Nucleoside analog cytotoxicity
-focus on enzyme regulation, metabolism, and development of resistance

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“Science is nothing but developed perception, interpreted intents, common sense rounded out and minutely articulated.”

George Santayana

“Science is facts; just as houses are made of stones, so is science made of facts; but a pile of stones is not a house and a collection of facts is not necessarily science.”

Henri Poincare

“The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them.”

Sir William Bragg
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Enzyme activity measurement, papers I-V

RNA interference, papers I, III & IV

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Gene expression microarray, papers III & V

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Results and discussion

Expression and activity of nucleoside analog metabolizing enzymes and correlation with nucleoside analog cytotoxicity (papers I & II)

Downregulation of nucleoside analog phosphorylating enzymes in order to elucidate their involvement in activation (papers III & IV)

Characterization of a nucleoside analog resistant cell line using gene expression microarray analysis (paper V)

Conclusions

Future perspectives

Acknowledgments

References
Abstract

The aim of this thesis was to determine the role of nucleoside analog activating and deactivating enzymes in nucleoside analog metabolism and resistance development. Nucleoside analogs are anti-cancer drugs and are often used to treat different leukemias, attributably to presence of high levels of nucleoside analog activating enzymes in hematopoietic cells. More recently some of the newer analogs have been used successfully to treat solid tumors as well.

We have used human leukemic cell lines, and isolated cells from patients with leukemia, to investigate the nucleoside analog activating enzymes deoxycytidine kinase (dCK) and deoxyguanosine kinase (dGK) and some of the deactivating enzymes called 5´-nucleotidases (5´-NTs). We have measured mRNA expressions and enzymatic activities and correlated them with the cytotoxic response to nucleoside analogs and changes in cell cycle progression. We optimized and evaluated a siRNA-transfection method and decreased the activities of dCK and dGK in two different cell lines in order to find out more about their respective contribution to activation of these drugs. An expression microarray analysis of a nucleoside analog resistant cell line was also performed in order to clarify which genes are involved in development of resistance.

We found that expressions and activities of dCK and dGK were not correlated The enzyme activities of activating and deactivating enzymes changed during cell cycle progression, giving actively proliferating cells a more favorable enzymatic profile with regard to nucleoside analog cytotoxicity.

The activities of dCK and dGK could be reduced transiently in leukemic and solid tumor cell lines, thereby confer either resistance or increased sensitivity to nucleoside analogs to variable degrees. Expression microarray analysis was used to evaluate the effect of the transfection method and the specificity of siRNA. We concluded that cells tolerated the transfection well without major effects on gene expression, and considered the siRNA used to be specific to its target.
An expression microarray experiment on a nucleoside analog-induced resistant cell line revealed a hypomethylating capacity of the drug and induction of fetal hemoglobin and a multidrug resistance efflux pump as a result of the hypomethylation. This pump should not be affected by nucleoside analoges since they are not a substrate of it, and upregulation of the pump unfortunately renders the cells highly cross-resistant to different types of drugs. Our preliminary data supports our theory that it may be upregulated in order to help excrete hemoglobin that otherwise would be toxic to the cells.
Nukleosidanaloger är en grupp cytostatika som framför allt används för att behandla olika typer av leukemi men som på senare år även används mot vissa solida tumörer. Dessa läkemedel liknar de naturliga byggstenarna i DNA men när läkemedlet inkorporeras i den växande DNA-strängen avstannas processen och cellen dör. Cancerceller växer oftast okontrollerat men många av kroppens friska celler växer också fort och kommer även de att påverkas av nukleosidanalogerna. Denna ospecifika effekt gör att biverkningarna blir kraftiga. Även det faktum att cancercellerna behöver olika mycket av läkemedlet för att dö gör att vissa celler får för liten dos, överlever behandlingen och blir mer motståndskraftig mot den, dvs utvecklar resistens. Vid resistensutvecklingen minskar ofta de nukleosidanalogaktiverande enzyme rna sin aktivitet men det finns även andra sätt för en cell att bli resistent.

I denna avhandling studeras de enzym som är viktiga för att göra nukleosidanaloger aktiva inne i cellen så att de får en celldödande effekt och även de enzym som kan inaktivera dessa läkemedel. Vi har studerat detta i celler från leukemipatienter och i så kallade cellinjer som vi odlar. Vi har tittat på enzymernas aktivitet i förhållande till nukleosidanalogernas effekt och sett att celler som aktivt delar sig har större kapacitet att svara på nukleosidanalogbehandling samt att aktiviteten av aktiverande enzym skiljer sig kraftigt åt mellan olika patienter med leukemi, vilket delvis kan förklara varför vissa patienter har mycket svårare att tillgodogöra sig behandlingen. Vi har även utarbetat en metod för att minska aktiviteten av enzym i celler för att studera vad avsaknaden av enzym gör för effekten av nukleosidanaloger, och sett att celler kan bli både känsligare eller mer resistenta mot nukleosidanalogbehandling beroende på vilket enzym och i vilken cellinje man utför nedregleringen. I en studie analyserade vi celler som utvecklat resistens mot en nukleosidanalog och fann att de uttryckte stora mängder hemoglobin och en drogresistenspump som inte borde påverkas av nukleosidanaloger. Vi tror att pumpen uppregleras för att cellen ska göra sig av med hemoglobinet som
annars skulle vara skadligt för den i för stora mängder men tyvärr också leder till att celler som blir resistenta mot nukleosidanaloger även blir resistenta mot andra typer av läkemedel, så kallad korsresistens.
Papers Presented in the Thesis


III Optimization and evaluation of electroporation delivery of siRNA in the human leukemic CCRF-CEM cell line, Anna Fyrberg and Kourosh Lotfi, Cytotechnology, DOI 10.1007/s10616-010-9309-6

IV The role of deoxyguanosine kinase for nucleoside analog activation in leukemic and solid tumor cell lines, Anna Fyrberg, Freidoun Albertioni, Kourosh Lotfi, manuscript

V Induction of fetal hemoglobin and ABCB1 gene expression in 9-β-D-arabinofuranosylguanine- resistant MOLT-4 cells, Anna Fyrberg, Curt Peterson, Bertil Kågedal, Kourosh Lotfi, submitted
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AML</td>
<td>acute myeloid leukemia</td>
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<td>ALL</td>
<td>acute lymphoid leukemia</td>
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<tr>
<td>AraC</td>
<td>cytarabine, 1-β-D-arabinofuranosylcytosine, Cytosar®</td>
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<tr>
<td>AraG</td>
<td>9-β-D-arabinofuranosylguanine, administered as nelarabine, Atriance®</td>
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<tr>
<td>CAFdA</td>
<td>clofarabine, 2-chloro-2′-arabino-fluoro-2′-deoxyadenosine, Evoltra®</td>
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<tr>
<td>CdA</td>
<td>cladribine, 2-chloro-2′-deoxyadenosine, Leustatin®</td>
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<td>CLL</td>
<td>chronic lymphoid leukemia</td>
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<td>CML</td>
<td>chronic myeloid leukemia</td>
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<tr>
<td>cN-I</td>
<td>cytosolic 5′-nucleotidase I</td>
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<td>cN-II</td>
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<tr>
<td>dCK</td>
<td>deoxycytidine kinase</td>
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<td>dCyd</td>
<td>deoxycytidine</td>
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<tr>
<td>dFdC</td>
<td>gemcitabine, 2′2′-difluorodeoxycytidine, Gemzar®</td>
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<td>dGK</td>
<td>deoxyguanosine kinase</td>
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<tr>
<td>dGuo</td>
<td>deoxyguanosine</td>
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<tr>
<td>dNT-I</td>
<td>5′(3′)-deoxyribonucleotidase I</td>
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<tr>
<td>dNTP</td>
<td>deoxyribonucleotide (incorporated into DNA)</td>
</tr>
<tr>
<td>FaraA</td>
<td>2-fluoro-9-β-arabinosyladenine, administered as fludarabine, Fludara®</td>
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<tr>
<td>5′-NT</td>
<td>5′-nucleotidase</td>
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<tr>
<td>NTP</td>
<td>ribonucleotide (incorporated into RNA)</td>
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<td>P-gp</td>
<td>P-glycoprotein</td>
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<td>Abbreviation</td>
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<tr>
<td>RNAi</td>
<td>RNA interference</td>
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<tr>
<td>RR</td>
<td>ribonucleotide reductase</td>
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<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
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<td>TK2</td>
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Introduction

Leukemia is a disease of the white blood cells, leukocytes, which normally develop into two different lineages; the myeloid lineage giving rise to erythrocytes, thrombocytes, granulocytes, and monocytes, and the lymphoid lineage developing into lymphocytes. Leukemia is roughly classified into acute or chronic and into myeloid or lymphoid. Acute leukemia is characterized by immature cells which are blocked in their differentiation, called blasts, whereas in chronic leukemia, the malignant cells resemble the normal ones but do not function properly and the onset of the disease is often slower than for acute leukemia. The four main types of leukemia are therefore based on its nature and origin, and are divided into acute myeloid leukemia (AML), acute lymphoid leukemia (ALL), chronic myeloid leukemia (CML), and chronic lymphoid leukemia (CLL). Leukemia arises due to inactivation of genes involved in the control of differentiation and growth, which may be due to spontaneous or inherited mutations [1], or through epigenetic gene silencing due to environmental factors [2].

In Sweden, approximately 1000 persons are diagnosed with leukemia each year. It affects both sexes and all ages, with a slightly higher prevalence in men (the Swedish Cancer Society). Although the overall survival rate for leukemia has improved remarkably during the last 20 to 30 years, there is still a great need for better diagnoses and treatment strategies. The highest survival rate is for children with ALL and adult patients with acute leukemias have the worst prognosis (the Swedish Cancer Society).

Several different types of drugs are used in cancer therapy but the class of deoxyribonucleoside analogs, or just nucleoside analogs, are particularly common in the treatment of leukemia, but have also been used more recently to treat different solid tumors [3, 4]. This is due to the presence of high levels of nucleoside analog activating enzymes in hematopoietic cells and leukemias derived from these cells, as well as to abundant expression of transporters for these drugs [5]. Nucleoside analogs are often used in combination with other drugs with different mechanisms of action, so-called combination...
regimens. Although nucleoside analogs serve as the cornerstone in the treatment of most leukemias, major obstacles to successful treatment are the development of resistance and the severe side effects of nucleoside analogs on normal tissues.
**Background**

**Nucleoside analogs**
Nucleoside analogs are prodrugs, highly similar to natural deoxyribonucleosides (Fig. 1), and require phosphorylation in order to become active and cytotoxic to cells. The enzymes performing this activation are localized in the cytosol and in the mitochondria and are abundantly expressed in lymphoid tissues, but also in cells containing large amounts of mitochondria, such as neurons [6].

![Diagram of nucleoside analogs](image)

**Fig. 1** The natural deoxyribonucleosides adenosine, guanosine, cytidine, and thymidine, and clinically used nucleoside analogs. Analogs of thymidine are not shown since they are used as anti-viral drugs and not discussed here.
Toxic side effects of nucleoside analog treatment therefore include bone marrow suppression with immune system deprivation and neurotoxicity due to large amounts of activated drugs in these tissues. Since the main effect of nucleoside analogs is DNA synthesis inhibition [7], every cell that is rapidly proliferating is subjected to nucleoside analog cytotoxicity, making these drugs rather nonspecific in their target. Some nucleoside analogs are however active in non-proliferating cells as well [8].

A common obstacle to successful treatment of leukemia is the development of resistance. Patients may be resistant to nucleoside analog chemotherapy prior to treatment, so-called inherited resistance, or may progressively develop resistance to the drugs, acquired resistance. Some of the known resistance mechanisms include decreased or increased activities of nucleoside analog metabolizing enzymes [9-12]. It is necessary to thoroughly study the expression and activity of these enzymes and correlate this with nucleoside analog cytotoxicity, and to determine resistance mechanisms to these compounds in order to improve treatment combinations and strategies, optimize doses to avoid resistance, and to develop new drugs with higher specificity and less severe side effects.
Nucleoside Analog Activating Enzymes

Nucleoside analog activating enzymes belong to the group of salvage enzymes providing deoxyribonucleotides (dNTPs) for DNA synthesis in resting or G1 cells which is needed for DNA repair and mitochondrial DNA replication [7]. These enzymes phosphorylate and activate deoxyribonucleosides to corresponding deoxyribonucleotides. In contrast, the de novo synthesis of DNA precursors is carried out by the ribonucleotide reductase (RR) enzyme, which has the capacity to reduce the diphosphates of ribonucleotides (NTPs) to the corresponding diphosphates of dNTPs, acting predominantly in cycling cells [7]. The salvage enzymes are, however, also abundantly expressed in hematologic cells due to their need of dNTPs for their rapid proliferation, and the deoxyribonucleosides used for making dNTPs are derived from extracellular nutrients and degraded DNA. Once transported into the cells, either passively or most frequently through nucleoside transporters due to their hydrophilic nature, the deoxyribonucleosides are phosphorylated by the salvage enzymes to monophosphates and thereby trapped intracellularly [7]. The monophosphates are further phosphorylated to di- and triphosphates and these give rise to the cytotoxic effects attributed to nucleoside analogs. The effects involve incorporation into DNA leading to chain termination, and inhibition of RNA synthesis, DNA polymerases [13-15], and the RR enzyme, [12, 15, 16] and finally induction of apoptosis [17]. The enzymes that phosphorylate nucleosides to monophosphates are considered to be the key regulatory step in the formation of nucleotides since the formation of di- and triphosphates usually is not rate-limiting or specific for different deoxyribonucleotide substrates [7].

The most important of these enzymes with regard to nucleoside analog activation is deoxycytidine kinase (dCK, EC 2.7.1.74). The other salvage enzymes are deoxyguanosine kinase (dGK, EC 2.7.1.113), thymidine kinase 1 (TK1, EC 2.7.1.21), and thymidine kinase 2 (TK2, EC 2.7.1.21). TK1 is the only enzyme with strictly cell-cycle dependent activity [18], and is involved in the activation of
anti-viral drugs [19] and hence is not discussed in this thesis focusing on anti-cancer drugs.

**Deoxycytidine kinase**

The cDNA of dCK was cloned in 1991 by Chottinger and co-workers [20] and the gene is localized on chromosome 4q13.3-21.1 [21]. It is a cytosolic enzyme [22] but may localize to the nucleus when over-expressed in cell lines [23]. Low activity of dCK is found in non-lymphoid solid tissues, with increasing levels in malignant cells [24]. Interestingly, when screening patients with pancreatic cancer for dCK using immunostaining, significantly higher dCK levels were found in older patients and in males [25]. dCK mRNA expression and activity vary widely in clinical samples from leukemia patients, but also in different cell lines, and a ten- or hundred-fold range or more in dCK activity is not an uncommon phenomenon [26, 27]. It is probably the most important enzyme for nucleoside analog activation since its expression is high in different hematopoietic cells [28], it has broad substrate preferences [7], and its decreased activity in patients or cell lines confers resistance to nucleoside analogs [12, 24, 29, 30].

The synthesis of dNTPs for DNA synthesis takes place in close relation to DNA replication and several of the enzymes needed for making these building blocks are essentially synthesized during the cell cycle. Alterations in the dNTP pools not motivated by physiological demands may lead to mutations, chromosomal aberrations, and mutagenesis, [31] and it is crucial that the activities of the enzymes responsible for maintaining dNTP pools are in balance. Although dCK/dGK belong to the same family, their activities are less influenced by the cell cycle. Although they do not meet the criteria for being cell-cycle-regulated, at least dCK activity has been shown to increase 3 to 15-fold in the S-phase of different cells [32, 33].

The natural substrates of dCK are deoxycytidine (dCyd), deoxyadenosine (dAdo), and deoxyguanosine (dGuo) [20] with $K_m$ values of 1, 120, and 150 μM, respectively [34]. The activity of this
enzyme can be enhanced after treatment with different deoxyribonucleosides [35], nucleoside analogs [36], genotoxic agents [37], and UV- and γ-irradiation [38, 39]. The level of dCK activation seems to differ between different leukemic cell lines and compared to normal lymphocytes. Treating cells with nucleoside analogs may either decrease, modestly increase, or markedly increase dCK activity [37]. It has been shown that the activity of dCK is regulated by reversible phosphorylation, and that treatment of dCK over-expressing cells with protein phosphatase leads to a profound decrease in dCK activity [40], while protein kinase inhibitors can activate the enzyme [41]. The dCK enzyme is negatively feedback regulated by the triphosphate of dCyd (dCTP) [7] so that high levels of this end-product inhibits the enzyme responsible for its initial phosphorylation.

**Deoxyguanosine kinase**

The dGK cDNA was characterized in 1996 by Johansson and Karlsson [42], and the DGUOK gene is located on chromosome 2p13 [43] and shows a cDNA sequence homology of 57 % with the DCK gene [42]. In contrast to dCK, dGK is strictly localized to the mitochondria [44]. It phosphorylates dGuo, deoxyinosine (dIno), dCyd, and dAdo [45] with $K_m$ values of 4, 13, 330, and 460 µM [46]. dCK and dGK proteins are both dimers of approximately 60 and 58 kDa, respectively, [20, 34, 45] and the two enzymes show 48 % homology in their amino acid sequences [42, 47]. High dGK activity has been found in such tissues as spleen, skin, and resting- and mitogen-stimulated lymphocytes [45]. The activity of dGK is 10-fold higher than that of dCK in both human and bovine brain tissue, as a consequence of the high content of mitochondria [45] making the brain especially vulnerable to impaired dGK activity [48]. Tissues deficient in dGK may be partly compensated by the activity of dCK, since the two enzymes have over-lapping substrate specificities [48]. However, the opposite may not be true since leukemic patients with low dCK enzyme activity did not show increased levels of dGK activity as compensation [49]. While total loss of the cytosolic enzymes in clinical samples has not been described, deactivational
mutations in the dGK gene can lead to a hepatocerebral form of mitochondrial DNA depletion syndrome [43].

**Thymidine kinase 2**
Thymidine kinase 2 is mapped to chromosome 16q22 and its cDNA sequence shows approximately 30% homology with dCK and dGK cDNA [50]. This constitutively expressed enzyme [7] is located in the mitochondria in all tissues and phosphorylates thymidine, deoxyuridine, and dCyd [50]. It is also involved in the activation of pyrimidine nucleoside analogs as well as anti-viral drugs [19, 50]. TK2 knock-out mice are normal at birth, but they suffer from growth retardation, hypothermia, and abnormalities in heart and skeletal muscle, brain, spleen and kidney, and die before they are 30 days old due to mitochondrial depletion in affected organs [51]. In humans, deactivational deletions in the TK2 gene leads to a myopathic form of mitochondrial DNA depletion syndrome [48].

**Ribonucleotide Reductase**
The RR enzyme is not directly involved in the activation of nucleoside analogs but may modulate the effect of these drugs by altering the intracellular NTP/dNTP pools, which can increase or decrease the effect of nucleoside analog di- and triphosphates. This enzyme provides the cell with all four deoxyribonucleotides that are needed for DNA synthesis, and is the rate-limiting enzyme in this process. It consists of two non-identical subunits, the large RRM1, which is constitutively expressed, and the smaller RRM2 subunit which is cell-cycle-regulated [52]. The RRM1 subunit is expressed in all tissues, while RRM2 is absent in heart, brain, and muscle tissue [53]. In G1- or non-proliferating cells, the optional p53-induced R2 protein (p53R2) subunit substitutes for the RRM2 subunit, [54, 55], and is involved in the p53-dependent check-point of the cell cycle in response to DNA damage [56]. Mutations in the gene coding for the p53R2 subunit can cause mitochondrial DNA depletion similar to what can be seen with dGK and TK2 mutations [53].
Imbalanced dNTP pools may be mutagenic for the cells, eventually resulting in apoptosis or cancer, and therefore these pools must be tightly controlled by the RR enzyme [57]. The enzymes is activated upon binding of ATP to the allosteric activity site, and inhibited upon binding of dATP [52]. The effect of nucleoside analogs on the intracellular NTP pools varies widely and is clearly cell-line-dependent and therefore this effect on the RR enzyme differs [58]. Some nucleoside analogs affect the intracellular NTP levels to a very large extent, particularly depleting the CTP pools with decreased dCTP levels as a consequence, and activation of dCK [58].

### Nucleoside Analog Deactivating Enzymes

There are enzymes that oppose the activating capacity of the deoxyribonucleoside kinases described above, and these are localized on the cellular surface, in the cytosol, and in the mitochondria. They are called 5´-nucleotidases and seven different human enzymes have been described [59], but we have worked with only three of them and they will be discussed here. The 5´-NTs work in the opposite direction hydrolyzing the monophosphates to deoxyribonucleosides or ribonucleosides, and are among several other enzymes important for nucleoside analog deactivation. The 5´-NTs form substrate cycles with the deoxyribonucleoside kinases, maintaining a balance between phosphorylated and unphosphorylated deoxyribonucleosides and ribonucleosides in response to rapid changes in metabolism and physiological changes (Fig. 2) [60]. The net effect of these cycles may be anabolic or catabolic. Potentially, development of inhibitors to these enzymes should enhance the effect of nucleoside analogs but no such drugs are available at the moment other than at the experimental level.

### Cytosolic 5´-nucleotidase IA

The cytosolic 5´-nucleotidase IA (cN-IA, NM_032526) uses a broad range of substrates but has a preference for monophosphates of adenosine, cytidine, and guanosine [61], and as the name suggests, it
is located in the cytosol. It is most likely involved in resistance to nucleoside analogs since when over-expressed in cell lines, the cells become less sensitive to several nucleoside analogs [61]. It is abundantly expressed in the heart, and brain, as well as in skeletal muscle [62], where it is assumed to be involved in muscle contraction [63].

Cytosolic 5’-nucleotidase II
The cytosolic 5’-nucleotidase II (cN-II, NM_012229) enzyme preferentially hydrolyzes inosine and guanosine monophosphates [64, 65]. It shows high activity in lymphoblastoid cells [66], although it is ubiquitously expressed throughout different tissues [67]. Studies have shown that pretreatment levels of cN-II mRNA are significantly
lower in patients responding to nucleoside analog treatment [68], indicating that this enzyme may be involved in resistance to nucleoside analogs. It appears to be involved in cellular sensitivity to all kinds of nucleoside analogs [69, 70], at least in cell lines, and show activity against several nucleoside analog monophosphates [59]. On the other hand, this enzyme has been shown to possess phosphotransferase activity [71] i.e. it can basically activate a deoxyribonucleoside to deoxyribonucleotide as deoxyribonucleoside kinases would do. Some speculate that rather than conferring resistance to nucleoside analogs, this enzyme is a marker of disease aggressiveness [72]. There are studies showing that a high cN-II/dCK ratio is relevant to nucleoside analog resistance both in leukemic patients [73], and in cell lines [70, 74].

5′(3′)-deoxyribonucleotidase

The cytosolic 5′(3′)-deoxyribonucleotidase (dNT-I) is also ubiquitously expressed and prefers all deoxyribonucleotide monophosphates except deoxycytidine monophosphate [75]. It is believed to form substrate cycles with deoxyribonucleoside kinases, such as dCK, thereby helping to maintain the dNTP levels in the cells [76]. However, although decreased mRNA levels of this enzyme in leukemic blasts from AML patients at diagnosis correlated with a shorter disease-free survival, it may still be involved in resistance. Since this enzyme also possesses phosphotransferase activity it may be involved in the activation of drugs, and hence the poor effect with low dNT-I levels [77]. It may also be involved in inactivation and reduction of dCTP, and with low levels of dNT-I there may be more dCTP competing with the triphosphate of active cytidine analogs, and hence a decreased cytotoxic effect [77].

Mechanisms of Resistance to Nucleoside Analogs

Resistance to the cytotoxic effects of nucleoside analogs is often accompanied by decreased activity of the activating enzymes dCK
and/or dGK thereby causing a smaller amount of active di- and triphosphates of the particular drug in use.

A total loss of dCK in cells isolated from patients treated with nucleoside analogs has not been observed [78, 79], while a nearly complete loss of dCK is common in nucleoside analog-resistant cell lines [80]. It has been suggested that the dCK enzyme loses its activity as its promoter is methylated, and that demethylating agents such as 5-azadeoxycytidine (5-aza) [80] or even some nucleoside analogs [80] can demethylate and hence reexpress dCK activity, at least in a proportion of the resistant cells. Alternatively spliced and inactive forms of dCK have been found by some [29], and particularly in resistant cells [81], but not by us [27]. Nucleoside analogs predominantly phosphorylated by dCK appear to be able to decrease dCK enzyme activity without affecting dGK [82], while nucleoside analogs mainly activated by dGK have a tendency to decrease dCK activity as well [11].

Since nucleoside analogs are mainly transported into cells by special nucleoside transporters, loss of expression of these transporters may also confer resistance. Yet, another resistance mechanism is altered activity of the RR enzyme, since this enzyme maintains the intracellular NTP/dNTP pools. If the RR activity for some reason increases, the natural dNTPs will compete with the dNTPs of activated nucleoside analogs, with a smaller cytotoxic effect thereof [12, 83, 84].

**Multidrug resistance**

Nucleoside analog treatment may also, and rather frequently, induce a multidrug resistance phenotype. The multidrug resistance gene (mdrl or ABCB1) encodes a drug efflux pump on the cellular surface called P-glycoprotein (P-gp) which is responsible for excretion of compounds toxic to the cells, such as different drugs. Nucleoside analogs are not considered to be substrates of this pump [85]. It may nevertheless be highly upregulated in cells treated with nucleoside analogs, which render the cells cross-resistant to compounds such as anthracyclines [11], that are transported through this mechanism.
However, there is one report of increased intracellular retention of the nucleoside analog cytarabine (AraC) in leukemic cell lines when treated with a P-gp inhibitor, suggesting that AraC, and possibly other nucleoside analogs, may actually be a substrate of this pump under certain conditions [86]. It also seems that 9-β-D-arabinofuranosylguanine (AraG) [11] and AraC can upregulate this pump, and in one study using AraC in blasts from AML and CML, the nucleoside analog was more potent in upregulating P-gp than were traditional substrates of this pump [87]. Withdrawal of the P-gp-inducing drug reduces P-gp expression and makes the cells drug-sensitive again. However, this takes several months, and the P-gp over-expressed phenotype is easily re-induced [88]. Cells should theoretically sustain their sensitivity to nucleoside analogs in P-gp overexpressing cells. Even so, there are reports about cells with induced P-gp levels, along with unaltered dCK levels, that were resistant to nucleoside analogs [89]. An inverse correlation between the ABCB1 transcription and methylation of the gene has been found [90, 91] and ABCB1 may be induced when treating cells with hypomethylating drugs.

**Down-regulation of enzymes and effect on nucleoside analog activation**

Due to the importance of nucleoside analog metabolizing enzymes in the development of resistance, it is necessary to study the lack of these enzymes. In a study by Beauséjour et al., hammerhead ribozymes were designed to inactivate dCK mRNA and thereby decrease dCK activity in murine leukemic cells. Despite a transfection efficiency of 80%, the reduction in dCK enzyme activity was less than 20% and cells did not show altered sensitivity to AraC compared to control cells [92]. The long-term aim of the study was to suppress dCK in hematopoietic stem cells and make them resistant to nucleoside analog therapy to avoid the bone marrow suppression that otherwise occurs during the treatment of leukemia [92]. In still another study, dGK was down-regulated by approximately 30% in HeLa cells, and the aim was to investigate the effect of reduced dGK activity on mitochondrial (mt)DNA. No effects were seen on mtDNA.
in exponentially growing cells, but resting cells tended to die and showed up to 50% reduction in mtDNA content [93]. No resistance to the dGK-activated nucleoside analog AraG was seen, but rather an increased phosphorylation due to increased expression of dCK in the siRNA treated cells [93]. In yet another study, a human leukemia cell line was depleted of mitochondrial DNA, but did not lose the expression or activity of either dGK or TK2, and retained its sensitivity to nucleoside analogs [94].

**Overcoming resistance**
Attempts have been made to conjugate nucleoside analogs with different lipid compounds and make them independent of nucleoside transporters for cell entry [95]. A phospholipid conjugate of the nucleoside analog gemcitabine (dFdC) has been promising and shows an effect in cells resistant to nucleoside analogs due to decreased dCK expression [95] and may constitute the future of nucleoside analog drug design. Another approach is to use a prodrug that enters the cells and releases the monophosphate intracellularly, irrespective of the presence of activating enzymes [96]. Another option is to deliver proteins directly into cells in contrast to the traditional gene therapy, and attempts have been made with a multisubstrate deoxyribonucleoside kinase from *Drosophila melanogaster*, which was successfully delivered into cells using protein-lipid complexes and rendered the cells more sensitive to nucleoside analogs [97].
Purine Nucleoside Analogs

Cladribine
Cladribine (CdA, Fig. 1), as well as other adenosine analogs, exerts toxicity both in non-dividing and rapidly proliferating cells [8], and therefore differs from other nucleoside analogs which are toxic mainly to proliferating cells. Its effect in non-dividing lymphocytes has been attributed to its capacity to decrease intracellular nicotinamide adenine dinucleotide (NAD) pools, among others, with subsequent DNA strand breaks and inhibition of RNA synthesis, glycolysis, and ATP generation [98]. It is used primarily to treat hairy-cell leukemia, CLL, and lymphomas [99], but also conditions such as psoriasis [100] and multiple sclerosis [101]. Adenosine analogs show particularly high toxicity in cells with high dCK levels and low levels of 5’-nucleotidases, independently of their proliferative capacity, [8] and high levels of cN-IA [61] and cN-II [59] confers resistance to CdA. A study made on cells from leukemia patients showed a correlation between dCK levels and the effect of CdA [102], but a lack of correlation has also been found in both patients and cell lines [103]. CdA is phosphorylated by dGK as well, [7] and its activity is enhanced when dGK is over-expressed in cell lines [104].

Fludarabine
Fludarabine is the name of the soluble 5’-monophosphate of the nucleoside 2-fluoro-9-β-arabinosyladenine (Fara A, Fig. 1) that is given to patients, particularly those with CLL, and hydrolyzed in the blood by plasma phosphatases and membrane-bound ecto-nucleotidase (e-NT) to Fara A. When taken up into the cells it is once again phosphorylated to its monophosphate form presumably by dCK [105], but also by dGK since cells with reduced dGK activity show resistance to this drug [106]. There are studies indicating that cells resistant or cross-resistant to FaraA has lost the ability to phosphorylate dCyd, and hence lost dCK activity [107, 108]. On the other hand, cells resistant to dFdC and cross-resistant to several
nucleoside analogs were not resistant to FaraA, although these cells basically had lost all dCK activity. The reason for FaraA resistance [12], as well as for dFdC resistance [109], has also been shown to involve altered activity of the RR enzyme, and the resistance to FaraA therefore seems to differ from the resistance mechanisms to the other adenosine analogs [12]. Increased levels of cN-IA confer moderate resistance against FaraA [61], and increased levels of e-NT may also make cells less sensitive to FaraA.

**Clofarabine**

Clofarabine (CAFdA, Fig. 1) is, like CdA and FaraA, an adenosine analog, and incorporates the best properties of its two predecessors [4]. It was brought into use in 2006 for the treatment of relapsed or refractory pediatric ALL [4]. CAFdA shows toxicity to a variety of tumors, including non-small cell lung cancer, colon, central nervous system, melanoma, ovarian, renal, prostate, and breast cancer [4], as well as AML, CML, and myelodysplastic syndrome [110]. Toxic effects of adenosine analogs are attributed to incorporation into DNA, inhibition of DNA polymerases, inhibition of RR, and for CAFdA and FaraA, incorporation into RNA [111]. The triphosphate of CAFdA is more potent than the triphosphates of CdA or FaraA in substituting for the natural deoxyriboadenosine triphosphate dATP in the apoptosis protease activating factor-1 (Apaf-1) complex eventually leading to apoptosis [112]. CAFdA and the other adenosine analogs can enhance the effect of dCK, either directly by affecting the enzyme itself or by inhibition of the RR enzyme, leading to decreased dNTP pools, and especially dCTP that otherwise inhibits dCK [113]. Clinical trials combining CAFdA and AraC have been rather successful in acute leukemia, showing that CAFdA pretreatment enhances the AraC triphosphate levels, and since these two drugs also have non-overlapping toxicities, they may constitute a future treatment combination strategy [114]. CAFdA has been shown to hypomethylate DNA and induces the expression of cancer-testis antigens which are of interest as drug targets since they are almost exclusively expressed by malignant tissues [115].
All adenosine analogs are phosphorylated by dCK to different extents, they may, however, be rather potent substrates of dGK as well, and may be useful in tissues and cells with high dGK activity, since the uptake of radiolabeled CdA, FaraA, and CAFdA in one study was high in skin [116] and since melanocytes harbor many mitochondria [117].

**Nelarabine**

Nelarabine is a soluble substance demethylated in the blood by adenosine deaminase to the less soluble drug 9-β-D-arabinofuranosylguanine (AraG, Fig. 1). It can be activated to its monophosphate by both dCK and dGK [118], and due to a higher accumulation of its triphosphate in T-cells it also displays higher toxicity to T-cell malignancies [119]. When first developed in the 1950’s, it was created to mimic the purine nucleoside phosphorylase deficiency disease, which is a genetic condition depleting the individual of T-cells, with subsequent immune deficiency [6]. When activated, its acute cytotoxicity is mediated by incorporation into nuclear DNA [120], but it is also incorporated into mitochondrial DNA [121] due to its site of phosphorylation [122]. Resistance to AraG is due to decreased activity of both dCK, and dGK, and induction of multidrug resistance genes [11] as well as increased levels of cN-II and dNT-I [59].
Pyrimidine Nucleoside Analogs

Cytarabine
AraC (Fig. 1) constitutes, together with anthracyclines, the cornerstone treatment of acute myelogenous leukemia (AML). Its cytotoxicity is due to inhibition of DNA polymerases and incorporation into DNA [111]. Resistance mechanisms to AraC may include decreased dCK activity [123], decreased expression of dNT-I [77] or cN-IA [61], and increased expression of cN-II, although this enzyme is not directly involved in the deactivation of AraC, but serves as a prognostic factor in AML patients [124]. AraC has the ability to induce P-gp expression, at least in cell lines, which is a less good characteristic, because of its combination therapy with anthracyclines which are substrates of this pump [125]. Although highly similar to the cytidine analog dFdC, one study reported a lack of cross-resistance between these two nucleoside analogs in a CdA-resistant AML cell line, indicating that dFdC may be potent in AraC-resistant cells [69] or even a superior choice before AraC in the treatment of AML [126].

Gemcitabine
dFdC (Fig. 1) is one of the nucleoside analogs used to treat solid malignancies such as head and neck cancer, non-small cell lung cancer, small cell lung cancer, and ovarian, and pancreatic cancer [127-132]. It is usually given together with other chemotherapeutic agents with different mechanisms of actions and side effects, and the most successful combination regimen with dFdC is currently with cisplatin [133, 134]. DFdC exerts its cytotoxic effects in several ways. The diphosphate can inhibit the RR enzyme, leading to decreased deoxyribonucleotide pools, and hence less competition for dFdC [135] or inhibition of CTP synthetase or dCMP deaminase, leading to decreased dCTP pools, increased dCK activity, and dFdC self-potentiation [135, 136]. The triphosphate of dFdC can also be incorporated into RNA instead of CTP [137]. Resistance to dFdC has been shown to involve a decrease in dCK enzyme activity [3, 82],
reduced transportation [5], an over-expression of ribonucleotide reductase subunits, [83, 84] increased activity of the deactivating enzyme cytidine deaminase [138], a decrease in TK2 activity [83, 139], an increase in cN-IA and dNT-I [59], or alterations in DNA polymerase activity [140]. There are however, examples of dFdC-resistant cell lines without significantly decreased dCK levels [83, 141]. dFdC is also, in contrast to the cytosine analog AraC, a substrate for the TK2 enzyme [142].
Aims of the Thesis

As the title of this thesis indicates, the aim was to investigate the enzymes responsible of the metabolism of nucleoside analogs, and correlate to nucleoside analog cytotoxicity, and to determine mechanisms of resistance to these drugs.

Specific aims:

✓ To determine if the expression and activity of nucleoside analog activating enzymes in cells from patients with CLL can be used to predict the patient’s sensitivity to nucleoside analogs prior to treatment.

✓ To investigate the regulation of activities of nucleoside analog activating and deactivating enzymes during cell cycle progression, and correlate this with nucleoside analog cytotoxicity.

✓ To develop a method for transient downregulation of dCK and dGK in leukemic and solid tumor cell lines, and investigate the effects on nucleoside analog activation.

✓ To characterize a nucleoside analog resistant cell line using gene expression microarray analysis.
Material and Methods

Materials used in the studies in this thesis are mostly established immortal cell lines, or, as in the case of paper I, peripheral blood mononuclear cells from patients with CLL. Methods used include common molecular biology and chemical techniques such as real-time PCR, Western blot, HPLC, and flow cytometry. Other techniques used in several of the studies included in this thesis are discussed below.

Cell lines
The advantages of using cell lines are that they are readily accessible, form a homogenous cell population and can be maintained for a long time in culture. It is, however, important to screen them routinely for infections and to be aware of genetic changes that can occur during long-term culture, which can alter cell characteristics. It may also be difficult to transfer data obtained from cell lines to clinical use for the patients. Cell lines may not reflect the true nature of the disorder they represent since leukemia cells or tumors in vivo are interspersed with other cells to form a heterogeneous cell population with which they interact.

The cell lines used were primarily established human leukemia cell lines derived from the American Type Culture Collection and represent three of the most common leukemia disorders that we know of. The CCRF-CEM (CEM, CCL-119) and MOLT-4 (CRL-1582) cell lines are both derived from patients with ALL of T-cell origin. The HL60 (CCL-240) cell line represents a patient with AML, and the K562 (CCL-243) cell line was obtained from a CML patient. These cell lines were chosen because they are used by several other research groups and large amounts of data that are available have been generated from working with them and, because we also have many years of experience from working with these cells. We have also generated variants of these cell lines resistant to several nucleoside analogs as well as other anti-cancer drugs. We also used the metastatic malignant melanoma cell line Sk-mel-28 (HTB-72) as
well as RaH3 and RaH5 which were created from primary cutaneous melanoma cells. The lung adenocarcinoma epithelial cell line A549 (CCL-185) was also used in one study.

**Drug Cytotoxicity Measurement (MTT), papers II-V**

The cytotoxicity of different drugs, especially nucleoside analogs, was evaluated in different cell lines using the colorimetric 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay described by Mosmann [143]. This method utilizes the fact that the mitochondrial respiratory chain as well as other electron transport systems, can reduce the yellow MTT salt to insoluble formazan crystals bearing a purple color inside the cells [144, 145]. These crystals can be detected and quantified using a spectrophotometer after solubilization of the crystals. This will give an estimated number of intact mitochondria present in the cells and serve as a measurement of the number of living cells in the sample. Several different concentrations of the particular drug in use are employed and as a control, cells grown with no drug are used. One important issue to bear in mind is the confluency or density of the cells. The control cells, and often the cells grown with low concentrations of drug, will continue to proliferate during the 72 hrs that it takes for the experiment to proceed. If cells are plated too dense, they will die from a lack of space and nutrition, and will not be able to serve as a reliable control. The effectiveness of the drug or compound in causing death is reflected in the amount of color in the wells and can be compared to cells grown with no drug. The half maximal inhibitory concentration (IC50-value) can be determined by plotting a dose-response, or survival curve (Fig. 3).

![Fig. 3](image-url) The principles of MTT assay and the generation of a survival curve.
Enzyme activity measurement, papers I-V

Enzyme activities of dCK and dGK were measured in all five papers using a method described by Ives and Wang [146] using an anion-exchange paper technique. This method utilizes the fact that nucleoside monophosphates are trapped intracellularly, or in this case on the filter paper disk, while nucleosides can be washed away. By letting the enzymes in a crude protein extract phosphorylate a substrate \textit{in vitro} that is highly specific for the particular enzyme studied, and applying the reaction mixture containing enzyme, substrate, and product to an anion-exchange paper, and washing away everything but the monophosphates, the monophosphates can be quantified on the paper (Fig. 4). This will reflect the activity of the enzyme. In order to detect the products, it is necessary that the substrate added to the reaction is radio-labeled with an isotope. Since dCK, dGK, TK1, and TK2 have overlapping substrate specificities, it may be necessary to add an additional non-labeled substrate to the reaction that can occupy an enzyme so that it does not participate in the phosphorylation of the radio-labeled substance. When measuring dCK activity using dCyd as a substrate, an excess of thymidine is used to occupy the TK2 enzyme, which prefers thymidine as a substrate but also has the ability to phosphorylate dCyd. When quantifying dGK using dGuo, an excess of dCyd is added, because otherwise dCK will be able to phosphorylate dGuo. The quantification of the monophosphates (as well as di- and triphosphates) may also be done using HPLC.

\begin{figure}[h]
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\includegraphics[width=0.5\textwidth]{fig4.png}
\caption{Basic principle of enzyme activity measurement using $^3$H-labeled substrates and filter discs.}
\end{figure}
When performing this assay it is important to use an optimal amount of enzyme, an appropriate concentration of the substrate, and appropriate reaction times (the time for the enzyme to phosphorylate its substrate). Therefore, it is important to test the linearity of the reaction whenever different samples (cell lines or patient samples) or substrates are used.

**RNA interference, papers I, III & IV**

RNA interference (RNAi) is a method that attracted much attention in 2006 because the discoverers of this method, Andrew Fire and Craig Mello, were awarded the Nobel Prize in Physiology or Medicine. This phenomenon of post-transcriptional gene silencing was discovered by accident in petunia plants in which transgenes were found to be able to suppress mRNA coding for flower pigmentation [147]. Fire and Mello then studied the ability of double-stranded (ds)RNA to induce sequence-specific gene silencing, or RNAi, in the nematode *Caenorhabditis elegans* [148]. Later, it has been shown that RNAi is a naturally occurring cellular process for post-transcriptional gene regulation, and protection from viral infection and genetic damage in virtually all species [149].

Experimentally, RNAi can be induced by small interfering RNA (siRNA) which can be delivered into cells by several means, including vector-based systems that continuously produce siRNA to suppress a gene, or as siRNA molecules directly transfected into the cell, giving rise to transient suppression of an mRNA and its protein product. Irrespective of the source, all siRNA follow the same pathway in the cell leading eventually to gene silencing. The siRNA molecules, 20 to 25 nucleotides (nt) long, assemble into a complex known as the RNA-induced silencing complex (RISC) in the cytosol, and, using the siRNA anti-sense strand as a guide, it will find the target mRNA, hybridize to it, and degrade it [150] (Fig. 5). If longer dsRNA molecules of more than 30 nt would be introduced into a cell, it will initiate an anti-viral response with inhibition of protein synthesis and RNA degradation. Long dsRNA molecules therefore have to be processed by the dicer enzyme into shorter molecules in order to be active in gene silencing.
When designing siRNA molecules for *in vitro* use in cell lines, several parameters need to be considered regarding length, thermal stability, sequence, base pair content, sequence specificity, and mRNA target accessibility [151]. Genes with highly stable or abundantly expressed protein products may, however, be difficult to silence even with the most potent siRNA molecules [152].

Today, many biotechnology companies specialize in developing ready-to-use validated siRNA molecules with at least 80% target knock-down efficiency if used with an appropriate transfection method to introduce the siRNA into the cells. In the studies included in this thesis, hematopoietic cell lines were used for siRNA experiments, and since these cells are usually of the “hard-to-transfect” type, we concentrated on optimizing the transfection method rather than optimizing the siRNA molecules, and therefore used validated ready-to-use siRNA.

**Fig. 5** The basic steps involved in transient RNAi. DsRNA enters the cells, and the anti-sense strands assemble with the RISC-complex and guides the binding to homologous mRNA and degrades it. This will subsequently lead to less protein product formed by that particular mRNA as illustrated here by Western blot. Modified picture from RNAi Therapeutics: How Likely, How Soon? Robinson R PLoS Biology Vol. 2, No.1,e28doi:10.1371/journal.pbi.o.0020028.
Electroporation, papers I, III & IV

Electroporation is a technique for introducing molecules through the cell membrane, although the membrane is naturally impermeable to the molecules in use. An electric field strength is applied to the cells which transiently opens up pores in the cell membrane through which siRNA may enter. Larger cells require low field strengths and vice versa, and adding different reagents in the electroporation media, such as dimethyl sulfoxide [153] or serum [154], can help the cells reseal and avoid lysis. A number of different parameters can be optimized in order to increase transfection efficiency, such as field strength, pulse length, number of pulses, electroporation- and post-electroporation media, temperature, cuvette size, and cell density, among others.

Electroporation can be tough on the cells. DNA strand breaks and cell rupture induced by the electroporation procedure frequently occur but can be minimized if the protocol is optimized [155]. Traditional lipid-mediated transfection reagents and viral vectors may also be highly toxic to cells. Viral vectors may induce pathogenicity and immunogenicity or transform the cells by insertional mutagenesis. Plasmid vectors do not have these abilities but have shown limited efficiency in hematopoietic cells, which is also the case with lipid-based transfection reagents [156].

Gene expression microarray, papers III & V

Gene expression microarray analysis is a tool for whole-genome studies of the expression of known genes. In this thesis, the Affymetrix platform was used together with two different types of arrays. In study V, the GeneChip® Human Genome (HG) Focus Array was used. It analyzes the expression of approximately 8,500 of the most well characterized human genes. A gene, or a target sequence, is represented by 11 to 20 oligonucleotide probes, 25 nt in length, spanning roughly 600 bases of the target sequence. For each of the oligonucleotides there is one that is a perfect match (PM) of the target sequence, and one that is mismatched (MM) in its central position. The latter one serves as a control for nonspecific hybridization of the target of the array.
The second type of array used was the GeneChip® Human Gene 1.0 ST Array, which includes probes for all human genes. More than 750,000 oligonucleotides of 25 nt in length represent approximately 28,000 genes on the array. This array does not rely on a perfect match or mismatch since every oligonucleotide should make a perfect match with the target sequence.

The procedure of preparing the RNA samples used in the microarray experiment is the same for the two types of array. After isolation, RNA has to be reverse-transcribed to ds complementary DNA (cDNA). In the second reaction round, the cDNA serves a template for making biotin-labeled antisense mRNA, called cRNA, or the target RNA. Before hybridization of the RNA to the array, it is heated and fragmented, resulting in cRNA fragments 25 to 200 bases long. The target RNA is mixed in a cocktail containing bacterial RNA, serving as hybridization controls, and is then injected into the array, and hybridized to the oligonucleotides on the array during heating. The next step is staining of the chip array with a fluorescent streptavidin-phycoerythrin molecule binding to biotin in the cRNA. The arrays are then scanned and processed using special software provided by the manufacturer. The next challenge is to process all the data obtained from the arrays, which is done using bioinformatics (Fig. 6).

**Fig. 6** The different steps of a gene expression microarray experiment, starting with isolation of total RNA, and ending with extensive bioinformatics. Validation and verification of genes of interests are also usually necessary, employing real-time PCR, Western blot, siRNA or over-expression of genes in cell lines.
The algorithms used to analyze microarray data are included in the analysis software and we chose to use the robust multiarray average (RMA) algorithm [157]. This could be used for both types of arrays since it does not use the information from the MM probes used in the HG Focus Array. The signal strength from the MM probes may exceed that of the PM probes leading to absurd amounts of negative expression values which may not be absolutely true. RMA corrects the background signal, and, normalizes, and summarizes the probe level information across all arrays to correct for array biases, and compare gene expressions levels between arrays. Significantly differentially expressed genes were then found using the Welch’s t test, followed by the Benjamin-Hochberg post hoc test. Samples were filtered on expression, \( p \)-value computation was asymptotic, and the cut-off limit was drawn at 0.05.

The advantage of the RMA algorithm, compared to others, is less false positive data, reduction of noise, and persistent fold-change estimations. Disadvantages may be loss of information, especially at very low differences in expression (false negative data), and since the normalization process of this algorithm assumes that the data are normally distributed, it may hide true biological differences. However, for fold-change analysis as used in our studies, it is an ideal algorithm (Agilent Technologies, Inc., Santa Clara, CA, USA).

Gene expression microarray analysis has developed into a standard approach to determine genetic differences between normal and malignant tissue in order to find new therapeutic targets, to investigate the mechanisms of action of drugs, as well as on- and off-target effects of drugs or therapies [158].

**Statistics**

Statistical analyses were performed using the GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). In paper I, data were expressed as median value, and the non-parametric Spearman’s rank correlation test was used to compare relationships between expression and activity of dCK and dGK.
In additional papers, data were expressed as means and standard deviations and analyzed using Student’s t-test. A \( p \) value of \( \leq 0.05 \) was considered significant for both tests.
Results and discussion

Knowledge about the metabolism of nucleoside analogs and development of mechanisms of resistance to these chemotherapeutic drugs is crucial in trying to optimize the treatment of leukemia. Too high drug doses may, in a highly chemoresponsive individual, lead to toxic side effects and sometimes death, while administration of the same dose to a patient with a metabolism favoring inactivation and elimination of nucleoside analogs can lead to the development of resistance to these as well as to other compounds.

Expression and activity of nucleoside analog metabolizing enzymes and correlation with nucleoside analog cytotoxicity (papers I & II)

Attempts have been made to individualize the dose and choice of nucleoside analogs based on the patients’ individual capacity to assimilate with the treatment. Some studies suggest that measuring mRNA expression levels of nucleoside analog metabolizing enzymes prior to treatment may predict clinical sensitivity to these drugs [73, 77, 159], while others have suggested in vitro cytotoxicity measurements with the patient’s cells in combination with different drugs prior to treatment to find the best drug and dose [160]. We can identify problems using these approaches, such as difficulties in culturing primary cells from patients, and a lack of correlation between mRNA expression and enzymatic activities of nucleoside analog metabolizing enzymes. In paper I, we therefore looked at the enzymes involved in the activation of nucleoside analogs, and especially the purine nucleoside analogs CdA, FaraA, and CAFdA, in cells from patients with CLL, prior to treatment. This was done in order to determine approaches for predicting the sensitivity to nucleoside analogs.

We measured the activities of dCK and dGK in peripheral blood mononuclear cells from CLL patients using several substrates, both the natural deoxyribonucleosides and nucleoside analogs phosphorylated by these enzymes. There was a good correlation between the enzyme activities measured using the natural substrate
and the analogs for both enzymes. However, there were large interindividual differences in dCK and dGK enzyme activities between the 53 CLL patients, with dCK differing more than 200-fold, and dGK up to 27-fold between patients (Fig. 7A). There was also a wide range of dCK mRNA expression in the samples (0.011 to 0.189), and no correlation between dCK mRNA expression and activity (p=0.35, Fig. 7B). Although others have reported on alternatively spliced forms of dCK in cells from patients with leukemia [29], thereby explaining reduced sensitivity to nucleoside analogs in some patients, no such spliced variants could be found in this study.

Since dCK, and, to some extent, dGK, are responsible for the phosphorylation and activation of these drugs, and since their activities differ several fold, it is obvious that when receiving the same standard therapeutic dose, the amount of active drug in the patients’ leukemia cells must be highly individual.

In order to see if dCK mRNA and activity could be suppressed simultaneously, we used siRNA to downregulate the mRNA and activity of dCK in the T-ALL cell line CEM, which we were able to do to equal degrees, with mRNA and enzyme activity closely following each other. However, following the dCK mRNA expression and activity in cells electroporated without siRNA (mock transfected) we could see stable mRNA levels but markedly decreased enzymatic activity. We speculate that this may due to the decreased proliferation that were observed of electroporated cells,
since dCK activity has been shown to be higher in proliferating cells [32]. This suggests different regulatory mechanisms for dCK mRNA and activity, and different cell cycle dependency.

Resistance to nucleoside analog treatment and low mRNA and protein expression levels of dCK has been reported in patients with both acute and chronic leukemia [73, 102], while other studies have shown no such findings [161, 162]. With or without correlations, we emphasize that the determination of phenotype, by measuring the enzyme activity, would provide the most correct picture of the capacity for nucleoside analog metabolism in an individual. The obvious lack of correlation between mRNA and activity makes it inappropriate to use quantitative PCR in order to identify chemo-responsive patients prior to treatment.

To elucidate if cell cycle phase could explain the variability in activity of different enzymes that we can see in both cell lines and in primary cells, the effect of cell cycle progression on the activity of nucleoside analog-metabolizing enzymes was determined in different leukemic cell lines. Cells were synchronized in G₀/G₁-phase applying serum deprivation for 24 h with subsequent adding of serum. Different enzyme activities were then measured after 24 h, as presented in paper II. The T-ALL cell lines CEM and MOLT-4, and the AML cell line HL60 showed the highest activities of dCK and dGK and also the highest increase in enzyme activity during proliferation, compared to the CML cell line K562 (Fig. 8A and B). The activity of cN-I decreased in all four cell lines after adding serum and subsequent cell cycle progression, while the activity of cN-II increased in MOLT-4 but decreased in K562 cells. The dNT-I activity increased in all cells but K562, where it decreased.

The cytotoxicity of different nucleoside analogs was highest in the cells that showed high total 5´-NT activities but also had the highest activities of dCK and dGK, and the highest induction of these enzymes during proliferation, and included the CEM, MOLT-4, and HL60 cell lines.
Proliferating cells may therefore have a more favorable enzymatic profile reflected by the increased dCK/dGK levels and decreased cN-I levels. Attempts to recruit cells into S-phase during nucleoside analog treatment have been done using hematopoietic growth factors among others [163], and our data support that this may be a useful strategy. Knowledge concerning the regulation of these enzymes is crucial for the optimal use of nucleoside analogs as well as the development of new and more efficient analogs. It is also important to determine whether the net effect of 5’-NTs on different substrates is anabolic or catabolic, since the cells that showed the highest sensitivity to nucleoside analogs also showed the highest 5’-NT activities.

**Fig. 8** A: The activity of dCK measured in four different cell lines at resting and proliferative state. B: The activity of dGK measured in the same cells.

Downregulation of nucleoside analog phosphorylating enzymes in order to elucidate their involvement in activation (papers III & IV)

Several groups have downregulated the expression and activity of nucleoside analog metabolizing enzymes to elucidate their respective contributions and involvements in nucleoside analog activation or deactivation. Attempts to do so were also made here as presented in
papers III and IV. Since most published work was done in adherent solid tumor cell lines, the first achievement was to optimize a transfection method for introducing sequence-specific siRNA into a leukemic cell line. After trying three different cell lines (CEM, MOLT-4, HL60), the T-cell acute lymphoblastic leukemia cell line CEM, was selected, since it seemed most suitable to transflect with the highest effect on dCK downregulation. SiRNA delivery was optimized using dCK siRNA, since this is an abundantly expressed enzyme in hematopoietic cells, whose reduced activity should be able to confer some degree of resistance to nucleoside analogs.

We managed to downregulate dCK mRNA and enzyme activity by approximately 75 to 80%, 24 hrs after transfection (Fig. 9). Electroporation parameters were optimized so that cell death was reduced to about 10%.

![Fig 9 Optimization of electroporation parameters in order to introduce siRNA to the CEM cell line resulted in a protocol where dCK mRNA expression and activity were reduced with approximately 75-80% initially.](image)

We performed a gene expression microarray analysis on the electroporated and siRNA-transfected cells. To our knowledge, we are the first ones to report on which genes are affected by the electroporation process itself. More than 120 genes were 2-fold or more differentially expressed after electroporation of CEM cells. However, looking above a 4-fold difference, only ten genes were altered, and these were all upregulated. They were genes involved in apoptosis, fatty acid metabolism, cholesterol biosynthesis, and glucose metabolism, and probably were upregulated to help the cells increase their glucose uptake, repair their membranes, or go into apoptosis, if irreversibly damaged by the electroporation process. In the cells transfected with dCK siRNA, only dCK was downregulated.
Two genes were upregulated, but their function was unknown (Table I).

Table I A whole-genome expression microarray experiment was conducted on cells in order to investigate the effect of electroporation on gene expression and also the effects of the negative control and dCK siRNA.

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<td>4.3</td>
<td>up</td>
<td>C21orf90</td>
<td>chromosome 21 open reading frame 90</td>
</tr>
<tr>
<td>AK125918</td>
<td>4.9</td>
<td>up</td>
<td>GBE1</td>
<td>glycogen branching enzyme</td>
</tr>
<tr>
<td>BC107897</td>
<td>4.6</td>
<td>up</td>
<td>SC4MOL</td>
<td>sterol-C4-methyl oxidase-like 3</td>
</tr>
<tr>
<td>BC083514</td>
<td>6.0</td>
<td>up</td>
<td>HMGCS1</td>
<td>3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1</td>
</tr>
<tr>
<td>BC001880</td>
<td>7.0</td>
<td>up</td>
<td>INSIG1</td>
<td>insulin induced gene 1</td>
</tr>
</tbody>
</table>

| electroporated vs negative control 2-fold |     |            |             |                                                       |
| NC_001807     | 2.1 | down       | TRNF        | mitochondrion complete genome                         |

| negative control vs dCK 1.7-fold |     |            |             |                                                       |
| BC103764      | 1.7 | down       | DCK         | deoxycytidine kinase                                  |
| AF493523      | 1.8 | up         | REXO1L1     | REX1. RNA exonuclease 1 homolog                       |
| BC130374      | 1.7 | up         | GAGE13      | G antigen 13                                         |

The efficiency of nucleoside analog activation was altered in the siRNA-transfected cells, and the sensitivity to CdA, AraC, and CAFdA was decreased, indicating their dependency on dCK for activation. The sensitivity to AraG and FarA was more or less unchanged, reflecting other routes of activation and mechanisms of resistance to these drugs.

We also worked with nucleoside analogs and solid tumors since some analogs, such as dFdC and CAFdA are used quite successfully in various solid malignancies. Screening of a panel of different malignant melanoma cell lines showed that they were equally sensitive to at least two of the three nucleoside analogs CAFdA, FarA, and dFdC as leukemic cell lines. These analogs can be activated by dCK and dGK [46], as well as TK2 in the case of dFdC [123] Although the activities of the enzymes did not significantly
differ, we could show that the mRNA and protein expressions of dGK (Fig. 10) were higher in the melanoma cell lines than in the leukemic cell line CEM.

![Western blot data](image)

**Fig. 10** Western blot data clearly showing different expression of dCK and dGK in CEM cells compared to the RaH3 and RaH5 cell lines, and partly to the Sk-Mel-28 cell line.

To elucidate the role of dGK in the activation of nucleoside analogs in melanoma cells, the optimized protocol for siRNA delivery was used in order to silence the dGK enzyme in a malignant melanoma cell line expressing the highest dGK levels. Downregulation of dGK using siRNA in the CEM cell line (Fig. 11 A) and in the melanoma cell line RaH5 (Fig. 11 B) led to different effects on nucleoside analog activation in the cells. Downregulation of dGK in CEM cells did not increase the activity of dCK and TK2 as compensation, but these activities were rather decreased in the cells. Therefore, CEM cells showed decreased sensitivity to CAFdA, FaraA, and dFdC (Fig. 11 C). When reducing the dGK levels in the RaH5 cells, the activity of dCK decreased while the activity of TK2 increased, and made the cells more sensitive to the nucleoside analogs (Fig 11 D). TK2 strictly activates pyrimidines, such as dFdC [142], and the slightly higher activation of CAFdA and FaraA can probably not be due to the increased TK2 activity but must be due to other compensatory mechanisms. Both dGK and TK2 are localized in the mitochondria, and they should be able to substitute for each other to some extent, as they seem to do in the melanoma cell line. In CEM cells, dCK is the
most predominant enzyme and is probably most important for the activation of nucleoside analogs, and that might be the reason why these cells did not upregulate the other deoxyribonucleoside kinases as compensation, even though the activity of dGK decreased.

We believe that the method of downregulation of nucleoside analog-metabolizing enzymes can be useful when trying out the activity of new analogs and different combination regimens in cell lines. This technique makes it possible to create a cell line with inherited resistance to nucleoside analogs, due to decreased activity of activating enzymes, and is much faster than creating nucleoside analog-induced resistant cell lines.

**Fig. 11** A Downregulation of dGK in the CEM cell line and in the RaH5 cell line B. The effect of dGK down-regulation rendered the CEM more resistant to the nucleoside analogs tested C, while RaH5 cells became more sensitive D. \( p \leq 0.05 \)
Characterization of a nucleoside analog resistant cell line using gene expression microarray analysis (paper V)

In cell lines that have been made resistant to nucleoside analogs, variable degrees of dCK and/or dGK reduction have been demonstrated, and these cells show several-fold higher degrees of resistance than siRNA-treated cells, or cells from patients resistant to nucleoside analog treatment. This may be due to the long and continuous, and rather high concentration of drug applied to these cells because when making them resistant, the goal is not to kill all cells, but the majority of cells, eventually leading to a clone of cells with reduced sensitivity to the drug in use. In study V, we were determined to characterize mechanisms of resistance to the nucleoside analog AraG in the human leukemic cell line MOLT-4 using gene expression microarray analysis.

The cells had previously been made resistant to AraG, by incubating them with increasing concentrations of the drug for several months to obtain a cell line resistant to AraG at a concentration of 900 nM [11]. The cells showed reduced dCK and dGK activities and were resistant to several nucleoside analogs as well as to the anthracycline daunorubicin. The gene expression microarray analysis revealed more than 1000 genes that were 2-fold or more differentially expressed in the resistant cells compared to sensitive cells. Among the top-ten most upregulated genes were several fetal hemoglobin genes, and the ABCB1 gene coding for the multidrug resistance protein P-gp (Table II). Since drugs that hypomethylate DNA can induce fetal hemoglobin [164] by affecting the beta-globin locus on chromosome 11p15.5 [165], we tested whether short-term incubation with AraG could induce global hypomethylation in MOLT-4 cells, and in the lung adenocarcinoma cell line A549. We used the antimetabolite 6-mercaptopurine as a reference drug in the experiments, since it has shown global hypomethylation of DNA in MOLT-4 cells using the same assay for measuring global methylation status as we used [166]. The A549 cell line was used as a control, since AraG shows very limited activity against solid tumors. AraG significantly induced hypomethylation in both cell lines, and fetal hemoglobin gamma and the ABCB1 gene were also induced. Nucleoside analogs are not considered to be substrates of this drug efflux pump and
hence should not affect its expression [85], but have been shown able to induce its expression in cell lines [125]. We therefore speculated if the hemoglobin gamma and ABCB1 genes are connected to each other in some way, and if the P-gp pump could be upregulated in order to excrete hemoglobin waste products from the cells that would otherwise be toxic.

Table II List of genes differentially expressed between sensitive and resistant MOLT-4 cells showing the eight most upregulated genes as well as genes involved in hemoglobin regulation and metabolism (KLF1, BCL11A, BCL11B, BLVRB), transportation (ABCB6), as well as genes traditionally thought of being involved in nucleoside analog-resistance (DCK, DGUOK (dGK)).

<table>
<thead>
<tr>
<th>genebank</th>
<th>FC</th>
<th>gene symbol</th>
<th>gene description</th>
<th>gene ontology</th>
<th>chr</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_003352</td>
<td>433</td>
<td>HBZ</td>
<td>Hemoglobin beta</td>
<td>oxygen transport</td>
<td>16p13.3</td>
</tr>
<tr>
<td>NM_000134</td>
<td>411</td>
<td>HBG1</td>
<td>Hemoglobin gamma</td>
<td>oxygen transport</td>
<td>11p15.5</td>
</tr>
<tr>
<td>NG_015153</td>
<td>225</td>
<td>ABCB1</td>
<td>P-glycoprotein, multidrug resistance</td>
<td>membrane transporter</td>
<td>7q21.12</td>
</tr>
<tr>
<td>NM_008336</td>
<td>195</td>
<td>DLK2</td>
<td>Delta-like 1 homolog</td>
<td>tumor marker</td>
<td>14q32</td>
</tr>
<tr>
<td>TS359</td>
<td>183</td>
<td>HBA1/2</td>
<td>Hemoglobin alpha 1/2</td>
<td>oxygen transport</td>
<td>16p13.3</td>
</tr>
<tr>
<td>NM_208594</td>
<td>147</td>
<td>PRADE</td>
<td>Preferentially expressed antigen in melanoma</td>
<td>inhibits myeloid differentiation</td>
<td>22q11.22</td>
</tr>
<tr>
<td>NM_0013180</td>
<td>137</td>
<td>MBX4</td>
<td>Membrane-spanning 4-domain</td>
<td>hematopoietic cell specific</td>
<td>11q12.1</td>
</tr>
<tr>
<td>NM_008380</td>
<td>116</td>
<td>HBE1</td>
<td>Hemoglobin epsilon 1</td>
<td>oxygen transport</td>
<td>11p15.5</td>
</tr>
<tr>
<td>US4604</td>
<td>290</td>
<td>KLF1</td>
<td>Kruppel-like factor 1</td>
<td>(erythroid)</td>
<td>involved in β- and γ-globin expression</td>
</tr>
<tr>
<td>NM_000712</td>
<td>188</td>
<td>BLVRB</td>
<td>Blister vescicle B</td>
<td>hemoglobin degradation</td>
<td>19q13.1</td>
</tr>
<tr>
<td>NM_005859</td>
<td>5.7</td>
<td>ABCBC3</td>
<td>ATP-binding cassette, sub-family C, member 6</td>
<td>porphyria hemoglobin synthesis</td>
<td>2q96</td>
</tr>
<tr>
<td>NM_128376</td>
<td>-12</td>
<td>BCL11B</td>
<td>B-cell CLL/lymphoma 11 B</td>
<td>hematopoietic cell development</td>
<td>14q12.2</td>
</tr>
<tr>
<td>NM_022059</td>
<td>-8.6</td>
<td>BCL11A</td>
<td>B-cell CLL/lymphoma 11A (death finger protein)</td>
<td>hematopoietic cell development</td>
<td>2p16.1</td>
</tr>
<tr>
<td>NM_000706</td>
<td>-4.3</td>
<td>DCK</td>
<td>Dectin-1/like kinase</td>
<td>nucleotide and nucleoside metabolism</td>
<td>4q15.3</td>
</tr>
<tr>
<td>NM_001929</td>
<td>1.6</td>
<td>DGUOK</td>
<td>Deoxyguanosine kinase</td>
<td>nucleotide and nucleoside metabolism</td>
<td>2p13</td>
</tr>
</tbody>
</table>

In young rats, inhibitors of P-gp have been shown to increase the entry of bilirubin, a degradation product of hemoglobin, into the brain [167]. In order to test our hypothesis, we incubated MOLT-4/AraG- resistant cells containing high levels of fetal hemoglobin and P-gp with inhibitors of this pump. The cells proliferated more slowly and decreased their expression of hemoglobin gamma and increased their expression of ABCB1. This may be a consequence of the cells trying to decrease the hemoglobin content in order to avoid cytotoxicity when the function of P-gp is reduced, and increasing the expression of ABCB1 to compensate for the inhibition of P-gp.
Leukemia develops due to inactivation of genes controlling differentiation and growth [2] and this may be a consequence of epigenetic silencing of the genes. DNA molecules are extensively modulated after being synthesized and these modulations include transfer of methyl groups to adenine or cytosine bases, leading to transcriptional repression of genes. These genes may become reactivated by such inhibitors of DNA methylation as 5-aza or even AraC [168, 169]. Treating cells with 5-aza results in global hypomethylation of DNA, including the beta globin locus that appears to be especially sensitive to the impact of hypomethylating agents and may serve as a marker for global DNA hypomethylation [165, 170, 171]. These genes are silenced shortly after birth through methylation as the hemoglobin changes from fetal to adult forms, a process known as the fetal switch [164, 172]. Substances that are able to induce fetal hemoglobin include several chemotherapeutic drugs.

On the basis of the results generated in this thesis it appears that traditional mechanisms of resistance to nucleoside analogs, such as altered activities of nucleoside analog metabolizing enzymes, induce rather modest changes in nucleoside analog efficiency while altered activity of P-gp and a multidrug resistance phenotype seems more important for resistance and cross-resistance. However, most data were generated in cell lines and conclusions may be difficult to apply to patients. On the other hand, dCK activity differed more than 200-fold in the CLL patients, and we know that 50 to 70% of patients on the same standardized treatment with purine nucleoside analogs, initially respond to treatment with approximately 30% going into complete remission (Swedish Cancer Society). This may well be linked to the inter-individual activity of dCK, among other things.

When decreasing the activity of dCK or dGK using siRNA, the degree of resistance was usually less than 5-fold, while in nucleoside analog resistant cells, the degree of resistance may be 100- or 1000-fold, although the effects on dCK and dGK can be rather modest. This indicates that these enzymes are important for the development of resistance, but that other mechanisms are of importance as well. It is also difficult to draw conclusions from the resistant cell lines since they can tolerate a higher dose of nucleoside analog than would be
tolerated by a patient, who would suffer from severe side effects if the dose is increased too much.

SiRNA studies may give important insight into which enzymes are involved in the metabolism of different nucleoside analogs, especially if new analogs are created. Gene expression microarray studies on resistant cell lines and on patients prior to and during treatment may enhance our knowledge of additional resistance mechanisms, but also mechanisms of drug action. It is interesting that DNA hypomethylation by AraG in the MOLT-4 cells rendered the cells highly resistant to nucleoside analogs, when there are hypomethylating drugs such as 5-aza that are used successfully in the treatment of leukemia, where the goal is to hypomethylate and reexpress different genes [173]. Further studies are therefore needed to find out which genes are hypomethylated and reexpressed when patients are treated with nucleoside analogs and other drugs with hypomethylating capacity.
Conclusions

This thesis focuses on the intracellular activation of the cytotoxic anti-cancer drugs nucleoside analogs, and resistance mechanisms to them, which are of importance for the effect of nucleoside analog treatment, both in cell lines and in patients. Some general conclusions from the studies are as follows:

The expression and activity of the nucleoside analog activating enzymes dCK and dGK vary widely in leukemia patients, and there is no correlation between expression and activity, suggesting post-transcriptional regulation of these enzymes. Therefore, if used in clinical practice to guide physicians, the activities of the enzymes should be determined using natural substrates or nucleoside analogs.

The activities of both nucleoside analog-activating and deactivating enzymes differ in indolent and proliferating cells. A cell with high levels of 5′-nucleotidase activity may still be highly sensitive to nucleoside analogs if capable of inducing the activities of dCK and dGK so as to lead to a net anabolic effect on nucleoside analogs.

Transient siRNA transfection using electroporation is a useful and specific tool for studying nucleoside analog metabolism, and downregulation of dCK confers different degrees of resistance on clinically relevant nucleoside analogs.

Nucleoside analogs that can be efficiently activated by dGK as well as dCK may constitute a useful complement in the treatment of malignant cells expressing high levels of dGK due to high mitochondrial content, such as in melanocytes.
The nucleoside analog AraG is able to hypomethylate DNA and induce the induction of fetal hemoglobin and the multidrug resistance gene ABCB1 encoding a drug efflux pump. The increase in ABCB1 may be linked to the ability for this pump to transport hemoglobin waste out of the cells. Potent hypomethylating drugs should be used with caution together with drugs serving as a substrate of this pump.
Future perspectives

I am now turning my focus on investigating nucleoside analog metabolizing enzymes more thoroughly in leukemia patients, rather than cell lines. I will look particularly at genetic variants of the different enzymes, so-called single nucleotide polymorphisms, and correlate these variants with response and survival.

Another study I am planning is to collect sequential samples from patients with AML before treatment and a few days after treatment and study correlations between therapeutic response and gene expression. I am also planning on detecting the level of hypomethylation and to characterize which genes are affected and activated by the treatment. By using the patients as their own controls the inter-individual differences in gene-expression are smaller, since only 10 to 20% of the variability seen in expression microarrays is due to the disease, while 80 to 90% is due to normal genetic variability between patients. Genes of interest will be thoroughly investigated using real-time PCR, Western blotting, siRNA, and different genetic variants created by site-directed mutagenesis.

We will continue to investigate the relationship between induction of fetal hemoglobin and multidrug resistance using inhibitors of the induction of hemoglobin production. Treating cells with inhibitors together with nucleoside analogs and other cytotoxic agents should, if our theory is true, lead to less hemoglobin and less ABCB1 induction.
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