4.0 software (Applied Biosystems). Patient samples with *FLT3*-ITD (internal tandem duplication) was identified using PCR, as previously described[192].

Sequencing of exons 5–9 on *TP53* was performed on a capillary sequencer (MegaBASE 1500, GE Healthcare, Uppsala, Sweden) using DYEnamic ET Dye Terminator Kits (US81090, GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions. Staden Software was used for sequence assembly. All sequences were scored manually for the presence of mutations.

For fluorescence *in situ* hybridization (FISH) analyses, fresh bone marrow cells were cultured for 2 days, and Vysis (Abott-Vysis Inc., Downers Grove, IL, USA) was used as *TP53* probe. Loss of one signal in less than 5% of the cells was not regarded as representing a clone.

STATISTICS

Paper I

Student's *t*-test for paired samples was used to compare the expression of topo IIα in S/G2/M versus G0/G1, and Fisher's exact test was used to compare clinical response between patients with more or less than 20% topo IIα-positive cells in G0/G1.

Paper II

The Mann-Whitney test was used to compare mRNA and protein expression in the groups defined by clinical response, cytogenetic risk and *de novo*/secondary AML. Survival curves were calculated according to Kaplan-Meier, and the log rank test was used for comparison of survival. Student's *t*-test for independent groups was used to compare chemosensitivity *in vitro* in different groups of patient samples. Statistics were calculated using SPSS 11.5 for Windows (SPSS Inc, Chicago, Illinois).

Paper III

The Mann-Whitney test was used to compare gene expression in the two groups defined by clinical response. Survival curves were calculated according to Kaplan-Meier. The log-rank test was used to compare survival between responders with high and low expression of BCRP mRNA. To control for the effects of age and white blood cell count (WBC), a multivariate Cox regression analysis was performed with BCRP mRNA expression, age and WBC as explanatory variables. Statistics were calculated using SPSS 11.5 for Windows (SPSS Inc, Chicago, IL, USA).

Paper V

Kaplan–Meier analysis was used to estimate overall survival (OS) and the log-rank test to determine significance. Multivariate analysis was performed using the Cox

proportional hazards model. Student's unpaired *t*-test was used to compare groups in terms of differences in sensitivity in the *in vitro* drug panel.

RESULTS

TOPO IIα PROTEIN EXPRESSION AND CELL CYCLE PHASES (PAPER I)

In the patient samples, the majority of cells were in the G0/G1 cell cycle phases (mean 88% SD ±5.3%), rather than in the S/G2/M phases (mean 11% SD ±5.3%).

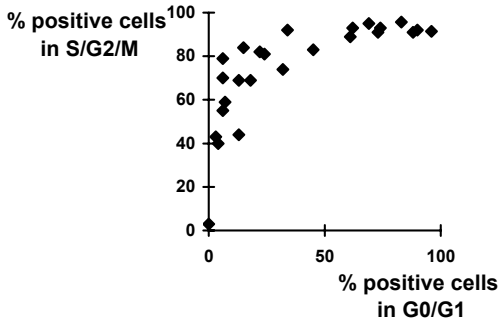

Figure 5. Expression of topo IIα in cells in S/G2/M versus G0/G1 cell cycle phases.

The control samples from ten healthy volunteers had a mean of 2.3% topo IIα positive cells. The overall expression of topo IIα in the patient samples varied between 0% and 99% positive cells, with a mean of 36%. The mean expression of topo IIα was higher in S/G2/M (76% positive cells) than in G0/G1 (39%) ($p<0.001$). Twenty-one of 25 samples displayed >50% topo IIα positive cells in S/G2/M, whereas 9 showed >50% and further 5 showed >20% topo IIα positive cells in G0/G1. All samples with >20% topo IIα positive cells in G0/G1 also displayed >70% positive cells in S/G2/M (Figure 5).

Thirteen patients were evaluable for clinical response. Six of eight patients with >20 % topo IIα positive cells in G0/G1 entered CR, while only one of five patients with <20 % positive cells entered CR ($p=0.10$) (Figure 6).

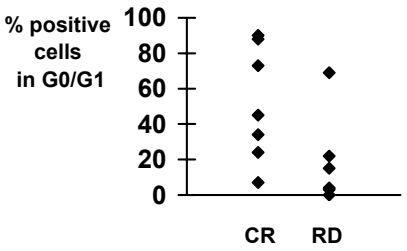

Figure 6. Expression of topo IIα in G0/G1 versus clinical response.

TOPO IIα EXPRESSION AND CYTOTOXICITY *IN VITRO* (PAPER II)

Topo IIα mRNA

Topo IIα mRNA expression varied widely, with relative values ranging from 0.04 to 15.32 arbitrary units (median 1.10) and displaying a skewed distribution. No statistically significant differences were found between topo IIα mRNA expression in samples from patients in different cytogenetic risk groups or from patients with *de novo* versus secondary AML. There was also no association between topo IIα mRNA expression and either age or WBC.

Topo IIα protein

The overall expression of topo IIα varied, with a median of 23% positive cells (range 0-99%) and a bimodal distribution. Cells in the S/G2/M cell cycle phase expressed topo IIα to a higher extent than cells in the G0/G1 cell cycle phase; median 70.5% (range 0-98%) positive cells versus 20.5% (range 0-99%).

Overall, samples from patients with secondary AML (n=9) showed topo IIα positivity with a median of 6% (range 0-95%), while samples from patients with *de novo* AML (n=45) were topo IIα positive with a median of 24% (range 0-99%) (p=0.04). For cells in G0/G1, the median was 6% (range 0-96%) for secondary AML and 32% (range 0-99%) for *de novo* AML (p=0.04), while the corresponding figures for cells in S/G2/M were 55% (range 0-91%) for secondary AML and 74% (range 0-98%) for de novo AML (n.s.). There was no correlation between age or WBC and percentage of topo IIα positive cells nor were there any statistically significant differences between cytogenetic risk groups.

Topo IIα mRNA and protein

Topo IIα mRNA expression and percentage of topo IIα positive cells were determined in 24 patients. There was no correlation between mRNA expression and percentage of topo IIα positive cells.

Cytotoxicity in vitro

Patient samples with low topo IIα mRNA expression tended to be less sensitive to topoisomerase poisons, but not to other drugs (Figure 7). This tendency was stronger for samples with a low percentage of topo IIα protein positive cells, and reached statistical significance for daunorubicin and etoposide (Figure 8). Since RT-PCR and flow cytometry were performed in different patient groups, it was not possible to compare the groups tested for topo IIα mRNA and percentage of topo IIα protein positive cells, respectively.

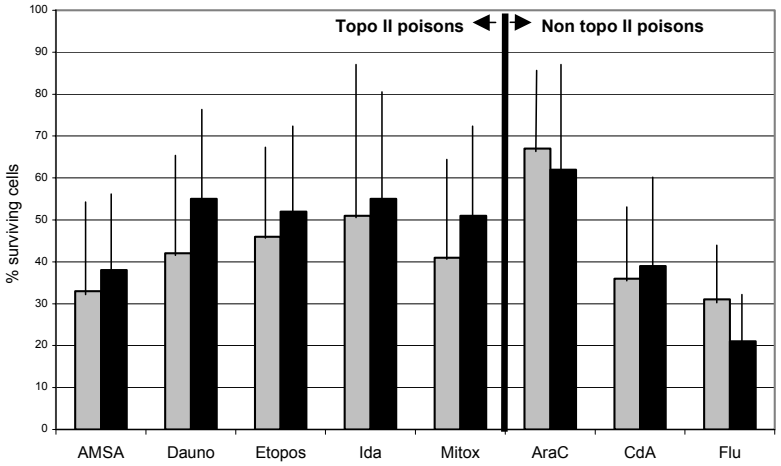

Figure 7. In vitro sensitivity to different cytostatic drugs in patient samples with high (grey bars) or low (black bars) expression of topo IIα mRNA. The groups of high and low expression were defined by dividing the group of patients tested for each drug into two groups, with median expression as cut-off. Sensitivity is expressed as a percentage of living cells after incubation with the drug, i.e. a relatively low percentage indicates relatively more sensitive cells. Vertical lines indicate standard deviation for each sample. AMSA=amsacrine (n=44), Dauno=daunorubicin (n=46), Etopos=etoposide (n=31), Ida=idarubicin (n=44), Mitox=mitoxantrone (n=43), AraC=cytarabine (n=37), CdA=cladribine (n=31), Flu=fludarabine (n=25).

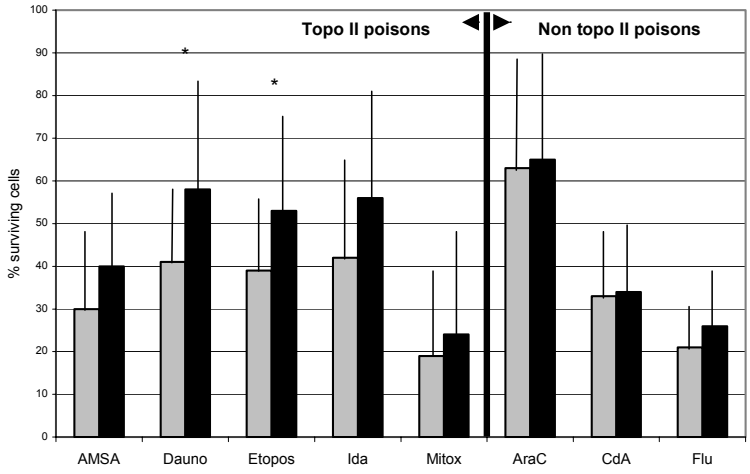

Figure 8. In vitro sensitivity to different cytostatic drugs in patient samples with high (grey bars) or low (black bars) percentage of topo IIα protein-positive cells. The groups of high and low expression were defined by dividing the group of patients tested for each drug into two groups, with median percentage as cut-off. Sensitivity is expressed as a percentage (mean value of the group) of living cells after incubation with the drug, i.e. a relatively low percentage indicates relatively more sensitive cells. Vertical lines indicate standard deviation for each sample. AMSA=amsacrine (n=39), Dauno=daunorubicin (n=37), Etopos=etoposide (n=38), Ida=idarubicin (n=38), Mitox=mitoxantrone (n=32), AraC=cytarabine (n=34), CdA=cladribine (n=29), Flu=fludarabine (n=32). The difference in sensitivity was statistically significant for daunorubicin and etoposide (p=0.02 and p=0.04).

TOPO IIα EXPRESSION AND CLINICAL OUTCOME (PAPER II)

Of the patients tested for topo IIα mRNA expression, 49 were evaluable for reponse to induction treatment; 37 were classified as responders and 12 as non-responders. Median topo IIα mRNA expression was almost the same in the responder group (1.26; range 0.04-15.32) as in the non-responder group 1.08; 0.24-6.13).

Survival was investigated by dividing the patients into two groups with the median topo IIα mRNA expression as cut-off. Median survival was 21 months (95% CI: 3-39) in the group with low topo IIα mRNA expression and 15 months (95% CI: 6-25) in the group with high topo IIα mRNA expression (n.s.). Similar results were obtained when patients who were not evaluable for response to induction therapy were excluded (data not shown).

Of the patients tested for topo IIα protein expression, 40 were evaluable for response to induction treatment; 30 were classified as responders and 10 as non-responders. There was no significant difference in percentage of topo IIα positive cells overall between samples from responders and non-responders (median 35% and 25% positive cells respectively) and the same was true for cells in G0/G1 (29% and 18.5%) and S/G2/M (67.5% and 71.5%). Survival was compared between patients with low and high topo IIα expression, using the median (23% positive cells) as cut-off. Median survival was 7 months (95% CI: 0-16) in the group with <23% positive cells and 10

months (95% CI: 6-14) in the group with >23% positive cells (n.s.). Results regarding survival were similar when patients who were not evaluable for response to induction treatment were excluded (data not shown).

BCRP mRNA EXPRESSION IN AML (PAPER III)

The expression of BCRP mRNA varied widely, with a skewed distribution. BCRP mRNA expression is presented relative to the BCRP mRNA expression of the drug-sensitive cell line MCF-7 (Figure 9). Ten patient samples expressed more BCRP mRNA than the MCF-7 cells (2.30–76.20 times as much) and seven expressed almost as much (0.5–1.5 times) as the MCF-7 cells. Twenty-three samples expressed less BCRP mRNA than MCF-7 cells and ten of these expressed <0.05 times the expression of MCF-7 cells. There was no association between BCRP mRNA and age, WBC or cytogenetic risk group.

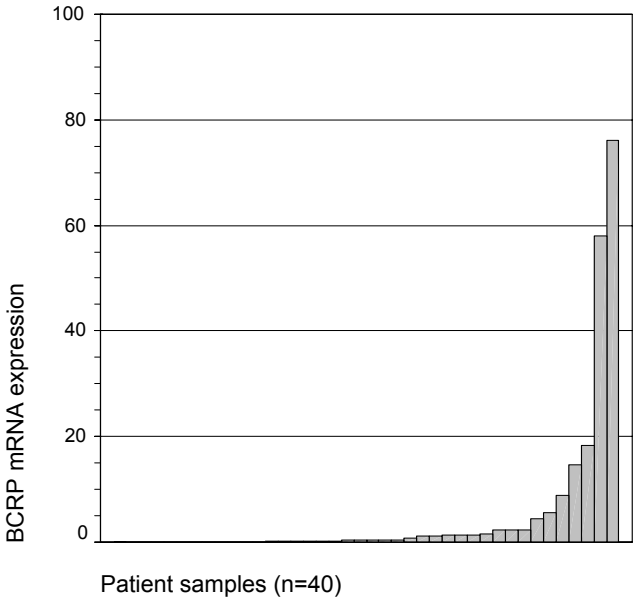

Figure 9. BCRP mRNA expression in 40 AML patient samples. The expression is given relative to BCRP mRNA expression of the MCF-7 cell line. The expression was highly variable, and a majority of patients (n=23) expressed <0.5 times the level expressed by MCF-7 cells.

BCRP mRNA EXPRESSION AND CLINICAL OUTCOME (PAPER III)

Twenty-eight patients were classified as responders and 12 as non-responders. Median BCRP mRNA expression was higher in the responder group (0.30 [range 0.00–76.20] times the expression in MCF-7 cells) than in the non-responder group

(0.12 [range 0.00–2.30] times the expression of MCF-7 cells) but the difference was not statistically significant (p=0.6, Figure 10).

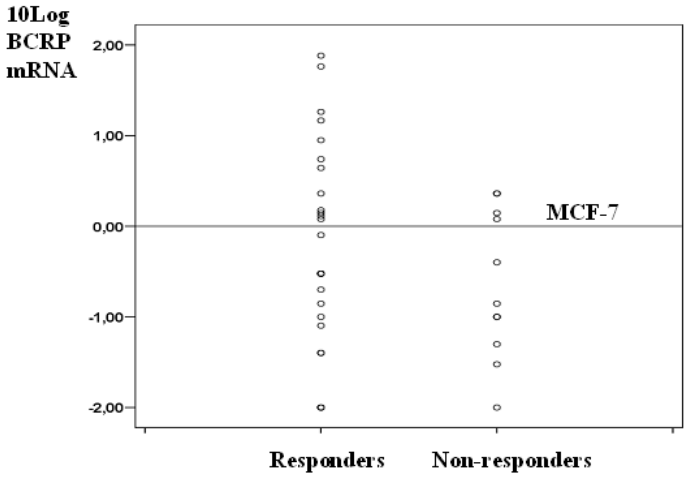

Figure 10. BCRP mRNA expression in responders (n=28) and non-responders (n=12). BCRP mRNA expression is presented as ^{10}Log and related to BCRP mRNA expression of cell line MCF-7, indicated by the horizontal line.

To investigate a possible association between BCRP mRNA expression and overall survival, we divided the patients into two groups with the median BCRP expression as cut-off. Mean survival was 60 months (SEM 14 months) for the low BCRP mRNA group and 45 months (SEM 14 months) for the high BCRP mRNA group, but the difference was not statistically significant (p=0.2) (Figure 11). The corresponding median survival times were 26 months (95% CI 0-57 months) and 15 months (95% CI: 8-21 months).

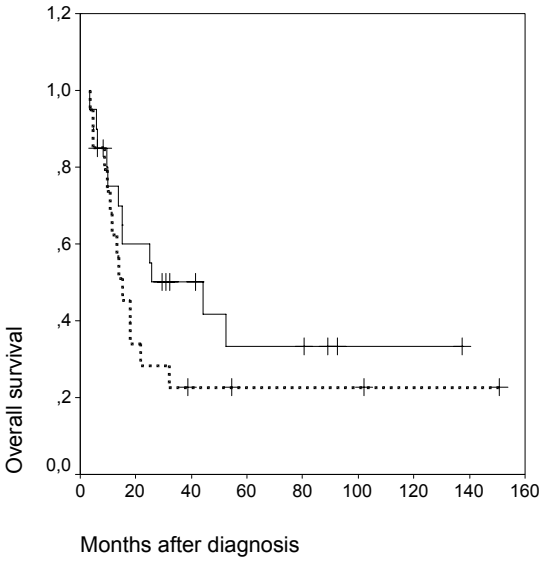

Figure 11. Overall survival for the 20 AML patients with high BCRP expression (dotted line) and the 20 AML patients with low BCRP mRNA expression (continuous line). There was no statistically significant difference in survival between the two groups (mean 60 months, SEM 14 months vs. mean 45 months, SEM 14 months) (p=0.2).

When survival in the 28 responders was analyzed separately, the 14 patients with low BCRP mRNA expression had a significantly longer survival (mean 74 months, SEM 16 months) than the 14 patients with high BCRP mRNA expression (mean 38 months, SEM 15 months), p=0.047 (Figure 12). The corresponding median survival times were 52 months (95% CI: 15-90 months) and 18 months (95% CI: 0-26 months), respectively. In this subgroup two patients had a favourable karyotype (inv 16 and t15;17, respectively) and 23 patients had an intermediate karyotype, based on the cytogenetic risk groups derived from the MRC AML 10 Trial[15]. Cytogenetic data were missing in the remaining three patients. Karyotype was therefore considered not to be a confounding factor in this subgroup.

For further analysis of the responder subgroup, a Cox regression analysis was performed. The patients were categorized into groups based on median BCRP (median value in this subgroup was 0.30 (range 0.00–76.20), age (median age was 66 years [range 22–79 years]) and WBC (median value was 18 x 10^9/L [range 0–108 x 10^9/L]). In a multivariate analysis, high BCRP mRNA expression was associated with a hazard ratio for death of 3 (95% CI: 1.1–8.8), while age and WBC did not significantly influence the risk of death (hazard ratios: 1.4 and 1.6, respectively).

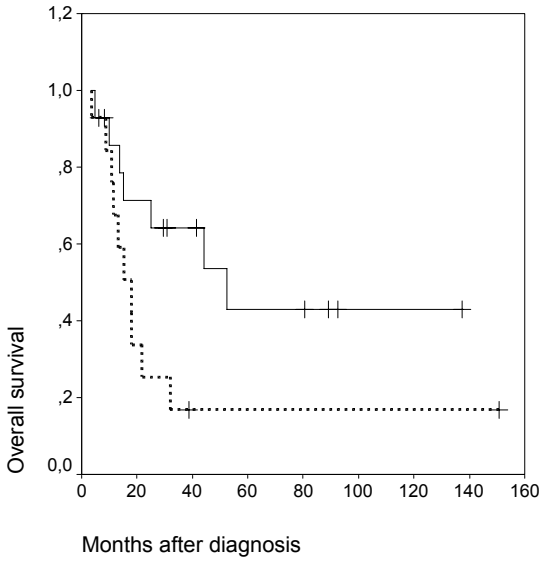

Figure 12. Survival of the 28 patients classified as responders. In this patient group, the 14 patients with low BCRP mRNA expression (continuous line) had a statistically significantly longer survival (mean 74 months, SEM 16 months) than the 14 patients with high BCRP mRNA expression (mean 38 months, SEM 15 months) (dotted line) (p=0.047).

PGP, BCRP AND GSTII mRNA DURING DRUG EXPOSURE *IN VITRO* (PAPER IV)

HL-60 S: Exposure to cytarabine for 10 minutes resulted in an increase in Pgp mRNA expression to 1.7 times that of cells cultured in RPMI alone (Figure 13), and this increased level remained unchanged throughout 36 hours of incubation. No rapid increase in Pgp mRNA expression was observed in HL-60 S cells after incubation with daunorubicin (Figure 13), but an increase was observed after 24 hours. For GSTπ mRNA, incubation with daunorubicin showed a trend towards increased levels from the initiation of treatment (Figure 13) and a 1.8-fold increase was seen after 12 hours. After incubation with cytarabine a minor decrease in the GSTπ mRNA level was seen after 10 minutes incubation (Figure 14), and the subsequent time points also showed only minor changes.

HL-60 R0.5: A 3.1-fold increase in Pgp mRNA expression was detected after 10 minutes exposure to cytarabine (Figure 13). This increased expression declined and after 2 hours no increase was evident. After incubation with daunorubicin for 10 minutes, a 2.8-fold increase in Pgp mRNA expression was observed (Figure 13) after which the expression declined. After 4 hours there was no further increase compared to cells cultured in RPMI alone. Exposure to daunorubicin resulted in a 2.4-fold increase in GSTπ mRNA after 10 minutes (Figure 14) and the increased levels were sustained throughout the incubation time. A 3.4-fold increase in GSTπ mRNA was observed after 10 minutes exposure to cytarabine (Figure 14). This increase declined during the incubation time and was undetectable after 36 hours.

HL-60 R5: After incubation with cytarabine for 10 minutes, a 1.9-fold increase in Pgp mRNA expression was observed (Figure 13). Following a period of decline the expression further increased to 2.4-fold at 12 hours and 4.5-fold at 36 hours. At all time points during the 36 hours of incubation with daunorubicin, only negligible changes in Pgp mRNA expression was observed in comparison to cells cultured in RPMI alone. Exposure to daunorubicin resulted in a negligible increase in GSTπ mRNA after 10 minutes (Figure 14) but after 1 hour of exposure the levels of GSTπ mRNA were 1.5-fold higher than in cells cultured in RPMI alone and further increased during the incubation time to a maximum of 4.0-fold after 36 hours. After 10 minutes exposure to cytarabine there was a 4.0-fold increase in GSTπ mRNA (Figure 14). This increase remained and further increased to 5.8-fold after 36 h.
BCRP mRNA was undetectable in all three cell lines, regardless of exposure time, throughout the experiment.

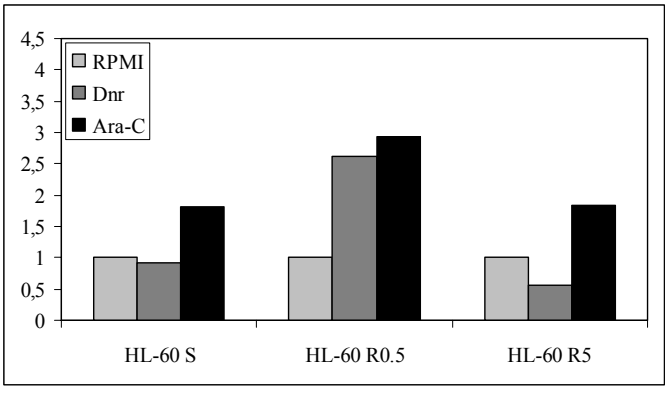

Figure 13. Expression of Pgp mRNA, determined by real-time RT-PCR, in HL-60 S, HL-60 R0.5 and HL-60 R5 cells after 10 min culturing in RPMI (grey bar) or after 10 min incubation with daunorubicin (dnr, dark grey bar) or cytarabine (Ara-C, black bar). The mRNA expression in cells cultured in RPMI was arbitrary set to 1 for each cell line so the mRNA levels are only comparable within the cell line.

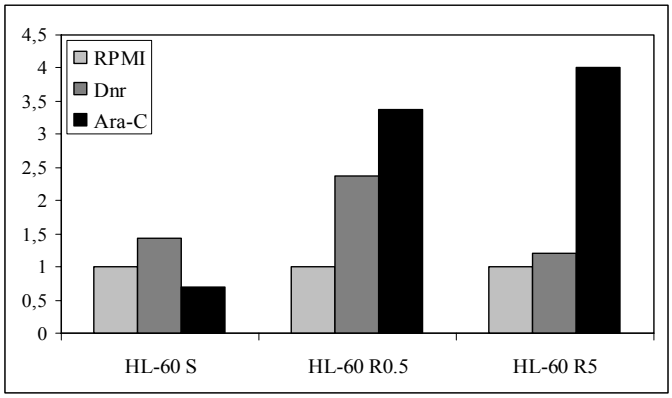

Figure 14. Expression of GSTπ mRNA, determined by real time RT-PCR, in HL-60 S, HL-60 R0.5 and HL-60 R5 cells after 10 min culturing in RPMI (grey bar) or after 10 min incubation with daunorubicin (dnr, dark grey bar) or cytarabine (Ara-C, black bar). The mRNA expression in cells cultured in RPMI was arbitrary set to 1 for each cell line so the mRNA levels are only comparable within the cell line.

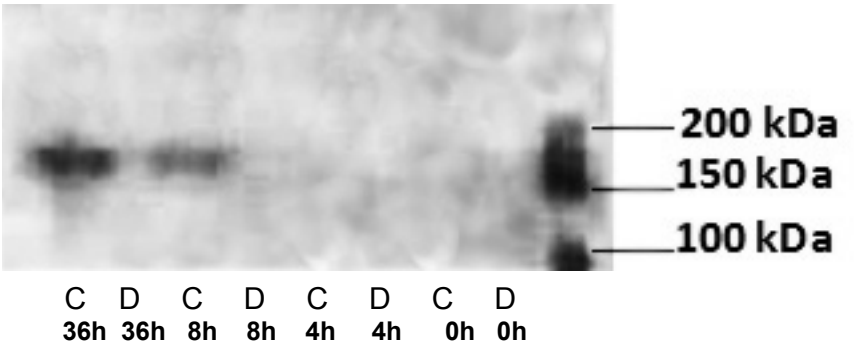

Figure 15. Expression of Pgp in HL-60 S cells, determined by Western blot at 0, 4, 8 and 36 h after initiation of drug exposure. An increase in Pgp from undetectable to detectable levels was seen after 8 hours of incubation with cytarabine (A), while incubation with daunorubicin (D) did not result in any detectable Pgp during 36 h.

PGP PROTEIN DURING DRUG EXPOSURE *IN VITRO* (PAPER IV)

Western blot

In HL-60 S cells, incubation with cytarabine resulted in detectable Pgp protein expression after 8 hours of exposure, while no Pgp was detectable during 36 hours of exposure to daunorubicin (Figure 15).

The high levels of Pgp expressed by HL-60 R0.5 and HL-60 R5 cells from the start of incubation did not change throughout the entire 36 hours of exposure to cytarabine or daunorubicin.

Flow cytometry

All results for HL-60 S have been adjusted to viable cells only. Cell death in HL-60 R0.5 and HL-60 R5 cells was negligible during 24 hours.

HL-60 S cells did not display any detectable Pgp protein when cultured in RPMI, while HL-60 R0.5 and HL-60 R5 cells expressed 1.9-fold and 3.2-fold levels of Pgp respectively, compared to HL-60 S (Figure 16).

In HL-60 S cells exposed to cytarabine or daunorubicin, no increase in Pgp expression was detected during 24 hours of incubation.

In HL-60 R0.5 cells incubated with cytarabine, a 1.2-fold increase in Pgp expression was seen after 24 hours, as compared to cells cultured in RPMI. There was no increase in Pgp expression, in cells exposed to daunorubicin (Figure 17).

HL-60 R5 cells showed a 1.3-fold increase in Pgp expression after 24 hours of exposure to cytarabine (Figure 18), but no increase after exposure to daunorubicin.

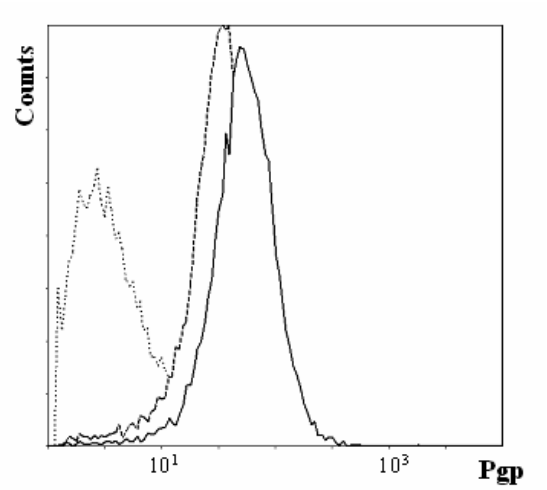

Figure 16. Pgp protein expression in HL-60 S (dotted line), HL-60 R0.5 (broken line) and HL-60 R5 (continuous line) as determined by flow cytometry. The 1.9- or 3.2-fold Pgp levels expressed by HL-60 R0.5 and R5, as compared to the Pgp-negative HL-60 S cell line, correspond to an 80- and 858-fold resistance to daunorubicin, respectively, as compared to the HL-60 S cell line[186].

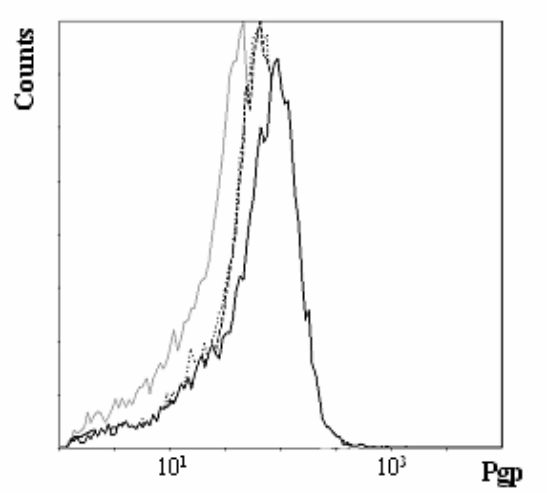

Figure 17. Pgp protein expression determined by flow cytometry in the HL-60 R0.5 cell line before incubation (grey line), after 24 h culturing in RPMI (dotted line), after 24 h incubation with daunorubicin (broken line) and after 24 h incubation with cytarabine (continuous line).

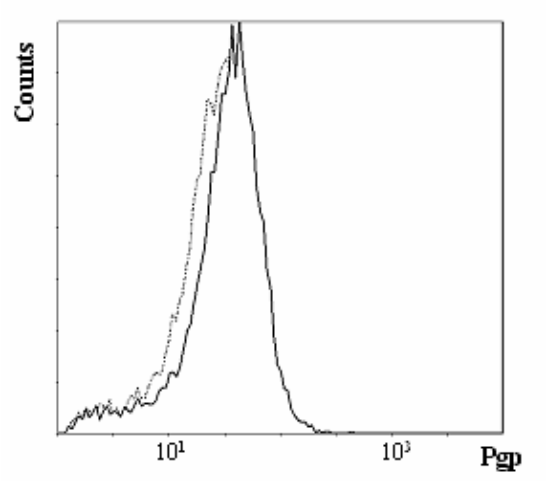

Figure 18. Pgp protein expression determined by flow cytometry in the HL-60 R5 cell line after 24 h culturing in RPMI (dotted line) and after 24 h incubation with cytarabine (continuous line).

$P14^{ARF}$ mRNA IN AML WITH NORMAL KARYOTYPE (PAPER V)

Real-time RT-PCR was performed in all 57 cases. However, in 9 patients the level of $p14^{ARF}$ mRNA could not be determined because of either inadequate material or technical error. $P14^{ARF}$ mRNA expression displayed a skewed distribution with a median value of 0.05. The 25^{th} and 75^{th} percentiles of $p14^{ARF}$ expression were 0.006 and 0.26 respectively. Values were categorized as being above or below the 75^{th} percentile for the study population. Twelve patients showed high expression of

$p14^{ARF}$ (>0.26) and 36 displayed low expression (Table VI). The expression of $p14^{ARF}$ in relation to *NPM1/FLT3* status is shown in Table V.

Mutated *FLT3*-ITD (*FLT3*-ITD+) was detected in 21 out of 57 patients (37%) and mutated *NPM1* (NPM1+) in 29 (51%). Eighteen patients (32%) were found to be *NPM1*+/*FLT3*-ITD- (mean age 65 [38–82], 3-year OS: 71%). Ten patients were found to be *NPM1*- /*FLT3*-ITD+ (mean age 59 [43–82], 3-year OS: 24% (Table V). FISH analysis of *TP53* was performed in 38 patients. Hemizygous *TP53*-deleted clones were not found in any of the patients. Sequencing of exons 5–9 of the *TP53* gene was performed in 15 patients without any evidence of mutation.

Table V. *NPM1/FLT3* status versus 3-year OS and $p14^{ARF}$ levels

	3-year OS (%)	<0.26 rtPCR/ $p14^{ARF}$ % (*n*)	≥0.26 rtPCR/ $p14^{ARF}$ % (*n*)	Unknown rtPCR/ $p14^{ARF}$ (*n*)	Sex male/ female	Total % (*n*)
NPM1+, *FLT3*-	71	39 (14)	17 (2)	2	8/10	32 (18)
NPM1+, *FLT3*+	31	17 (6)	17 (2)	3	5/6	19 (11)
NPM1-, *FLT3*-	26	30 (11)	41 (5)	2	6/12	32 (18)
NPM1-, *FLT3*+	24	14 (5)	25 (3)	2	4/6	17 (10)
All	36	100 (36)	100 (12)	9	23/34	100 (57)

Table VI. $P14^{ARF}$ levels versus 3-year OS and *NPM1/FLT3* status

	3y OS (%)	n	Mean age (range)	*NPM1*+, *FLT3*- (n)	3y OS (%)	*NPM1*+,*FLT3*- not included (n)	*3y OS (%)*
$p14^{ARF}$ <0.26	31	36	64 (20–84)	14	63	22	12*
$p14^{ARF}$ ≥0.26	65	12	61 (35–79)	2	100	10	54
Total	36	48	63 (20–84)	16	70	32	28

*Survival was estimated at 2.5 years

IMPACT OF $P14^{ARF}$ mRNA EXPRESSION ON SURVIVAL (PAPER V)

Overall survival was analyzed in all patients (n=57). Patients treated by means of allogeneic stem cell transplantation (n=4) were censored at time of transplantation.

As expected, *NPM1*+/*FLT3*-ITD- patients showed better OS compared with all the others (p=0.027) (Figure 19A). The overall median survival time for all patients was 15 months, with 3-year OS of 36%. Using 0.26 as a cut-off point for $p14^{ARF}$ mRNA expression, 3-year OS was 65% in patients with high expression (n=12) and only 31% in those with low expression (n=36) (p=0.046) (Figure 19B). When *NPM1*+/*FLT3*-ITD- patients were excluded and the remaining patients separated into groups according to the expression of $p14^{ARF}$, the difference in OS remained significant (*p*=0.012, 3-year OS 54% *vs.* 12%) (Figure 19C and Table VI).

Cox's proportional hazard model was used to analyze the prognostic impact of $p14^{ARF}$ mRNA level and *NPM1*/*FLT3* status, and to exclude age and sex as confounding factors. Age (>60 years) was a predictor of outcome as regards OS in univariate analysis but it did not reach significance, and sex was not relevant with respect to OS. The adverse effect of a low level of $p14^{ARF}$ mRNA was shown to be independent of age and sex, and the same was true for *NPM1*/*FLT3* status (Table VII).

Table VII. Risk of death in terms of age, sex, p14ARF level and *NPM1/FLT3* status – a Cox regression model

Variable*	Patients (n)	Hazard ratio (95% CI) - risk of death	SE	p
Age (>60/<60 years)	57	1.01 (1.20–8.60)	0.01	0.158
Sex (male/female)	57	1.06 (0.32–1.73)	0.38	0.815
$p14^{ARF}$ <0.26/≥0.26	48	3.83 (1.13–9.44)	0.26	0.025
Absence/presence of NPM1+/FLT3-	57	4.86 (1.17–9.23)	0.51	0.004

*All variables were entered in the model as binary categorical variables

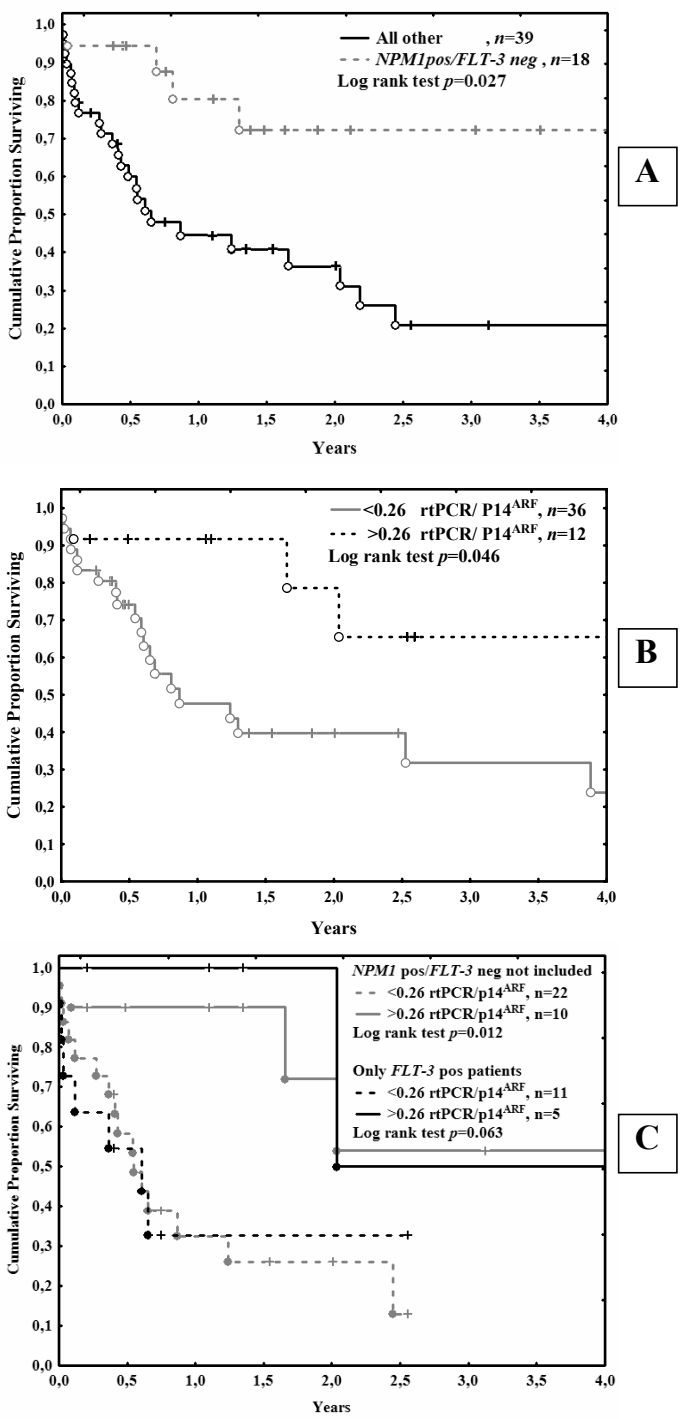

Figure 19. Overall survival of patients with: A) *NPM1*+/*FLT3*-ITD- *vs.* all other combinations (p=0.027); B) high and low expression of $p14^{ARF}$ mRNA (p=0.046); C) high and low expression of $p14^{ARF}$ mRNA with *NPM1*+/*FLT3*-ITD- patients excluded (p=0.012). Patients treated by means of BMT were censored at transplantation date.

$P14^{ARF}$ mRNA AND CYTOTOXICITY *IN VITRO* (PAPER V)

Conventional cytostatics

Initially, we tested the sensitivity of AML cells to chemotherapeutic drugs commonly used in the treatment of AML. When survival rates were calculated in comparison with untreated control cells, cytarabine killed on average 40% of the cells in samples with <0.26 $p14^{ARF}$ mRNA and 45% of the cells in samples with ≥0.26 $p14^{ARF}$ mRNA compared with untreated control cells (Figure 20A). The corresponding values for the other drugs were 52% and 58% for daunorubicin, 50% and 59% for etoposide and 56% and 65% for mitoxantrone. None of the differences were statistically significant.

PRIMA-1

Cells were then exposed to PRIMA-1 (5, 10 and 20 μM), which induced dose-dependent cytotoxic effects in all AML samples (Figure 20B). In contrast to the picture with conventional cytostatics, leukemic cells with low expression of $p14^{ARF}$ mRNA were more sensitive to PRIMA-1 5 μM than cells with high expression (p=0.046). The same tendency was seen at 10 μM (p=0.087), while PRIMA-1 20 μM showed very high toxicity in both groups without any obvious difference.

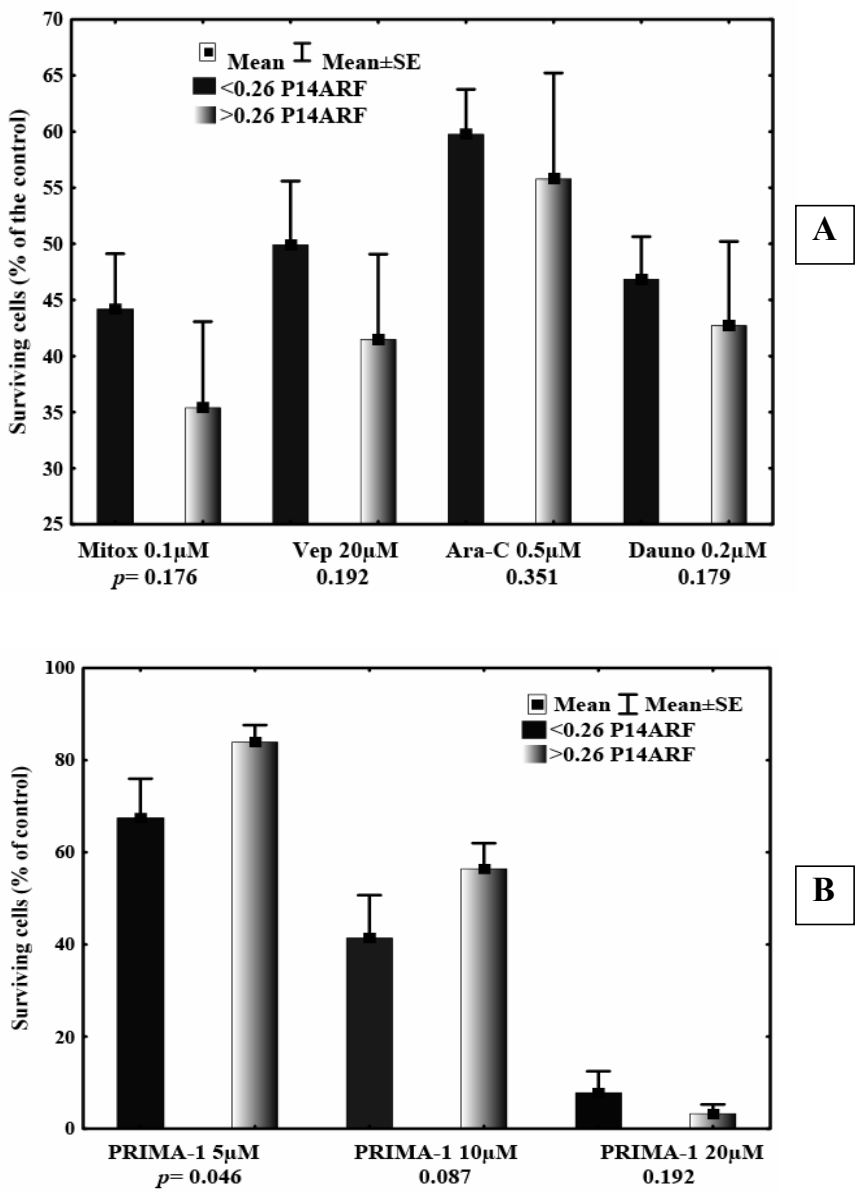

Figure 20. Cytotoxicity of a panel of antileukemic drugs on myeloblasts from AML patients. Samples with high expression of $p14^{ARF}$ mRNA are compared with those having low expression (A). Cytotoxicity of PRIMA-1 in AML cells with high *vs.* low expression of $p14^{ARF}$ mRNA (B).

DISCUSSION

TOPO IIα (PAPERS I-II)

Topo IIα in relation to cell cycle

In normal cells, topo IIα is mainly expressed in S/G2/M phases[137,193]; it is however possible that this enzyme could be differentially regulated in tumor cells[138,139]. There are also data indicating that topoisomerase poisons, in addition to their predominant effect in S/G2/M, also could have an effect in the G1 phase[154-156].

The study reported in paper I was initiated to investigate the expression of topo IIα in blood or bone marrow samples from patients with acute leukemia (22 AML, 3 ALL). A two-parameter flow cytometry method enabled us to separate cells according to DNA content. Diploid cells were categorized as G0/G1 and cells with more DNA content as S/G2/M, but it was not not possible to distinguish cells in active cell cycle (G1) from quiescent cells (G0). The majority of cells were shown to be in G0/G1. This is in accordance with other data on cell cycle distribution of leukemic cells[156], which also showed that a majority of G0/G1 cells were in G0. We found that topo IIα was significantly expressed in G0/G1, although, as expected, cells in S/G2/M displayed positivity for topo IIα to a higher degree.

Kaufmann *et al.*[152] investigated topo IIα content by Western blot in leukemic blasts from 140 patients with newly diagnosed AML and found overall low levels. Immunoperoxidase staining of marrow mononuclear cells from 40 of these patients showed a marked heterogeneity in topo IIα expression; in the vast majority of leukemic blasts the topo IIα was diminished or absent. The relation between the percentage of topo IIα positive cells and the percentage of S phase cells was determined in 11 patients. The frequency of topo IIα positive cells tended to increase as the number of cycling cells increased and, in contrast to our results, the authors concluded that there was no evidence of abnormal expression of topo IIα in relation to cell cycle in AML. However, our results, showing more topo IIα positive cells in S/G2/M, are in agreement with the finding that these cells also have the most pronounced expression. On the other hand, flow cytometry could be a more sensitive method than immunoperoxidase staining and we thus conclude that topo IIα is significantly expressed in the G0/G1 cell cycle phases in acute leukemia.

Although the nonselective effects of the cytostatic drugs constitute a major problem and dose-limiting factor in treatment of acute leukemia, these drugs show an obvious selectivity for malignant cells. The reason for this selectivity is not clear. The topo IIα content of the G0/G1 cells may give a partial explanation, regarding the effect of topoisomerase poisons.

Topo IIα and drug sensitivity in vitro

As already mentioned, interaction with topo IIα is considered the main cytotoxic mechanism exerted by anthracyclins as well as by amsacrine or etoposide[40]. However, results concerning the relationship between topo IIα expression or activity

and drug sensitivity are inconsistent. Regarding leukemic cell lines, topo IIα mRNA, protein and activity have been positively correlated to drug sensitivity by some authors[143,146], while others have reported contradictory results[194,195]. As for clinical AML samples, studies of topo IIα protein[152] or activity[195] and drug sensitivity *in vitro*, have been negative. Our results suggest an association between relatively high topo IIα mRNA expression and sensitivity to topoisomerase poisons *in vitro*, and show a statistically significant association between a relatively high percentage of topo IIα protein positive cells and sensitivity to daunorubicin and etoposide, both topoisomerase poisons, *in vitro*. These findings support the relevance of topo IIα in drug resistance in acute leukemia and the specific role for topo IIα as the main target for anthracyclines[38].

Topo IIα and clinical outcome

The analysis of paper I suggests that there may be an association between low percentage of topo IIα positive cells in G0/G1 and resistant disease, defined as not having achieved CR after at most two induction courses. However, the group of patients evaluable for clinical response in this paper was small and we were unable to verify this finding in our extended material (presented in paper II) of topo IIα protein expression in 40 patient samples or in the 49 patient samples in which topo II mRNA was determined. This negative result is in agreement with other studies which have investigated topo IIα mRNA[149-151,153]or protein[152] regarding response to induction treatment.

We also found no association between topo IIα mRNA expression and OS. This is in accordance with Galmarini *et al.*[150], who investigated the expression of topo IIα mRNA by RT-PCR in blood or bone marrow samples from 123 patients with newly diagnosed AML. The authors chose to consider expression of topo IIα mRNA as a dichotomous variable, that is samples were considered positive or negative for topo IIα expression, with 37% being positive. Galimberti *et al.*[149] conducted an RT-PCR study of 35 AML patients, 43 % of whom were positive for topo IIα by RT-PCR and again found no association with OS. Lohri *et al.*[153] also used RT-PCR to determine topo IIα mRNA expression in samples from AML patients at diagnosis in 57 patients. Like us, they used median expression of topo IIα mRNA as a cut-off for survival comparisons. However, in contrast to our results, they found that low topo IIα mRNA expression was associated with shorter OS. While our study included 58 patients tested for topo IIα mRNA, only 49 of them were evaluable for clinical response to induction treatment and so our material was more limited. Lohri *et al* investigated a younger patient population with median age 43, while our evaluable patients were markedly older with median age 60. Response rates may also have differed since Lohri *et al.* reported complete and partial response of 88%, while CR rate was 75% in our material. Galmarini *et al.* reported a median age of 57 and a CR rate 79%, while Galimberti *et al.* reported a median age of 62 and a CR rate of 54%. Thus, one reason for diverging results may be higher age in the negative studies, including our own.

Besides the advantage of determining protein instead of mRNA, percentage of topo IIα protein positive cells could be assumed to be a more sensitive measurement than topo IIα mRNA expression, since heterogeneity between cells is taken into consideration to some extent. However, in our material of 60 patients investigated for percentage of topo IIα positive cells we found no association with survival, and this remained the case when the 40 patients evaluable for response to induction treatment were analyzed separately. There are no similar data on topo IIα protein expression and survival available for comparison.

Another factor which may have obscured the clinical impact of topo IIα is that we determined topo IIα mRNA and protein, while post-translational modifications could influence protein activity. Data indicate that cell-cycle specific phosphorylation is important in regulation of human topo IIα enzymatic activity[158,160]. Formation of "cleavable complexes" by topo II poisons was reduced in a mutated HL-60 leukemic cell line, with impaired phosphorylation of topo IIα, compared to unmutated HL-60[157]. Another mechanism that could enhance enzymatic activity is the formation, together with other proteins, of a "toposome" during mitosis[159].

From a theoretical point of view, it would be reasonable to assume that topo IIα expression or activity would have an impact on remission rate rather than on survival, at least if the enzyme is determined on average and the distribution of the enzyme is relatively homogeneous. *In vitro* studies, like our own, suggest that topo IIα is likely to influence the effect of topoisomerase poisons; and high average expression of topo IIα would presumably make initial debulking treatment more successful, meaning that the patient would enter CR more readily. Although CR is a prerequisite for long-term survival and cure, the emerging knowledge of leukemic stem cells and their putative importance for relapse, as outlined by Huff *et al*[196], makes it less likely that average topo IIα expression at diagnosis would have an impact on OS, at least for patients entering CR.

Topo IIα mRNA versus protein

In our material of 24 patients samples tested for both topo IIα mRNA and protein, there was no correlation between the two. A correlation is not necessarily to be expected, since the real time RT-PCR method determines topo IIα mRNA expression on average, while the flow cytometry method used in this study determines the proportion of cells that are classified as topo IIα positive.

BCRP (PAPER III)

Expression of BCRP in AML samples

In our study, BCRP mRNA expression was determined in relation to the BCRP mRNA level of the drug sensitive breast cancer cell line MCF-7, a technique also employed by Ross *et al.*[121] in a previous study of 21 patients with acute leukemia. Our material of 40 patients displayed BCRP mRNA levels similar to those presented by Ross *et al*. with a majority of patient samples expressing less BCRP mRNA than

MCF-7 cell samples. This is also in accordance with results reported by Suvannasankha *et al.*[124], who determined BCRP mRNA expression at diagnosis in AML (n=31), indirectly in relation to MCF-7 cells. Abbott *et al.*[113] investigated the expression of BCRP mRNA in samples from 40 patients with newly diagnosed AML in relation to BCRP mRNA levels of two cell lines, one drug-sensitive and one drug-resistant. The vast majority of samples expressed less BCRP mRNA than the drug-sensitive cell line; only two samples displayed BCRP mRNA levels exceeding those found in the drug-resistant cell line.

BCRP expression in AML has also been determined at the protein level. Suvannasankha *et al.*[124] found that 48% of the tested samples were stained by at least one of the three chosen antibodies against BCRP and that the cells were heterogeneously stained. Abbott *et al.*[113] also used flow cytometry in three of their patient samples and found very small BCRP-positive subpopulations. Van der Kolk *et al.*[127] and van der Pol *et al.*[122] used flow cytometry to determine BCRP protein expression in 38 and 25 samples, respectively, from patients with newly diagnosed AML and found that a majority displayed levels below the expression of BCRP in MCF-7 cells, which is in accordance with our results regarding BCRP mRNA.

BCRP expression and CR rate

We did not find any difference in BCRP mRNA expression between responders and non-responders to induction chemotherapy. This is in agreement with results reported by van den Heuvel-Eibrink *et al.*[128], who determined BCRP mRNA in samples from 154 AML patients >60 years, and Damiani *et al.*[130], who investigated BCRP protein in samples from 72 AML patients with normal karyotype. Both studies demonstrated a significant co-expression of BCRP with *MDR1* and Pgp, respectively; and co-expression of BCRP mRNA and *MDR1* mRNA was associated with low CR rate[128]. In contrast, Benderra *et al.*[68,129], who investigated both BCRP mRNA[129] (n=149) and protein activity[68] (n=85), demonstrated an association between high levels of BCRP mRNA or protein and low CR rate.

BCRP mRNA and survival

In our material, using the median expression of BCRP mRNA as cut-off, we could not demonstrate any statistically significant impact on OS. However, when responders to induction therapy were analyzed separately, a statistically significant difference in OS was revealed; high BCRP mRNA expression was associated with shorter OS, and this finding was suggested to imply a predictive value for prognosis in AML. Our findings were corroborated by Benderra *et al.*[129], who demonstrated that high BCRP mRNA expression was associated with shorter 4-year OS. However, this finding was only true regarding the patients receiving daunorubicin and mitoxantrone but not regarding the patients receiving idarubicin . The same authors also found that BCRP activity in the same way was a predictor for OS (all patients received daunorubicin in this study)[68]. Damiani *et al.*[130] found no predictive value for BCRP

regarding OS and van den Heuvel-Eibrink *et al.*[128] found no association between BCRP mRNA and survival in elderly patients with AML.

BCRP and clinical outcome in AML – general considerations

So far, studies of BCRP expression and clinical outcome have yielded conflicting results to some extent. In the early reports on BCRP in AML, average expression was shown to be in the range of or below the level found in the drug-sensitive cell line MCF-7, suggesting that BCRP has no clinical importance in AML. This is, however, based on the assumption that BCRP is homogeneously expressed among AML blasts. As the AML blast population has been shown to consist of different subpopulations with different phenotypes[9-11], it is reasonable to assume that BCRP is also heterogeneously distributed. This has already been demonstrated for the stem cell-like blasts[108,183] and thus clinical relevance for BCRP cannot be ruled out.

Our study of BCRP mRNA did not reveal any clear association with clinical outcome, although a predictive value for OS was suggested in the subgroup of responders to induction chemotherapy. We included 40 AML patients with a mean age of 57 years and with a CR rate of 70%. Our results in all patients are in agreement with those of van den Heuvel-Eibrink *et al.*[128], who included 154 AML patients with a mean age 67 years and a CR rate of 52%. In contrast, Benderra *et al.*[129] demonstrated a predictive value for BCRP mRNA in a study of 149 AML patients with a CR rate 66%. The mean age (52 years) in this patient cohort was considerably lower than in the study by van den Heuvel-Eibrink *et al*. and also, although less evidently so, than in our study. Benderra *et al.*[129] included only *de novo* AML, while we, like van den Heuvel-Eibrink *et al*., included a minority of secondary AML (10% and 20%, respectively).

Our results cannot be directly compared to the above mentioned BCRP protein data[68,130] but it may be noted that Damiani *et al.*[130] found no overexpression of BCRP protein in non-responders to induction chemotherapy, nor any association between BCRP expression and OS. In contrast, Benderra *et al.*[68] found that BCRP activity was significantly higher in non-responders and also demonstrated an association of BCRP with OS. Age seems to be fairly comparable in these studies, while, in addition to different ways of determining BCRP expression, Damiani *et al.* only included patients with normal karyotype. However, Damiani *et al.* did find that BCRP-positivity predicted shorter event-free survival, that is, in accordance with our results, BCRP expression was shown to be of clinical importance in the initially drug-sensitive group of patients.

Expression of Pgp or other ABC proteins were not included in our study, while the four above-mentioned clinical studies[68,128-130] in adult AML did include other ABC transporters to a variable extent. A correlation between BCRP mRNA and Pgp mRNA expression in AML has been demonstrated earlier[197]. Van den Heuvel-Eibrink *et al.*[128] found that co-expression of Pgp mRNA and BCRP mRNA was associated with lower CR rate, and the same pattern was evident in the two studies by Benderra

et al.[68,129], while Damiani *et al.*[130] found no such association. Benderra *et al.*[68] also found that MRP3 activity was associated with CR rate, both alone and in combination with Pgp and BCRP. Concerning other clinical and biological variables, van den Heuvel-Eibrink *et al.* found a positive association between BCRP mRNA expression and secondary AML or CD34 expression, while WBC was negatively associated. Age and karyotype were not correlated with BCRP mRNA expression, a result in agreement with the other three studies, which in addition found no association with CD34 and WBC. Our own study showed no associations between BCRP mRNA and age, WBC, karyotype or secondary AML (CD34 expression was not registered).

The finding that BCRP in combination with other markers also had a predictive value in the elderly cohort[128] supports the clinical relevance of BCRP in AML. Further support is provided by Wilson *et al.*[19], who used gene expression profiling in samples from patients with newly diagnosed *de novo* or secondary AML to identify a cluster of patients with very poor prognosis, distinguished by high expression of BCRP. We suggest that BCRP mRNA expression could be of predictive value in AML. Taken together, the above-mentioned studies support this notion.

As mentioned earlier, wild-type and mutated BCRP have different substrate profiles[115]. Suvannasankha *et al.*[124] found that all of their 31 AML samples contained wild-type BCRP. The clinical studies, discussed above, did not determine the mutational status of BCRP, but one interesting finding of Benderra *et al.*[129] was that the association of BCRP mRNA expression with clinical outcome was evident in patients treated with mitoxantrone and daunorubicin but not in patients treated with idarubicin. This supports earlier findings that idarubicin is not a substrate for BCRP[77,113] and implies either that daunorubicin is a substrate of wild-type BCRP or that mutated forms of BCRP could be expressed in AML. In our material, a minority of patients (n=11) received idarubicin in the induction courses. Excluding patients treated with idarubicin did not reveal any more evident association between BCRP mRNA and clinical outcome. In the patient cohort reported by van den Heuvel-Eibrink *et al.*[128], all patients received daunorubicin as induction therapy.

PGP, BCRP AND GSTII EXPRESSION DURING DRUG EXPOSURE *IN VITRO* (PAPER IV)

Expression of Pgp at diagnosis in AML has been extensively studied, and has been shown to be a prognostic marker, while the initial short-term effect of cytostatic drugs on Pgp expression *in vitro* and *in vivo* has been studied to a more limited extent[95-99,122]. The main objective of paper IV was to investigate the effect of daunorubicin and cytarabine on Pgp, BCRP and GSTπ expression in the drug-sensitive leukemic HL-60 (S) cell line and in two-drug resistant sublines, HL-60 R0.5 and HL-60 R5. The drug concentrations used (daunorubicin 0.2 μM and cytarabine 0.5 μM) were chosen to be clinically relevant[187].

Cytarabine

An increase in Pgp mRNA was evident after only ten minutes incubation with cytarabine in all three cell lines. The finding of this very rapid upregulation of Pgp mRNA is supported by previously reported data showing that Pgp mRNA is upregulated in human sarcoma cells exposed to doxorubicin *in vivo* for 20 minutes[198]. Our finding of increased Pgp mRNA was also confirmed on the protein level in HL-60 S cells; Western blot showed detectable Pgp protein after 8 hours exposure to cytarabine. In the resistant cell lines HL-60 R0.5 and R5, an increase in Pgp protein was detected by flow cytometry after 24 hours. As expected, Pgp was detectable from the start in these two resistant cell lines, and Western blot showed no obvious increase.

Our results are in contrast to data reported by Yague *et al.*[199], who found that short-term exposure of the leukemic cell line K562 to cytotoxic agents, including cytarabine, induced increased Pgp mRNA, while no increase in Pgp protein was detected due to a "translational block". In that study, increase in mRNA was shown to be achieved by stabilization of mRNA rather than by enhanced transcription. However, in comparison with our study, Yague *et al.* used a much higher concentration of cytarabine (100 μM).

Daunorubicin

The three HL-60 cell lines S, R0.5 and R5 were also incubated with daunorubicin. The effects of daunorubicin on Pgp mRNA and Pgp protein expression were more limited than the effects of cytarabine. An increase in Pgp mRNA expression was seen after 10 minutes in the drug-resistant cell line HL-60 R0.5. There was no Pgp mRNA increase in HL-60 R5, while an increase was seen in HL-60 S cells after 24 hours. It is reasonable that daunorubicin 0.2 μM had no effect on the HL-60 R5 cells, which are resistant to more than 5 μM daunorubicin[94] while it did show some effect on HL-60 R0.5 cells, which are resistant to more than 0.5 μM daunorubicin[94].

Induction of Pgp mRNA as well as Pgp protein expression and function by short-term exposure to daunorubicin has been previously demonstrated in leukemic cell lines[95,97,98]. A study by Hu *et al.*[97], using the drug-resistant leukemic cell line CEM/A7R and daunorubicin, bears some resemblance to our study. An increase in Pgp mRNA expression was demonstrated after 4 hours incubation with daunorubicin, which is in accordance with our data for HL-60 R0.5, although we monitored Pgp mRNA very closely initially and found an increase after only 10 minutes of incubation. By flow cytometry, Hu *et al.*[97] showed an increase in Pgp protein expression after 24 hours of continuous exposure to daunorubicin, while we found no increase in either of the cell lines. However, Hu *et al*.[97] used a higher concentration of daunorubicin than we did (2.6 μM vs. 0.2 μM) and that higher concentration actually exceeded IC50 for the CEM/A7R cell line (0.10 μM). From previous experience we know that HL-60 R0.5 and HL-60 R5 cells survive constant culturing in the drug

concentrations used, and cell death was negligible during the 36 hours of culturing. Thus, relative to our resistant cell lines, we used a much lower concentration of daunorubicin. Baker *et al.*[98] demonstrated an increase in Pgp mRNA after 8 hours exposure of the cell line CEM-Bcl2 to 0.35 μM daunorubicin, while Pgp protein expression was not detectable after 24 hours. In that study, the methylation status of the *MDR1* gene promoter region was shown to be crucial for the ability of a cell line to increase Pgp mRNA expression in response to drug exposure. The differences between the studies discussed here and our own results regarding time to initial response and level of response to drug exposure could probably, apart from measuring at different time points, be explained by differences in cell lines and drug concentrations.

Clinical samples

Hu *et al.*[99] determined Pgp protein expression and function by flow cytometry in myeloid leukemia patient samples exposed *in vitro* to daunorubicin and cytarabine. An increased expression, which correlated well to Pgp function, was evident after 16 hours exposure to both drugs in both Pgp-negative and Pgp-positive samples. In terms of cytarabine, this is in accordance with our results from all cell lines; however, we detected no increase in Pgp protein expression during exposure to daunorubicin. The daunorubicin concentration (0.14 μM) used by Hu *et al.*[99] was similar to the concentration used in our study (0.2 μM), while the cytarabine concentration was about a tenth of ours (0.04 μM vs. 0.5 μM).

It is not possible to directly compare our data with those from myeloid leukemia patient samples in relation to the drug concentrations used, since clinical samples consist of a heterogeneous blast population expressing different levels of Pgp[183] and drug resistance. On the other hand, just as Hu *et al.*[99] demonstrated upregulated Pgp protein expression in both Pgp-negative and Pgp-positive cells, our results regarding cytarabine were evident in both the Pgp-negative HL-60 S cells and the Pgp-positive HL-60 R0.5 and R5 cells.

One difference between these *in vitro* studies (including our own) and the clinical situation is that daunorubicin in the clinic is not given continuously over several hours, but usually as a 1 hour infusion. This would suggest a weaker clinical effect of daunorubicin on Pgp expression. On the other hand, cytarabine is often given as a continuous infusion in the clinical setting, and both our results and the results reported by Hu *et al.*[99] indicate the possibility that increased Pgp expression could be induced by cytarabine during induction chemotherapy.

Hu *et al*[99] found that concomitant treatment with daunorubicin and cytarabine *in vivo* induced Pgp protein expression in leukemic blasts four hours after start of chemotherapy in one patient[99]. In contrast, van der Pol *et al.,* who determined Pgp function in an efflux study using flow cytometry, did not find any increase 8 hours after start of induction chemotherapy in 10 of 11 patients with newly diagnosed AML[122]. Interestingly, 10 of these patients received cytarabine alone or in

combination with fludarabine as induction therapy, and only one received an anthracycline. Obviously, increased expression of Pgp protein without concomitant increase in Pgp function would not be of clinical significance. Support for this possibility was reported by Volm *et al.*[200], who detected increased levels of Pgp within a few hours of doxorubicin treatment of a murine leukemic cell line *in vivo* in ascites, but found no concomitant increase in Pgp function; drug resistance emerged during repeated doxorubicin exposure for weeks. However, data regarding the possible effect of chemotherapy on initial expression of Pgp and on drug resistance *in vivo* are far too limited to allow anything but speculation.

BCRP and GSTπ

We also investigated the short-term effect of drug exposure on BCRP and GSTπ mRNA expression. All three cell lines were negative for BCRP mRNA, regardless of drug exposure. We found a marked increase in GSTπ mRNA after 10 minutes incubation with cytarabine in both HL-60 R0.5 and R5 cells, while no increase was seen in HL-60 S cells. After 10 minutes incubation with daunorubicin, an increase in GSTπ was found in HL-60 R0.5 cells but not in R5 cells, while an increase was seen after 12 hours in HL-60 S cells. Based on the findings that GSTπ is an inhibitor of c-Jun N-terminal kinase 1 (JNK1)[201], it has been suggested that elevated levels of GSTπ might have an anti-apoptotic effect via JNK1, which is involved in apoptotic signalling via the MAP kinase pathway[131]. This would provide an explanation for the resistance to non-GSTπ substrates such as daunorubicin and cytarabine[202] that is displayed by GSTπ overexpressing cells, that is these cells are not resistant to specific drugs but to apoptosis induced by various drugs. Our results might reflect a general response to stress (for example exposure to cytostatic drugs), in leukemic cells, making them less prone to apoptosis.

Conclusion and general considerations

We were able to verify an induction of Pgp, more rapid than previously shown, in leukemic cells *in vitro* by short-term exposure to the non-Pgp substrate cytarabine. One could speculate that induction of Pgp mRNA and protein, as well as GSTπ mRNA, might be a general response to an ominous stimulus rather than a specific response to cytarabine. Environmental insults, such as heat shock or exposure to sodium arsenite have been shown to induce a transient increase in Pgp mRNA and Pgp protein expression in an adenocarcinoma cell line within a matter of hours[203]. Chaudary *et al.*[95] demonstrated that increased Pgp mRNA expression, induced by cytarabine in leukemic cell lines, was retained for several weeks; however, the longer duration of drug exposure in that study (12-72 hours) means that the results may be due to selection of Pgp-positive cells rather than induction of previously Pgp-negative cells. Hu *et al.*[96] noted that the elevation of Pgp mRNA induced by 5-FU, also a non-Pgp substrate, returned to baseline within 72 hours, while the Pgp mRNA elevation induced by anthracyclines was stable for at least three weeks. Long-term exposure of the CEM-CCRF cell line to cytarabine induced resistance but no elevation of Pgp[70],

supporting the theory that there is a difference between Pgp induction by short-term exposure and induction/selection by long-term exposure. Nevertheless, even a transient increase in Pgp protein, if accompanied by drug resistance, could be of importance during induction chemotherapy. This problem could possibly be circumvented or minimized by post-poning the first dose of cytarabine during induction chemotherapy. There is, however, no doubt that the overall benefit of combining cytarabine and anthracyclines by far exceeds the possible disadvantage[204]. An alternative to altered drug scheduling could be attempts to inhibit the emergence of Pgp-mediated drug resistance by adding drugs which interfere with signalling pathways involved in regulation of Pgp mRNA or Pgp protein expression[205].

P14^{ARF} mRNA IN AML WITH NORMAL KARYOTYPE (PAPER V)

P14^{ARF} and prognosis

TP53 mutations have been reported in about 10% of cases of newly diagnosed AML, and have been shown to be associated with clinical drug resistance and poor prognosis[168-170]. However, the function of p53 protein can also be affected by alterations of the ARF-HDM2-p53 pathway[206]. P14^{ARF} stabilizes p53 via degradation of HDM2[177], and consequently inactivation of p14^{ARF} is believed to result in p53 inactivation.

In a study reported by Müller-Tidow *et al*, the expression of p14^{ARF} mRNA was shown to be a predictor of survival in two independent patient samples (n=78 and n=57) of karyotypically unselected AML[178]. The authors used a cut-off defined as the median p14^{ARF} mRNA expression in the first patient sample. When this cut-off limit was used in the second patient sample, 42% of the patients were classified as expressing low levels of p14^{ARF} mRNA, while 58% expressed high levels. As in the first patient sample, low expression of p14^{ARF} was associated with worse prognosis in the second patient sample. In our material of AML with normal karyotype, the data were not normally distributed, and so we used the quartiles of p14^{ARF} mRNA expression to analyze a possible association with prognosis. We found that p14^{ARF} mRNA expression levels below the 75th percentile were associated with worse prognosis and a role for the ARF-HDM2-p53 pathway in drug resistance in AML with normal karyotype is suggested by our data.

The large subgroup of normal karyotype AML patients is heterogeneous in terms of prognosis. *NPM1* and *FLT3*-ITD mutations are currently used as prognostic tools for risk-adapted therapy, specifically in decision-making regarding allogeneic stem cell transplantation. Patients with mutated *NPM1* and wild type *FLT3* (*NPM1*+/*FLT3*-ITD-) have a more favorable prognosis than other patients with normal karyotype, and are generally not considered for allogeneic stem cell transplantation in first CR. In our material, 39 of 57 patients (68%) did not belong to the group who were *NPM1*+/*FLT3*-ITD- at diagnosis, and p14^{ARF} was also significantly associated with prognosis in this subgroup. Hence, p14^{ARF} mRNA levels could possibly add to the prognostic information and assist decision-making in the heterogeneous patient group

which remains when the prognostically favorable NPM1+/FLT3-ITD- patients are excluded.

Wild-type NPM1 is localized to the nucleolus and is implicated in several cellular processes. It has been shown recently that NPM1 binds to and stabilizes $p19^{ARF}$ (the mouse protein corresponding to the human $p14^{ARF}$)[207]. Mutated NPM1, on the other hand, relocalizes $p19^{ARF}$ to the cytoplasm, thereby inhibiting the stabilizing effect of $p19^{ARF}$ on p53[208,209]. This mechanism is believed to have importance in leukemogenesis in *NPM1*-positive cases. In our material, many of the *NPM1+/FLT3*-ITD- patients displayed low levels of $p14^{ARF}$ mRNA but still had a favorable outcome in relation to the other patients with low $p14^{ARF}$ mRNA expression. This suggests that *NPM1* status is superior to $p14^{ARF}$ mRNA expression level as a prognostic marker.

In vitro cytotoxicity

Leukemic cells with low expression of $p14^{ARF}$ mRNA were less sensitive to exposure to conventional cytostatic drugs compared to samples with high expression, although the differences were not statistically significant. One could speculate that impaired apoptosis, mediated by the ARF-HDM2-p53 pathway, could be the reason for poorer response to these drugs *in vitro*.

In contrast to the picture with conventional cytostatics, leukemic cells with low expression of $p14^{ARF}$ were more sensitive to PRIMA-1 5 μM than cells with high expression (p=0.046). The same tendency was seen at 10 μM (p=0.087), while PRIMA-1 20 μM showed very high toxicity in both groups without any obvious difference.

PRIMA-1 is believed to exert its cytotoxic effect by restoring the conformation and function of mutated p53, although there are data indicating a p53-independent cytotoxic effect[210,175]. In AML, mutated *TP53* is mostly found in cases of secondary AML and complex karyotype, and rarely in AML with normal karyotype[168,211-215]. In our study, FISH analysis of *TP53* and sequencing of the same gene revealed no signs of deletion or mutation. Even though small *TP53* deletions cannot be completely ruled out by FISH and *TP53* mutational status was not determined in all patients in this study of normal karyotype de novo AML, it is reasonable to assume that *TP53* was not mutated in the vast majority of the samples. The greater effect of PRIMA-1 on samples with low expression of $p14^{ARF}$ mRNA might indicate a p53 function-dependent effect of PRIMA-1 other than restoration of mutated p53. We did not measure intracellular p53 levels and hence one can only speculate that PRIMA-1 increased down-regulated p53. Some support for this notion comes from previously presented data by Nahi *et al.*[216], who showed a cytotoxic synergy between PRIMA-1 and another compound, RITA (reactivation of p53 and induction tumor cell apoptosis). RITA, by interfering with HDM-2 mediated degradation, restores the levels of wild type p53. The synergy between PRIMA-1 and RITA was evident in AML cells with and without loss of chromosome 17, that is probably also in cells with wild-type p53.

CONCLUSIONS

- topoisomerase IIα is significantly expressed in G0/G1 cells in AML
- low topoisomerase IIα protein expression in samples from patients with acute leukemia is associated with resistance *in vitro* to daunorubicin and etoposide
- no association between topo IIα mRNA or protein expression and response to induction chemotherapy or survival was found
- BCRP mRNA was detected in blood or bone marrow samples from patients with AML but the majority expressed less BCRP mRNA than the drug sensitive breast cancer cell line MCF-7
- no statistically significant association between BCRP mRNA expression and response to induction treatment or survival was shown
- in the group of responders, high expression of BCRP mRNA was statistically significantly associated with shorter survival, suggesting a predictive value of BCRP mRNA expression in AML
- short-term exposure of cytarabine induced Pgp mRNA and protein expression in leukemic cell lines with different basal levels of drug resistance
- low expression of $p14^{ARF}$ mRNA was sigificantly associated with shorter OS in AML with normal karyotype
- *in vitro,* AML cells expressing low levels of $p14^{ARF}$ mRNA tended to be more sensitive to PRIMA-1 than cells expressing high levels, suggesting a specific effect of this compound in cells with low expression of $p14^{ARF}$

FUTURE PERSPECTIVES

Our failure to demonstrate the theoretically expected clinical relevance of topo IIα and BCRP could have several explanations. The relatively small patient numbers are one obvious possibility, as is the heterogeneous patient material. As mentioned, age could also be of importance since the studies indicating a predictive value for topo IIα[153] and BCRP[129], respectively, investigated younger patients than those included in both our own study and the other negative studies. Another general problem is the difficulty of independently evaluate certain mechanisms. The different drugs have multiple mechanisms of action, the efflux pumps are promiscuous regarding substrates, the different proteins involved in drug resistance are probably heterogeneously distributed among the leukemic cells, and AML patients receive at least two cytostatic drugs with different modes of action. Furthermore, an increase at the mRNA level is probably a prerequisite for, but is not necessarily equivalent to, an increase of protein or protein activity. As well as including a sufficient number of patients, studies in this field should preferably determine multiple markers of drug resistance at different expression levels. Another way would be to study subpopulations within the leukemic clone since it is probable that the drug resistance profile of leukemic stem cells is important for long term results of chemotherapy.

Investigation of the short-term effects of cytostatic drugs is a less well-explored field of drug resistance in AML. Initial increase of drug resistance *in vitro* or *in vivo* could be a prognostic marker in itself; but the main question is its clinical relevance, and whether an initial increase in drug resistance could be avoided or minimized by, for example, different scheduling of the induction course. Together with previously reported data, our results form a basis for further studies regarding the clinical relevance of early induction of Pgp expression and function during induction chemotherapy.

In the era of emerging targeted drug therapy in hematological malignancies, one could argue that further scientific efforts in the field of conventional cytostatic drugs will prove to be futile. However, the short-term survival of newly diagnosed AML patients and the often slowly initiated effect of new targeted drugs will probably make the use of unselective cytostatic drugs indispensable for a long time. Besides, a substantial minority of AML patients do not achieve CR at all, which probably makes them inaccessible for more sophisticated therapy, and so attempts to improve conventional chemotherapy are imperative.

Altered disposition to apoptosis, which in this thesis is represented by low expression of $p14^{ARF}$ mRNA, is a more general mechanism of drug resistance than either efflux pumps or specific drug targets such as topo IIα and is likely to be of importance independently of the used cytostatic drugs. An association with survival was demonstrated in our modest number of AML patients with normal karyotype. The suggested specific effect of PRIMA-1 on leukemic cells with low $p14^{ARF}$ expression provides motivation for further *in vitro* studies regarding the effect of this compound in leukemic cells with not only mutated p53 but also with drug resistance due to alterations of the ARF-MDM2-p53 pathway.

ACKNOWLEDGEMENTS

There are several people to whom I want to express my gratitude for guidance, help and encouragement during the years I have been struggling with this thesis:

Ulf Tidefelt, my tutor, for introducing me to the scientific field and for your gentle guidance, enduring patience and recurrent enthusiasm.

Christer Paul, my co-tutor, for your generosity, interest and constructive criticism.

Mats G Karlsson, my co-tutor, for support with methodological guidance, laboratory facilities and bone marrow samples.

Hareth Nahi, my co-author, for your encouraging interest and remarkable efficiency.

Allan Sirsjö and **Hilja Strid**, my co-authors, for sharing your methodological expertise.

Malin Prenkert and **Elisabet Tina**, my co-authors, for skilful work and many fruitful discussions; and for letting me participate at the lab, under your close supervision.

Sofia Bengtzén, my co-author**,** and **Kerstin Jönsson-Videsäter**, for skilful work and for keeping order in the freezers.

My other co-authors, **Ingrid Dahlman, Stefan Deneberg, Monica Hermansson, Martin Höglund, Lotta Mossberg, Lars Möllgård, Esbjörn Paul, Richard Rosenquist, Klas G Wiman** and **Dick Wågsäter**, for expertise and contributions.

Eva-Hellström-Lindberg, Director of Department of Medicine, Karolinska Institutet, Huddinge, for valuable support.

Anders Magnuson, for comprehensible statistical advice.

Margareta Landin and the staff at the Medical Library, Örebro University Hospital, for kind help during many years, providing articles and helping with PubMed strategies and EndNote management.

Hand Fredlund, my mentor, for pleasant lunch-meetings.

Olle Linder, Head of Division of Hematology, Örebro University Hospital, and my former clinical tutor, for sharing your great knowledge and experience in clinical hematology.

Former and present colleagues and staff at the Divison of Hematology, Örebro University Hospital, for pleasant day-to-day collaboration in the clinic.

This thesis was supported by grants from Örebro County Council Research Committee and the Swedish Cancer Society.

REFERENCES

1 Deschler B, Lubbert M. Acute myeloid leukemia: epidemiology and etiology. *Cancer* 2006; **107**: 2099-107.

2 Astrom M, Bodin L, Tidefelt U. Adjustment of incidence rates after an estimate of completeness and accuracy in registration of acute leukemias in a Swedish population. *Leuk Lymphoma* 2001; **41**: 559-70.

3 Jemal A, Murray T, Ward E *et al.* Cancer statistics, 2005. *CA Cancer J Clin* 2005; **55**: 10-30.

4 Estey E, Dohner H. Acute myeloid leukaemia. *Lancet* 2006; **368**: 1894-907.

5 Bennett JM, Catovsky D, Daniel MT *et al.* Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. *Br J Haematol* 1976; **33**: 451-8.

6 Bennett JM, Catovsky D, Daniel MT *et al.* Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group. *Ann Intern Med* 1985; **103**: 620-5.

7 Brunning R, Matutes E, Harris N *et al.* World Health Organization Classification of Tumours. Pathology & Genetics. Tumours of Haematopoietic and Lymphoid Tissues. In: (Jaffe E, Harris N, Stein H et al., eds). Lyon: IARC Press. 2001; 75-107.

8 McKenna RW. Multifaceted approach to the diagnosis and classification of acute leukemias. *Clin Chem* 2000; **46**: 1252-9.

9 Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med* 1997; **3**: 730-7.

10 Knaust E, Porwit-MacDonald A, Gruber A *et al.* Heterogeneity of isolated mononuclear cells from patients with acute myeloid leukemia affects cellular accumulation and efflux of daunorubicin. *Haematologica* 2000; **85**: 124-32.

11 Macedo A, Orfao A, Gonzalez M *et al.* Immunological detection of blast cell subpopulations in acute myeloblastic leukemia at diagnosis: implications for minimal residual disease studies. *Leukemia* 1995; **9**: 993-8.

12 Astrom M, Bodin L, Nilsson I *et al.* Treatment, long-term outcome and prognostic variables in 214 unselected AML patients in Sweden. *Br J Cancer* 2000; **82**: 1387-92.

13 Lowenberg B. Prognostic factors in acute myeloid leukaemia. *Best Pract Res Clin Haematol* 2001; **14**: 65-75.

14 Mrozek K, Heerema NA, Bloomfield CD. Cytogenetics in acute leukemia. *Blood Rev* 2004; **18**: 115-36.

15 Grimwade D, Walker H, Oliver F *et al.* The Importance of Diagnostic Cytogenetics on Outcome in AML: Analysis of 1,612 Patients EnteredInto the MRC AML 10 Trial. *Blood* 1998; **92**: 2322-33.

16 Bienz M, Ludwig M, Leibundgut EO *et al.* Risk assessment in patients with acute myeloid leukemia and a normal karyotype. *Clin Cancer Res* 2005; **11**: 1416-24.

17 Mrozek K, Dohner H, Bloomfield CD. Influence of new molecular prognostic markers in patients with karyotypically normal acute myeloid leukemia: recent advances. *Curr Opin Hematol* 2007; **14**: 106-14.

18 Bullinger L, Dohner K, Bair E *et al.* Use of gene-expression profiling to identify prognostic subclasses in adult acute myeloid leukemia. *N Engl J Med* 2004; **350**: 1605-16.

19 Wilson CS, Davidson GS, Martin SB *et al.* Gene expression profiling of adult acute myeloid leukemia identifies novel biologic clusters for risk classification and outcome prediction. *Blood* 2006; **108**: 685-96.

20 Cheson BD, Bennett JM, Kopecky KJ *et al.* Revised recommendations of the International Working Group for Diagnosis, Standardization of Response Criteria, Treatment Outcomes, and Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia. *J Clin Oncol* 2003; **21**: 4642-9.

21 de Lima M, Strom SS, Keating M *et al.* Implications of potential cure in acute myelogenous leukemia: development of subsequent cancer and return to work. *Blood* 1997; **90**: 4719-24.

22 Kimby E, Nygren P, Glimelius B. A systematic overview of chemotherapy effects in acute myeloid leukaemia. *Acta Oncol* 2001; **40**: 231-52.
23 Tallman MS, Gilliland DG, Rowe JM. Drug therapy for acute myeloid leukemia. *Blood* 2005; **106**: 1154-63.
24 Bennett JM, Young ML, Andersen JW *et al.* Long-term survival in acute myeloid leukemia: the Eastern Cooperative Oncology Group experience. *Cancer* 1997; **80**: 2205-9.
25 Mayer RJ, Davis RB, Schiffer CA *et al.* Intensive postremission chemotherapy in adults with acute myeloid leukemia. Cancer and Leukemia Group B. *N Engl J Med* 1994; **331**: 896-903.
26 Bloomfield CD, Lawrence D, Byrd JC *et al.* Frequency of prolonged remission duration after high-dose cytarabine intensification in acute myeloid leukemia varies by cytogenetic subtype. *Cancer Res* 1998; **58**: 4173-9.
27 Appelbaum FR. Haematopoietic cell transplantation as immunotherapy. *Nature* 2001; **411**: 385-9.
28 Blaise D, Vey N, Faucher C *et al.* Current status of reduced-intensity-conditioning allogeneic stem cell transplantation for acute myeloid leukemia. *Haematologica* 2007; **92**: 533-41.
29 Oliansky DM, Appelbaum F, Cassileth PA *et al.* The role of cytotoxic therapy with hematopoietic stem cell transplantation in the therapy of acute myelogenous leukemia in adults: an evidence-based review. *Biol Blood Marrow Transplant* 2008; **14**: 137-80.
30 Yanada M, Matsuo K, Emi N *et al.* Efficacy of allogeneic hematopoietic stem cell transplantation depends on cytogenetic risk for acute myeloid leukemia in first disease remission: a metaanalysis. *Cancer* 2005; **103**: 1652-8.
31 Juliusson G, Billstrom R, Gruber A *et al.* Attitude towards remission induction for elderly patients with acute myeloid leukemia influences survival. *Leukemia* 2006; **20**: 42-7.
32 Appelbaum FR, Gundacker H, Head DR *et al.* Age and acute myeloid leukemia. *Blood* 2006; **107**: 3481-5.
33 Grimwade D, Walker H, Harrison G *et al.* The predictive value of hierarchical cytogenetic classification in older adults with acute myeloid leukemia (AML): analysis of 1065 patients entered into the United Kingdom Medical Research Council AML11 trial. *Blood* 2001; **98**: 1312-20.
34 Leith CP, Kopecky KJ, Godwin J *et al.* Acute myeloid leukemia in the elderly: assessment of multidrug resistance (MDR1) and cytogenetics distinguishes biologic subgroups with remarkably distinct responses to standard chemotherapy. A Southwest Oncology Group study. *Blood* 1997; **89**: 3323-9.
35 Plesa C, Chelghoum Y, Plesa A *et al.* Prognostic value of immunophenotyping in elderly patients with acute myeloid leukemia: a single-institution experience. *Cancer* 2008; **112**: 572-80.
36 Wahlin A, Markevarn B, Golovleva I *et al.* Prognostic significance of risk group stratification in elderly patients with acute myeloid leukaemia. *Br J Haematol* 2001; **115**: 25-33.
37 Kantarjian H, O'Brien S, Cortes J *et al.* Results of intensive chemotherapy in 998 patients age 65 years or older with acute myeloid leukemia or high-risk myelodysplastic syndrome: predictive prognostic models for outcome. *Cancer* 2006; **106**: 1090-8.
38 Gewirtz D. A Critical Evaluation of the Mechanisms of Action Proposed for the Antitumor Effects of the Anthracyline Antibiotics Adriamycin and Daunorubicin. *Biochem Pharmacol* 1999; **57**: 727-41.
39 Rabbani A, Finn RM, Ausio J. The anthracycline antibiotics: antitumor drugs that alter chromatin structure. *Bioessays* 2005; **27**: 50-6.
40 Liu LF. DNA topoisomerase poisons as antitumor drugs. *Annu Rev Biochem* 1989; **58**: 351-75.
41 Nitiss JL, Beck WT. Antitopoisomerase drug action and resistance. *Eur J Cancer* 1996; **32A**: 958-66.
42 A systematic collaborative overview of randomized trials comparing idarubicin with daunorubicin (or other anthracyclines) as induction therapy for acute

myeloid leukaemia. AML Collaborative Group. *Br J Haematol* 1998; **103**: 100-9.
43 Von Hoff DD, Rozencweig M, Layard M *et al.* Daunomycin-induced cardiotoxicity in children and adults. A review of 110 cases. *Am J Med* 1977; **62**: 200-8.
44 Arlin Z, Case DC, Jr., Moore J *et al.* Randomized multicenter trial of cytosine arabinoside with mitoxantrone or daunorubicin in previously untreated adult patients with acute nonlymphocytic leukemia (ANLL). Lederle Cooperative Group. *Leukemia* 1990; **4**: 177-83.
45 Hiddemann W, Kreutzmann H, Straif K *et al.* High-dose cytosine arabinoside and mitoxantrone: a highly effective regimen in refractory acute myeloid leukemia. *Blood* 1987; **69**: 744-9.
46 Arlin ZA, Feldman EJ, Mittelman A *et al.* Amsacrine is safe and effective therapy for patients with myocardial dysfunction and acute leukemia. *Cancer* 1991; **68**: 1198-200.
47 Legha SS, Keating MJ, McCredie KB *et al.* Evaluation of AMSA in previously treated patients with acute leukemia: results of therapy in 109 adults. *Blood* 1982; **60**: 484-90.
48 Ho AD, Brado B, Haas R *et al.* Etoposide in acute leukemia. Past experience and future perspectives. *Cancer* 1991; **67**: 281-4.
49 Kufe D, Spriggs D, Egan EM *et al.* Relationships among Ara-CTP pools, formation of (Ara-C)DNA, and cytotoxicity of human leukemic cells. *Blood* 1984; **64**: 54-8.
50 Major PP, Egan EM, Beardsley GP *et al.* Lethality of human myeloblasts correlates with the incorporation of arabinofuranosylcytosine into DNA. *Proc Natl Acad Sci U S A* 1981; **78**: 3235-9.
51 Momparler RL. Effect of cytosine arabinoside 5'-triphosphate on mammalian DNA polymerase. *Biochem Biophys Res Commun* 1969; **34**: 464-71.
52 Kees UR, Ford J, Dawson VM *et al.* Development of resistance to 1-beta-D-arabinofuranosylcytosine after high-dose treatment in childhood lymphoblastic leukemia: analysis of resistance mechanism in established cell lines. *Cancer Res* 1989; **49**: 3015-9.
53 Steuart CD, Burke PJ. Cytidine deaminase and the development of resistance to arabinosyl cytosine. *Nat New Biol* 1971; **233**: 109-10.
54 Herzig RH, Wolff SN, Lazarus HM *et al.* High-dose cytosine arabinoside therapy for refractory leukemia. *Blood* 1983; **62**: 361-9.
55 Robak T. Purine nucleoside analogues in the treatment of myleoid leukemias. *Leuk Lymphoma* 2003; **44**: 391-409.
56 Estey EH, Thall PF, Cortes JE *et al.* Comparison of idarubicin + ara-C-, fludarabine + ara-C-, and topotecan + ara-C-based regimens in treatment of newly diagnosed acute myeloid leukemia, refractory anemia with excess blasts in transformation, or refractory anemia with excess blasts. *Blood* 2001; **98**: 3575-83.
57 Juliusson G, Hoglund M, Karlsson K *et al.* Increased remissions from one course for intermediate-dose cytosine arabinoside and idarubicin in elderly acute myeloid leukaemia when combined with cladribine. A randomized population-based phase II study. *Br J Haematol* 2003; **123**: 810-8.
58 Faderl S, Gandhi V, O'Brien S *et al.* Results of a phase 1-2 study of clofarabine in combination with cytarabine (ara-C) in relapsed and refractory acute leukemias. *Blood* 2005; **105**: 940-7.
59 Kantarjian H, Gandhi V, Cortes J *et al.* Phase 2 clinical and pharmacologic study of clofarabine in patients with refractory or relapsed acute leukemia. *Blood* 2003; **102**: 2379-86.
60 Faderl S, Ravandi F, Huang X *et al.* A randomized study of clofarabine versus clofarabine plus low-dose cytarabine as frontline therapy for patients age >= 60 years with acute myeloid leukemia and high-risk myelodysplastic syndrome. *Blood* 2008.
61 Kantarjian HM, Jeha S, Gandhi V *et al.* Clofarabine: past, present, and future. *Leuk Lymphoma* 2007; **48**: 1922-30.

62 Estey E, Kornblau S, Pierce S *et al.* A stratification system for evaluating and selecting therapies in patients with relapsed or primary refractory acute myelogenous leukemia. *Blood* 1996; **88**: 756.
63 McLornan DP, McMullin MF, Johnston P *et al.* Molecular mechanisms of drug resistance in acute myeloid leukaemia. *Expert Opin Drug Metab Toxicol* 2007; **3**: 363-77.
64 McKenna SL, Padua RA. Multidrug resistance in leukaemia. *Br J Haematol* 1997; **96**: 659-74.
65 Dean M, Rzhetsky A, Allikmets R. The human ATP-binding cassette (ABC) transporter superfamily. *Genome Res* 2001; **11**: 1156-66.
66 Higgins CF. ABC transporters: from microorganisms to man. *Annu Rev Cell Biol* 1992; **8**: 67-113.
67 de Jonge-Peeters SD, Kuipers F, de Vries EG *et al.* ABC transporter expression in hematopoietic stem cells and the role in AML drug resistance. *Crit Rev Oncol Hematol* 2007.
68 Benderra Z, Faussat AM, Sayada L *et al.* MRP3, BCRP, and P-glycoprotein activities are prognostic factors in adult acute myeloid leukemia. *Clin Cancer Res* 2005; **11**: 7764-72.
69 Steinbach D, Lengemann J, Voigt A *et al.* Response to chemotherapy and expression of the genes encoding the multidrug resistance-associated proteins MRP2, MRP3, MRP4, MRP5, and SMRP in childhood acute myeloid leukemia. *Clin Cancer Res* 2003; **9**: 1083-6.
70 Beck WT, Mueller TJ, Tanzer LR. Altered surface membrane glycoproteins in Vinca alkaloid-resistant human leukemic lymphoblasts. *Cancer Res* 1979; **39**: 2070-6.
71 Juliano RL, Ling V. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim Biophys Acta* 1976; **455**: 152-62.
72 Riordan JR, Ling V. Purification of P-glycoprotein from plasma membrane vesicles of Chinese hamster ovary cell mutants with reduced colchicine permeability. *J Biol Chem* 1979; **254**: 12701-5.
73 Callen DF, Baker E, Simmers RN *et al.* Localization of the human multiple drug resistance gene, MDR1, to 7q21.1. *Hum Genet* 1987; **77**: 142-4.
74 Horio M, Gottesman MM, Pastan I. ATP-dependent transport of vinblastine in vesicles from human multidrug-resistant cells. *Proc Natl Acad Sci U S A* 1988; **85**: 3580-4.
75 Schinkel AH, Mayer U, Wagenaar E *et al.* Normal viability and altered pharmacokinetics in mice lacking mdr1-type (drug-transporting) P-glycoproteins. *Proc Natl Acad Sci U S A* 1997; **94**: 4028-33.
76 Litman T, Druley TE, Stein WD *et al.* From MDR to MXR: new understanding of multidrug resistance systems, their properties and clinical significance. *Cell Mol Life Sci* 2001; **58**: 931-59.
77 Ross DD, Doyle LA, Yang W *et al.* Susceptibility of idarubicin, daunorubicin, and their C-13 alcohol metabolites to transport-mediated multidrug resistance. *Biochem Pharmacol* 1995; **50**: 1673-83.
78 Hait WN, Choudhury S, Srimatkandada S *et al.* Sensitivity of K562 human chronic myelogenous leukemia blast cells transfected with a human multidrug resistance cDNA to cytotoxic drugs and differentiating agents. *J Clin Invest* 1993; **91**: 2207-15.
79 Campos L, Guyotat D, Archimbaud E *et al.* Clinical significance of multidrug resistance P-glycoprotein expression on acute nonlymphoblastic leukemia cells at diagnosis. *Blood* 1992; **79**: 473-6.
80 Leith CP, Kopecky KJ, Chen IM *et al.* Frequency and clinical significance of the expression of the multidrug resistance proteins MDR1/P-glycoprotein, MRP1, and LRP in acute myeloid leukemia: a Southwest Oncology Group Study. *Blood* 1999; **94**: 1086-99.
81 Pirker R, Wallner J, Geissler K *et al.* MDR1 gene expression and treatment outcome in acute myeloid leukemia. *J Natl Cancer Inst* 1991; **83**: 708-12.
82 van den Heuvel-Eibrink MM, van der Holt B, te Boekhorst PA *et al.* MDR 1 expression is an independent prognostic factor for response and survival in de novo acute myeloid leukaemia. *Br J Haematol* 1997; **99**: 76-83.

83 Wood P, Burgess R, MacGregor A *et al.* P-glycoprotein expression on acute myeloid leukaemia blast cells at diagnosis predicts response to chemotherapy and survival. *Br J Haematol* 1994; **87**: 509-14.
84 Baer MR, George SL, Dodge RK *et al.* Phase 3 study of the multidrug resistance modulator PSC-833 in previously untreated patients 60 years of age and older with acute myeloid leukemia: Cancer and Leukemia Group B Study 9720. *Blood* 2002; **100**: 1224-32.
85 Greenberg PL, Lee SJ, Advani R *et al.* Mitoxantrone, etoposide, and cytarabine with or without valspodar in patients with relapsed or refractory acute myeloid leukemia and high-risk myelodysplastic syndrome: a phase III trial (E2995). *J Clin Oncol* 2004; **22**: 1078-86.
86 Gruber A, Bjorkholm M, Brinch L *et al.* A phase I/II study of the MDR modulator Valspodar (PSC 833) combined with daunorubicin and cytarabine in patients with relapsed and primary refractory acute myeloid leukemia. *Leuk Res* 2003; **27**: 323-8.
87 List AF, Kopecky KJ, Willman CL *et al.* Benefit of cyclosporine modulation of drug resistance in patients with poor-risk acute myeloid leukemia: a Southwest Oncology Group study. *Blood* 2001; **98**: 3212-20.
88 Liu Yin JA, Wheatley K, Rees JK *et al.* Comparison of 'sequential' versus 'standard' chemotherapy as re-induction treatment, with or without cyclosporine, in refractory/relapsed acute myeloid leukaemia (AML): results of the UK Medical Research Council AML-R trial. *Br J Haematol* 2001; **113**: 713-26.
89 Mahadevan D, List AF. Targeting the multidrug resistance-1 transporter in AML: molecular regulation and therapeutic strategies. *Blood* 2004; **104**: 1940-51.
90 Matsouka P, Pagoni M, Zikos P *et al.* Addition of cyclosporin-A to chemotherapy in secondary (post-MDS) AML in the elderly. A multicenter randomized trial of the Leukemia Working Group of the Hellenic Society of Hematology. *Ann Hematol* 2006; **85**: 250-6.
91 van der Holt B, Lowenberg B, Burnett AK *et al.* The value of the MDR1 reversal agent PSC-833 in addition to daunorubicin and cytarabine in the treatment of elderly patients with previously untreated acute myeloid leukemia (AML), in relation to MDR1 status at diagnosis. *Blood* 2005; **106**: 2646-54.
92 Fisher GA, Lum BL, Hausdorff J *et al.* Pharmacological considerations in the modulation of multidrug resistance. *Eur J Cancer* 1996; **32A**: 1082-8.
93 van den Heuvel-Eibrink MM, Wiemer EA, de Boevere MJ *et al.* MDR1 gene-related clonal selection and P-glycoprotein function and expression in relapsed or refractory acute myeloid leukemia. *Blood* 2001; **97**: 3605-11.
94 Jonsson K, Dahlberg N, Tidefelt U *et al.* Characterization of an anthracycline-resistant human promyelocyte leukemia (HL-60) cell line with an elevated MDR-1 gene expression. *Biochem Pharmacol* 1995; **49**: 755-62.
95 Chaudhary PM, Roninson IB. Induction of multidrug resistance in human cells by transient exposure to different chemotherapeutic drugs. *J Natl Cancer Inst* 1993; **85**: 632-9.
96 Hu XF, Slater A, Rischin D *et al.* Induction of MDR1 gene expression by anthracycline analogues in a human drug resistant leukaemia cell line. *Br J Cancer* 1999; **79**: 831-7.
97 Hu XF, Slater A, Wall DM *et al.* Rapid up-regulation of mdr1 expression by anthracyclines in a classical multidrug-resistant cell line. *Br J Cancer* 1995; **71**: 931-6.
98 Baker EK, Johnstone RW, Zalcberg JR *et al.* Epigenetic changes to the MDR1 locus in response to chemotherapeutic drugs. *Oncogene* 2005; **24**: 8061-75.
99 Hu XF, Slater A, Kantharidis P *et al.* Altered multidrug resistance phenotype caused by anthracycline analogues and cytosine arabinoside in myeloid leukemia. *Blood* 1999; **93**: 4086-95.
100 Michelutti A, Michieli M, Damiani D *et al.* Effect of fludarabine and arabinosylcytosine on multidrug resistant cells. *Haematologica* 1997; **82**: 143-7.

101 Nakayama M, Wada M, Harada T *et al.* Hypomethylation status of CpG sites at the promoter region and overexpression of the human MDR1 gene in acute myeloid leukemias. *Blood* 1998; **92**: 4296-307.
102 Knutsen T, Rao VK, Ried T *et al.* Amplification of 4q21-q22 and the MXR gene in independently derived mitoxantrone-resistant cell lines. *Genes Chromosomes Cancer* 2000; **27**: 110-6.
103 Chen Y-N, Mickley LA, Schwartz AM *et al.* Characterization of adriamycin-resistant human breast cancer cells which display overexpression of a novel resistance-related membrane protein. *The Journal of Biological Chemistry* 1990; **265**: 10073-80.
104 Doyle LA, Ross DD, Sridhara R *et al.* Expression of a 95 kDa membrane protein is associated with low daunorubicin accumulation in leukaemic blast cells. *Br J Cancer* 1995; **71**: 52-8.
105 Doyle LA, Yang W, Abruzzo LV *et al.* A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proc Natl Acad Sci U S A* 1998; **95**: 15665-70.
106 Mao Q, Unadkat JD. Role of the breast cancer resistance protein (ABCG2) in drug transport. *AAPS J* 2005; **7**: E118-33.
107 Zhou S, Schuetz JD, Bunting KD *et al.* The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat Med* 2001; **7**: 1028-34.
108 Scharenberg CW, Harkey MA, Torok-Storb B. The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. *Blood* 2002; **99**: 507-12.
109 Allikmets R, Schriml LM, Hutchinson A *et al.* A human placenta-specific ATP-binding cassette gene (ABCP) on chromosome 4q22 that is involved in multidrug resistance. *Cancer Res* 1998; **58**: 5337-9.
110 Jonker JW, Buitelaar M, Wagenaar E *et al.* The breast cancer resistance protein protects against a major chlorophyll-derived dietary phototoxin and protoporphyria. *Proc Natl Acad Sci U S A* 2002; **99**: 15649-54.
111 Raaijmakers MH, de Grouw EP, Heuver LH *et al.* Breast cancer resistance protein in drug resistance of primitive CD34+38- cells in acute myeloid leukemia. *Clin Cancer Res* 2005; **11**: 2436-44.
112 Litman T, Brangi M, Hudson E *et al.* The multidrug-resistant phenotype associated with overexpression of the new ABC half-transporter, MXR (ABCG2). *J Cell Sci* 2000; **113**: 2011-21.
113 Abbott BL, Colapietro AM, Barnes Y *et al.* Low levels of ABCG2 expression in adult AML blast samples. *Blood* 2002; **100**: 4594-601.
114 Komatani H, Kotani H, Hara Y *et al.* Identification of breast cancer resistant protein/mitoxantrone resistance/placenta-specific, ATP-binding cassette transporter as a transporter of NB-506 and J-107088, topoisomerase I inhibitors with an indolocarbazole structure. *Cancer Res* 2001; **61**: 2827-32.
115 Honjo Y, Hrycyna CA, Yan QW *et al.* Acquired mutations in the MXR/BCRP/ABCP gene alter substrate specificity in MXR/BCRP/ABCP-overexpressing cells. *Cancer Res* 2001; **61**: 6635-9.
116 Rabindran SK, He H, Singh M *et al.* Reversal of a novel multidrug resistance mechanism in human colon carcinoma cells by fumitremorgin C. *Cancer Res* 1998; **58**: 5850-8.
117 Rabindran SK, Ross DD, Doyle LA *et al.* Fumitremorgin C reverses multidrug resistance in cells transfected with the breast cancer resistance protein. *Cancer Res* 2000; **60**: 47-50.
118 Allen JD, van Loevezijn A, Lakhai JM *et al.* Potent and specific inhibition of the breast cancer resistance protein multidrug transporter in vitro and in mouse intestine by a novel analogue of fumitremorgin C. *Mol Cancer Ther* 2002; **1**: 417-25.
119 Xia CQ, Liu N, Miwa GT *et al.* Interactions of cyclosporin a with breast cancer resistance protein. *Drug Metab Dispos* 2007; **35**: 576-82.
120 Qadir M, O'Loughlin KL, Fricke SM *et al.* Cyclosporin A is a broad-spectrum multidrug resistance modulator. *Clin Cancer Res* 2005; **11**: 2320-6.

121 Ross DD, Karp JE, Chen TT *et al.* Expression of breast cancer resistance protein in blast cells from patients with acute leukemia. *Blood* 2000; **96**: 365-8.
122 van der Pol M, Broxterman H, Pater J *et al.* Function of the ABC transporters, P-glycoprotein, multidrug resistance protein and breast cancer resistance protein, in minimal residual disease in acute leukemia. *Haematologica* 2003; **88**: 134-47.
123 Nakanishi T, Karp JE, Tan M *et al.* Quantitative analysis of breast cancer resistance protein and cellular resistance to flavopiridol in acute leukemia patients. *Clin Cancer Res* 2003; **9**: 3320-8.
124 Suvannasankha A, Minderman H, O'Loughlin KL *et al.* Breast cancer resistance protein (BCRP/MXR/ABCG2) in acute myeloid leukemia: discordance between expression and function. *Leukemia* 2004; **18**: 1252-7.
125 van den Heuvel-Eibrink MM, Wiemer EA, Prins A *et al.* Increased expression of the breast cancer resistance protein (BCRP) in relapsed or refractory acute myeloid leukemia (AML). *Leukemia* 2002; **16**: 833-9.
126 Steinbach D, Sell W, Voigt A *et al.* BCRP gene expression is associated with a poor response to remission induction therapy in childhood acute myeloid leukemia. *Leukemia* 2002; **16**: 1443-7.
127 van der Kolk DM, Vellenga E, Scheffer GL *et al.* Expression and activity of breast cancer resistance protein (BCRP) in de novo and relapsed acute myeloid leukemia. *Blood* 2002; **99**: 3763-70.
128 van den Heuvel-Eibrink MM, van der Holt B, Burnett AK *et al.* CD34-related coexpression of MDR1 and BCRP indicates a clinically resistant phenotype in patients with acute myeloid leukemia (AML) of older age. *Ann Hematol* 2007; **86**: 329-37.
129 Benderra Z, Faussat AM, Sayada L *et al.* Breast cancer resistance protein and P-glycoprotein in 149 adult acute myeloid leukemias. *Clin Cancer Res* 2004; **10**: 7896-902.
130 Damiani D, Tiribelli M, Calistri E *et al.* The prognostic value of P-glycoprotein (ABCB) and breast cancer resistance protein (ABCG2) in adults with de novo acute myeloid leukemia with normal karyotype. *Haematologica* 2006; **91**: 825-8.
131 Townsend DM, Tew KD. The role of glutathione-S-transferase in anti-cancer drug resistance. *Oncogene* 2003; **22**: 7369-75.
132 Tidefelt U, Elmhorn-Rosenborg A, Paul C *et al.* Expression of glutathione transferase pi as a predictor for treatment results at different stages of acute nonlymphoblastic leukemia. *Cancer Res* 1992; **52**: 3281-5.
133 Alberts B, Johnson A, Lewis J *et al.*, eds. *Molecular biology of the cell*, 4th edn.: Garland Sciences. 2002.
134 Chung TD, Drake FH, Tan KB *et al.* Characterization and immunological identification of cDNA clones encoding two human DNA topoisomerase II isozymes. *Proc Natl Acad Sci U S A* 1989; **86**: 9431-5.
135 Miller KG, Liu LF, Englund PT. A homogeneous type II DNA topoisomerase from HeLa cell nuclei. *J Biol Chem* 1981; **256**: 9334-9.
136 Tsai-Pflugfelder M, Liu LF, Liu AA *et al.* Cloning and sequencing of cDNA encoding human DNA topoisomerase II and localization of the gene to chromosome region 17q21-22. *Proc Natl Acad Sci U S A* 1988; **85**: 7177-81.
137 Woessner RD, Mattern MR, Mirabelli CK *et al.* Proliferation- and cell cycle-dependent differences in expression of the 170 kilodalton and 180 kilodalton forms of topoisomerase II in NIH-3T3 cells. *Cell Growth Differ* 1991; **2**: 209-14.
138 Tanoguchi K, Sasano H, Yabuki N *et al.* Immunohistochemical and two-parameter flow cytometric studies of DNA topoisomerase II alpha in human epithelial ovarian carcinoma and germ cell tumor. *Mod Pathol* 1998; **11**: 186-93.
139 Villman K, Ståhl E, Liljegren G *et al.* Topoisomerase II-alpha Expression in Different Cell Cycle Phases in Fresh Human Breast Carcinomas. *Mod Pathol* 2002; **15**: 486-91.

140 Nicklee T, Crump M, Hedley DW. Effects of topoisomerase I inhibition on the expression of topoisomerase II alpha measured with fluorescence image cytometry. *Cytometry* 1996; **25**: 205-10.
141 Azarova AM, Lyu YL, Lin CP *et al.* Roles of DNA topoisomerase II isozymes in chemotherapy and secondary malignancies. *Proc Natl Acad Sci U S A* 2007; **104**: 11014-9.
142 Chin KV, Chauhan SS, Pastan I *et al.* Regulation of mdr RNA levels in response to cytotoxic drugs in rodent cells. *Cell Growth Differ* 1990; **1**: 361-5.
143 Deffie AM, Batra JK, Goldenberg GJ. Direct correlation between DNA topoisomerase II activity and cytotoxicity in adriamycin-sensitive and -resistant P388 leukemia cell lines. *Cancer Res* 1989; **49**: 58-62.
144 Eijdems EW, Borst P, Jongsma AP *et al.* Genetic transfer of non-P-glycoprotein-mediated multidrug resistance (MDR) in somatic cell fusion: dissection of a compound MDR phenotype. *Proc Natl Acad Sci U S A* 1992; **89**: 3498-502.
145 Fry AM, Chresta CM, Davies SM *et al.* Relationship between topoisomerase II level and chemosensitivity in human tumor cell lines. *Cancer Res* 1991; **51**: 6592-5.
146 Ritke MK, Yalowich JC. Altered gene expression in human leukemia K562 cells selected for resistance to etoposide. *Biochem Pharmacol* 1993; **46**: 2007-20.
147 Webb CD, Latham MD, Lock RB *et al.* Attenuated topoisomerase II content directly correlates with a low level of drug resistance in a Chinese hamster ovary cell line. *Cancer Res* 1991; **51**: 6543-9.
148 Chen M, Beck WT. DNA topoisomerase II expression, stability, and phosphorylation in two VM-26-resistant human leukemic CEM sublines. *Oncol Res* 1995; **7**: 103-11.
149 Galimberti S, Testi R, Guerrini F *et al.* The clinical relevance of the expression of several multidrug-resistant-related genes in patients with primary acute myeloid leukemia. *J Chemother* 2003; **15**: 374-9.
150 Galmarini CM, Thomas X, Calvo F *et al.* In vivo mechanisms of resistance to cytarabine in acute myeloid leukaemia. *Br J Haematol* 2002; **117**: 860-8.
151 McKenna SL, West RR, Whittaker JA *et al.* Topoisomerase II alpha expression in acute myeloid leukaemia and its relationship to clinical outcome. *Leukemia* 1994; **8**: 1498-502.
152 Kaufmann SH, Karp JE, Jones RJ *et al.* Topoisomerase II levels and drug sensitivity in adult acute myelogenous leukemia. *Blood* 1994; **83**: 517-30.
153 Lohri A, van Hille B, Bacchi M *et al.* Five putative drug resistance parameters (MDR1/P-glycoprotein, MDR-associated protein, glutathione-S-transferase, bcl-2 and topoisomerase IIalpha) in 57 newly diagnosed acute myeloid leukaemias. Swiss Group for Clinical Cancer Research (SAKK). *Eur J Haematol* 1997; **59**: 206-15.
154 D'Arpa P, Beardmore C, Liu LF. Involvement of nucleic acid synthesis in cell killing mechanisms of topoisomerase poisons. *Cancer Res* 1990; **50**: 6919-24.
155 Potter AJ, Rabinovitch PS. The cell cycle phases of DNA damage and repair initiated by topoisomerase II-targeting chemotherapeutic drugs. *Mutat Res* 2005; **572**: 27-44.
156 Jedema I, Barge RM, Nijmeijer BA *et al.* Recruitment of leukemic cells from G0 phase of the cell cycle by interferons results in conversion of resistance to daunorubicin. *Leukemia* 2003; **17**: 2049-51.
157 Chikamori K, Grabowski DR, Kinter M *et al.* Phosphorylation of serine 1106 in the catalytic domain of topoisomerase II alpha regulates enzymatic activity and drug sensitivity. *J Biol Chem* 2003; **278**: 12696-702.
158 Ishida R, Takashima R, Koujin T *et al.* Mitotic specific phosphorylation of serine-1212 in human DNA topoisomerase IIalpha. *Cell Struct Funct* 2001; **26**: 215-26.
159 Lee CG, Hague LK, Li H *et al.* Identification of toposome, a novel multisubunit complex containing topoisomerase IIalpha. *Cell Cycle* 2004; **3**: 638-47.

160 Wells NJ, Fry AM, Guano F *et al.* Cell cycle phase-specific phosphorylation of human topoisomerase II alpha. Evidence of a role for protein kinase C. *J Biol Chem* 1995; **270**: 28357-63.
161 McBride OW, Merry D, Givol D. The gene for human p53 cellular tumor antigen is located on chromosome 17 short arm (17p13). *Proc Natl Acad Sci U S A* 1986; **83**: 130-4.
162 Nelson WG, Kastan MB. DNA strand breaks: the DNA template alterations that trigger p53-dependent DNA damage response pathways. *Mol Cell Biol* 1994; **14**: 1815-23.
163 Chen X, Ko LJ, Jayaraman L *et al.* p53 levels, functional domains, and DNA damage determine the extent of the apoptotic response of tumor cells. *Genes Dev* 1996; **10**: 2438-51.
164 Ronen D, Schwartz D, Teitz Y *et al.* Induction of HL-60 cells to undergo apoptosis is determined by high levels of wild-type p53 protein whereas differentiation of the cells is mediated by lower p53 levels. *Cell Growth Differ* 1996; **7**: 21-30.
165 Anensen N, Haaland I, D'Santos C *et al.* Proteomics of p53 in diagnostics and therapy of acute myeloid leukemia. *Curr Pharm Biotechnol* 2006; **7**: 199-207.
166 Honda R, Tanaka H, Yasuda H. Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. *FEBS Lett* 1997; **420**: 25-7.
167 Vousden KH, Lu X. Live or let die: the cell's response to p53. *Nat Rev Cancer* 2002; **2**: 594-604.
168 Fenaux P, Preudhomme C, Quiquandon I *et al.* Mutations of the P53 gene in acute myeloid leukaemia. *Br J Haematol* 1992; **80**: 178-83.
169 Stirewalt DL, Kopecky KJ, Meshinchi S *et al.* FLT3, RAS, and TP53 mutations in elderly patients with acute myeloid leukemia. *Blood* 2001; **97**: 3589-95.
170 Wattel E, Preudhomme C, Hecquet B *et al.* p53 mutations are associated with resistance to chemotherapy and short survival in hematologic malignancies. *Blood* 1994; **84**: 3148-57.
171 Lai JL, Preudhomme C, Zandecki M *et al.* Myelodysplastic syndromes and acute myeloid leukemia with 17p deletion. An entity characterized by specific dysgranulopoiesis and a high incidence of P53 mutations. *Leukemia* 1995; **9**: 370-81.
172 Nahi H, Lehmann S, Bengtzen S *et al.* Chromosomal aberrations in 17p predict in vitro drug resistance and short overall survival in acute myeloid leukemia. *Leuk Lymphoma* 2008; **49**: 508-16.
173 Bykov VJ, Issaeva N, Selivanova G *et al.* Mutant p53-dependent growth suppression distinguishes PRIMA-1 from known anticancer drugs: a statistical analysis of information in the National Cancer Institute database. *Carcinogenesis* 2002; **23**: 2011-8.
174 Bykov VJ, Issaeva N, Shilov A *et al.* Restoration of the tumor suppressor function to mutant p53 by a low-molecular-weight compound. *Nat Med* 2002; **8**: 282-8.
175 Nahi H, Merup M, Lehmann S *et al.* PRIMA-1 induces apoptosis in acute myeloid leukaemia cells with p53 gene deletion. *Br J Haematol* 2006; **132**: 230-6.
176 Mao L, Merlo A, Bedi G *et al.* A novel p16INK4A transcript. *Cancer Res* 1995; **55**: 2995-7.
177 Zhang Y, Xiong Y, Yarbrough WG. ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. *Cell* 1998; **92**: 725-34.
178 Muller-Tidow C, Metzelder SK, Buerger H *et al.* Expression of the p14ARF tumor suppressor predicts survival in acute myeloid leukemia. *Leukemia* 2004; **18**: 720-6.
179 Bonnet D. Normal and leukaemic stem cells. *Br J Haematol* 2005; **130**: 469-79.
180 Goodell MA, Brose K, Paradis G *et al.* Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J Exp Med* 1996; **183**: 1797-806.

181 Goodell MA, Rosenzweig M, Kim H *et al.* Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. *Nat Med* 1997; **3**: 1337-45.
182 Wulf GG, Wang RY, Kuehnle I *et al.* A leukemic stem cell with intrinsic drug efflux capacity in acute myeloid leukemia. *Blood* 2001; **98**: 1166-73.
183 de Figueiredo-Pontes LL, Pintao MC, Oliveira LC *et al.* Determination of P-glycoprotein, MDR-related protein 1, breast cancer resistance protein, and lung-resistance protein expression in leukemic stem cells of acute myeloid leukemia. *Cytometry B Clin Cytom* 2008.
184 Raaijmakers MH, de Grouw EP, van der Reijden BA *et al.* ABCB1 modulation does not circumvent drug extrusion from primitive leukemic progenitor cells and may preferentially target residual normal cells in acute myelogenous leukemia. *Clin Cancer Res* 2006; **12**: 3452-8.
185 Ho MM, Hogge DE, Ling V. MDR1 and BCRP1 expression in leukemic progenitors correlates with chemotherapy response in acute myeloid leukemia. *Exp Hematol* 2008; **36**: 433-42.
186 Jonsson K, Dahlberg N, Tidefelt U *et al.* Characterization of an anthracycline-resistant human promyelocyte leukemia (HL-60) cell line with an elevated MDR-1 gene expression. *Biochem Pharmacol* 1995; **49**: 755-62.
187 Sundman-Engberg B, Tidefelt U, Liliemark J *et al.* Intracellular concentrations of anti cancer drugs in leukemic cells in vitro vs in vivo. *Cancer Chemother Pharmacol* 1990; **25**: 252-6.
188 Kaufmann SH, McLaughlin SJ, Kastan MB *et al.* Topoisomerase II levels during granulocytic maturation in vitro and in vivo. *Cancer Res* 1991; **51**: 3534-43.
189 Mollgard L, Tidefelt U, Sundman-Engberg B *et al.* In vitro chemosensitivity testing in acute non lymphocytic leukemia using the bioluminescence ATP assay. *Leuk Res* 2000; **24**: 445-52.
190 Rhedin AS, Tidefelt U, Jonsson K *et al.* Comparison of a bioluminescence assay with differential staining cytotoxicity for cytostatic drug testing in vitro in human leukemic cells. *Leuk Res* 1993; **17**: 271-6.
191 Thiede C, Koch S, Creutzig E *et al.* Prevalence and prognostic impact of NPM1 mutations in 1485 adult patients with acute myeloid leukemia (AML). *Blood* 2006; **107**: 4011-20.
192 Andersson A, Johansson B, Lassen C *et al.* Clinical impact of internal tandem duplications and activating point mutations in FLT3 in acute myeloid leukemia in elderly patients. *Eur J Haematol* 2004; **72**: 307-13.
193 Heck MM, Hittelman WN, Earnshaw WC. Differential expression of DNA topoisomerases I and II during the eukaryotic cell cycle. *Proc Natl Acad Sci U S A* 1988; **85**: 1086-90.
194 Brown GA, McPherson JP, Gu L *et al.* Relationship of DNA topoisomerase II alpha and beta expression to cytotoxicity of antineoplastic agents in human acute lymphoblastic leukemia cell lines. *Cancer Res* 1995; **55**: 78-82.
195 Gieseler F, Glasmacher A, Kampfe D *et al.* Topoisomerase II activities in AML and their correlation with cellular sensitivity to anthracyclines and epipodophyllotoxines. *Leukemia* 1996; **10**: 1177-80.
196 Huff CA, Matsui W, Smith BD *et al.* The paradox of response and survival in cancer therapeutics. *Blood* 2006; **107**: 431-4.
197 Galimberti S, Guerrini F, Palumbo GA *et al.* Evaluation of BCRP and MDR-1 co-expression by quantitative molecular assessment in AML patients. *Leuk Res* 2004; **28**: 367-72.
198 Abolhoda A, Wilson AE, Ross H *et al.* Rapid activation of MDR1 gene expression in human metastatic sarcoma after in vivo exposure to doxorubicin. *Clin Cancer Res* 1999; **5**: 3352-6.
199 Yague E, Armesilla AL, Harrison G *et al.* P-glycoprotein (MDR1) expression in leukemic cells is regulated at two distinct steps, mRNA stabilization and translational initiation. *J Biol Chem* 2003; **278**: 10344-52.
200 Volm M, Mattern J, Pommerenke EW. Time course of MDR gene amplification during in vivo selection for doxorubicin-resistance and during reversal in murine leukemia L 1210. *Anticancer Res* 1991; **11**: 579-85.

201 Adler V, Yin Z, Fuchs SY *et al.* Regulation of JNK signaling by GSTp. *EMBO J* 1999; **18**: 1321-34.
202 Tew KD. Glutathione-associated enzymes in anticancer drug resistance. *Cancer Res* 1994; **54**: 4313-20.
203 Chin KV, Tanaka S, Darlington G *et al.* Heat shock and arsenite increase expression of the multidrug resistance (MDR1) gene in human renal carcinoma cells. *J Biol Chem* 1990; **265**: 221-6.
204 Gahrton G. Treatment of acute leukemia--advances in chemotherapy, immunotherapy, and bone marrow transplantation. *Adv Cancer Res* 1983; **40**: 255-329.
205 Shtil AA. Emergence of multidrug resistance in leukemia cells during chemotherapy: mechanisms and prevention. *J Hematother Stem Cell Res* 2002; **11**: 231-41.
206 Brooks CL, Gu W. Dynamics in the p53-Mdm2 ubiquitination pathway. *Cell Cycle* 2004; **3**: 895-9.
207 Bertwistle D, Sugimoto M, Sherr CJ. Physical and functional interactions of the Arf tumor suppressor protein with nucleophosmin/B23. *Mol Cell Biol* 2004; **24**: 985-96.
208 Colombo E, Martinelli P, Zamponi R *et al.* Delocalization and destabilization of the Arf tumor suppressor by the leukemia-associated NPM mutant. *Cancer Res* 2006; **66**: 3044-50.
209 den Besten W, Kuo ML, Williams RT *et al.* Myeloid leukemia-associated nucleophosmin mutants perturb p53-dependent and independent activities of the Arf tumor suppressor protein. *Cell Cycle* 2005; **4**: 1593-8.
210 Cory AH, Chen J, Cory JG. Effects of PRIMA-1 on wild-type L1210 cells expressing mutant p53 and drug-resistant L1210 cells lacking expression of p53: necrosis vs. apoptosis. *Anticancer Res* 2006; **26**: 1289-95.
211 Christiansen DH, Andersen MK, Pedersen-Bjergaard J. Mutations with loss of heterozygosity of p53 are common in therapy-related myelodysplasia and acute myeloid leukemia after exposure to alkylating agents and significantly associated with deletion or loss of 5q, a complex karyotype, and a poor prognosis. *J Clin Oncol* 2001; **19**: 1405-13.
212 Herzog G, Lu-Hesselmann J, Zimmermann Y *et al.* Microsatellite instability and p53 mutations are characteristic of subgroups of acute myeloid leukemia but independent events. *Haematologica* 2005; **90**: 693-5.
213 Merlat A, Lai JL, Sterkers Y *et al.* Therapy-related myelodysplastic syndrome and acute myeloid leukemia with 17p deletion. A report on 25 cases. *Leukemia* 1999; **13**: 250-7.
214 Sterkers Y, Preudhomme C, Lai JL *et al.* Acute myeloid leukemia and myelodysplastic syndromes following essential thrombocythemia treated with hydroxyurea: high proportion of cases with 17p deletion. *Blood* 1998; **91**: 616-22.
215 Haferlach C, Dicker F, Herholz H *et al.* Mutations of the TP53 gene in acute myeloid leukemia are strongly associated with a complex aberrant karyotype. *Leukemia* 2008; **22**: 1539-41.
216 Nahi H, Selivanova G, Lehmann S *et al.* Mutated and non-mutated TP53 as targets in the treatment of leukaemia. *Br J Haematol* 2008; **141**: 445-53.