IDENTIFICATION AND MODULATION OF DRUG RESISTANCE IN ACUTE MYELOID LEUKEMIA

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IDENTIFICATION AND MODULATION OF DRUG RESISTANCE IN ACUTE MYELOID LEUKEMIA

IDENTIFICATIE EN MODULATIE VAN CYTOSTATICA RESISTENTIE IN ACUTE MYELOIDE LEUKEMIE

PROEFSCHRIFT

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IDENTIFICATION AND MODULATION OF DRUG RESISTANCE IN ACUTE MYELOID LEUKEMIA

CONTENTS

CHAPTER 1

INTRODUCTION

1.1 Hematopoiesis and leukemia

A continuous, strictly organised process of blood cell production or hematopoiesis normally takes place in the bone marrow. The human hematopoietic system is capable of replacing the normal daily loss of peripheral blood cells, and will adapt the blood cell formation to increased demands such as bleeding or infection. This system is capable of maintaining a balance between cell loss and formation. The different types of blood cells that are normally present in the peripheral blood, are derived from committed progenitor cells (1-4). The compartment of these committed progenitor cells is maintained by a small population of pluripotent stem cells (2-3,5). Besides the ability of the stem cells to give rise to committed progenitor cells, they are capable of self-renewal (2,4-5). The process of proliferation and differentiation is regulated by cellular interaction, i.e. the micro-environment in the bone marrow, and several regulatory glycoproteins, the hematopoietic growth factors (HGFs) (3,6-8).

Malignant transformation of hematopoietic cells somewhere during their development and leading to the accumulation of immature hemopoietic cells is referred to as leukemia. According to the clinical presentation, the leukemias are divided in acute and chronic leukemias. Acute leukemias, if untreated, will lead to death within several weeks or months, while patients with untreated chronic leukemias often may survive for several years. Depending on the cell lineages involved, a further distinction in myeloid or lymphoid leukemias can be made (9). In this thesis, acute myeloid leukemia is subject of investigation.

1.2 Acute myeloid leukemia (AML)

AML is a clonal disease, that is characterized by the expansion of an immature, abnormally differentiated, myeloid cell population in the bone marrow and peripheral blood (10-12). The excessive proliferation of leukemic cells, that generally fail to differentiate into functional mature blood cells, will eventually result in suppression of the normal hematopoiesis. As a consequence, immature leukemic cells appear in the marrow and peripheral blood and replace normal functional blood cells. Eventually, this will result in anemia, granulocytopenia, and thrombocytopenia. Without treatment, the median survival of untreated AML has been reported to be 2 months (13). Death has usually been caused by infectious and/or bleeding complications. Although the leukemic cells are the progeny of a single transformed malignant cell, morphological analysis and immunophenotyping have shown that the leukemic cells may have a partly retained ability

to differentiate (14-17). This is apparent as in a heterogeneity of the leukemic cell population within each patient.

1.2.1 Epidemiology of AML

The overall incidence of AML in the Netherlands is 2.5 per 10^5 persons per year (18). A comparison with international data shows geographical differences in incidence rates. An overall incidence has been reported varying from 1 to 3 per 10^5 each year (19-21). The incidence rates are greater for males than females (18-21), and the incidence increases with age, from below 1 per 10^5 persons each year during childhood and young adulthood, to over 10 per 10^5 persons each year after the age of 70. The median age of AML patients at diagnosis is over 60 years of age (9).

At present, the exact cause of AML is not known. Nevertheless, several risk factors have been reported in literature. Certain genetic syndromes, e.g. Down's syndrome and Fanconi's anemia, show a predisposition for AML development (20,22-23). Exposure to ionizing radiation and a number of chemicals have shown to be associated with the development of leukemia. Long-term follow-up studies of survivors of the atomic bomb explosions have indicated an increased risk of leukemia development (24). Also, patients who received low doses of radiation, e.g. for benign disorders, have increased risks to develop leukemia (24,25). Exposure to chemicals like benzene, pesticides, and other organic solvents are associated with increased risk of development of AML (20,26-28). Previous chemotherapy treatment also seems to predispose to AML development (29,30). An increased incidence of AML among cigarette smokers has also been suggested several times (31-33).

1.2.2 Treatment and prognosis

The aim of antileukemic therapy is the eradication of the malignant cells. Treatment strategies have been developed by using combinations of cytostatic drugs (34-36). With these protocols complete remissions (CR) are obtained in the majority of the AML patients. Nevertheless, only a part of the AML patients are long-term survivors. Especially treatment outcome of elderly AML patients, i.e. patients over the age of 60, is most unsatisfactory (37-38). The introduction of bone marrow transplantation (BMT) and intensive postinduction chemotherapy have improved long-term disease-free survival in young and middle aged adult patients with AML. Other treatment options like using differentiating agents, and hemopoietic growth factors may also be beneficial for some patients with AML.

Chemotherapy

Conventional chemotherapy can be distinguished according to remission induction and post-remission chemotherapy. For remission induction therapy, one or two cycles of a combination of cytosine arabinoside (Ara-C) (39), and an anthracycline (40), e.g. daunomycine, and one or two other drugs, like mitoxantrone (41), amsacrine (42) or etoposide (43) are generally used. Following this approach, about two-thirds of the AML patients enter CR (34-35,44-45). Whenever CR has been achieved, this does not mean that the patient has been cured. Residual leukemic cells often remain and may cause recurrence of AML. These AML cells are undetectable with standard cytomorphology techniques. In order to eliminate residual leukemic cells, post-remission chemotherapy is used.

Three different forms of post-remission chemotherapy can be distinguished, i.e. consolidation or early intensification, maintenance chemotherapy, or bone marrow transplantation. For consolidation one or more courses of therapy are given soon after achievement of a CR. In some schedules this can be followed by additional chemotherapy given over a period of 6 to 12 months. With maintenance chemotherapy, frequent courses are administered over a period of months or years. Although post-remission treatment has been shown to prolong the median duration of CR (46), there is no general agreement which treatment form should be preferred (35).

The median duration of CR ranges from 10 to 18 months in most studies (9,35). However, age is an important negative prognostic factor (37,47). Since the median age of AML patients is over 60 years, improvement of AML treatment in this group has remained a major goal.

Bone marrow transplantation

An effective therapy for AML is bone marrow transplantation (BMT). There are two modalities, autologous BMT and allogeneic BMT. For autologous BMT (ABMT), the patient's own BM or peripheral blood cells potential as a source of hematopoietic stem cells can be taken and cryopreserved once the patient has achieved CR. The autologous cells are reinfused later following total body irradiation and/or high dose chemotherapy as stem cell rescue. In allogeneic BMT, the stem cell graft comes from a HLA identical sibling or from an unrelated compatible donor.

The best results with allogeneic BMT have been obtained with patients transplanted during their first CR. Prolonged disease-free survival may be achieved in 40 to 70% of these patients (35,48). Allogeneic BMT is the most active antileukemic therapy, reducing relapse to approximately 20% of CR cases (35,49). Since mortality from allogeneic BMT

increases with age, patients older than 55 years of age are usually not considered eligible for this form of therapy (50). Graft versus host (GVH) reactivity might occur as the result of the infusion of the donor T-lymphocytes. Although removal of the T-cells from the graft prior to transplantation may prevent GVH disease, the rate of graft rejection and leukemia relapse may increase after this procedure in case no special measures are taken (35,51-52).

Because of the absence of GVH reactions, ABMT is less toxic than allogeneic BMT. Nevertheless, there are two major disadvantages associated with this post-remission treatment. First, graft versus leukemia reactions associated with allogeneic BMT is unlikely to occur. Second, in ABMT, there is a risk of reinfusing residual leukemic cells. In vitro attempts have been made to eliminate the residual leukemic cells in autografts, but the results appear to be controversial (35,53). Although allogeneic BMT seems to be a more effective therapeutic option in AML treatment than ABMT, comparative prospective trials are required to recognise the most effective therapy form (34-35,50). Recently, a study has been published comparing the outcome of allogeneic, autologous bone marrow transplantation and intensive chemotherapy (54). Allogeneic and autologous BMT resulted in better disease-free survival rates than chemotherapy treatment. Interpretation of the results of this study can be troubled for several reasons. First, median age of the AML patients differed between the three treatment groups in favour of the BMT treatment groups (55). Second, post-remission chemotherapy consisted of only one chemotherapy cycle, which could well be sub-optimal (55-56). Therefore, this study does not allow a definite conclusion whether BMT is superior to intensive post-remission chemotherapy.

Other treatment possibilities

Leukemic cells have at least partially lost the ability to differentiate. A possible approach for treatment is to induce differentiation of leukemic cells towards mature non-proliferating cells. Several agents, e.g. Ara-C (57), retinoic acid (58), dimethylsulfoxide (59), are capable of differentiation induction in vitro. In a distinct AML subgroup, i.e. AML-M3 with a specific cytogenetic abnormality in the leukemic cells, t(15;17)(q22;q21), all-trans-retinoic acid has been found to be effective in differentiation induction and can result in CR (60-62). Nevertheless, chemotherapy remains necessary to prevent relapse (60-61).

The role of hematopoietic growth factors in enhancing the cytotoxic effects of chemotherapeutic drugs by proliferation induction will be discussed extensively in §1.7.1 and the chapters 4, 5, and 6 of this thesis.

Immunological approaches of AML treatment have focused on stimulating lymphokine-activated killer (LAK) cells by interleukin-2 (IL-2) to enhance lysis of leukemic cells. The

number of patients included in studies using IL-2 for the treatment of relapsed AML patients are small so that the results are still preliminary (63-65).

1.3 Drug resistance

As discussed in §1.2.2, about one-third of the AML patients do not attain a CR with conventional chemotherapy treatment. In patients following relapse of AML, new complete response rates can be attained in approximately 40% of cases (9,34). Extensively pretreated patients are less likely to respond and are less likely to enter CR. Failure of conventional chemotherapy can be caused by existing or developing resistance for the relevant cytostatic drugs. Several mechanisms of drug resistance have been proposed and some have been identified. Resistance to a single agent like Ara-C, but also cross-resistance of several structurally unrelated drugs (multidrug resistance [MDR]) have been reported in cell lines and in clinical tumour samples (66-67). In the next paragraphs, the drug resistance mechanisms that might be involved in resistance for AML chemotherapy are described. The resistance mechanisms and related drugs are summarized in table 1.

1.4 Resistance to cytosine-arabinoside (Ara-C)

As described in §1.2.2, one of the most effective anti-leukemic agents is Ara-C, and for this reason, this drug is included in virtually all AML treatment regimens. The mechanisms of drug action and resistance to Ara-C have been extensively studied and reported.

Ara-C metabolism

Ara-C is the synthetic 2'-α hydroxy derivative of deoxycytidine. Ara-C enters the cell via a nucleoside carrier (68), although simple diffusion across the cell membrane has also been described (69). After entering the cell, Ara-C is phosphorylated to its mono-, di-, and triphosphate forms by a series of kinase enzymes (Figure 1) (70-71). The triphosphate form, Ara-CTP, is considered to be the metabolically active component (71-72). In this process, the first phosphorylation step, from Ara-C to Ara-CMP, is catalyzed by the enzyme deoxycytidine kinase (dCk), and has shown to be rate-limiting (73). Two major mechanisms of cytotoxicity of Ara-CTP have been proposed. First, Ara-CTP has shown to inhibit DNA polymerases by competing with its natural substrate deoxycytidine triphosphate (dCTP) (74). Second, small amounts of Ara-CTP are incorporated into DNA

Table 1. Drug resistance in acute myeloid leukemia

Type of resistance	Drugs involved	Mechanisms	Page
Ara-C resistance	Cytosine-arabinoside (Ara-C)	- Kinetic resistance - Deoxycytidine kinase levels/activity (↓)* - Rapid deamination - dCTP pools (↑)* - DNA polymerases activity (↓) - DNA repair (↑)	16
Typical MDR†	Several unrelated drugs: - Anthracyclines, e.g.: daunomycin, doxorubicin - Epipodophyllotoxins, e.g.: etoposide - Vinca-alkaloids, e.g.: vincristine, vinblastin - Other, e.g.: actinomycine-D, paclitaxel, colchicine, mitoxantrone, amsacrine	- *MDR*1 overexpression	16
MRP‡	Several unrelated drugs: - like typical MDR (similar, but not identical pattern)	- Expression of MRP‡	21
A-typical MDR	Several unrelated drugs: - like typical MDR (similar, but not identical pattern)	- Expression of p-110 - Decreased/altered activity of Topoisomerase IIα	21 22
Other mechanisms	Several drugs like: - Anthracyclines - Alkylating agents - Cisplatin	- Gluthation S-transferase - Glutathione peroxidase - Glutathione levels (↑) - Methallotionine	23

* ↓ or ↑, decreased or increased.
† MDR, multidrug resistance.
‡ MRP, multidrug-resistance-associated protein

(75-77), where the incorporated Ara-C residues behave as relative chain terminators (78). Other mechanisms that have been suggested to be involved with cytotoxicity are effects on mitochondrial functions (79), induction of apoptosis (80-81), and inhibition of DNA ligase (82).

Mechanisms of resistance to Ara-C

Several mechanisms of resistance for Ara-C have been described. Since Ara-C mainly acts during active DNA synthesis, non-cycling cells will be relatively protected from the cytotoxic effects. This form of resistance is designated as kinetic resistance. Defects in Ara-C metabolism may also cause Ara-C resistance (pharmacological resistance). The main cause of pharmacological resistance is impaired Ara-CTP formation. Impaired incorporation into the DNA and enhanced DNA repair mechanisms might also contribute to Ara-C resistance.

Possible causes for impaired Ara-CTP formation may relate to the failure of Ara-C to enter the cells. This has been described as a cause of resistance in cell lines (83). Since intracellular Ara-C accumulation is only limited by the transmembrane transport rate at extracellular concentrations below 10 μM in AML cells, this possibility is only important in treatment with low-dose Ara-C (84). Second, low levels or altered activity of dCk may cause reduced phoshorylation of Ara-C to Ara-CMP, and will also result in reduced Ara-CTP formation (85-86). Third, increased levels of cytidine deaminase or dCMP deaminase enhance deamination of Ara-C and Ara-CMP to the inactive metabolites Ara-U and Ara-UMP (87). As a result, Ara-CTP accumulation will be less. Finally, intracellular dCTP pools have a regulatory role in Ara-C metabolism, e.g. as regards i) negative feedback on dCK activity, ii) allosteric activation of dCMP deaminase, iii) competition with Ara-CTP for incorporation into DNA (88-89). Therefore, elevated intracellular dCTP pools will suppress Ara-C phosphorylation. Some of the possible mechanisms of resistance for Ara-C are summarized in Figure 1.

Even if Ara-CTP formation and retention are not reduced, resistance for Ara-C can occur. Mutations in DNA polymerases may result in a failure to recognise Ara-CTP as a substrate (90). Also, enhanced DNA repair enzyme activity that removes Ara-C residues from the DNA has been described (91).

1.5 Typical multidrug resistance

Cell lines expressing cross-resistance for several structurally unrelated drugs were first described by Biedler *et al* in 1970 (92). Others have shown that MDR was associated with the (over)expression of a 170-kDa membrane protein (P-glycoprotein [P-gp]), and a decrease of intracellular drug accumulation (93-94). Several cell lines have been made resistant to cytostatic drugs which display this phenotype. Usually, these resistant cell lines contain an amplified gene, the *MDR*1 gene (95). Increased *MDR*1 expression without gene amplification has also been reported, especially at low levels of resistance (96). The *MDR*1 gene has been mapped on the long arm of chromosome 7 (97). Transfection

experiments have shown that expression of a full length cDNA clone of the human *MDR*1 gene in drug sensitive cells confers the MDR phenotype to these cells (98).

The MDR pump, P-gp, is a member of the ATP-binding cassette (ABC) superfamily of transport systems. These forms of transport systems have been found in several organisms, including bacteria, yeasts, plants, insects, and mammals. P-gp is a transmembrane glycoprotein consisting of two similar, but not identical domains. Each domain has six transmembrane segments, and one intracellular ATP binding site (99). P-gp is considered to be an unidirectional, energy (ATP) dependent efflux pump. Although the drugs that are a substrate for P-gp are structurally unrelated, they are usually hydrophobic, have a molecular weight of 300-900, and enter the cell by diffusion. Drugs that form a substrate for P-gp are summarized in Table 1. There is no cross resistance for alkylating agents (e.g. cyclophosphamide) or antimetabolites like Ara-C.

The exact mechanism of drug transport is still unclear. Extrusion of drugs through a channel formed by transmembrane segments has been suggested (100). Alternatively, a "flippase" model has been proposed (101). In this model, drugs intercalating with the lipid bilayer will be removed by P-gp before entering the cytoplasm. Previous studies have shown that binding of vinblastine to the P-gp molecule was inhibited by daunomycin, but not by colchicine (102-103). This suggest that different parts of the P-gp molecule may participate in transport of different drugs. Typically, the transport function of P-gp can be inhibited by several agents which will be discussed in §1.7.2

In humans, another P-gp isoform has been identified. This P-gp form is encoded by the *MDR*3 gene which is located at the long arm of chromosome 7 (104-105). The two forms of P-gp have highly homologous amino acid sequences of about 80%. Nevertheless, expression of *MDR*3 gene does not seem to be associated with drug resistance (106).

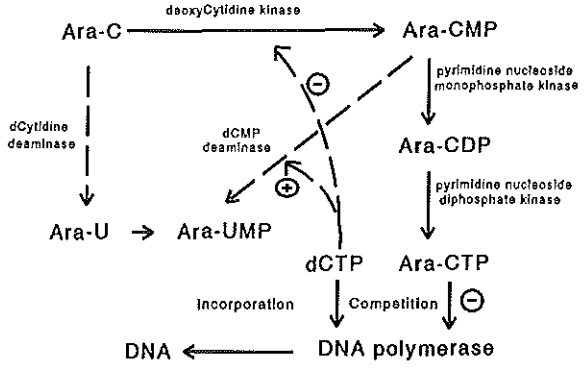

Fig. 1: Metabolic pathways of Ara-C metabolism. Normal phosphorylation and incorporation of Ara-C (—); inactivation to metabollically in active forms(---). (-), (+); Negative or positive feedback respectively.

Table 2. Characteristics of monoclonal antibodies used for P-glycoprotein detection.

Monoclonal antibody	Epitope	*MDR*1 specific
JSB-1	Intracellular	No
C219	Intracellular	No
C494	Intracellular	Yes
MRK-16	Extracellular	Yes
4E3	Extracellular	Yes
MoAb 57	Extracellular	Yes

References (107-112)

MDR mediated through *MDR*1 (P-gp) expression is named typical or classical MDR. Cross-resistance for several drugs has also been found without *MDR*1 expression. This form of MDR is referred to as atypical MDR.

Several techniques can be used in order to determine *MDR*1 expression. At RNA level, semi-quantitative polymerase chain reaction (PCR), quantitative RNase protection assay, RNA-RNA hybridisation methods, slot- or Northern blotting have been employed to detect and/or quantify the mRNA content. These assays are suitable for *MDR*1 detction in total cell populations. Specific RNA probes have also been used for in situ detection of *MDR*1 expression. The lack of correlation between *MDR*1 mRNA levels and P-gp expression is a disadvantage of RNA assays in *MDR*1 detection. Western blotting can be used for P-gp detection in total cell populations. P-gp expression in individual cells can be studied by immunocytochemistry using P-gp specific monoclonal antibodies (MoAbs). Some of these MoAbs are specific for P-gp encoded by the *MDR*1 gene, while others also recognise P-gp encoded by the *MDR*3 gene (107-112). Table 2 presents some of the characteristics of the MoAbs that can be used for P-gp detection. Functional analysis of P-gp expression is used to study the effects of P-gp expression on the intracellular accumulation or efflux of the substrates (e.g. cytostatic drugs) tested. This assay has been done by flowcytometric analysis of accumulation or efflux of fluorescent P-gp substrates like daunomycin, doxorubicin or rhodamine. Another possibility is to determine functional P-gp expression by assessing accumulation or efflux of tritiated cytostatic drugs.

*MDR*1 expression in normal tissues

*MDR*1 is expressed in several human tissues (113). Although *MDR*1 mRNA transcripts can be found in many organs, especially high levels have been found in the adrenal medulla, kidney, lung, liver, jejunum, and colon (114). Immunocytochemistry studies have shown the distribution of P-gp in these tissues. In the liver, P-gp is found in the bile

canalicular surface of hepatocytes, in the intestine on the mucosal surface, and in the kidney on the luminal surface of the brush border of proximal tubules (115). The localisation of P-gp suggests that pumping xenobiotic substances out of the cell is a physiological function of P-gp. Since P-gp is also associated with volume-regulated chloride channels, osmotic regulation might be another physiological function (116-117).

*MDR*1 is also expressed in normal hematological tissues. In PB, expression of *MDR*1 mRNA has been found in subsets of B- ($CD19^+$) and T-lymphocytes ($CD4^+$, $CD8^+$), natural killer (NK) cells ($CD56^+$), and monocytes ($CD14^+$) but not in granulocytes ($CD15^+$)(118-119). In BM, *MDR*1 expression has been found in lymphoid cells ($CD19^+$) and $CD34^+$ hemopoietic progenitor cells (120-121). Chaudhary and Roninson showed the functionality of P-gp expression in $CD34^+$ hemopoietic progenitor cells using rhodamine efflux studies (120). Reversal of *MDR*1 expression by addition of verapamil has provided strong evidence for P-gp functionality in these cells. This might explain the relative resistance of hemopoietic progenitor cells for chemotherapy.

*MDR*1 expression in hematological and non-hematological malignancies

*MDR*1 expression has been demonstrated in many solid malignancies. Especially tumours derived from tissues that normally express *MDR*1, were found to be *MDR*1 positive (122-123). Solid tumours that are *MDR*1 negative or express *MDR*1 only at low levels include ovarian, lung, bladder, oesophageal, head and neck cancer (122). High *MDR*1 expression has been found in tumours of patients who have previously been treated with cytostatic drugs (122). This suggests that besides intrinsic *MDR*1 expression, *MDR*1 positivity can also be induced following exposure to drugs. Selection of *MDR*1 positive cells during chemotherapy treatment could explain the increase of *MDR*1 positive cells following treatment. Other investigators have found that various anticancer agents appear capable of activating *MDR*1 transcription (124-125).

Overexpression of *MDR*1 in hematological malignancies has been reported by several investigators (122,126-131). Almost all hematological malignancies show detectable *MDR*1 expression. In tissue samples of previously treated patients and with clinically resistant disease, *MDR*1 expression is often elevated.

In untreated AML, the percentages of *MDR*1 positive AML samples has been reported to vary from 17 to 71% (132-138). The large variation may be caused by differences in sensitivity of the techniques used to assess *MDR*1 expression and differences in thresholds set for positivity. It may also relate to patient selection in those studies. For instance, in cell samples from patients with relapsed AML, *MDR*1 expression is significantly greater as compared to that of newly diagnosed AML (139-140).

As indicated before, normal stem cells have a low level of P-gp expression. In AML

and myelodysplastic syndromes (MDS), P-gp expression is associated with CD34 positivity (141-142). In one of our own studies, we were able to demonstrate that P-gp in $CD34^+$ AML is predominantly expressed by the $CD34^+$ blast cells (138) (chapter 2). Drenou *et al* reported enhanced rhodamine efflux in $CD34^+$ AML as compared with $CD34^-$ cells, although no specific estimation of P-gp expression was included in the latter study (143). The results of these studies suggest that the physiological expression of P-gp in normal hemopoietic stem cells can be preserved through leukemogenesis. This may explain the relatively high *MDR*1 expression found in untreated AML.

The functional expression of P-gp has also been the subject of study. In one report, daunomycin accumulation was roughly proportional to the level of P-gp expression among a series of AML cell samples (144). Another study reported no correlation of P-gp expression and daunomycin accumulation (145). Expression of other drug transport systems might cause this discrepancy. Although rhodamine accumulation and efflux appears more convenient to study the "pump" function of P-gp (146), functional assays using clinically relevant drugs can provide important information of the P-gp function.

Prognostic value of *MDR*1 expression in AML

Several investigators have reported the clinical significance of *MDR*1 expression (129,132-139,147-152). In most of these studies, *MDR*1 expression was found to be an unfavorable prognostic factor (132-139,147,149-150,152). In newly diagnosed AML, CR rates were significantly less in patients with *MDR*1 positive AML. Pirker *et al* reported also a significant decrease in overall and disease-free survival in *MDR*1 positive AML (136). Currently, most of the available evidence would indicate that *MDR*1 expression is associated with poor prognosis in AML.

CD34 expression has also been associated with a poor prognosis in AML (138,145,152-153). In several studies, a subgroup of patients with an extreme poor prognosis could be identified by comparing the outcome of chemotherapy with P-gp and CD34 expression (138,145,152). In chapter 2 of this thesis, the possible explanation of this observation will be discussed. In chapter 3, the prognostic value of Pgp expression in AML is further addressed.

1.6 A-typical multidrug resistance

The term a-typical multidrug resistance is used for MDR that is not associated with the expression of P-gp. A-typical MDR includes several different mechanisms that are possibly associated with drug resistance. These mechanisms may involve expression of

multidrug associated protein (MRP), expression of p-110 (LRP-56), and topoisomerase-IIα mediated resistance.

1.6.1 Multidrug resistance associated protein (MRP)

In 1992, a novel resistance-associated gene overexpressed in a P-gp negative, multidrug resistant, small cell lung carcinoma cell line (H69AR) was described by Cole *et al* (154). This MRP gene encodes for a 190 kDa membrane bound glycoprotein (p190) (155), and is located on chromosome 16 at band p13.1 (154). Based on its primary sequence and its secondary structure, MRP, like *MDR1*, is a member of the ATP-binding cassette (ABC) superfamily of transport systems. In cell lines, MRP overexpression is associated with gene amplification (156-157). Transfection experiments showed increased drug resistance (155). MRP mRNA is found in a variety of human normal tissues, especially in muscle, lung, testes and peripheral blood mononuclear cells (154,157-158). The analysis of subcellular fractions of a resistant cell line, located the presence of p190 primarily in the endoplasmic reticulum but also, at lower levels in plasma membranes (155). The physiological function of MRP expression is still unclear. MRP might play a role in cellular osmotic regulation, since it appears be associated with ion channels (159).

The expression of MRP has also been the subject of study in leukemia. Using a sensitive RNase protection assay, 15 of 21 samples (70%) of chronic lymphocytic leukemia were found to have a relative high MRP gene expression (160). In the same study, low or absent MRP expression was found in multiple myeloma, acute lymphocytic and chronic myelocytic leukemia. For AML, 3 of 16 samples were found to be positive. Another study reported MRP overexpression in 9 of 17 AML samples (52%), using a highly sensitive semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) (161). No significant differences in MRP mRNA expression between chemotherapy sensitive or resistant AML patients have been found in one study (162). Increased MRP expression in leukemic cells of AML patients at relapse has been described by others (163-164). The clinical relevance of MRP overexpression, however, remains unclear. In chapter 3 of this thesis, MRP overexpression in untreated AML is discussed in relation to other resistance mechanisms and clinical outcome.

1.6.2 Expression of p110

The overexpression of a protein with a molecular weight of 110-kDa (p110) has been found in drug resistant tumour cell lines using the MoAb LRP-56 (165). These cells appear to be cross-resistant to vincristin, etoposide, and doxorubicin. In these cell lines,

the *MDR*1 gene is not overexpressed, although drug accumulation deficiencies are present. Immunocytochemical studies have shown that staining with the MoAb LRP-56 is primarily present in the outer cytoplasmic zone of the cells. This suggests that LRP recognizes an epitope that is closely related to endoplasmatic reticular/lysosomal structures. Staining with the plasma membrane of the drug resistant cells was less prominent. In normal tissues, p110 expression is found in epithelial cells as well. LRP-56 staining was especially found in epithelia with excretory or secretory functions, like bronchial, renal and adrenocortical cells. A physiological role as a transport protein is assumed.

In a serie of 56 AML samples (34 untreated, 22 relapsed AML samples), LRP-56 was detected in 36% of cases, and appeared to be associated with the clinical response after chemotherapy (166). In chapter 3 of this thesis, LRP-56 expression has been studied and the results are discussed with respect to other mechanisms of drug resistance.

1.6.3 Expression of topoisomerase II

Topoisomerases (topo) are nuclear enzymes that are involved in modifying and controlling the topological state of DNA (167). Two major types have been identified. One type, topo I, functions by breaking one DNA strand and resealing the break through the complementary strand of DNA (167). The second type, topo II, catalyzes coordinated breakage and resealing of double-stranded DNA (167-168). During this ATP-dependent process, the enzyme is covalently linked to the DNA, and allows passage of another DNA strand. The transient covalent enzyme-DNA complex (cleavable complex) can be stabilized by several anti-neoplastic drugs including epipodophylotoxins and intercalating agents (169-170). As a consequence, this results in DNA lesions that eventually lead to cell death. Topo II gene mutations and reduced topo II levels have been associated with drug resistance (171-172). In mammalian cells, two isoforms of topo II, topo IIα (170-kDa) and topo IIß (180-kDa), have been identified (173). They are encoded by different genes (174), and are differentially regulated during cellular proliferation (175-176). In vitro experiments have shown that topo IIα is more sensitive than topo IIß to some of the topo II-interactive drugs, e.g. amsacrine (175). Therefore, especially alterations in topo IIα levels or activity can account for drug resistance. Nevertheless, the topo IIß isoform has found to be depleted in a myeloid cell line that was made resistant with mitoxantrone (177). The precise role of topo IIß in resistance still has to be elucidated (178).

In AML, a large variation in topo IIα and topo IIß expression is observed (179-181). In one of these studies, the results of topo II expression in AML samples, obtained from 140 untreated and 57 treated AML patients were presented (179). No relation of topo II expression with in vitro and in vivo drug sensitivity was found. No topo II gene mutations

accounting for resistance could be identified either. Due to the relatively small sample size, results of other studies are difficult to interprete.

1.6.4 Other mechanisms drug resistance

Beside the mechanisms of drug resistance introduced above, several more or less non-specific detoxification mechanisms have been identified that might be involved in drug resistance. Among these are altered (increased) intra-cellular levels of glutathione (GTH), glutathione S-transferases (GSTs), glutathione peroxidase, and metallothionein (131,182-183). Of these, only (over)expression of GSTs in AML has been seriously studied.

GSTs are encoded by four genes. Normally, they play an important role in the detoxification of drugs, toxins, and carcinogens by catalyzing the conjugation of these compounds to reduced glutathione (184). Resistance to anthracyclines might be linked to GST overexpression. Increased expression of one of the GST subgroups, GSTπ, has been found in hematological malignancies, including AML (131,185-186). In one study, decreased GSTπ expression was correlated with poor initial treatment results and decreased remission duration (187). This correlation was not found by other investigators (185,188).

At present, it remains unclear whether this group of drug resistance mechanisms is clinically relevant in the development of drug resistance in AML.

1.7 Modulation of drug resistance

1.7.1 Modulation of Ara-C metabolism

Attempts have been made to modulate or reverse the causes of drug resistance. As described in §1.4, resistance for Ara-C can be caused by kinetic and/or pharmacological resistance. Much effort has been made to enhance Ara-C cytotoxicity in vitro by inducing proliferation of AML cells in order to overcome kinetic resistance. Several hemopoietic growth factors (HGFs) have been used for this purpose (189). Enhanced cytotoxicity to Ara-C has been described for granulocyte colony-stimulating factor (G-CSF) (190-194), interleukin-3 (IL-3) (193,195-197), granulocyte-macrophage colony-stimulating factor (GM-CSF) (193,198-200), and stem cell factor or C-kit ligand (201). Also, enhanced Ara-C cytotoxicity by combined exposure of cells to Ara-C and combinations of HGFs has been reported (191,201-206). Despite differences of cell-culture conditions and experimental design of these studies, enhanced cytotoxicity by combined HGF and Ara-C exposure of AML cells has been reported consistently. Some studies did not find these

effects (191,194,207). Although it is generally believed that increased numbers of AML cells in S-phase are induced by HGF stimulation which may condition for enhanced cytotoxicity. Effects of HGF stimulation on Ara-C metabolism, i.e. enhanced Ara-CTP formation and Ara-C incorporation into DNA, have also been described (208-211). Experiments comparing the effects of HGF stimulation plus Ara-C exposure in AML cells and normal bone-marrow cells show quantitative differences of Ara-C metabolism, resulting in increased intracellular dCTP pools in normal hemopoietic progenitor cells but not in AML cells (212-214). As a consequence, normal hemopoietic progenitors are relatively protected from Ara-C cytotoxicity as compared with leukemic cells. The concept of in vitro modulation of cytotoxic drugs, and especially Ara-C, by concurrent HGF stimulation will be discussed in the chapters 4, 5, and 6 of this thesis.

Clinical trials using GM-CSF in combination with induction chemotherapy in order to modulate Ara-C resistance, have also been published (215-216). One study included 18 newly diagnosed AML patients, and reported a high CR rate (83%), and an early recovery of bone-marrow aplasia (215). No data of the long-term follow-up were available. One other study, reported by Estey *et al* in 1992, included 56 newly diagnosed AML patients, and has shown a decreased CR rate as a result of a significant frequency of basic mortality, and a lower survival probability as compared to the historical control group. Büchner presented preliminary data about a trial that included 63 newly diagnosed AML patients, and showed beneficial effects of GM-CSF priming on the remission duration (217). G-CSF before, during and after induction chemotherapy did not show beneficial effects on CR rate and disease-free survival in a study that included 50 relapsed or primary resistant AML patients as compared with placebo treated controls (218). The results of these trials indicate that HGF combined with induction chemotherapy should be used with great caution. These studies are still of a preliminary nature. At present time the position of HGFs in AML therapy has not been established.

Attempts to reduce mortality in elderly AML by shortening the neutropenic period after chemotherapy treatment using HGFs have been reported (219,220). No effect on survival has been found using G-CSF (219) or GM-CSF (220). Explanations for these results can be the use of suboptimal chemotherapy treatment or differences in biological behavior of the disease in elderly AML patients (221).

The purine nucleoside analogue 9-ß-D-arabinofuranosyl-2-fluoroadenosine (F-Ara-A; fludarabine) is an effective agent for the treatment of chronic lymphocytic leukemia (222-223).

Like Ara-C, fludarabine has to be phosphorylated to its cytotoxic triphosphate form (F-Ara-ATP) (224). The rate-limiting step in this process is catalyzed by the enzyme deoxycytidine kinase (dCK) (225). In vitro, fludarabine has been shown to potentiate ara-C metabolism (226-227). Cell lines pretreated with fludarabine accumulate Ara-CTP at a faster rate and to a greater concentration as compared with cells that were treated with

Ara-C alone (226-227). Synergistic cytotoxic effects of Ara-C on clonogenic AML cells pretreated with fludarabine have also been described (228). The potentiation of Ara-C metabolism by fludarabine can be explained by a direct and indirect stimulatory effect of F-Ara-ATP on dCk (229). Since fludarabine is inactive in dCk deficient cells (225), its role in modulating pharmacological resistance to Ara-C probably will be of limited value.

Clinical trials combining fludarabine and Ara-C treatment have shown that Ara-CTP formation in circulating AML cells is increased by fludarabine (230). Increased remission rates obtained with this form of treatment in relapsed AML patients with initial remissions >1 year have been reported (14/20 with fludarabine vs 9/23 without fludarabine) (231). Major side effects that are associated with fludarabine treatment are neurotoxicity (232), cytopenia (233), and impaired T-cell function (234). Especially sustained effects on the number of CD4 positive cells have been reported (235-236). Randomised studies would be needed to determine the value of the combined fludarabine and Ara-C treatment in AML.

1.7.2 In vitro modulation of typical MDR

Cell lines expressing the MDR phenotype have been used to determine the modulating capacity of many agents in vitro. Two possible approaches of *MDR*1 reversal by these agents can be distinguished. First, some agents like *MDR*1 specific anti-sense oligonucleotides (237), and protein C kinase inhibitors like staurosporine are capable of down-regulating *MDR*1 expression (238-240). The reduced *MDR*1/P-gp expression will decrease functional resistance. Another approach to modulate the P-gp function is inhibiting the interaction of P-gp with cytostatic drugs by several so called reversing agents. Direct interaction of P-gp and MDR reversing agents has been demonstrated with competitive binding experiments using [^{3}H]azidopine or tritiated cytostatic drugs (241-243). It is currently accepted that reversal agents can restore drug accumulation by competing with cytostatic drugs for P-gp binding sites. These agents include calcium channel blockers (244-245), calmodulin inhibitors (241,246), immunosuppressive agents (242,245,247-250), quinolines (251), indole alkaloids (252), detergents (253-254), steroids (255), and antiestrogens (256) (Table 3). Several of these reversing agents have common chemical features like a planar aromatic domain and two amino groups, one of which has a cationic charge at physiological pH (257). Not all modulators meet these criteria, but all are highly lipophilic (258-259). Synergistic effects by combining several modulators like verapamil and cyclosporin or other modifiers have been described (256,260-263). This observation suggests that the exact mechanism of drug reversal is not identical for all reversing agents. Therefore, the exact mechanism of modulation remains to be determined. Table 3 shows some examples of these modulating agents and the effective concentrations to reverse MDR in vitro (264). *MDR*1 specific monoclonal

antibodies have also shown to modulate P-gp function (265). The potent protein C kinase inhibitor staurosporin and its derivates can not only downregulate the *MDR*1 gene expression, but also inhibit drug binding (266-267). The underlying mechanism is inhibition of P-gp phosphorylation thereby interfering with its function (268), rather than competing for P-gp bindingsites.

P-gp modulators have also shown to be effective in clinical specimens of AML patients (269-272). It has been demonstrated in these studies that drug accumulation can be restored by modulators (269). Enhanced cytotoxicity in AML has been reported by others, although cytotoxic effects were not always correlated to P-gp expression in the AML samples (270-271). This may indicate that other resistance mechanisms can also be important in drug resistance in AML. Also, the effects of P-gp modulators combined with cytostatic drugs have been tested on normal hemopoietic progenitors (272-273). The results of these experiments have shown no or only moderate increased cytotoxic effects (273-274). Only one study compared the drug-modulation effects of verapamil and R-verapamil on normal and leukemic progenitor cells (272). Nevertheless, other studies should be done to compare the modulating effects on normal and leukemic cells in vitro.

1.7.3 In vivo modulation of typical MDR

Several agents that have been shown to reverse MDR in vitro have been tested in clinical trials. Of these, verapamil has been studied extensively in hematological and in non-hematological malignancies (275-286). Especially in hematological malignancies, the results of these phase I or II trials are encouraging. Increased responses and better survival have been reported when patients with myeloma (279,285) or non-Hodgkin's lymphoma (279,285), were treated with a combination of verapamil and cytostatic drugs. Trials in patients treated with verapamil have shown serious, dose-limiting cardiotoxic effects (275-276,284,286). Because of the serious side effects associated with verapamil treatment, other, less toxic modulators have been evaluated. Another calcium antagonist, bepridil, has been used in 14 patients with therapy-resistant, solid malignancies (287). No major response was observed. A possible explanation could be the low P-gp expression in the tumor samples tested before treatment with bepridil and anthracyclines.

Trifluoperazine, a calmoduline inhibitor was evaluated for MDR reversal in vivo in one study (288). The patient group consisted of patients with doxorubicin resistant malignancies (e.g. breast-, ovarian-, gastric carcinomas, and sarcomas). It was found that this reversing agent was well tolerated with extrapyramidal side effects as dose limiting toxicity. Several patients responded to this form of therapy.

Promising results are obtained with cyclosporine (CsA) as a drug-resistance modifier. Sonneveld and Nooter reported the in vitro restoration of daunomycin accumulation by cyclosporin in *MDR*1-positive leukemic cells obtained from a relapsed AML patient (289).

Table 3. MDR reversing agents in vitro

Class of agent	Examples of agents	
Calcium channel blockers	Verapamil (6-10 μM); Nifedipine (35 μM);	Bepridil (4μM) Dex-Niguldipine (10μM)*
Calmodulin inhibitors	Trifluoperazine (3-5 μM); Fluphenazine (3 μM);	Prochlorperazine (4 μM) trans-Flupenthixol (3-5 μM)
Immunosuppressive agents	Cyclosporin A (0.8-2 μM)*; FK-506 (3 μM)*	SDZ PSC 833 (0.1-1 μM)*
Quinolines	Quinidine (10μM);	Chloroquine (10-50 μM)
Indole alkaloids	Reserpine (5 μM);	
Detergents	Cremaphor EL (1:10,000);	Tween 80 (1:1,000)
Steroids	Progesterone (2 μM);	Megestrol (5 μM)
Antiestrogens	Tamoxifen (3-10 μM)	

Concentrations in parenthesis refer to the effective concentrations to reverse MDR in vitro.
* Clinically relevant agents.

This patient was treated with reinduction therapy and cyclosporin, and a transient elimination of the *MDR*1-positive leukemic clone was observed in vivo. A phase I/II study in poor-risk AML showed a particular high response rate (62%) (290). The most prominent side effect noted was a transient hyperbilirubinemia but no elevated serum liver enzymes. The hyperbilirubinaemia might be caused by the inhibition of the physiological P-gp expression in the bile canaliculi. Altered pharmacokinetics of cytostatic drugs associated with CsA treatment, i.e. a dose-dependent decrease in drug elimination, has been reported (291). This is most likely caused by cyclosporin induced impaired function of physiologically expressed P-gp in the liver and kidney. Therefore, increased plasma cytostatic drug concentrations, caused by the inhibition of physiological P-gp function, can also in addition to MDR modulation alter the outcome of these clinical trials. In drug-resistant myeloma, cyclosporin has also shown to induce responses without serious nephrotoxic or cardiovascular side effects (292).

Other modulators that are used in clinical trials include tamoxifen (293-294), quinine (295), quinidine (296), and nifedipine (297).

The overall results of these trials clearly indicate that drug-resistance modifiers can be clinically useful in order to circumvent MDR. Dex-niguldipine, cyclosporin A, FK-506 and SDZ PSC 833 seem to be the most clinically relevant reversing agents since effective concentrations without serious side effects can be achieved in vivo. Randomised,

prospective phase III studies are needed for further evaluation.

1.8 Aim of this study

Resistance to chemotherapy is an important cause of treatment failure in acute myeloid leukemia. Several mechanisms of primary or acquired resistance may be involved in clinically refractory disease. In this study, we have analyzed the frequency and clinical relevance of Ara-C mediated resistance and multidrug resistance as potential causes of resistance in a series of de novo AML patients. In addition, we have explored the possible role of other drug resistance mechanisms for treatment failure. We have made an attempt to identify the phenotype of AML cells which potentially escape drug-induced cell kill and thus may represent residual tumor cells that eventually give rise to relapse. Finally, the potential beneficial role of drug-resistance modifiers and hematopoietic growth factors was investigated since these agents may represent a new approach to modulate drug-resistant AML cells and circumvent resistance.

In chapter 2 the prognostic value of *MDR1* overexpression in newly diagnosed AML is described. Also, the association of P-gp expression with an immature immunophenotype (CD34 expression) is discussed.

In chapter 3 various drug-resistance mechanisms are examined in 38 patients with untreated AML. A comparison with relevant clinical prognostic factors is made to assess clinical relevancy of the in vitro parameters of drug resistance.

In chapter 4 the effects of HGF stimulation on the cytotoxicity of Ara-C and mafosfamide, a non S-phase specific drug, on untreated clonogenic AML cells are described. The relationship between HGF enhanced cytotoxicity and cell-cycle activation is determined.

In chapter 5, we investigated the role of HGF stimulation on Ara-C cytotoxicity in relapsed or primary refractory AML. The results are compared with those obtained with AML cells of the same patients with untreated disease. In some samples, the effects of HGF stimulation on Ara-C metabolism has also been studied. This has been done in order to make a distinction between kinetic and metabolic effects of HGF stimulation on Ara-C action.

In chapter 6, the effects of HGF stimulation on cytotoxicity of several drugs are described. The results obtained with two different assays that claim to be of clinical relevance, i.e. the MTT assay and the clonogenic assay, are compared. The differences in the results using these assays are discussed.

In chapter 7 the results and relevance of the presented experimental data are discussed.

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CHAPTER 2

PREDOMINANCE OF FUNCTIONAL MULTIDRUG RESISTANCE (*MDR1*) PHENOTYPE IN CD34$^+$ ACUTE MYELOID LEUKEMIA CELLS.

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SUMMARY

The expression of the *MDR*1-encoded P-170 glycoprotein (P-170) associated with clinical multidrug resistance (MDR) was investigated in 52 consecutive patients with untreated acute myeloid leukemia (AML). P-170 expression was analyzed in correlation with CD34 expression and clinical response. Thirty of 52 patients expressed P-170 (58%). Eight of 30 P-170$^+$ as compared to 16 of 22 P-170$^-$ patients achieved a complete remission (CR) (27% vs 73%, p=0.003). In 21 of 30 p-170$^+$ patients, expression of the CD34 antigen was observed in greater than 10% of the blast cells, as compared with 14 of 22 P-170$^-$ patients (70% vs 64%, p>0.05). The CR rate of CD34$^+$ and CD34$^-$ patients was 31% and 76%, respectively (p=0.006). In AMLs that simultaneously expressed both P-170 and CD34, the CR rate was worse as compared with those negative for P-170 and CD34 (5% vs 63%, p=0.004). In 12 patients (8 P-170$^+$, 4 P-170$^-$) CD34 and P-170 expression were further characterized by double fluorescence studies. It was shown that P-170$^+$ cells were largely, but not exclusively, restricted to the CD34$^+$ cell population. For the 8 P-170$^+$ AML samples, the median ratio of P-170$^+$/P-170$^-$, in CD34$^+$ cells was 4.845 (range: 0.60 - 25.00) as compared with 0.135 (range: 0.02 - 0.67) in CD34$^-$ cells. In these 12 AML samples, the presence of functional resistance as defined by reduced daunorubicin accumulation was evaluated in CD34$^+$ and CD34$^-$ AML cells. In 8 of 8 P-170$^+$ patients, intracellular daunorubicin accumulation in CD34$^+$ AML blast cells was lower than in CD34$^-$ cells, and it increased after cyclosporin addition. No difference of intracellular daunorubicin accumulation was observed between CD34$^+$ and CD34$^-$ AML cells of 4 P-170$^-$ patients. These data indicate that P-170 expression in AML with a heterogeneous CD34$^+$ phenotype seems predominantly present in CD34$^+$ AML blast cells.

INTRODUCTION

Resistance to chemotherapeutic agents is an important factor of treatment failure in acute myeloid leukemia (AML). At present, the exact mechanism of clinical drug resistance is not clear. Multidrug resistance (MDR) is a phenotype of resistance to several structurally unrelated, natural cytotoxic agents such as anthracyclines, vinca alkaloids and epipodophyllotoxins. MDR is associated with overproduction of a 170-kD membrane glycoprotein (P-170) which is encoded by the *MDR*1 gene located on the long arm of chromosome 7 (1). Overexpression of P-170 is associated with lower intracellular retention of the involved drugs in vitro, mediated through enhanced membrane efflux. It has been shown that transfection of the *MDR*1 gene into drug-sensitive cells confers the

MDR phenotype (2). Recent studies have indicated that MDR in vivo is induced by treatment with anthracyclines and vinca alkaloids in several hematologic malignancies. However, in untreated AML patients the expression of P-170 is present in 22.7% to 71% of de novo AML (3-6). In some studies, expression of P-170 was a negative prognostic factor for response, and it was associated with the presence of an immature AML phenotype (4-10).

In normal human bone marrow (BM), genomic P-170 expression is predominantly present in CD34$^+$, rhodamine dull precursor cells and in T-lymphocytes (11,12). It is not known if P-170 overexpression in AML is restricted to certain subpopulations of cells, and if it is involved in drug resistance in these cells. Separate analysis of P-170 and CD34 expression in AML indicates that both are unfavourable prognostic parameters (13).

In the present study, we report that, in AML with an immature, CD34$^+$ phenotype, *MDR*1 expression associated with reduced intracellular daunorubicin retention can be shown in CD34$^+$, but not in CD34$^-$ blast cells.

MATERIALS AND METHODS

Patients. Fifty-two consecutive patients with untreated AML were included in the study. Patients with prior hematologic disorders or known exposure to carcinogens were excluded. After informed consent, BM aspirates were taken for evaluation of the AML phenotype and other studies. Histologic classification was performed on May-Grünwald-Giemsa-stained bone marrow smears according to the French-American-British (FAB) criteria (14).

After diagnosis, the patients were treated according to the protocol of the Dutch Hematology-Oncology Group Protocol for Acute Leukemia (HOVON-4; 22 patients) or the protocol for elderly AML patients (HOVON-9/11; 30 patients), respectively. Remission induction treatment in HOVON-4 consisted of daunorubicin (45 mg/m^2 intravenously (i.v.), days 1-3) and cytosine-arabinoside (200 mg/m^2, i.v., days 1-7) followed by a second induction course of amsacrine (120 mg/m^2, i.v., days 4-6) and cytosine-arabinoside (2 g/m^2, i.v., days 1-6) and a consolidation course of mitoxantrone (10 mg/m^2, i.v., days 1-5) plus etoposide (100 mg/m^2, i.v., days 1-5).

Remission induction treatment in HOVON-9/11 consisted of 2 courses of daunorubicin (30 mg/m^2, i.v., days 1-3) or mitoxantrone (8 mg/m^2, i.v., days 1-3) combined with cytosine-arabinoside (200 mg/m^2, i.v., days 1-7).

The remission status following induction therapy was evaluated using the FAB criteria (14).

Cell samples. Samples from BM were collected at diagnosis in heparinized tubes. Mononuclear cells (MNC) were isolated from the samples by Ficoll-Hypaque density gradient centrifugation (density 1.077 g/ml, Pharmacia, Uppsala, Sweden). Further purification of AML blast cells was performed by E-rosette -and adherence depletion. After these steps, greater than 95% of the cells were of the AML blast phenotype. These MNC were used for CD34 marker analysis and for P-170 evaluation.

Cytogenetics and immunological marker analysis. Cytogenetic analysis of AML samples were performed by standard techniques (15). Chromosomes were identified by banding techniques according to the ISCN (16).

Immunological marker analysis was performed as described previously (17).

Analysis of *MDR1* (gene) expression. For determination of the P-170 expression of the cells, three different methods were used.

Immunocytochemistry. P-170 staining on cytospin slides was performed using a panel of monoclonal antibodies (MoAbs), i.e., C219 (Centocor, Malvern, PA), JSB1 (Dr. R. Schepers, Free University, Amsterdam, The Netherlands), and MRK16 (Hoechst, Amsterdam, The Netherlands). Of these, MRK16 is considered specific for *MDR1* glycoprotein. Each antibody binds to a different epitope of the P-glycoprotein. Therefore, staining with 3 Moab excludes false-negative samples due to epitope shedding.

Fresh BM AML cells were washed twice in phosphate-buffered saline (PBS) and cytocentrifuge slides were prepared. The slides were fixed in acetone/formalin phosphate buffer (50/25/25%), soaked in 50 mM Tris in 0.02% Tween 20 for 5 minutes, and incubated with 10% normal rabbit + 1% normal goat serum for 30 minutes. C219, JSB1 or MRK16 or isotype-matched controls ($IgG2^a$ [C219, MRK16] or IgG1 [JSB1]; Coulter Clone, Hialeah, FL) were added for 60 minutes at 37°C. Anti-mouse immunoglobulin was added and, after washing, the slides were incubated with alkaline phosphatase substrate (APAAP) for 60 minutes at 37°C and washed three times. The cells were counterstained using Papanicolaou's solution 1[a] /Harris' hematoxylin solution (Merck, Darmstadt, Germany).

For positive samples, an arbitrary cut-off was set at greater than 30% of the blast cells staining independently with all three Moabs with absence of IgG matched control staining (18). For each sample 500 cells were counted on duplicate slides.

Flowcytometric determination of P-170 and CD34 expression. Cells were washed in PBS supplemented with 2% bovine serum albumin (BSA), 10% rabbit serum (Gibco, Paisley, UK), 0.1% goat serum (Gibco) and 0.1% pooled AB serum. All supplements were heat inactivated at 56°C for 30 min. For the fixation of the cells, two fixation methods were compared. For one method, 70% ethanol containing 50 mM glycine (pH 2)

was used for 20 minutes at -20°C. For the other method, 35% ethanol containing 50 mM glycine (pH 2) was used for 50 seconds at 4°C. After washing the cells, a cell suspension of $10x10^6$ cells/ml was prepared. Fifty μl of the cell suspension was incubated with 25 μl MRK16 plus 25 μl C219 for 30 minutes at 0°C. After washing, the cell pellet was resuspended and incubated with 50 μl 1:20 (v/v) diluted fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulin antiserum (Dakopatts, Glostrup, Denmark). Next, the cells were washed and incubated with 25 μl normal mouse serum (1:100) for 5 minutes and phycoerythrin (PE)- conjugated HPCA-2 (anti-CD34 PE; Becton & Dickinson [B&D], San Jose, CA) was added for 30 minutes at 0°C. An irrelevant, isotype matched Moab was used as negative control. After washing, 5000 events were counted using a FACScan flowcytometer (B&D). Both FITC and PE fluorescence signals were logarithmically amplified. The blast cell population was gated using scatter parameters. Data analysis was performed using FACScan software (B&D).

RNAse protection assay. Total RNA was isolated from nucleated cells by the lithium-chloride-urea method. Quantitative detection of MDR gene transcripts was performed by use of the RNAse protection assay. The assay was performed as described (8). Ten micrograms of total RNA was hybridized with ^{32}P-UTP-RNA probes under standard conditions, followed by RNAse-A treatment. Hybridized probes were visualized by electrophoresis on a denaturing 6% acrylamide gel, followed by autoradiography. For RNAse protection, a 301-nucleotide *MDR*1 cDNA fragment (positions 3498 to 3801) and a 310-nucleotide *MDR*3 cDNA fragment (positions 2599 to 2900) were used as previously described (8). Because the *MDR*3 probe can detect several fragments in the RNAse protection assay, a 310-nucleotide fragment for splice variant A + B and a 220-nucleotide fragment for splice variant C were used. These cDNA probes are specific for *MDR*1 and *MDR*3 respectively. This *MDR*3 probe does not hybridize with *MDR*1-specific RNA. A human γ-actin probe was included in all RNAse protection assays as a control for RNA recovery. The mRNA levels were quantitated by densitometric scanning of the autoradiographs as described previously (8). The signal obtained with a 10 μg total RNA sample of KB-8-5 cells which are colchicine resistant, *MDR*1 mutants of the KB parent cell line was assigned an arbitrary expression level of 30 U.

Daunorubicin (DNR) accumulation. DNR (Rhône-Poulenc Pharma, Amstelveen, The Netherlands) was diluted in distilled water to a concentration of 0.2 mM. Cyclosporin (CsA; Sandoz, Basel, Switzerland) was disolved in ethanol at 0.84 mM.

To determine DNR accumulation in CD34$^+$ and CD34$^-$ AML blast populations, a suspension of AML cells (10^6 cells/ml) in Iscove's Modified Dulbecco's Medium (IMDM; Gibco) supplemented with 10% foetal calf serum (FCS; Gibco) was prepared. DNR was added at 2 μM and incubated at 37°C. At various intervals, samples (0.5 ml)

were taken and immediately put on ice. After washing at 4°C, the cell pellets were resuspended in 50 μl PBS/BSA. Twenty μl of undiluted FITC-conjugated MoAb HPCA-2 (anti-CD34 FITC; B&D) was added and incubated for 30 minutes at 0°C. After washing the cells, they were kept on ice until analysis. When drug accumulation was studied using CsA, the same procedure was followed except that the cells were preincubated with or without 0.84 μM CsA for one hour at 37°C.

DNR fluorescence was measured using a FACScan flowcytometer at 488 nm excitation, calibrated using PE coated plastic beads (Calibrite Beads; B&D). Both FITC and DNR fluorescence were logarithmically amplified. DNR accumulation was measured in $CD34^+$ and $CD34^-$ subpopulations by the use of "live gating". Data analysis was performed using histogram analysis of the Lysys software program (B&D). The accumulation of DNR was expressed in arbitrary units by calculating the mean fluorescence distribution of each population in each sample. A correction for the background fluorescence of the $CD34^+$ and $CD34^-$ cells was made.

Cell sorting and fluorescence in situ hybridization (FISH). One AML sample with a cytogenetic abnormality, monosomy 7, that was used for the double immunofluorescence staining for P-170 and CD34 and for DNR uptake studies, was also sorted in $CD34^+$ and $CD34^-$ cell populations after labeling with HPCA-2 PE. In the sorted populations, FISH technique was used to ensure that the $CD34^+$ and $CD34^-$ cell populations were derived from the leukemic clone with the monosomy 7. Cell sorting was performed on a FACS II (Sunnyvale, CA) at a rate of 2500 cells per second using a single argon laser tuned at 488 nm (350 mW). After sorting, cytospins were prepared and dried overnight. Next, the slides were fixed in methanol (Merck) for 5 minutes, rinsed with water, and air dried.

Table 1. Clinical response of patients according to AML blast cell phenotype.

	P-170*		CD34†		P-170/CD34	
	Negative	Positive	Negative	Positive	Neg/Neg	Pos/Pos
CR	16	8	12	12	5	1
no CR	6	22	5	23	3	20
	p=0.003		p=0.006		p=0.004	

* $P\text{-}170^+$ defined as more than 30% of blast cells staining with three P-170-specific MoAbs (C219 + JSB1 + MRK16) with negative aspecific antibody control on cytospin slides.
† $CD34^+$ samples defined as more than 10% of blast cells staining with MoAb.

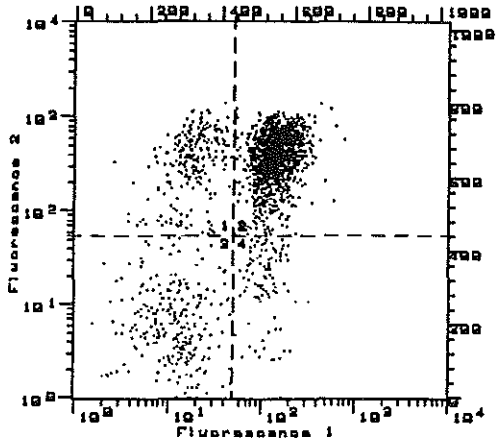

Fig.1 : Dot plot analysis of double fluorescence labelling in one AML sample (no. 6, Table 3). Green fluorescence (FITC; Fluorescence 1; P-170) is shown on the X-axis, red fluorescence (PE; Fluorescence 2; CD34) on the Y-axis. The quads were set by using IgG1 and IgG2a as negative controls.

A biotin-labeled DNA probe specific for the α-satellite sequences on the centromeric region of the chromosome 7 (p7t1) (19), kindly provided by Dr. P. Devilee (University of Leiden, The Netherlands), was used at a concentration of 5 ng/μl. Denaturation and hybridization procedures have been described previously (20). The hybridized probe was detected by using fluorescein labelled avidine (FITC; Vector Laboratories, Burlingame). On each slide, 200 cells were scored. One CD34$^+$ AML sample without a monosomy 7, was also sorted in CD34$^+$ and CD34$^-$ populations, and was used as a control for the FISH technique.

Statistical analysis. Correlations were calculated using the Chi-Square frequency test. Analysis of variance was performed by the Cox proportional test. The Mann Whitney *U* test was used for statistical analysis of DNR accumulation between CD34$^+$ and CD34$^-$ populations.

RESULTS

Correlation of clinical data with P-170 and CD34 expression. Fifty-two consecutive patients with untreated AML were evaluated. The clinical characteristics were as follows: age, 16-78 yr (median, 62 yr); 28 males and 24 females. The FAB classifications were M0, 1 patient; M1, 13 patients; M2, 21 patients; M3, 2 patients; M4, 11 patients; M5, 2 patients; M6, 2 patients.

All 52 patients could be evaluated for response. Of 30 patients over 60 years of age, 13 achieved a complete response, as compared to 11 of 22 patients under 60 years of age (43% vs 50%; not significant [n.s.]).

Expression of *MDR*1 was studied by staining of P-170 on blast cells by C219, JSB1, and MRK16 in all patients. In this group of 52 patients, 30 were P-170$^+$ (58%). In 10 randomly chosen P-170$^+$ patients, the results of P-170 analysis were compared with RNAse protection assay to exclude coexpression of *MDR*3.

All 10 patients had increased *MDR*1 mRNA levels, varying from 5 to 56 arbitrary units (a.u.)(median 18 a.u.), as compared to 30 a.u. in a 10 μg total RNA sample of the colchicine resistant KB-8-5 cell line. No *MDR*3 mRNA expression was noted.

P-170 positivity strongly correlated with a poor response to chemotherapy. Eight of 30 P-170$^+$ patients achieved a complete response as compared with 16 of 22 P-170$^-$ patients (27% vs 73%, p=0.003). There was no correlation of P-170 expression with age, sex, or FAB classification.

Thirty-five of 52 patients (67%) had a significant proportion (>10%) CD34$^+$ blast cells. Patients expressing CD34 positivity had a lower complete remission (CR) rate as compared to CD34$^-$ patients (31% vs 76%, p=0.006; Table 1).

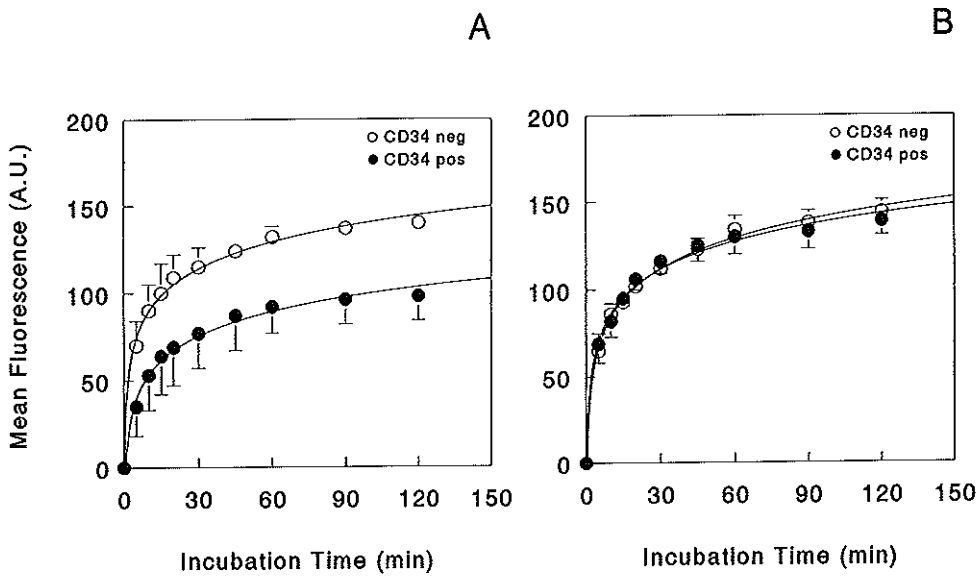

Fig. 2: Mean intracellular concentration of daunorubicin in AML blasts (CD34$^+$/CD34$^-$ cells) of 8 P-170 positive patients (A) and 4 P-170 negative patients (B).

Twenty-one of 30 P-170$^+$ patients had more than 10 percent CD34 positive blast cells, as compared with 14 of 22 P-170$^-$ patients (70% vs 64%, n.s.). In the patients with double expression of P-170 and CD34 (21 of 52), 1 of 21 attained a CR as compared with 5 of 8 P-170/CD34$^-$ patients (5% vs 63%, p=0.004).

Cytogenetic analysis showed abnormal karyotypes in 29 of the 52 AML samples. Abnormal karyotype was associated with P-170 expression (p=0.003), and with a decreased CR rate (p=0.014), but not with CD34 expression (p=0.09). These data are summarized in Table 2.

Flowcytometric detection of P-170 and CD34 expression. In 12 patient samples, the relationship between P-170 expression and CD34 expression was further investigated. In these 12 samples the CD34 expression varied from 20 to 83% of the AML blasts (mean: 57%). Because Ficoll-Hypaque-separated, T-cell-and adherent cell-depleted BM was used, the AML blast population was highly concentrated and amounted to more than 95% of either sample. Immunological marker analysis showed that the percentage of CD2$^+$, CD3$^+$ or CD19$^+$ cells did not exceed 5%. Consequently, in these patients it was possible to analyze the CD34$^+$ and CD34$^-$ AML cells without significant interference of residual normal cells. Using the FISH technique on the CD34$^+$ and CD34$^-$ samples of 1 AML sample (no. 4; Table 3) with monosomy 7 showed no cells with two fluorescence

Table 2. Distribution of cytogenetic abnormality in relation with clinical response, CD34 expression, and P-170 expression in 52 cases of AML.

Cytogenetic Abnormality	Total (n=52)	CD34^{+*}	P-170$^+$†	CR‡
None	23	12	8	15
t(8;21)(q22;q22)	2	2	0	2
inv(16)(p13q22)	2	2	1	1
t(15;17)(q22;q12-21)	2	1	1	2
Trisomy 8	2	2	2	0
Monosomy 7/del(7)(q22-q36)	4	4	4	0
Monosomy 7 or del(7)(q22-q36)/Other	6	6	6	0
Other	11	6	8	4

* CD34 positive samples were defined as more than 10% of blast cells staining with MoAb.
† P-170 positive samples defined as more than 30% of blast cells staining with three P-170 specific MoAb's (C219, JSB1, MRK16) with negative non-specific antibody control on cytospin slides.
‡ CR, Complete Remission.

signals in the cells scored in both cell populations. This indicates that the monsomy 7 was present in both cell populations. The AML sample that was used as a control, showed in 94% of the $CD34^+$ cells and in 92% of the $CD34^-$ cells two signals.

In some AML samples, the fixation with 70% ethanol/50mM glycine for 20 minutes at -20°C was shown to decrease the CD34 positivity as compared to nonfixed AML samples. The fixation procedure using 35% ethanol/50 mM glycine for 50 seconds at 4°C did not negatively effect CD34 expression (data not shown). Therefore, we preferred the latter fixation procedure for the P-170 and CD34 flowcytometric detection. Using a CD34/C219 + MRK16 double fluorescence technique, P-170 labeling was investigated in $CD34^+$ and $CD34^-$ populations separately. Figure 1 shows the double expression in 1 of these patients. $P\text{-}170^+$ staining was predominantly found in $CD34^+$ but not in $CD34^-$ cells of 7 of 8 $P\text{-}170^+$ patients (Table 3). In 1 AML sample (no. 8; Table 3), P-170 expression was almost equally expressed in $CD34^+$ (ratio: 0.60) and $CD34^-$ blast population (ratio: 0.67). In 4 patients who were $P\text{-}170^-$ with APAAP, only small numbers of cells, equally divided among $CD34^+$ and $CD34^-$ cells, stained for C219 and MRK16 (Table 3).

Table 3. *MDR1* expression in $CD34^+$ and $CD34^-$ AML subpopulations.

	CD34-*			CD34+		
Pt.	P-170+ (%)	P-170- (%)	Ratio	P-170+ (%)	P-170- (%)	Ratio
1.	1	48	0.02	38	13	2.92
2.	13	44	0.30	41	2	20.50
3.	4	53	0.08	34	9	3.77
4.	5	43	0.12	50	2	25.00
5.	2	45	0.04	47	6	7.83
6.	5	12	0.42	71	12	5.92
7.	4	25	0.16	48	23	2.10
8.	8	12	0.67	30	50	0.60
9.†	1	27	0.04	13	59	0.22
10.†	1	22	0.05	3	74	0.04
11.†	6	60	0.01	7	27	0.27
12.†	7	73	0.01	9	11	0.82

Percentage values are of the total AML cell population after T-cell and adherence depletion.

* $CD34^-$ samples were defined as less than 10% of blast cells staining with MoAb.

† $CD34^+$, but $P\text{-}170^-$ AML samples using P-170 APAAP staining on BM cytospins.

Daunorubicin accumulation experiments. In order to evaluate if P-170 overexpression in CD34$^+$ cells could have a functional relationship to drug resistance, the intracellular concentration of daunorubicin as measured by its fluorescence was assessed in CD34$^+$ and CD34$^-$ cell populations of 12 patients (8 P-170$^+$, 4 P-170$^-$). The net cumulative, intracellular retention of daunorubicin was lower in CD34$^+$ cells as compared to CD34$^-$ cells in the P-170 expressing patients ($p<0.001$), but not in P-170$^-$ AML samples (Fig. 2). Addition of CsA (1 μg/ml in 0.84 μM) led to an increase of the intracellular daunorubicin concentration of CD34$^+$ cells up to a level comparable with that in CD34$^-$ cells in two analyzed P-170 expressing patients (Fig. 3).

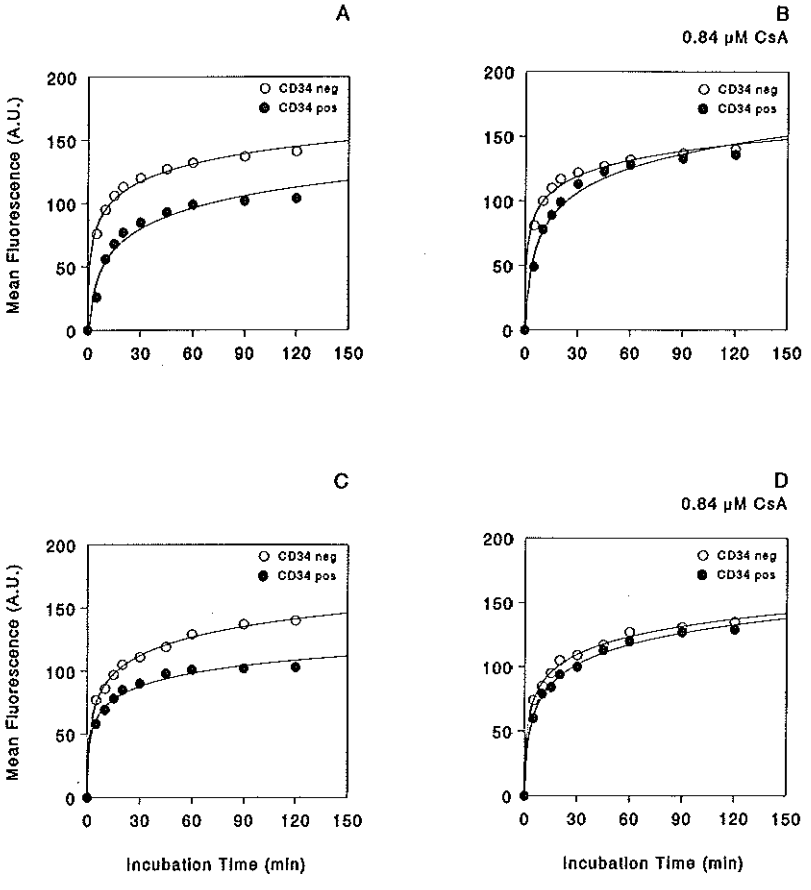

Fig. 3: Intracellular concentration of daunorubicin in AML blasts (CD34$^+$/CD34$^-$ cells) of two P-170 positive AML samples without (A,C) and with 0,84 μM cyclosporin (B,D).

DISCUSSION

Expression of the *MDR*1-encoded P-170 glycoprotein has been recognized as a mechanism of pleiotropic drug resistance in refractory tumours. More recently, several studies have shown that P-170 overexpression is observed in a significant proportion of untreated AML patients (3-10). Some of these patients have secondary leukemias, and therefore the P-170 induction in their AML cells may be related to prior toxic exposure. However, many patients with P-170 overexpression did not have a history of preceding myelodysplasia or of cytotoxic and/or radiation therapy. The present study shows that 58% of untreated AML are MDR^+. However, in many cases only a proportion of AML blast cells were found to stain positive when studied on cytospin slides. We therefore wished to investigate wether P-170 expression concorded with a specific AML phenotype. CD34 is a frequently expressed marker of AML and it predicts for a poor therapeutic outcome (13). In the present study, 35 of 52 patients had CD34 expression in part of the blast cells, and many of these (21 of 35) were $P\text{-}170^+$. Also, in 8 patients with $CD34^-$ AML, no P-170 expression was found. We therefore examined P-170 expression in $CD34^+$ and $CD34^-$ subpopulations of AML cells.

In 7 out of 8 $P\text{-}170^+$ patients, P-170 staining was primarily demonstrated in $CD34^+$ but not or to a small extent in $CD34^-$ cells. In $CD34^+$ cells, P-170 overexpression was functional, as can be concluded from the lower intracellular daunorubicin retention in $CD34^+$ cells. Although other factors may also lead to lower intracellular drug retention (21), evidence for the role of P-170 in $CD34^+$ cells is provided by the modulation of daunorubicin retention by CsA. Addition of CsA restores the lower intracellular daunorubicin concentrations of $CD34^+$ cells to a level observed in $P\text{-}170^-$, $CD34^-$ cells.

Because normal blood cells have been demonstrated to express P-170 (12,22), residual normal blood cells might interfere with the results obtained with flow cytometry and DNR accumulation experiments. However, because all samples following T-cell and adherence depletion consisted of greater than 95% blast cells, this possibility seems unlikely. Using FISH techniques with a centromere probe for chromosome 7, we were able to show that within such a purified population, the $CD34^-$ cell population were derived from the leukemic clone in 1 patient. Because in normal BM CD34 expression is present in less than 5% of the cells, it can be assumed that the $CD34^+$, $p\text{-}170^+$ cells are indeed AML cells in these patients.

To determine whether P-170 and CD34 expression were independent prognostic factors in these patients, the presence of a cytogenetic abnormality was also evaluated. Cytogenetic abnormalities such as trisomy 8 and abnormal or absent chromosome 7 are associated with a poor prognosis, while other abnormalities such as t(8;21)(q22;q22), inv(16)(p13q22), and t(15;17)(q22;q12-q36) have a favourable prognostic value (23-24. In our study, the CR rate for the AML patients with a unfavourable cytogenetic abnormality

(trisomy 8, absent or abnormal chromosome 7, Other, Table 2) was significantly lower ($p=0.014$) when compared with AML patients with normal karyotypes. This might be due to P-170 expression, because this was more frequently expressed in AML with unfavourable cytogenetic abnormalities (87%) as compared with AML without a cytogenetic abnormality or with a favourable cytogenetic abnormality (both 30%). CD34 expression was not correlated with cytogenetic abnormalities.

These data indicate that in untreated AML of a heterogeneous phenotype, a subpopulation of cells can be recognized that has a potentially lower sensitivity to the majority of cytostatic drugs commonly used in AML. It may be assumed that CD34$^+$ AML cells include possibly clonogenic AML cells. Therefore, the presence of CD34$^+$/P-170$^+$ cells may indicate a poor chance of CR and a high probability of refractory relapse. Using P-170/CD34 double fluorescence the presence of this subset of cells can be recognized.

A study in normal BM precursor cells has shown that rhodamine dull, CD34$^+$ cells may also stain with MRK16 (11). In myeloid cells of a later differentiation stage like promyelocytes and granulocytes, P-170 expression could not be detected (12,22). This could explain why P-170 overexpression is so frequently observed in untreated AML with an immature CD34$^+$ phenotype (3-4,6,25), while in other tumours it is largely restricted to pretreated cells. Expression of the *MDR*1 phenotype in untreated CD34$^+$ AML subpopulations may have been conserved through leukemic mutagenesis. Interestingly, the more mature, CD34$^-$ AML subpopulations of CD34$^+$ AML samples lost P-170 expression, or only expressed the *MDR*1 phenotype to a small extent.

This study indicates that P-170 expression is a predominant phenomenon in CD34$^+$ AML cells. By examination of these resistant subpopulations of cells, their presence can be monitored throughout therapy. On the basis of this, alternative therapy directed against P-170$^+$ cells can be instituted, e.g. by addition of CsA or other MDR modulating agents (26-27).

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CHAPTER 3

MULTIDRUG RESISTANT CELLS WITH HIGH PROLIFERATIVE CAPACITY DETERMINE RESPONSE TO THERAPY IN ACUTE MYELOID LEUKEMIA.

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SUMMARY

High spontaneous proliferation of acute myeloid leukemia (AML) in vitro is an unfavorable, tumor-specific prognostic factor. We investigated the frequency of drug-resistant tumor cells with high proliferating capacity in *de novo* AML and analyzed the expression of multiple resistance parameters in relation to the response to chemotherapy and overall survival. Thirty-eight patients were included in this study. P-glycoprotein (P-gp) expression was found in 28/38 patients and was associated with lower intracellular accumulation of DNR ($p=0.0001$). Thirty-five out of 38 patients treated with 1-2 regimens of daunorubicin (DNR)/cytarabine (Ara-C), 57% attained a complete remission (CR). Failure to achieve a CR correlated with autonomous growth ($p=0.0064$), CD34 and P-gp expression alone ($p=0.0005$ and $p=0.048$ respectively), and with simultaneous expression of P-gp and CD34 ($p=0.0001$), but not with expression of the non-P-gp drug resistance associated protein (p110), the multidrug resistance-associated protein (MRP), Ara-CTP formation or Ara-C incorporation, respectively. AML cells with CD34/P-gp double expression were more frequently observed in samples with high autonomous growth ($p=0.003$). The median survival was 6 months in $CD34^+/P\text{-}gp^+$ patients as compared with 15 months in other AML patients ($p=0.003$). In patients with *de novo* AML who fail on chemotherapy, a population of autonomously proliferating, immature AML cells with a multidrug resistant phenotype can be recognized. These cells thus are primary resistant to chemotherapy and have the potential for rapid regrowth, leading to resistant disease.

INTRODUCTION

In acute myeloid leukemia (AML), several factors have been established which determine the outcome of remission-induction therapy such as advanced age, CD34 surface marker expression, and specific cytogenetic abnormalities, i.e. deletions of chromosomes 5 and 7 (1-3). Recently it was shown that autonomous growth of AML cells in vitro has a negative impact on the prognosis of patients with AML (4,5). High autonomous growth may represent agressive leukemia, that is associated with a low complete response (CR) rate and with a high probability of recurrence. It is not known why AML patients with high cytokine-independent growth respond poorly to chemotherapy. One explanation is that proliferating AML cells would lead to rapid regrowth of the leukemia after treatment, in particular if these cells exhibit a drug-resistant phenotype. Alternatively, it is conceivable that the increased proliferation rate in AML's with high autonomous proliferation would be associated with an increased spontaneous mutation frequency and the development of drug-resistant leukemic clones (4,6).

Ara-C and anthracyclines are cytostatic drugs that are usually included in AML treatment regimens. The biological activity of the single most effective anti-leukemic drug Ara-C is determined by the phosphorylation to Ara-CTP. Ara-CTP is an inhibitor of DNA polymerase and competes with deoxycytidine triphosphate (dCTP) (7,8). Ara-C resistance may be caused by reduced activation or rapid deamination or high endogenous dCTP pools (9-15).

Resistance to anthracyclines such as daunorubicin may be associated with overexpression of drug transport proteins. In resistant cells overexpression of several ATP-binding cassette (ABC) transport proteins has been identified. The most important of these is P-glycoprotein (P-gp) which is an energy-dependent, transmembrane efflux pump, that is encoded by the *MDR*-1 gene (16-18). P-gp overexpression is associated with decreased intracellular concentrations of daunorubicin. Other proteins associated with anthracycline resistance in cell lines include the non-P-glycoprotein drug resistance associated 110-kDa protein (p110) and a 190-kDA multidrug resistance-associated protein designated MRP (19-21).

In this study the most important parameters for resistance to Ara-C and daunorubicin were prospectively determined in AML cells and analyzed together with the proliferative characteristics. The phenotype of proliferating, resistant AML cells was examined in relation to clinical response to therapy.

MATERIALS AND METHODS

Patients. In this study, 38 newly diagnosed patients (22 males, 16 females, median age: 54 years) with primary AML without a prior dysplastic phase were included. After informed consent, a bone marrow (BM) aspirate and/or peripheral blood (PB) were taken for diagnostic evaluation and other studies. AML was classified cytologically according to the French-American-British (FAB) criteria (22) (M1:8, M2:10, M3:3, M4:11, M5:6). Cytogenetic analysis was carried out by standard banding techniques (Prof. Dr. A. Hagemeijer, Dept. of Cell Biology and Genetics, Erasmus University, Rotterdam, The Netherlands).

Following diagnosis, 35 patients were treated with standard chemotherapy induction regimens (Table 1). The remission status following induction therapy was evaluated after the second course of chemotherapy. Three patients were not treated because of refusal (n=1), or infection/hemorrhage (n=2).

Table 1. Remission induction chemotherapy protocols used in AML treatment

	Remission induction chemotherapy	
Patient's age (years)	First course	Second course
<60 years	Cytarabine: 200 mg/m^2, days 1-7 Daunomycin: 45 mg/m^2, days 1-3	Cytarabine: 2000 mg/m^2, days 1-6 Amsacrine: 120 mg/m^2, days 4-6
>60 years	Cytarabine: 200 mg/m^2, days 1-7 Daunomycin: 30 mg/m^2, days 1-3 OR Mitoxantrone: 8 mg/m^2, days 1-3	Cytarabine: 200 mg/m^2, days 1-7 Daunomycin: 30 mg/m^2, days 1-3 OR Mitoxantrone: 8 mg/m^2, days 1-3

AML sample preparation. Mononuclear cells from bone marrow or blood were separated by Ficoll-Isopaque (Nycomed, Oslo, Norway) centrifugation. All samples were depleted of T-lymphocytes by E-rosetting, and of adherent cells as described (4). The experiments were performed under serum-free conditions (23).

Autonomous growth of AML. To determine autonomous proliferation, DNA synthesis was determined after three days of culture of AML cells in serum-free medium . Briefly, 100 μl of AML cell suspensions (2x10^5 cells/ml) were plated in round-bottomed, 96-well microtiter plates (Greiner, Nurtingen, Germany) and incubated for 2 days. Next, tritiated thymidine (^{3}HTdR; 2 Ci/mmol; Amersham, UK) was added (0.1 μCi/well) and incubated overnight. After harvesting the cells on nitrocellulose filters, radioactivity was determined. As a control for background ^{3}HTdR incorporation, for each sample irradiated AML cells (30 Gy) were used. The experiments were performed in triplicate and the results were expressed as log transformed mean dpm values. The results were expressed as low, intermediate or high autonomous growth, according to published criteria (4).

Ara-CTP formation. Intracellular Ara-CTP formation was determined by incubating AML cells (10^6/ml) with 10^{-5}M Ara-C (Upjohn, Kalamazoo, MI, USA). After 4 hours of incubation, the cells were washed, and extracted twice with 300 μl 60% methanol at 37°C

for 20 minutes. The supernatants were collected and analysed by high performance liquid chromatography (HPLC).

HPLC analysis of intracellular dCTP and Ara-CTP pools in AML cells. Immediately before analysis, extracted AML samples were treated with periodate oxidation to remove endogenous intracellular ribonucleotide pools according to a method described by Garrett and Santi (24). Aliquots of periodate treated samples were injected onto a 100 mm x 46 mm Mono-Q column (Pharmacia, Uppsala, Sweden) and eluted with an ammonium phosphate buffer. The HPLC equipment consisted of a Spectra-Physics HPLC system (Spectra-Physics Analytical, Fremont, CA, USA). The method used to quantify dCTP and Ara-CTP levels was adapted from Danks (25). Briefly, a gradient of two buffer solutions A and B was prepared. Buffer A and B consisted of 25 mM (pH 3.5) and 750 mM (pH 3.5) ammoniumphosphate (Suprapur [Merck, Darmstadt, Germany]) respectively. Step 1 was 5 % buffer B increased to 25% in 15 minutes (min). Step 2 was an increase of buffer B to 100% in 20 min, and was maintained for 5 min. Step 3 was a decrease of buffer B to 0% in 5 min, and maintained for 5 min. The flow rate was 1.5 ml/min throughout. UV absorbence was detected at a wavelength of 280 nM. Separation of the two components was >1.5 min. Peak areas were used for quantification of concentrations in this assay.

Ara-C incorporation. Ara-C incorporation into DNA was determined by incubating AML cells (0.45 x 10^6 cells/well, triplo) with 10^{-5}M tritiated Ara-C (^{3}H-Ara-C; 31 Ci/mmol, Amersham). After 4 hours, the cells were precipitated using 2.5% trichloric acetic acid (TCA; Merck). Before precipitation, 50 μl of an RNA solution (1%) was added as a co-precipitate. After washing three times (2.5% TCA), the pellets were solubilized with 500 μl Soluene (United Technologies Packard, Groningen, The Netherlands). Scintillation fluid was added, and radioactivity was determined. The amount of Ara-C incorporated was calculated and expressed as pmol/10^6 cells.

Immunocytochemical analysis of the P-glycoprotein and p110 membrane pumps. P-gp staining was performed on cytospin slides by separate staining on two slides with the monoclonal antibodies (MoAb) C219 and C494 (Centocor, Malvern, PA, USA). P-110 expression was analysed using MoAb LRP-56 (kindly donated by Dr R Schepers, Free University, Amsterdam, The Netherlands).

Purified bone marrow or blood AML blast cells were washed twice in phosphate-buffered saline (PBS) and cytocentrifuge slides were prepared. The slides were fixed in acetone/formalin phosphate buffer (50/25/25%), soaked in 50 mM Tris in 0.02% Tween 20 for 5 minutes and incubated with 10% normal rabbit + 1% normal goat serum for 30 minutes. C219, C494, LRP-56 or isotype matched controls (IgG2^a [C219, C494]; IgG2^b for LRP-56) were added for 60 minutes at 37°C. Anti-mouse immunoglobulin was added and after washing, the slides were incubated with Alkaline Phosphatase substrate

(APAAP) for 60 minutes at 37°C and washed three times. The cells were counterstained using Papanicolaou's solution 1[9] /Harris' hematoxylin solution (Merck).

Cells in specific samples were only considered positive if they stained with both Moabs (P-glycoprotein) or with LRP56 in the absence of IgG matched control staining. For each sample 500 cells were counted on duplicate slides.

CD34 analysis. For analysis of CD34 by flowcytometry the phycoerythrin (PE)-conjugated MoAb HPCA-2 (anti-CD34PE, Becton & Dickinson (BD), San Jose, CA, USA) was used. Samples containing more than 10% CD34 cells were considered positive (26,27).

Analysis of CD34 and P-gp double expression. The procedure used for double fluorescence of CD34 and P-gp was performed as described before (1). AML cells were fixed with 35% ethanol containing 50 mM glycine (pH=2) for 50 seconds at 4°C. After washing, the cells were incubated with a mixture of MRK16 (Hoechst, Amsterdam, The Netherlands) and C219 for 30 minutes at 0°C. After washing the cell pellet was resuspended and incubated with 1:20 (v/v) diluted fluorescein isothiocyanate (FITC) conjugated rabbit anti-mouse immunoglobulin antiserum (DAKOPATTS, Glostrup, Denmark). After washing, the AML cells were incubated with normal mouse serum (1:100) for 5 minutes, and PE conjugated HPCA-2 (anti-CD34 PE; Becton & Dickinson [BD]) was added for 30 minutes at 0°C. An irrelevant, isotype matched Moab was used as negative control. After washing, 5000 events were counted using a FACScan flowcytometer (BD). Both FITC and PE fluorescence signals were logarithmically amplified. The blast cell population was gated using scatter parameters. Data analysis was performed using FACScan software (BD).

Daunorubicin (DNR) accumulation experiments. DNR (Rhône-Poulenc Pharma, Amstelveen, the Netherlands) was diluted in distilled water to a concentration of 0.2 mM, and stored in aliquots at -80°C.

To determine DNR accumulation, a suspension of AML cells (10^6 cells/ml) in Iscove's Modified Dulbecco's Medium (IMDM; Gibco) supplemented with 10% Fetal Calf Serum (FCS; Gibco) was prepared. DNR was added at 2 μM and incubated at 37°C. After 90 minutes, the cells were immediately put on ice, washed at 4°C, and put on ice until analysis. An incubation period of 90 minutes was chosen since the intracellular DNR accumulation has usually reached a plateau by then.

DNR fluorescence was measured using a FACScan flowcytometer at 488 nm excitation (BD). The instruments settings were calibrated using phycoerythrin (PE) coated plastic beads (Calibrite Beads, BD). DNR fluorescence was logarithmically amplified. Data analysis was performed using histogram analysis of the Lysys software program (B&D).

Table 2. Clinical significance of various parameters in AML

		No. of cases	CR (%)	p-value
Age	≤60 yrs	22	63%	>0.05
	>60 yrs	13	46%	
FAB	M1	8	88%	
	M2	8	63%	
	M3	3	0%	>0.05
	M4	10	40%	
	M5	6	67%	
Autonomous growth				
	Low	12	83%	
	Intermediate	8	75%	0.0064
	High	15	27%	
Cytogenetics				
	Normal	22	64%	>0.05
	Abnormal	13	46%	

Statistical analysis: Fisher's exact test.
CR, complete remission

The accumulation of DNR was expressed in arbitrary units by calculating the mean fluorescence distribution of the cell population in each sample. A correction for the background fluorescence was made.

RNase protection assay for MRP. Total RNA was isolated from nucleated cells by the lithium-chloride-urea method, and isolation of genomic DNA was performed by standard procedure. The RNase protection assay was performed as described elsewhere (28). Briefly, 10 μg of total RNA were hybridized under standard conditions with α-^{32}P labeled RNA transcripts complementary to sequences (nucleotides 240-484) at the 5' end of the MRP mRNA (19,28). (Kindly provided by Dr. G.J.R. Zaman and Prof. Dr. P. Borst, Netherlands Cancer Institute, Amsterdam). Radiolabeled protected probes were visualized by electrophoresis through a denaturing 6% acrylamide gel, followed by autoradiography. A human γ-actin probe was always included as control for RNA integrity and recovery. All individual experiments included tRNA, GLC4 and GLC4/ADR cell lines as controls (20). The bands were quantitated by densitometric scanning of the autoradiographs and the amount of MRP mRNA relative to the amount of γ-actin was calculated subsequently. Double and even triple bands were obtained with the MRP probe, probably due to incomplete trimming of the protected probe by RNase A (28) and the final MRP mRNA levels were calculated on the basis of the cumulative values of the separate bands.

Statistical analysis. For a comparison of the distribution of a parameter (e.g. CD34) between the three different groups of autonomous growth, a one-way of variance (ANOVA) test was used. Fisher's exact test and the Mann-Whitney *U* test were used to the outcome of the in vitro experiments with the clinical data.

RESULTS

Patients and treatment results. Thirty-eight consecutive patients (22 males, 16 females, median age 54 yr) with untreated *de novo* AML were included in this analysis. Three patients were not treated because of refusal (n=1) or infection/hemorrhage (n=2). Fifty-seven per cent of the patients attained a complete remission according to the FAB criteria (Table 2).

Autonomous growth. ^{3}HTdR incorporation was determined in purified AML samples. The incorporated counts varied from 220 to 38900 DPM/0.2 x 10^4 cells (mean: 4465; median: 1794 dpm/0.2 x 10^4 cells). Based on these counts the samples from 12 AML patients exhibited low autonomous growth, 10 individuals had intermediate, and the cells of 16 patients showed high autonomous proliferation.

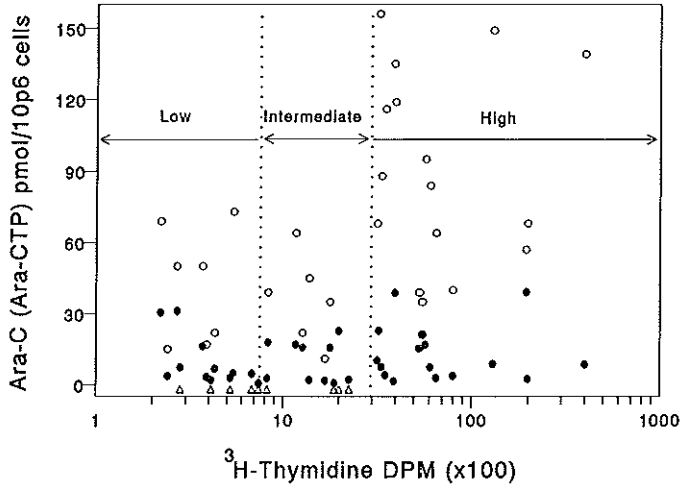

Fig. 1: Ara-CTP concentration (○) and Ara-C incorporation (●)(pmol/10^6) vs autonomous growth (dpm x 100/2x10^4 cells) in 38 AML samples. The dotted lines indicate the division in low, intermediate, and high autonomous growth. In 9 samples, Ara-CTP concentration was below the detection level (△).

Ara-C metabolism.

Intracellular dCTP pools and Ara-CTP formation. The lower detection limit of the procedure used to quantify dCTP and Ara-CTP pools was 5 pmol/10^6 cells. In all samples, dCTP pools were below the detection limit. The intracellular levels of Ara-CTP varied from less than 5 pmol/10^6 (n=9) to 156 pmol/10^6 cells (median: 39.5 pmol/10^6 cells). As shown in Fig.1, Ara-CTP formation was significantly greater in AML samples with an high autonomous growth as compared to the AML samples with a low autonomous growth ($p<0.0001$).

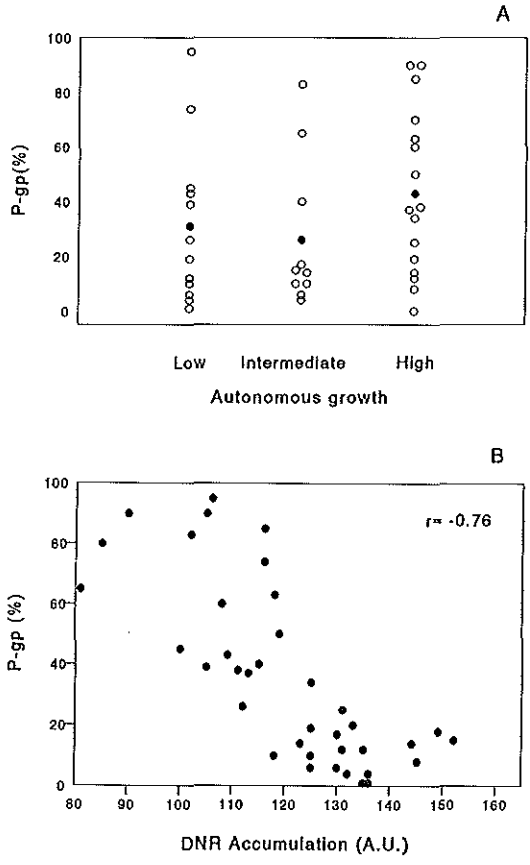

Fig. 2: A) P-gp expression (% of positive cells) according to low, intermediate, and high autonomous growth in 38 AML samples (○); mean percentage of P-gp positive cells (●). P-gp expression was determined by immunocytochemical studies using C219 and C494 monoclonal antibodies. B) DNR accumulation (arbitrary units, [A.U.]) in these samples after incubation with 2 μM DNR for 90 minutes.

Ara-C incorporation. The Ara-C incorporation into DNA as measured in the acid insoluable fraction of the cell pellet varied from 0.67 to 39.1 pmol/10^6 cells (mean: 11.17 pmol/10^6 cells; median: 7.39 pmol/10^6 cells). The amount of Ara-C incorporation did not correlate with autonomous proliferation (Fig. 1). When comparing the total amount of Ara-CTP which was formed after Ara-C exposure with the amount of ^{3}H-Ara-C recovered from DNA, only 5-60 % of Ara-CTP was incorporated. No significant differences among the three AML groups with low, intermediate or high autonomous growth were found in this respect.

Typical MDR (P-gp) expression.

Expression of membrane P-glycoprotein. The percentage of P-gp positive, leukemic blast cells at diagnosis as determined by immunocytochemistry varied from 0% to 95% (median: 30%). Using a standard staining of 2 x 2 cytospin slides with 2 different P-gp antibodies a high correlation between the results of C219 and C494 was found (r=0.96). P-gp expression according to low, intermediate or high autonomous growth is shown in Figure 2a. Cells which expressed P-gp were equally distributed among these three subgroups of patients.

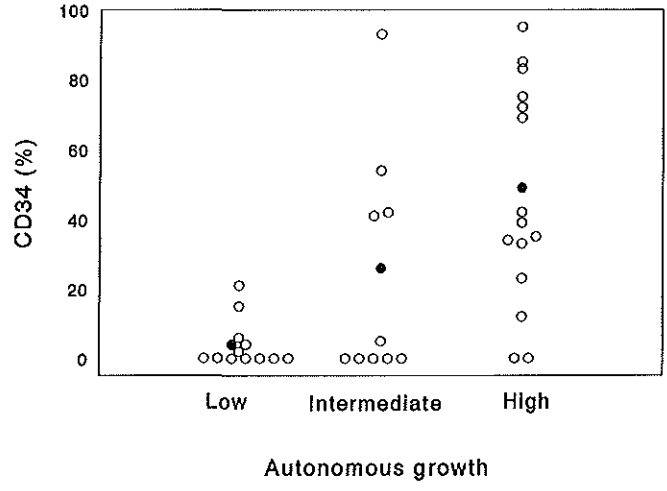

Fig. 3: CD34 expression according to low, intermediate, and high autonomous proliferation (n=38). (●) Mean percentage of CD34 positivity; (○) % of CD34 positive cells in each AML sample.

Table 3. Clinical significance of P-glycoprotein expression in CD34 positive blast cells in untreated *de novo* AML.

AML phenotype	Pgp >1% CD34>1%	Pgp >5% CD34>5%	Pgp >10% CD34>10%	Pgp >20% CD34>20%	Pgp >30% CD34>30%	Pgp >40% CD34>40%
	Complete Remissions observed					
Pgp+, CD34+	7/21	5/18	5/17	3/14	2/9	0/5
Pgp+, CD34-	12/13	12/14	8/10	5/7	5/8	3/5
Pgp-, CD34+	0/0	1/1	1/1	1/2	1/4	4/7
Pgp-, CD34-	1/1	2/2	6/7	11/12	12/14	13/18
Overall p-value	0.0006	0.0006	0.0001	0.001	0.019	n.s.

Complete response rates with standard remission-induction therapy in 35 patients with different proportions of P-glycoprotein and CD34 positive blasts.
N.s.; not significant.

DNR accumulation studies. The presence of functional, P-gp mediated drug resistance was investigated by analyzing the intracellular accumulation of daunomycin in AML blasts of these 38 patients. The accumulation of DNR ranged from 81 to 152 A.U. (mean value: 121 A.U.; median value: 124 A.U.,respectively). Since DNR accumulation is reduced in cells expressing P-gp at greater density, DNR cellular content was compared with the results of P-gp expression. A negative correlation between DNR accumulation and P-gp expression was observed (r= -0.76) (Fig. 2b). DNR accumulation was significantly decreased in P-gp positive AML samples as compared with P-gp negative samples (p=0.0001).

CD34 expression. The percentage of Ficoll concentrated AML blast cells which showed CD34 expression varied from 0 to 95% (mean: 28%; median: 13.5%). AML samples with high autonomous growth more frequently expressed CD34 than samples with intermediate or low autonomous growth (p=0.0012). The CD34 expression according to low, intermediate, and high autonomous growth is shown in Figure 3. Because more than 10% of CD34 positive cells in AML samples can be accurately detected, we prefer to use 10% as a cut-off level for positivity.

P-gp and CD34 co-expression. A large variation of CD34 and P-gp expression was observed. In Table 3 the proportion of cells positive for either marker is shown. Of 18 AML samples with >10% CD34+ and P-gp+ cells, 13 cases showed high autonomous growth while 5 CD34+/P-gp+ specimens had low/intermediate growth (p=0.003). Of 7 AML samples with <10% CD34+ and P-gp+ cells, only 1 case had high spontaneous growth (Table 4). Seven of 13 samples expressing >10% P-gp and CD34 were used for flowcytometric double fluorescence experiments. In these samples, the proportions of P-gp$^+$ and P-gp$^+$ AML cells in the CD34$^+$ and CD34$^+$ subsets of AML cells were calculated. The ratio of P-gp$^+$ vs P-gp$^-$ cells in the CD34$^-$ blast cells was 0.39 (range 0.04-1.0) as opposed to 4.28 (range 2.0 - 7.83) in the CD34$^+$ population ($p< 0.001$). These results indicate that P-gp is predominantly expressed in a subset of AML cells which also express CD34.

p110 expression. The expression of p110, another potential intracellular membrane drug transporter was determined by immunocytochemistry using the MoAb LRP-56. p110 expression was detected in 8 out 36 samples tested (22%). The proportion of p110 positive cells ranged from 0 to 83% (mean 13 %; median value 3%, respectively). P110 expression did not correlate with autonomous growth or CD34 expression.

MRP expression. Twenty-four out of 38 patient's samples were used for mRNase protection assay of MRP based on bulk RNA extracted from the cells. The MRP mRNA expression levels ranged from 0.8 to 8.3 arbitrary units (a.u.) (mean $\pm$ SD: 4.0 $\pm$ 2.5 a.u.) as compared to 6.4 $\pm$ 3.0 a.u. in mononuclear cells from normal volunteers and 100 a.u. in the GLC4/ADR cell line. None of these samples was considered to have MRP overexpression.

Clinical outcome and parameters of drug resistance. Thirty-five of the 38 AML patients who were treated with induction chemotherapy including daunorubicin and Ara-C, were evaluated to determine the probability of complete remission and overall survival. Twenty patients (57%) achieved a complete remission with 1 or 2 remission-induction courses. In this group of patients, several unfavourable prognostic factors for achieving a remission could be identified, i.e., i) the presence of a high autonomous proliferation (p=0.0064); ii) CD34 expression (p=0.0005); iii) P-gp expression (p=0.048); iv) CD34/P-gp simultaneous expression (p=0.0001). Of 18 patients with >10% CD34$^+$ and P-gp$^+$ AML blasts 5 achieved a complete response as compared with 15/18 other patients. At a follow-up of one year, 4/17 CD34$^+$ and P-gp$^+$ patients stayed in first remission as compared with 10/18 other patients.At the time of analysis the median survival of 18 CD34$^+$/P-gp$^+$ patients was 6 months as compared to 15 months in the other patients (p=0.003)(Fig.4).

Table 4. P-gp and CD34 expression in relation to autonomous growth in AML (n=38)

	Low	Intermediate	High	Total
$P\text{-gp}^+/CD34^+$	1	4	13	18
$P\text{-gp}^+/CD34^-$	7	3	1	11
$P\text{-gp}^-/CD34^+$	1	0	1	2
$P\text{-gp}^-/CD34^-$	3	3	1	7
	12	10	16	38

$p=0.003$

The other prognostic factors analyzed, like sex, age, white cell count, percentage of bone marrow blast cells, FAB subtype or abnormal karyotype did not predict for the outcome of response in this group of patients, although patients with higher age had a lower response rate. Other potential mechanisms of drug resistance such as MRP mRNA expression, p110 expression, amount of Ara-CTP formation and Ara-C incorporation did not correlate with clinical outcome ($p>0.05$).

DISCUSSION

In this prospective study, we examined the question of wether pharmacologic parameters which are characteristic of drug resistance have a prognostic significance for response. We also wanted to know if primary drug resistance is frequently observed in AML blasts with a high autonomous proliferation. The presence of multidrug resistant, proliferating AML cells may explain why refractory disease is present in many AML patients who relapse after chemotherapy. Therefore we have studied several potential mechanisms of drug resistance in AML blast cells, such as (over)expression of membrane drug transporter molecules and suboptimal Ara-C activation/incorporation. These mechanisms were compared with in vitro autonomous growth and clinical outcome of remission-induction therapy.

In fresh clinical samples of AML, overexpression of P-gp is observed in a significant proportion of cells and it leads to lower intracellular accumulation of daunorubicin. The accumulation of daunorubicin in AML samples is inversely correlated with the number of P-gp positive cells, and this would explain why P-gp is a significant negative prognostic factor in this and other studies (1,29-30). However, patients with P-gp expression were equally divided among the three groups with different autonomous growth characteristics. Evidently, it is important to know if AML cells which are capable of rapid regrowth after chemotherapy have enhanced growth in vitro and express P-gp. It may be assumed that

cells with a high frequency of cell divisions are prone to mutations associated with the probability of spontaneous drug resistance (6). However, this would not explain why a particular mechanism of drug resistance such as MDR is frequently observed in untreated AML. Alternatively, among various phenotypic subpopulations of AML cells, $CD34^+$ cells are considered an immature progenitor cell phenotype (27). In this study, the presence of CD34 cells correlated with high autonomous growth. Within the population of $CD34^+$ cells P-gp positive cells were present, and these $CD34^+$ AML blast cells were associated with high spontaneous proliferation and poor prognosis.

P-gp overexpression may be conserved during leukemic transformation of normal marrow $CD34^+$ cells, which have the MDR1 phenotype (1). $CD34^+$ AML cells are potentially capable to proliferate and at the same time they are relatively protected from the effects of chemotherapy. From our data it appears that the presence of more than 1% of these cells at diagnosis predicts for a poor response. For practical purposes it seems that a cut-off level of 10% positive cells can be used since this percentage of cells is easily detected, and also has a highly significant negative impact on response. Thus, the presence of such cells at diagnosis has significant prognostic relevance with respect to the probability to achieve a complete remission and durable disease-free survival.

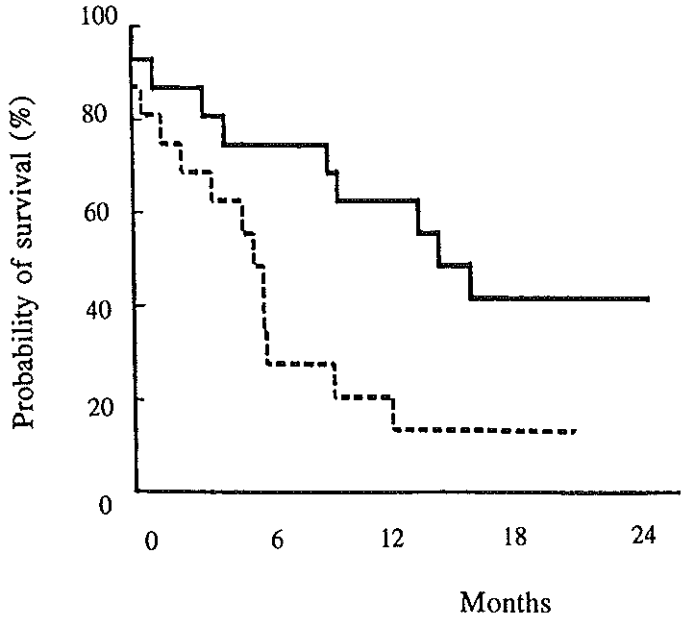

Fig. 4: Actuarial survival according to the simultaneous expression of CD34 and P-gp in 38 patients with untreated AML, treated with induction chemotherapy. Patients with >10% CD34/P-gp blast cells (-----) are compared with all other patients (——).

In this group of patients, other drug transport molecules seem of minor importance in untreated AML. Both MRP and p110 were expressed at background levels, and no correlation with the presence of an immature stem cell phenotype or high proliferation capacity was observed. Since these molecules, unlike P-gp are not overexpressed in normal bone marrow cells, apparently they are not preserved in AML cells and are not involved in primary drug resistance. Recently, it was found that in a mixed group of primary and secondary AML, MRP was overexpressed in 0/18 patients and p110 in one third of the patients (30). Therefore, one may conclude that P-gp is the major drug transporter in untreated AML, and that it can be associated with an immature, highly proliferative stem cell phenotype.

The other major antileukemic drug used in induction chemotherapy is Ara-C. The present study shows that Ara-C phosphorylation to its active form Ara-CTP is significantly greater in AML cells with high autonomous proliferation. This may result from increased metabolic activity in AML cells with high spontaneous growth. Although intra-cellular Ara-CTP formation is essential for Ara-C incorporation in AML blasts, the higher Ara-CTP levels in cells with high autonomous growth did not result in a higher incorporation of Ara-C into DNA in these cells. The most likely explanation for this observation is that incorporated Ara-C is rapidly diluted in proliferating cells. Therefore, high Ara-CTP levels may reflect primarily the metabolic activity of cells with high autonomous growth. High Ara-CTP combined with a low level of Ara-C incorporation indicates that the active metabolite cannot be optimally incorporated into a rapidly expanding population of tumour cells. This finding concurs with the clinical observation that high-dose Ara-C is more effective than normal dose in patients with unfavourable or relapse AML (12). In conclusion, this study provides evidence that high autonomous growth is associated with the presence of a multidrug-resistant phenotype in subsets of AML cells with a high proliferative capacity. The presence of these resistant, autonomously proliferating cells may explain why patients are refractory to remission-induction therapy in primary AML. Although few patients with $CD34^+/P\text{-}gp^+$ AML may attain a CR, residual cells are present in these patients, ultimately leading to relapse. Therefore, these cells may represent a primary target for the use of non-cytotoxic drugs which modulate P-glycoprotein mediated drug efflux such as cyclosporin.

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CHAPTER 4

ENHANCED CHEMOSENSITIVITY OF CLONOGENIC BLASTS FROM PATIENTS WITH ACUTE MYELOID LEUKEMIA BY G-CSF, IL-3 OR GM-CSF STIMULATION.

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SUMMARY

Exposure to hemopoietic growth factors (HGFs) induces proliferation of clonogenic acute myeloid leukemia (AML) cells. Recruitment of quiescent, clonogenic blasts may improve the cytotoxic effects of cell-cycle-specific drugs like cytosine-arabinoside (Ara-C). Because other studies have shown heterogeneous effects of HGF and Ara-C incubation, we analyzed the individual effects of granulocyte colony-stimulating factor (G-CSF), interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF) combined with an S-phase and non-phase specific cytostatic agent on clonogenic blasts of 14 newly diagnosed AML patients under standardized, serum-free conditions. AML cells were incubated for 24 hours with titrated concentrations of Ara-C (10^{-8}, 10^{-7}, 10^{-6}M) or Mafosfamide (Maf; 0, 1.0, 10 or 20 μg/ml) following preincubation for 48 hours with or without G-CSF, IL-3 or GM-CSF, starting at 24 hours prior to chemotherapy exposure. AML colony forming cells (AML-CFU) were then determined in semi-solid culture in the presence of the same growth factor. The results showed significantly enhanced cytotoxicity of Ara-C to AML-CFU following stimulation by G-CSF ($p<0.002$ at 10^{-8}M, $p<0.002$ at 10^{-7}M, and $p<0.01$ at 10^{-6}M Ara-C), IL-3 ($p<0.002$ at 10^{-8}M, $p=0.001$ at 10^{-7}M and $p<0.01$ and 10^{-6}M Ara-C) and GM-CSF ($p=0.01$ at 10^{-8} M, $p<0.01$ at 10^{-7}M and $p<0.002$ at 10^{-6}M Ara-C). A moderate but significant enhancement of Maf cytotoxicity by HGF was also observed ($p<0.05$ at Maf 1.0 μg/ml by IL-3 and GM-CSF and $p<0.05$ by GM-CSF at 10 μg/ml Maf). Ara-C cytotoxicity to normal bone marrow progenitors was enhanced significantly only by G-CSF ($p=0.02$ at 10^{-8}M, $p=0.01$ at 10^{-7}M and $p<0.01$ at 10^{-6}M Ara-C), and by GM-CSF at 10^{-7}M Ara-C ($p=0.05$). However, the effect of HGF stimulation as studied by bromodeoxyuridine (BrdU) incorporation during the first or second 24 hours of HGF stimulation, did not explain the difference between poor and good HGF enhanced Ara-C cytotoxicity, indicating that other cellular changes than cell cycle activation as the consequence of growth factor stimulation are responsible for enhanced cytotoxicity. Our findings indicate that combining HGFs, and especially IL-3, with chemotherapy may be useful in the AML treatment.

INTRODUCTION

Acute myeloid leukemia (AML) is clonal disorder (1), where a limited population of neoplasmic precursor cells sustains cell renewal and proliferation of the malignancy. The processes of self-renewal and proliferation are under the control of a group of specific glycoproteins, the hematopoietic growth factors (HGFs) (2-3). The target of chemotherapy is the elimination of the leukemic progenitors. Several drugs with great anti-leukemic activity, e.g. cytosine arabinoside (Ara-C), are mainly effective in actively cycling cells. Because some leukemic precursor cells probably belong to a non-cycling leukemic cell

population with a prolonged generation time (4-5), these leukemic cells may escape cell death by cytostatic drugs. Recruitment of leukemic cells from a quiescent state into proliferation, e.g. by hemopoietic growth factor stimulation may enhance the cytotoxicity of Ara-C to AML blasts. Several investigators have reported that the sensitivity of leukemic blasts to Ara-C can be altered by culturing the cells in vitro in the presence of recombinant human granulocyte colony-stimulating factor (G-CSF) (6-8), recombinant human interleukin-3 (IL-3) (6-7,9-11), recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) (6-8,12-13) or combinations of these HGFs (7,14-15). Also, effects of these cytokines on Ara-C metabolism have been described (16-19). Other studies showed that the effects may be heterogeneous (20,21).

The effects of HGFs and Ara-C sensitivity on clonogenic AML cells were reported in 10 studies (8-14,18,20-21). In only 4 of these studies, 10 or more AML cases were used to studie HGF altered cytotoxicity (10-11,18,21). In 2 of these reports, also blasts of relapsed and therefore previously treated patients were studied (18,21). The clonogenic assays were performed in serum containing cultures in all studies, plating efficiencies were determined in the presence of conditioned media (CM) like PHA-CM or 5637-CM or a mixture of HGFs in 7 studies (8,10-12,14,18,20). Three investigators report effects after preculturing AML cells in the presence of 5637-CM for several days (8,20-21). One of the studies reported the individual effects of 3 HGFs on the Ara-C sensitivity using the same AML samples, however no control cultures using cells without HGF and Ara-C incubation were performed (21). We therefore wanted to examine the effects of individual G-CSF, IL-3 and GM-CSF stimulation on drug-induced cytotoxicity to clonogenic cells in a series of newly diagnosed AMLs, under serum-free, highly standardized conditions. In order to evaluate the mechanism of altered cell-cycle kinetics of the clonogenic AML cells, we used the cell-cycle-specific drug Ara-C and compared it with the non-cycle-specific, alkylating agent mafosfamide (Maf). Bromodeoxyuridine (BrdU) incorporation studies were done to examine the efficacy of HGF stimulation on cell proliferation. To assess the clinical value of the results of the clonogenic assays, we compared the results of Ara-C sensitivity in vitro with the clinical outcome of the treatment in these patients following remission induction chemotherapy (e.g. Ara-C plus daunomycin).

MATERIALS AND METHODS

Patients and therapy. Fourteen adult patients with newly diagnosed AML classified according to the criteria of the French-American-British (FAB) committee (22), were included in the study (Table 1). After bone marrow sampling, 12 patients were treated with one or two courses of daunomycine and Ara-C or with m-Amsacrine (m-AMSA) and Ara-C remission induction chemotherapy according to the AML protocol of the Dutch Hemato-Oncology Cooperative Group HOVON, or to the protocol AML-11 of the EORTC

Table 1. Clinical characteristics, treatment and response.

Pt	Age (Sex)	FAB	WBC*	Mat†	Blast (%)	Treatment	Outcome 1st cycle	Outcome 2nd cycle
1	66(F)	M2	5.3	BM	66	Ara-C + DNR; Ara-C + DNR	PR‡	CR‡
2	56(F)	M2	33	BM	78	Ara-C + DNR; m-AMSA + Ara-C	PR	PR
3	45(M)	M2	7.6	BM	75	Ara-C + DNR; m-AMSA + Ara-C	PR	CR
4	53(F)	M1	56.2	PB	95	Ara-C + DNR; m-AMSA + Ara-C	CR	CR
5	42(M)	M4	90	BM	68	Ara-C + DNR; m-AMSA + Ara-C	PR	PR
6	43(F)	M1/M2	26.2	BM	70	Ara-C + DNR; m-AMSA + Ara-C	Res‡	Res
7	60(M)	M1	252	PB	97	No treatment	-	-
8	34(F)	M1/M2	83	PB	80	Ara-C + DNR; m-AMSA + Ara-C	CR	CR
9	64(F)	M1	300	PB	89	No treatment	-	-
10	67(M)	M2	17.7	BM	31	Ara-C + DNR; Ara-C + DNR	CR	CR
11	26(M)	M4	86	BM	90	Ara-C + DNR; m-AMSA + Ara-C	CR	CR
12	68(M)	M2	7.7	BM	33	Ara-C + DNR	CR	-
13	51(F)	M1/M2	256	BM	82	Ara-C + DNR; m-AMSA + Ara-C	Resist.	Resist.
14	60(F)	M4	6.8	BM	31	Ara-C + DNR	CR	-

*WBC, white blood count (x10^9/L).
†Mat, Material used for the experiments; BM, bone marrow; PB, peripheral blood.
‡PR, partial remission; CR, complete remission. Resist., Resistant to therapy.
M or F, male or female.

Leukemia Group. One patient refused therapy, the other patient died before the start of the chemotherapy. After each course, the effect was evaluated. Complete remission (CR) was defined as normal bone marrow cellularity with less than 5% blast cells including monocytoid cells and normal peripheral blood counts. Partial remission (PR) was characterized as bone marrow containing less then 25% blasts and normal blood counts or moderate pancytopenia with less than 5% circulating blast cells. The clinical results of treatment are shown in Table 1.

AML and bone marrow cells. Bone marrow or peripheral blood from the patients was obtained after informed consent. Bone marrow aspirates were obtained from the posterior iliac spine and collected in tubes containing non-toxic heparinized Hank's balanced salt solution (HBSS). Mononuclear cells from peripheral blood or bone marrow were separated by Ficoll-Isopaque (Nycomed, Oslo, Norway) centrifugation. T-lymphocytes were removed by E-rosette Ficoll separation (22). Adherent cells were removed by incubating the cells (1 x 10^6 nucleated cells/ml) in serum-free medium for 1 hour in plastic culture flasks (Costar, Cambridge, MA) at 37°C, in a fully humidified atmosphere with 5% CO_2 in air.

The T-cell and adherence depleted cells were cryopreserved in Iscove's modified Dulbecco's medium (IMDM; Gibco, Paisley, UK) supplemented with 10% dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany) and 20% fetal calf serum (FCS; Gibco) using a temperature controlled freezer (Kryo 10, Planer Biomed, UK) and were subsequently stored in liquid nitrogen.

Normal bone marrow was obtained from five healthy volunteers.

Drugs. Ara-C (Upjohn, Kalamazoo, MI) was dissolved in phosphate-buffered saline (PBS) and stored at -80°C. Final dilutions were made in culture medium immediately before use. Mafosfamide (Maf; ASTA-Z 7557; ASTA-Werke, Bielefeld, FRG) was freshly dissolved in culture medium immediately before use.

Hematopoietic growth factors. Recombinant human G-CSF was obtained from Amgen (Thousand Oaks, CA) and used at a concentration of 100 ng/ml. Recombinant human IL-3 and GM-CSF were donated by Sandoz (Basle, Switzerland) and both used at a concentration of 10 ng/ml. These concentrations were found to be optimal, as determined by dose-response studies using clonogenic assays on normal, T-cell and adherent cell depleted bone marrow cells.

Cell culture methods. Purified AML cells were cultured in serum-free medium as described (24). The suspension cultures consisted of IMDM supplemented with 15 mg/ml dialysed bovine serum albumin (BSA; Cohn fraction V, Sigma, St Louis, MO), 2.8 μg/ml linoleic acid (Sigma), 7.8 μg/ml cholesterol (Sigma), 1 μg/ml insulin (Sigma), 7.7 μM iron saturated human transferrin (Behring Institute, Marburg, Germany), 0.1 mM 2-mercaptoethanol (Merck), and 60 μg/ml gentamycin (Gibco). The cell suspensions were plated in 24-wells cell culture plates (Costar) at a concentration of 0.5 x 10^6 cells/ml, in the presence or absence of G-CSF, IL-3 or GM-CSF and incubated for 24 hours at 37°C and 5% CO_2 in a fully humidified atmosphere. Subsequently, the appropriate doses of Ara-C (final concentrations: 0, 10^{-8}, 10^{-7} or 10^{-6}M) and Maf (final concentrations: 0, 1, 10 or 20 μg/ml) were added to the cultures. After 24 hours, the cells were carefully resuspended and a fixed volume (0.2 ml) was taken of each sample. The cells were washed three times in HBSS and each sample was subsequently suspended in 1 ml IMDM containing 0.9 % methylcellulose (Fluka, Switzerland) supplemented with the same additives as used in the suspension cultures. This mixture was plated in triplicate in 24-wells plates (Costar), 0.25 ml/well. Cells exposed to a growth factor in suspension were cultured in the presence of the same growth factor in the clonogenic assays. Cells that were not incubated with a growth factor in suspension were cultured in the methylcellulose cultures in the presence of G-CSF, IL-3 or GM-CSF. Identical volume fractions of each of the suspension cultures were taken to be plated in colony cultures. Thus, before plating, cell numbers were not adjusted for cell death or cell growth, in

order to make results directly comparable. After 7 to 9 days of incubation (37°C, 5% CO_2), aggregates containing 10 cells or more were scored using an inverted microscope. AML cases were defined as having a good cytotoxic response if an absolute increase of killing of AML-CFU of more than 25% was achieved at two different tested Ara-C concentrations in suspension cultures with a certain HGF, as compared with the cultures that did not contain HGF addition. From the obtained results, also IC_{50} values could be calculated. The IC_{50} was defined as the concentration of the cytostatic drug that resulted in 50% inhibition of colony formation. In some cases, pooled colonies were evaluated for morphology in order to verify that the cells in the colonies were blasts.

Normal bone marrow cultures. To compare the effects of growth factor stimulation before and during Ara-C incubation between AML cells and normal hemopoietic progenitors, the same procedure was applied to normal bone marrow cells. However, to obtain optimal culture conditions, serum containing cultures had to be performed. The suspension cultures contained IMDM supplemented with 20% FCS, 1 mg/ml BSA, 30 μM egg lecithin (Merck), 7.7 μM iron saturated human transferrin, 100 μM 2-mercaptoethanol and 60 μg/ml gentamycin. The clonogenic assays consisted of IMDM containing 0.9% methylcellulose supplemented with the same additives as described for the suspension cultures. The cultures were performed in 35 x 10 mm tissue culture dishes (Costar), 1 ml per well plated. After 10 days of incubation, the number of colonies (more than 50 cells) were scored.

BrdU incorporation. To evaluate the effect of HGF stimulation by bromodeoxyuridine (BrdU) incorporation in the first or second 24 hours of incubation, AML cells (0.5 x 10^6/ml) were cultured in serum-free medium in the presence or absence of G-CSF, IL-3 or GM-CSF at 37°C and 5% CO_2 in air. Of each patient, one sample was cultured for 24 hours in the presence of 10 μM BrdU/Fluorodeoxyuridine (FdU;Amersham, UK). After 24 hours, the cells were washed and cytospin slides were prepared. The other sample of the cell suspensions was incubated with BrdU/FdU after a preincubation period of 24 hours in the presence or absence of HGFs. After 24 hours, the cells were washed and cytospin slides were prepared. After air-drying the slides overnight, they were fixed in methanol at 4°C for 30 minutes. After rinsing in PBS, the slides were subsequently exposed to 15 μl anti-BrdU and nuclease (Amersham) for one hour at 20°C. The reactivity of anti-BrdU was visualized by use of a fluorescein isothiocyanate (FITC) conjugated rabbit anti-mouse immunoglobulin antiserum (Dakopatts, Glostrup, Denmark). The fluorescence staining was evaluated with a Zeiss microscope equipped with phase contrast facilities (Carl Zeiss, Oberkochen, Germany). One hundred cells were counted for each determination, and each sample was counted in duplicate.

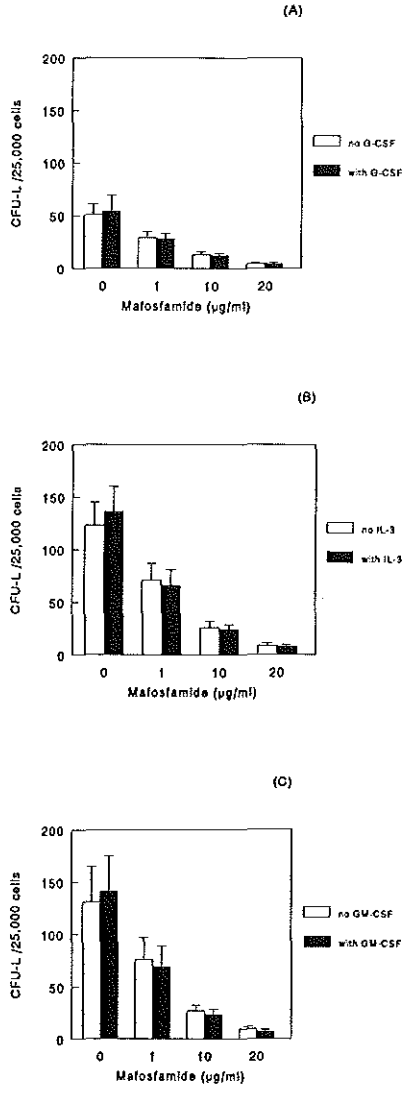

Fig. 1: The effect of G-CSF (a), IL-3 (b), and GM-CSF (c) pre- and costimulation on mafosfamide cytotoxicity in clonogenic AML cells (AML-CFU) per 25,000 cells plated (triplicate cultures) as compared to non pre- and costimulated cells. The values shown are the mean of 14 AML samples +/- SD. o, no HGF, • HGF present.

Statistical Analysis. For a comparison between the AML treatment groups, Student's *t*-test for paired samples was used.

RESULTS

AML clonogenic assays. We investigated the effect of G-CSF, IL-3 and GM-CSF (pre- and co-) stimulation on the cytotoxicity of Ara-C and Maf to clonogenic AML cells. Ara-C was used as a cycle-specific drug, Maf as a non-cycle-specific drug. Table 2 shows the effects of HGF pre- and costimulation on Ara-C cytotoxicity. Controls were cells not stimulated in suspension, and subsequently plated with the appropriate HGF. This resulted in different number of colonies for the three HGFs (Table 2, column 1). HGF prestimulation of the cells without cytostatic drug (control suspension cultures) resulted in a moderate increase of colony-forming cells for all three tested hemopoietic growth factors (Table 2, columns 1 and 2). Incubation of AML cells without HGF for 24 hours followed by incubation with varying concentrations of Ara-C for another 24 hours induced significant kill of clonogenic cells (Table 2, columns 3, 5 and 7). When AML cells were prestimulated with HGF for 24 hours in the presence of Ara-C, a significantly enhanced killing of AML clonogenic cells was observed (Table 2, columns 4, 6 and 8). The latter increase of Ara-C cytotoxicity was apparent in association with G-CSF, IL-3 and GM-CSF and at all tested Ara-C concentrations as compared with Ara-C alone. However, enhancement of cytotoxicity was quantitatively different between the patients. As defined in Materials and Methods, we distinguished patients who responded well to HGF stimulation (good cytotoxic response in vitro) from those with a poor cytotoxic response in vitro. Nine of 14 cases responded well to G-CSF stimulation, 13 of 14 cases to IL-3, and 8 of 14 to GM-CSF (Table 3). Enhancement of Ara-C cytotoxicity by HGF was highly significant when G-CSF, IL-3, or GM-CSF were supplemented to the suspension cultures.

The effects of HGF stimulation of AML cells on Maf cytotoxicity are shown in Figure 1. Cytotoxicity to Maf was only slightly enhanced when the cells were co-cultured with HGF. The increase of cell killing was seen only at low concentrations of Maf ($p<0.05$ at Maf 1.0 μg/ml by IL-3 and GM-CSF, respectively, and $p<0.05$ by GM-CSF at 10 μg/ml Maf). Alteration of Maf cytotoxicity to AML clonogenic cells by the various HGFs did not differ.

BrdU incorporation. The BrdU incorporation in absence or presence of G-CSF, IL-3 or GM-CSF during the first or second 24 hours of incubation was determined in 9 of the 14 AML samples. If no growth factor was present in suspension culture, a two-fold increase (from 7% to 16%) of BrdU-labeled nucleated cells was observed. Using G-CSF, IL-3 or GM-CSF, the absolute increase of BrdU incorporation was 4.3, 4.1 and 3-fold

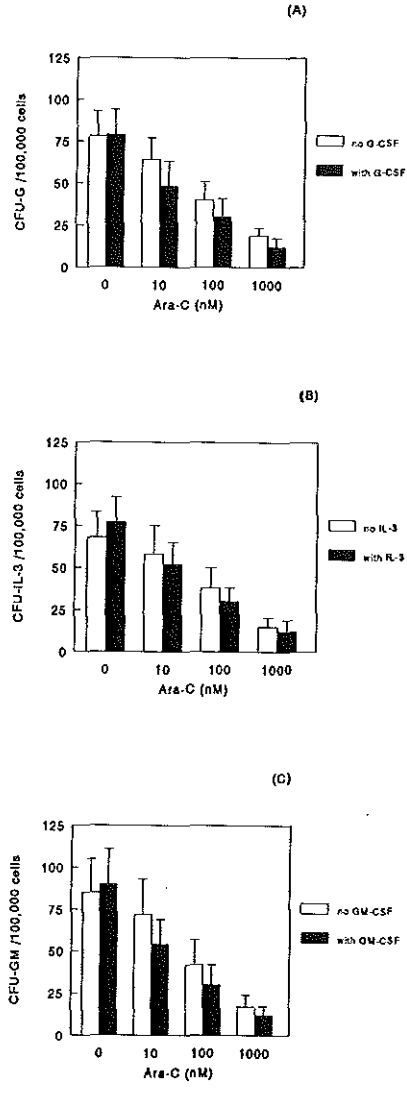

Fig. 2: The effect of G-CSF (a), IL-3 (b), and GM-CSF (c) pre- and costimulation on Ara-C cytotoxicity in normal bone marrow progenitors (CFU-G, CFU-IL-3, CFU-GM) per 100,000 cells plated (triplicate cultures) as compared to non pre- and costimulated cells. The values shown are the mean of 14 AML samples +/- SD. o, no HGF, • HGF present.

Table 2. Ara-C cytotoxicity to clonogenic AML cells in adjunct to stimulation by G-CSF, IL-3 or GM-CSF.

		Cytotoxicity with Ara-C at							
		0 M		10^{-8}M		10^{-7}M		10^{-6}M	
		No HGF	+ HGF	No HGF	+ HGF	No HGF	+ HGF	No	+ HGF
Column no.		1	2	3	4	5	6	7	8
G-CSF	Mean	51	54	28 (45)	23 (57)	17 (67)	12 (78)	6.2 (88)	3.4 (94)
	SEM	11	13	4.4	4.7	2.9	2.3	1.3	0.8
	Median	35	34.5	22	16	15	8	5	4
	p	>0.05		<0.002		<0.002		<0.01	
IL-3	Mean	123	136	95 (23)	64 (53)	60 (51)	31 (77)	26 (79)	10 (93)
	SEM	22	24	17	14	12	10	6	3.3
	Median	82	83	61	32.5	34	15.5	16	5.5
	p	<0.05		<0.002		=0.001		<0.01	
GM-CSF	Mean	133	140	98 (26)	61 (56)	55 (59)	30 (79)	20 (85)	9.1 (94)
	SEM	34	34	23	15	12	9.2	5.4	4.1
	Median	95	99	79.5	41.5	32.5	19	17	4
	p	>0.05		=0.01		<0.01		<0.002	

Values represent the mean number of colonies (AML-CFU) of 14 cases of AML. Suspension cultures were performed in the presence or absence of HGF (48 hrs) and Ara-C (24 hrs). After washing, a fixed volume of cells was plated in the presence of the corresponding HGF (triplicate wells). Values in parentheses represent the kill of AML-CFU (%) as compared with control cultures without Ara-C pre-incubation.

Table 3. Clinical outcome to chemotherapy and HGF induced Ara-C mediated cytotoxicity.

Pt.	Clinical Response	G-CSF	IL-3	GM-CSF
1	I*	-‡	+§	-
2	I	+	+	+
3	I	-	+	+
4	II†	-	+	+
5	I	-	+	-
6	I	+	+	+
7	No treatment	+	+	+
8	II	+	+	+
9	No treatment	+	+	-
10	II	+	+	+
11	II	-	+	-
12	II	+	+	-
13	I	+	-	-
14	II	+	+	+

*I, Clinical response to chemotherapy defined as no complete remission after one cycle.
†II, Clinical response to chemotherapy defined as complete remission after one cycle.
‡-, Poor cytotoxic response in vitro, defined as an increase of AML-CFU kill of less than 25% after HGF (pre) stimulation
§+, Good cytotoxic response in vitro, defined as an increase of AML-CFU kill of 25% or more following HGF (pre) stimulation.

respectively. No differences of BrdU labeling were observed between AML samples with a good or a poor HGF induced enhancement of Ara-C cytotoxicity in vitro (Table 4).

Normal bone marrow cultures. The effects of HGF stimulation on Ara-C cytotoxicity to normal bone marrow progenitors are summarized in Figure 2. Prestimulation with HGF without Ara-C did not significantly increase the number of marrow colonies induced by G-CSF, IL-3 or GM-CSF, respectively. Significant increases of precursor cell killing by Ara-C preincubation with HGF compared to Ara-C exposure without HGF, were only noted in case of G-CSF ($p=0.02$ at 10^{-8}M, $p=0.01$ at 10^{-7}M and $p<0.01$ at 10^{-6}M Ara-C) and GM-CSF ($p=0.05$ at 10^{-7}M Ara-C), but not with GM-CSF at the other Ara-C concentrations, nor with IL-3.

Comparison of clinical outcome and in vitro response to Ara-C. The clinical response to chemotherapy is shown in Table 1. Of the 12 patients treated with chemotherapy, 6 patients achieved a complete remission (group II, Table 3) and 6 patients did not reach CR after one cycle (group I, Table 3). Good or poor in vitro response of HGF induced Ara-C cytotoxicity was not related to the clinical response of the chemotherapy (Table 3).

Table 4. % BrdU positive AML cells following stimulation by G-CSF, IL-3 or GM-CSF.

	no HGF	G-CSF		IL-3		GM-CSF	
Incubation time (hours)	Total of 9 AML cases (n=9)	Good cytotoxic response* (n=5)	Poor cytotoxic response* (n=4)	Good cytotoxic response (n=9)	Poor cytotoxic response (n=0)	Good cytotoxic response (n=5)	Poor cytotoxic response (n=4)
0-24h	7±1.6	7±1	9±3	12±2.9	-	13±4.5	13±1.7
24-48h	16±2.4	30±15	40±11	49±5.8	-	34±6.4	46±8.8
p		NS†				NS	

Average results ± SEM of 9 AML cases classified according to the cytotoxic response to Ara-C and HGF pre-incubation. The percentage of BrdU-positive cells of each AML case was obtained by counting 100 cells in duplicate slides of each sample.

*Good and poor cytotoxic responses: see Table 2.

†NS, not significant.

DISCUSSION

In this study, we investigated the individual effects of G-CSF, IL-3 and GM-CSF stimulation before and during cytostatic incubation of clonogenic AML cells. A period of 24 hours of HGF prestimulation prior to cytostatic incubation was selected because, with this exposure, significant recruitment of AML cells has been reported in vitro and in vivo (18,25-26). BrdU incorporation studies showed a three- to fourfold increase in BrdU-positive cells during the second 24 hours of HGF stimulation. This increment exceeds the increase of BrdU-positive cells seen in culture with no HGF. Following unstimulated culture in vitro, the increase of BrdU-positive cells is twofold. The latter "spontaneous" increase is most likely caused by autocrine cell-cycle activation of AML cells (27). Because AML cells were incubated for 24 hours in the presence of BrdU, the possibility of the formation of BrdU-positive daughter cells can be responsible for the high percentages of BrdU-positive cells scored. However, these experiments clearly indicate that the effects of HGF stimulation were most eminent from 24 to 48 hours after start of HGF incubation. The possibility remains of course that a period longer than 24 hours of HGF stimulation would permit to recruit the entire quiescent leukemic precursor cell population. However, following prolonged exposure, mechanisms of cell maturation and the generation of progeny may interfere with the direct quantitative assay of AML-CFU inoculated in culture.

Incubation of AML cells with HGF before and during cytostatic incubation induced a significantly greater cell kill by Ara-C but not by Maf. This suggests that changes in the cell cycle by HGF stimulation could be largely responsible for increased sensitivity to Ara-C. However BrdU analysis suggested that other factors than pure cell-cycle activation by HGF stimulation would contribute to evaluated Ara-C cytotoxicity. Another explanation why BrdU incorporation did not correlate with the enhanced cell kill might be the fact that BrdU incorporation was studied among the total AML cell population while cytotoxicity enhancement was studied in clonogenic subpopulations. Although all three HGFs were effective in increasing Ara-C cytotoxicity, a large patient to patient variability was observed. We were able to identify a group of AML cases that showed a better in vitro response than others. Comparison of the effects of G-CSF, IL-3 and GM-CSF in good and poor cytotoxic responders in vitro, showed that G-CSF was less effective than IL-3 and GM-CSF in enhancing Ara-C cytotoxicity. The number of AML's responsive to GM-CSF was 8 out of the 14, as compared to 13 out of 14 samples which responded well to IL-3. Five AMLs with a good response to IL-3, did not react to GM-CSF. This is of particular interest, since IL-3 and GM-CSF are known to bind to common or closely related cell surface receptors (28-29).

HGF may also interfere with the intracellular Ara-C metabolism needed for Ara-C-mediated cytotoxicity (16-18). The enhancement of Ara-C cytotoxicity by combined HGF (pre-) incubation, was more evident at higher Ara-C concentrations. The efficacy of HGF

combined with Ara-C to inhibit AML colony formation may therefore be improved using higher doses of Ara-C. Because Ara-C also effects the self-renewal capacity of the leukemic cells (30), it is be possible that the HGF enhanced cytotoxicity is underestimated. Experiments to determine secondary plating efficiencies would be required to investigate this possibility.

The clinical effects of GM-CSF treatment before and during chemotherapy as reported recently by Estey *et al* resulted in a lower CR rate and a lower survival survival probability (31). Our in vitro data indicate that IL-3 is more effective in enhancing Ara-C sensitivity than GM-CSF. This finding is supported by other data in vitro, suggesting that GM-CSF may stimulate mature blast progenitors rather then recruiting undifferentiated blasts (32). The clinical studies of IL-3 combined treatment are currently on their way.

Further, normal bone marrow samples (n=5) were used to evaluate the effects of combined Ara-C and HGF (pre-) incubation on myeloid progenitors. The results of these experiments showed less enhancement of Ara-C cytotoxicity towards normal bone marrow (NBM) precursors by HGF in comparison to clonogenic AML cells. GM-CSF (pre-) incubation caused the largest enhancement of Ara-C toxicity to normal myeloid progenitors, while no or modest responses were found with IL-3. Although enhanced cell kill by G-CSF was modest, these effects were just significant ($p<0.05$). This observed preferential kill of leukemic versus normal clonogenic cells is in agreement with observations done with other test systems (11,14). Although differences in cell-cycle kinetics of normal and leukemic precursors may provide an explanation (11), differences in intra-cellular Ara-C metabolism in leukemic and normal hemopoietic cells after HGF stimulation might offer a probable mechanism for this observation (16-19). Briefly, combined HGF and Ara-C incubation can cause a significant expansion of intracellular dCTP pools in normal bone marrow progenitors but not in leukemic cells, thereby antagonizing Ara-C metabolism through mechanisms as inhibition of deoxycytidine kinase (33), inhibition of Ara-C DNA incorporation (34-35), and stimulation of dCMP deaminase (36). If other factors than cell-cycle activation would determine increased cell kill, this would also explain the lack of correlation between BrdU positivity induced by HGF stimulation and enhancement of Ara-C cytotoxicity to clonogenic AML cells as demonstrated.

Contradictory results about the correlation of in vitro data and in vivo outcome of chemotherapy have been reported (37-38). In our study, we found no correlation between the cytotoxic response of Ara-C in vitro and the outcome of the chemotherapy among a small cohort of patients. This might be explained by the fact that HGF stimulation was not included in the treatment regimens of the patients.

In conclusion, HGF stimulation increases the cytotoxic effects on clonogenic AML cells in vitro. IL-3 seems to be more effective than G-CSF and GM-CSF. The effects on normal hematopoietic progenitors are minor compared with the effects on clonogenic AML cells. These observations indicate that combining HGF, especially IL-3, with

chemotherapy may be useful in the treatment of a subgroup of AML patients.

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CHAPTER 5

HEMATOPOIETIC GROWTH FACTOR STIMULATION AND CYTARABINE CYTOTOXICITY IN VITRO: EFFECTS IN UNTREATED AND RELAPSED OR PRIMARY REFRACTORY ACUTE MYELOID LEUKEMIA CELLS.

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SUMMARY

Stimulation of clonogenic acute myeloid leukemia (AML) cells by hematopoietic growth factors (HGF) is capable in enhancing cytosine-arabinoside (Ara-C) cytotoxicity in vitro. Until now, it is not known to what extent Ara-C cytotoxicity can be restored by HGF stimulation in previously treated AML samples. Therefore, we studied the individual effects of the hematopoietic growth factors granulocyte colony-stimulating factor (G-CSF), interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulation on the sensitivity of clonogenic leukemic cells from 6 patients with newly diagnosed AML. These results were directly compared with the oucome using AML samples from the same patients at relapse or primary refractory AML. In 1 patient, a sample of the second relapse was also studied. The results were expressed as IC_{50} values, and were used to calculate sensitivity ratios, defined as the ratio of the IC_{50} value with drug exposure alone and with HGF plus Ara-C. In AML samples treated with Ara-C and no HGF , IC_{50} values of Ara-C in relapsed/refractory AML were greater than IC_{50} values of AML cells at diagnosis. Addition of either HGF enhanced the Ara-C cytotoxicity for the relapsed samples significantly (for G-CSF $p=0.036$, IL-3 $p=0.036$ and for GM-CSF $p=0.036$). The values of Ara-C sensitization of AML samples due to HGF at relapse did not significantly differ from those at diagnosis. However, enhancement of Ara-C cytotoxicity to AML progenitors by IL-3 or GM-CSF stimulation was significantly less in the cell specimens from AML recurrence patients as compared with the original diagnosis samples. In three AML samples at diagnosis and at their relapse, Ara-C incorporation into the DNA was determined. IL-3 stimulation enhanced Ara-C incorporation in all samples tested. Nevertheless, Ara-C incorporation in the relapsed samples was significantly less than in that in the diagnosis samples of the same patients. A good correlation between Ara-C incorporation and BrdU incorporation was found. The results indicate that HGF stimulation in relapsed/refractory AML enhances Ara-C cytotoxicity, but not to the level that was observed with AML samples at diagnosis.

INTRODUCTION

The overall, long term survival of acute myeloid leukemia (AML) with chemotherapy has only marginally improved in recent years (1-4). In vitro and in vivo data have shown that stimulation of AML cells by human recombinant hematopoietic growth factors (HGF) like granulocyte colony-stimulating factor (G-CSF), interleukin-3 (IL-3), or granulocyte-macrophage colony-stimulating factor (GM-CSF) may stimulate AML cells into cycle (5-10). The recruitment of cycling, previously "dormant" AML cells by HGF stimulation may render these more sensitive to cytostatic drugs. Therefore, HGF may be beneficial in the treatment of AML. Several investigators have reported better cytotoxic effects in vitro

if HGF and cytostatic drugs are combined as compared to drug exposure alone (6,8,11-18). Especially the cytotoxic effects of phase-specific anti-leukemic drugs like cytosine-arabinoside (Ara-C), can be enhanced by HGF stimulation (19-20). From a clinical point of view, the use of HGF may be considered in order to improve the efficacy of chemotherapy in refractory AML. Only few data are available about HGF stimulation in combination with cytostatic drug in AML cells of relapsed or refractory patients (11,13). Also, it is not known for example if HGF alters the cytotoxicity of Ara-C to the same extent in untreated and relapsed/refractory AML cells. Therefore, we studied the modulation of Ara-C cytotoxicity by pre- and co stimulation by G-CSF, IL-3, or GM-CSF in clonogenic AML cells obtained form samples of the same patients at diagnosis and at relapse or refractory stage after chemotherapy treatment. A possible difference of the effects of these HGFs on the cell-cycle status of the AML cells at diagnosis or at relapse was studied by Bromodeoxyuridine (BrdU) incorporation experiments. We have chosen Ara-C as a model drug because it is a phase-specific drug, and it is included in AML treatment regimens.

In order to make a distinction between kinetic and metabolic effects of HGFs on Ara-C, the effect of HGF stimulation on intra-cellular Ara-C metabolism was studied. This was done by determining Ara-C incorporation into the DNA, an important parameter for Ara-C metabolism (21-22).

MATERIALS AND METHODS

Patients and Therapy. Pretreatment AML samples of 6 adult patients and of their relapse or primary refractory AML, classified according to the criteria of the French-American-British (FAB) classification (23), were included in the study. In one AML patient samples obtained at diagnosis, at first and at second relapse respectively, were studied. The clinical data are summarized in Table 1.

AML Sample Preparation. Bone marrow (BM) or peripheral blood (PB) from the patients was obtained after informed consent. BM aspirates were obtained from the posterior iliac crest and collected in tubes containing non-toxic heparinized Hank's balanced salt solution (HBSS). Mononuclear cells from PB or BM were separated by Ficoll-Isopaque (Nycomed, Oslo, Norway) centrifugation. T-lymphocytes were removed by E-rosette Ficoll separation (24). Adherent cells were removed by incubating the cells (1×10^6 nucleated cells/ml) in serum-free medium for 1 hour in plastic culture flasks (Costar, Cambridge, MA) at 37°C, in a fully humidified atmosphere with 5% CO_2 in air. The T-cell and adherence depleted cells were cryopreserved in Iscove's modified Dulbecco's medium (IMDM; Gibco, Paisley, UK) supplemented with 10% dimethyl sulfoxide

Table 1. Clinical Characteristics and Treatment History.

Pt	Age/sex	Presentation	FAB	WBC*	Mat	% Blasts	Previous Treatment (drugs)	Clinical response
1[a]	66/F	Diagnosis	M2	5.3	BM	66	-	CR[†]
1[b]	67/F	1[st] Relapse	M2	3.7	BM	59	Ara-C, DNR	CR
1[c]	67/F	2[nd] Relapse	M2	61	PB	80	Ara-C, DNR, MTX	Refractory
2[a]	56/F	Diagnosis	M2	33	BM	75	-	CR
2[b]	56/F	Relapse	M2	3.4	BM	59	Ara-C, DNR, m-AMSA	Refractory
3[a]	42/M	Diagnosis	M4	90	PB	68	-	Refractory
3[b]	43/M	Refractory	M4	390	BM	97	Ara-C, DNR, m-AMSA, MTX, VP-16, Hydrea	Refractory
4[a]	68/M	Diagnosis	M2	7.7	BM	35	-	CR
4[b]	69/M	Relapse	M2	12	PB	65	Ara-C, DNR	Refractory
5[a]	51/F	Diagnosis	M1/M2	256	BM	82	-	Refractory
5[b]	52/F	Refractory	M2	131	PB	90	Ara-C, DNR, m-AMSA, MTX, VP-16, Hydrea	Refractory
6[a]	60/F	Diagnosis	M4	6.8	BM	33	-	CR
6[b]	61/F	Relapse	M4	8	BM	60	Ara-C, DNR	CR

*WBC ($x10^9$/L).

†CR, Complete remission.

(DMSO; Merck, Darmstadt, FRG) and 20% fetal calf serum (FCS; Gibco) using a temperature controlled freezer (Kryo 10, Planer Biomed, UK) and were subsequently stored in liquid nitrogen.

Cytosine-Arabinoside (Ara-C). Ara-C (Upjohn, Kalamazoo, Michigan) was dissolved in phosphate-buffered saline (PBS) and stored in aliquots at -80°C. ^{3}H-Ara-C (specific activity: 31 Curies/mmol) was obtained from Amersham (Buckinghamshire, UK). Final dilutions were made in culture medium immediately before use.

Hematopoietic Growth Factors. Recombinant human G-CSF was obtained from Amgen (Thousand Oaks, CA) and used at a concentration of 100 ng/ml. Recombinant human IL-3 and GM-CSF were donated by Sandoz (Basle, Switzerland) and both used at a concentration of 10 ng/ml. These concentrations were found to be optimal, as determined by dose-response studies using clonogenic assays on normal, T-cell and adherent cell depleted bone marrow cells.

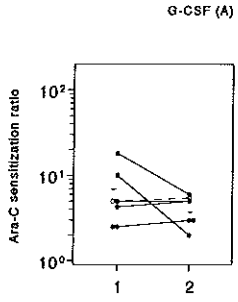
Cell Culture Methods. Purified AML cells were cultured in serum-free medium as described (25). The suspension cultures consisted of IMDM supplemented with 15 mg/ml dialysed bovine serum albumin (BSA; Cohn fraction V, Sigma, St Louis), 2.8 μg/ml linoleic acid (Sigma), 7.8 μg/ml cholesterol (Sigma), 1 μg/ml insulin (Sigma), 7.7 μM iron saturated human transferrin (Behring Institute, Marburg, FRG), 0.1 mM 2-mercaptoethanol (Merck, Darmstadt, FRG), and 60 μg/ml gentamycin (Gibco). The cell suspensions were plated in 24-wells cell culture plates (Costar) at a concentration of 0.5 x 10^5 cells/ml, in the presence or absence of G-CSF, IL-3 or GM-CSF and incubated for 24 hours at 37°C and 5% CO_2 in a fully humidified atmosphere. Subsequently, the appropriate doses of Ara-C (final concentrations: 0, 0.01, 0.1, 1 μM) were added to the cultures. After 24 hours, the cells were carefully resuspended and a fixed volume (0.2 ml) was taken from each sample. The cells were washed three times in HBSS and each sample was subsequently suspended in 1 ml IMDM containing 0.9 % methylcellulose (Fluka, Switzerland) supplemented with the same additives as used in the suspension cultures. This mixture was plated in triplicate in 24 wells-plates (Costar), 0.25 ml/well. Cells exposed to a growth factor in suspension were cultured in the presence of the same growth factor in the clonogenic assays. Cells that were not incubated with a growth factor in suspension were cultured in the methylcellulose cultures in the presence of G-CSF, IL-3 or GM-CSF. Identical volume fractions of each suspension culture were taken to be plated in colony cultures. Thus, before plating, cell numbers were not adjusted for cell death or cell growth, in order to make results directly comparable. After 7 to 9 days of incubation (37°C, 5% CO_2), aggregates containing 10 cells or more were scored using an inverted microscope. Pooled colonies were evaluated for morphology in order to verify the number

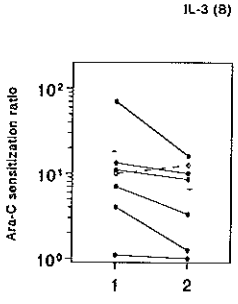
of blast cells in the colonies. From the results, IC_{50} values defined as the concentration of the cytostatic drug that resulted in 50% inhibition of colony formation, were derived. Ara-C sensitization ratios were calculated by the equotion: IC_{50} without HGF stimulation/IC_{50} for Ara-C with HGF stimulation.

BrdU Incorporation. To evaluate the effect of HGF stimulation on the proliferation status of cells, bromodeoxyuridine (BrdU) was added in the first or second 24 hours of incubation with AML cells (0.5 x 10^6/ml) under serum-free conditions in the presence or absence of G-CSF, IL-3 or GM-CSF at 37°C and 5% CO_2 in air. Of each patient, one sample was cultured for 24 hours in the presence of 10 μM BrdU (Amersham, UK). Next, these cells were washed and cytospin slides were prepared. After a preincubation of 24 hours with or without HGFs, BrdU was added to the other sample of the cell suspensions for another 24 hours. Following the BrdU incubation, cells were washed and cytospin slides were prepared. After air-drying the slides overnight, they were fixed in methanol at 4°C for 30 minutes. After rinsing in PBS, the slides were subsequently exposed to 15 μl anti-BrdU and nuclease (Amersham) for 1 hour at 20°C. The binding of anti-BrdU was visualised by use of a fluorescein isothiocyanate (FITC) conjugated rabbit anti-mouse immunoglobulin antiserum (DAKOPATTS, Denmark). The fluorescence staining was evaluated with a Zeiss microscope equipped with phase contrast facilities (Carl Zeiss, Oberkochen, FRG). One hundred cells were counted in duplicate for each determination.

Ara-C incorporation. The effects of HGF stimulation on Ara-C incorporation into the DNA was determined by culturing AML cells in the presence or absence of G-CSF, IL-3 or GM-CSF under serum-free conditions as described above. After 24 hours of HGF incubation, ^{3}H-Ara-C was added to the cells at various concentrations (0.01, 0.1, and 1 μM). After another 24 hours, the cells were harvested on nitrocellulose filters using a cell harvester and radioactivity was determined.

Statistical analysis. Student's *t* test for paired samples was used to compare the effects of HGF stimulation on Ara-C cytotoxicity. The Mann-Whitney *U* test for two tailed testing was used to compare the results of the experiments using newly diagnosed and relapsed/refractory AML samples.





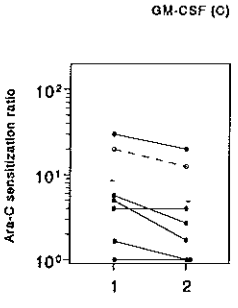
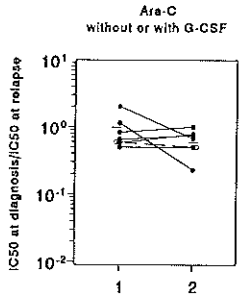
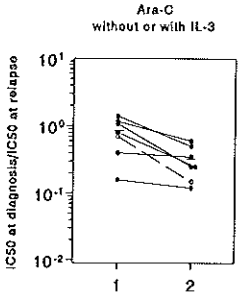


Fig. 1: Ara-C sensitization ratios using G-CSF (A), IL-3 (B) or GM-CSF (C) for AML at diagnosis (1) and for relapsed/refractory AML (2). Ara-C sensitization ratios were defined as the ratio of IC_{50} values for drug exposure alone and the IC_{50} values for drug and HGF exposure. The dotted line represents the Ara-C sensitization ratios for the first and second relapse of AML no. 1. (-) Mean value.





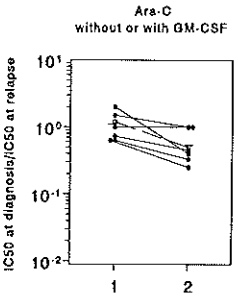


Fig. 2: Ara-C IC_{50} ratios for leukemic cells at diagnosis and at relapse or refractory AML cells of the same patients without (1) or with (2) HGF pre- and costimulation. The dotted line represents the ratios for the first and second relapse of AML no. 1. (-) Mean value.

Table 2. IC_{50} values of Ara-C (μM) with or without pre- and costimulation with G-CSF, IL-3 or GM-CSF in AML samples at diagnosis and in relapsed/refractory AML samples.

		AML at diagnosis		Relapse/refractory AML	
(Pre-) incubation with HGF		no	yes	no	yes
G-CSF	Mean	0.125	0.015	0.182	0.04
	Median	0.055	0.012	0.10	0.02
	Range	0.025 - 0.40	0.01 - 0.04	0.03 - 0.35	0.01 - 0.18
	p	0.036		0.03	
IL-3	Mean	0.245	0.067	0.612	0.398
	Median	0.13	0.01	0.650	0.04
	Range	0.040 - 0.70	0.01 - 0.35	0.05 - 1.00	0.01 - 1.00
	p	0.036		0.036	
GM-CSF	Mean	0.152	0.052	0.170	0.101
	Median	0.075	0.01	0.170	0.02
	Range	0.012 - 0.40	0.01 - 0.25	0.012 - 0.55	0.01 - 0.55
	p	0.036		0.04	

Values represent the IC_{50} values for Ara-C (μM) calculated from the results obtained from clonogenic assays.

RESULTS

Clonogenic Assays. The IC_{50} values of Ara-C with or without in clonogenic AML cells obtained at presentation or in relapsed/primary refractory AML, are summarized in Table 2. Evaluation of the HGF effect was performed by calculation of the Ara-C sensitization ratios. The results show significantly enhanced Ara-C cytotoxicity in untreated AML using G-CSF ($p=0.036$), IL-3 ($p=0.036$) or GM-CSF ($p=0.036$) pre- and costimulation. In relapsed/refractory samples, Ara-C cytotoxicity was also significantly enhanced by HGF stimulation with G-CSF ($p=0.03$), IL-3 ($p=0.036$), and GM-CSF ($p=0.04$). Comparison of the effects of HGF pre- and costimulation on Ara-C cytotoxicity on AML cells at presentation in the same patients and at relapse/refractory disease showed that the mean Ara-C sensitization ratios decreased from 6.7 at diagnosis to 3.8 at relapse with G-CSF, from 17 to 7.5 with IL-3 and from 11.2 to 5.3 with GM-CSF respectively ($p>0.05$) (figure 1).

In six patients, who were analyzed sequentially at diagnosis and at relapse, a ratio of Ara-C IC_{50} values at diagnosis AML versus relapsed/refractory AML was calculated. The

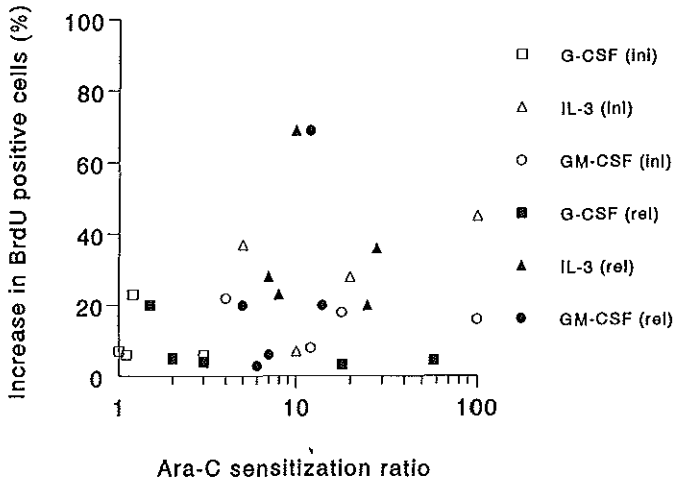


Fig. 3: Increase in BrdU-positive cells (%), during the second 24 hrs of HGF stimulation as compared to unstimulated cells versus Ara-C sensitivity ratios.

mean IC_{50} ratios in AML samples pretreated with Ara-C alone but subsequently plated in the presence of a HGF, were 0.89 with G-CSF, 0.82 in cells cultured with IL-3, and 1.1 in AML cells cultured with GM-CSF. The same ratios were calculated in HGF stimulated cells significantly decreased in case of IL-3 (mean: 0.32, $p=0.02$) or GM-CSF (mean: 0.56, $p=0.036$) pre- and costimulation, but not with G-CSF (mean: 0.68, $p>0.05$). In 3 out of 7 AML samples using IL-3 or G-CSF, and 4 out 7 for GM-CSF, HGF stimulation did not enhance Ara-C cytotoxicity to the level that could be reached with the AML cells obtained at presentation. The ratios of IC_{50} values with HGF pre- and costimulation obtained in AML at diagnosis and at relapse are shown in Figure 2.

BrdU Incorporation. In 4 AML samples at initial diagnosis and relapsed/refractory AML, the percentage of BrdU-positive cells during the first or second 24 hours of HGF stimulation was determined. During the first 24 hours of HGF incubation, no significant increase of BrdU positivity was observed as compared with non-HGF incubated cells (data not shown). The mean percentages of BrdU-positive cells during the next 24 hours of the culture was 21% (range: 9-90%) for unstimulated cells. In the presence of HGF

stimulation, the values increased to 38% (range: 14-100%) for G-CSF, 53% (range: 19-100%) for IL-3, and 45% (range: 13-100%) for GM-CSF. Although all HGF increased the percentage of BrdU-labeled cells above the level of unstimulated samples during the second 24 hours, this increase of BrdU-positive cells did not correlate with the enhanced cytotoxicity. No differences of HGF stimulation on BrdU incorporation between newly diagnosed or relapsed/refractory AML were found. The results of the BrdU incorporation study are shown in Figure 3.

Ara-C incorporation. In AML cells obtained from three patients at presentation and at relapse, the effects of HGF stimulation on Ara-C incorporation were determined. In three patients tested, a marked difference of spontaneous incorporation in AML cells obtained at presentation and at relapse. Using IL-3 pre- and costimulation, a twofold increase in Ara-C incorporation was observed. With G-CSF or GM-CSF, only a moderate increase of Ara-C incorporation was found, and Ara-C incorporation was not enhanced to the level that was observed in untreated AML samples. In the tested AML samples, BrdU incorporation did not significantly differ at presentation or relapse. These data are summarized in figure 4a (newly diagnosed AML) and in figure 4b (relapsed AML).

Ara-C cytotoxicity, evaluated in a clonogenic assay, showed for IL-3 pre- and costimulation enhanced Ara-C cytotoxicity in all samples that were used for the Ara-C incorporation experiments. Using G-CSF, enhanced cytotoxicity was found in 2 out of 3 newly diagnosed samples, and in 1 of 3 relapse sample. With GM-CSF, this was found in 1 of 3 newly diagnosed samples, and in 1 of 3 relapse samples.

DISCUSSION

In vitro studies reporting about the effects of HGF (pre-) stimulation on the effect of chemotherapy in relapse or refractory AML are scarce (11,13). Also, no data are available about the effects of HGF stimulation on the possibly developed resistance for Ara-C resistance after chemotherapy. Therefore, we investigated the role of HGF pre- and costimulation on Ara-C cytotoxicity in clonogenic blasts from the same patients at diagnosis and at relapse or primary refractory stage of the disease.

The results of the clonogenic assays show that, in control cultures without Ara-C exposure, IL-3 induced more colonies than G-CSF and GM-CSF (data not shown). Since sub-optimal stimulation of the AML cells may possibly interfere with the effectivity of a cycle-specific drug like Ara-C, this might explain the differences in IC_{50} values for the three HGFs used. We observed enhanced Ara-C cytotoxicity using prestimulation with G-CSF, IL-3 as well as with GM-CSF in newly diagnosed AML and relapsed or refractory AML. Although the effects of HGF stimulation on Ara-C cytotoxicity, expressed as Ara-C sensitivity ratios, are less evident in relapsed or refractory AML, the difference with

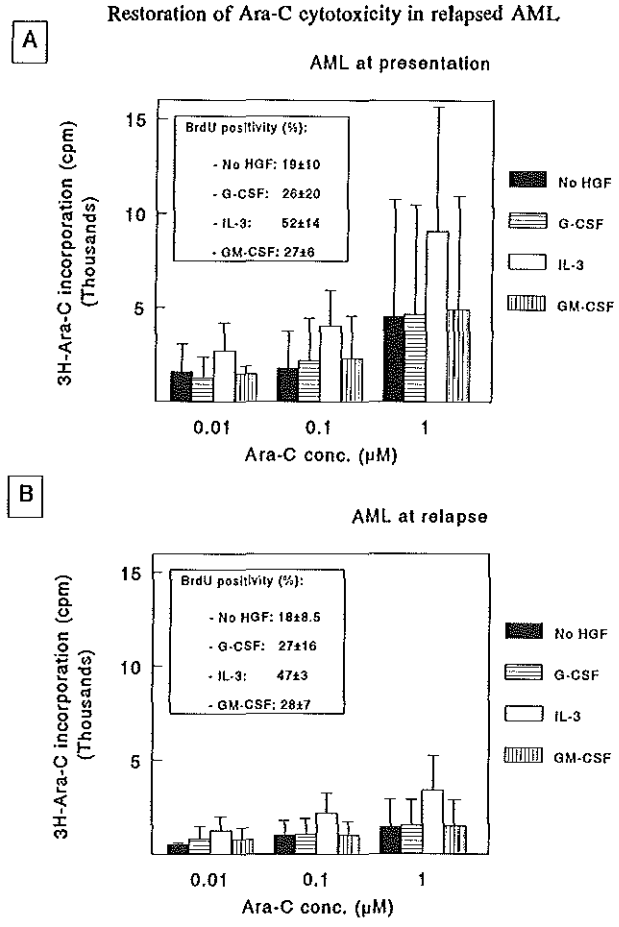


Fig.4: Mean ^{3}H-Ara-C incorporation (±SD) in 3 AML samples at diagnosis (A) and at relapse (B). AML cells were cultured in the presence or absence of HGFs for 24 hrs, and subsequently cultured with various Ara-C concentrations for another 24 hrs. The mean percentages of BrdU-positive cells during the second 24 hrs of culture as determined in another experiment are also shown.

newly diagnosed AML samples is not significant. As a consequence, sensitization by HGF stimulation is not altered in relapsed/refractory AML cells as compared with newly diagnosed AML samples. IC_{50} values of Ara-C combined with IL-3 or GM-CSF in untreated AML were significantly lower than in relapsed or refractory AML samples of the same patients. As a result, HGF stimulation does not enhance Ara-C cytotoxicity to the level that can be obtained by HGF stimulation of newly diagnosed AML cells. This indicates that relapsed AML clones have developed a relative resistance to Ara-C that can not be fully restored by HGF stimulation. The Ara-C resistance is not caused by kinetic resistance, t.i. a decreased proliferative response to HGF stimulation, since the percentage

of BrdU-positive cells after HGF exposure in AML at diagnosis does not significantly differ from those in relapsed or primary refractory AML. Also, the Ara-C incorporation experiments have shown a decrease in incorporation in relapsed AML samples indicating that the AML clones have developed resistance to Ara-C that could not be overcome by HGF stimulation. These observations can possibly be explained by the assumption that pharmacological resistance to this drug, e.g. caused by decreased intracellular levels of deoxycytidine kinase (dCk) is involved. This enzyme catalyses the first- and rate-limiting step in the phosphorylation of Ara-C to it's metabolically active form Ara-CTP, and decreased levels are associated with Ara-C resistance. Determination of intracellular dCk levels should be done in order to study this phenomena. Other possible causes of pharmacological Ara-C resistance are rapid deamination of Ara-C to Ara-U, increased intracellular levels of dCTP that compete with Ara-CTP for incorporation into the DNA, altered DNA polymerase activity and/or enhanced DNA repair mechanisms (26-28).

Recent reports have shown that HGF exposure in the presence of Ara-C may enhance phosphorylation of Ara-C to Ara-CTP (29-30). In addition, Ara-C incorporation into the DNA was shown to be enhanced by HGF stimulation, unrelated to the number of cells in S-phase (29). In our small series in which Ara-C incorporation was studied, a twofold increase in the mean Ara-C incorporation using IL-3 stimulation is found, but not with G-CSF and GM-CSF. In these samples BrdU positivity also shows a twofold increase when IL-3 is used. These results suggest that enhanced Ara-C metabolism by HGF is clearly associated with cell-cycle activation. The reason why others did not find a correlation between Ara-C and BrdU incorporation, can be found in a different approach to determine recruitment. In our study, we have analyzed the total percentage of BrdU-positive cells during HGF stimulation by adding BrdU for 24 hours to the cell cultures. In this way, BrdU positivity is determined during the same period of time as the co-cultured AML cells are exposed to Ara-C. Other studies only looked at BrdU positivity at various short time intervals.

Recently, Löwenberg *et al* reported that a high level of autonomous growth in AML is associated with highly aggressive leukemia (31). Since we have used relapsed or primary resistant AML samples, this can possibly explain the high percentages of BrdU-positive cells scored even when no HGF was added to the cultures. The lack of correlation of BrdU incorporation with the enhancement of Ara-C cytotoxicity has also been reported by other investigators (11,29,32-33). Since BrdU incorporation experiments are studied in the total AML cell population and cytotoxicity is determined in clonogenic AML cells, this can explain the lack of correlation of BrdU positivity and cytotoxicity.

Finally, we conclude that this study demonstrates that HGF pre- and costimulation is able to enhance Ara-C cytotoxicity in vitro. Nevertheless, HGF stimulation does not seem to be able in enhancing Ara-C cytotoxicity to the extent that pharmacological resistance for Ara-C can be overcome in vitro.

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CHAPTER 6

ENHANCED CHEMOSENSITIVITY IN ACUTE MYELOID LEUKEMIA BY HEMATOPOIETIC GROWTH FACTORS: A COMPARISON OF THE MTT ASSAY WITH A CLONOGENIC ASSAY.

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SUMMARY

The MTT assay, a colorimetric assay, is found to be suitable for chemosensitivity testing. Recently, it has been suggested that hematopoietic growth factors (HGF) may enhance the effects of cytostatic drugs in acute myeloid leukemia (AML). Therefore, we studied the effects of Granulocyte Colony-Stimulating Factor (G-CSF), Interleukin 3 (IL-3) and Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) combined with cytosine-arabinoside (Ara-C), daunorubicin (DNR), mitoxantrone (MXT) or etoposide (VP-16) by using the MTT assay. The results were compared with in vitro clonogenic assays. Briefly, AML cells of nine patients were incubated in the presence or absence of G-CSF, IL-3 or GM-CSF under serum-free conditions for 24 hours. Next, for the MTT assay, Ara-C (final dilution range: 0.0024-240 μg/ml), DNR (final dilution range: 0.5-3.2 μg/ml), MXT (final dilution range: 0.5-3.2 μg/ml) or VP-16 (final dilution range: 0.1-100 μg/ml) were added and incubated for 48 hours. Cell survival was determined and IC_{75} values (75% reduction as compared to control cultures) were calculated. For clonogenic assays, the the three lowest drug concentrations were used. After 48 hours, the clonogenic response was determined in serum-free, semi-solid cultures with G-CSF, IL-3 or GM-CSF. The results obtained by the MTT assay showed no significant enhancement of cytotoxicity by HGF on cytostatic drug preincubated cells as compared to cytostatic drugs alone. The results obtained by the clonogenic assays showed increased cytotoxicity of Ara-C combined with G-CSF, IL-3 or GM-CSF. The median IC_{75} values of Ara-C decreased from 0.056 to 0.0168 μg/ml with G-CSF ($p=0.01$), from 0.108 to 0.0168 μg/ml with IL-3 ($p=0.004$) and from 0.12 to 0.0204 μg/ml for GM-CSF ($p=0.02$). Only moderate enhanced cytotoxicity was observed when VP-16 was combined with IL-3 ($p=0.036$) or GM-CSF ($p=0.036$), but not with G-CSF. No enhanced cytotoxicity of DNR and MXT to clonogenic AML cells was found when these agents were combined with HGF stimulation. The results indicate that the MTT assay underestimates HGF enhanced cytotoxicity of Ara-C or VP-16 to clonogenic cells. Therefore, the assay is not useful for accurately detecting differences of clonogenic response due to the proliferative status of cells. In this paper, the potential explanations for the failure of the MTT assay are discussed.

INTRODUCTION

The MTT assay, originally described by Mosmann (1) in 1983, is a colorimetric assay based on the ability of living cells to reduce the tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) to a formazan product. This assay has been used for in vitro drug sensitivity testing in several hematological malignancies including acute and chronic myeloid leukemia (2-4) and acute lymphoblastic and chronic lymphocytic leukemia (5-7). The cytotoxic effects of cytostatic drugs in acute myeloid

leukemia (AML) may be enhanced by hemopoietic growth factors (HGFs). This phenomenon has been studied in vitro using clonogenic assays (8-18) and other proliferation assays like ^{3}H-thymidine uptake (19) or the determination of cell viability (17,20). However, it is unclear whether screening by the MTT assay can be utilized to detect an enhancement of cytotoxicity by HGFs. Therefore, we evaluated the MTT assay for cell kill of AML cells in culture in the presence of recombinant human Granulocyte-Colony Stimulating Factor (G-CSF), recombinant human Interleukin-3 (IL-3) or recombinant human Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) combined with cytostatic drugs. Effective antileukemic drugs like cytosine-arabinoside (Ara-C), daunorubicin (DNR), mitoxantrone (MXT) and etoposide (VP-16) were added to AML cell culture with or without HGF pre- and costimulation. The results were compared with those of a clonogenic assay.

Ara-C is not only effective in reducing terminal divisions of AML blast cells, as can be determined by primary colony formation (PE_1), but is also capable in suppressing the self-renewal capacity of clonogenic AML cells (18,21). The additional aim of this study was to evaluate the effect of HGF and Ara-C preincubation on the self-renewal capacity of clonogenic AML cells. The self-renewal capacity was determined by secondary plating efficiencies (PE_2) in methylcellulose cultures.

Since the MTT assay does not distinguish between cell death of proliferating (clonogenic) versus non-cycling cells, we compared the results with those of primary and secondary plating efficiency of clonogenic cells.

MATERIALS AND METHODS

Preparation of AML cell suspensions. Bone marrow or peripheral blood from 9 AML patients was obtained after informed consent. The AML cases were classified according the criteria of the French-American-British (FAB) committee (22). The clinical characteristics are shown in Table 1. Bone marrow aspirates were obtained from the posterior iliac crest and collected in tubes containing non-toxic heparinized Hank's balanced salt solution (HBSS). Mononuclear cells from peripheral blood or bone marrow were separated by Ficoll-Isopaque (Nycomed, Oslo, Norway) centrifugation. T-lymphocytes were removed by E-rosette Ficoll separation (23). Adherent cells were removed by incubating the cells (1×10^6 nucleated cells/ml) in serum-free (SF) medium for 1 hour in plastic culture flasks (Costar, Cambridge, MA) at 37°C, in a fully humidified atmosphere with 5% CO_2 in air.

The T-cell and adherence depleted cells were cryopreserved in Iscove's modified Dulbecco's medium (IMDM; Gibco, Paisley, UK) supplemented with 10% dimethyl sulfoxide (DMSO; Merck, Darmstadt, FRG) and 20% fetal calf serum (FCS; Gibco) using a temperature controlled freezer (Kryo 10, Planer Biomed, UK) and were subsequently

stored in liquid nitrogen.

Drugs. Ara-C (Upjophn, Kalamazoo, Michigan), Daunorubicin (DNR; Rhone-Poulenc Pharma, France) and Mitoxantrone (MXT; Cyanamid, Mont-Saint-Guibert, Belgium) were dissolved in sterile distilled water to a concentration of 1 mg/ml and stored as aliquots at -80°C. Etoposide (VP-16; Bristol laboratories, Syracuse, New York) was stored as a stock solution of 5 mg/ml at -80°C. Dilutions were made in phosphate buffered saline (PBS) immediately before use.

Hematopoietic Growth Factors. Recombinant human G-CSF was obtained from Amgen (Thousand Oaks, CA) and used at a concentration of 100 ng/ml. Recombinant human IL-3 and GM-CSF were kindly provided by Sandoz (Basle, Switzerland) and both used at concentrations of 10 ng/ml. These concentrations were found to be optimal, as determined by dose-response studies using clonogenic assays on normal, T-cell and adherence depleted bone marrow cells.

Cell Culture Conditions. Purified AML cells were cultured in serum-free (SF) medium as described (24). The suspension cultures consisted of IMDM supplemented with 15 mg/ml dialysed bovine serum albumin (BSA; Cohn fraction V, Sigma, St Louis), 2.8 μg/ml linoleic acid (Sigma), 7.8 μg/ml cholesterol (Sigma), 1 μg/ml insulin (Sigma), 7.7 μM iron saturated human transferrin (Behring Institute, Marburg, FRG), 0.1 mM 2-mercaptoethanol (Merck, Darmstadt, FRG) and 60 μg/ml gentamycin (Gibco). The semi-solid cell cultures consisted of 0.9% methylcellulose (Fluka, Switzerland) supplemented with the same additives as described above.

A period of 24 hours HGF stimulation before cytostatic incubation was chosen because this period of HGF stimulation increases the number of AML cells in S-phase (25), thereby increasing the susceptibility to S-phase specific drugs.

BrdU Incorporation. To evaluate the proliferative status of the AML cells during the first or second 24 hours of HGF incubation, AML cells (0.5 x 10^6/ml) of 5 AML patients were cultured in SF medium in the presence or absence of G-CSF, IL-3 or GM-CSF at 37°C and 5% CO_2 in air. Of each patient, one sample was cultured for 24 hours in the presence of 10 μM Bromo-deoxyuridine/Fluoro-deoxyuridine (BrdU/FdU)(Amersham, UK). After 24 hours, the cells were washed and cytospin slides were prepared. After an incubation for 24 hours with or without HGFs, BrdU/FdU was added to the other sample of the cell suspensions. Following the BrdU/FdU incubation for 24 hours, the cells were washed and cytospin slides were prepared. After air-drying the slides overnight, they were fixed in methanol at 4°C for 30 minutes. After rinsing in PBS, the slides were subsequently exposed to 15 μl anti-BrdU and nuclease (Amersham) for one hour at 20°C. The reactivity of anti-BrdU was visualized following labelling with fluorescein

isothiocyanate (FITC) conjugated rabbit anti-mouse immunoglobulin antiserum (Dakopatts, Denmark). The fluorescence staining was evaluated with a Zeiss microscope equipped with phase contrast facilities (Carl Zeiss, Oberkochen, FRG). One hundred cells were counted for each determination, and each sample was scored in duplicate.

MTT Assay. The direct in vitro cytotoxicity of the cytostatic drugs Ara-C, DNR, MXT or VP-16 in absence or presence of G-CSF, IL-3 or GM-CSF on AML cells, was determined by preparing cell suspensions containing 0.5 x 10^6 cells per ml in SF-medium with or without an additional HGF. Next, 200 μl aliquots of the suspension were dispensed into 96-well flat-bottomed microtiter plates (Costar). After 24 hours incubation, the appropriate doses of Ara-C (final concentration: 0.0024, 0.024, 0.24, 2.4, 24, 240 μg/ml), DNR (final conc.: 0.05, 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 μg/ml), MXT (final conc.: 0.05, 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 ng/ml), VP-16 (final conc.: 0.1, 0.5, 1, 5, 10, 50 and 100 μg/ml) or solvent control were added to the wells in a volume of 10 μl. In these experiments 3 replicate wells were used at each point. The plates were incubated for 48 hours. Then, 30 μl of a 5 mg/ml solution of MTT (Sigma) was added to each well and the plates were incubated for another 5 hours. Next the plates were centrifuged for 10 minutes at 500 g. After removing the supernatants, the formazan crystals were dissolved with 100 μl DMSO. The optical density (OD) of the wells was measured with a Titertek multiscan plate reader (Flow Laboratories, Finland) at 540 nm. The percentage of leukemic cell survival was defined as:

(mean OD treated wells/OD untreated control wells) x 100%.

The IC_{75}, defined as the drug concentration that results in 25% cell survival, was derived by calculating the point where the dose-response curve crosses the 25% cell survival limit.

Table 1. Clinical characteristics.

Pt	Age/sex	Presentation	FAB	WBC*	Mat	% Blasts	% Blasts used in experiments†
1	60/M	Diagnosis	M1	252	PB	97	97
2	57/M	Relapse	M1	43	BM	87	93
3	34/F	Diagnosis	M1/M2	83	PB	80	95
4	67/M	Diagnosis	M2	18	BM	31	77
5	68/M	Diagnosis	M1	33	PB	78	88
6	45/M	Diagnosis	M2	8	BM	75	95
7	67/F	Relapse	M2	61	PB	80	98
8	51/M	Diagnosis	M4	14	BM	94	94
9	51/F	Diagnosis	M1/M2	256	BM	82	98

*WBC, (x10^9/L).

†Blast percentages after T-cell -and adherence depletion.

Clonogenic Assays. The cell suspensions were plated in 24-wells cell culture plates (Costar) at a concentration of 0.5 x 10^6 cells/ml, in the presence or absence of G-CSF, IL-3 or GM-CSF and incubated for 24 hours at 37°C and 5% CO_2 in a fully humidified atmosphere. Subsequently, the appropriate doses of Ara-C (final concentrations: 0.0024, 0.024 and 0.24 μg/ml), DNR (final conc.: 0.05, 0.1 and 0.2 μg/ml), MXT (final conc.: 0.05, 0.1 and 0.2 μg/ml), VP-16 (final conc.: 0.1, 0.5, and 1.0 μg/ml) or solvent control were added to the cultures. After 48 hours, the cells were carefully resuspended and a fixed volume (0.2 ml) was taken of each sample. The cells were washed three times in HBSS and each sample was subsequently suspended in 1 ml semi-solid medium. This mixture was plated in triplicate in 24 wells-plates (Costar), 0.25 ml/well. Cells exposed to a growth factor in suspension were cultured in the presence of the same growth factor in the clonogenic assays. Cells that were not incubated with a growth factor in suspension were plated in methylcellulose cultures with either G-CSF, IL-3 or GM-CSF. Identical volume fractions of each of the suspension culture were taken to be plated in colony cultures. Thus, before plating, cell numbers were not adjusted for cell death or cell growth, in order to make results directly comparable. After 7 to 9 days of incubation (37°C, 5% CO_2), aggregates containing 10 cells or more were scored using an inverted microscope. The results were used to calculate IC_{75} values, i.e. the drug concentration resulting in a 75% reduction of colony formation as compared with control cultures.

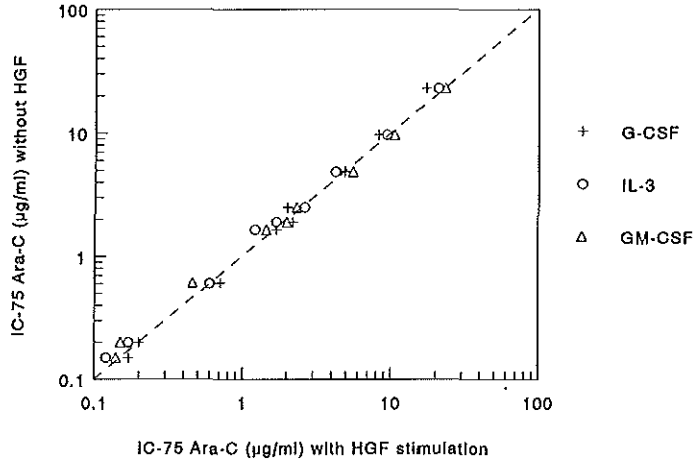

Fig. 1: Relationship between Ara-C cytotoxicity (IC_{75}) with or without G-CSF, IL-3 or GM-CSF stimulation values as determined by the MTT assay in 9 AML samples. The dotted line represents the function of equivalent IC_{75} values of Ara-C with or without HGF stimulation.

Secondary plating efficiencies (PE_2) of Ara-C exposed AML cells were determined in three AML cases by pooling colonies and subsequent plating of 0.25 x 10^5 viable cells per well in the presence of the same HGF as was present in clonogenic assays from which the colonies were derived. Cultures were performed in triplicate wells under the same conditions as described above. Pooled colonies were evaluated for morphology in order to verify that the cells in the colonies were blasts.

Statistical Analysis. For a comparison between the IC_{75} values of the AML treatment groups, the Mann-Whitney *U* test for two tailed testing was used.

RESULTS

AML cells. The mean percentage of blast infiltration in the marrow the AML cases was 78%, the median value 80% (range: 31-97%). The blast percentages used in the experiments following depletion of adherent cells and E-rosetting T-cells ranged from 77%-98% (median: 95%) (Table 1).

BrdU incorporation. The BrdU incorporation in absence or presence of G-CSF, IL-3 or GM-CSF during the first or second 24 hours of incubation was determined using 5 AML samples. When no growth factor was present in the suspension culture, an increase of the median value from 13% to 23% of BrdU-labelled nucleated cells was observed. Using G-CSF, IL-3 or GM-CSF, the absolute increase of median BrdU incorporation was 4.6, 4.3 and 3.4-fold respectively. The results are summarized in Table 2.

MTT assay. The mean optical density (OD) values of the chemotherapy untreated control wells of the nine AMLs varied from 0.765 ± 0.25 when no HGF was added, to 0.770 ± 0.22, 0.834 ± 0.282 and 0.783 ± 0.243 when the cells were cultured in the presence of G-CSF, IL-3 or GM-CSF respectively. The results of the MTT assay using Ara-C with or without HGF stimulation are shown in Fig. 1. If the cells were preincubated with Ara-C without HGF, the IC_{75} values varied from 0.17 - 23 μg/ml Ara-C. Pre- and costimulation with G-CSF, IL-3 or GM-CSF did not significantly enhance Ara-C cytotoxicity. The IC_{75} values of DNR without HGF stimulation varied from 0.125 to 1.5 μg/ml. Incubating the cells with G-CSF, IL-3 or GM-CSF did not significantly alter the IC_{75} values of DNR.

The IC_{75} values of MXT without HGF addition varied from 0.05 to 1.6 μg/ml. No significant enhancement of cytotoxicity by HGF was apparent. Using VP-16, the IC_{75} values of VP-16 varied from 56 to 100 μg/ml. HGF pre- and costimulation did not alter VP-16 cytotoxicity measurably. The IC_{75} values of DNR, MXT and VP-16 are summarized in Table 3.

Table 2. % BrdU-positive AML cells following stimulation with G-CSF, IL-3 or GM-CSF.

Incubation time (hrs)	0-24 Hours				24-48 Hours			
	no HGF	G-CSF	IL-3	GM-CSF	no HGF	G-CSF	IL-3	GM-CSF
Mean±SD	26±32	29±39	32.2±35	34.2±36	34.4±32	51±31	64±22	57±28
Median	13	10	15	16	23	46	64	54

Results of BrdU incorporation in leukemic cells of 5 AML cases in the presence or absence of HGF. BrdU was present in the cell suspensions for 24 hrs. The percentage of BrdU-positive cells of each AML case was obtained by counting 100 cells in duplicate slides of each sample.

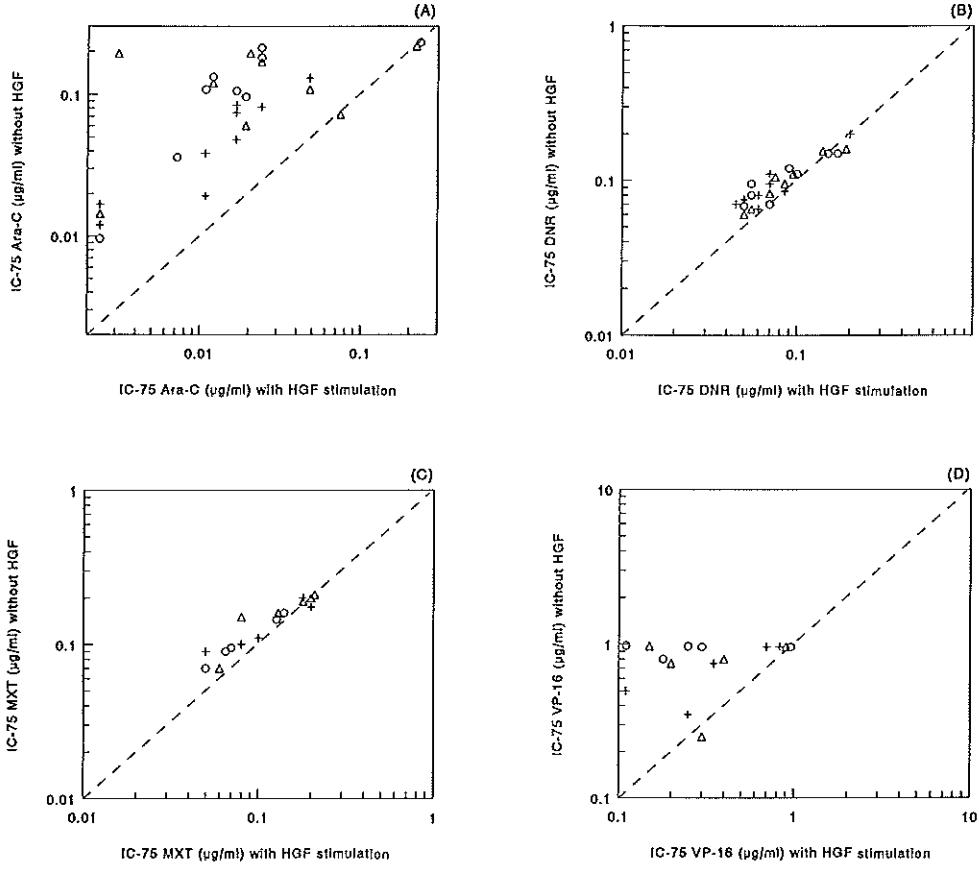

Fig. 2: Relationship between of Ara-C (A), DNR (B), MTX (C) or VP-16 (D) cytotoxicity (IC_{75}) to clonogenic AML cells with or without G-CSF (+), IL-3 (o) or GM-CSF (Δ) stimulation. Using DNR, in two AML samples, using MTX and VP-16, in one AML sample the IC_{75} values could not be calculated. The dotted line represents the function of equivalent IC_{75} values.

Clonogenic assays. The effects of HGF stimulation combined with Ara-C and DNR on the colony formation of AML cells were studied in 9 AML patients and with MTX and VP-16 in six AML patients.

Ara-C cytotoxicity with G-CSF, IL-3 as well as GM-CSF was enhanced as compared with Ara-C alone (Fig. 2A). The median IC_{75} value for Ara-C without HGF preincubation was 0.056 μg/ml (range: 0.012-0.13 μg/ml) for G-CSF, 0.108 μg/ml (range:0.0096-0.234 μg/ml) for IL-3, and 0.12 μg/ml (range:0.0144-0.2224 μg/ml) for GM-CSF. Using Ara-C with HGF preincubation, these values decreased to 0.0168 μg/ml (range: 0.0024-0.048 μg/ml) for G-CSF (p=0.01), 0.0168 μg/ml (range: 0.0024-0.234 μg/ml) for IL-3 (p=0.004), and to 0.0204 μg/ml (range: 0.0024-0.2244 μg/ml) for GM-CSF (p=0.02).

Preincubation of AML cells with HGF did not significantly enhance DNR or MTX cytotoxicity (p>0.05) (Fig. 2B and 2C).

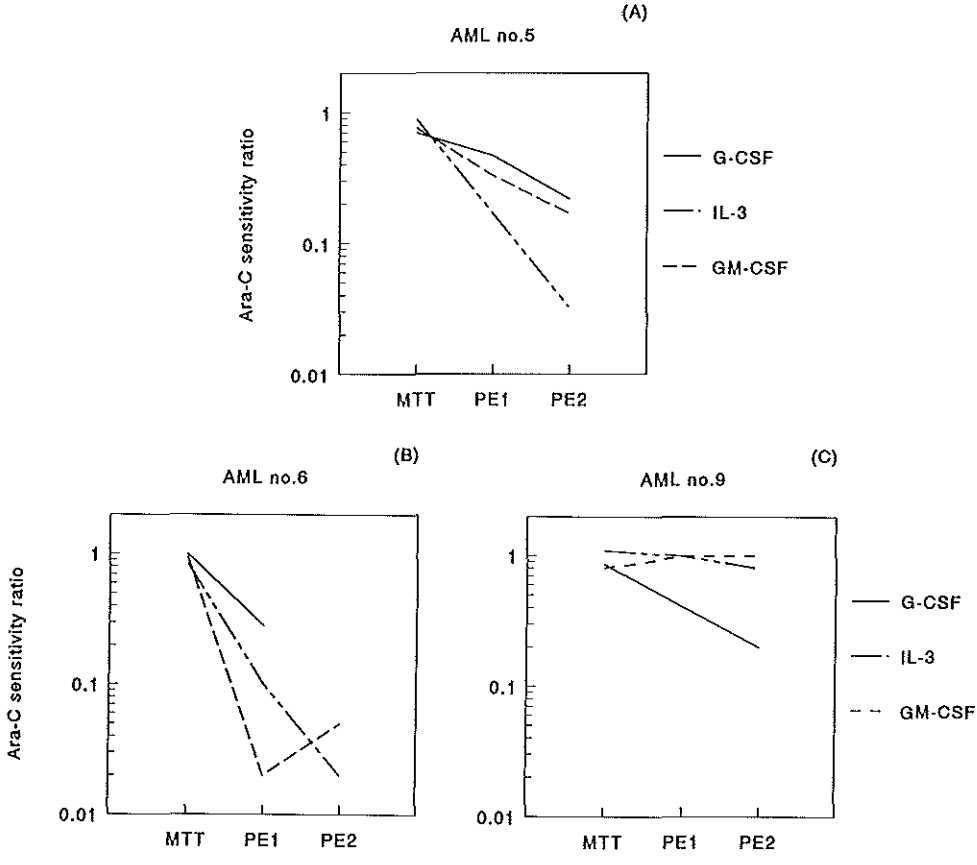

Fig. 3: The effect of HGF stimulation on the Ara-C sensitivity ratios in three AML samples as determined by the MTT assay and clonogenic assays (PE_1 and PE_2).

Table 3. IC_{75} values of daunorubicin, mitoxantrone and VP-16 in the presence or absence of G-CSF, IL-3 or GM-CSF.

Drug		No HGF	G-CSF	IL-3	GM-CSF
Daunorubicin (μg/ml)	Mean	0.66	0.67	0.7	0.67
	Median	0.62	0.65	0.65	0.61
	Range	0.125-1.5	0.15-1.6	0.14-1.7	0.16-1.5
Mitoxantrone (μg/ml)	Mean	0.74	0.81	0.81	0.84
	Median	0.78	0.78	0.84	0.8
	Range	0.05-1.6	0.065-2.0	0.06-1.8	0.07-1.9
VP-16 (μg/ml)	Mean	80	82	83	86
	Median	80	84	82	90
	Range	56-100	58-100	64-100	62-100

IC_{75} values in 9 AML samples as determined by the MTT assay using DNR, MXT or VP-16.

IC_{75} values of VP-16 with no prestimulation varied from 0.35 to >1.0 μg/ml for G-CSF (median: 0.88 μg/ml), from 0.8 to >1.0 μg/ml for IL-3 (median: 1.0 μg/ml) and from 0.35 to >1.0 μg/ml for GM-CSF (0.9 μg/ml). When clonogenic AML cells were HGF pre- and costimulated the IC_{75} values of VP-16 ranged from 0.1 to >1.0 μg/ml (median: 0.6 μg/ml) for G-CSF ($p>0.05$ as compared, from 0.1 to >1.0 μg/ml (median: 0.27 μg/ml) for IL-3 ($p=0.036$) and from 0.15 to >1.0 μg/ml (median:0.25 μg/ml) for GM-CSF ($p=0.036$) (Fig. 2D).

Secondary plating efficiencies (PE_2). In three AML samples secondary plating efficiencies (PE_2) were determined. Two AML samples (no. 5 and 6) were selected because Ara-C cytotoxicity in clonogenic cells (PE_1) was enhanced by HGF addition. The third AML sample (no. 9) showed no significant enhanced Ara-C cytotoxicity by HGF preincubation.

The IC_{75} (PE_2) values for AML sample no. 5 using Ara-C addition alone as compared to Ara-C and HGF pre- and costimulation decreased from 0.0108 to 0,0024 μg/ml for G-CSF, from 0.144 to 0.0048 μg/ml for IL-3 and from 0.1152 to 0.0192 μg/ml Ara-C for G-CSF. Using AML sample no. 6, the IC_{75} (PE_2) values decreased from 0.12 to 0.0024 μg/ml when AML cells were preincubated with IL-3 and Ara-C, and from 0.18 to 0.0096 μg/ml for GM-CSF and Ara-C preincubation as compared to Ara-C alone. Replating experiments with G-CSF dependent AML cells did not show colony formation in the methylcellulose cultures. The IC_{75} (PE_2) values using AML sample no. 9, decreased from 0.12 to 0.024 μg/ml Ara-C for G-CSF, from 0.24 to 0.192 μg/ml for IL-3, and remained 0.24 μg/ml for GM-CSF. In figure 3, the relative sensitivity ratios for Ara-C are shown for the three AML samples using the MTT assay and the clonogenic assays (PE_1 and PE_2). The relative sensitivity ratio was defined as the ratio of the IC_{75} values for Ara-C and HGF exposure of the AML cells and the IC_{75} values for Ara-C exposure alone.

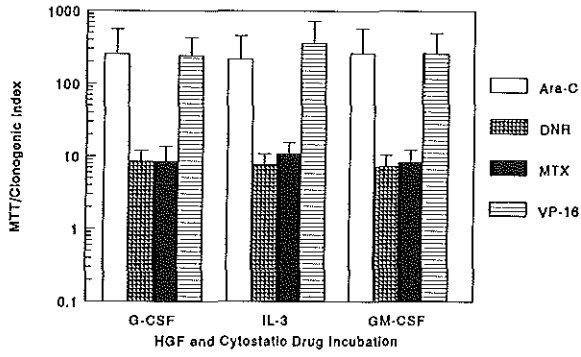

Fig. 4: The MTT/Clonogenic index for Ara-C, DNR, MXT and VP-16 using HGF stimulation. The index was calculated by dividing the IC_{75} values obtained by the MTT assay and the clonogenic assay for each sample tested. The values represent the mean of the indices and SD.

MTT assay vs Clonogenic assay. For a comparison of the IC_{75} data obtained with the MTT assay and clonogenic assays, the MTT/Clonogenic index of each drug combined with HGF stimulation was estimated for each experiment (Fig. 4). The results of the MTT assay do not correlate with those of the clonogenic assay, especially when cycle specific agents as Ara-C and VP-16 combined with HGF stimulation were used.

DISCUSSION

This paper describes the effects of HGF and cytostatic drug preincubation on AML cells as evaluated by the MTT assay and a clonogenic assay. Investigators using the MTT assay reported that non-malignant cells might influence the test results in cytotoxicity experiments (3,5). However, efforts to separate out the AML blasts from the cell suspensions were not made in these reports. The blast fraction of the AML samples used in our experiments was purified following depletion of T-lymphocytes and adherent cells, so that AML samples usually contained more than 90% AML blasts. A period of 48 hours of drug exposure was selected because this exposure time has been reported to be adequate for cytotoxicity testing using the MTT assay (2,4).

BrdU incorporation studies showed a 1.8-fold increase of the median BrdU incorporation in non HGF incubated AML cells. Using HGF stimulation, a 3.4 to 4.6 increase in median BrdU incorporation was observed. Nevertheless, the effects of cytostatic drug and HGF preincubation as compared to cytostatic drug exposure alone showed no significant enhanced cytotoxicity using the MTT assay for the drugs tested. When the effects were evaluated by clonogenic assays (PE_1), significant enhancement of cytotoxicity by HGF when combined with Ara-C preincubation as compared to Ara-C incubation alone could be demonstrated ($p=0.01$, 0.004, and 0.02 for G-CSF, IL-3, and GM-CSF resp.). A comparatively small but significant enhancement of VP-16 cytotoxicity was found in case of IL-3 ($p=0.036$) and GM-CSF ($p=0.036$) pre- and costimulation. No increased cytotoxicity was observed using the non-cycle specific drugs DNR or MXT. The enhanced cytotoxic effect of HGF stimulation can be partly explained by recruiting non-dividing, potentially clonogenic AML cells. Once these cells enter cell cycling, these cells become more susceptible for phase-specific drugs like Ara-C and VP-16. The finding that Ara-C toxicity was more enhanced than VP-16 toxicity by HGF stimulation could indicate that factors other than pure cell cycle related are responsible for the increased cytotoxicity. Changes in Ara-C metabolism by HGF stimulation have indeed been suggested (8,25-26). Briefly, HGF stimulation was shown to enhance intracellular Ara-C phosphorylation and incorporation into the DNA, unrelated to the number of cells in S-phase (26). Because DNR and MXT act as intercalating agents, their cytotoxic effects do not depend on S-phase activation of the cells. Consequently, it may be expected that combining DNR with HGF does not lead to enhanced cytotoxicity in the clonogenic assays. The discrepancy between the results of the MTT assay and the clonogenic assay (PE_1) in HGF mediated cytotoxicity might be caused by several factors. First, the MTT assay is an assay based on the ability of metabolically living cells to convert MTT to a coloured formazan product. Therefore, the results of this assay are not only a measure for cell viability, but also for the metabolic activity of cells. Jabbar *et al* (27) reported that the growth inhibitory effects of interferons were underestimated by the MTT assay. This was in part caused by an alteration of metabolic activity in the cells induced by interferon. The metabolic activity and proliferative status of cells are influenced by HGFs (11). This could result in an underestimation of the HGF and Ara-C or VP-16 effect by the MTT assay. However, no significant change of the optical density of the control cultures in the presence of HGFs was noted. Therefore, this possibility appears unlikely. Another explanation could be that Ara-C causes a mitotic arrest that does not necessarily lead to immediate cell death, and thus is not detected with the MTT assay. This explanation is supported by observations that Ara-C not only effectively reduces terminal division of AML cells but also inhibits the self-renewal capacity (18,21). The MTT assay as we used it, gives a measure for the direct cytotoxic effects of cytostatic drugs. The long term effects of cytostatic drugs in cell populations can not be assessed by using the MTT assay.

The effects of HGF combined with Ara-C on the self renewal capacity of clonogenic

AML cells was tested in three AML samples by analysis of the secondary plating efficiencies. In two patients the PE_2 values were lower after Ara-C and HGF preincubation as compared with Ara-C alone, thereby indicating that the self renewal capacity of the clonogenic AML cells was increasingly impaired by combined Ara-C and HGF incubation. In the other AML sample the Ara-C cytotoxicity (PE_1) was not enhanced by combined HGF incubation. The cytotoxic effects of HGF and Ara-C preincubation on the self renewal capacity of the clonogenic cells in this patient were not enhanced by IL-3 and GM-CSF, and to a small extent by G-CSF. The effects on the PE_2 might be underestimated since cell numbers and viability of the pooled colonies were adjusted before plating. However, this observation indicates that in this AML case, kinetic resistance to Ara-C could not be overcome by HGF pre- and costimulation.

Dye-exclusion assays are used for in-vitro chemosensitivity testing in hematological malignancies (28). These assays have shown to correlate well with the MTT assay (3,29). The outcome of dye exclusion assays have been compared with clonogenic assays and seemed to have a good correlation, although dye exclusion assays gave lower estimates of cell kill than clonogenic assays (30-31), except with Ara-C (32). Data on a direct comparison of clonogenic assays and the MTT assay are scarce (30), and not a single study has been reported on the comparison of the MTT assay with clonogenic assays in AML. In our evaluation, we found a poor correlation between the results of the MTT assay and the clonogenic assay. As discussed, explanations like differences in the cytotoxic mechanism of drugs in short or long term evaluation might be responsible. The observation that the increase in BrdU-positive cells following HGF stimulation was not reflected by an increased cytotoxicity as determined by the MTT assay, seems to indicate that the MTT assay is not capable in accurately detecting changes in cytotoxicity. Nevertheless, usefulness of the MTT assay in predicting enhanced cytotoxicity caused by other than cell cycle related processes, e.g. by multidrug-resistance modifiers, has been suggested recently (33).

Finally, we conclude that although the MTT assay may be useful in chemosensitivity testing, it does not seem suitable to evaluate the HGF enhanced cytotoxicity of clinically relevant drugs in AML, a disease characterized by a large compartment of proliferating cells.

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CHAPTER 7

GENERAL DISCUSSION

AND

SUMMARY

Drug resistance may be an important cause of chemotherapy treatment failure in patients with acute myeloid leukemia (AML). In vitro experiments have identified different mechanisms involved in drug resistance (Introduction). In this respect, the expression of the *MDR*1 gene has been studied most extensively in AML. Moreover, *MDR*1 drug resistance appears one of the most important forms of drug resistance in newly diagnosed AML. Several investigators have studied *MDR*1 expression in untreated AML (1-24). The results of these and our studies raise several questions. First, a variety of assays has been used to detect and quantify *MDR*1 expression. Has the application of available detection methods influenced the results and the interpretation these data? Which technique is to be preferred? Second, what can be concluded from these studies with respect to the significance of *MDR*1 expression in drug resistance in AML? Third, in vitro, several modulators of *MDR*1 expression have been developed (see also chapter 1: introduction). If *MDR*1 expression is clinically relevant, the question arises wether *MDR*1 modulators can be of benefit in AML chemotherapy.

7.1 *MDR*1 expression in AML: controversies

Convincing evidence has been gathered that *MDR*1 expression is indeed an independent, unfavourable prognostic factor in AML. Holmes and West published a meta-analysis on the results of several studies and concluded that P-gp expression is associated with a relative risk of 0.68 to achieve complete remission in AML (25). The experimental work described in chapters 2 and 3 confirms that *MDR*1 expression has a negative prognostic value in AML. Some authors were not able to establish a negative correlation between *MDR*1 expression and clinical outcome of remission-induction chemotherapy (9,15,18). Also, comparing *MDR*1 expression in untreated AML among several studies, a large variation has been reported. These discrepancies may in part relate to differences among detection methods of *MDR*1 expression and the selection of parameters for scoring of *MDR*1 positivity.

Detection methods

Many assays can be used to detect *MDR*1 (over)expression at RNA, protein or functional level (see also chapter 1, p. 18). Methods to measure *MDR*1 (over)expression described in this thesis included the mRNase protection assay, immunocytochemical staining of leukemic cells, flowcytometry and daunorubicin accumulation assays.

RNase protection assays are usually used to detect *MDR*1 overexpression at mRNA level. They served as a control for our immunocytochemical studies. There are at least three major pitfalls with regard to the use of these RNA methods. The first potential disadvantage of the RNase protection assays is their sensitivity. Even very low levels of *MDR*1 expression are detected, perhaps too low to confer clinical resistance. As a consequence, problems with the interpretation of the test results can occur. Another highly sensitive technique like RT-PCR may meet the same problem. This might explain the observation done by Gruber *et al* (RNAse protection assay) and Paietta *et al* (RT-PCR) that *MDR*1 expression could not be recognised as a negative prognostic factor in untreated AML (9,18). Nevertheless, one study showed a decrease in CR rate and disease-free survival using the RNAse protection assay (6). A second limitation of these techniques relates to the fact that *MDR*1 expression is determined in cells without regard of the phenotypical differences. No insight can be obtained in the *MDR*1 expression levels in distinct subpopulations of leukemic cells. This information has shown to be clinically relevant (chapters 2 and 3). Third and less importantly, *MDR*1 expression at mRNA and protein level do not always correlate (10,20). *MDR*1 RNA assays are highly sensitive but may not be specific for clinical resistance. Detection assays that detect P-glycoprotein could provide more direct information as regards the *MDR*1 phenotype. Therefore, we suggest that the RNA assays should be limited to be used as an assay for *MDR*1 (over)expression in combination with other techniques.

Immunocytochemical studies using several monoclonal antibodies (MoAbs) have shown that P-gp expression is a significant negative prognostic factor for the probability to achieve CR (chapters 2 and 3). Also, immunocytochemistry is capable in showing variation in *MDR*1 expression within one AML sample. A major disadvantage of this time consuming technique is the relatively small amount of cells that can be screened within one AML sample. Nevertheless, immunocytochemical studies have been shown to be very useful in *MDR*1 detection. Flowcytometry can circumvent some of the disadvantages of immunocytochemical assays. In addition, using flowcytometric techniques, significantly greater numbers of cells can be rapidly screened. Using dual fluorescence, subpopulations in AML samples can be studied. In fact, ideally one would like to examine the level of *MDR*1 expression in leukemic progenitor cells that are responsible for the maintainance of the malignancy. A difficulty is that most MoAbs recognize an intracellular epitope of the P-gp molecule. If one uses these MoAbs, fixation procedures of leukemic cells are necessary. These fixation procedures may introduce artefacts which might complicate the interpretation of the results of the experiments. Several MoAbs, like MRK-16 and 4E3, recognize epitopes of the extracellular loops of the MDR molecule and can therefore avoid the use of fixation procedures. These MoAbs (MRK-16, 4E3) only recognize P-gp encoded by the *MDR*1 gene, while others like C219 also recognise P-gp encoded by the *MDR*3 gene. In this thesis, we did not apply MRK-16 or 4E3 in flowcytometry using

unfixed cells.

Functional tests are considered important for evaluation of P-gp (over)expression. First, the relation between P-gp expression and intracellular drug accumulation can be studied. Second, the effect of P-gp modulators can be measured in functional assays. In this thesis, we used daunorubicin accumulation because this drug was included in the treatment regimens of the AML patients included in these studies. Others have used rhodamine as a fluorescent dye (26). Rhodamine is known to be non-cytotoxic, and a good substrate for the P-gp molecule. The use of $DiOC_2$ as a dye in efflux studies has also been described (27). Although rhodamine and $DiOC_2$ can be useful in accumulation/efflux studies, studying cytostatic drug accumulation/efflux has the advantage that the affinity of individual drugs for the P-gp molecule can be tested in the tumor cells, analogous to the clinical treatment.

In conclusion, flowcytometry using unfixed AML cells to detect P-gp expression combined with a functional assay provide appropriate methods to assess *MDR*1 expression. It is essential that quantification of *MDR*1 expression is standardized among international laboratories. The diverse assays may be calibrated using cell lines or AML samples.

CD34 and *MDR*1 expression in AML

As described in chapter 2, P-gp is predominantly expressed in CD34 positive AML cells. Recently, this observation has also been confirmed by others (17,23,26-27). Leith *et al* also reported the strong correlation of functional DiOC2 efflux with both CD34 and *MDR*1 expression (27,28). This may be a major mechanism by which the cells protect themselves against the effects of certain cytostatic agents. The immature, CD34 positive, leukemic population is assumed to be clinically relevant because this population contains leukemic progenitor cells. As in their normal hematopoietic counterparts, *MDR*1 can also be expressed in immunophenotypically more mature leukemic cells. P-gp positivity in CD34 negative AML has also been observed (chapter 2). Especially the presence of leukemic cells co-expressing P-gp and CD34, even if low in number, appears to have impact on prognosis. When *MDR*1 expression has been studied without assessing CD34 expression, the correlation between *MDR*1 expression and clinical prognosis in AML may be confounded. This may account for the absence of clinical significance of *MDR*1 expression as has been reported by some investigators.

Our findings may also imply that, like *MDR*1 expression, other forms of drug resistance like MRP and p110 expression should be studied in the clinically relevant subpopulations of AML samples. These kinds of experiments can further contribute to our knowledge of drug resistance in AML.

Autonomous growth of leukemic cells in vitro is also known to be an important negative prognostic factor. As shown in chapter 3, a correlation between CD34 expression is often associated with autonomous growth. Another negative prognostic factor like monosomy 7 seemed to be associated with *MDR*1 overexpression (chapter 2). From these data, the question arises if these factors are independent prognostic factors in AML. Future studies using multivariate analysis of data could elucidate this question.

*MDR*1 expression and clinical significance

The clinical relevance of *MDR*1 expression in most studies is based on the probability of attaining complete remission after remission induction chemotherapy. Considering this endpoint, most studies identified *MDR*1 expression as a negative prognostic factor. From a clinical point of view, the negative prognostic value of *MDR*1 expression is also relevant with regard to overall survival and disease-free survival (DSF). As shown in chapter 3, P-gp and CD34 double expression is associated with a significantly shorter overall survival in AML patients expressing both markers. Data concerning disease-free survival have been reported by several investigators (6,15-16,19). Although three studies recognized P-gp as a negative prognostic factor (6,15,19), no difference in disease-free survival between the *MDR*1 positive and negative AML group has been observed in two other reports (15,19). Relatively low numbers of AML patients included in these studies may explain that the difference in DFS between P-gp positive and negative AML cases was not always significant. In one of the two studies, several patients (10 out of 62) were treated with allogeneic BMT which confounded the analysis of the role of P-gp expression on long-term survival in AML following chemotherapy (15). Hunault *et al* recently showed prelimenary results of a multivariate analysis study in a group of 72 AML newly diagnosed AML patients (29). In this study *MDR*1 overexpression was a powerfull negative prognostic factor for remission induction and survival.

We conclude that *MDR*1 expression is associated with a poor outcome of chemotherapy. However, data on *MDR*1 expression in relation to long term, disease-free survival are still needed to establish the full clinical impact of *MDR*1 expression. Future prospective studies in larger numbers of AML patients are likely to give a definite answer to this question.

7.2 Drug resistance and treatment development

Identification of drug resistance has prompted attempts to reverse it and improve treatment outcome.

Modulation of drug resistance by hematopoietic growth factors

In the studies presented in the chapters 4, 5, and 6, we have shown that the individual effects of HGF pre- and costimulation enhanced Ara-C cytotoxicity in AML. Especially IL-3 as compared with G-CSF and GM-CSF, was shown to be effective in enhancing Ara-C cytotoxicity. Although HGF stimulation could enhance Ara-C cytotoxicity in relapsed or refractory AML, pharmacological Ara-C resistance could not be overcome by HGF stimulation (chapter 5). Only few clinical trials which have used HGF in order to prime leukemic cells for chemotherapy in vivo have been published. Despite the promising in vitro results, in these studies clinical outcome did not improve following additional treatment with the growth factors G-CSF (30) or GM-CSF (31-32). Several factors might have contributed to in vitro-in vivo discrepancy. First, small numbers of AML patients were included in some the studies. Second, historical controls instead of randomised controls were used in two of these reports (31-32). Third, in one study, chemotherapy treatment regimens differed between the HGF treated AML patient group and the controls (32). Fourth, serum HGF levels after administration might be insufficient for optimal stimulation of the AML cells. In one study, G-CSF is used in a group of refractory AML patients. Since HGF stimulation can not overcome pharmacological resistance for Ara-C (see chapter 5), this might explain the disappointing outcome of this study. It is obvious that no convincing conclusions can be drawn from these studies. Carefully planned clinical studies are necessary to assess the value of HGF addition in AML chemotherapy treatment. Ideally, these studies should include randomised non HGF exposed controls and sufficient numbers of AML patients for statistical analysis. Löwenberg *et al* has shown preliminary results of such a study using GM-CSF during and after chemotherapy treatment in elderly patients with AML (33). Response rates were not improved in this study. Because remission-induction chemotherapy includes simultaneous treatment with different kind of drugs, attention should be paid to the sequence in which these drugs are used. As a matter of fact, HGF stimulation especially enhances Ara-C cytotoxicity (chapter 4, 5). It is conceivable that simultaneous addition of other, non S-phase specific drugs like daunorubicin, would prevent leukemic cells to enter S-phase. This would antagonise an optimal action of HGF on Ara-C cytotoxicity and as a result any possible beneficial effect of HGF stimulation would be lost.

Modulation of *MDR*1 function

In chapter 2, cyclosporin was used to study MDR reversal in leukemic subpopulations. Several other agents have also been shown to reverse MDR in vitro (Introduction). Some of these agents have been used in clinical trials. The results of modulator studies suggest

that P-gp can be modulated and that the clinical significance of *MDR*1 expression can be reversed. Nevertheless, the interpretation of these studies is complicated for several reasons. First, P-gp modulating agents also affect the physiological P-gp function in normal tissues like bile canaliculi and kidney. Therefore, drug clearance can be altered and, as a consequence, this may result in increased plasma $t_{1/2}$ and/or increased plasma drug concentrations. Bartlett *et al* reported decreased clearance of drugs and metabolites in a group of patients with incurable malignancies on cyclosporin therapy (34). Whether the beneficial effect of MDR modulators is caused by altered pharmacokinetics or indeed by modulation of *MDR*1 expression in leukemic cells remains to be determined. Second, phase I and II studies do not include a randomized control group of patients treated with conventional chemotherapy only. Therefore, randomized phase III studies will allow a definite conclusion of the effectivity of P-gp modulators in the chemotherapy treatment. In these studies, perhaps it will be necessary to reduce the dose of cytostatic drugs in patients who are also treated with modulating agents in order to compensate for the altered pharmacokinetic profile and increased plasma exposure.

Future studies should focus on the following aspects in order to optimize modulation of MDR.

Development of other drugs with no or low affinity to the P-gp molecule(s). One example that this approach might be of important value is the use of a new anthracycline, idarubicin (Ida), in AML treatment. Clinical studies have indicated that the response rates and the disease-free survival are better in AML patients who received idarubicin. A possible explanation is that idarubicin is more antileukemic because it is a comparatively poor substrate for the P-glycoprotein molecule. In vitro drug accumulation studies comparing daunorubicin with idarubicin, show that the intracellular daunorubicin accumulation was restored by combining daunorubicin with cyclosporin in *MDR*1 expressing cell lines. However, the intracellular concentrations of idarubicin were not influenced by cyclosporin (35). Intermediate restoration of drug accumulation was found in case of idarubicinol, a cytotoxic metabolite of Ida. Therefore, it was concluded that the intracellular accumulation of Ida, and to a lesser extent idarubicinol, is only minimally affected by P-gp mediated efflux. In vitro cytotoxicity assays have shown that DNR but not Ida cytotoxicity could be enhanced by CsA, again confirming a difference in affinity of DNR and Ida for P-gp bindingsites. These findings are especially important since DNR has been replaced by Ida for remission induction chemotherapy in several recent AML treatment protocols.

Several drugs, e.g. Ara-C, methotrexaat, and alkylating agents, are not at all involved in drug resistance caused by *MDR*1 (over)expression. Intelligent use of these agents in AML chemotherapy may contribute to a more favourable response in MDR patients.

Efforts may be made to develop new MDR modulators. These new class modulating agents should exert a highly selective effect on P-gp expression or function without major

side effects in vivo like immunosuppression or tissue toxicity (e.g. cardio- or nephrotoxicity). An example of such an agent is the molecule SDZ PSC 833, a non-immunosuppressive cyclosporin D analogue that is currently entering clinical trials (36).

Since *MDR*1 expression is the clinically most relevant cause of drug resistance in AML, carefully planned studies are urgently needed to optimize chemotherapy treatment in patients with AML in the future.

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SAMENVATTING

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

De behandeling van AML bestaat uit het toedienen van cytostatica, eventueel gevolgd door beenmerg transplantatie. Na chemotherapie kunnen met behulp van standaard cytomorphologische technieken, bij ongeveer 2/3 van de behandelde AML patiënten geen leukemie cellen in bloed of beenmerg meer worden aangetoond. Men spreekt dan van het bereiken van een complete remissie. Desondanks treedt er bij het merendeel van deze patiënten toch terugkeer, een recidief, van de ziekte op. Een recidief AML blijkt met cytostatica moeilijker te behandelen.

Het falen van conventionele chemotherapie kan veroorzaakt worden door het bestaan of het ontwikkelen van resistentie voor de relevante cytostatica. Verschillende mechanismen van cytostatica resistentie worden verondersteld hierbij betrokken te zijn. Het doel van het onderzoek dat in dit proefschrift beschreven wordt, is na te gaan of resistentie tegen de cytostatica die bij de leukemie behandeling worden gebruikt reeds aanwezig zijn bij de start van de therapie. Tevens werd de vraag gesteld of bepaalde patiënten die slecht zullen reageren op de behandeling kunnen worden geïdentificeerd door onderzoek naar resistentie van de AML cellen. Geprobeerd werd om de AML cellen te identificeren die mogelijk aan de werking van cytostatica kunnen ontsnappen en zodoende uiteindelijk verantwoordelijk zijn voor het optreden van een recidief. Tevens werd de mogelijkheid bekeken om deze AML cellen door middel van farmacologische behandeling gevoeliger te maken voor de werking van cytostatica, m.a.w. de mogelijkheid tot moduleren van cytostatica resistentie.

In hoofdstuk 1 worden de diverse mechanismen betreffende cytostatica resistentie besproken. Een reeds eerder onderzocht mechanisme is resistentie voor cytarabine (Ara-C), een veel gebruikt en effectief anti-leukemisch middel. Een ander mechanisme vormt het voorkomen van bepaalde eiwitten in leukemie cellen die de intracellulaire concentratie van diverse cytostatica kunnen verlagen. Omdat het hier gaat om resistentie tegen verschillende, niet gerelateerde cytostatica (o.a. anthracyclines, epipodophylotoxines en vinca alkaloiden), wordt gesproken van "multidrug resistance" (MDR). Glycoproteinen die hierbij betrokken kunnen zijn, zijn P-glycoproteine (P-gp, P-170) dat gecodeerd wordt door het *MDR*1 gen. Deze vorm van MDR wordt ook wel klassiek of typische MDR genoemd. Andere transport eiwitten die een rol bij MDR kunnen spelen zijn multidrug

resistance-associated protein (MRP) en p110. MDR kan ook geassocieerd zijn met kwalitatieve of kwantitatieve veranderingen in het celkern enzym topoisomerase II (topo II) en het versterkt tot expressie komen van diverse detoxificatie mechanismen. Tevens worden de mogelijkheden tot in vitro en in vivo cytostatica modulatie besproken.

Een van de klinisch meest belangrijke oorzaken van cytostatica resistentie in onbehandelde AML blijkt het voorkomen van een specifiek, celmembraan gebonden glycoproteine (P-gp, P-170) op AML cellen (hoofdstuk 2). De resultaten lieten zien dat binnen de totale populatie leukemie cellen een subpopulatie leukemie cellen kan voorkomen met een onrijp immunophenotype, d.w.z. CD34 expressie, die P-gp tot expressie te brengen. Met behulp van daunomycine (cytostaticum) accumulatie experimenten kon worden aangetoond dat deze P-gp expressie inderdaad gepaard ging met een verminderde intracellulaire accumulatie. Aangenomen wordt dat binnen de onrijpe, CD34 positieve leukemie cellen, zich de leukemische stamcellen bevinden die verantwoordelijk zijn voor het handhaven van de leukemie. Uit de resultaten afkomstig van experimenten zoals beschreven in hoofdstuk 2, blijkt dat het mogelijk is dat juist deze cellen relatief goed beschermd zijn tegen de werking van cytostatica. De aanwezigheid van kleine percentages CD34 en P-gp positieve leukemie cellen bleek een negatief effect op de prognose te hebben (hoofdstuk 3). Andere oorzaken voor cytostatica resistentie zoals het voorkomen van andere eiwitten die cytostatica uit de cel kunnen verwijderen zoals p110 and MRP, bleken niet van klinisch belang. Ook parameters voor Ara-C resistentie bleken niet te correleren met de klinische respons van de patiënten op chemotherapie. Hieruit is dan ook geconcludeerd dat P-gp expressie de belangrijkste oorzaak is van klinisch relevante cytostatica resistentie in nieuw gediagnostiseerde AML. Daarnaast werd gevonden dat er een duidelijke relatie is tussen twee andere prognostische factoren, te weten CD34 expressie en autonome groei van leukemie cellen.

Resistentie tegen cytostatica d.m.v. P-gp expressie kan worden beinvloedt (modulatie) door toevoeging van sommige niet cytotoxische farmaca zoals ciclosporine, verapamil en SDZ PSC 833. Deze stoffen interfereren met de functie van het P-gp molecuul waardoor bepaalde cytostatica niet meer de leukemie cel uit kunnen worden gepompt. De intracellulaire cytostatica concentratie kan dan weer het niveau bereiken van leukemie cellen die geen P-gp tot expressie brengen (hoofdstuk 2). Dit heeft tot gevolg dat het celdodend effect van de cytostatica op de P-gp positieve leukemie cellen toeneemt.

Het verbeteren van de Ara-C cytotoxiciteit wordt beschreven in de hoofdstukken 4, 5 en 6. Ara-C is een effectief antileukemisch middel dat gedurende de S-fase, de fase in de cel cyclus waarin DNA replicatie plaats vindt, werkzaam is. Door leukemie cellen aan te zetten tot proliferatie, d.m.v. stimulatie met hematopoietische groeifactoren, werd gekeken of de Ara-C cytotoxiciteit inderdaad was toegenomen. Dit effect werd bestudeerd door middel van clonogene assays, aangezien de clonogene, leukemische stamcellen

verantwoordelijk worden gehouden voor het in stand houden van de leukemie. Vooral stimulatie van leukemie cellen voor en tijdens Ara-C expositie met IL-3, en in mindere mate GM-CSF en G-CSF, bleken in vitro effectief om de cytotoxiciteit te verhogen. De gemeten toename van de cytotoxiciteit was niet gecorreleerd met het percentage cellen dat in S-fase was. Er werd geen verhoogde effect van groeifactor stimulatie op mafosfamide cytotoxiciteit, een niet S-fase specifiek cytostaticum, gevonden (hoofdstuk 4). Tevens werd het effect van groeifactor stimulatie op Ara-C cytotoxiciteit in AML cellen van patiënten met een recidief bestudeerd (hoofdstuk 5). Alhoewel de Ara-C cytotoxiciteit door IL-3 en GM-CSF stimulatie in recidief AML kon worden verhoogd, was dit effect significant minder in vergelijking met het effect op leukemie cellen van dezelfde patiënten bij diagnose. Bij leukemie cellen van drie patiënten werd de inbouw van Ara-C in het DNA, een parameter voor het Ara-C metabolisme, bepaald. Ara-C inbouw in de recidief AML cellen werd verhoogd door IL-3 stimulatie. Desalniettemin bleek ook de Ara-C inbouw in de leukemie cellen significant minder in vergelijking met de AML cellen van dezelfde patiënten bij het stellen van de diagnose. De resultaten tonen aan dat groeifactor stimulatie van recidief AML cellen de cytotoxiciteit van Ara-C wel kunnen verhogen, maar niet tot het niveau dat in de AML cellen van nog niet behandelde patiënten kon worden bereikt.

Naast clonogene assays kunnen ook andere assays gebruikt worden om de effectiviteit van cytostatica te beoordelen. Een test die hiervoor geschikt zou kunnen zijn is de MTT test. Deze test maakt gebruik van het feit dat levende cellen in staat zijn om het tetrazoliumzout MTT om te zetten in een formazan produkt. Dit formazan produkt kan op colorimetrische wijze gekwantificeerd worden. De hoeveelheid gevormd formazan vormt dan een maat voor de vitaliteit van cellen. Het effect van groeifactor stimulatie op Ara-C cytotoxiciteit in AML cellen werd in hoofdstuk 6 met zowel een clonogene als de MTT assay bestudeerd. Verschillende cytostatica, te weten Ara-C, daunomycine, etoposide en mitoxantrone, werden voor de experimenten gebruikt. De resultaten verkregen met de MTT test lieten geen toename in cytotoxiciteit zien bij groeifactor gestimuleerde AML cellen. De resultaten verkregen met clonogene assays lieten wel een significante toename zien van Ara-C cytotoxiciteit in G-CSF, GM-CSF of IL-3 gestimuleerde AML cellen. Het falen van de MTT test kan betekenen dat deze test niet geschikt is voor het detecteren van cel cyclus gerelateerde processen.

In hoofdstuk 7 volgt tenslotte een samenvatting en discussie van de resultaten van het onderzoek dat is beschreven in dit proefschrift. Verschillende aspecten komen aan bod, zoals de waarde van de diverse methodes om *MDR*1 expressie te detecteren, de klinische relevantie van *MDR*1 expressie bij AML en de nieuwe ontwikkelingen om cytostatica resistentie bij patiënten met AML te moduleren.

Abbreviations

ABC	:ATP-binding cassette
ABMT	:autologous bone marrow transplantation
AET	:2-amino-ethylisothiouronium bromide
AML	:acute myeloid leukemia
Ara-C	:cytosine arabinoside
Ara-CMP	:cytosine arabinoside monophosphate
Ara-CDP	:cytosine arabinoside diphosphate
Ara-CTP	:cytosine arabinoside triphosphate
ATP	:adenosinde triphosphate
BM	:bone marrow
BMT	:bone marrow transplantation
BrdU	:bromo-deoxyuridine
BSA	:bovine serum albumine
CD	:cluster of differentiation/cluster of designation
CFU	:colony forming unit
CLL	:chronic lymphocytic leukemia
CM	:conditioned medium
CR	:complete remission
CSF	:colony-stimulating factor
CsA	:cyclosporin
dCk	:deoxycytidine kinase
dCTP	:deoxycytidine triphosphate
DNA	:deoxyribonucleic acid
DNR	:daunomycin/daunorubicin
DMSO	:dimethylsulfoxide
F	:female
FAB	:French-American-British cytomorphological classification of acute leukemias
F-Ara-A	:fludarabine
F-Ara-ATP	:fludarabine triphosphate
FCS	:fetal calf serum
FITC	:fluorescein isothiocyanate
FISH	:fluorescent in situ hybridization
FdU	:fluoro-deoxyuridine
FSC	:forward scatter
G-CSF	:granulocyte colony-stimulating factor
GM-CSF	:granulocyte-macrophage colony-stimulating factor
GST	:glutathion S-transferase
GTH	:glutathion
GVH	:graft-versus-host
HBSS	:Hank's balanced salt solution
HGF(s)	:hematopoietic growth factor(s)
Ida	:idarubicin
Ig	:immunoglobulin
IL	:interleukin
IMDM	:Iscove's modified Dubecco's medium

IV	:intravenously
kDa	:kilo Dalton
LAK cell	:lymphokine-activated killer cell
Maf	:mafosfamide
m-AMSA	:amsacrine
MDR	:multidrug resistance
MDS	:myelodysplastic syndrome
MGG	:May-Grunwald-Giemsa
MNC	:mononuclear cells
MoAb	:monoclonal antibody
MRP	:multidrug resistance associated protein
MTT	:methyl tetrazolium bromide
MXT	:mitoxantrone
ND	:not determined
NK cell	:natural killer cell
OD	:optical density
P-170	:P-glycoprotein
PB	:peripheral blood
PBS	:posphate-buffered saline
PE	:phycoerythrin
PE_1	:primary plating efficiency
PE_2	:secondary plating efficiency
P-gp	:P-glycoprotein
PR	:partial remission
RT-PCR	:reverse-transcriptase polymerase chain reaction
SF	:serum-free
SRBC	:sheep red blood cells
SSC	:sideward scatter
TCA	:trichloric acetic acid
Topo	:topoisomerase
VP-16	:etoposide
WBC	:white bloodcell count

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