Pharmacological and clinical studies of new ways to improve cytostatic treatment of acute myelocytic leukemia

-in vitro and in vivo studies

by

Christina Löfgren

Stockholm 2005
ABSTRACT

Treatment failure in refractory or relapsed disease is often caused by resistance towards Ara-C or daunorubicin, the two drugs mainly used in the treatment of acute myeloid leukemia (AML). This thesis comprises studies of resistance mechanisms regarding Ara-C and the newer nucleoside analogues CdA and fludarabine, improvement of treatment outcome after enhancement of tumour Ara-C susceptibility by concomitant treatment with GM-CSF and the effect of liposomal formulation on leukemic cell uptake of daunorubicin. The main objective was to find ways to improve the cytostatic treatment of AML.

First, different anthracycline resistant cell lines were evaluated for resistance towards Ara-C and possible resistance mechanisms. In a HL 60 doxorubicin resistant cell line, expressing high levels of MDR-1 gene and protein we found cross-resistance to Ara-C, reduced dCK activity, decreased 5'NT activity and only half the uptake and retention of Ara-C compared to wild type cells indicating higher efflux of the drug in resistant cells, probably via the P-gp. This was confirmed, as the sensitivity to Ara-C was increased in combination with reversing agents CsA and PSC, measured by cytotoxicity, DNA fragmentation and caspase-3-like activity. Likewise, in K 562 cell lines with acquired resistance to daunorubicin and vincristine cross-resistance to Ara-C was found. These cells expressed P-gp but not MRP1-6. Decreased dCK (20 – 45%) activity was confirmed, reductions in dCK activity and protein levels in concordance with a decrease in dCK mRNA. In contrast to the HL 60 cell line elevated 5'NT activity resulting in several fold increased 5’NT/dCK enzyme ratios in resistant cells was found. Next, leukemic cells from 170 AML patients were evaluated for in vitro-activity and cross-resistance patterns of the purine analogues cladribine and fludarabine, and Ara-C using the ATP-assay. After incubation with clinically relevant concentrations about 25% of the samples were highly sensitive for Ara-C or CdA/fludarabine. CdA was significantly more active than Ara-C (p<0.05) but not more than fludarabine. The cytotoxicity of CdA correlated strongly to fludarabine (r=0.82,p<0.0001) but less toward Ara-C (r=0.49,p=0.002). A significant number of Ara-C resistant cells were sensitive to CdA/fludarabine.

To study if addition of growth factor increases the effect of Ara-C, in a phase III randomised study, patients aged > 64 years with newly diagnosed AML received a 3-day MEA protocol + the addition of prior and concomitant GM-CSF. This regimen induced CR in 64.5%, with no difference between the treatment arms. With GM-CSF remission duration was shorter (6 vs. 13 months, ns) as was the median OS (9 vs. 14 months, ns). The median time to neutrophil recovery was significantly shorter in the GM-CSF treatment arm (17 vs. 25 d, p=0.03) as was the number of septic episodes.

Liposomal daunorubicin, DaunoXome, was compared to conventional daunorubicin with respect to plasma and intracellular pharmacokinetics. Following DaunoXome the peak values and plasma AUC were more than 100 times higher than after administration of free daunorubicin (AUC: 176.16 vs. 0.98µM x hour) but the intracellular AUCs were comparable (759.5 vs. 715.03µM x hour). Intracellular concentrations after DaunoXome peaked later and half as high as after daunorubicin. After DaunoXome or daunorubicin plasma clearance was 0.001 or 0.4 µmol/h respectively. The volume of distribution was 5.5 L for DaunoXome vs. 3640 L for daunorubicin indicating low tissue affinity for the liposomal formulation.

In conclusion cross-resistance to Ara-C is found in cell lines with acquired resistance towards anthracyclines, expressing high levels of P-gp. This is in part explained by increased 5’NT/dCK enzyme ratios in the resistant cells but the addition of reversing agents restores Ara-C sensitivity, indicating efflux via P-gp as a separate mechanism of resistance. In leukemic cells from patients in vitro sensitivity to CdA or Fludarabine correlates strongly to Ara-C, however as this correlation is not complete, CdA and fludarabine can play a role as alternatives or in combination with Ara-C. Addition of GM-CSF to anti leukemic therapy shortens ANC recovery and reduces septicemia but does not enhance treatment outcome compared to chemotherapy alone. Liposomal daunorubicin, DaunoXome, gives two-log higher plasma but similar intracellular concentrations of daunorubicin and active metabolite daunorubicinol compared to administration of free daunorubicin indicating comparable antileukemic effects.

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“You know that medicines, when well used, restore health to the sick; they will be well used when the doctor together with his understanding of their nature is shall understand also what man is, what life is, and what constitution and health are. Know these well and you will know their opposites; and when this is the case you will know well how to devise a remedy.”

- Leonardo da Vinci, *Codex Atlantico*

*To my Love, and our children*
LIST OF PAPERS

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


III. Löfgren, C., Albertioni, F., and Paul, C. High activity and incomplete cross-resistance of nucleoside analogues cladribine and fludarabine versus ara-C on leukemic cells from patients with AML. *(Submitted)*


* The authors contributed equally to this article.

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**ABBREVIATIONS**

<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ALL</td>
<td>Acute lymphatic leukemia</td>
</tr>
<tr>
<td>AML</td>
<td>acute myeloid leukemia</td>
</tr>
<tr>
<td>ANLL</td>
<td>acute non-lymphatic leukemia</td>
</tr>
<tr>
<td>APAF-1</td>
<td>apoptosis activating factor 1</td>
</tr>
<tr>
<td>Ara-C</td>
<td>cytosine arabinoside, cytarabine</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the curve</td>
</tr>
<tr>
<td>CdA</td>
<td>2-chloro2’deoxyadenosine, cladribine</td>
</tr>
<tr>
<td>CR</td>
<td>complete remission</td>
</tr>
<tr>
<td>CyA</td>
<td>cyclosporine A</td>
</tr>
<tr>
<td>dCK</td>
<td>deoxycytidine kinase</td>
</tr>
<tr>
<td>dGK</td>
<td>deoxyguanosine kinase</td>
</tr>
<tr>
<td>DiSC</td>
<td>differential staining cytotoxicity assay</td>
</tr>
<tr>
<td>dnr</td>
<td>daunorubicin</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>dXm</td>
<td>liposomal daunorubicin, DaunoXome</td>
</tr>
<tr>
<td>F-araA</td>
<td>9-β-D-arabinofuranosyl-2-fluoroadenine, fludarabine</td>
</tr>
<tr>
<td>FLT-3</td>
<td>FMS like receptor tyrosine kinase</td>
</tr>
<tr>
<td>FMCA</td>
<td>fluorometric microculture cytotoxicity assay</td>
</tr>
<tr>
<td>hCNT</td>
<td>human concentrative nucleoside transporter</td>
</tr>
<tr>
<td>hENT</td>
<td>human equilibritve nucleoside transporter</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>ICU</td>
<td>intensive care unit</td>
</tr>
<tr>
<td>kD</td>
<td>kiloDalton</td>
</tr>
<tr>
<td>LRP</td>
<td>lung resistance protein</td>
</tr>
<tr>
<td>MDR</td>
<td>multidrugresistance</td>
</tr>
<tr>
<td>MRP1-6</td>
<td>multidrugresistance associated protein 1-6</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide</td>
</tr>
<tr>
<td>NA</td>
<td>nucleoside analogues</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>5’NT</td>
<td>5’nucleotidase</td>
</tr>
<tr>
<td>OS</td>
<td>overall survival</td>
</tr>
<tr>
<td>P-gp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PSC</td>
<td>the cyclosporine analogue PSC 833, Valspodar</td>
</tr>
<tr>
<td>RR</td>
<td>ribonucleotide reductase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>TK1</td>
<td>thymidine kinase 1</td>
</tr>
<tr>
<td>WBC</td>
<td>white blood counts</td>
</tr>
</tbody>
</table>
INTRODUCTION

Leukemia was identified and designated by Virchow (originally as “leukämie” new-Latin, derived from Greek meaning “white blood”) in 1845 (Virchow 1845). However, Bennet made an independent report the same year (Bennet 1845), and the first modern report of a patient with leukemia appeared earlier, in 1827 (Velpeau). An acute form of leukemia was described by Friedreich in 1857, and recognised as a separate disease in 1889 by Ebstein, but not until 1931 could the distinction between ALL and ANLL be made.

Untreated leukemia is rapidly fatal usually within two months (Tivey 1954). However, prolonged survival due to successful chemotherapy was achieved when aminopterin, a folic acid antagonist, induced complete remission in patients with acute leukemia in 1948 (Farber et al 1948). In 1959 a new antimetabolite, Cytarabine (Ara-C), was synthesised inducing complete remission in about 30%. Thenceforth, a number of active drugs of different origin were tested and with the discovery of the anthracycline antibiotics more effective drugs were brought into practice. The success of cancer chemotherapy is limited in part due to insufficient tumour cell selectivity of the drugs, leading to severe side effects from toxicity to normal tissue, and in part due to intrinsic or acquired resistance to cytostatic drugs. The concept of drug resistance is based on the work by Goldie and Coldman (Goldie and Coldman 1979).

The introduction of combination chemotherapy was a breakthrough in antileukemic treatment and standard chemotherapy regimens since then consists of Ara-C and an anthracycline, most often daunorubicin, the so-called “3+7” regimen leading to remission rates around 70%. The rationale for combination therapy is based on four principles; tumour specific activity of the chosen drugs, different biochemical targets, differences in toxicity profiles to normal tissue and lack of cross-resistance between the drugs.

ACUTE MYELOID LEUKEMIA (AML)

The incidence of leukemia increases with increasing age, 2.5-5.4/100000 (Astrom, et al 2000), which in Sweden translated into 944 new cases during the three years 1997-99 according to the Swedish registry for acute leukemia. More than 70% of the patients were over 70 years at diagnosis. The etiology of AML is still largely unknown. However, environmental factors such as exposure to high doses of irradiation (i.e. survivors of atomic detonations, subjects to therapeutic irradiation or by occupation) are recognised. Chronic benzene exposure is another accepted risk factor. Genetic factors seem to play a role. AML can evolve from certain hematological disorders such as myeloproliferative diseases and myelodysplastic syndrome. Chemotherapy, especially alkylating agents or topoisomerase II inhibitors, is associated with an increased risk for developing AML. These so-called secondary leukemias are associated with a poorer outcome compared to primary AML.

AML is a malignant disorder characterized by clonal expansion of immature myeloid hematopoetic cells (myeloblasts) with impairment of normal hematopoesis leading to anemia, infections and bleeding disorders. Leukemic infiltration can produce organ specific symptoms. Hyperleukocytosis, extremely elevated WBC, can occur and is associated with multiorgan failure, and bleeding disorders.

Diagnosis of AML is based on morphological identification of leukemic myeloblasts exceeding 30% of all cells in a bone marrow smear. Further classification is based on cytochemical staining, flow cytometry and cytogenetic analysis of myeloblasts. Until recently
the classification developed by the French-American-British committee (FAB-classification) was most widely applied (Bennet, et al 1976). Table 1.

**Table 1.** FAB classification of AML.

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Code</th>
</tr>
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<tbody>
<tr>
<td>Myeloblastic leukemia minimally differentiated</td>
<td>M0</td>
</tr>
<tr>
<td>Myeloblastic leukemia without maturation</td>
<td>M1</td>
</tr>
<tr>
<td>Myeloblastic leukemia with maturation</td>
<td>M2</td>
</tr>
<tr>
<td>Hypergranular promyelocytic leukemia</td>
<td>M3</td>
</tr>
<tr>
<td>Microgranular variant (M3v)</td>
<td></td>
</tr>
<tr>
<td>With bone marrow eosinophilia (M4EO)</td>
<td>M4</td>
</tr>
<tr>
<td>Myelomonocytic leukemia</td>
<td>M5</td>
</tr>
<tr>
<td>Monocytic leukemia</td>
<td></td>
</tr>
<tr>
<td>Monoblastic leukemia (M5A)</td>
<td></td>
</tr>
<tr>
<td>Monocytic leukemia (M5b)</td>
<td></td>
</tr>
<tr>
<td>Erythroleukemia</td>
<td>M6</td>
</tr>
<tr>
<td>Megakaryoblastic leukemia</td>
<td>M7</td>
</tr>
</tbody>
</table>

Based on studies that have revealed biological and prognostic important categories of AML, with implications on treatment, a new classification system has been developed by the World Health Organization. This takes into account clinical information, morphology, cytochemistry, immunophenotyping, and cytogenetic and molecular analysis. Furthermore, only 20% blasts are required (in the bone marrow) for the diagnosis according to the WHO (Brunning, et al 2001). Table 2.

**Table 2.** The WHO classification of AML.

<table>
<thead>
<tr>
<th>Subtype</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1. AML with recurrent cytogenetic abnormalities</td>
<td></td>
</tr>
<tr>
<td>t(8;21)(q22;q22)</td>
<td></td>
</tr>
<tr>
<td>inv(16)(p13q22) or t(16;16)(p13;q22)</td>
<td></td>
</tr>
<tr>
<td>t(15;17)(q22;q12)</td>
<td></td>
</tr>
<tr>
<td>11q23 abnormalities</td>
<td></td>
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<tr>
<td>2. AML with multilineage dysplasia</td>
<td></td>
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<tr>
<td>With prior myelodysplastic syndrome</td>
<td></td>
</tr>
<tr>
<td>Without prior myelodysplastic syndrome</td>
<td></td>
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<tr>
<td>3. AML and myelodysplastic syndromes, therapy related</td>
<td></td>
</tr>
<tr>
<td>Alkylating agent-related</td>
<td></td>
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<tr>
<td>Topoisomerase-II inhibitor-related</td>
<td></td>
</tr>
<tr>
<td>Other types</td>
<td></td>
</tr>
<tr>
<td>4. AML not otherwise categorized</td>
<td></td>
</tr>
<tr>
<td>(classification according to FAB criteria)</td>
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</table>
TREATMENT OF AML AND OUTCOME (PROGNOSTIC AND RISK FACTORS)

Antileukemic therapy comprises induction therapy aiming at complete remission (CR) and postremission treatment. Complete remission is defined as 5% leukemic blasts in a normocellular bone marrow. Unless CR is achieved survival is generally shorter than a few weeks or months. To achieve long time survival and cure additional therapy, consolidation or postremission treatment is necessary due to the presence of residual disease.

The most widespread induction treatment regimen for AML consists of daunorubicin 45-60 mg/m²/day intravenously for three days in combination with Ara-C 100-200 mg/m²/day for 7 days. Depending on the age of the patient and on other biological characteristics, 50-80% will achieve CR (Dillman, et al 1991, Yates, et al 1982). A variety of alternative regimens consisting of these two drugs in combination with one of more cytostatic have been in trial. Randomised trials have suggested that idarubicin and mitoxantrone be superior to daunorubicin. These results are difficult to interpret since the equipotent doses for these anthracyclines have yet to be established. Intensifications of the induction therapy through use of higher doses of Ara-C or adding a third drug such as etoposide of thioguanine have generally failed to improve long-term outcome (Kimby, et al 2001, Rowe and Tallman 1997).

Postremission therapy is essential to prevent relapse. Therapeutic options in younger patients (<60 years) are chemotherapy, autologous stem cell transplantation (autoSCT) and allogeneic stem cell transplantation (alloSCT). AlloSCT show the lowest relapse rates, but is associated with a high early mortality due to organ toxicity, infections and graft-versus-host disease (GVHD). AutoSCT also reduces the relapse risk but to a lesser degree than alloSCT and with lesser toxicity.

The choice of postremission therapy is guided by prognostic or risk factors. Different cytogenetic abnormalities give information on prognosis in AML (Burnett, et al 2000, Grimwade, et al 2001). Patients can be divided into subgroups depending on the karyotype-cytogenetic prognostic groups. Cytogenetic aberrations t(8;21), t(15;17), inv(16) or del(16) are defined as associated to favourable prognosis. As intermediate risk related karyotype are regarded a normal karyotype or t(6;9), +8 or +21 and as associated with adverse risk t(9;12), -5, del(5), -7, del(7), del(11) or complex (>3) aberrations . Another major prognostic factor is the speed of initial clearance of leukemic blasts from the bone marrow after the first course of chemotherapy (Burnett, et al 2000). Associated with poor prognosis are factors such as high leukocyte count and thrombocytopenia at diagnosis, CD34 expression, biphenotypical leukemias, tandem repeat mutations in the FLT3 receptor gene, and expression of the multidrug resistance phenotype.

CHEMOTHERAPY IN AML

Nucleoside analogues

Cytosine arabinoside, Ara-C, Cytarabine

Ara-C, the cornerstone of anti-leukemic treatment, is one of the most effective agents in the treatment of acute myelogenous leukemia and is incorporated into virtually all standard induction treatment regimens for this disease, generally in combination with an anthracycline. Ara-C is also a component of consolidation and maintenance after remission is attained. It is used mainly in hematological malignancies especially leukemia.
Ara-C is a deoxycytidine analogue that differs from deoxycytidine by a substitution of the hydrogen atom in the 2’position in the pentose group by a hydroxyl group. Figure 1.

**Figure 1.** Chemical structure of Ara-C, cladribine and fludarabine.

Administered at high doses, Ara-C is taken up in the cell by a carrier-mediated process shared by physiologic nucleosides and by passive diffusion. Ara-C crosses into CNS. In the cell, Ara-C has to be phosphorylated to a triphosphate form, (Ara-CTP) to become toxic. Ara-CTP competes with deoxycytidine triphosphate, dCTP, for incorporation into DNA, resulting in a block of DNA synthesis. Ara-C competitively inhibits the function of DNA polymerases α and (when present in high intracellular concentrations) β. Other biochemical actions of Ara-C have been described, including inhibition of ribonucleotide reductase (RR) and formation of ara-CDP-choline, an inhibitor of membrane glycoproteins and glycolipids. The phosphorylation of Ara-C is catalysed by 3 different kinases, in which the rate-limiting step is performed by deoxycytidine kinase (dCK). Mitochondrial deoxyguanosine kinase (dGK) does not efficiently phosphorylate Ara-C. In plasma, Ara-C is deaminated by cytidine deaminase (CD) to the inactive metabolite Ara-U, which is rapidly eliminated. (Ho 1973). Plasma levels of Ara-C correlate poorly with cellular levels of the active metabolite Ara-CTP (Liliemark, et al 1985). The phosphorylation of Ara-C to Ara-CTP can be saturated which is demonstrated by the fact that the same dose administered as a continuous infusion or subcutaneous injection will accomplish higher levels of Ara-CTP than a bolus injection (Liliemark, et al 1985, Plunkett, et al 1987). In general the inhibition of cell growth correlates well with the degree of incorporation of Ara-C into cellular DNA. Decreased activities of the carrier for Ara-C or activity of dCK, increased formation of dCTP by RR, increased catabolism of Ara-C through the action of CD, and increased activity of 3’ to 5’ exonuclease (removes the Ara-CMP from the DNA) have been suggested as mechanisms of resistance to Ara-C (Feng, et al 2000).

In induction regimens Ara-C is usually administered intravenously with doses of 100 mg/m² – 3g/m² infused daily for 5-7 days (Plunkett and Gandhi 1996). Peak plasma concentrations of
10 µmol/L and 150 µmol/L are achieved after doses of 100 mg/m² and 3 g/m² resp. Terminal drug half-life is 30-150 minutes. Steady state levels of Ara-C in plasma are proportional for dose rates up to 2 g/m²/day leading to steady state plasma levels app. 5 µmol/L. Above this level, the deamination rate is saturated and plasma levels rise unpredictably (Donehower, et al 1986).

2-chlorodeoxyadenosine, CdA, Cladribine
Substitution of chlorine at the 2 position of deoxyadenosine produces CdA or cladribine. Figure 1. CdA was synthesized by Christensen et al. (Christensen, et al 1972) and has been clinically developed at the Scripps Clinic, USA (Piro, et al 1994). CdA is toxic to both dividing and indolent lymphoid malignancies (Carson, et al 1983) as well as autoimmune disorders (Bryson and Sorkin 1993, Schirmer, et al 1997). CdA is relatively resistant to enzymatic deamination by adenosine deaminase but has limited oral bioavailability due to degradation by either bacterial nucleoside phosphorolysis or acid hydrolysis to chloroadenine (Liliemark, et al 1992). CdA is transported into the cells by the hCNT2 carrier. Inhibition of nucleoside transport potentiates CdA toxicity by blocking efflux via hENT1. CdA needs intracellular activation through phosphorylation to CdA-MP by dCK (Eriksson, et al 1991) and dGK (Wang, et al 1993). CdA cytotoxicity is due to intracellular accumulation of CdATP after phosphorylation of CdAMP by nucleoside monophosphate kinase and nucleoside diphosphate kinase. 5’nucleotidase (5’NT) dephosphorylates CdAMP and the accumulation of CdATP depends on the ratio of these enzymes in the cells (Kawasaki, et al 1993). Incorporation of CdATP into DNA (Avery, et al 1989), inhibition of DNA polymerase, inhibition of RR, activation of poly (ADP-ribose) synthetase leading to depletion of NAD, direct binding of CdATP to the apoptotic protease activating factor –1 (APAF-1) and the activation of the caspase-9 and caspase-3 pathways are mechanisms proposed for activity (Leoni, et al 1998). The mechanisms behind CdA resistance are: decreased nucleoside transport, decreased activity or deficiency of dCK (Mansson, et al 1999, Orr, et al 1995), altered intracellular pools of competing nucleotides, altered regulation of RR, and increased drug inactivation by 5’NT (Schirmer, et al 1998). However, a reduction in dCK activity is probably the major determinant of CdA resistance (Mansson, et al 1999, Orr, et al 1995).
In AML, CdA is usually administered intravenously either as continuous infusion at doses of 5 - 8.9 mg/m² /24 h for 3-5-(7) days or as 2-hour infusion of 5 mg/m² daily for 3-5 days. Peak plasma concentrations are higher after 2-hour infusion (100-400 nM) (Liliemark and Juliusson 1991) than after continuous infusion (10-50nM) (Santana, et al 1992). A dose-dependent clearance has been reported, with clearance decreasing with increasing dosages from 3.5 – 10.5 mg/m²/day. After 5mg/m²/2h AUC is 0.59-0.76 mol/L x h and the terminal half-life 9.9-13.4 hrs. Oral bioavailability is roughly 37-51% (Albertioni, et al 1993, Liliemark, et al 1992, Liliemark and Juliusson 1995). Subcutaneous administration gives a high peak concentration of short duration with AUC identical to i.v. infusion and bioavailability of 100% (Liliemark, et al 1992, Sonderegger, et al 2000).

9-β-d-arabinofuranosyl-2-fluoroadenosine monophosphate, fludarabine, Fludara®
Over the past decade, fludarabine has become an effective agent for the treatment of lymphatic disorders (chronic lymphocytic leukemia (CLL), prolymphocytic leukemia, and indolent non-Hodgkins lymphoma).
Fludarabine was developed through chemical modifications of adenosine-arabinoside (ara-A), designed to avoid the rapid deamination of ara-A by adenosine deaminase. Figure 1. The 2-fluorodervative of ara-A is relatively resistant to deamination and retains cytotoxic activity, but is poorly soluble in water, which is why the monophosphate form is used in the clinic. After intravenous administration, fludarabine phosphate is rapidly dephosphorylated by ecto 5'NT in plasma to the nucleoside 2-fluoro-ara-A (F-ara-A) prior to entering the cell. This compound enters cells via carrier-mediated transport and is phosphorylated to its active form F-ara-ATP. F-ara-A has a common pathway with CdA in terms of activation by dCK. Phosphorylation, initiated by deoxycytidine kinase, is necessary for the cytotoxic and therapeutic activity of fludarabine. F-ara-ATP inhibits several intracellular enzymes important in DNA replication, including DNA polymerase, RR (Plunkett, et al 1990), DNA primase and DNA ligase I. In addition fludarabine is incorporated into DNA, inducing effective chain termination, primarily at the 3' end of DNA. Incorporation of fludarabine into DNA is required for drug-induced DNA fragmentation. Fludarabine is usually administered intravenously with doses of 20-30 mg/m² infused daily for 3-5 days (Plunkett and Gandhi 1996). Peak plasma fludarabine concentrations of 0.3 to 1.0 mg/L are achieved after doses of 80 – 250 mg fludarabine. Wide variations in terminal drug half-life (7 – 33 hrs) and AUC are found. F-ara-A is excreted mainly in urine with no metabolites. Clearance is linear, 73.53 mL/min/m². Oral bioavailability is roughly 75% and oral formulations are available.

Thioguanine, 6-thioguanine, (6-TG)
The guanine analogue thioguanine is used in remission induction and maintenance treatment of AML. Thioguanine is converted to 6-thioguanosine monophosphate (TGMP) by HGPRT. TGMP is subsequently incorporated into RNA and DNA in its deoxytriphosphate form. Incorporation of fraudulent nucleotides is believed to be the primary mechanism of cytotoxicity, triggering apoptosis by a process involving the mismatch repair pathway. Thioguanine is converted to its inactive metabolite 6-thiohypoxantine by guanase. Thioguanine inactivation is not dependent on xantine oxidase (in contrast to 6-MP, another guanine analogue, primarily used in maintenance treatment of childhood ALL). Thus, detoxification of thioguanine is not blocked by allopurinol, a xantine oxidase inhibitor. Furthermore thioguanine is methylated by thiopurine methyltransferase (TPMT) resulting in a substantially less active and less toxic product. The TPMT gene is cloned and eight polymorphisms associated with reduced enzyme activity have been defined. The bioavailability after oral administration of thioguanine is roughly 30%. Peak plasma levels vary, 0.03-5μmol/L as does the median half-life, around 90 minutes. Clearance of drug (0.6 –1L/min/m²) appears to be dose-dependent, suggesting saturation at doses exceeding 10 mg/m²/hour. Plasma concentration of 4 –10 mmol/L can be achieved.

Anthracyclines
Daunorubicin
Two independent research groups discovered the first anthracyclines in clinical use, daunorubicin and doxorubicin, in two different Streptomyces species in the early 1960s (Dimarco, et al 1964, Dubost, et al 1963). These drugs are composed of a four-ring chromophore attached to the amino sugar, daunosamine. In addition the chromophore
contains hydroxyquinone functionality on the middle two rings, rendering the intense red fluorescence of these drugs. Figure 2.

**Figure 2.** Chemical structure of daunorubicin.

In contrast to the nucleoside analogues anthracyclines have activity against solid tumors as well as against hematological malignancies. The anthracyclines are weak bases and the lipid solubility of daunorubicin is higher than that of doxorubicin. Anthracyclines are taken up by facilitated diffusion but pumped out in an energy dependent manner, involving P-gp, MRP and LRP (see mechanisms of resistance).

After entering the cell most of the anthracycline drug localizes in the nucleus of sensitive tumour cells. The drugs are to a variable degree metabolised in the liver mainly through reduction of the side chain by aldo-keto reductase to the corresponding alcohol, such as daunorubicinol. Urinary excretion is of minor importance. Plasma pharmacokinetics of daunorubicin best fits into a two or three compartment model with a rapid initial half-life followed by a slower elimination phase. Clearance is 1.2 L/kg/h and the volume of distribution 30-50 L/kg indicating pronounced tissue affinity.

The concentration of the active metabolite daunorubicinol exceeds that of daunorubicin within a few minutes after administration (Paul, et al 1980). Daunorubicin accumulates in the leukemic cells reaching concentrations that exceed the plasma concentration 400 – 4000 times (Paul, et al 1989). The anthracycline daunorubicin has antimitotic and cytotoxic activity through a number of proposed mechanisms of action; intercalation between base pairs, inhibition of topoisomerase II activity, inhibition of polymerase activity, regulation of gene expression and by free radical damage to DNA (Minotti, et al 2004). Cardiac toxicity, a main dose-limiting factor in anthracycline treatment is a drug-specific side effect manifested in its most severe form by fatal congestive heart failure that may occur either during therapy or months to years after therapy. The resultant congestive cardiomyopathy correlates with peak plasma anthracycline concentrations as well as with the cumulative dose administered (Theodoulou and Hudis 2004) and an accumulated anthracycline dose exceeding 550mg/m² for doxorubicin or 750 mg/m² for daunorubicin increases the risk for cardiac toxicity (Lefrak, et al 1973, Lehmann, et al 2000). The clinical effect differs between daunorubicin and doxorubicin despite the structurally close relationship. Daunorubicin is most effective in AML but apart for some activity against lymphomas, rhabdomyosarcoma and neuroblastoma not a useful agent against solid tumours. In contrast doxorubicin is effective against a broad
spectrum of tumours, including hematological malignancies; it is the most effective drug available for treatment of many solid tumours.

In induction regimens daunorubicin is usually administered at dosed 40-60 mg/m²/day.

**Liposomal formulation of daunorubicin; DaunoXome®**

Lipid formulations of anthracyclines were designed primarily to overcome cardiac toxicity (Forssen 1997). It has also been reported that liposomal encapsulation might be less susceptible to extrusion by MDR protein than native daunorubicin (Michieli, *et al* 1999, Wang, *et al* 1999). Liposomal daunorubicin, DaunoXome® (dXm) by Swedish Orphan, is a formulation of a solution of the citratic salt of daunorubicin encapsulated within lipid vesicles, composed of a bilayer of disterylphosphatidylcholine and cholesterol of about 45nm in diameter (Forssen 1997). The liposomes enter the extra vascular space (e.g. tumour) through fenestrated or defect endothelium where the liposomes discharge the internalised daunorubicin either intracellularly after endocytosis (Forssen 1997) or extracellularly after spontaneous rupture. The current indication for dXm is first line cytotoxic therapy for advanced HIV associated Kaposis Sarkoma (KS) (Gill, *et al* 1995, Tulpule, *et al* 1998). Presently dXm is also entered into clinical trials in acute myeloid leukemia.

Despite anthracyclines exerting their effects intracellularly, presently there are no studies on the intracellular pharmacokinetics of daunorubicin after administration of DaunoXome, in the target, the tumor cell.

**GM-CSF**

Granulocyte-macrophage colony stimulating factor (GM-CSF) is produced predominantly by T-lymphocytes and mesenchymal cells, but in small amounts also by macrophages (Akashi *et al*, 1991). GM-CSF is a glycoprotein that stimulates the proliferation and differentiation of granulocyte and macrophage progenitor cells and also induces the growth of stem cells *in vitro* in a majority of patients with AML (Asano, *et al* 1987, Griffin, *et al* 1986, Sundman-Engberg, *et al* 1996). Treatment with GM-CSF has been shown to reduce the neutropenic period after cytostatic treatment as well as number of days with fever and days in hospital. Since pretreatment with GM-CSF stimulates the proliferation of leukemic blasts (Bettelheim, *et al* 1991, Estrov, *et al* 1992) this might increase leukemic blast susceptibility to ara-C induced apoptosis. GM-CSF *in vitro* affects the therapeutic index by increasing the ara-CTP to dCTP ratio and the ara-C incorporation into DNA. However, it has been cautioned that this stimulation of leukemic blasts might also predispose for early relapse.

**Drug resistance mechanisms**

Despite the high initial complete remission rate with conventional chemotherapy a majority (30-80%) of the patients will relapse and die of their disease, moreover 15% do not respond to initial therapy. Therefore long-term survival with AML is only 20% (Champlin and Gale 1987). Many patients become resistant to therapy at relapse, some are resistant already at diagnosis.

Resistance to the so-called naturally occurring anti-cancer drugs such as anthracyclines and epipodophyllotoxins, depends partly on the occurrence of multidrug resistance. The mechanisms underlying multidrug resistance (MDR) have been extensively studied. Alterations in drug target, metabolism and transport, altered cellular repair mechanisms and defect cellular apoptosis have been described. Several separate forms of MDR have been
characterized in detail; Classical MDR (P-glycoprotein (P-gp), non-P-gp MDR, vault-related MDR (VR-MDR, LRP), and atypical MDR. Transport mediated drug resistance is the most studied form of MDR in AML (McKenna and Padua 1997).

The expression of P-gp, a cell membrane localized transport protein, in leukemic cells is associated with a significantly poorer outcome in AML (Leith, et al 1999). P-glycoprotein is a 170-kDa cell membrane protein encoded by the mdr1 gene located on the long arm of chromosome 7. It binds various anti-cancer agents to reduce intracellular accumulation through an energy dependent efflux mechanism from the cells (Bradshaw et al 1990). P-gp is a member of a family of transport proteins known as ATP-binding cassette (ABC) transporters.

The non-P-gp MDR phenotype is caused by overexpression of the multidrug resistance-associated protein (MRP, mrp gene), which is a related family member and had been characterised in drug-resistant cancer cells. The MRP family consists of at least six members of which three, mrp1, mrp2, and mrp3 can transport MDR drugs and could be involved in MDR (Scheffer, et al 2000). The mrp gene encodes a 190k-da membrane-bound glycoprotein that probably functions similar to P-gp, mediating rapid efflux of cytotoxic drugs from the cell. LRP is the human major vault protein, first identified in non-small cell lung cancer cell line, selected for anthracycline resistance (Scheffer, et al 1995). LRP is a part of ribonucleoprotein complexes together with two other high molecular weight proteins and a small RNA molecule and located in the cytoplasm, nuclear membrane or nuclear pore complex (Kedersha and Rome 1986). LRP has a role in the transport of doxorubicin between the nucleus and the cytoplasm (Kitazono, et al 1999).

Atypical MDR is associated with alterations e.g. mutations, in topoisomerase II, a nuclear enzyme that catalyses the unlinking of the DNA strands by making transient strand breaks and allowing DNA to rotate around or traverse through these breaks (Wang 1996).

More than one MDR phenotype can contribute to resistance and additional MDR phenotypes can emerge under advancing drug selection leading to high levels of resistance.

The enzyme dCK normally phosphorylates deoxycytidine to deoxycytidine monophosphate (dCMP), but in cancer treatment it is also important for the initial phosphorylation of nucleoside analogues such as CdA and Ara-C. It has formerly been shown to be decreased or completely missing in cell lines with a resistance to nucleoside analogues (Bhalla, et al 1984, Kees, et al 1989). Large pools of deoxynucleoside triphosphates are also known to decrease the activity of dCK due to feedback inhibition, and substances such as CdA, which inhibit ribonucleotide reductase, have been shown to induce an upregulation of dCK (Laliberte and Momparler 1994).

**IN VITRO CHEMOSENSITIVITY TESTS**

Several in vitro tests have been developed in order to estimate the effect of, or resistance to cytostatic drugs in tumor samples.

**Clonogenic assays** give an estimation of the toxicity on proliferating cells but since they rely on the proliferative capacity of the tumor cells, in vitro G0 cells might not be accurately measured. Tumor cells are cultured on soft agar. The tests are labour intense and time consuming, in addition the result is not available until after 10-14 days.

**Cell proliferation assays** are based on the incorporation of radioactive precursors of DNA and RNA in the proliferative fraction for 1-4 days.
Total cell kill assays
The differential staining cytotoxicity assay (DiSC) combines culturing and staining for viability and morphological assessment. After incubation and culture for four days cells are vital stained and counterstained after centrifugation and then assessed against an internal standard. The method is labour intense and relies on subjective counting in the microscope. However malignant cells can be separated from non-malignant cells. A good correlation with both response and survival in de novo AML patients has been shown (Tidefelt, et al 1989) (Staib et al 2000).

The MTT assay (Black et al 1954, Mosmann et al 1983) and the FMCA assay (Larsson et al 1990) determine the amount of surviving cells based on the metabolic activity within the cells. In these assays the cells are cultured usually as continuous infusions, together with the cytostatic drugs, often in IC50 concentrations, in 96-well culture dishes. In the MTT assay the viability is assessed by the ability of the living cells to convert 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) into an insoluble formazan dye. The optical density of the colored product is measured in a spectrophotometer at 540nm. In the FMCA assay the non-fluorescent substrate fluorescein diacetate (FDA) is hydrolysed to a fluorescent derivative, fluorescein measured in a fluorometer. Pieters et al (1989) have shown good correlation in leukemia between the DiSC and the MTT-assay in leukemia.

The Bioluminescence ATP assay is also based on cellular metabolic activity but measures cellular ATP content. ATP and luciferase result in light emittance, the amount of ATP is calculated. Since the ATP levels are constant in a given living cell it can be used as an indirect method for measuring cell growth or death. In similarity with the MTT and FMCA assays cells are cultured with cytotoxic drugs. ATP is rapidly degraded leading to prompt depletion if the respiratory cycle is disturbed in aerobic cells (Lundin, et al 1986). This assay has shown good correlation with the DiSC and clonogenic assays and has been used in AML and solid tumors (Rhedin, et al 1993). In contrast to the MTT and FMCA assays, in our hands, in this ATP based assay drug concentrations as well as the drug incubation times are chosen so as to mimic the in vivo situation.
THE PRESENT STUDY

AIMS

The main objective of this thesis was to find ways to improve the cytostatic treatment in AML as seen from a clinicians’ perspective. The standard induction treatment is Ara-C in combination with daunorubicin. The effect of this treatment is limited by drug resistance and by side effects. These statements raise multiple questions. What are the mechanisms of Ara-C resistance? Do the newer nucleoside analogues differ from Ara-C regarding effect on leukemic cells from patients? Is the antileukemic effect of Ara-C i.e. clinical outcome improved if the leukemic cells are primed into S-phase by adding GM-CSF to the treatment? Is liposomal encapsulation of daunorubicin more efficient in AML? The answers were sought performing studies with the following aims.

Specific aims:

- To clarify the observed cross-resistance to Ara-C for the human promyelocytic subline, HL60/R10, resistant to doxorubicin and to investigate whether the resistance to Ara-C could be reversed by reversing agents such as verapamil or cyclosporines.
- To clarify the biochemical and molecular mechanisms behind the cross-resistance to nucleoside analogues in four erythroleukemic cell lines with acquired resistance to the anthracycline daunorubicin and to the vinca alkaloid vincristine, expressing p-glycoprotein.
- To evaluate in vitro activity of novel purine analogues in leukemic cells from patients with adult myeloblastic leukemia and (eventual) patterns of resistance comparing CdA and fludarabine to Ara-C and daunorubicin.
- To evaluate the effect of addition of GM-CSF to Ara-C, etoposide and mitoxantrone (MEA) in patents older than 60 years and with newly diagnosed AML with respect to remission rate, remission duration, survival, infectious complications and neutrophil recovery.
- To investigate the intracellular pharmacokinetic properties of a liposomal daunorubicin, DaunoXome® in patients with newly diagnosed AML.
**MATERIALS AND METHODS**

All studies where patient or patient samples are involved have been approved by the ethical Committees at Huddinge University Hospital, Örebro University Hospital and in paper IV, at all participating centres.

**Patient samples and sample collection**

In the evaluation of nucleoside analogues (paper III) samples from AML patients were included in the analysis. In the pharmacokinetic study (paper V) 14 patients with de novo AML were included.

In the *in vitro* study (paper III) bone marrow was collected and mononuclear cells were isolated by density gradient centrifugation with Lymphoprep (Nycomed, Oslo, Norway).

In the pharmacokinetic study (paper V) peripheral blood was drawn in heparinized tubes before and at specified intervals after the daunorubicin or DaunoXome infusion. Separation of leukemic cells and plasma was done by centrifugation of the cells, the plasma was removed and frozen at -80°C, mononuclear cells (>85% myeloblasts) were isolated as above. After determination of cell number and cell volume with a Coulter Multisizer (M/SZRII) 0.5 - 1 ml plasma was added and the cell sample was stored at -80° C until analysis.

**Cell lines (paper I and II)**

In the studies presented in this thesis two different cell lines were utilized: the human myeloid leukemia cell lines HL 60 (paper I) and K 562 (paper II). Cells were cultured in RPMI medium, containing fetal calf serum (10%), and L-glutamine at 37ºC with 5% CO₂. Cells were made resistant by repeated exposure to increasing concentrations of the respective anticancer drugs. Prior to experiments, cells were subcultured twice without drug.

**In vitro chemosensitivity assays (paper I, II and III)**

**MTT** (paper II). Soluble MTT is cleaved by mitochondrial dehydrogenases in living cells and is converted to unsoluble formazan dye. Cells were incubated at 37°C with drug for 72 hours. MTT (5mg/mL) was added and cells were further incubated for four hours and the formazan crystals were solubilized by adding SDS. The absorbance was measured spectrophotometrically using an ELISA plate reader at 540 nm with the reference at 650 nm. The cell survival was expressed as IC50 values, the drug concentration used for 50% cell survival.

**Bioluminescence ATP** (paper III). The cells were incubated in the medium consisting of 1.8 mL RPMI 1640 supplemented with 1% L-glutamine and 10% fetal calf serum and 0.2 mL of the cytostatic drug and with the cytostatic drug at final drug concentrations as follows: daunorubicin 0.2µM for 1 h. Ara-C 0.5µM continuously, CdA 0.05µM continuously, fludarabine 2 µM continuously. All incubations were performed in quadruple and with two drug free controls. All samples were then incubated at 37°C for 4 days. Extraction of ATP was performed by mixing equal volumes of cell suspension and trichloracetic acid. The bioluminescence assay was performed automatically in a Bio orbit Photometer as previously described (Rhedin, *et al* 1993). A cuvette with cells was placed in the photometer, ATP monitoring reagent was dispensed automatically and the resulting light emission was measured. The amount of ATP was calculated with correction for the blanks. With this procedure the light emitted is proportional to the amount of ATP in the sample. The results
were given as nmol ATP/sample. The percentage ATP in a sample when compared to the
drug free control was then calculated.

**Determination of the triphosphate formation of CdA and Ara-C by HPLC (paper I)**
HPLC methods were used to determine the amount of active metabolites of CdA in cells (Reichelova, et al 1996). Cells were preincubated with analogues for 1-2 hours. Nucleotides were extracted with a solution of perchloric acid containing triethylammonium phosphate. The nucleotides were separated using isocratic HPLC with a C18 column and the mobile phase consisted of triethylammonium phosphate buffer and 11% methanol. F-ara-A and Ara-C nucleotides were separated using gradient elution and a Partisil-10 SAX column. The gradient solutions consisted of: Solvent A: 0.005M NH₄H₂PO₄, pH 2.8 and solvent B: 0.75M NH₄H₂PO₄, pH 3.5. The concentration of Ara-CTP was measured at 280nm and CdATP and F-ara-ATP at 265nm.

**Activities of kinases and nucleotidases (paper I and II)**
The kinases dCK, TK1 and TK2 and the nucleotidases high Kₘ 5'-NT were measured using radio labelled substrates as previously described (Spasokoukotskaja, et al 1995, Spychala and Mitchell 1994). For kinases the cells were extracted, thawed and centrifuged. The supernatant was used as the source of protein for the kinase assays. The protein (2-3µg) was assayed in a reaction mix containing 50mM tris-HCl (pH 7.6), 5mM MgCl₂, 5mM ATP, 4mM dithiothreitol, 10mM sodium fluoride and substrates. The concentration for each substrate used in the assays was approximately 10 times higher than the Kₘ value for the respective kinase. Prior to the experiments the linearity of the assay was tested. After the assay, the reaction mix was spotted onto Whatman DE81 filters, to which the phosphorylated fraction bound.

For high Kₘ 5'-NT activity, inosine monophosphate was used as a substrate as previously described (Spychala and Mitchell 1994) and 3 mM ATP was added to activate the enzyme. Separation of inosine monophosphate and the products from dephosphorylation (inosine and hypoxantine) were separated on a thin layer chromatography plate (PEI-cellulose) and developed in 1-butanol:H₂O:methanol:NH₄ (60:20:20:1 vol/vol). The enzyme activity was expressed as pmol/million cells/min or pmol/mg cellular protein/min.

**Deoxyribonucleotidase triphosphate pools (paper I)**
The intracellular amounts of dNTP pools were determined using a DNA-polymerase-based method (Sherman and Fyfe, 1989). Cells were extracted by 70% methanol, evaporated and dissolved in milliQ water. The dNTP pools were determined using DNA-polymerase and synthetic oligonucleotide primers as described. One of four dNTPs was radiolabeled and its degree of incorporation into its newly synthesised DNA was detected. The elution of ³H-labeled oligonucleotides from the DEAE-papers was improved by shaking with 2M NaOH. In addition to the DNA polymerase-based assay for dNTP pools, an HPLC based method was used. The dNTPs were extracted with 0.4M perchloric acid and the ribonucleotides were removed by peroxidase oxidation as previously described (Griffig et al 1989). The respective dNTP was measured by HPLC using a Partisil-10 SAX anion column with gradient solution as described above, for F-ara-TP and Ara-CTP.
Calcein uptake (paper I and II)
The fluorogenic dye calcein aceroxymethyl (calcein AM) is a hydrophobic substance that rapidly penetrates the plasma membrane. In the cytoplasm calcein AM is cleaved by endogenous esterases to form hydrophilic, fluorescent calcein. Calcein AM is a substrate for P-gp. Cells overexpressing P-gp will accumulate less calcein than cells with normal expression of P-gp. CsA can reverse the increased calcein efflux in P-gp positive cells.

Western blot analysis (paper I and II)
The western blot technique was used to determine protein levels of dCK, dGK, P-gp, mrp 1-6, LRP, topoisomerase II. The protein mixtures were electrophoretically separated in SDS-polyacrylamide gels and transferred to polyvinylidene difluoride or nitrocellulose membranes. Membranes were blocked with 5% dry milk to reduce non-specific binding. Primary antibodies were added to the membrane to identify the protein of interest. The secondary horseradish peroxidase (HRP)-conjugated antibodies were added and the protein of interest was visualized using enhanced chemiluminescence (ECL).

RT-PCR and real time quantitative PCR (paper I and II)
The mRNA levels of various genes were determined using semi-quantitative PCR or real time quantitative PCR (RQ-PCR). For semi-quantitative PCR β2-mikroglubulin was used as internal control. The PCR product for the gene of interest was electrophoresed in 1.5% agarose gels and visualised as ethidium bromide fluorescent bands. For RQ-PCR two primers and a dual labelled fluorogenic probe were used. During the PCR reaction the probe is cleaved by the Taq polymerase and the fluorogenic reporter is separated from the quencher. The fluorescence increases in proportion to the amount of PCR product. The reactions were performed and the fluorescence detected using an ABI Prism 7700 Sequence detection System (Applied Biosystems, Foster City, CA).

DNA fragmentation (paper I)
DNA fragmentation was detected by propidium iodide staining and fluorescence activated cell sorting (FACS). Cells were pelleted by centrifugation and resuspended in a solution containing propidium iodide. The red fluorescence intensity of propidium iodide was detected by FL-3 (exited at 568nm) by FACS analysis.

Caspase-like activity (paper I and II)
Caspase activity was measured according to a previously described method (Nicholson et al 1995). The synthetic substrates DEVD-AMC and LEHD-AMC were used for caspase-3 and caspase-9, respectively. Caspases cleave the preferred substrate and the fluorochrome AMC is liberated and fluorescence emitted. Cells were added to a microtiterplate and lysed and thereafter diluted in the reaction buffer containing the respective substrate (50µM). AMC liberation was measured during 30 min, every 70 seconds in a fluorometer. The caspase activities were calculated using a standard curve with free AMC and expressed as pmol/min.

Cytostatic treatment (paper III, IV and V)
In paper III, patients were treated with a variety of regimens depending on whether induction of remission or palliative treatment was intended. Some patients did not receive any treatment
at all. In most cases treatment consisted of Ara-C in various doses with or without additional drugs such as anthracyclines or etoposide.

In paper IV, MEA - treatment; cytarabine 1 g/m² twice daily as a two-hour infusion, mitoxantrone 12 mg/m² daily as a one-hour infusion and etoposide 200 mg/m² daily as a one-hour infusion. Patients in randomization arm 2 were in addition given GM-CSF 200 µg/m² s.c once daily, beginning one day prior to the start of chemotherapy and continued until absolute neutrophil count (ANC) >1.0 x10⁹/L. The first consolidation treatment consisted of MEA as described above, albeit only one day of mitoxantrone. A second consolidation course consisting of m-AMSA 90 mg/m², as a one-hour infusion for four days, was given with GM-CSF as previously. For patients remaining in complete remission after consolidation therapy a second randomization was performed between maintenance therapy with thioguanine 160 mg weekly, or no further (maintenance) treatment.

In paper V, patients were randomized to a modified 3+ 7 regimen as follows; Ara-C 200 mg/m² as a continuous intravenous infusion for seven days. DaunoXome 50 mg/m², as a 1-hour intravenous infusion either on day 1 or 3, and daunorubicin (Cerubidine®) 50 mg/m², as 1-hour infusions on alternate days 1 or 3 and on day 5. If a second induction course was needed the patients were treated with daunorubicin on days 1-3 and Ara-C days 1-7 at doses described above. As consolidation treatments were recommended, HiDAC (high dose Ara-C 2g/m²/2hours twice daily for four days), ACE (four days of amecrine 150 mg/m², Ara-C 200 mg/m², and etoposide 110 mg/m²), HiDAC and finally ida/Ara-C (idarubicin 10 mg/m² for two days and Ara-C 1g/m² for three days). Based on the standard prognostic markers for AML such as cytogenetics, patients in remission were evaluated for autologous or allogeneic stem cell transplantation if suitable donors were available.

**Drug determination (paper V)**

Plasma and intracellular concentrations of daunorubicin were determined by HPLC (Tidefelt, et al 1994). The patient samples and six quality control samples diluted in plasma were thawed and sonicated. Standard curves for daunorubicin and daunorubicinol were prepared. A 0.2 mL aliquot of plasma or cell sample was transferred to a glass tube. To each tube was added chloroform: methanol (4:1), 0.1M Sodiumborate buffer pH 8.3 and internal standard i.e. doxorubicin (except for blank samples). The chloroform/MeOH phase was evaporated under nitrogen and the drug extract was dissolved in mobile phase (0.05M KH₂PO₄ buffer with acetronile). 100µl from each sample were injected in to the HPLC system. We used a LiCroCART® 125-4 Column (endcapped Merck), a LiCroCART 4-4 LiChrospher® precolumn with a Jasco HPLC-pump PU 980 and a Jasco Spectrofluorometer FP 920 (wavelength ex 485nm, em 560nm). The flow rate was 1.0 mL/min.

**Pharmacokinetic evaluation (paper V)**

In paper V intracellular and plasma peak levels, retention, half-lives and AUC for intracellular concentration versus time were calculated using the Win Nonlin, non-linear estimation program, V 02.A, Scientific Consulting Inc.

**Statistical analysis**

Statistical analysis was performed with Statview software (papers I and II) and Statistica (Papers III-V). The data was expressed as mean and standard deviation. A p-value of <0.05 was considered as significant.
RESULTS AND DISCUSSION

Cross resistance to cytosine arabinoside in a multidrug resistant human promyelocytic cell line selected for resistance to doxorubicin: implications for combination chemotherapy (paper I)

The aim was to clarify the observed cross-resistance to Ara-C for the human promyelocytic subline, HL60/R10, resistant to doxorubicin and to investigate whether the resistance to Ara-C could be reversed by reversing agents such as verapamil and cyclosporines.

Results and discussion

First we confirmed, by Western blot analysis of P-gp and calcein accumulation, previous characterization of the subline with expression of high mRNA levels of the MDR-1 gene and protein. Moreover, the cells did not express MRP or LRP. As levels of topoisomerase II α and β, were essentially the same in these cell lines, we found also this resistance mechanism unlikely. This P-gp expressing doxorubicin resistant subline showed cross-resistance to vinca alkaloids and epipodophyllotoxins, but also to Ara-C by a factor 8 and to other purine analogues e.g. CdA (but to a lesser degree). Although upregulation of P-pg by anthracyclines and Ara-C has been described (Hu, et al 1999) and may be a general response for leukemic cells to cytotoxic stress, we proceeded to verify or exclude different mechanisms of Ara-C resistance as contributing factors to the observed cross-resistance to Ara-C. Table I:1.

### Table I:1. Biological and biochemical characterization of wild type and doxorubicin resistant HL 60 cells.

<table>
<thead>
<tr>
<th>% in S-phase</th>
<th>HL60/wt</th>
<th>HL60/r</th>
</tr>
</thead>
<tbody>
<tr>
<td>% in G1-phase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% in G2-phase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>triphosphates (µmol/l):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10µmol/l CdA</td>
<td>29±1</td>
<td>21±0.4**</td>
</tr>
<tr>
<td>10µmol/l Ara-C</td>
<td>359±28</td>
<td>111±4**</td>
</tr>
<tr>
<td>dCK (Ara-C as substrate)*</td>
<td>299±13</td>
<td>189±6**</td>
</tr>
<tr>
<td>dCK (Ara-C as substrate + THU)</td>
<td>282±22</td>
<td>200±10**</td>
</tr>
<tr>
<td>dCK + dGK (CdA as substrate)</td>
<td>345±28</td>
<td>351±74</td>
</tr>
<tr>
<td>dGK (CdA as substrate)</td>
<td>318±14</td>
<td>209±18**</td>
</tr>
<tr>
<td>Thymidine kinase 1 and 2</td>
<td>111±24</td>
<td>103±18</td>
</tr>
<tr>
<td>5’Nucleotidase – ATP</td>
<td>810±24</td>
<td>569±22**</td>
</tr>
<tr>
<td>5’Nucleotidase + ATP</td>
<td>11747±625</td>
<td>8185±191**</td>
</tr>
</tbody>
</table>

Standard deviation of at least two separate experiments.

**P< 0.05 compared with wild type cells. #Enzyme activities expressed as pmol/mg protein/min.

The resistant subline had a reduced dCK activity of about 35% and we found that 70% less triphosphates (Ara-CTP) were accumulated compared to HL60/wt. However, as HL60/r10 retained as much as 190 pmol/mg/min of dCK activity it is improbable that this is the only factor explaining the low sensitivity to nucleoside analogues. Furthermore, CdA is phosphorylated by both dCK and the mitochondrial enzyme dGK, and there was no difference in activity between the two cell lines regarding CdA phosphorylation.
Tetrahydouridine (THU) inhibition of cytidine deaminase, did not affect the kinase activity measured as crude extract. The lower number of cells undergoing apoptosis in HL60/R10 cells seemed to correspond to the reduced uptake of Ara-C for resistant cells and thus it seems improbable that the resistant cells had defective apoptotic machinery. 5'NT activity was decreased in R10 as compared to wild type cells excluding this as a cause for resistance. Increased pools caused by mutation or altered regulation of ribonucleotide reductase has been shown to cause nucleoside analogue resistance. The sensitivity to Ara-C was dependent of the size of the deoxynucleoside triphosphate pools, but the pools were almost unchanged for the resistant clone and thus not a contributor to resistance. Table I:1. The cytotoxicity assay showed that there was no resistance to tubericidin in R10 indicating that there was no defect/ decrease in nucleoside transport. The uptake and retention of Ara-C in resistant cells was only half of that in the parental cells indicating that R10 cells effluxed the drug faster. Moreover a significantly lower incorporation of Ara-CTP due to lower accumulation was found in R10 cells. Lower dCK activity can partly explain this as unphosphorylated Ara-C may leave the cell, but the higher efflux rate probably also depends on an active export pump probably the P-pg. To investigate this possibility we used reversing agents, Cyclosporine A (CsA), Cyclosporine analogue PSC and verapamil (Ver). The sensitivity (measured by cytotoxicity) to Ara-C increased in combination with CsA/PSC, as was the amount of cells undergoing apoptosis (measured by DNA fragmentation and caspase-3-like activity). However verapamil did not enhance sensitivity to Ara-C. Fig I:A and I:B.

**Fig I:A and I:B.** Cytotoxicity of Ara-C on HL-60 R10 cells without (A) and with (B) reversing agents.

The next study was performed to further investigate the mechanisms behind resistance against nucleoside analogues in another multidrug resistant cell line.

**Mechanisms of cross-resistance between nucleoside analogues and vincristine or daunorubicin in leukemic cells (Paper II)**

The aim of this study was to investigate the molecular and biochemical mechanisms behind the cross-resistance to nucleoside analogues of four erythroleukemic, K 562 cell-lines with acquired resistance to daunorubicin and vincristine. Four K 562 leukemia cell lines were
chosen. Two of them were resistant to vincristine at different degrees and the other two were selected for resistance to daunorubicin.

**Results and discussion**

The resistant cell lines displayed low sensitivity as well as low caspase-3-like activity upon treatment with daunorubicin, vincristine and nucleoside analogues. All resistant cell lines were highly resistant to Ara-C in clinically relevant concentrations. The resistant cell lines accumulated more calcein after co-incubation with P-gp inhibiting drugs. The presence of P-gp in the resistant strains was confirmed using Western blot with a specific monoclonal antibody, JSB1. The parental cells were devoid of P-gp. There was no difference in protein expression of MRP1-6 in resistant and wild type cell lines. This implicates that both daunorubicin and vincristine induced MDR1 expression in these resistant cells.

We found that the dCK activity measurements using crude cell extract of resistant strains (deoxycytidine as a substrate for phosphorylation) implied alterations in phosphorylating capacity rather than a transport defect. For this purpose the cellular dCK protein concentration was analysed by western blot and the mRNA quantified using real time quantitative PCR. The results show a clear decrease of dCK activity in the resistant strains (Figure II:1A), where vincristine resistant strains exhibit a decrease of 30-45%. In daunorubicin resistant strains the activity was as little as one fifth of the sensitive cell line level of enzyme activity. The latter values are in agreement to those obtained in another study of a K562 cell line with acquired
resistance to daunorubicin (Grant, et al 1995). We confirmed dCK activity by a western blot assay to measure the dCK protein. This showed a clear decrease of dCK level in vincristine as well as in daunorubicin resistant cell lines, although the difference compared with the sensitive cells was much greater for the two latter (figure II:1B). Reductions in dCK activity and protein levels were in concordance with a decrease in dCK mRNA (Figure II:1C).

We found the size of the amplified gene as expected – 834 bp, implying that truncated mRNA as a reason for cross-resistance does not apply in this case (figure II:1D).

Elevated expression of several 5’ nucleotidases has been shown to lead to drug resistance in several studies mainly in vitro (Lotfi, et al 2001), but the clinical relevance is yet unclear.

However, in this study we found elevated levels of 5’-NT activity using IMP as substrate, resulting in several fold increased 5’-NT/dCK enzyme ratios in resistant cells (figure II:2). The 5’-NT mRNA levels using the real time quantitative PCR method showed comparable results as with the wild type cells.

The results from these papers prompted us to study the cross-resistance pattern for different nucleoside analogues and daunorubicin on leukemic cells from patients with AML.

**High activity and incomplete cross-resistance of nucleoside analogues cladribine and fludarabine versus Ara-C on leukemic cells from patients with AML (Paper III)**

The purpose was to evaluate in vitro activity and cross-resistance patterns of the purine analogues cladribine and fludarabine and the pyrimidine analogue cytarabine on leukemic cells from 170 patients with AML using a bioluminescence assay.

**Results and discussion**

We have used an ex vivo method to investigate the relation between a certain drug concentration and its effect on the malignant cell. In contrast to other assays using IC50 concentrations, which come close to in vivo plasma peak levels, we mimic the drug concentration found intracellularly in vivo during therapy and using concentrations and incubation times as in the clinic, for the different drugs.

Approximately 25% of the samples were highly sensitive to Ara-C or CdA/fludarabine. Fig III:1. Cells from patients with secondary leukemia were slightly more sensitive to Ara-C than cells from de novo or relapsed AML.

We found that CdA was significantly more active than Ara-C (p < 0.05) but not more than fludarabine. Fig III:1. The cytotoxicity of CdA correlated strongly to fludarabine (r =0.82, p<0.0001) but less towards Ara-C (r =0.49, p=0.002). The cells were also tested for daunorubicin. The correlation between CdA or fludarabine and daunorubicin was weak (r=0.37 and 0.44) and there was an absence of correlation between daunorubicin and Ara-C (r=0.3, p=0.08).

In this study we found a strong correlation between CdA and fludarabine and Ara-C, in accordance with findings of other groups (Kristensen, et al 1994). The weak correlation to daunorubicin indicates different mechanisms of resistance between anthracyclines, well-known substrates for ABC-transport proteins such as P-gp and MRP, and these nucleoside analogues. As discussed above in vitro studies indicate that down-regulation of dCK is a mechanism of resistance for Ara-C and CdA, but this is not shown for fludarabine as it is a weak substrate for dCK, which might explain the correlation in sensitivity between Ara-C and CdA. The strong correlation between CdA and fludarabine is more difficult to explain, but both drugs are strong inhibitors of ribonucleotide reductase.
45 samples were highly resistant to Ara-C and of those, 5 samples were highly sensitive and another 18 samples intermediately sensitive to CdA. 4 and 8 samples were sensitive to fludarabine. Thus the correlation between the nucleoside analogues is not complete; a significant number of Ara-C resistant cells are sensitive to CDA or fludarabine. This indicates that CdA and fludarabine can play a role as alternatives or in combination with Ara-C.

In contrast to previous studies by our and other groups (Kristensen, et al 1994, Tidefelt, et al 1989) we did not find correlates between in vitro effects of these drugs, clinical outcome and prognostic markers such as cytogenetic abnormalities or FAB group. Many of the patients included in this study did not receive treatment with curative intent if at all, due to high age or concomitant illness. When used, induction regimens were conventional and did not contain cladribine or fludarabine in more than a few cases. Furthermore, in our assay, each drug is tested as a single agent to make it possible to deduce similarities or differences in resistance patterns, in the clinical setting combination chemotherapy is standard.

**Fig III:1.** Cytotoxicity of CdA, Ara-C and fludarabine on leukemic cells from patients expressed as % growth as compared to a control sample.

Another way to increase the activity of nucleoside analogues can be to synchronize and increase the number of leukemic cells in S-phase by treatment with growth factors. To investigate this was the objective of the next paper

**Granulocyte-macrophage colony-stimulating factor to increase efficacy of mitoxantrone, etoposide and cytarabine in previously untreated elderly patients with acute myeloid leukemia: a Swedish multicenter randomised trial. (Paper IV)**

The aim of this study was to study the impact on remission rate and survival of addition of GM-CSF to an intensive induction schedule consisting of MEA given to elderly AML patients and to evaluate the influence of maintenance treatment with thioguanine on survival. This was performed as a double randomised study with two induction/consolidation arms and two arms of maintenance treatment. 110 patients with de novo AML 64 years and older were randomised.
Results and discussion
In this study the 3-day MEA protocol induced an overall remission rate of 64.5%. Despite the higher median age in our study (77 years), this is well in accordance with the remission rates achieved in elderly patients in previously published studies (Rowe, et al 1995, Shepherd, et al 1993). Several reasons for the higher remission rate in our study can be found. First, patients with high leukocyte counts over 50x10^9/L were excluded. A high WBC count at diagnosis is generally regarded as a marker for poor prognosis. Second, only patients with de novo AML were included. High risk MDS or AML with antecedent hematological disorder such as MDS are associated with a poorer response to chemotherapy (Bernstein, et al 1996, Estey, et al 1997). This has recently been confirmed in a separate study of 93 patients, median age 72 years) with high-risk MDS and MDS-AML, showing an overall CR rate of 43% after 6-thioguanine, cytarabine, daunomycine (TAD) chemotherapy (Hast, et al 2003). Finally, we reported a lower number of toxic deaths, 12% within the first 30 days, than reported in other studies that treated elderly patients 5-10 years younger than the patients in our study (Kalaycio and Andresen 2001, Rowe, et al 1995, Stone and D T Berg 1995). This might be due to a selection bias as only patients considered fit for intensive treatment were included in our study, excluding those patients with severe organ dysfunction or concomitant severe disease.

The addition of GM-CSF did not augment the number of CR compared to treatment with MEA only. Without MEA 36/55 (65%) entered complete remission compared to 35/55 (64%) (n.s.) with the addition of GM-CSF. The median age of patients entering remission was 78.8 years and the oldest patient was 94 years old. In a multivariate analysis gender, FAB subgroups or karyotypic risk profiles were not significantly associated with differences in remission rates.

The median remission duration was 13 months for patients that did not receive GM-CSF and 6 months with GM-CSF with 10% and 18% in continuous complete remission at 6 years. The median overall survival for all patients was 12 months with 12 % alive after 5 years. Without GM-CSF the median OS was 14 months compared to 9 months with GM-CSF treatment and with 10% and 8% alive after 6 years (p=0.07, log-rank). Figure IV.

The median relapse free survival was short and did not differ between the two treatment arms. In both arms we found an unusually high number of verified septicaemias; 39 episodes in 19 GM-CSF treated patients as compared to 46 episodes in 31 patients in the non GM-CSF arm (p=0.05), possibly since elderly patients are more prone to and susceptible to septicaemia after intensive treatment. The significantly lower number of septic episodes in the GM-CSF treated arm did not translate into a better survival, also this in accordance with previous findings.

Thirty patients were randomised a second time, in remission; 16 were randomised to maintenance therapy with 160 mg thioguanine weekly. There was no difference regarding the median remission duration for these patients, 18 months, compared to 16 months for those without maintenance. For patients receiving maintenance therapy the median OS was 28 months versus 16.5 months for not receiving maintenance (p=0.5, n.s, log-rank)

We refrained from further analysis of the effect of maintenance treatment with thioguanine since due to early relapse during consolidation only 30 patients were evaluable.
Figure IV. Overall survival after treatment with or without the addition of GM-CSF.

In the first four papers we studied mechanisms behind resistance to nucleoside analogues and ways to improve their effect against AML. The next paper focused on investigating the possibility to improve the antileukemic effect of the other major AML drug – daunorubicin, by enveloping it in a drug carrier.

**Higher plasma but not intracellular concentrations after infusion with liposomal daunorubicin compared to free daunorubicin in adult acute myeloid leukemia (paper V)**

This was designed as a pharmacokinetic study where liposomal daunorubicin, dXm was to be compared to free daunorubicin (dnr) with respect to plasma and intracellular pharmacokinetics. The active metabolite of daunorubicin, daunorubicinol was also measured. Remission rate, survival, bone marrow toxicity and other toxicity were recorded, but not intended for detailed analysis. Complete pharmacokinetic profiles were obtained for 13 of the 14 included patients as in one patient the sampling was interrupted due to treatment in the ICU. Due to a prolonged intracellular retention than expected the intended inter-individual comparison of intracellular pharmacokinetics could not be performed.

**Results and discussion**

The CR rate in our study (11/14, 79%) was comparable to that reported after standard induction chemotherapy in AML and the toxicity was similar albeit the study was not designed to evaluate the clinical effects of DaunoXome®. We found pronounced differences in plasma pharmacokinetics between the two formulations despite large inter-individual variations in plasma peak concentration, AUC, clearance and volume of distribution. Table V:1.

**Table V:1**

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The plasma peak levels and the AUC of dnr were 100-fold higher after infusion of liposomal daunorubicin (34.3µM) than after free daunorubicin, (0.29µM) which was in accordance with previous studies (Forssen 1997, Gill, et al 1995). The plasma levels of daunorubicinol, reflecting the metabolism of dnr, was similar for the two formulations, (0.14 and 0.13µM respectively, n.s.) which is in accordance with the findings of Pea et al (2003) (Pea, et al 2003) but in contrast to those of Bellott et al (2001) where the plasma concentration of dol after infusion of dXm appeared to be lower than found after conventional administration of dnr (Bellott, et al 2001). In plasma no apparent carry over effect from day 1 to day 3 was seen. The initial and terminal half-lives for dnr and dol were slightly but significantly (p<0.05) longer after infusion of dXm compared to after free dnr (1.9 and11.6 vs. 1.1 and 9.6 hours). We also found that the liposomal encapsulation gave a markedly reduced distribution volume, 5.5 L compared to 3640 L after infusion of free dnr, indicating a lower tissue affinity that might explain the proposed lower cardiotoxicity of liposomal daunorubicin (Drummond, et al 1999, Gill, et al 1995). The plasma clearance was considerably slower, 0.001L/h, after dXm vs. 0.4 L/h after dnr (p<0.005). This was also true for the plasma clearance of the metabolite dol, 0.06 vs. 0.1 L/hr. Plasma AUC of the parent drug was increased 100-fold and the AUC of dol was two fold increased after infusion of dXm compared to free dnr.

In contrast to what was seen in plasma, after infusion of the liposomal formulation, intracellular peak concentrations were lower, peaked 1-2 hours later and lasted longer before declining, resulting in a different time vs. concentration profile compared to free dnr as illustrated in fig V:1. The much higher plasma levels of dnr after infusion of dXm (106.2µM) than after infusion of free dnr (45.58µM) did not translate into higher intracellular AUC. The intracellular peak levels of the active metabolite dol were comparable between the two formulations. The intracellular half-life for dnr was 10.23 hrs after infusion of free dnr and 10.24 hrs after infusion of dXm. Dol half-lives were comparable on day 1 around 17 hrs and prolonged on day 3, especially if dXm was given on day 1 (23 hrs). There was an evident carry over effect from day 1 to day 3 for the intracellular dnr, an accumulation that prevented us from making an interindividual statistical analysis.

**Figure V:1.** Plasma (A) and intracellular (B) concentrations of dnr and dol after infusion of DaunoXome or daunorubicin.
GENERAL DISCUSSION
The overall aim for this thesis was to find ways to improve treatment results for patients with AML, especially those that do not respond to conventional treatment or the patients with relapsed disease. In these situations resistance to one or both components of standard induction treatment, Ara-C and daunorubicin, is a clinically important issue. In the first four papers different studies on Ara-C are presented concerning molecular mechanisms behind Ara-C resistance in cell lines, cross-resistance patterns of leukemic cells from patients and finally a randomised trial on an attempt to improve the effect of Ara-C on clinical outcome. The fifth paper focuses on the “other leg” of AML 3+7 induction treatment, daunorubicin.

Possible biochemical and molecular mechanisms behind cross-resistance to nucleoside analogues, mainly Ara-C but also in some extent to CdA were pursued in studies on two different cell-lines, HL 60 and K 562 with acquired resistance to anthracyclines and for K 562 also to vincristine. These cell lines were also highly resistant to Ara-C and to a lesser degree to CdA. We were able to confirm presence of MDR1-gene and protein in the resistant cell lines. Our results show that over-expression of MRP, LRP or topoisomerase α or β are unlikely as mechanisms of resistance. However, P-gp over-expression can not fully explain the insensitivity to nucleoside analogues since they are not substrates of this protein, as shown in a study where K 562 cells transfected with MDR1 cDNA remained sensitive to Ara-C (Hait, et al. 1993). The substrates of P-gp are not undisputedly established though, and as shown, paper I indicated that Ara-C on the contrary does work as a P-gp substrate. In addition, other studies have presented results showing that Ara-C (Hu, et al. 1999) and 9-beta-D-arabinofuranosylguanine (Lotfi, et al. 2002) upregulate the expression of P-gp, implying that over expression of the MDR1 gene might be a general response to certain forms of cytotoxic stress. We found reduced dCK activity in all resistant strains. We confirmed dCK activity by a western blot assay to measure the dCK protein. Reductions in dCK activity and protein levels were in concordance with a decrease in dCK mRNA. Recent studies describe expression of several alternatively spliced dCK mRNA constructs in AML patients and in leukemic cell lines (Veuger, et al. 2000). Low dCK activity leads to less formation of active drug intracellularly. It seems likely that this mechanism is responsible for the major part of the resistance to nucleoside analogues. Loss of activity can depend on several defects in the cell such as deletions or inactivating mutations of the gene (Owens, et al. 1992). We found the size of the amplified gene as expected – 834 bp, implying that truncated mRNA as a reason for cross-resistance does not apply in this case (figure II:1D).

Although dCK defects in daunorubicin- and vincristine-resistance selected cells were identified as major contributors to the resistant phenotype to nucleoside analogues, we analysed whether other known mechanisms of nucleoside analogue resistance could make additional contributions. These cells were cross-resistant to Ara-C as well as to CdA and the latter drug is not deaminated by deoxycytidine deaminase. Therefore the possibility that elevated levels of deoxycytidine or deoxycytidine monophosphate deaminase might be resistance factors were not further investigated. In K 562 resistant cell lines we found elevated levels of 5’NT activity resulting in several fold increase in 5’NT/dCK enzyme ratios in resistant cells. This is in agreement with the observation that 5’-NT/dCK ratio might be an important factor in predicting resistance to NAs (Galmarini, et al. 2004, Galmarini, et al. 2003, Kawasaki, et al. 1993). In contrast, in resistant HL 60 cells, 5’NT activity was decreased.
To further investigate the possibility of P-gp related efflux as a cause for Ara-C resistance we analysed the cytotoxicity after addition of P-gp reversals and we found that the effect of Ara-C increased in presence of Cyclosporine A or PSC but not verapamil. PSC has been shown to act in part by a P-gp independent mechanism to alter apoptosis, through activation of ceramide synthetase. CsA causes alterations in the membrane fluidity. But in this study only the P-gp over-expressing cells were influenced by the addition of reversing agents, indicating that the effect was somehow linked to P-gp in these cells.

In conclusion, P-gp induction through acquired resistance to anthracyclines and vincristine also confers resistance to Ara-C and to some extent to CdA. This resistance can be reversed by cyclosporine analogues. However, these resistant cells also express reduced dCK activity, protein and mRNA. Elevated levels of 5’NT activity was found in K 562 but not in HL 60 resistant cells adding increased 5’NT/dCK enzyme ratio as an additional mechanism for Ara-C resistance.

These in vitro studies give important information regarding mechanisms of Ara-C resistance in cell lines with acquired resistance to anthracyclines. The cell lines were also cross-resistant to CdA. However clinical reports showed that patients with refractory AML after treatment with Ara-C and daunorubicin could attain complete remissions after treatment with regimens containing the purine analogues CdA or fludarabine. (Santana, et al 1991, Spriggs, et al 1986, Strickland, et al 1999) The clinical role of these analogues has yet to be determined as well as the clinical relevance of the observed cross-resistance to Ara-C in cell lines. This prompted us to evaluate the in vitro effect of nucleoside analogues on leukemic cells from patients with AML with focus on the resistance patterns of CdA and of fludarabine compared to Ara-C but also to daunorubicin. The samples in this study are taken at time of diagnosis, or relapse, on consecutive patients during the study period and are thus representative for the population of patients with AML in the recruitment area of our hospital.

For most cytostatic drugs conventional therapeutic drug monitoring (TDM) is associated with a number of complications. The drugs are mainly given as short infusions for a few days, thus no plasma steady states are achieved. Furthermore, they are often highly concentrated intracellularly with small or no relations between plasma and target cell, the malignant cells (Tidefelt, et al 1989). In order to further increase the clinical relevance in this way to monitor the effect of cytotoxic drugs we have based our incubation concentrations not only on plasma drug levels in patients but also in in vivo intracellular concentrations in the leukemic cells (Gandhi and Plunkett 2002, Lillemark and Juliusson 1991, Tidefelt, et al 1989).

For nucleoside analogues an additional problem is that they need activation by intracellular phosphorylation. CdA and fludarabine are potent inhibitors of ribonucleotide reductase and augment ara-CTP formation (Galmarini, et al 2001). Thus these drugs not only have antileukemic potential in themselves but also by potentiating effects of Ara-C, thereby possibly overcoming Ara-CTP resistance. The results of our study verify that in vitro CdA and fludarabine have a high activity against leukemic cells from patients with AML. In fact the average toxicity on these cells were higher than for Ara-C (p<0.001). The finding that leukemic cells from patients with secondary or relapsed leukemia were slightly more sensitive to Ara-C is in concordance with previous findings of Möllgård et al (Möllgard, et al 2003) and might depend on a higher proliferative status of the leukemic cells in these leukemias and the cell-cycle specificity of Ara-C.
It is also evident that other factors such as polymorphism in metabolism and secretion can be of importance for the antileukemic effect of these drugs. Taking these facts together with the variation of treatment protocols and of treatment intensity administered to these patients into account it is not surprising that we did not find any correlations between sensitivity to these drugs and remission rates.

In these leukemic cells there was a strong correlation for CdA, fludarabine and Ara-C. However the cross-resistance was not complete; we found no correlation between the effects of daunorubicin and the other drugs indicating different mechanisms of resistance other than P-gp and MRP, between anthracyclines and these nucleoside analogues. Furthermore, a significant number of the 45 highly Ara-C resistant samples were highly or moderately sensitive to CdA or fludarabine, which is of clinical interest and indicates differences in resistance mechanisms between the nucleoside analogues. In vitro studies on cell lines indicate that absence or down regulation of dCK, one of the mechanisms for Ara-C and CdA resistance, is not valid for fludarabine (Mansson, et al 2003). This could be an explanation for the weaker correlation between Ara-C and fludarabine than for Ara-C and CdA. Our finding of a strong correlation between CdA and fludarabine is more difficult to explain but both are strong inhibitors of ribonucleotide reductase. Thus, in vitro CdA and fludarabine in clinically used concentrations are at least as active agents against AML as Ara-C and that cross-resistance of CdA or fludarabine to Ara-C is not complete. This indicates that in some cases CdA and fludarabine could be of clinical value as single agents or in combination with Ara-C.

The pharmacological properties of Ara-C present two theoretical targets for attempts to increase the cytotoxic effect and thereby improve the clinical outcome: it is cell cycle specific and needs intracellular activation by dCK. Based on the assumption that GM-CSF by inducing cell proliferation (Bettelheim, et al 1991, Estrov, et al 1992) prime the cells into S-phase and thereby render them more susceptible to Ara-C a phase III randomised trial evaluating the clinical effect of Ara-C with the addition of GM-CSF was initiated. Furthermore in vitro GM-CSF has been described to augment Ara-CTP to dCTP ratio (Hiddemann, et al 1992). The addition of GM-CSF was described to have additional benefits such as shorter time to neutrophil recovery, less septicaemia and thereby possibly a benefit on survival. In our study, the addition of GM-CSF did not augment the number of CR compared to treatment with MEA only. There was a tendency towards shorter remission duration in the GM-CSF treated arm. The overall survival did not differ between the two groups but here was a tendency toward shorter survival in the GM-CSF treated arm which is in accordance with other studies where GM-CSF was added from day 10 after start of chemotherapy (Rowe, et al 1995, Shepherd, et al 1993, Stone, et al 2001). A proposed explanation for this is an increased risk for early relapse caused by stimulation of the malignant clone during GM-CSF treatment after chemotherapy, during the neutropenic period. We found a small but significant reduction of the time to neutrophil recovery for the GM-CSF treated patients, 17 d vs. 25 d (p=0.03) but even so, in both arms we found an unusually high number of verified septicaemias; 39 episodes in 19 GM-CSF treated patients as compared to 46 episodes in 31 patients in the non GM-CSF arm (p=0.05), possibly since elderly patients are more prone to and susceptible to septicaemia after intensive treatment. The significantly lower number of septic episodes in the GM-CSF treated arm did not translate into a better survival, also this in accordance with previous findings.
In summary, intensive cytostatic treatment is feasible in patients in good physical condition with low leukocyte counts, giving lasting remission with acceptable toxicity even in very advanced age. The addition of GM-CSF did not improve remission duration or survival despite reduced time to neutropenic recovery as well as neutropenic septicaemia. However, the addition of GM-CSF prior and concomitant to chemotherapy does not improve the outcome of AML in the elderly.

Multidrug resistance could theoretically be, at least partially, circumvented by dose-escalation. Unfortunately this is not possible for the second cornerstone of leukemia treatment, daunorubicin, due to dose-dependent fatal cardiotoxicity. This side effect also limits the number of courses applicable to one patient and thus in advanced relapse, or in patients with concomitant congestive heart disease, daunorubicin, even if efficient is no longer an option. In previous studies we found that administering doxorubicin complex-bound to DNA as a drug carrier increased the doxorubicin uptake in the leukemic cells as well as the long-term survival for patients with AML (Paul, et al. 1989, Paul, et al. 1991); and the DNA-complex also had a decreased cardiotoxicity.

Liposomal formulations were designed to minimize anthracycline side effects especially cardiotoxicity (Forssen 1997) and have been alleged efficacy in P-gp positive tumours (Mamot, et al. 2003, Wang, et al. 1999). The current indication for liposomal daunorubicin DaunoXome is first line cytotoxic therapy for advanced HIV associated Kaposis Sarkoma and DaunoXome is also entered in clinical trials in AML. In different studies plasma levels after DaunoXome were 200-800 times higher than after free daunorubicin (Forssen 1997, Gill, et al. 1995). However, the pharmacokinetic properties have not been studied in detail on adult patients with AML. This is the first report about intracellular, target cell, pharmacokinetics of the drug, comparing liposomal daunorubicin to free daunorubicin with respect to plasma and intracellular pharmacokinetics during standard induction treatment with a 3+ 7 regimen due to AML.

In concordance with earlier studies we found pronounced differences in plasma pharmacokinetics. In contrast after infusion of liposomal daunorubicin the much higher plasma levels of daunorubicin did not translate into a higher AUC. Peak intracellular concentrations were 200 fold higher than peak concentrations in plasma after infusion of free dnr due to rapid uptake. But after infusion of dXm the intracellular peak concentrations and plasma were comparable indicating a slower uptake. Intracellular AUC of dnr was 100 fold that of plasma after infusion with free dnr in contrast to after infusion with dXm where the intracellular increase was only 3 – 5 times.

One possible explanation for this difference in intracellular time versus concentration curve is the liposomal encapsulation. The liposomal daunorubicin is slowly released from the liposomes either extracellularly by spontaneous rupture of liposomes or intracellularly after endocytosis (Forssen 1997). This results in a slow release of drug even though administered as a short time infusion. In this study we used the same dose (50 mg/m²) of liposomal and free daunorubicin. Even though this yields a 2-log higher plasma concentration after liposomal daunorubicin compared to free daunorubicin since daunorubicin exerts its effect intracellularly, based solely on the intracellular exposure (AUC), these could be regarded as comparable doses regarding efficacy and risk for bone marrow toxicity. If the different intracellular pharmacokinetic properties of dXm, with a lower peak and a prolonged retention, is advantageous for treatment of leukemia is not clear. Differences in anthracycline efficacy
against acute leukemia have been associated to the intracellular peak-values reached (Tidefelt, et al 1994), the lower peak-value after dXm might indicate an inferior effect compared to native dnr. On the other hand, for other drugs it has been shown that a more prolonged plasma level exposition is favourable for the clinical effect (Alvan, et al 1999). It can be speculated, but not shown in our study, that the liposomal formulation makes daunorubicin more suitable for treatment of solid tumours than for leukemia.
CONCLUSIONS
Some general conclusions were drawn from this thesis:

- The cross-resistance to Ara-C for P-gp-expressing cell lines with acquired anthracycline resistance can be explained by two mechanisms: downregulation of the ara-C activating enzyme dCK sometimes combined with increased 5’NT activity leading to increased 5’NT/dCK enzyme ratios and an increased efflux that could be influenced by CsA and PSC (valspodar).

- In in vitro testing on leukemic cells from patients the cross-resistance between the nucleoside analogues is not complete; a significant number of Ara-C resistant cells are sensitive to CDA or fludarabine. This indicates that Cda and fludarabine can play a role as alternatives to or in combination with Ara-C in the treatment of AML.

- The addition of GM-CSF prior to and in combination with induction treatment does not improve the effect of Ara-C. The addition reduces the time to neutrophil recovery, as well as neutropenic sepsicaemia but does not improve remission duration or total survival. Thus the addition of GM-CSF before during and after chemotherapy does not improve the outcome of AML in the elderly. However, intensive cytostatic treatment is feasible in patients in good physical condition with low leukocyte counts, giving long-lasting remission with acceptable toxicity even in very advanced age.

- With liposomal daunorubicin 100-fold higher plasma concentrations are reached than after free daunorubicin but the intracellular exposure in leukemic cells from patients with AML is comparable and thus there is no pharmacologic evidence that DaunoXome, at equal dose, is superior to ordinary daunorubicin for treatment of acute leukemia.
WHAT NOW? - FUTURE PROSPECTS

In this thesis some, but not all of the surmised mechanisms of resistance have been perused. Loss of dCK activity leads to less intracellular formation of active drug. This loss of activity can depend on several defects in the cell such as deletions or inactivating mutations of the gene. Alternative splicing has been suggested as a cause of inactivation. Hypermethylation of the CpG island in the dCK promoter has been shown to correlate with Ara-C resistance. It would therefore be interesting to investigate the anthracycline-resistant strains of HL60 and K 562 in that respect.

In previous in vitro studies we found that substances such as arsenic and selenite in low concentrations are effective inducers of apoptosis in multidrugresistant AML cells. These findings should be further explored in the clinic. Another question is the role of altered apoptotic response as a resistance mechanism. PKC activation opposes apoptosis in hematopoietic cells. Increased protein kinase C (PKC) activity is observed in leukemic cells in response to Ara-C in vitro, perhaps due to Ara-C induction of diacylglycerol, which in turn induces PKC activity. The cytotoxic action of Ara-C might partially depend on relative effects on the PKC pathway. To study the impact of PKC-inhibitors on cellular Ara-C, CdA and fludarabine metabolism on sensitive and resistant cell lines and on leukemic patients is another project for the immediate future.

In our in vitro experiments on leukemic cells from patients a possible reason for the lack of correlation between in vitro sensitivity data and clinical outcome is that CdA or fludarabine in combination with Ara-C has not been used as induction treatment. We have conducted such a study. 65 patients with refractory or relapsed AML have received FLAG-ida and are currently evaluated for remission rate, remission duration and survival as well as hematologic toxicity. For these patients we also have corresponding in vitro chemosensitivity data. Frozen samples of leukemic cells from some of these patients will be evaluated regarding NA metabolism (dCK, 5’NT, NTP, RR) and resistance factors and compared to clinical outcome (remission vs. non-remission).

Activation of the apoptotic pathway in response to DNA damage, in a p53 requiring process, is responsible for cytotoxicity of chemotherapeutic drugs and radiation. The p53 tumor suppressor gene is mutated in almost 50% of cancers at diagnosis leading to drug resistance. Recently, the compound p53-dependent reactivation and induction of massive apoptosis (PRIMA-1) has been shown to induce cytotoxic effects and apoptosis in human tumour cells by restoration of the transcriptional activity of mutated p53. This is believed to be mediated by a change in the conformation of mutated p53 protein, restoring DNA binding and activation of p53 target genes. We are presently conducting a study of the effects of PRIMA-1 and commonly used anti-leukemic drugs on AML cells. Another substance of interest is RITA (reactivation of p53 and induction of tumor cell apoptosis) that binds to p53 and induces its accumulation in tumor cells. RITA has been shown to induce expression of p53 target genes and massive apoptosis in various tumor cells lines expressing wild-type p53.

Furthermore the role of genetic polymorphism for daunorubicin and Ara-C metabolism is not studied in detail. Polymorphism, or genetic variation that can be found as traits in the population, leads to different effects of the encoded protein. If the polymorph gene is engaged in drug metabolism, depending on which variant the individual carries the effect, and side effects, of the drug could be less or more than with the wild type gene. Thus, polymorphism is yet another factor to be reckoned regarding resistance to cytostatic drugs. Daunorubicin is metabolised mainly into its alcohol form by a member of the ketoalldoreductase family. We
are now planning a project to study whether this gene is subject to polymorphism. Neither has the role of polymorphism of cytochrome P been evaluated for these cytostatic agents.

**SUMMARY IN SWEDISH; SAMMANFATTNING PÅ SVENSKA**


**I delarbete I och II** utvärderades två olika typer av leukemi cell-linjer med resistens för antracykliner avseende samtidig resistens mot Ara-C och möjliga mekanismer för denna. Dessa cell-linjer skiljer sig åt dels avseende sorten av leukemi de härstammar från men också
genom att de utvecklat resistens mot olika antracykliner. I en resistent HL 60 celllinje med högt uttryck av P-gp fann vi även resistens mot Ara-C. Aktiviteten av deoxycytidinkinas, dCK, var nedsatt. Därutöver fann vi endast hälften så mycket Ara-C i resistaenta celler jämfört med i icke-resistenta ursprungsceller. Detta visade högre utflöde av Ara-C i resistaenta celler, eventuellt via P-gp. Detta kunde i sin tur bekräftas genom att tillsats av substanser som blockerar P-gp ökade de resistaenta cellernas känslighet för Ara-C. På liknande vis fanns en korsresistens för Ara-C i K 562 celler. Även dessa celler uttryckte P-gp och nedsatt dCK aktivitet, men in i motsats till HL 60 celler förelåg hos dessa resistaenta K 562 celler en förhöjd 5’nukleotidas (5’NT) aktivitet vilket resulterade i flerfaldigt förhöjda 5’NT/dCK enzymkvoter ledande till en kraftigt minskad aktivering av Ara-C. *Sammanfattningsvis* visade försöken i delarbete I och II att kors-resistens mot Ara-C i P-gp-uttryckande cell-linjer kan förklaras av två mekanismer; nedreglering av det Ara-C aktiverande enzymet dCK, ibland i kombination med ökad 5’NT aktivitet och ett ökat utflöde som kan påverkas av hämmare av P-gp pumpen.


**I delarbete IV**, studerades om effekten av Ara-C kunde ökas genom att till behandling med cellgift ge tillägg av tillväxtfaktorn GM-CSF före, under och efter behandlingen. GM-SCF stimulerar blodceller till delning, tillväxt, vilket dels teoretiskt gör dem mer känsliga för cellgifter vilka är verksamma under delningsfas som t ex. Ara-C, men GM-CSF leder även till snabbare återhämtning av normala vita blodkroppar efter behandling och därmed teoretiskt mindre risk för allvarliga infektioner. Detta skulle innebära att mer känsliga personer, t ex. patienter över 60 år, skulle kunna ges en kraftigare behandling och därmed eventuellt kunna botas från sjukdomen. I vår studie lottades patienterna till antingen en Ara-C innehållande behandling, s.k. MEA-kur, eller MEA i kombination med GM-CSF. Behandlingen ledde till remission hos 64,5% av patienterna men utan skillnad i överlevnad mellan alternativen. Tiden till dess att antalet neutrofila vita blodkroppar i blodet (mått på benmärgsfunktion liksom infektionskänslighet) normaliserats var betydligt kortare för dem som behandlats med GM-CSF, 17 jämfört med 25 dagar (p=0,03). *Konklusionen* blev således att tillägg av GM-CSF inte förbättrar effekten av behandling med Ara-C. Tillägget minskar visserligen tiden till återhämtning liksom antalet allvarliga infektionskomplikationer men förbättrar inte varaktigheten av remission eller total överlevnad. Vi kunde också visa att det var möjligt att ge äldre patienter i god fysisk kondition intensiv cellgiftsbehandling med acceptabla biverkningar, resulterande i långvariga remissioner även i mycket hög ålder.
Slutligen studerades i delarbete V, det ”andra benet” i vår standardbehandling nämligen daunorubicin. Ett sätt att komma runt effekten av multidrogresistens skulle kunna vara att öka dosen läkemedel så att P-gp pumpen inte hinner med. Tyvärr är det inte möjligt när det gäller daunorubicin pga. dosberoende hjärtbiverkan. Genom att kapsla in daunorubicin i fettbubblor, liposomer, till dess att dessa tas upp av tumörcellerna skyddar man teoretiskt andra celler från daunorubicinets skadliga verkan samtidigt effekten ökas genom att en större mängd läkemedel per given dos skulle kunna komma till målcellen. Hur stor del av det på så vis inkapslade daunorubicinet som verkligen kommer till målcellen har inte tidigare studerats. Vi jämförde liposomalt daunorubicin, DaunoXome, med vanligt daunorubicin med avseende på koncentrationer i plasma och intracellulär, i leukemiceller. 14 patienter behandlades med en modifierad ”3+7” där DaunoXome ersatte en av de tre daunorubicindoserna. Före under och efter behandling togs blodprover och såväl plasma som leukemiceller analyserades med avseende på innehåll av daunorubicin. Vi fann att efter DaunoXome var plasmanivåerna mer än 100 gånger högre än efter vanligt fritt daunorubicin men de intracellulära nivåerna var likvärdiga. Vi fann att daunorubicin fördelade sig i kroppens alla vävnader medan DaunoXome var starkt koncentrerat till blodbanan. Således ger liposomalt daunorubicin i form av DaunoXome likvärdig koncentration inne i leukemiceller trots hundrafalt högre plasmakoncentration jämfört med konventionellt fritt daunorubicin.

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REFERENCES


Men vad lär dig vad morgonstjärnan är?
Koranen: Sura 86
In Japan the crane is a symbol of good-fortune and of long life together with the turtle, pine and bamboo. The bird was perceived the messenger between heaven and earth, the link between man and the spiritual world. In the concept of “thousand cranes” is the Japanese tradition that as physical proof of compassion and love, fold one thousand paper cranes to a sick relative or friend.

My daughter Emelie folded over 100 paper cranes when her primary school participated in a World Peace Project for Children one of the “thousand cranes peace networks” connecting people and activities to promote peace, non-violence and tolerance, all emanating from the same source and based on the principle that the determination of the individual is what makes the difference. Working at our kitchen table she told me the story about Sadako Sasaki, a young Japanese girl, who developed leukemia in 1955, from the effects of the radiation caused by the bombing of Hiroshima. While hospitalised, her friend reminded her of the Japanese legend that if she folded a thousand paper cranes, the gods might grant her wish to be well again. With hope and determination Sadako began folding. She reached her goal of a thousand cranes but never remission. The point is that she never gave up, she continued folding paper cranes until her death less than three months later. After her death and inspired by her determination her friends dreamt of building a monument for Sadako and all the other children killed by the atom bomb. Today, paper cranes is sent from all over the world to Sadako’s monument. At the bottom of the statue of Sadako and a golden crane in Hiroshima Peace Park an inscription reads: This is our cry, This is our prayer, Peace in the world.