On mechanisms of drug resistance in acute myeloid leukemia
I humbly dedicate this work to my family.
On mechanisms of drug resistance in acute myeloid leukemia
ABSTRACT


In this thesis focus has been to increase the knowledge and understanding of some of the mechanisms responsible for drug resistance in acute myeloid leukemia, as well as identify possibilities to predict drug resistance at diagnosis. We have studied the intracellular behavior of cytostatic drugs and their main metabolites (paper I) and the cellular response to cytostatic drugs (paper III). A new flow cytometry in vitro chemosensitivity assay was developed, to enable identification of viable myeloid cells and determination of drug sensitivity (paper II). Finally, possible new markers involved in drug resistance were investigated (paper IV).

In conclusion we found that idarubicin and daunorubicin are equally toxic at the same intracellular concentrations. The contribution of the main metabolites to the cytotoxic effects of idarubicin and daunorubicin, in both drug sensitive and drug resistant human myeloid leukemia cells, is low. It is most likely the pharmacokinetic properties of idarubicin and daunorubicin that confer their main cytotoxic effect. With the new flow cytometry chemosensitivity assay we selectively identified viable CD13/CD33 expressing myeloid cells and found that the cytotoxicity results correlated to clinical parameters, such as secondary AML and resistant disease. Short-term exposure of leukemia cell lines with different levels of drug resistance to ara-C revealed that Pgp mRNA and protein expression levels, as well as GSTπ mRNA levels, were rapidly up-regulated. Clinically, this up-regulation may be of importance for the sequential scheduling of daunorubicin and ara-C during the induction treatment of AML. CRIM1 has never been studied in the context of drug resistance before. We show for the first time that baseline expression of CRIM1 mRNA is much higher in drug resistant leukemia cells compared to drug sensitive cells. We also found a co-variance between CRIM1 and Pgp mRNA expression levels in leukemia cell lines with different levels of drug resistance, suggesting that CRIM1 may be useful as a marker of drug resistance.

Keywords: Acute myeloid leukemia, Chemosensitivity, CRIM1, Cytarabine, Daunorubicin, Drug resistance, Glutathione-S-transferase π, P-glycoprotein.
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Malin Prenkert, Clinical Research Center, Örebro University Hospital, SE-701 85 Örebro, Sweden.
SVENSK SAMMANFATTNING

Det övergripande syftet med avhandlingsarbetet var att på olika sätt studera resistenta och känsliga leukemiceller för att öka kunskapen om mekanismerna bakom cytostatikaresistens. I delarbete I undersöktes skillnaden i intracellulärt cytostatikaupptag och cytotoxisk effekt mellan idarubicin, daunorubicin och de huvudmetaboliter. I delarbete II utvecklades en flödescytometrisk metod för att selektivt bestämma effekten av cytostatika på myeloiska blaster från patienter diagnosticerade med AML. I delarbete III var syftet att kartlägga förändringar över tid i uttryck av markörer med känd relevans för cytostatikaresistens (Pgp, GST\(\pi\) och BCRP) på mRNA- och proteinnivå efter exponering för cytostatika. I delarbete IV slutligen, studerades CRIM1, ett transmembranprotein som hittills är outforskat i samband med AML. Syftet med den studien var att påvisa eventuella skillnader i uttryck av CRIM1 i cytostatikakänsliga celler och celler genom överuttryck av Pgp som är höggradigt resistenta. Även skillnader i uttryck av Smad5, BMP4 och BMP7 studerades i dessa celler.


När känsliga och resistenta celler exponerades för ara-C fann vi att det skedde en mycket snabb uppreglering av Pgp på mRNA-nivå. Detta trots att ara-C inte anses vara ett substrat för Pgp. Efter 8 timmars exponering såg vi dessutom ett Pgp-proteinuttryck i känsliga celler som normalt inte uttrycker Pgp. Exponering för ara-C gav även ett ökat uttryck av GST\(\pi\) i resistenta celler men inte i känsliga. Resultatet kan ha klinisk betydelse för i vilken ordning man administrerar daunorubicin och ara-C vid behandling av AML.

CRIM1, Smad5 och BMP4 uttrycktes mycket högre i resistenta celler än i känsliga, på mRNA-nivå. Efter exponering för daunorubicin eller ara-C ökade uttrycket i de känsliga cellerna men inte i de resistenta. Fler studier, speciellt med syfte att studera uttryck av CRIM1 på proteinnivå, behövs för att utreda vilken roll CRIM1 spelar vid uppkomst av cytostatikaresistens.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ABCB1</td>
<td>gene encoding Pgp</td>
</tr>
<tr>
<td>ABCG2</td>
<td>gene encoding BCRP</td>
</tr>
<tr>
<td>AML</td>
<td>acute myeloid leukemia</td>
</tr>
<tr>
<td>Ara-C</td>
<td>cytarabine</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5’-triphosphate</td>
</tr>
<tr>
<td>BCRP</td>
<td>breast cancer resistance protein</td>
</tr>
<tr>
<td>BM</td>
<td>bone marrow</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CR</td>
<td>complete remission</td>
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<tr>
<td>CRIM1</td>
<td>cysteine rich transmembrane bone morphogenetic protein regulator 1 (chordin-like)</td>
</tr>
<tr>
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<td>cyclosporine A</td>
</tr>
<tr>
<td>dCK</td>
<td>deoxycytidine kinase</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>DNR</td>
<td>daunorubicin</td>
</tr>
<tr>
<td>FMCA</td>
<td>fluorometric microculture cytotoxicity assay</td>
</tr>
<tr>
<td>GSTπ</td>
<td>glutathione-s-transferase π</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>50% inhibitory concentration</td>
</tr>
<tr>
<td>IDA</td>
<td>idarubicin</td>
</tr>
<tr>
<td>JNK1</td>
<td>the c-Jun N-terminal kinase 1</td>
</tr>
<tr>
<td>MAP</td>
<td>mitogen-activated protein kinase pathway</td>
</tr>
<tr>
<td>MDR</td>
<td>multidrug resistance</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MRP</td>
<td>multidrug resistance associated protein</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide</td>
</tr>
<tr>
<td>PB</td>
<td>peripheral blood</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer solution</td>
</tr>
<tr>
<td>Pgp</td>
<td>permeability-glycoprotein</td>
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<td>Rh123</td>
<td>rhodamine 123</td>
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<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>Topo IIα</td>
<td>topoisomerase IIα</td>
</tr>
</tbody>
</table>
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FLOW CYTOMETRY CHEMOSENSITIVITY TESTING (PAPER II)
INTRODUCTION

Leukemia is a condition characterized by the uncontrolled proliferation of white blood cells in the bone marrow. The disease is characterized by the formation of blasts, or immature cells, which do not function properly and can invade other tissues. Though the caus e of leukemia is not yet fully understood, risk factors such as exposure to radiation or various chemicals and smoking tobacco have been identified.

In recent decades prognosis has improved considerably but, nonethe- less, long-term survival remains poor. Cytostatic drugs enter cells via passive diffusion, thus leading to inadequate drug levels for effective treatment. Before then leukemia was considered a fatal disease.

The central cause for this lack of response to cytostatic drugs is the development of resistance against the drugs, which results in drug resistance. This thesis investigates some of the key mechanisms involved in drug resistance and how they can be used to enable identification of viable myeloid cells and determination of drug sensi-tivity and chemosensitivity.

This thesis is divided into four papers, each focused on specific aspects of the mechanisms involved in drug resistance. Paper I investigates the intracellular localization of cytostatic drugs and their metabolites. Paper II explores the effects of different drugs on myeloid cells, while Paper III examines the expression of markers involved in drug resistance during exposure to cytostatic drugs. Paper IV focuses on the expression of CRIM1, Smad5, BMP4, and BMP7.

CONCLUSIONS

The results presented in this thesis have shown that cytostatic drugs interfere with the "cutting and pasting" of topo-isomerase I, which is involved in the replication of DNA. This leads to the formation of defective DNA copies that cannot be repaired by the cell. Other cytostatic drugs interfere with the "cutting and pasting" of topo-isomerase II, which is involved in the replication of DNA. This leads to the formation of defective DNA copies that cannot be replicated by the cell.

The expression of CRIM1, Smad5, BMP4, and BMP7 was found to be upregulated in cells treated with cytostatic drugs. This upregulation is likely to contribute to the development of drug resistance.

ACKNOWLEDGEMENTS

I would like to thank my thesis advisor, Dr. Jane Doe, for her guidance and support throughout the course of this research. I would also like to thank the members of my research group for their help and support.

REFERENCES


INTRODUCTION

Leukemia was first described in the mid 19th century by European physicians who had observed that their patients had abnormally high levels of white blood cells. In 1845 a German physician named Rudolph Virchow referred to the condition as “weisses blut”, or white blood. Virchow introduced the term “leukemia”, from the Greek words “leukos” and “heima” which means “white blood”, to describe the disease. Leukemia is a blood or bone marrow cancer that comprises a large spectrum of diseases all characterized by an abnormal growth of blood cells. Though the cause of leukemia is not yet fully understood, risk factors such as exposure to radiation or various chemicals and smoking tobacco have been identified.

Since the 1960s when the first anthracyclines were introduced, leukemia has been treated with cytostatic drugs. Before then leukemia was considered a fatal disease. In recent decades prognosis has improved considerably but, nonetheless, long-term survival remains poor. Cytostatic drugs enter cells via passive diffusion and exert their effects intracellularly. Some cytostatic drugs function by binding to DNA and preventing DNA transcription or replication, whilst others function by cross-linking to DNA strands to produce defective DNA copies. Other cytostatic drugs interfere with the “cutting and pasting” of topoisomerases by rendering the cuts permanent, resulting in cell death. Yet others produce free radicals that poison the cells or cause chromatin aggregation, which induces apoptosis. Some patients are cured by this cytostatic drug treatment but the majority of patients respond incompletely or not at all. The central cause for this lack of response to cytostatic drugs is the development of drug resistance, which is either present at diagnosis or induced during treatment.

This thesis investigates some of the key mechanisms involved in drug resistance and possible assays to predict drug resistance at diagnosis. This was achieved by first studying the intracellular behavior of cytostatic drugs and their metabolites (paper I) and the cellular response to cytostatic drugs (paper III). Secondly, a new flow cytometry in vitro chemosensitivity assay was developed, to enable identification of viable myeloid cells and determination of drug sensitivity (paper II). Finally, new putative markers involved in drug resistance were identified (paper IV).
ACUTE MYELOID LEUKEMIA

The most common form of leukemia is acute myeloid leukemia (AML), which is a group of malignancies characterized by clonal expansion of different lineage-specific hematopoietic precursor cells in the bone marrow (BM). This expansion leads to a lack of balance in the differentiation, proliferation and self-renewal systems of normal hematopoiesis. Even though AML is considered a rare disease, approximately 450 adults are diagnosed every year in Sweden. The incidence is slightly higher in men than in women, i.e. 3 male cases for every 2 female cases and the median age at diagnosis is 70 years.

Leukemia cells lose the ability to respond to normal regulators which eventually leads to fatal infections, bleeding, bruising and fever. Untreated, AML is fatal within a few months. With current therapies approximately 40-50% of younger age (i.e. < 60 years) patients are cured. However, the majority of AML patients are over the age of 60 years and for them long-term survival (> 5 years) is only 10-15%.

A combination treatment with an anthracycline, mainly daunorubicin (DNR), combined with cytarabine (ara-C), has been the cornerstone of AML treatment since the 1960s when it was first introduced. There has been significant but modest improvement in survival, especially in younger AML patients, yet much remains to be done to improve overall survival rates.

Several new targeted therapy strategies are being introduced, which include monoclonal antibodies and small molecule inhibitors. In contrast to traditional cytotoxic chemotherapy, which works through inhibition of all rapidly dividing cells, targeted therapy either interferes directly with specific molecules or delivers cytostatic drugs to cells that express specific molecules. For example, conjugation of ozogamicin (gemtuzumab, Mylotarg®) to a CD33 antibody enables drug “delivery” directly to the target myeloid leukemia cells. Another example is the protein farnesyltransferase inhibitor tipifarnib (Zarnestra®), which enters the cell and competitively inhibits intracellular signaling of tyrosine kinases. However, to date none of these targeted therapies have proven more effective than the anthracycline/ara-C combination in AML treatment, with the exception of the use of all-trans retinoic acid (ATRA) in acute promyelocytic leukemia treatment, which has improved survival substantially.
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Cytostatic drugs

Cytostatic drugs work through the inhibition of cell division, either by inhibiting DNA synthesis or by damaging the DNA template. During many intracellular processes, DNA undergoes conformational and topological changes. To enable these changes the cell uses topoisomerases, which can relax supercoiled DNA, unlink intertwined DNA circles and religate double-stranded DNA that has been cut. Hence, targeting topoisomerase will inhibit DNA synthesis. The site of action for the cytostatic drugs known as topoisomerase poisons, is illustrated in Figure 1. The nucleoside analogs have high similarities to normal nucleosides and are therefore competitively incorporated into DNA during proliferation, resulting in damaging to the DNA template (Figure 1).

Topoisomerase poisons

The first anthracycline, daunorubicin (DNR), was introduced in 1962. It was isolated simultaneously by two independent groups; as rubidomycin, isolated from Streptomyces coeruleorubidus by a French group, and as daunomycin, initially isolated from the Streptomyces peucetius, which was found in a soil sample collected in Apulia in Italy. The name daunorubicin was chosen to reflect the dual origin. The anthracyclines were introduced as antibiotics, but soon proved to also have anti-tumor properties. Clinical trials with DNR showed that it had high cardiac toxicity and researchers began the search for less toxic but equally or more effective analogs. Doxorubicin is a 14-hydroxylated version of DNR that was identified in 1969. Idarubicin is a semi-synthetic derivative of daunomycin.
daunomycin\textsuperscript{32}. None of the new analogs have been proven to be more efficient than the two original anthracyclines, DNR and doxorubicin, although some differences in toxicity have been identified\textsuperscript{33}.

Structurally the anthracyclines consist of a planar hydrophobic tetracycline ring that is linked to an amino sugar. Anthracyclines possess quinone moieties that allow them to participate in electron transfer reactions and generate oxygen free radicals. At physiological pH the anthra-cyclines are positively charged, which favors intercalation into DNA\textsuperscript{16}. The anthracyclines are weak bases with high lipid solubility. They are highly reactive in solution and enter the cell by passive diffusion\textsuperscript{7-11}. Anthracyclines have several possible mechanisms of action, including intercalation into DNA, free radical formation, DNA cross-linking, interaction with chromatin and most important, poisoning of topoisomerases, and thereby damage to DNA with subsequent apoptosis\textsuperscript{12, 17}. Anthracyclines are metabolized in the liver and excreted in the bile. Notably, idarubicin is metabolized more rapidly than the other anthracyclines. The red fluorescent properties of the anthra-cyclines are useful, for example, in flow cytometry analysis.

The search for anthracycline analogs with less cardio toxicity resulted in the identification of mitoxantrone, which is an amino anthrancenedione. Like the anthracyclines, mitoxantrone acts through the poisoning of topoisomerase and therefore the production of double-stranded DNA breaks. It also engages in the intercalation into DNA but, unlike the anthracyclines, it is less prone to contribute to the generation of free oxygen radicals\textsuperscript{16, 34}.

Amsacrine is an aminoacridine derivative consisting of a fused planar ring system that can be intercalated into DNA and thereby alter the minor groove proportions. Like the anthracyclines, amsacrine also inhibits topoisomerase\textsuperscript{35}. No cumulative cardiac toxicity by amsacrine has been shown\textsuperscript{36}.

The epipodophyllotoxin, etoposide is a semi-synthetic derivative of podophyllotoxin. Etoposide is believed to form a complex with topoisomerase II\textsubscript{α} and DNA, and thereby induce breaks and prevent DNA repair. Unlike the anthracyclines, mitoxantrone and amsacrine, etoposide does not intercalate into DNA\textsuperscript{35, 37-38}.

Mechanisms of action

Poisoning of topoisomerase II\textsubscript{α}. Topoisomerase II\textsubscript{α} is an intracellular enzyme with the ability to modify the topology of double-stranded DNA during replication and transcription. Topoisomerase II\textsubscript{α} forms a covalent complex with DNA but in the presence of topoisomerase poisons the breakage-rejoining reaction is interfered with and the topoisomerase II\textsubscript{α}-DNA complex becomes stabilized. This stabili-
Intercalation into DNA. When the anthracyclines bind to DNA the planar ring system is inserted between the bases of the double-stranded DNA. The positively charged amino sugar on the drug molecule cross-links to the negatively charged phosphate groups of the DNA, and forms a strong complex. With the drug intercalated the DNA primer becomes useless, which interferes with DNA and RNA synthesis\textsuperscript{12, 15, 38}.

Formation of free radicals. Anthracyclines can be reduced by one or two electron reduction to form reactive oxygen species (ROS), including oxygen free radicals, hydroxyl radicals, and hydrogen peroxide. These radicals damage DNA, mRNA, proteins and lipids and may also account for the cardiac toxicity of the drugs\textsuperscript{16-18}.

DNA cross-linking. Anthracyclines are able to form adducts or DNA cross-links with a covalent bond to one DNA-strand and a hydrogen bond to the other strand, which results in a double-strand stabilization. This binding results in minimal distortion of the DNA helix and therefore is poorly recognized and not removed by repair proteins\textsuperscript{39-40}.

Interaction with chromatin. In the cell nucleus, DNA is associated with a variety of proteins making a nucleoprotein complex called chromatin. Intercalation of anthracyclines starts at the linker DNA regions and results in unfolding of the chromatin conformation. As a consequence, the subunits of DNA lose their stability, which causes aggregation that precedes the chromatin fragmentation that is characteristic of apoptotic cells\textsuperscript{12, 41}.

Nucleoside analogs

Cytarabine (ara-C) was first synthesized in Europe in 1950 and introduced into clinical medicine in 1963. It is a synthetic pyrimidine nucleoside, and an antimetabolite, and differs from normal cytidine and deoxycytidine with respect to the sugar moiety\textsuperscript{42}. Ara-C enters the cell either by active transport by the human equilibrative nucleoside transporter, or by diffusion. Intracellularly, ara-C is either rapidly deaminated to a much less active metabolite, or undergoes a three step phosphorylation to become the active drug ara-CTP. In the first step ara-C is phosphorylated into ara-C monophosphate (ara-CMP) by cytoplasmic deoxycytidine kinase (dCK). In the second and third steps ara-CMP is phosphory-
lated into ara-C diphosphate (ara-CDP), which is subsequently phosphorylated into the active metabolite ara-C triphosphate (ara-CTP). These two later steps are carried out by pyrimidine kinases. The primary action of ara-C is to inhibit nuclear DNA synthesis, and this can occur via three main mechanisms, i.e. 1) inhibition of replication of DNA due to incorporation of ara-C into the replication-initiation primer, 2) retardation of DNA-chain elongation due to ara-C incorporating into DNA and 3) ara-C inhibiting DNA primase. All of these mechanisms may be dose dependent. At present it is still unclear whether additional, as yet unidentified, mechanisms may be involved.

The synthetic purine analogs, cladribine and fludarabine, resemble the nucleoside adenosine and like ara-C they are phosphorylated intracellularly by dCK and incorporated into DNA. Both cladribine and fludarabine are potent inhibitors of ribonucleotide reductase and human DNA polymerases. They are directly toxic to non-dividing cells because of their ability to confer DNA strand breaks and prevent repair.

TREATMENT OF AML

AML treatment is divided into induction therapy and consolidation therapy. The aim of induction therapy is to achieve complete remission (CR), defined as ≤5% blasts in the BM. Consolidation therapy, on the other hand, aims to improve treatment outcome since it is well-recognized that even when in CR, the majority of patients have residual disease that will lead to relapse and eventually death.

Induction therapy
A combination of DNR (45-60 mg/m², days 1-3) and ara-C (100 mg/m²/day, continuous infusion days 1-7) has been used as an induction regime since the 1960s. Attempts to improve outcome by adding a third drug or intensifying the dose has resulted in increased toxicity but little or no gain in survival. 75-90% of patients aged 18-60 years treated with induction therapy will achieve CR, however a majority of these patients will relapse and only 40-50% will survive longer than 5 years. In older patients (>60 years of age) the survival rate is even lower with only 10-15% surviving more than 5 years.
Consolidation therapy
Consolidation or post-remission treatment consists of repeated courses of
anthracyclines or anthracycline analogs together with ara-C and sometimes a
third drug such as etoposide. Based on individual risk factors, allogeneic stem
cell transplantation may be used to improve the treatment outcome. Allogeneic
stem cell transplantation has been shown to reduce the risk of relapse in pa-
tients in the intermediate risk group, probably due to the “graft-versus-leukemia”
effect. However, data on the benefits of allogeneic stem cell transplantation are
contradictory\textsuperscript{49-50}.

Resistance to cytostatic drugs
Even though CR can be achieved in a mean of 70\% of all patients treated with
induction therapy, a majority of these patients relapse within a three year period
and long-term survival, (i.e. >5 years) remains low\textsuperscript{5}. The cause of this poor
long-term survival is mainly drug resistance, which is either intrinsic in patients
who do not respond to induction treatment, or acquired during treatment with
cytostatic drugs\textsuperscript{51-52}. Resistance of leukemia cells to cytostatic drugs occurs
through several different mechanisms, including expression of one or more
energy-dependent transporters that detect and eject drugs, induction of drug-
detoxifying mechanisms, increased or altered drug targets, decreased mem-
brane permeability and/or insensitivity to drug-induced apoptosis\textsuperscript{53}. These me-
chanisms are summarized in Figure 2.

After treatment with cytostatic drugs it is a common clinical finding that pa-
tients develop cross-resistance towards a broad spectrum of structurally unre-
lated drugs, including anthracyclines, epipodophyllotoxins, vinca alkaloids, and
taxanes, to which the cells have never been exposed\textsuperscript{54-55}. The cell’s ability to
protect itself from toxins is an essential biological function. Protection against, or
resistance towards, a single class of cytostatic drugs can be achieved in several
ways, but for multidrug resistance (MDR) the main mechanism is active drug
transport out of the cell\textsuperscript{56}. Multidrug transporters take advantage of the
common properties drugs need to cross the cell membrane. Most compounds transported by the multidrug transporters are small, planar, lipophilic molecules, like many of the cytostatic drugs. Hence, it is a major challenge to overcome this MDR without sacrificing the ability of the drug to enter the cell and exert its effect intracellularly. The multidrug transporters belong to a number of distinct transporter superfamilies of which the ABC (adenosine triphosphate (ATP) - binding cassette) superfamily is the most important in the context of AML. In AML the MDR phenotype has been associated with clinical resistance and poor treatment outcome.

**Drug transport**

*ATP-binding cassette (ABC-)* proteins. The ABC superfamily is a large family of transmembrane proteins that was first defined in the 1980s. In humans, there are 49 known members of this family that efflux compounds in an active ATP-dependant manner. However, these proteins are present in normal tissues and involved not only in drug efflux but also in moving nutrients, hormones and other biologically important molecules into and out of cells, and across plasma membranes.
membranes\textsuperscript{65-67}. To translocate substrates, these proteins require a minimum of four domains, i.e. two transmembrane domains and two nucleotide-binding domains, plus energy which is derived from ATP\textsuperscript{68-70}. In this context transporter protein substrates are defined as compounds that are transported and inhibitors are compounds that restrict the function of the transporters. The ABC-proteins are organized in seven subfamilies, i.e. ABCA – ABCG, as described below\textsuperscript{71-72}.

- The \textit{ABCA} subfamily contains some of the largest ABC genes (>2100 amino acids). Two members of this family have been extensively studied; ABCA1 which is involved in cholesterol transport and high-density lipoprotein synthesis, and ABCA4 which transports vitamin A derivatives.
- The \textit{ABCB} subfamily is unique in that it contains both full and half transporters. The ABCB1 protein is the most extensively studied and in AML and multidrug resistance the most important member of this family. ABCB1, or permeability glycoprotein (Pgp) is further described below.
- The \textit{ABCC} subfamily contains proteins with a diverse functional spectrum. Of these, the ABCC1, ABCC2 and ABCC3 (or multidrug resistance related proteins (MRP1-3)) transport drug conjugates to glutathione and other organic anions.
- The \textit{ABCD} subfamily function in the regulation of very long fatty acid transport.
- The \textit{ABCE} and \textit{ABCF} subfamilies have ATP-binding domains but no transmembrane domains and are not known to be involved in any cross-membrane transport.
- The \textit{ABCG} subfamily contains half transporters that function as homodimers. The most relevant of the ABCG proteins, in the context of AML, is the ABCG2 or breast cancer resistance protein (BCRP), which is further described below.

In this thesis, focus will be on Pgp (ABCB1) and BCRP (ABCG2).

\textit{Permeability glycoprotein}. The permeability glycoprotein (Pgp, ABCB1) is one of the most well characterized proteins that has been linked to multidrug resistance. It was first found to be over-expressed in cell lines selected for resistance to colchicin and vinblastin and was believed to alter the permeability of the cell membrane, hence the name\textsuperscript{73-75}.
Pgp is the protein product of the MDR1 gene on chromosome 7q21. It has a molecular mass of 170 kDa and contains 1280 amino acids. Pgp is a transmembrane glycoprotein found in several normal human tissues such as liver, kidney, pancreas, colon, jejunum and placenta, as well as in numerous cancers. Pgp consists of two similar halves, joined by a linker region. Each half forms a total of six transmembrane domains and one cytoplasmic domain with ATPase activity (the ATP binding cassette) that hydrolyses ATP during molecular efflux. Both halves interact to form a single transporter. This interaction is necessary for functional drug transport. Even though the physiological role of Pgp is not yet fully understood, it is generally accepted that Pgp functions as an energy-dependent drug efflux pump, either as a "hydrophobic vacuum cleaner" or as a flippase that reduces the intracellular concentrations of a wide range of hydrophobic, but otherwise structurally unrelated, drugs and xenobiotics. Binding of a drug results in activation of one of the ATP-binding domains, and the subsequent hydrolysis of ATP causes a major change in the configuration of Pgp, which results in release of the drug into the extracellular space. The substrates are transported against a concentration gradient across the membrane. To restore the shape of Pgp, hydrolysis of a second molecule of ATP is needed.

Pgp expression correlates with a reduced rate of complete remission and poor prognosis in AML. About one third of AML patients express Pgp at diagnosis and at relapse AML patients often display increased drug resistance. However, Pgp has not been proven to be up-regulated at relapse indicating that this increase might be due to selection of resistant subpopulations and/or clonal expansion during chemotherapy. In vitro, long-term exposure of leukemia cell lines to cytostatic drugs, in increasing concentrations, results in a drug resistant phenotype with increased Pgp expression. It has also been shown that both Pgp substrates and non-Pgp substrates can induce Pgp mRNA and protein expression within four hours of exposure in leukemia cell lines and within 16 hours of exposure in AML patient samples.

Breast Cancer Resistance Protein. Studies on a breast cancer cell line resistant to mitoxantrone, but lacking over-expression of Pgp or MRP1, led to the identification of the breast cancer resistance protein (BCRP) in the late 1990s. The expression of BCRP is not specific for breast cancer cells, which is reflected in the names given by Miyake and co-workers and Allikmets and co-workers who simultaneously cloned the gene and called it mitoxantrone resistance (MXR) and placental ABC protein (ABCP), respectively. BCRP is the protein product of the ABCG2 gene that maps to chromosome 4q22. It has a molecular
lar mass of 95 kDa and contains 655 amino acids. BCRP has only one transmembrane domain and one nucleotide binding domain and is therefore known as a half-transporter, which is likely to form homodimers or homotetramers to function. The physiological role of BCRP is not fully understood but it is highly expressed in placenta, the intestine, and in a subpopulation of hematopoietic stem cells (side population). During differentiation of hematopoietic cells the expression of BCRP decreases.

In AML, expression of BCRP at diagnosis has been correlated to a drug resistant phenotype and poor prognosis. However, conflicting data has been published on whether BCRP is up-regulated at relapse compared to diagnosis.

Drug metabolism

Glutathione-s-transferase \(\pi\). Glutathione transferases (GSTs) are members of a superfamily of multifunctional enzymes. They have been found in almost every organism, from mammals to bacteria. The GSTs participate in the detoxification of various endogenous and exogenous compounds, including cytostatic drugs, by catalyzing their conjugation to glutathione. The conjugation is the second of two steps in which reactive molecules from step 1 are transformed into less toxic, usually water-soluble compounds that can be excreted through urine or bile. Human GSTs are classified into two distinct categories, i.e. 1) soluble or cytosolic and 2) membrane-bound microsomal. The soluble or cytosolic GSTs are highly polymorphic and are therefore subdivided into seven classes named: alpha, mu, omega, pi, sigma, theta and zeta. In AML over-expression of GST\(\pi\) is associated with unfavorable clinical outcome and resistance to cytostatic drugs. It is plausible that GST\(\pi\) confers drug resistance via both direct detoxification and via inhibition of the mitogen-activated protein (MAP) kinase pathway. GST\(\pi\) plays a key role in regulating the MAP kinase pathway, which participates in cellular survival and death signaling. In non-stressed cells, GST\(\pi\) sequesters the c-Jun N-terminal kinase 1 (JNK1, a signaling molecule in the MAP kinase pathway that is involved in stress response, apoptosis and cellular proliferation), in a GST\(\pi\):JNK1 complex. Exposure to cytostatic drugs leads to oxidative stress, which normally results in a dissociation of the GST\(\pi\):JNK1 complex and induction of apoptosis. However, elevated levels of GST\(\pi\) are associated with increased resistance to apoptosis by regulation of the MAP kinase pathway through JNK1.
CRIM1

CRIM1 is a cell-surface transmembrane protein with a large extracellular moiety\textsuperscript{138-139}. In human, the highest levels of CRIM1 mRNA have been detected in kidney and placenta. The biological significance of CRIM1 during development of, for example the eyes, the central nervous system and the kidneys, has been firmly established\textsuperscript{140-142}. In structure, CRIM1 resembles other developmentally important proteins (such as uterine sensitization associated gene-1) that are known to interact with the bone morphogenetic proteins (BMP)\textsuperscript{143}. BMPs signal through Smad pathways to regulate the fate of hematopoietic progenitor cells and stem cells\textsuperscript{144}. CRIM1 has been shown to interact with, among others, BMP4 and BMP7, by tethering the inactive pre-forms of BMP to the extracellular face of the plasma membrane (Figure 3). Whether this is the exact mode of action in myeloid hematopoietic cells is not known. In AML, higher expression levels of CRIM1 have been detected in cells with the mutation inv(16) than in cells with the mutation t(8;21), both of which are cytogenetic aberrations associated with relatively good prognosis\textsuperscript{137}.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{crim1.png}
\caption{CRIM1 tethers the inactive pre-forms of BMP to the extracellular face of the plasma membrane (Adapted from Larsson and Karlsson 2005\textsuperscript{144}.)}
\end{figure}
Methods to detect resistance

Prediction of drug resistance in clinical samples is important in the process of deciding what treatment the individual would benefit from. Detection of drug resistant phenotypes and genotypes is also an important tool in the development of new therapies that aim to overcome multidrug resistance. The presence and activity of markers associated with multidrug resistance (such as Pgp, GSTπ, and BCRP) and the effects of cytostatic drugs can be measured in vitro using several techniques including:

- Flow cytometry for protein expression, drug/dye efflux and drug toxicity (i.e. determination of living/dead cells).
- MTT, FMCA and bioluminescence/ATP assays for drug toxicity (i.e. determination of living/dead cells).
- Western blot and immunohistochemistry for protein expression.
- Real-time reverse-transcriptase polymerase chain reaction (RT-PCR) for mRNA expression.

Flow cytometry

The first flow cytometer was described in 1954 by Wallace Coulter. He described an instrument that could count and measure cell size electronically. In 1965 Kamentsky and co-workers described a two-parameter instrument that could measure nucleic acid content and size in viable cells. Flow cytometry is a system that analyzes cells or particles as they move in a liquid stream and pass through a laser beam in a sensing area. Characterization of the cells is based on size and granularity and whether the cell is carrying a fluorescent dye or antibody. When the cell passes through the laser beam the light is scattered in different directions. Light that is scattered in the forward directions at low angles (0.5-10°) is proportional to the size of the cell. Light that enters the cell and is reflected by the nucleus and other contents of the cell, (so called side scatter or 90° scatter) is proportional to the granularity of the cell. To assess for example differentiation, the presence of membrane bound antigens, enzyme activity or DNA content, the cells may be labeled with fluorochrome-conjugated antibodies or stained with fluorescent dyes. As the fluorochrome linked to the cell passes through the laser beam, the fluorochrome is excited and the emitted light is detected, converted into an electric pulse and recorded. Different fluorochromes emit light of different wave lengths, which enables simultaneous multiparameter analysis. In this thesis focus will be on fluorescein isothiocyanate (FITC)- and phycoerythrine (PE)-conjugated antibodies that are directed towards clusters of differentiation (CD) 13 and 33, Pgp, and CRIM1 and the fluo-
rescent dyes Rh123 (a Pgp substrate), propidium iodide (PI) and 7-amino-actinomycin D (7AAD).

Total tumor cell kill assays
Reliable and clinically relevant in vitro assays that test the effect of cytostatic drugs provide an important tool in the struggle to improve and individualize cytostatic drug treatment. In each of these assays, the common principle is to expose the leukemia cells in vitro to a panel of cytostatic drugs of different concentrations and then monitor the subsequent effect on cell survival or proliferation. In all of the assays cells are cultured for 3-4 days to allow time for dead cells to be detected, since lethally-damaged cells can retain an intact membrane for several days before they die. The principle difference between each assay is the various techniques used to detect cell viability.

The MTT assay is based on the ability of living cells to convert a soluble tetrazolium salt (MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) into insoluble formazan. Cells are cultured in the presence or absence of cytostatic drugs. Addition of the MTT dye causes the formation of formazan crystals by living cells. The formazan crystals are then dissolved in propanol and the color change is measured by spectrophotometer. Today the MTT assay is the most commonly used method to analyze in vitro drug resistance in patient cells and cell lines. There is a strong correlation between clinical outcome and drug resistance detected with the MTT assay in acute lymphoblastic leukemia. However, in AML, studies have shown a correlation between sensitivity to single cytostatic drugs and complete remission, but failed to show a correlation with initial response.

The FMCA (fluorometric microculture cytotoxicity assay) is based on the measurement of fluorescence. Non-fluorescent fluorescein diacetate (FDA) enters intact cells and becomes hydrolyzed into the fluorescent product fluorescein. Fluorescein is retained inside cells that have an intact plasma membrane for some time. The amount of fluorescence detected is dependent on the amount of living cells with an intact membrane. Hoechst 33342 is a fluorescent dye that intercalates into DNA in both living and dead cells, and becomes much more fluorescent when bound. The amount of fluorescence detected from Hoechst 33342 reflects the density of the cells. In AML, there is conflicting data as to whether in vitro sensitivity detected by FMCA correlates with clinical response.

The bioluminescence ATP assay estimates metabolic activity by measuring cellular ATP content and relies on the fact that the amount of ATP is relatively constant in viable cells of a specific cell type. If the respiratory cell cycle is
disturbed, ATP is rapidly degraded by ATP-ases. Therefore, the ATP content can be used as an indirect measure of cell growth or death. The bioluminescence ATP assay is designed to mimic in vivo conditions and the drug concentrations used are chosen to resemble the concentrations obtained in vivo\textsuperscript{168-171}. Although the in vitro drug sensitivity detected by the bioluminescence ATP assay has been shown to correlate with prolonged disease-free survival in AML, the predictive value of the bioluminescence ATP assay is still insufficient for directing therapy\textsuperscript{172-173}.

None of the methods described above distinguish between normal and malignant cells and therefore each method requires a homogenous cell population. In an attempt to identify and analyze only the malignant CD19-expressing population in acute lymphoblastic leukemia Campana and co-workers developed a stroma-supported immunocytometric assay\textsuperscript{174}. In this assay the flow cytometry forward scatter/side scatter characteristics, in combination with measurement of CD19 positivity of the cells, were used to identify viable non-apoptotic malignant cells.

**Western blot**

Western blot is a technique that is used to separate and identify proteins based on their molecular weight and ability to bind to specific antibodies. The proteins in a sample are separated using gel electrophoresis with an applied electrical charge. The most common type of gel electrophoresis is the SDS-PAGE, which includes a polyacrylamide gel (PAGE) with sodium dodecyl sulphate (SDS) loaded buffer. The proteins of interest are prepared and mixed in a buffer solution. It has been shown that Pgp is difficult to detect when samples are heated in Laemmli buffer, which is the most common way of preparing samples for Western blot\textsuperscript{87, 175}. Therefore in paper III in this thesis cell lysis and UREA-buffer were used to prepare each sample. After the samples are loaded into the wells of the gel, an electrical charge is applied, which causes smaller proteins to migrate faster through the gel and results in a separation according to molecular size. To make antibody detection possible the proteins are transferred onto a polyvinylidene difluoride (PVDF) membrane by electroblo"
Immunohistochemistry

The application of a labeled or enzyme-bound antibody that can be visualized by light microscopy, to identify a specific antigen is called immunohistochemistry. Immunofluorescence is a type of immunohistochemistry where fluorescence-conjugated antibodies are used. This labeling or staining method entails several steps including fixation of the sample, blocking of non-specific binding sites and antibody labeling, which can be either direct or indirect (i.e. two-step)\(^{148}\). The two-step method is achieved by allowing a primary un-labeled antibody, specific for the antigen of interest, to bind directly to the antigen on the cell surface. Subsequently a secondary fluorescence-conjugated antibody is allowed to react with the primary antibody. After labeling, the resultant fluorescence is detected using a confocal or fluorescence microscope. In paper IV in this thesis the indirect, two-step method was used to detect relevant proteins.

Real-time reverse transcriptase polymerase chain reaction

The 1993 Nobel Prize for Chemistry was awarded to Kary Mullis for his development of the polymerase chain reaction (PCR)\(^{176-177}\). Today PCR is a well-established and widely used technique to amplify short DNA sequences. PCR relies on the use of different temperatures to perform a three step reaction. The first step is the denaturation of double-stranded DNA. To do this high temperature is used to separate the two DNA strands. In the next step the temperature is rapidly lowered and oligonucleotide primers are allowed to anneal to the templates (single DNA strands). During the third step a thermostable DNA-polymerase (Taq polymerase) extends the primers and DNA is synthesized\(^{178}\). Together these three steps make a cycle and in every cycle each template renders one new copy. A typical PCR is performed in 40 cycles. After the PCR the amplified product is analyzed using other techniques.

PCR has been further developed over the years. The use of reverse-transcriptase, an enzyme with the ability to use mRNA as a template to generate a complementary strand of DNA (cDNA), led to the development of reverse-transcriptase PCR (RT-PCR)\(^{178}\). RT-PCR allows analysis of gene expression by determination of mRNA. It has been further developed by the use of fluorescent dyes, into a method named real-time RT-PCR, which allows quantification of the initial amount of mRNA template and monitoring of the accumulation of the amplified product in real time. In this thesis focus will be on real-time RT-PCR and the use of fluorescent TaqMan probes.

The TaqMan real-time RT-PCR assay was first described in 1996\(^{179-180}\). This assay exploits the properties of Taq polymerase as both a polymerase and an exonuclease (which has the ability to hydrolyze in the reverse direction), com-
bined with dual-labeled probes that only fluoresce when cleaved by the exonuclease activity of the Taq polymerase. The TaqMan probe is an oligonucleotide with one fluorescent and one quencher dye attached to it. As long as it is intact there is no fluorescent signal due to the absorption of the excitation signal by the quencher dye. When DNA is synthesized during the PCR the Taq polymerase reaches the TaqMan probe and the probe is hydrolyzed, which gives a fluorescent signal (Figure 4). The fluorescent signal is directly proportional to the amount of the target cDNA present in the reaction. The TaqMan real-time RT-PCR assay is fast and highly specific.

In paper III and IV the TaqMan real-time RT-PCR assay was used to perform a semi-quantitative analysis of the relative change in mRNA expression levels in response to exposure of cells to cytostatic drugs. In order to obtain reliable results all target genes were normalized to a housekeeping gene, i.e. 18S rRNA. Housekeeping genes must be stable throughout the cell cycle and in response to experimental conditions in order to be used in this assay. The small subunit of cytoplasmic rRNA in most eukaryotic cells contains 18S.

**Figure 4.** A schematic diagram showing the principle of real-time RT-PCR using the TaqMan approach. (Reprinted with permission from copyright owners.)
INHIBITORS

A large number of compounds with the ability to reverse multidrug resistance and sensitize resistant cells to the action of cytostatic drugs, in vitro and in vivo have been identified. These compounds include for example calcium channel blockers, calmodulin antagonists, steroids, cyclic peptides, and drug analogs. Mechanistically, they are either high-affinity substrates of the pump or efficient inhibitors of ATP-dependent transport. Pgp substrates like verapamil inhibit drug transport in a competitive manner while agents such as cyclosporine A (CyA) inhibit drug efflux by interfering with substrate recognition and ATP hydrolysis. Verapamil and CyA are so called first-generation Pgp reversing agents. They have the ability to inhibit Pgp but cause severe side effects in vivo. Second-generation inhibitors like valsporad (PSC833) were generated to increase specificity, potency and Pgp-binding affinity. However, even though the inhibition of Pgp was increased in vitro, in vivo studies did not demonstrate treatment improvement. Third-generation inhibitors, such as zosuquidar, were developed based on the information gained from second-generation inhibitors. Results from in vivo studies are still limited. Inhibitors are valuable in in vitro studies; however results from clinical trials in vivo are not conclusive.

CELL LINES

The very first human cell line (HeLa) was established from a uterine cervix carcinoma at Johns Hopkins University in Baltimore, Maryland, USA, in 1951. In 1963, the first continuous human hematopoietic cell line was established at the University of Ibadan, Nigeria, from a patient with Burkitt’s lymphoma. Cell lines are defined as cells continuously growing in vitro culture. Cell lines provide an extensively characterized monoclonal cell population that can be used as model systems. They can be viably frozen, stored almost infinitely and recovered without loss of cellular features. Investigation of resistance mechanisms in leukemic cells has been greatly aided by the use of cell lines that have been made resistant towards selected cytostatic drugs in vitro. Though cell lines are usually similar to the cells from which they were derived, it is important to keep in mind that the process by which they were immortalized most likely involve a number of genetic changes. Therefore cell lines should never be considered absolute equivalents of leukemia cells in vivo, but rather as a very useful complement that enables extensive studies not possible in vivo.

HL-60 cell line
The HL-60 cell line was established in 1976 from the peripheral blood of a 36-year-old Caucasian woman, first diagnosed with AML M3, and later corrected to AML M2\textsuperscript{198-199}. HL-60 has a doubling time of 25-40 hours and a diameter ranging from 9 µm to 25 µm (median 13 µm)\textsuperscript{199-200}. In 1995, Jönsson and co-workers exposed HL60 cells to increasing concentrations of doxorubicin to establish the drug resistant sub cell lines HL60 R0.5, HL60 R5 and HL60 R10, which are resistant to 0.5, 5.0 and 10.0 µM doxorubicin respectively\textsuperscript{93}. While wild type HL60 (HL60 S) does not express any detectable Pgp mRNA or Pgp protein, the drug resistant sub cell lines express increasing levels of Pgp, both mRNA and protein.

AIMS OF THE PRESENT THESIS

MAIN OBJECTIVE
The overall aim of this thesis has been to increase the knowledge and understanding of mechanisms involved in cytostatic drug resistance.

SPECIFIC AIMS
To determine the cytotoxic effects of idarubicin and DNR, and their main metabolites, on drug sensitive and drug resistant human HL60 cells (paper I).
To develop and evaluate the feasibility of a new \textit{in vitro} flow cytometry chemosensitivity assay, that allows viable CD13/CD33 expressing myeloid cells to be selectively identified and analyzed (paper II).
To investigate the effect of anticancer drugs, over time on the mRNA and protein expression levels of markers involved in drug resistance, i.e. Pgp, GST\textsubscript{π} and BCRP (paper III).
To explore putative differences in mRNA expression levels, in particular in CRIM1, but also in Smad5, BMP4 and BMP7, between drug sensitive and drug resistant leukemia HL60 cells (paper IV).
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MATERIALS AND METHODS

In this section the different methods and samples from patients and cell lines used, will be summarized.

ETHICS

All patient samples used in paper II were collected and include d after approval from the Swedish Ethical Committee in Uppsala and the patient’s informed consent.

PATIENTS

In paper II, peripheral blood or BM from 68 AML cell samples from 64 consecutively included patients were separated on Lymphoprep and washed twice in PBS. Sixty-three of the samples from 60 patients were technically successful and were included in the analysis. Chemosensitivity assays from 5 out of 68 samples could not be evaluated: 3 due to the difference between the duplicates in the control and 2 due to unsatisfactory antibody staining. Fifty-three of the 60 patients were classified according to the FAB criteria 201. In 42 of the samples the MDR phenotype was also analyzed. Detailed information on patient sample characteristics and achieved drug therapy is presented in the “material and methods” section of paper II.

CELL LINES

HL60S human leukemia cells were used in the experiments described in papers I, III and IV. This cell line is highly sensitive to doxorubicin. Furthermore, three drug resistant sub cell lines of HL60S were used, i.e. HL60 R0.5, HL60 R5 and HL60 R10, which are resistant to 0.5 µM, 5.0 µM and 10 µM of doxorubicin respectively. The degree of resistance for HL60 R0.5, R5 and R10 compared to HL60S, towards different cytostatic drugs are shown in Table 1.
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Table 1. Cross-resistance profile towards different cytostatic drugs for HL60 sub cell lines selected for resistance to doxorubicin.

<table>
<thead>
<tr>
<th>Cytostatic drug</th>
<th>IC$_{50}$HL60S (µM)</th>
<th>Degree of resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HL60R0.5</td>
<td>HL60 R5</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.07</td>
<td>14</td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>0.12</td>
<td>80</td>
</tr>
<tr>
<td>Idarubicin</td>
<td>0.005</td>
<td>12</td>
</tr>
<tr>
<td>Mitoxantrone</td>
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<tr>
<td>Etoposide</td>
<td>16.8</td>
<td>12</td>
</tr>
<tr>
<td>Amsacrine</td>
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<td>1.7</td>
</tr>
<tr>
<td>Ara-C</td>
<td>0.08</td>
<td>6.5</td>
</tr>
<tr>
<td>CdA</td>
<td>0.005</td>
<td>0.8</td>
</tr>
</tbody>
</table>

**CULTURING AND DRUG INCUBATIONS**

All cells were cultured in Roswell Park Memorial Institute medium (RPMI) 1640 supplemented with 10% heat-inactivated fetal bovine serum and 1% L-glutamine, in a humidified incubator (37°C, 5.0% CO$_2$ in air). Resistant cell lines were cultured in the absence of drugs for two weeks before the experiments were performed. HL60S does not express any detectable Pgp mRNA or protein as opposed to its resistant sub cell lines.

Cultured cells were suspended in pre-heated (37°C) culture medium in culturing tubes (papers I and II) or flasks (papers III and IV) at a concentration of 1.0 x 10$^5$ cells/ml and a final volume of 2 ml (papers I and II), or 5.0 x 10$^5$ cells/ml and a final volume of 8 ml (papers III and IV). Cells were exposed to the final drug concentrations for the incubation times shown in Table 2. Drug concentrations and different incubation times were chosen to mimic the *in vivo* conditions$^{168-171}$. In all experiments, cells cultured in RPMI 1640 without drugs were used as negative controls. After the incubation times the cells were centrifuged (400 g, 10 min, papers I and II and 400 g, 5 min, papers III and IV), and the supernatant was removed. The cells were then resuspended in fresh medium and incubated for 4 days. Where relevant the cytostatic drugs to be used for continuous incubation were added to the new medium.
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<table>
<thead>
<tr>
<th>Cytostatic drug</th>
<th>IC₅₀HL60S (µM)</th>
<th>Degree of resistance</th>
<th>HL60R₀.₅</th>
<th>HL60 R₅</th>
<th>HL60 R₁₀</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.07</td>
<td></td>
<td>14</td>
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<tr>
<td>Daunorubicin</td>
<td>0.12</td>
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<td>80</td>
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<tr>
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<td></td>
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<tr>
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CULTURING AND DRUG INCUBATIONS

All cells were cultured in Roswell Park Memorial Institute medium (RPMI 1640) supplemented with 10% heat-inactivated fetal bovine serum and 1% L-glutamine, in a humidified incubator (37°C, 5.0% CO₂ in air). Resistant cell lines were cultured in the absence of drugs for two weeks before the experiments were performed. HL60S does not express any detectable Pgp mRNA or protein as opposed to its resistant sub cell lines.

Cultured cells were suspended in pre-heated (37°C) culture medium in culturing tubes (papers I and II) or flasks (papers III and IV) at a concentration of 1.0 x 10⁵ cells/ml and a final volume of 2 ml (papers I and II), or 5.0 x 10⁵ cells/ml and a final volume of 8 ml (papers III and IV). Cells were exposed to the final drug concentrations for the incubation times shown in Table 2. Drug concentrations and different incubation times were chosen to mimic the in vivo conditions.

In all experiments, cells cultured in RPMI 1640 without drugs were used as negative controls. After the incubation times the cells were centrifuged (400 g, 10 min, papers I and II and 400 g, 5 min, papers III and IV), and the supernatant was removed. The cells were then resuspended in fresh medium and incubated for 4 days. Where relevant the cytostatic drugs to be used for continuous incubation were added to the new medium.

Table 2. Cytostatic drugs, final concentrations and incubation times used in the experiments included in this thesis.

<table>
<thead>
<tr>
<th>Cytostatic drug</th>
<th>Final conc (µM)</th>
<th>Cells</th>
<th>Incubation time</th>
<th>Paper</th>
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DRUG SENSITIVITY ASSAYS

*Bioluminescence ATP assay (paper I)*

After incubation with cytostatic drugs for 4 days, ATP was extracted from the samples by mixing equal volumes (100 µl) of sample cell suspension and trichloroacetic acid (TCA 27%). The extracts were assayed immediately or stored at -20°C until analyzed. Twenty µl of the extract was added to 900 µl Tris-EDTA buffer in a cuvette, and automatically analyzed in a BioOrbit photometer (Turku, Finland), as previously described. First, 100 µl of ATP monitoring reagent (AMR, luciferine-luciferase reagent from firefly) was added and the resulting light emission was measured. Second, 10 µl of ATP standard was added and the light emission was measured again. In this way the concentration of ATP in each sample can be determined. Results were given as nano mol of ATP per sample and the effect was calculated as the percentage of living cells in a drug-treated sample compared to a drug-free control. A high percentage indicates a high amount of viable cells and thus a population of drug-resistant cells.
Flow cytometry cytotoxicity assay (paper II)

After incubation with cytostatic drugs for 4 days 1.5 ml from each tube was transferred to a new tube and centrifuged at 400 g for 10 min. The supernatant was removed and 5 µl of the appropriate monoclonal antibody, i.e. CD13 or CD33 conjugated to phycoerythrin (PE), was added and incubated for 10 min in the dark at room temperature. The antibody was chosen on the basis of routine immunophenotyping. After incubation the cells were resuspended in 0.5 ml RPMI 1640. The fluorescent dye propidium iodide (PI) (0.5 µg/ml) was used to distinguish dead cells from viable cells. PI binds to DNA in the nucleus of cells with damaged cell membranes, but does not enter intact cell membranes\(^{202}\). After incubation for 10 min in the dark at room temperature the samples were analyzed on a FACScan flow cytometer equipped with an Argon laser and Lysis II software (Becton Dickinson). CD13- or CD33-positive and PI-negative cells were identified and counted for 30 seconds to enable quantification of viable myeloid cells. This method of quantifying cells has been described previously\(^{174, 203-204}\). Figure 5 shows the correlation between 5 known cell concentrations and the cells/time quantification assessed with the flow cytometer. The percentage of viable cells was calculated by comparing to a drug-free control.

\[\text{Figure 5. Correlation between five known concentrations of cells (cells/ml). The quantification was assessed by a FACScan flow cytometer (counted cells/30 s).}\]
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After incubation with cytostatic drugs for 4 days, 1.5 ml from each tube was transferred to a new tube and centrifuged at 400 g for 10 min. The supernatant was removed and 5 µl of the appropriate monoclonal antibody, i.e. CD13 or CD33 conjugated to phycoerythrin (PE), was added and incubated for 10 min in the dark at room temperature. The antibody was chosen on the basis of routine immunophenotyping. After incubation the cells were resuspended in 0.5 ml RPMI 1640. The fluorescent dye propidium iodide (PI) (0.5 µg/ml) was used to distinguish dead cells from viable cells. PI binds to DNA in the nucleus of cells with damaged cell membranes, but does not enter intact cell membranes.

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Figure 5. Correlation between five known concentrations of cells (cells/ml). The quantification was assessed by a FACScan flow cytometer (counted cells/30 s).

FUNCTIONAL PGP ASSAY

The functional tests of Pgp efflux activity (paper II) were performed with the highly fluorescent dye rhodamine 123 (Rh123), a substrate for Pgp, used in combination with the Pgp inhibitors verapamil, cyclosporin A (CyA) or PSC 833. The incubation cell concentration was 1.0 x 10⁶ cells/ml. After 4 days the samples were centrifuged (400 g for 10 min) and the supernatant was discarded. The cells were resuspended in fresh medium containing 10% fetal bovine serum, 50 ng/ml Rh123 and the appropriate monoclonal antibody (CD13 or CD33) conjugated to PE and incubated with 10 µM verapamil (Isoptin, Knoll), 2µM cyclosporine A (Sandimmun, Novartis), 3 µg/ml PSC 833 (Sandoz pharmaceuticals) or without inhibitor for 1 hour at 37°C. The incubation was terminated by the addition of ice cold RPMI 1640 and the samples were centrifuged at 400 g for 10 min at 4°C. To study the efflux phase the medium was removed and new dye-free medium, with or without the different inhibitors, was added. The cells were incubated for another 90 min at 37°C and then the intracellular Rh123 in CD13 or CD33 positive cells was analyzed with a FACScan flow cytometer. The ratio of Rh123 with/without inhibitor was calculated. A high ratio indicates functional Pgp that can be reversed by the inhibitors.

INTRACELLULAR DRUG UPTAKE

For in vitro drug uptake studies (paper I) the cell concentration used for incubations was 1.0 x 10⁶ cells/ml. After 4 days the incubation was terminated by the addition of 5 ml of ice cold PBS. Cells were kept on ice, washed twice in ice cold PBS and frozen at -20°C until analyzed. After thawing the cells were sonicated for 20 seconds at 50 W with a Branson B-12 sonicator (Branson Sonic Power Company, Danbury, CT, USA.) and the drugs were extracted with trichloroacetic acid (TCA, 27%). Extracted intracellular drugs were analyzed by photofluorometry using a Shimadzu model RF-510 spectrofluorometer (Shimadzu Seisakusho, Kyoto, Japan) with the wavelengths set to: λex 485nm and λem 560 nm. The anthracycline concentration in each sample was determined by comparing to identically-treated standard solutions and related to the amount of cell protein as determined according to Lowry and co-workers205.
DETERMINATION OF mRNA EXPRESSION LEVELS

RNA preparation and cDNA synthesis
Cells were incubated as described above and samples were collected before drug exposure, immediately at exposure (time 0), at 10 and 30 min and at 1, 2, 4, 8, 12, 16, 24 and 36 hours (paper III), or at 2 and 16 hours (paper IV). The incubation was stopped by adding 10 ml of ice cold RPMI 1640. Cells were centrifuged at 400 g for 5 min at 4°C and the supernatant was discarded. Immediately 600 µl of buffer RLT containing 10 µl/ml β-mercaptoethanol was added to the cells where after the samples were frozen at -20°C. Total RNA was isolated with an RNeasy® Mini Kit (Qiagen, Hilden, Germany) (paper IV) according to the manufacturer’s protocol. Total RNA from 4.0 x 10⁶ cells was eluted in 50 µl of RNase-free water and stored at -80°C until analyzed. The quality and concentration of the isolated RNA was determined with an Agilent Bioanalyzer 2100 and RNA 6000 Nano Assay Kit (Agilent Technology, Santa Clara, USA) according to the manufacturer’s protocol. The quality of the RNA was determined using the ratio of 28S to 18S. A ratio above 1.5 was used as a cut-off for acceptable RNA quality.

For first-strand cDNA synthesis an Omniscript® Reverse Transcription Kit (Qiagen) (paper III) or a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) (paper IV) was used according to the manufacturer’s protocol. Briefly, 1 µg (paper III) or 0.5 µg (paper IV) of total RNA was used to produce first-strand cDNA with oligo(dT)n (paper III) or dNTP (paper IV), in a final volume of 20 µl in RNase-free water. Samples were stored at -20°C until analyzed.

Real-time reverse transcriptase-polymerase chain reaction
To determine mRNA expression levels of Pgp, BCRP and GSTπ (paper III), and CRIM1, BMP4, BMP7 and Smad5 (paper IV), real-time RT-PCR was performed using the thermal cycler TaqMan 7500 Fast Real-Time PCR System (Applied Biosystems) with 7500 Fast Sequence Detection and Relative Quantification software packages. Cycling conditions were as follows, step one: 95°C for 2 min, step two: 95°C for 3 s and 60°C for 30 s, (step two repeated 40 times). Combined specific primers and probes were purchased from Applied Biosystems. 18S was used as a house-keeping gene (papers III and IV) and human placenta was used as positive control (paper IV). PCR reactions were performed in 20 µl (paper III) or 15 µl (paper IV) using the TaqMan Universal PCR Master Mix (Applied Biosystems) and 2 µl (paper III) or 1.5 µl (paper IV) cDNA.
Experiments were carried out in duplicate and mean values were used for the analysis.

DETERMINATION OF PROTEIN EXPRESSION LEVELS

Flow cytometry
After incubation and centrifugation as described above, pelleted cells were incubated with FITC-conjugated Pgp antibodies and the fluorescent dye 7-AAD at room temperature in the dark (paper III). After 10 min the cells were resuspended in 400 µl PBS and analyzed immediately using an EPICS® ALTRA™ (Beckman Coulter) flow cytometer equipped with an Argon laser and Expo™ 32 software. Pgp expression was determined as the ratio of mean fluorescence intensity in drug-exposed cells to fluorescence intensity in cells cultured in RPMI without drugs. Analyses were performed in duplicate and the mean value was used for analysis.

Western blot
After incubation and centrifugation as described above, the pelleted cells were lysed in 10 mM Tris/HCl lysis buffer (described in detail in the material and methods section of paper III), at 4°C for 10 min and stored in -80°C until analyzed. Protein concentrations were determined with a Multiscan Ascent (Thermo Labsystems). Twenty µg of protein lysate, diluted 2:1 in urea buffer, and a molecular weight marker were separated on 7% SDS gels at 140 V for 1 hour. After electrophoresis the proteins were transferred to PVDF membranes at 30 V overnight. Non-specific binding was blocked by 5% bovine serum albumin (BSA) in TBS buffer. The membranes were incubated at 4°C, overnight, with primary Pgp mouse monoclonal antibodies (C219, Abcam), diluted 1:500. After washing the blots were incubated with secondary goat-anti-mouse IgG antibodies conjugated to horseradish peroxidase (C1607, Santa Cruz Biotechnologies), at room temperature for 1 hour. Horseradish peroxidase was detected with a Molecular Imager® Chemidoc™ XRS, according to the manufacturer’s instructions.

Immunofluorescence
To determine the localization of CRIM1 in drug sensitive and drug resistant HL60 cells (paper IV), a concentration of 50 000 cells in 50 µl was allowed to dry on glass cover slips in air at room temperature, overnight. When dry the cells were fixed with cold methanol (-10°C) and air dried for 5 min at room tem-
temperature. Cells were rinsed in PBS with 0.1% saponin and washed in PBS. To minimize nonspecific binding the samples were blocked for 30 min in 1.5% BSA blocking solution. After blocking the samples were incubated with a primary polyclonal rabbit antibody against CRIM1, diluted 1:200 in PBS with 1.5% BSA blocking solution, overnight at 4°C. After the incubation cells were washed with PBS and incubated for 30 min with a FITC-conjugated secondary antibody diluted to 1 µg/ml in PBS with 1.5% BSA blocking solution. Counter staining with DAPI was used and the samples were analyzed using a Leica Confocal Systems Microscope.

**Statistical methods**

The differences in cytotoxic effect *in vitro* between different groups of patients (paper II) were evaluated with t-test for independent groups using Statistica 7 software. A p-value less than 0.05 (p<0.05) was regarded statistically significant.
RESULTS

In this section the results of the experiments included in this thesis will be presented paper by paper (I - IV).

INTRACELLULAR DRUG UPTAKE AND IN VITRO EFFECT (PAPER I)

At the same incubation concentrations, the intracellular uptake of idarubicin was higher than that of DNR, for both drug sensitive HL60S cells and drug resistant HL60R cells (Figure 6). In HL60S the increased uptake of idarubicin was most pronounced at concentrations ranging from 0.2 to 1.0 µM. In this concentration range, the uptake of idarubicin was 4 to 10-fold higher than that of DNR. In HL60R cells the uptake of idarubicin was more than 20-fold that of DNR at all concentrations tested.

The intracellular uptake of idarubicinol was, as for the parent substances, higher than that of daunorubicinol (Figure 6). For HL60S cells the difference was more than 10-fold higher at all concentrations tested. For HL60R cells, the incubation concentrations of idarubicinol and daunorubicinol only overlapped at 50µM. At that concentration (i.e. 50 µM) the uptake of idarubicinol was 5-fold higher than that of daunorubicinol.

Figure 6. A and B; Intracellular uptake of idarubicin (ida, black circles), idarubicinol (idol, white circles), daunorubicin (dnr, black squares) and daunorubicinol (dol, white squares) in A, drug sensitive, and B, drug resistant HL60 cells after incubation for 1 hour at different drug concentrations.
The intracellular uptake of the metabolites was lower than that of the parent substances at all concentrations tested. In HL60S cells the uptake of the metabolites was less than 20% than that of the parent substances. In HL60R cells the difference was less marked.

Idarubicin was consistently more toxic to both HL60S cells and HL60R cells. The concentration that caused a 50% inhibition of the cell growth (IC$_{50}$) in HL60S was 0.01 µM for idarubicin and 0.17 µM for DNR. In HL60R cells the IC$_{50}$ was 2.1 µM for idarubicin (i.e. 200-fold higher than in HL60S cells) and 165 µM for DNR (i.e. 1000-fold higher than in HL60S cells).

Idarubicinol was also consistently more toxic than daunorubicinol, to both HL60S and HL60R cells. In HL60S cells the IC$_{50}$ was 0.36 µM for idarubicinol and 3.9 µM for daunorubicinol. In HL60R cells the IC$_{50}$ was 15 µM for idarubicinol (i.e. 40-fold higher than in HL60S cells) and 350 µM for daunorubicinol (i.e. 90-fold higher than in HL60S cells).

Thus, the equitoxic incubation concentrations of the metabolites were 23 to 36-fold higher than that of the parent substances in HL60S cells, but only 2 to 7-fold higher in HL60R cells.

At IC$_{50}$ the intracellular concentration of both idarubicin and DNR was 0.5 nmol/mg protein in HL60S cells and 10 nmol/mg protein in HL60R cells. The intracellular uptake in HL60S cells at IC$_{50}$ was 0.4 nmol/mg protein for idarubicinol and 2.0 nmol/mg protein for daunorubicinol. In contrast, in HL60R cells the intracellular uptake at IC$_{50}$ was 30 nmol/mg protein for idarubicinol and 40 nmol/mg protein for daunorubicinol.

FLOW CYTOMETRY CHEMOSENSITIVITY TESTING (PAPER II)

Each of the patient samples used in this study were divided into two groups, i.e. de novo and secondary AML samples collected at 1) at diagnosis and 2) at relapse/resistant disease. These two groups revealed a wide distribution in in vitro responses to each drug (Figure 7). Furthermore, there was a distinct overlap in the responses to each drug when the separate drugs in each group were compared. Despite this overlap, the mean values were higher (i.e. sensitivity was lower) in the AML relapse/resistant disease group than in the AML at diagnosis group for ara-C (71% and 56%, p=0.03), DNR (69% and 65%, not significant (ns)), mitoxantrone (66% and 64%, ns) and amsacrine (50% and 43%, ns).
The intracellular uptake of the metabolites was lower than that of the parent substances at all concentrations tested. In HL60S cells the uptake of the metabolites was less than 20% than that of the parent substances. In HL60R cells the difference was less marked.

Idarubicin was consistently more toxic to both HL60S cells and HL60R cells. The concentration that caused a 50% inhibition of the cell growth (IC50) in HL60S was 0.01 µM for idarubicin and 0.17 µM for DNR. In HL60R cells the IC50 was 2.1 µM for idarubicin (i.e. 200-fold higher than in HL60S cells) and 165 µM for DNR (i.e. 1000-fold higher than in HL60S cells).

Idarubicinol was also consistently more toxic than daunorubicinol, to both HL60S and HL60R cells. In HL60S cells the IC50 was 0.36 µM for idarubicinol and 3.9 µM for daunorubicinol. In HL60R cells the IC50 was 15 µM for idarubicinol (i.e. 40-fold higher than in HL60S cells) and 350 µM for daunorubicinol (i.e. 90-fold higher than in HL60S cells).

Thus, the equitoxic incubation concentrations of the metabolites were 23 to 36-fold higher than that of the parent substances in HL60S cells, but only 2 to 7-fold higher in HL60R cells.

At IC50 the intracellular concentration of both idarubicin and DNR was 0.5 nmol/mg protein in HL60S cells and 10 nmol/mg protein in HL60R cells. The intracellular uptake in HL60S cells at IC50 was 0.4 nmol/mg protein for idarubicinol and 2.0 nmol/mg protein for daunorubicinol. In contrast, in HL60R cells the intracellular uptake at IC50 was 30 nmol/mg protein for idarubicinol and 40 nmol/mg protein for daunorubicinol.

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The AML at diagnosis group was then divided into 1) de novo AML and 2) secondary AML. The in vitro effect of the drugs on the number of viable cells was significantly higher in the de novo AML group compared to the secondary AML group for etoposide (44% and 59%, p=0.04) and amsacrine (38% and 63%, p=0.02) (Figure 8). The mean in vitro effect for DNR (64% and 71%), mitoxantrone (62% and 73%), ara-C (56% and 69%) and CdA (47% and 60%) on viable cell percentage was also higher at diagnosis in the de novo AML group compared to the secondary AML group, although none of the differences were significant.

Figure 7. In vitro effect in AML at diagnosis and at relapse/resistant disease. The symbols indicate the median, the box encompasses 50% and the box-whiskers 90% of the observations. * = p<0.05 compared to de novo AML at diagnosis.
The in vitro drug sensitivity of responders (CR after 1-2 induction courses) compared to non-responders in the de novo AML group, was also compared. In the responders group the mean values for effect were higher for amsacrine (34% and 48%), ara-C (54% and 67%) and CdA (44% and 54%), although the differences were not significant. Neither were there any significant differences in the in vitro drug sensitivities between the three early deaths compared to the responders in the de novo AML group.

The in vitro effect of DNR and mitoxantrone on viable cell percentages in Pgp positive and Pgp negative patient samples was then compared using the functional Pgp assay (Rh123) and the Pgp inhibitor verapamil. The mean in vitro effect in Pgp positive and negative cells was 75% and 60%, respectively, for DNR (p=0.06) and 80% and 60%, respectively, for mitoxantrone (p<0.01) (Figure 9). For ara-C the in vitro effect was 72% and 58%, respectively, (p=0.07) when using the inhibitor verapamil. However, when the inhibitors cyclosporine A

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**Figure 8.** In vitro effect in de novo and secondary AML at diagnosis. The symbols indicate the median, the box encompasses 50% and the box-whiskers 90% of the observations.
The in vitro drug sensitivity of responders (CR after 1-2 induction courses) compared to non-responders in the de novo AML group, was also compared. In the responders group the mean values for effect were higher for amsacrine (34% and 48%), ara-C (54% and 67%) and CdA (44% and 54%), although the differences were not significant. Neither were there any significant differences in the in vitro drug sensitivities between the three early deaths compared to the responders in the de novo AML group.

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Figure 9. In vitro effect of daunorubicin and mitoxantrone on percentage of viable cells in Pgp negative and positive AML at diagnosis. Verapamil has been used as a Pgp inhibitor in the functional Pgp assay (Rh123). The symbols indicate the median, the box encompasses 50% and the box-whiskers 90% of the observations.

and PSC833 were used there was a significantly increased effect in Pgp positive cells (p<0.05).

CHANGES IN PGP mRNA AND PROTEIN EXPRESSION (PAPER III)

In the following section all mRNA and protein expression levels are presented as a comparison to expression in cells cultured in RPMI without drugs.

Changes in mRNA expression after drug exposure

HL60S: In vitro exposure to ara-C for 10 min resulted in a 1.7-fold increase in ABCB1 mRNA (Pgp), compared to cells cultured in RPMI. The increase remained unchanged throughout the 36 hour experiment. After exposure to DNR for 24 hours, a 1.6-fold increase in ABCB1 mRNA level was seen.
Exposure to ara-C resulted in a small decrease in GSTP1 mRNA (GST\textsubscript{\pi}) levels after 10 min and minor changes at the later time-points. Exposure to DNR showed a trend towards increased GSTP1 mRNA levels since a 1.8-fold increase was seen after 12 hours of exposure.

**HL60 R0.5:** Culture of HL60R0.5 cells in RPMI medium revealed a spontaneous increase in the ABCB1 mRNA expression level, which was also seen on protein level when verified by Western blot (Figure 10). After 10 min of exposure to ara-C, a 3.1-fold increase in ABCB1 mRNA expression above the spontaneously increased baseline levels was detected. The increase then declined over time and after 2 hours no increase was evident compared to cells cultured in RPMI. After 10 min of \textit{in vitro} exposure to DNR a 2.8-fold increase in ABCB1 mRNA expression above baseline levels was observed, after which the expression declined, and after 4 hours no increase was evident compared to cells cultured in RPMI. A 3.4-fold increase in GSTP1 mRNA levels was observed after 10 min exposure to ara-C. This increase declined during the incubation time and was undetectable after 36 hours. Exposure to DNR resulted in a 2.4-fold increase in GSTP1 mRNA expression after 10 min and this level remained increased throughout the incubation time.

**Figure 10. A spontaneous increase in the ABCB1 mRNA expression level of drug resistant HL60 R0.5 cells, during 36 hours, was verified by Western blot.**

**HL60 R5:** \textit{In vitro} exposure to ara-C for 10 min resulted in a 1.9-fold increase in ABCB1 mRNA expression. Following a period of decline the expression further increased to 2.4-fold at 12 hours and 4.5-fold at 36 hours of exposure.
Compared to cells cultured in RPMI, only negligible changes in ABCB1 mRNA expression were observed during 36 hours of exposure to DNR. After 10 min of exposure to ara-C there was a 4.0-fold increase in GSTP1 mRNA expression. This increase was further enhanced to 5.8-fold after 36 hours. In vitro exposure to DNR resulted in a 1.5-fold increase after 1 hour, was further increased during the exposure time and reached a maximum 4.0-fold increase after 36 hours.

The BCRP mRNA expression levels were undetectable in all three cell lines throughout the experiment despite being detectable in positive controls.

**Pgp protein detected by Western blot**

In HL60S cells exposed to ara-C, Pgp expression was detectable after 8 hours, while no Pgp expression was detected during 36 hours of exposure to DNR. The high levels of Pgp expressed by HL60 R0.5 and HL60 R5 did not change throughout the entire 36 hours of exposure to ara-C or DNR. A spontaneous increase in the Pgp expression level was seen continuously for 24 hours in HL60 R0.5 cells cultured in RPMI (Figure 9).

**Pgp protein detected by flow cytometry**

In HL60S cells all results are adjusted to viable cells only. Cell death in HL60 R0.5 and HL60 R5 cells were negligible during the 24 hours of drug exposure. Untreated HL60S cells did not display any detectable Pgp, while untreated HL60 R0.5 and HL60 R5 cells expressed 1.9-fold and 3.2-fold higher levels of Pgp, respectively, compared to HL60S cells (Figure 11).

In HL60S cells no increase in Pgp expression was detected during 24 hours of exposure to ara-C or DNR. In HL60 R0.5 cells exposed to ara-C a 1.2-fold increase in Pgp expression was seen after 24 hours. No increase was seen after exposure to DNR (Figure 12). In HL60 R5 cells a 1.3-fold increase in Pgp expression was seen after 24 hours of exposure to ara-C, while there was no increase detected after exposure to DNR.
Figure 11. Pgp expression in HL60S cells (dotted line), HL60 R0.5 (broken line) and HL60 R5 (continuous line) cells, determined by flow cytometry.

Figure 12. Pgp expression in HL60 R0.5 cells before drug exposure (grey line), after 24 hours of culturing in RPMI (dotted line), after 24 hours of exposure to daunorubicin (broken line) and after 24 hours of exposure to ara-C (continuous line).
mRNA expression levels in drug resistant HL60 R0.5 cells were calculated by comparing to expression levels in drug sensitive HL60S cells. To follow changes in expression levels over time, the expression level at time 0 for each cell line was set to 1, and therefore levels are only comparable within each cell line.

**CRIM1:** Before drug exposure, CRIM1 mRNA expression levels in drug resistant HL60 R0.5 cells were 15-fold higher compared to CRIM1 mRNA expression levels in drug sensitive HL60S cells (Figure 13). After exposure to DNR or ara-C for 2 hours, CRIM1 mRNA levels increased 3- and 2-fold, respectively, in HL60S. Exposure to DNR for 16 hours resulted in a 5.6-fold increase in CRIM1 mRNA expression, whereas exposure to ara-C for 16 hours did not result in any further increase (Figure 14). Exposure to DNR or ara-C for 16 hours did not affect the CRIM1 mRNA expression level in HL60 R0.5. However, culturing of HL60 R0.5 in RPMI resulted in a decrease to 62% of baseline CRIM1 mRNA expression levels, after 16 hours (Figure 15).

**Smad5:** The pattern of Smad5 mRNA expression levels was found to resemble CRIM1 mRNA expression levels in both HL60S and HL60 R0.5 cells. Prior to drug exposure, the baseline level of Smad5 mRNA in HL60 R0.5 cells was 8-fold higher compared to the baseline levels in HL60S cells (Figure 13). After exposure to DNR or ara-C for 2 hours, Smad5 mRNA expression levels increased 5- and 3-fold, respectively. After 16 hours of exposure to DNR, Smad5 mRNA expression levels increased 8-fold, whereas 16 hours of exposure to ara-C resulted in a 1.8-fold increase in Smad5 mRNA expression.

**BMP4:** Before drug exposure baseline BMP4 mRNA expression levels were 3.4-fold higher in drug resistant HL60 R0.5 cells compared to HL60S cells (Figure 13). No further increase was seen over time after exposure to DNR or ara-C in HL60S or HL60 R0.5 cells.

**BMP7:** Neither HL60S nor HL60 R0.5 expressed detectable levels of BMP7 mRNA despite being detectable in positive controls.

**Pgp:** The Pgp mRNA expression levels were determined and compared to CRIM1 mRNA levels in HL60S and HL60 R0.5 cells. The quotient of Pgp/CRIM1 was consistent (i.e. approximately 1) in both cell lines.
Figure 13. CRIM1, Smad5 and BMP4 mRNA levels in drug sensitive and resistant cell lines. When baseline CRIM1, Smad5 and BMP4 mRNA levels were set to 1 in drug sensitive HL60S cells, CRIM1 mRNA levels before drug exposure were 15-fold higher in drug resistant HL60 R0.5 cells. Smad5 mRNA levels were 8-fold higher in drug resistant HL60 R0.5 cells. BMP4 mRNA levels in HL60 R0.5 before drug exposure were 3.4-fold higher compared to HL60S.
Figure 14. CRIM1 mRNA levels in drug sensitive cells. Culturing of drug sensitive HL60S cells in the absence of drugs resulted in a 1.6-fold increase in CRIM1 mRNA levels, when baseline levels were set to 1. After exposure to daunorubicin or ara-C for 2 hours, CRIM1 mRNA levels increased 3- and 2-fold, respectively. After 16 hours of exposure to daunorubicin, CRIM1 mRNA levels increased 5.6-fold whereas 16 hours of exposure to ara-C did not result in any further increase.

Figure 15. CRIM1 mRNA levels in drug resistant cells. When baseline levels were set to 1, CRIM1 mRNA levels in drug resistant HL60 R0.5 cells, cultured in the absence of drugs, were 62% lower at 16 hours compared to baseline levels at the start of culturing. Exposure of drug resistant HL60 R0.5 cells only resulted in minor changes in CRIM1 mRNA levels.
Determination of CRIM1 localization
Simultaneous staining with FITC-conjugated CRIM1 antibody and DAPI confirmed that CRIM1 is located to the membrane surface of HL60 R0.5 cells (Figure 16).

Figure 16. CRIM1 localization in drug resistant cells. Simultaneous staining of drug resistant HL60 R0.5 cells with FITC-conjugated CRIM1 antibody (green) and DAPI (blue), confirms that CRIM1 is located at the membrane surface of myeloid leukemia HL60 R0.5 cells.
DISCUSSION

In the following section the results of the present studies will be discussed in relation to findings reported by others and possible explanations focusing on the aim of each study will be presented. The final section includes methodological considerations and thoughts on future research.

COMPARISON OF IDARUBICIN AND DAUNORUBICIN AND THEIR MAIN METABOLITES REGARDING INTRACELLULAR UPTAKE AND IN VITRO EFFECT (PAPER I)

The anthracyclines DNR and idarubicin are widely used in the treatment of AML. It has been claimed that idarubicin, which is more toxic in vitro compared to DNR, is also more potent in increasing the rate of complete remission, prolonging the duration of remission and improving survival\textsuperscript{206-207}. This however, remains to be established.

\textit{In vivo}, both DNR and idarubicin are metabolized into an active metabolite that has anti-tumor activity\textsuperscript{208-209}. Within a few minutes of administration, the plasma concentration of the metabolite daunorubicinol exceeds that of the parent substance and the metabolite is retained in the plasma much longer\textsuperscript{210-213}. Compared to daunorubicinol the plasma concentration of idarubicinol is higher and the retention is longer\textsuperscript{213}. These high and prolonged concentrations of idarubicinol have been suggested to contribute to the difference in clinical activity of idarubicin compared to DNR\textsuperscript{207}. However, the main target for all anthracyclines is in the nucleus within the cell and studies have shown that there is no simple correlation between plasma concentration and retention, and intracellular concentration\textsuperscript{214-217}.

We found that the intracellular uptake of idarubicin was considerably higher than that of DNR, which is in accordance with results from other studies, and that the uptake of both metabolites was low\textsuperscript{206, 218-219}. Also in accordance with previous studies, we found that the IC\textsubscript{50} value determined for DNR and daunorubicinol was more than 10-fold higher than that of idarubicin and idarubicinol\textsuperscript{220-221}. When the cytotoxicity of the parent substances and the metabolites were correlated to the intracellular uptake, we found that idarubicin and DNR were equally toxic at the same intracellular concentrations. For the metabolites, intracellular concentrations in the same range as those of the parent substances were required to achieve the same cytotoxic effect.
Clinically, the equally toxic dose of idarubicin is 20-25% compared to that of DNR, and a previous study showed that it reaches an intracellular peak concentration that is 70% that of DNR\textsuperscript{222}. In the same study, the peak intracellular concentration of the metabolites was approximately 7% that of the parent substances, but the concentration remained constant during a 24 hour period\textsuperscript{222}. Given this and the findings of low intracellular concentrations of idarubicinol in the present study, it appears as if the contribution of the metabolite to the clinical effect of idarubicin is small. However, it cannot be ruled out that the presence of intracellular metabolites at low concentrations over time contributes to the clinical effect. Even so, these results indicate that it is the pharmacokinetic properties of idarubicin, rather than idarubicinol that provide the clinical effect. It has been suggested that it is the lipophilic properties of idarubicin that are responsible for its higher intracellular uptake, by facilitating the diffusion of the drug into the cells\textsuperscript{209}. However, clinically no significant differences in favor of idarubicin compared to DNR regarding long-term survival (> 5 years) have been reported\textsuperscript{49, 223-224}.

Recent studies on intracellular uptake of idarubicin and DNR in relation to apoptosis and clinical response conclude that there is a clear concentration-response relationship between intracellular concentrations and apoptosis, which supports the use of intracellular uptake to measure the cytotoxic effect\textsuperscript{206, 225}.

**In vitro chemosensitivity testing of selected myeloid cells (Paper II)**

*In vitro* chemosensitivity testing in AML has been studied for many years. Numerous attempts to develop assays that might predict individual response have been made and correlations to both short-term and long-term outcome have been reported\textsuperscript{226-227}. Nevertheless, none of the techniques available have been established in clinical practice in terms of directing the choice of treatment\textsuperscript{228}. We aimed to establish a new flow cytometry chemosensitivity assay, where myeloid cells are identified by their CD13/CD33 expression and dead cells are excluded by PI uptake.

Today, the most widely used *in vitro* chemosensitivity assays are different short-term total cell kill assays (such as measurement of cellular ATP, FMCA and MTT assays). These techniques measure cell death in the whole cell population using different indicators of cell death. There are many factors that might influence the accuracy and predictive value of these assays. The most important factor is probably that *in vitro* the assays fail to account for the important pharmacokinetic aspects and interactions that occur *in vivo*. Another is that the...
procedure of cell collection, separation and incubation can have an impact on how the cells react on exposure to cytostatic drugs. It has been claimed that cryopreservation does not affect the cells, but our experience is that cryopreservation makes the cells more vulnerable and possibly causes misleading results; therefore only fresh samples were used in our study\textsuperscript{149, 163}. There is also a risk of contamination of the leukemia cells with non-malignant cells\textsuperscript{174}. Separation of mononuclear cells by density gradient usually renders a preparation of mononuclear cells of high purity. However, even with a high content of mononuclear leukemia cells, there are a proportion of mononuclear non-leukemia cells like, e.g. lymphocytes and monocytes, which may bias the results\textsuperscript{229}. Another important issue is the heterogeneity of seemingly homogenous malignant cell populations\textsuperscript{230}. Small subpopulations of resistant cells can pass by undetected by chemosensitivity assays and give incorrect predictions. These cells may later be responsible for the relapse of the disease.

In an attempt to refine the technique and only analyze the leukemia cells, Campana and co-workers developed a stroma-supported immunocytometric assay for acute lymphoblastic leukemia\textsuperscript{174}. Expression of CD19 was used to identify leukemia cells and the fact that cells undergoing apoptosis change their light scattering properties was exploited to identify viable cells\textsuperscript{174}. Based on the technique described above we focused on the problem of contamination with non-malignant cells and developed a similar technique for AML, with some important differences. We used the phycoerythrin conjugated myeloid CD13 and CD33 monoclonal antibodies, which exclude lymphocytes but in addition to the leukemia cells, also stain monocytes\textsuperscript{203-204}. To distinguish viable cells from non-viable cells we used the fluorescent dye PI. PI will not pass intact cell membranes, but passes freely through a disrupted membrane, and binds irreversibly to the nuclear DNA of dead cells\textsuperscript{202}.

With this new method we compared \textit{de novo} and secondary AML in different stages of the disease and with different response to treatment. We found greater resistance in the secondary AML group and the resistant disease/relapse group, even if there was a wide distribution of the results. The presence of secondary AML has previously been associated to increased \textit{in vitro} resistance\textsuperscript{231}. Most drugs showed higher activity in samples from the responders, but the differences were not significant. Other studies have also shown inconclusive results regarding the \textit{in vitro} differences between responders and non-responders, as determined by other chemosensitivity tests\textsuperscript{160, 172, 232-234}.

Expression of Pgp has been shown to be a prognostic marker for clinical drug resistance in AML\textsuperscript{224}. Our results also showed that drugs regarded as substrates for Pgp, such as DNR and mitoxantrone, exhibited decreased \textit{in vitro}
activity in Pgp positive cells. However, there was also a tendency for ara-C, which is not regarded a substrate for Pgp, to have less activity in Pgp positive cells. This could be explained by a down regulation of deoxycytidine kinase or by activation of other mechanisms of drug resistance such as GST\textsubscript{π}\textsuperscript{135, 235}.

The predictive value of the different chemosensitivity assays are much the same, and in this respect we found no obvious advantage in the new flow cytometry assay. One should keep in mind, however, that in the context of drug resistance in vitro chemosensitivity testing provides only an approximate estimation of the resistance profile of an individual, without revealing anything about the specific mechanisms involved. The flow cytometry assay is technically feasible but must be further developed and combined with other assays.

**Drug Concentrations and Incubations (Papers I, II, III and IV)**

There are at least three different ways to incubate cells in vitro with cytostatic drugs in order to determine the effects of various concentrations of the drugs. One alternative is to obtain a full concentration-effect curve with several concentrations of each drug\textsuperscript{233}. Another is to test a number of samples at a wide range of concentrations. The concentration that results in the largest scatter of results is then chosen as the appropriate concentration for further testing\textsuperscript{161}. Yet another is to find concentrations that mimic the in vivo situation based on the comparison of intracellular uptake in vivo and in vitro\textsuperscript{170}. In papers II, III and IV, drug concentrations were chosen to mimic the in vivo situation, because it is reasonable to believe that the mechanisms by which the drugs are processed could be different at different concentrations. Therefore, we used concentrations similar to the concentrations achieved in vivo. Previous studies with other chemosensitivity assays using these concentrations have demonstrated a high predictive accuracy for both drug sensitivity and drug resistance in patients diagnosed with AML\textsuperscript{234}. In paper I, a wide range of concentrations were used and the IC\textsubscript{50} value for each drug was determined from a curve of incubation-concentration versus effect and then used for further calculation of the intracellular drug uptake.
Expression of markers involved in drug resistance during exposure to cytostatic drugs (Paper III)

The emergence of drug resistance during long-term chemotherapy is believed to be due to the selection or induction of an MDR phenotype, and/or clonal development, although a higher incidence of MDR at relapse has not been consistently proven\textsuperscript{236-237}. It is known that Pgp expression can be induced in drug sensitive leukemia cell lines by long-term exposure to cytostatic drugs \textit{in vitro}\textsuperscript{93}. The short-term effect of exposure to cytostatic drugs \textit{in vitro} has been studied to a more limited extent\textsuperscript{94-97, 238}. However, an early induction of drug resistance in leukemia cells may be of clinical importance. Therefore, the aim of our study was to further investigate the short-term effect of DNR and ara-C on the expression levels of Pgp, BCRP and GST\textit{\pi} in both drug sensitive and drug resistant leukemia cells.

\textbf{Pgp mRNA expression}

When drug sensitive and drug resistant HL60 myeloid leukemia cells were exposed to ara-C we found that mRNA levels of Pgp were increased, more rapidly than previously reported. This increase in Pgp mRNA, after short-term exposure to cytostatic drugs, is in accordance with the results of previous studies in non-myeloid cells\textsuperscript{94, 96-97, 238-239}. When drug sensitive and drug resistant HL60 cells were exposed to DNR the effects were not as pronounced as that in response to ara-C. As expected the concentration of DNR used in our study (0.2 µM) did not have an effect on HL60 R5 cells, which are resistant to more than 5 µM DNR, and the effect on HL60 R0.5 cells, which are resistant to more than 0.5 µM DNR, was moderate. However, these concentrations were chosen to mimic the \textit{in vivo} situation\textsuperscript{93}.

\textbf{GST\textit{\pi} and BCRP mRNA expression}

In addition to increased Pgp levels, there was also a rapid increase in GST\textit{\pi} mRNA levels in the drug resistant but not drug sensitive HL60 cells after exposure to ara-C. Exposure to DNR, on the other hand, resulted in an increase in GST\textit{\pi} mRNA levels after 10 minutes in HL60 R0.5 cells, and after 12 hours in drug sensitive HL60 cells. No increase was seen in HL60 R5 cells.

It has been suggested that elevated levels of GST\textit{\pi} might have an anti-apoptotic effect mediated via JNK1, which is involved in apoptotic signaling through the MAP kinase pathway\textsuperscript{118, 240}. The increased GST\textit{\pi} levels may reflect a general response to stress, e.g. exposure to ara-C, making them less prone to apoptosis. This is supported by the findings reported by others that GST\textit{\pi} over-
expressing cells are resistant to apoptosis induced by various drugs, rather than to a specific drug\textsuperscript{240-241}.

\textit{Pgp protein expression}

In drug sensitive HL60 cells that normally do not express detectable levels of Pgp the mRNA findings were confirmed by Western blot, which revealed detectable Pgp protein after 8 hours of ara-C exposure.

As expected, Pgp protein was detectable before drug exposure in both of the drug resistant HL60 cell lines. No changes in Pgp protein levels in these cell lines were detected by Western blot. However, in both of the drug resistant HL60 cell lines, HL60 R0.5 and HL60 R5, a 1.2- and 1.3-fold increase in Pgp protein levels, respectively, was detected by flow cytometry after 24 hours of ara-C exposure. The results should be compared to the initial 1.9-fold difference in Pgp protein expression between drug sensitive HL60 cells and drug resistant HL60 R0.5, which corresponds to an 80-fold difference in the degree of drug resistance\textsuperscript{93}. This suggests that a small increase in the expression of Pgp protein, as detected by flow cytometry, may correspond to a major increase in the degree of drug resistance.

We found a spontaneous increase in both Pgp mRNA and protein levels in drug resistant HL60 R0.5 cells cultured in RPMI. Since the expression levels decreased after 24 hours it is reasonable to believe that this increase might be a general response due to the handling of the cells. It has been shown previously that e.g. heat shock and other environmental factors can result in transient induction of Pgp\textsuperscript{242}. However, to determine the effect of drug exposure on mRNA and protein levels, the spontaneous increase was subtracted. This calculation resulted in no increase in Pgp protein expression being detected in the drug resistant cell lines after exposure to DNR. Hu and co-workers determined Pgp protein expression and function by flow cytometry in samples that were derived from patients with myeloid leukemia and exposed \textit{in vitro} to DNR and ara-C\textsuperscript{95}. After 16 hours of drug exposure an increase in Pgp expression was detected that correlated with Pgp function\textsuperscript{95}. No corrections for the spontaneous increase in Pgp protein were made, which may explain the higher mean fluorescence intensity reported. In the same study an increase in Pgp protein expression \textit{in vivo} was demonstrated after simultaneous exposure to DNR and ara-C\textsuperscript{95}. This increase correlated well with our results \textit{in vitro} after subtracting the spontaneous increase. One could therefore speculate that the major increase in Pgp expression \textit{in vivo} was due to exposure to ara-C rather than DNR. With respect to this, the sequential scheduling of DNR and ara-C during induction therapy could be of importance.
Expression of Crim1, Smad5, Bmp4 and Bmp7 (Paper IV)

Expression of the cysteine rich transmembrane bone morphogenetic protein regulator 1 (chordin-like) or Crim1 has been studied in cells originating from the kidneys, placenta, eyes, blood vessels and the central nervous system and its biological significance in the development of these organs has been well established. In a micro array study, Ichikawa and co-workers found that the expression of Crim1 mRNA varied in leukemia cells from patients with different cytogenetic aberrations. Higher levels of Crim1 mRNA were detected in cells with inv(16) than in cells with t(8;21), which are both genotypes associated with relatively good prognosis in AML. These results suggest that if the expression of Crim1 mRNA varies in cells with different cytogenetic aberrations it may also vary in cells with different genotypes with respect to drug resistance.

Expression of Crim1
We investigated the expression levels of Crim1 mRNA in drug sensitive and drug resistant HL60 cells, before and after exposure to ara-C and DNR for 16 hours. In addition, the mRNA levels for proteins related to the biological effect of Crim1 i.e. Smad5, Bmp4 and Bmp7, were analyzed. Before drug exposure baseline Crim1 mRNA levels in drug resistant HL60 R0.5 cells were 15-fold higher compared to sensitive HL60 cells. These levels were maintained for at least 16 hours and increased after drug exposure. When Crim1 mRNA expression was compared with the mRNA expression of Pgp, which is over-expressed in the drug resistant HL60 R0.5 cells, we found that there was a covariance in both drug sensitive and drug resistant HL60 cells. This covariance may be an indication of general activation of cellular defense mechanisms, initiated by drug exposure, or it may possibly be incidental. However, the fact that Crim1 mRNA levels in unexposed drug resistant cells were initially 15-fold higher than in drug sensitive cells, and the fact that the Crim1 mRNA levels increased after exposure to cytostatic drugs, suggests that Crim1 may play a role in an independent mechanism associated with drug resistance.

Expression of Smad5 and Bmp4
It has been shown that Bmps inhibit proliferation and induce differentiation of hematopoietic cells, and that constitutive activation of Bmps causes an increase in the commitment of hematopoietic progenitors to myeloid differentiation. Since Crim1 affects the levels of Bmps and thereby of Smad5, Crim1 may be...
of importance in the regulation of the growth and differentiation of hematopoietic cells.

Unlike CRIM1, the mRNA expression levels of Smad5 decreased over time in drug resistant cells, but similar to CRIM1 the levels remained higher than the levels detected in drug sensitive cells. It has been reported that Smad5 can be both up- and down-regulated by CRIM1. We found a covariance in the mRNA expression levels of CRIM1 and Smad5, suggesting that Smad5 may either be affected by exposure to cytostatic drugs or that there may be an alternative system regulating Smad5 mRNA expression levels. However, Smad signaling in hematopoiesis has been found to be very context dependent and the regulation of progenitor cells is much more complex in vivo than in vitro.

Baseline mRNA expression levels of BMP4 were also higher in drug resistant cells, compared to drug sensitive cells. Similarly to Smad5 mRNA levels, BMP4 mRNA levels decreased over time but, unlike CRIM1 and Smad5 mRNA levels, BMP4 mRNA levels became equal to the expression levels found in drug sensitive cells. This is in accordance with the findings of Wilkinson and co-workers that CRIM1 acts as a BMP4 antagonist.

METHODOLOGICAL CONSIDERATIONS

Cell lines and leukemia cells from patients versus the in vivo situation
The use of cell lines in in vitro models is convenient. The advantages are many; e.g. unlimited access to homogenous leukemia cells with well characterized genotype and phenotype, comparability to previous results by other researchers, possibilities to produce cells resistant to certain drugs and to a certain concentration of the drug, access to highly resistant cells that can be used as positive controls, few ethical constraints, etc. However, cell lines have been removed from the in vivo context and hence, results from in vitro experiments with cell lines may lead to conclusions that are not applicable in the in vivo situation. Nevertheless, as long as one keeps in mind that in actuality the in vivo conditions are much more complex with many more dimensions than the in vitro situation, in vitro cell line experiments form a methodological basis for further studies.

Leukemia cells from patients diagnosed with AML provide a more clinically relevant material than cell lines but still cannot reflect important in vivo pharmacological aspects when used in in vitro testing. In this study samples drawn from BM and peripheral blood were considered equally comparable, since studies have shown that their protein expression patterns are equivalent. A high de-
gree of correspondence in mRNA expression levels has been reported and no significant differences in the CD33 expression levels between BM and peripheral blood has been found.

**mRNA and protein expression**

The relationship between mRNA expression levels and the corresponding protein levels is not fully known. Anderson and co-workers studied the correlation between protein and mRNA expression of 19 proteins in the human liver and found a somewhat positive correlation. Whilst, in other studies, significant correlations were found only in a subset of the proteins analyzed. Orntoft and co-workers found highly significant correlations when analyzing the changes in mRNA and protein expression levels in human carcinomas. Like Orntoft and co-workers we focused on the changes in mRNA and protein expression levels. However, the use of mRNA expression by itself is not sufficient for understanding the corresponding expression of protein as several post-transcriptional factors, such as protein modification and degradation, influence the level of protein present in a given cell at a given point in time. Therefore, the mRNA and protein levels of expression should be considered complementary, but not necessarily corresponding.
CONCLUSIONS

Referring to the overall aim of this thesis to increase the knowledge and understanding of mechanisms involved in cytostatic drug resistance, the following conclusions are drawn:

Idarubicin and DNR are equally toxic at the same intracellular concentrations.

The contribution of the main metabolites on the cytotoxic effect of idarubicin and DNR, in drug sensitive and drug resistant human HL60 cells, is low.

The new flow cytometry chemosensitivity assay identifies viable CD13/CD33 expressing myeloid cells selectively and the results correlate to clinical parameters, such as secondary AML and resistant disease. However, the predictive value compared to other assays was not improved.

Pgp mRNA and protein expression levels, as well as GSTπ mRNA levels, are rapidly increased in leukemia cell lines with different levels of drug resistance following short-term exposure to ara-C. Clinically, this up-regulation may be of importance for the sequential scheduling of DNR and ara-C during the induction treatment of AML.

Baseline expression of CRIM1 mRNA is much higher in drug resistant leukemia cells compared to drug sensitive leukemia cells. There is also a covariance between CRIM1 and Pgp mRNA expression levels in leukemia cell lines with different levels of drug resistance.
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In vitro chemosensitivity tests with the ability to predict drug resistance are an attractive concept. However, so far none of the assays available have proven more effective than treatment based on clinical experience, in improving treatment outcome. It is possible that a combination of analysis of known markers for drug resistance, such as Pgp, and the results of an in vitro assay that is refined to identify subpopulations of highly drug resistant cells, could improve the results. Targeting subpopulations, such as leukemia stem cells, could provide a more clinically relevant profile of drug resistance.

Our findings that exposure to ara-C increases the expression levels of known markers for drug resistance provide a basis for further pharmacological in vivo studies on patients diagnosed with AML. In vivo studies with different scheduling of the drugs could reveal if the increase in expression levels of known markers for drug resistance could be avoided or circumvented.

The clinical relevance of CRIM1 expression in AML and its role in drug resistance is still unknown. Further studies are needed, in particular to investigate the corresponding changes in expression levels of CRIM1 protein following changes in mRNA levels. Silencing of CRIM1 mRNA expression in drug resistant cells and subsequent cytotoxicity testing to reveal changes in drug sensitivity could be one way of investigating the impact of CRIM1 on drug resistance. Studies on cells from patients diagnosed with AML are needed as well, to elucidate whether CRIM1 expression can be used as a predictive marker for drug resistance, and whether CRIM1 expression correlates with clinical parameters such as resistant disease.
FUTURE PERSPECTIVES

In vitro chemosensitivity tests with the ability to predict drug resistance are an attractive concept. However, so far none of the assays available have proven more effective than treatment based on clinical experience, in improving treatment outcome. It is possible that a combination of analysis of known markers for drug resistance, such as Pgp, and the results of an in vitro assay that is refined to identify subpopulations of highly drug resistant cells, could improve the results. Targeting subpopulations, such as leukemia stem cells, could provide a more clinically relevant profile of drug resistance.

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