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RESISTANCE MECHANISMS FOR NUCLEOSIDE ANALOGUES

-with focus on metabolism and apoptosis

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Stockholm 2002

ISBN 91-7349-330-9

Published and printed by Repro Print AB, Stockholm 2002

TO MY BELOVED FAMILY

ABSTRACT

The aim of the thesis was to elucidate the mechanisms underlying resistance to nucleoside analogues used in the treatment of leukemias, with focus on cellular metabolism and induction of apoptosis. Cladribine (CdA), Clofarabine (CAFdA), Fludarabine (Fara-A) and Nelarabine (Ara-G) are nucleoside analogues with activity against various types of leukemias.

CAFdA is a relatively new nucleoside analogue and we showed that CAFdA nucleotides were accumulated to a higher extent than CdA nucleotides in samples from patients with chronic lymphocytic leukemia (CLL) and acute myeloid leukemia (AML). CAFdA is more efficiently phosphorylated by deoxycytidine kinase (dCK) and CAFdA nucleotides are more slowly eliminated than CdA nucleotides.

As earlier indicated in patients, there is an absence of cross-resistance between CdA and Fara-A. In an acute myeloid leukemia cell line the mechanism of resistance to CdA was a deficiency in dCK. Fara-A resistant cells had another contributing factor to resistance, the deoxynucleoside triphosphate pools being altered, indicating a mutation or altered regulation of ribonucleotide reductase (RR). Further studies in a lymphoid leukemia cell line supported these findings, and we also demonstrated that Fara-A resistant cells had increased RR activity and protein levels of the R2 subunit of RR.

A real time quantitative PCR (RQ-PCR) method was established to measure mRNA levels of enzymes important in the metabolism of dCK, deoxyguanosine kinase (dGK) and high K_m 5´-nucleotidase (5´-NT). The RQ-PCR method was compared to semi-quantitative PCR and enzyme activity measurements and tested with samples from pediatric patients with acute lymphocytic or myeloid leukemias, and was shown to be a convenient and reliable tool in the measurement of these enzymes.

The major cause of resistance to CdA at the apoptotic level was a disturbed sensitivity to increased Ca^{2+} levels in the cytosol. Increased Ca^{2+} levels may induce changes in mitochondrial membrane potential ($\Delta\Psi_{mito}$). Accordingly, the increased Ca^{2+} levels and the following drop in $\Delta\Psi_{mito}$ are important events for CdA-induced apoptosis.

In another study we demonstrated that Ara-G-resistance was associated with perturbations in apoptotic events. Resistance to Ara-G correlated with upregulation of the anti-apoptotic protein Bcl-xL and downregulation of the Fas receptor.

Thus, our data demonstrate that CAFdA is more effectively accumulated in samples from CLL and AML patients. Important for CdA- and CAFdA-resistance is dCK and important for Fara-A-resistance in addition to dCK is RR. Apparent is also that abberations in apoptosis induced by nucleoside analogues may contribute to resistance.

LIST OF PUBLICATIONS AND MANUSCRIPTS

This thesis is based on the following papers:

I. Lotfi, K., Månsson, E., Spasokoukotskaja, T., Pettersson, B., Liliemark, J., Peterson, C., Eriksson, S., and Albertioni, F. Biochemical pharmacology and resistance to 2-chloro-2'-arabino-fluoro-2'-deoxyadenosine, a novel analogue of cladribine in human leukemic cells, Clinical Cancer Research, Sep;5(9):2438-44, 1999.

II. Månsson, E., Spasokoukotskaja, T., Sällström, J., Eriksson, S., and Albertioni, F. Molecular and biochemical mechanisms of fludarabine and cladribine resistance in a human promyelocytic cell line, Cancer Research, Dec 1;59(23):5956-63, 1999.

III. Månsson, E., Liliemark, E., Söderhäll, S., Gustafsson, G., Eriksson, S., and Albertioni, F. Real-time quantitative PCR assays for deoxycytidine kinase, deoxyguanosine kinase and 5´-nucleotidase mRNA measurement in cell lines and in patients with leukemia, Leukemia, 16:386-392, 2002.

IV. Chandra, J., Månsson, E., Gogvadze, V., Kaufmann, S. H., Albertioni, F., and Orrenius, S. Resistance of leukemic cells to 2-chlorodeoxyadenosine is due to a lack of calcium-dependent cytochrome c release, Blood, Jan 15;99(2):655-63, 2002.

V. Månsson, E., Flordal, E., Liliemark, J., Spasokoukotskaja, T., Elford, H., Lagercrantz, S., Eriksson, S., and Albertioni, F. Downregulation of deoxycytidine kinase in human leukemic cell lines resistant to cladribine and clofarabine and increased ribonucleotide reductase activity contributes to fludarabine resistance, Biochemical Pharmacology, ***In Press***, 2002.

VI. Månsson, E., Stridh, H., and Albertioni, F. Resistance to mitochondrial- and Fas-mediated apoptosis in human leukemic cells with acquired resistance to 9-β-D-arabinofuranosylguanosine, Biochemical and Biophysical Research Communications, Nov;298(3):338-44, 2002.

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ABBREVIATIONS

ADA	Adenosine deaminase
AIF	Apoptosis-inducing factor
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
APAF-1	Apoptosis protein activating factor 1
Ara-C	Cytosine arabinoside
Ara-G	9-β-D-arabinofuranosylguanine, Nelarabine
Ara-GMP	Ara-G monophosphate
Ara-GTP	Ara-G triphosphate
CAD	Caspase-activated DNase
CAFdA	2-chloro-2´-***arabino***-fluoro-2´-deoxyadenosine, Clofarabine
CAFdAMP	CAFdA monophosphate
CAFdATP	CAFdA triphosphate
CdA	2-chloro-2´-deoxyadenosine, Cladribine
CdAMP	CdA monophosphate
CdATP	CdA triphosphate
CGH	Comparative genomic hybridization
CLL	Chronic lymphoblastic leukemia
Didox	3,4-dihydroxybenzohydroxamic acid
dFdC	Difluorodeoxycytidine
dFdG	Difluorodeoxyguanosine
dCK	Deoxycytidine kinase
dGK	Deoxyguanosine kinase
DISC	Death-inducing signaling complex
dNTP	deoxynucleotide triphosphate
FADD	Fas-associated death domain
Fara-A	9-β-D-arabinofuranosyl-2-fluoroadenine, Fludarabine
Fara-AMP	Fara-A monophosphate
Fara-ATP	Fara-A triphosphate
hENT	Human equilibrative nucleoside transporter
HCL	Hairy cell leukemia
hCNT	Human concentrative nucleoside transporter
high K_m 5´-NT	cytosolic high K_m 5´-nucleotidase, cN-II
low K_m 5´-NT	cytosolic low K_m 5´-nucleotidase, cN-I
HPLC	High performance liquid chromatography
HU	Hydroxyurea
MDR	Multi-drug resistance
$\Delta\Psi_{mito}$	Mitochondrial transmembrane potential

MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide
NA	Nucleoside analogues
NAD	Nicotinamide adenine dinucleotide
NDPK	Nucleoside diphosphate kinase
NMPK	Nucleoside monophosphate kinase
NT	Nucleoside transporter
PARP	Poly(ADP-ribose) polymerase
P-gp	P-glycoprotein
PNP	Purine nucleoside phosphorylase
RR	Ribonucleotide reductase
RQ-PCR	Real-time quantitative PCR
SKY	Spectral karyotyping
TK1	Thymidine kinase 1
TK2	Thymidine kinase 2

INTRODUCTION

During the last 20 years the treatment of leukemias has improved tremendously, but leukemia still kills more than 100 000 people in the world each year. Leukemias arise from the malignant transformation of haematopoietic cells and are basically classified as either myeloid or lymphoid, and as acute or chronic. Chronic lymphocytic leukemia (CLL) is associated with a maturation arrest at an intermediate state of B-cell development, and is the most common form of adult leukemia in Western countries. In Sweden there are 300 diagnosed cases of CLL each year (Ringborg et al., 1998). Acute myeloid leukemia (AML) is associated with partially differentiated neutrophils. AML constitutes 40% of all leukemias in the Western countries. In Sweden there are 400 cases of acute leukemias per year (Ringborg et al., 1998). Before the era of chemotherapy acute leukemia was uniformly fatal but with the introduction of combination therapy, intensive care and stem cell transplantation therapy, the prognosis of leukemia has improved significantly during the past 20 years. Chemotherapy is the treatment of choice for leukemias and combination therapies, in which nucleoside analogues (NA) are often included.

NAs are analogues of endogenous nucleosides and are used in the treatment of leukemia and HIV. Many patients are cured with the treatment available but a large group of patients develop resistance to chemotherapy and are therefore difficult to cure. The mechanisms of resistance to NA have been studied intensely during the last decade. However, focus has been on the uptake and the metabolism of the drug and more recently, on the induction of programmed cell death. The aim of this thesis was to elucidate the mechanisms of resistance to NA, and to find predictive markers for acquired and intrinsic resistance. Based on this knowledge new combination therapies can be designed, and in the future, different treatments can be applied for patients depending on their profile of predictive markers.

BACKGROUND

Since the introduction in the late 1940s of the alkylating agent nitrogen mustard (HN_2), many anti-cancer drugs have been developed with the aim of killing cancer cells without harming other body cells. In the late 1950s, cytosine arabinoside (Ara-C, cytarabine) was synthesised (Walwick et al., 1959), and Ara-C is today one of the most widely used anti-cancer drugs (Tallman, 2001). Already in 1964 the guanosine derivative 9-β-D-arabinofuranosylguanine (Ara-G, Nelarabine) was first synthesized (Reist &

Goodman, 1964). Deficiency of purine nucleoside phosphorylase (PNP) was shown to lead to accumulation of deoxyguanosine and a specific T-lymphocyte defect (Giblett et al., 1975). Ara-G is resistant to PNP and selectively cytotoxic to T-lymphocytes.

2-chloro-2'-deoxyadenosine (CdA, Cladribine) was first synthesised and shown to have an anti-leukemic effect in 1972 (Christensen et al., 1972). The observation made by Giblett in 1972 that children with severe combined immunodeficiency disease (SCID) are deficient in the enzyme adenosine deaminase (ADA) opened the way for further development of nucleoside analogues (Giblett et al., 1972). ADA catalyzes the irreversible deamination of adenosine to produce inosine and ammonia. Lymphocytes have high levels of deoxycytidine kinase (dCK), the enzyme phosphorylating deoxyadenosine to dAMP and they have a low activity of 5´-nucleotidases (5´-NT). In the absence of ADA, lethal levels of dATP will accumulate in lymphocytes (Carson et al., 1977). This stimulated further search for new and effective NAs and led to the development of the halogenated adenosine derivatives CdA and 9-β-D-arabinofuranosyl-2-fluoroadenine (Fara-A, Fludarabine) that are used in the clinic today (Johnson, 2001). Another deoxyadenosine derivative, 2-chloro-2'-***arabino***-fluoro-2'-deoxyadenosine (CAFdA, Clofarabine), was synthesized in 1992 (Montgomery et al., 1992). CAFdA has shown promising anti-tumor activity and is now in clinical trials.

CHEMOTHERAPY

Chemotherapy can only cure a few types of cancers, including different types of leukemia. Chemotherapy is also used in an adjuvant setting against cancer with high frequency of micrometastasis in order to keep the patient disease-free. Because oral bioavailability may be poor, most cytotoxic drugs are administered intravenously to assure adequate plasma levels. In an attempt to individualize treatment with cytotoxic chemotherapy the dose is based on body-weight or body surface area. Chemotherapy is toxic to both tumor/leukemic cells and normal cells, and will therefore always give adverse effects. Toxicities associated with NAs are infections, especially opportunistic infections, immunosuppression such as myelosuppression, neutropenia and thrombocytopenia, and neurotoxicity (Johnson, 1996). It is difficult to adjust the dose of chemotherapy so that maximal effect and minimal toxicity is obtained. The first difficulty in achieving the proper plasma concentration is the individual's differential ability to metabolise cytostatic drugs. Secondly, many cytotoxic agents have a steep dose-response curve with a narrow therapeutic index. The response to cytotoxic drugs is often related to the area

under the concentration-vs-time curve (AUC) (Galpin & Evans, 1993). Furthermore, the effect of chemotherapy can only be measured long after the actual time of treatment, since relapses can occur several years after treatment.

SYNTHESIS OF DEOXYRIBONUCLEOTIDES

Deoxyribonucleotides are required in the cell as building blocks for DNA synthesis and repair. The synthesis of deoxyribonucleotides occurs in two different ways; ***de novo*** synthesis and via the salvage pathway (Figure 1). In ***de novo*** synthesis the enzyme ribonucleotide reductase (RR) reduces the ribonucleoside diphosphates to deoxyribonucleoside diphosphate (Reichard, 1988). In the salvage pathway, deoxyribonucleosides derived from nutrients and degraded DNA are transported into the cell. The deoxyribonucleosides are phosphorylated by deoxyribonucleoside kinases to deoxyribonucleoside monophosphate. The salvage enzymes are dCK, deoxyguanosine kinase (dGK), thymidine kinase 1 and 2, which aredescribed in detail below.

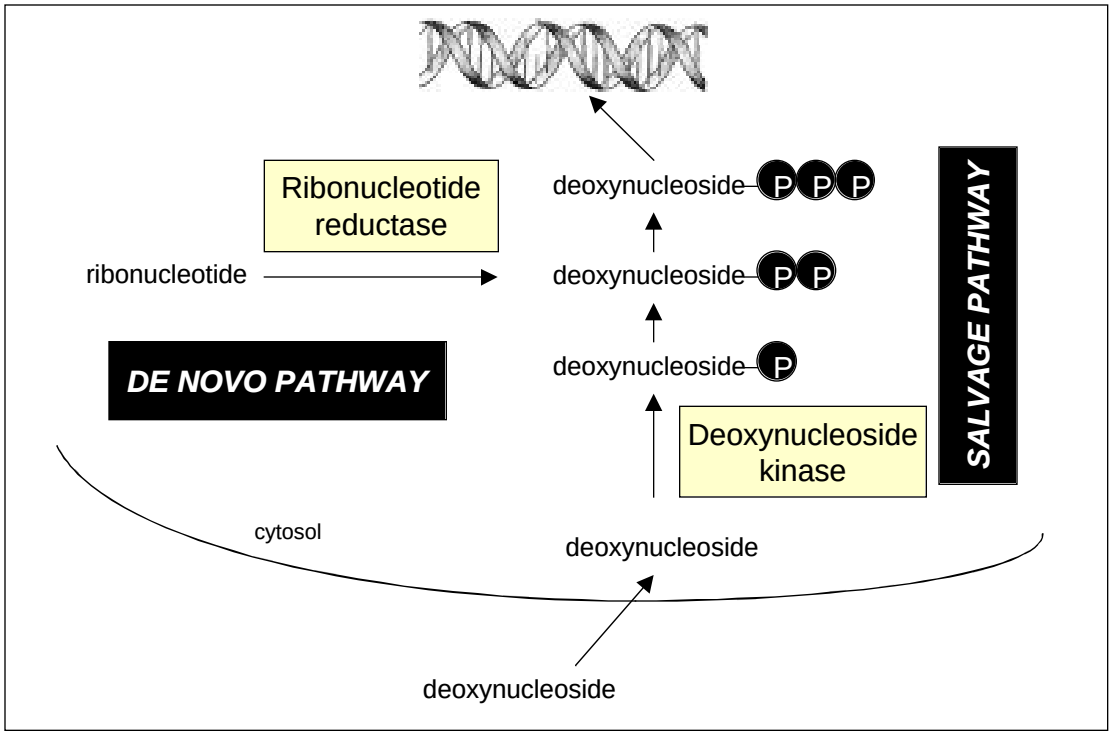

Figure 1. De novo and salvage pathways for synthesis of deoxyribonucleotides. In the de novo pathway, ribonucleotides are reduced by RR to the corresponding deoxyribonucleotide and in the salvage pathway, deoxyribonucleosides are phosphorylated by deoxynucleoside kinases to the

corresponding monophosphate. The deoxyribonucleoside triphosphates are incorporated into DNA.

NUCLEOSIDE ANALOGUES

Purine analogues share many characteristics; they have similar chemical structures, they are transported into the cell, phosphorylated by dCK or dGK and dephosphorylated by 5´-NTs. Although they have many similarities they may differ in their activity against different hematological malignancies.

Cladribine

The molecular structure of Cladribine (CdA, 2-chloro-2'-deoxyadenosine) differs from the naturally occurring nucleoside, deoxyadenosine, in the substitution of hydrogen by chlorine at the 2-position of the adenine ring, this confering resistance to deamination by ADA (Figure 2). CdA is effective in the treatment of HCL with an overall response rate of 95% (Piro et al., 1990; Rai, 1998). CdA has also been shown to be effective against other leukemias, including pediatric acute myeloid leukemia, CLL, low-grade lymphoma as well as autoimmune disorders (Beutler, 1994). CdA has been used and shown to have some efficacy in other diseases such as Langerhan cell histiocytosis, myofibromatosis and cutaneous T-cell lymphomas (Bouwhuis et al., 2002; Rodriguez-Galindo et al., 2002; Williams et al., 2002). The oral bioavailability of CdA is approximately 50% and is limited due to degradation by bacterial nucleoside phosphorolyses or acid hydrolysis to chloroadenine (Carson et al., 1984; Liliemark et al., 1992; Albertioni et al., 1993). When CdA was administered as a continuous infusion of 0.14 mg/kg for 24 hours, a steady-state plasma concentration of 22.5 nM was achieved (Liliemark & Juliusson, 1991). Elimination of CdA follows a two- or three-compartment model and 30-50% of the administered CdA is excreted unchanged in the urine during the first 24 hours (Liliemark et al., 1992; Albertioni et al., 1994). The CdA nucleotides accumulates rapidly in leukemic cells and the elimination half-life ($t_{1/2}$) was 14.6 hours for CdAMP and 9.7 hours for CdATP (Albertioni et al., 1998), and the drug appears to be completely cleared from the plasma 1 to 3 days after stopping its infusion (Carson et al., 1984; Liliemark & Juliusson, 1991).

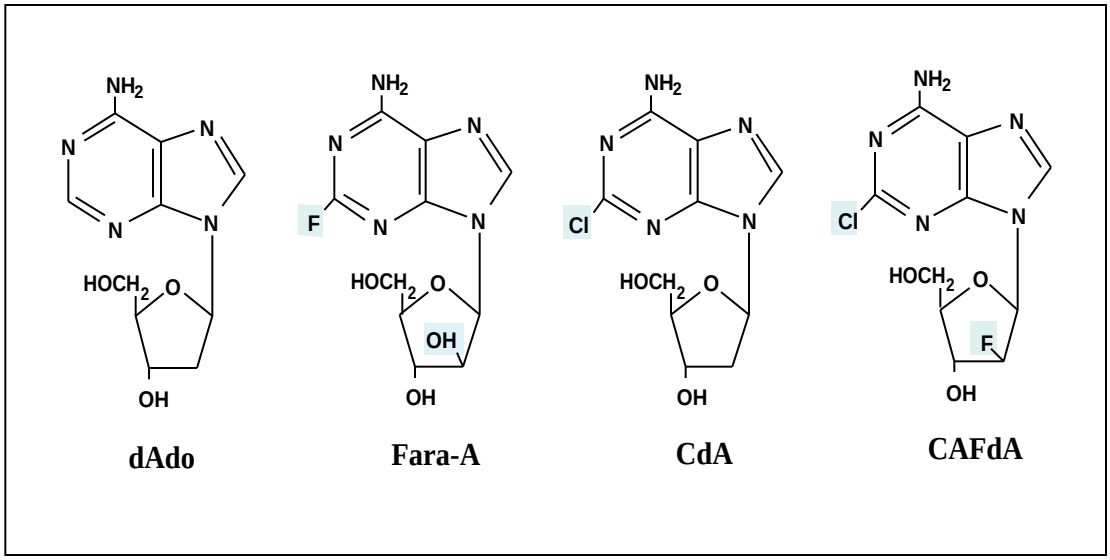

Figure 2. Chemical structure of deoxyadenosine (dAdo), Fara-A, CdA and CAFdA.

CdA is phosphorylated intracellularly by dCK and also by dGK, to CdA-monophosphate (CdAMP) (Wang et al., 1993). The cytotoxicity of CdA depends on accumulation of the triphosphate form of CdA (CdATP) (Griffig et al., 1989). In quiescent cells, CdATP interferes with the proper repair of DNA, leading to accumulation of DNA strand breaks, activation of poly(ADP-ribose) polymerase (PARP), depletion of nicotinamide adenine dinucleotide (NAD), resulting in depletion of ATP and a total disruption of cellular metabolism (Seto et al., 1985; Carson et al., 1988).

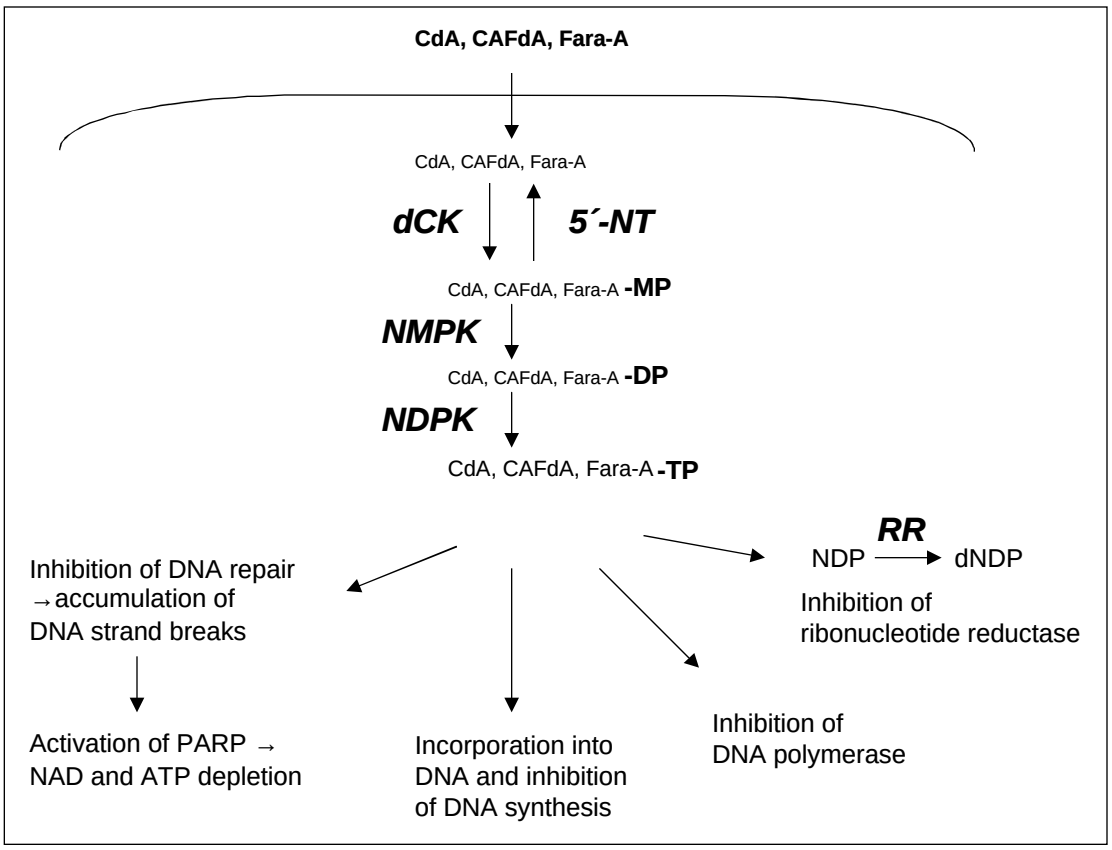

Figure 3. Schematic presentation of metabolism and mechanisms of action for CdA, CAFdA and Fara-A. Triphosphates of the respective NAs are accumulated, incorporated into DNA and inhibit DNA synthesis and DNA repair. The inhibition of DNA repair results in accumulation of DNA strand breaks, which activates poly(ADP-ribose) polymerase (PARP), leading to nicotinamide adenine dinucleotide (NAD) and ATP depletion. CdATP, CAFdATP and Fara-ATP inhibit DNA polymerases and RR.

Fludarabine

Fludarabine (Fara-A, 9-β-D-arabinofuranosyl-2-fluoroadenine) is a halogenated analogue of adenosine, with fluorine at the 2´-position of the adenine ring (Figure 2). Fara-A has major anti-tumor activity, especially against CLL (Adkins et al., 1997). For CLL, Fara-A is usually administered intravenously with doses of 20-30 mg/m^2 infused daily for 3-5 days (Plunkett & Gandhi, 1996). Treatment with Fara-A resulted in response rates of 25-30% for heavily pre-treated CLL patients and 70-80% for patients that were given Fara-A as first-line therapy (Grever et al., 1988; Keating et al., 1993). Recently, Fara-A was also reported to be effective in patients with Richter's syndrome or with refractory lymphoproliferative disorders (Tsimberidou et

al., 2002). The major toxicity of Fara-A is bone marrow suppression. The monophosphate form of Fara-A is used in the clinic, due to solubility problems with Fara-A. Fara-AMP is rapidly dephosphorylated by ecto 5´-NT in plasma, prior to entering the cell (Malspeis et al., 1990).

The major action of Fara-A is the inhibition of DNA synthesis, which is potentiated by the decrease of cellular dATP that results from inhibition of RR by Fara-ATP (Plunkett et al., 1990). Fara-A is also incorporated into RNA and may terminate RNA transcripts and interfere with their function as templates for translation (Spriggs et al., 1986; Huang & Plunkett, 1991). Fara-A has been shown to be a more effective activator of the apoptosome than are CdA and CAFdA (Genini et al., 2000b).

Clofarabine

A newer analogue of deoxyadenosine is Clofarabine (CAFdA, 2-chloro-2´-***arabino***-fluoro-2´-deoxyadenosine), which has a fluorine atom at the 2´-position of the adenine ring and a fluorine at the 2´-arabino position of the sugar molecule (Figure 2). The introduction of a fluorine at the 2´-arabino position increases its acid stability. The solubility problems associated with the administration of Fara-A may be overcome with CAFdA because of its greater solubility, and the low oral bioavailability of CdA may be overcome with CAFdA due to greater acid and enzymatic stability (Carson et al., 1992; Albertioni et al., 1995). CAFdA has cytotoxic effects in human cell lines (Secrist et al., 1988; Carson et al., 1992; Waud et al., 2000) and in Phase I clinical trials (Kozuch et al., 1999). To exert its cytotoxic effect, CAFdA has to be intracellularly phosphorylated by dCK, but may also be phosphorylated by dGK (Sjoberg et al., 1998).

Nelarabine

Nelarabine (Ara-G, 9-β-D-arabinofuranosylguanine) (Figure 4) is an analogue of guanosine that is resistant to degradation by PNP. Although Ara-G was already synthesized in 1964, its poor water-solubility has hampered it from being evaluated in clinical trials. The water soluble pro-drug of Ara-G, 2-amino-6-methoxypurine arabinoside (Nelarabine) was later developed. Nelarabine is converted to Ara-G in the plasma by ADA (Lambe et al., 1995). Ara-G is active against various hematological malignancies, e.g, T-cell ALL, T-lymphoid blast crisis, T-lymphoma and B-cell CLL (Gandhi et al., 1998b; Aguayo et al., 1999; Kisor et al., 2000). However, due to neurological

toxicity, clinical trials with Nelarabine have been terminated and its future as a clinically useful drug is uncertain.

Ara-G is phosphorylated to its monophosphate, predominantly by the mitochondrial enzyme dGK and also by dCK, and further to its active metabolite Ara-G triphosphate (Ara-GTP), which is incorporated into DNA and terminates DNA chain elongation, resulting in cell death (Wang et al., 1993; Rodriguez et al., 1997; Rodriguez & Gandhi, 1999). A critical role for mitochondria as a pharmacological target of Ara-G has been proposed since Ara-G is a better substrate for mitochondrial dGK than it is for the cytoplasmic/nuclear dCK (Sjoberg et al., 1998; Zhu et al., 1998)

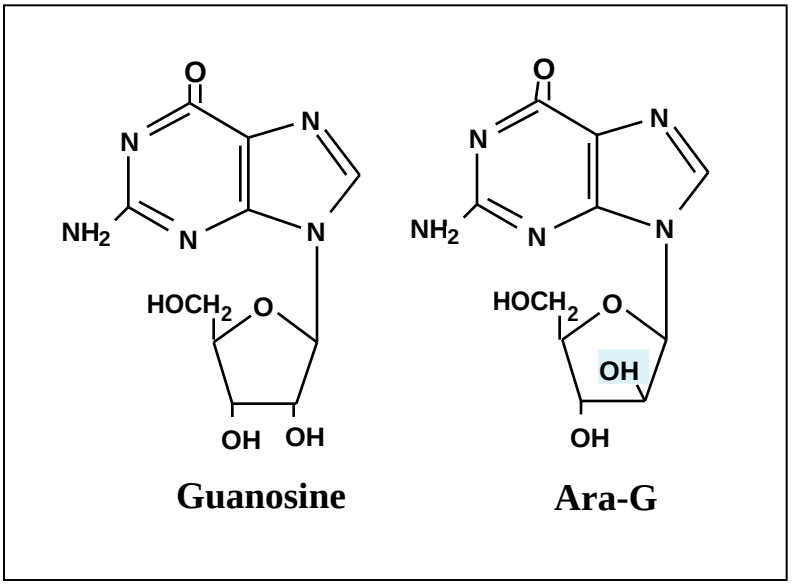

Figure 4. Chemical structure of guanosine and Ara-G.

TRANSPORTERS

Purine nucleosides and their analogues are hydrophilic and need to be transported into the cell by nucleoside transporters (NT). At least five different NTs have been identified and the NTs are defined as either equilibrative (hENT) or concentrative (hCNT). The equilibrative transporters mediate passive transport, both influx and efflux of nucleosides whereas the concentrative transporters mediate only influx which is driven by the sodium gradient across the cell membrane. The two cloned hENTs are hENT1, which is sensitive to inhibition of nitrobenzylmercaptopurine ribonucleoside (NBMPR), and hENT2 which is not sensitive to NBMPR (Griffiths et al., 1997a; Griffiths et al., 1997b). There are at least three hCNTs; hCNT1

transports pyrimidine nucleosides and adenosine more efficiently and hCNT2 mainly transports purine nucleosides (Schaner et al., 1999). It was recently shown that CdA may also be transported by hCNT2 to some extent (Lang et al., 2001). Ara-G is primarily transported by the NBMPR-sensitive hENT1 nucleoside transport system (Prus et al., 1990).

ENZYMES

NAs are administered as pro-drugs and their efficiency is dependent on the intracellular phosphorylation to the active phosphorylated compound. In mammalian cells, the anabolism is mediated by the cytosolic enzymes dCK, thymidine kinase 1 (TK1), the mitochondrial enzymes dGK and thymidine kinase 2 (TK2). The catabolism is mediated by 5´-NTs.

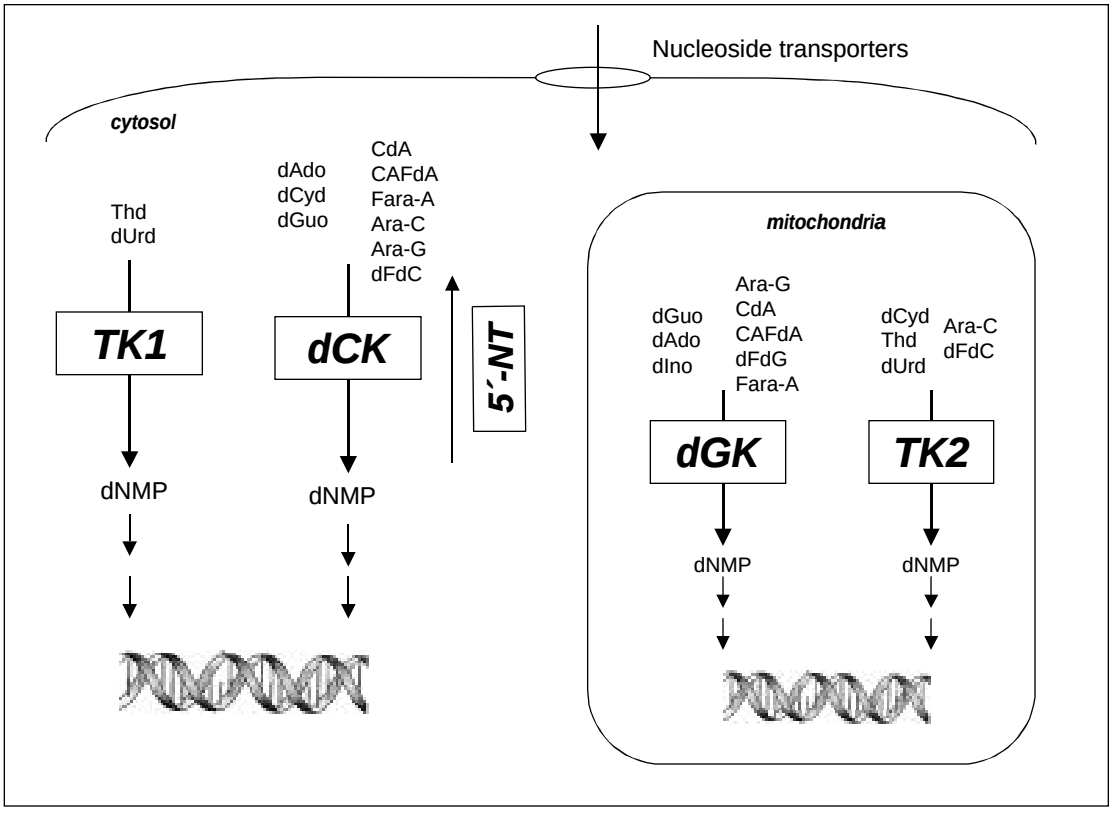

Figure 5. Deoxynucleoside kinases and nucleotidase and their substrates; endogenous nucleosides and nucleoside analogues.

ANABOLISM OF NUCLEOSIDE ANALOGUES

Deoxycytidine kinase

DCK (EC 2.7.1.74) is responsible for the initial phosphorylation of many NAs used in the treatment of hematological malignancies, such as Ara-C, dFdC, Fara-A and CdA (Plunkett & Saunders, 1991). dCK catalyzes the phosphorylation of 2´-deoxycytidine to its monophosphate form with ATP or UTP as phosphate donors (Momparler & Fischer, 1968; Datta et al., 1989). In 1991, the dCK gene was cloned encoding a 30.5 kDa protein (Chottiner et al., 1991). By isolating genomic clones of dCK, Song ***et al***., demonstrated that the coding region of dCK is composed of 7 exons (Song et al., 1993). dCK is located on chromosome 4q13.3-q21.1 (Stegmann et al., 1993a). Under physiological conditions dCK is a cytoplasmic enzyme, but when dCK is overexpressed it translocates to the nucleus (Johansson et al., 1997; Hatzis et al., 1998). dCK is constitutively expressed throughout the cell-cycle and is expressed at low levels in most tissues, with the exception of lymphoid cells in which it is expressed at particularly high levels (Arner et al., 1988; Spasokoukotskaja et al., 1995). The substrate specificity of dCK is very broad and dCK phosphorylates many endogenous substrates as well as NAs, summarized in Figure 5. dCK phosphorylates the natural deoxyribonucleosides dCyd, dAdo and dGuo, and purines have lower affinity for dCK than for dCyd (Arner & Eriksson, 1995). NAs are also phosphorylated efficiently by dCK, like CdA (K_m: 5 µM) (Eriksson et al., 1991), CAFdA (K_m: 14 µM) (Parker et al., 1999) and Fara-A (K_m: 213 µM) (Tseng et al., 1982). Treatment of cells with the NAs CdA, Fara-A, CAFdA and Ara-C has been reported to stimulate dCK activity, probably through post-translational modifications of the enzyme (Sasvari-Szekely et al., 1998; Spasokoukotskaja et al., 1998).

Deoxyguanosine kinase

Purine nucleosides can also phosphorylated by the mitochondrial enzyme dGK (EC 2.7.1.113). The dGK gene was mapped to chromosome 2p13 (Johansson et al., 1996). As summarized in Figure 5, dGK phosphorylates the natural substrates dGuo, dAdo and dIno with K_m-values of 7.6 µM, 60 µM and 12 µM, respectively (using recombinant dGK) (Wang et al., 1993; Sjoberg et al., 1998). Also phosphorylated by dGK are important NAs, e.g. Ara-G, CdA, CAFdA, difluorodeoxyguanosine (dFdG) and to some extent

Fara-A (Sjoberg et al., 1998). Ara-G is a substrate with high affinity for dGK (K_m: 7.6 µM), and higher efficiency (V_{max}/K_m) than dGuo (Wang et al., 1993). The cytotoxicity of NAs phosphorylated by dGK was enhanced when dGK was over-expressed in human sarcoma cells (Zhu et al., 1998). dGK can be relocated from the mitochondria to the cytosol during apoptosis (Jullig & Eriksson, 2001).

Thymidine kinase 1 and 2

TK1 and TK2 phosphorylate deoxynucleosides to deoxynucleotides. TK1 is the most well studied of the enzymes, is expressed at high levels during S-phase (Sherley & Kelly, 1988) and phosphorylates the natural substrates Thd and dUrd (Munch-Petersen et al., 1991). TK2 is located in mitochondria and has a broad substrate specificity (Figure 5), phosphorylating to some extent the clinically used anti-cancer analogues Ara-C and dFdC (Wang et al., 1999). TK1 was mapped to chromosome 17q25.2-q25.3 (Kuo et al., 1996; Petty et al., 1996) and TK2 to chromosome 16q22 (Johansson & Karlsson, 1997).

CATABOLISM OF NUCLEOSIDE ANALOGUES

5´-nucleotidases

5´-NTs dephosphorylate deoxynucleotides by hydrolysis of the ester bond, removing the phosphate group to form the corresponding deoxynucleoside. The 5´-NTs consist of a family of enzymes differing in cellular location and substrate specificity. The plasma membrane bound 5´-NT named ecto 5´-NT or CD73 degrades extracellular nucleotides to nucleosides, which can be transported into the cell and reused (Resta et al., 1998). Two other 5´-NTs are the cytosolic located high K_m 5´-NT (cN-II) and low K_m 5´-NT (cN-I). The high K_m 5´-NT is specific for purine monophosphates and is activated by ATP. The low K_m 5´-NT dephosphorylates pyrimidine monophosphates and is inhibited by ATP (Spychala et al., 1989). The 5'(3')-deoxyribonucleotidase (dNT-1) has recently been cloned (Rampazzo et al., 2000b). Another 5´-NT identified recently is the deoxy-specific dNT-2, which is located in the mitochondria (Rampazzo et al., 2000a). The 5´-NTs are a complex group of enzymes not yet fully investigated. Thus, the 5´-NT with the largest impact on NAs has not been identified. Recently, the high K_m 5´-NT (cN-1) was cloned

and characterized and shown to have a potential role in the metabolism of NAs (Hunsucker et al., 2001).

RIBONUCLEOTIDE REDUCTASE

RR is essential for the synthesis of the four deoxyribonucleotides required for DNA synthesis and to keep a balanced supply of dNTP pools in the cell. Mammalian RR is composed of two non-identical subunits, the R1 and the R2 subunits. The large R1 subunit is 85 kDa and contains the effector and specificity binding sites regulating the overall activity and the substrate specificity of the enzyme (Brown & Reichard, 1969). The small R2 subunit is 45 kDa and the activity of RR is dependent on R2 synthesis, which is regulated in a cell cycle dependentmanner. The R2 subunit contains an iron centre with a free radical which is essential for activity. During the enzymatic reaction of RR, an OH-group at the carbon-2´ of the ribonucleotide is replaced with a hydrogen atom. The biochemistry of the reaction mechanism of RR is reviewed in detail by Reichard (Reichard, 1988; Jordan & Reichard, 1998). As evident in Figure 1, RR reduces ADP, GDP, CDP and UDP to their corresponding deoxynucleotides, which are further phosphorylated by NDPK and can then be incorporated into DNA. The exception is dUTP, which is dephosphorylated by dUTPase to the monophosphate. Thymidylate synthase converts dUMP to dTMP, which is phosphorylated by thymidylate kinase and NDPK and can then be incorporated into DNA. Another possibility to form dTTP is to convert dCMP to dUMP catalyzed by dCMP deaminase. The overall activity of RR is controlled by ATP or dATP binding to the effector site (Figure 6). Binding of ATP to the effector site induces RR activity, while dATP negatively regulates the enzyme. ATP, dATP, dGTP or TTP control the substrate specificity by binding to the specificity site (Figure 6). ATP induces the formation of dCDP and dUDP, TTP induces the formation of dGDP and dGTP induces the formation of dADP (Reichard, 1988; Jordan & Reichard, 1998). The only dNTP not affecting the specificity of RR is dCTP; instead, dCTP acts allosterically on dCK (Reichard, 1988).

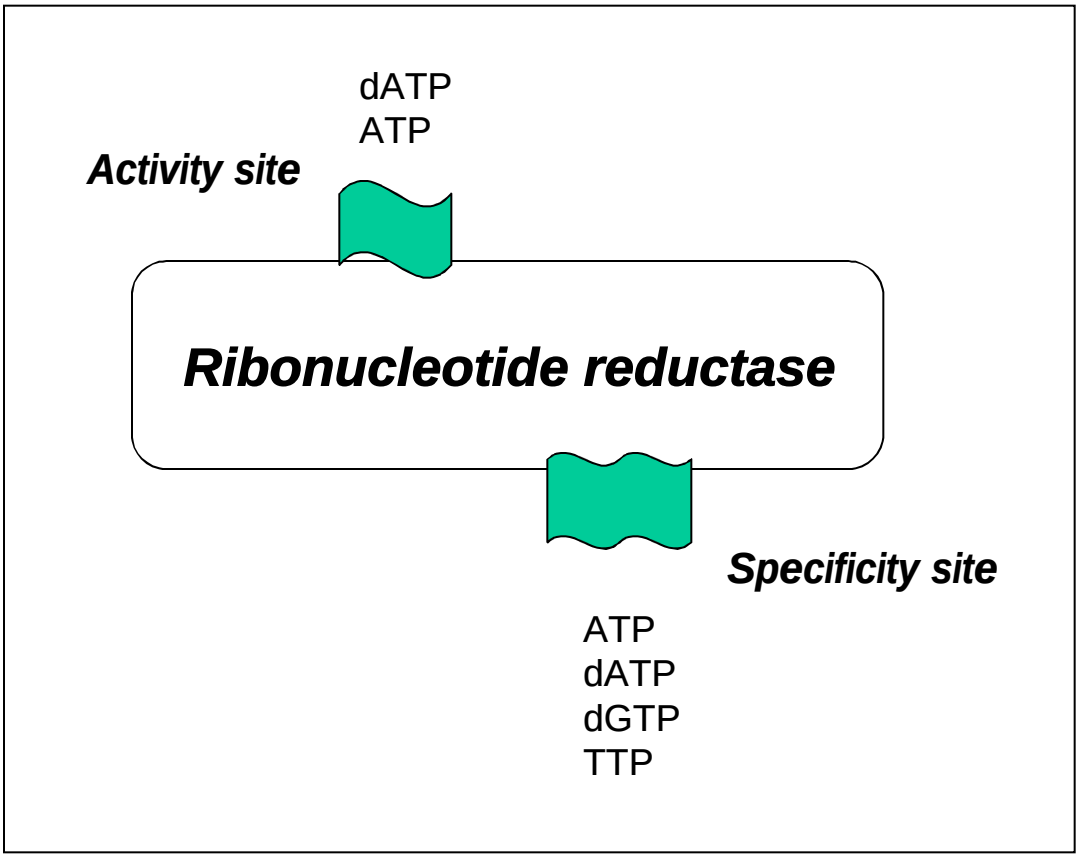

Figure 6. The enzyme RR consists of the R1 and R2 subunits. The R1 subunit contains the effector site and the specificity site. The overall activity of RR is regulated by ATP and dATP and the specificity is regulated by ATP, TTP and dGTP.

The NAs CdA, CAFdA and Fara-A have been demonstrated to inhibit RR and as a result of this the deoxynucleotide triphosphate (dNTP) pools are altered (Parker et al., 1991). This has been the strategy for combination therapy including Fara-A, CdA or Ara-G with Ara-C in the treatment of different leukemias (Gandhi et al., 1993; Gandhi et al., 1996; Rodriguez et al., 1997). Hydroxyurea (HU) inhibits RR by scavanging the free radical and is included in the treatment of chronic myelogenous leukemia. It has synergistic effects in combination with pyrimidine and purine analogues in the treatment of leukemia (Schilsky et al., 1992; Gandhi et al., 1998a). 3,4-dihydroxybenzohydroxamic acid (didox) is a polyhydroxy-substituted benzohydroxamate, which has been shown to be a more potent inhibitor of RR than is HU (Elford & van't Riet, 1985).

More recently, a new RR gene, p53R2, which is a directly targeted by p53, was discovered (Tanaka et al., 2000). The p53R2 gene exhibited a high

degree of identity to the small subunit R2 and is mainly involved in p53-regulated DNA repair. Mammalian p53R2 can form an active enzyme with the R1 subunit (Guittet et al., 2001).

APOPTOSIS

Apoptosis or programmed cell death is a process for controlled cell deletion. Errors in the onset of apoptosis can have devastating consequences for an organism. Apoptosis is distinguished from necrotic cell death by certain morphological criteria. Caspases (for cysteine aspartate-specific proteases) are important for apoptosis. They are proteases that exist in cells as inactive pro-enzymes (zymogens) and are activated by cleavage after Asp residues. Caspases cleave various cellular substrates, among them being lamins, that results in morphological changes (cell shrinkage, blebbing of the plasma membrane and formation of apoptotic bodies), apparent during apoptosis. PARP, which is involved in DNA repair, is also cleaved by caspases. The caspase-activated DNase (CAD) is essential for DNA fragmentation and is activated upon cleavage of the inhibitor of CAD (ICAD) by caspases (Liu et al., 1997).

Various agents cause apoptosis, among them being many anti-cancer drugs. Apoptosis is mainly mediated in two ways, via mitochondria and the formation of the apoptosome or via the death receptor Fas/TNF (tumor necrosis factor) pathway. Apoptosis can be mediated by mitochondria through the release of intermembrane located molecules such as cytochrome c and the apoptosis-inducing factor (AIF). Cytochrome c and dATP bind to APAF-1 that contains a CARD domain (caspase recruitment domain), which enables it to bind to procaspase-9. A complex termed the apoptosome is formed when APAF-1 /cytochrome c/dATP bind to caspase-9 (Li et al., 1997). Upon formation of the apoptosome-complex, procaspase-9 is cleaved at the Asp residue and the active form of caspase-9 cleaves procaspase-3, apoptosis being induced. As previously mentioned, AIF is also released from mitochondria upon loss of the mitochondrial transmembrane potential ($\Delta\Psi_{mito}$). AIF is released to the cytosol, but in contrast to cytochrome c, it translocates from the cytosol to the nucleus where it causes chromatin condensation and DNA fragmentation (Susin et al., 1999). As shown in Figure 7, NAs can induce apoptosis via the mitochondria and NA triphosphates can act as co-factors in the formation of the apoptosome (Leoni et al., 1998; Genini et al., 2000b; Marzo et al., 2001). The amount of dATP and cytochrome c present in the cells may be important for the induction of apoptosis. In cells treated with nucleoside

analogues, the triphosphate form of NAs are incorporated into DNA, causing DNA strand break accumulation, activation of poly-(ADP ribose) polymerase and p53. This leads to a depletion of nicotinamide adenine dinucleotide (NAD) and ATP and disruption of the mitochondrial integrity (Carson et al., 1988; Marzo et al., 2001).

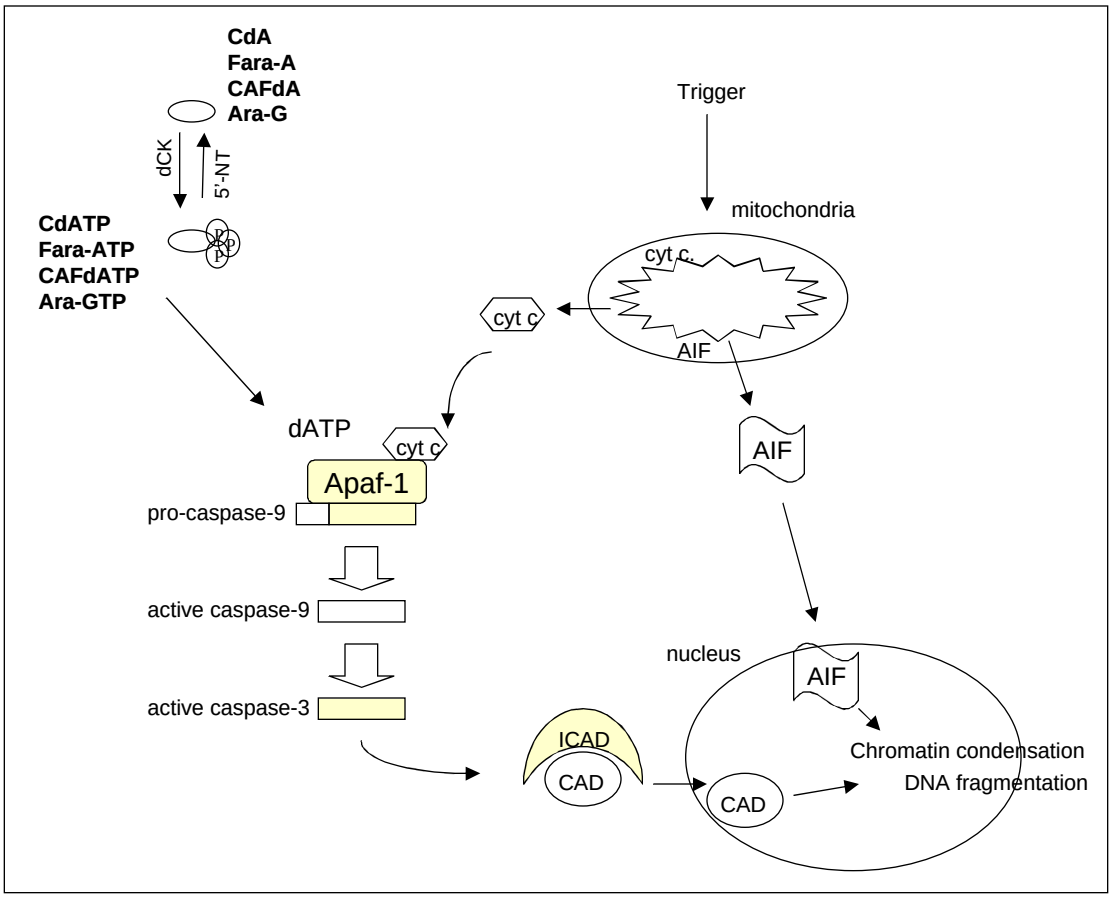

Figure 7. Mitochondrial- mediated induction of apoptosis. The triphosphte form of nucleoside analogues can substitute for dATP as a co-factor in the formation of the apoptosome. During induction of apoptosis via the mitochondria, cytochrome c is released into the cytosol and the apoptosome is formed. AIF is also released from the mitochondria but translocates to the nucleus where it causes chromatin condensation and DNA fragmentation.

The most studied death receptor is the Fas receptor (Figure 8). Apoptosis signalling begins with the binding of Fas ligand to the transmembrane Fas receptor, resulting in assembly of the death-inducing signaling complex (DISC). The DISC consists of the Fas receptor, FADD (Fas-associated death domain) and procaspase-8. Upon Fas ligation, FADD binds to the intracellular domain of the Fas receptor and recruits multiple procaspase-8 molecules,

caspase-8 being activated (Hengartner, 2000). Activated caspase-8 further activates caspase-3, culminating in DNA fragmentation and apoptotic cell death.

The mitochondrial and death receptor pathways of apoptosis are linked by the cleavage of the Bcl-2 family protein Bid. Caspase-8 is capable of cleaving Bid, and truncated Bid translocates to the mitochondria where it promotes the release of cytochrome c (Han et al., 1999). This can amplify the apoptotic response induced by the Fas ligand.

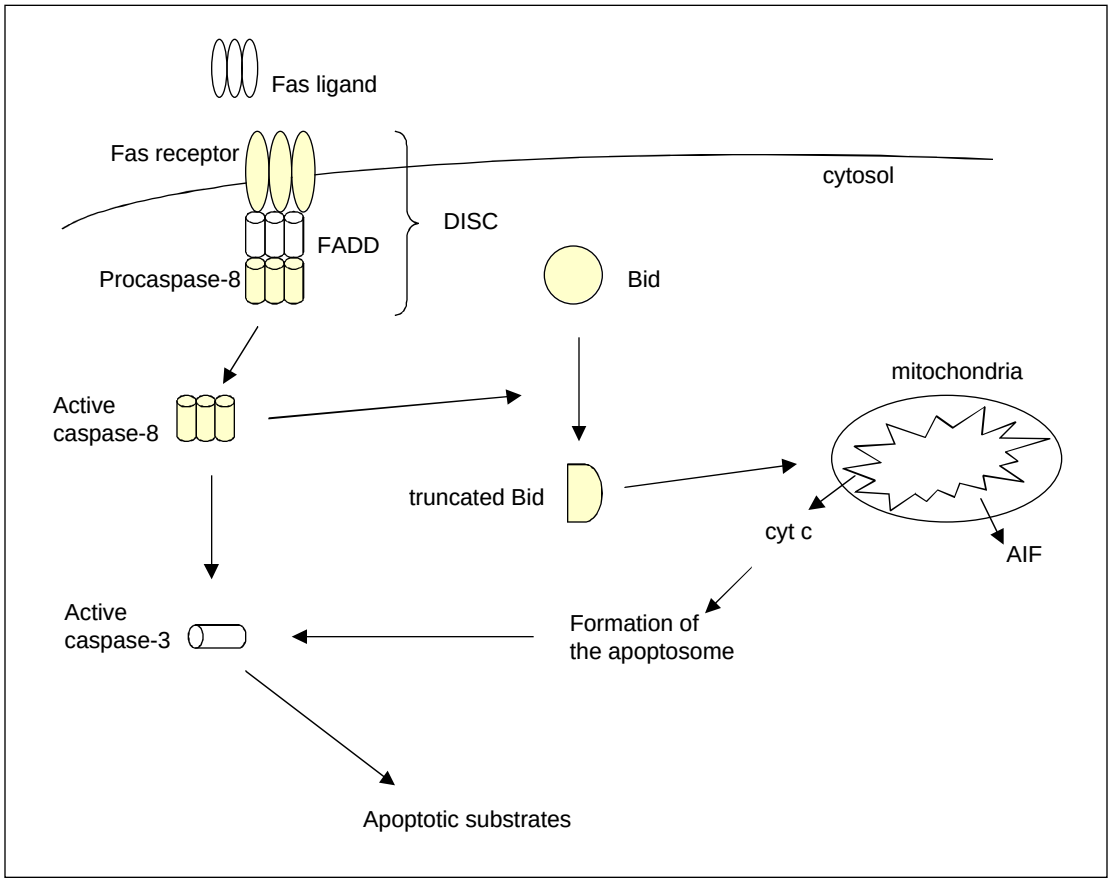

Figure 8. Induction of apoptosis mediated via the Fas pathway. Upon binding of the Fas ligand, the DISC is formed and caspase-8 is activated, resulting in the induction of apoptosis. Cross-talk with the mitochondrial- mediated pathway is possible since Bid is cleaved by caspase-8. Truncated Bid translocates to the mitochondria and interferes with mitochondrial integrity.

Regulation of apoptosis

The Bcl-2 family proteins include both pro- and anti-apoptotic proteins. All Bcl-2 family members contain at least one of the four conserved Bcl-2 homology domains BH1-BH4. Members with anti-apoptotic function are Bcl-2 and Bcl-xL. Bax, Bak, Bid and Bad are pro-apoptotic Bcl-2 proteins. All the pro-apoptotic Bcl-2 proteins contain the BH3 domain, which is required for the killing activity (Martinou & Green, 2001). The relative levels of the anti- and pro-apoptotic Bcl-2 proteins regulate the fate of the cell at the mitochondrial level.

Upon death signals, Bax is translocated from the cytosol to the mitochondria (Hsu et al., 1997), Bax oligomerizes and inserts into the outer membrane of the mitochondria (Gross et al., 1998; Eskes et al., 2000). The pore formed by Bax induces release of cytochrome c into the cytosol. The pore formation of Bax can be prevented by Bcl-2 and Bcl-xL (Desagher et al., 1999). The pro-apoptotic protein Bak is also activated in a similar way as is Bax. The over-expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL has been reported in many cell types to prevent the release of cytochrome c and induction of apoptosis (Vander Heiden et al., 1997; Yang et al., 1997). Dephosphorylated Bad is thought to block Bcl-xL and its anti-apoptotic effect (Green & Reed, 1998).

Induction of apoptosis by nucleoside analogues

Many chemotherapeutic agents, among them NAs, are DNA damaging agents, inducing apotosis. DNA damage caused by NA triphosphates induces expression of p53, leading to induction of pro-apoptotic proteins such as Bax (Miyashita et al., 1994; Gartenhaus et al., 1996). In resting cells high levels of NA triphosphates interfere with repair of DNA strand breaks. This activates PARP and NAD is depleted, resulting in decreased levels of ATP and, consequently, the induction of apoptosis (Carson et al., 1988).

CdATP may substitute for dATP and together with cytochrome c binds to Apaf-1, triggering the formation of the apoptosome-complex and resulting in activation of caspase-3 and finally in DNA fragmentation (Leoni et al., 1998). The capacity of different NA triphosphates to induce apoptosis through the Apaf-1-mediated pathway in a cell-free system demonstrated that Fara-A and CdA were more efficient than Ara-G in triggering apoptosis (Genini et al., 2000b). Moreover, CdA has also been shown to induce apoptosis through the Fas/FasL pathway in the MOLT-4 leukemia cell line (Nomura et al., 2000).

AIF contributes to chromatin condensation and fragmentation upon incubation with CdA (Marzo et al., 2001). NAs also has a proven direct effect on mitochondria and their function (Hentosh & Tibudan, 1997). An extended study suggests that CdA and CAFdA, but not Fara-A, interfere directly with mitochondria, thereby disturbing the mitochondrial integrity (Genini et al., 2000a). For Ara-G an earlier study concluded that the incorporation of Ara-GTP into DNA is the critical event mediating the induction of apoptosis (Rodriguez & Gandhi, 1999).

MECHANISMS OF RESISTANCE

Chemotherapeutic treatment of human cancers can induce or select drug-resistant cancer cells. Drug-resistance involves cellular selection, populations with resistant cells surviving and expanding during repeated cycles of chemotherapy. The leukemic cells can either be initially resistant or acquire resistance after repeated cycles of chemotherapy treatment.

At the molecular level there appear to be a number of mechanisms through which drug resistance can be acquired, as shown in Figure 9. The resistance can be due to insufficient concentration of the active NA in the cells, as a result of deficient uptake or metabolism of the drug. Resistance may also be due to the inability to achieve sufficient alterations in DNA strands or dNTP pools, either due to altered affinity for DNA polymerases or by decreased inhibition of RR. Apoptosis is the ultimate fate for the cells in successful chemotherapy and defects in the induction of the apoptotic machinery inevitably lead to resistance. Recently, cross-resistance between NAs and anti-tumor agents that are substrates for the multi-drug resistance protein has been reported (Grant et al., 1995; Mansson et al., 2001).

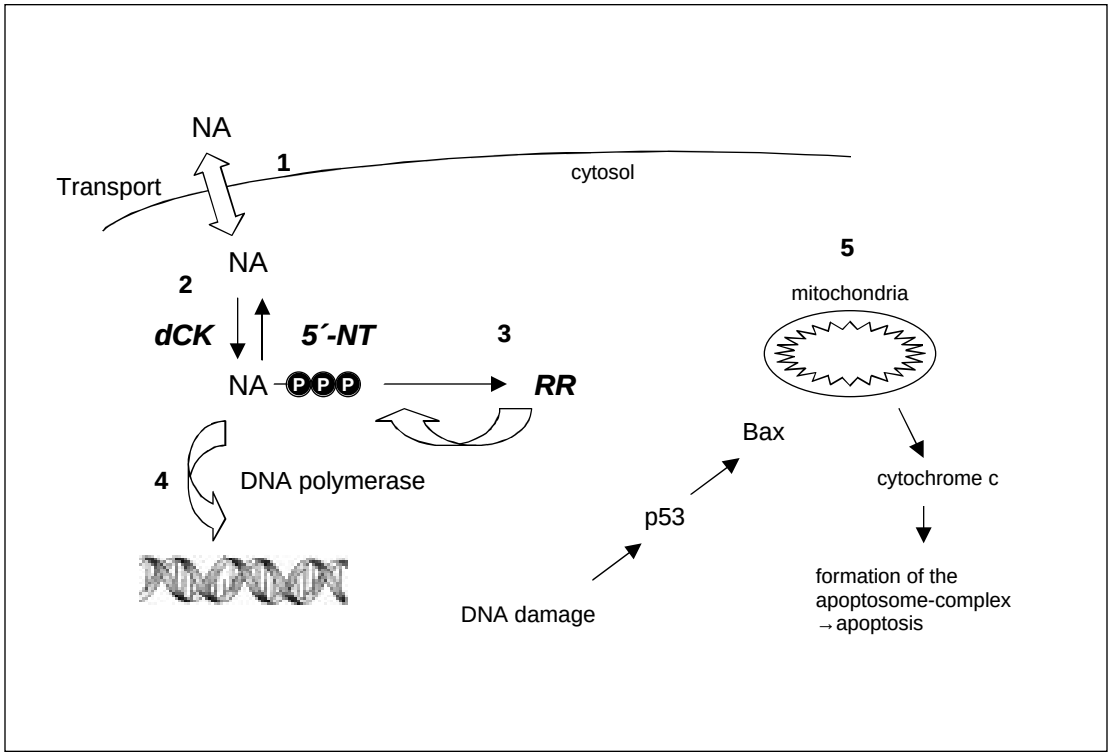

Figure 9. Possible mechanisms for resistance to nucleoside analogues. 1) Defect transport of NAs into the cell. 2) Deficiency of activating enzyme or upregulated deactivating enzyme. 3) Altered sensitivity of RR to the inhibitory effect of NAs. 4) Decreased inhibitory effect of NAs for DNA polymerases. 5) Defects in the induction of apoptosis.

Transporters

Deficiency in transport of NAs leads to insufficient concentrations of the drug and may cause resistance. The sensitivity to CdA, Fara-A and Ara-C correlated with the expression of hENT1 in blasts from acute leukemia patients (Gati et al., 1997; Gati et al., 1998). In AML and pediatric ALL patients a correlation between reduced nucleoside transport and poor response to Ara-C treatment has been demonstrated (Galmarini et al., 2002; Wright et al., 2002). ***In vitro*** studies indicated that resistance to Ara-C and CdA correlated with deficiency of nucleoside transport (Gati et al., 1997; Wright et al., 2002), but another study investigated cell lines resistant to CdA, Fara-A, Ara-C and dFdC that had no abberations in nucleoside transport (Dumontet et al., 1999) (Table 1). A recent study reported that the sensitivity to CdA was partially restored upon gene transfer of hCNT2 in resistant cells (Lang et al., 2001).

Table 1. Correlation of deficiency of nucleoside transport and resistance to nucleoside analogues in hematological malignancies and in different leukemic cell lines.

Drug	Cell line/Disease	Nucleoside transporter	Resistance	Reference
CdA, Fara-A, Ara-C	ALL, AML	hENT1	Yes	(Gati, 1998)
Ara-C	Childhood ALL	hENT1	Yes	(Wright, 2002)
	AML	hENT1	Yes	(Galmarini, 2002)
CdA, Fara-A, Ara-C, dFdC	K562	hENT1	No	(Dumontet, 1999)
Ara-C	CCRF-CEM	hENT1	Yes	(Gati, 1997)
CdA	CCRF-CEM	hENT1 (retention)	Yes	(Wright, 2002)
	CCRF-CEM (recombinant hCNT2)	hCNT2	Yes	(Lang, 2001)

Deoxycytidine kinase

Since dCK is essential in the activation of many NAs, acquired resistance to these substances has often been attributed to dCK deficiency. A list of studies conducted in cell lines and in patient samples is presented in Table 2. Several ***in vitro*** studies have shown that decreased gene expression, protein amount or dCK enzyme activity is important for resistance to NAs (Verhoef et al., 1981; Bhalla et al., 1984; Shewach & Mitchell, 1986; Stegmann et al., 1993b; Orr et al., 1995; Hapke et al., 1996; Bai et al., 1998; Dumontet et al., 1999; Curbo et al., 2001; Lotfi et al., 2002). Further evidence for the importance of dCK is the transfection of the dCK gene into dCK-deficient tumor cell lines, which restored the sensitivity to Ara-C and CdA (Hapke et al., 1996). A correlation between refractory patients and deficiency or low levels of dCK has been reported in patients with acute and chronic leukemias (Tattersall et al., 1974; Kawasaki et al., 1993; Arner et al., 1994; Stammler et al., 1997; Kakihara et al., 1998). However, other studies have concluded that there is no correlation between outcome of treatment and dCK levels (Leiby et al., 1987; Albertioni et al., 1998).

Table 2. Correlation of low dCK levels and resistance to nucleoside analogues in hematological malignancies and in different leukemic cell lines.

Drug	Cell line/Disease	Resistance	Reference
CdA	HCL/CLL	Yes	(Kawasaki, 1993)
	HCL/CLL	Yes	(Arnér, 1974)
	CLL	No	(Albertioni, 1998)
Fara-A	NHL	No	(Leiby, 1987)
Ara-C	AML	Yes	(Tattersall, 1974)
	Childhood ALL	Yes	(Kakihara, 1998)
	Childhood ALL	Yes	(Stammler, 1997)
CdA	W1L2 and L1210	Yes	(Orr, 1995)
Fara-A	JOK-1 and L1210	Yes	(Bai, 1998)
CdA, Fara-A, Ara-C	MCF-7, HT-29, H1437 (retroviral transfer)	Yes	(Hapke, 1996)
CdA, Fara-A, Ara-C, dFdC	K562	Yes	(Dumontet, 1999)
Ara-G	MOLT-4	Yes	(Schewach, 1986)
	MOLT-4	Yes	(Lotfi, 2002)
	CCRF-CEM	Yes	(Curbo, 2001)
Ara-C	CCRF-CEM	Yes	(Verhoef, 1981)
	HL60	Yes	(Bhalla, 1984)
	Rat AML model	Yes	(Stegmann, 1993)

The reason for the deficiency or down-regulation of dCK is still not known but one possibility is structural alteration of the dCK gene. Earlier studies have shown that structural alteration of the coding region of the dCK gene represents one possible resistance-mechanism for Ara-C in cell lines and in a small number of patient samples (Owens et al., 1992; Flasshove et al., 1994). Further studies of patients demonstrated that there are no mutations in the dCK gene in samples from relapsed patients or patients in remission (Stammler et al., 1997; Kakihara et al., 1998). A recent study reported a high incidence of alternatively spliced forms of dCK in AML patients resistant to Ara-C (Veuger et al., 2000). The hypothesis that the sliced variants were linked to resistance was further tested by transduction of the spliced variants, and was determined not to confer resistance (Veuger et al., 2002). One possible mechanism could be the DNA methylation of promotor regions which regulate gene expressions in mammalian cells. Treatment with demethylating agents induced re-expression in dCK deficient cells (Antonsson et al., 1987). Other studies found no DNA methylation in the areas upstream of the dCK gene, where the promotor is believed to be situated in a human cancer cell lines and AML patients (Leegwater et al., 1998; Dodge et al., 1998).

Deoxyguanosine kinase

Resistance to Ara-G is due, at least in part, to down-regulation of dGK (Lotfi et al., 2002). Not only the enzyme activity of dGK but also mRNA expression and protein levels were decreased in leukemic cells resistant to Ara-G (Lotfi

et al., 2002). The importance of dGK for resistance remains to be studied in patients.

5´-nucleotidase

5´-NTs are important enzymes in the metabolism of NAs, determining the amount of active drug in the cell, as presented in Table 3. It has been shown that CdA responders had significantly higher dCK levels and lower high K_m 5´-NT levels than non-responders in samples from patients with HCL or CLL (Kawasaki et al., 1993). An ***in vitro*** study reported that altered mRNA expression and specific activity of high K_m and low K_m 5´-NT correlated with resistance to CdA (Schirmer et al., 1998; Dumontet et al., 1999). High levels of high K_m 5´-NT is also an adverse prognostic factor in AML patients (Galmarini et al., 2001; Galmarini et al., 2002). Earlier studies have demonstrated that the response to other drugs, such as methotrexate and 6-mercaptopurine, correlates with the levels of ecto 5´-NT in pediatric ALL patients (Veerman et al., 1985; Pieters & Veerman, 1988). Nevertheless, overexpression of high K_m 5´-NT in human 293 cells did not increase the resistance to deoxyadenosine, deoxyguanosine or CdA (Gazziola et al., 1999; Rampazzo et al., 1999). A recent study demonstrated that expression of the low K_m 5´-NT, cN-1 in leukemic cell lines conferred resistance to CdA and dFdC (Hunsucker et al., 2001). Clearly, the relative involvement of the different 5´-NTs in clinical resistance to NAs remains to be studied.

Table 3. Correlation of high 5´-NT levels and resistance to nucleoside analogues in hematological malignancies and in different leukemic cell lines.

Drug	Cell line/Disease	5´-NT	Resistance	Reference
CdA	HCL/CLL	high Km 5'-NT	Yes	(Kawasaki, 1993)
Ara-C	AML	high Km 5'-NT	Yes	(Galmarini, 2001)
	AML	cytosolic 5'-NT	Yes	(Galmarini, 2002)
CdA	HL60	high Km 5'-NT	Yes	(Schirmer, 1998)
CdA, Fara-A, Ara-C, dFdC	K562	cytosolic 5'-NT	Yes	(Dumontet, 1999)
CdA	293 (recombinant high Km 5'-NT)	high Km 5'-NT	No	(Gazziola, 1999)
CdA, dFdC	Jurkat, HEK 293 (recombinant cN-I)	cN-I	Yes	(Hunsucker, 2001)

Ribonucleotide reductase

CdA, CAFdA and Fara-A target RR and as a result of this the dNTP pools are altered (Parker et al., 1988; Parker et al., 1991). Lack of the RR inhibitory effect or increased activity of RR may lead to resistance. RR may be insensitive to the allosteric regulation due to mutations or altered regulation of

the enzyme (Meuth & Green, 1974; Caras & Martin, 1988). Mutation in the allosteric activity site made cells resistant to the feedback control by dATP (Caras & Martin, 1988). Cells with acquired resistance to HU have a large increase in their RR activity, primarily caused by overproduction of the R2 subunit (containing the radical) (Akerblom et al., 1981; Wright et al., 1987). Large amounts of dNTP pools compete with nucleotide analogues for incorporation into DNA. As reported in Table 4, ***in vitro*** studies have shown that increased RR activity is an important determinator for resistance to the pyrimidine analogue Ara-C and dFdC (Meuth & Green, 1974; Dumontet et al., 1999; Goan et al., 1999), and that RR activity was slightly increased in a CdA and Fara-A resistant leukemic cell line (Dumontet et al., 1999).

Table 4. Correlation of high RR levels and resistance to nucleoside analogues in leukemic cell lines.

Drug	Cell line/Disease	Resistance	Reference
CdA, Fara-A, dFdC	K562	Yes	(Dumontet, 1999)
Ara-C	3T6	Yes	(Meuth, 1974)
dFdC	KB	Yes	(Goan, 1999)

The dCTP levels regulate dCK by feedback regulation, as high levels of dCTP can impair the cytotoxic effects of NAs (Ohno et al., 1988).

One rationale to overcome resistance caused by large amounts of dNTP pools is the use of inhibitors to RR, i.e. the radical scavengers HU or didox. HU has been used in combination with Ara-C on the human promyelocytic leukemia cell line HL60, resistant to Ara-C, this combination enhancing the cytotoxic effect of Ara-C (Bhalla et al., 1991).

Since CdA, CAFdA and Fara-A inhibit RR, the combination of one of these drugs with other NAs may increase the ratio of NA triphosphate to endogenous triphosphate. This would increase the DNA incorporation of NA. This strategy has been shown to be successful both ***in vitro*** and in patients (Gandhi & Plunkett, 1988; Gandhi et al., 1993; Gandhi et al., 1996; Rodriguez et al., 1997).

DNA polymerases

The NAs CdA, CAFdA and Fara-A inhibit DNA polymerase α, β, and γ to varying degrees (Parker et al., 1988; Parker et al., 1991). A possible mechanism of resistance to NAs could be the alteration of the binding site of the DNA polymerase, making it insensitive to the inhibitory effects of NAs. A decreased sensitivity of DNA polymerase to Ara-C and Fara-A was suggested to contribute to the resistance (TAnaka & Yoshida, 1982; Higashigawa et al., 1991).

Multidrug resistance

One cause of clinical resistance to many anti-cancer drugs is the overexpression of the multidrug resistance (MDR-1) gene, which encodes for the P-glycoprotein (P-gp) (Gottesman & Pastan, 1993; Ambudkar et al., 1999). This protein acts as an energy-dependent membrane efflux pump, since it has ATP-binding sites and structurally resembles bacterial transport proteins (Chen et al., 1986).

Multidrug resistance is resistance to various types of anti-cancer drugs such as vinca alkaloids, anthracyclines, epipodophyllotoxines and taxanes. Recent studies have shown that overexpression of P-gp or multi-drug resistance associated protein (MRP) may have a role in resistance to NAs. Cross-resistance to Ara-C has been observed in cells resistant to the anthracycline doxorubicin (Grant et al., 1995; Mansson et al., 2001). It has also been reported that Ara-C can upregulate MDR-1/P-gp expression in leukemic blasts (Hu et al., 1999), indicating that the P-gp upregulation may be a general response by leukemic cells to cytotoxic stress.

Apoptosis

NAs are believed to kill cells through apoptosis and defects in the onset of apoptosis may therefore cause resistance to the drugs. Since DNA damage caused by NA triphosphates is sensed by p53, mutations in the p53 gene may be one mechanism of resistance to chemotherapy (Wattel et al., 1994). The disturbance of mitochondrial integrity is suggested to be important for the onset of apoptosis by NAs (Marzo et al., 2001). Since the Bcl-2 family proteins are important regulators of mitochondrial integrity, dysregulation of these proteins might be involved in the mechanism of NA resistance. Indeed, Bax and Bcl-2 have been shown to correlate with clinical outcome in some studies (Zaja et al., 1998) but others failed to find a relationship (Thomas et

al., 1996; Morabito et al., 1997; Bromidge et al., 1998; Bosanquet et al., 2002).

THE PRESENT STUDY

AIMS

The aim of the present study was to study the mechanisms of action and especially the mechanism underlying resistance to the purine analogues CdA, Fara-A, Ara-G and the newly synthesized compound CAFdA.

Specific aims:

- To characterize the intracellular metabolism of CAFdA in human cell lines and in blood mononuclear cells isolated from patients with CLL and AML.

- To delineate the mechanism of resistance to CAFdA and compare it to that of CdA.

- To elucidate the mechanism of resistance to CdA and Fara-A in a myeloid cell line.

- To establish a real time quantitative PCR method to measure dCK, dGK and high K_m 5´-NT in cell lines and in samples from patients.

- To investigate the resistance mechanisms at the apoptotic level for CdA and Fara-A.

- To study the mechanisms of resistance to the purine analogues CdA, Fara-A and CAFdA. To study the strategy of reversal of Fara-A resistance in cell lines.

- To study the mechanism of resistance for Ara-G at the apoptotic level.

MATERIALS AND METHODS

Cell lines

In the studies presented in this thesis three different cell lines were utilized: the human myeloid leukemia cell line HL60 and the human lymphocytic cell lines CCRF-CEM and MOLT-4 (Table 5). Cells were cultured in RPMI medium (Gibco, Life Technologies Ltd), containing fetal calf serum (10%), penicillin (100 U/ml), streptomycin (100 µg/ml) and L-glutamine (2 mM) at 37°C with 5% CO_2. Cells were made resistant by repeated exposure to increasing concentrations of the respective anti-cancer drugs. Prior to experiments, cells were subcultured twice without drug and the cell number and cell size were determined by using a Coulter Multisizer apparatus (Coulter Electronics, Luton, UK).

Table 5. Parental cell lines and resistant sublines used in this study.

Parental cell line	Final concentration of selecting drug	Cell lines
Human acute T-lymphoblastic leukemia		CEM/wt
	25 nM, 100 nM, 1000 nM CdA	CEM/CdA
	150 nM, 1000 nM CAFdA	CEM/CAFdA
	1000 nM Fara-A	CEM/Fara-A
Human acute promyelocytic leukemia		HL60/wt
	150 nM CdA	HL60/CdA
	150 nM CAFdA	HL60/CAFdA
	2000 nM Fara-A	HL60/Fara-A
Human acute T-lymphoblastic leukemia		MOLT-4/wt
	500 nM Ara-G	MOLT-4/Ara-G 500
	900 nM Ara-G	MOLT-4/Ara-G 900

Patient samples

Blood from 14 CLL patients and 4 AML patients was collected in heparinized tubes. Bone marrow samples were obtained by aspiration from 32 childhood ALL patients and 3 childhood AML patients. Mononuclear cells were isolated by density gradient centrifugation with Lymphoprep (Nycomed, Oslo, Norway). Cells were kept in liquid nitrogen prior to RNA extraction. The studies were approved by the local ethics committee at Karolinska Hospital.

Chemosensitivity assay (MTT)

Cytotoxicity was measured using a tetrazolium salt based technique (MTT) (Mosmann, 1983). MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) is cleaved by mitochondrial dehydrogenases in living cells and is converted to formazan. Cells were incubated with 9-18 dilutions of the drug

and incubated at 37°C for 72 hours. MTT (5 mg/ml) was added and cells were further incubated for 4 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 IC_{50} values, the drug concentration used for 50% cell survival.

Determination of the triphosphate formation of CdA, CAFdA, Fara-A and Ara-C by HPLC

High Performance Liquid Chromatography methods were used to determine the amount of active metabolites of NAs in cells (Plunkett et al., 1980; Reichelova et al., 1996). Cells were preincubated with analogues for 1-2 hours. CdA and CAFdA 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. Fara-A and Ara-C nucleotides were separated using gradient elution and a Partisil-10 SAX column. The gradient solutions consisted of; Solvent A: 0.005 M $NH_4H_2PO_4$, pH 2.8 and solvent B: 0.75 M $NH_4H_2PO_4$, pH 3.5. The concentration of Ara-CTP was measured at 280 nm and CdATP, CAFdATP and Fara-ATP were measured at 265 nm.

Activities of kinases and nucleotidases

The kinases dCK, dGK, TK1 and TK2 and the nucleotidases high K_m 5´-NT were measured using radiolabelled substrates. For kinases, the cells were extracted, thawed and centrifuged. The supernatant was used as the source of protein for the kinase assays. The protein (2-3 µg) was assayed in a reaction mix containing 50 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 5 mM ATP, 4 mM dithiothreitol, 10 mM sodium fluoride and substrates. The concentration for each substrate used in the assays was approximately 10 times higher than the K_m value for the respective kinase. Prior to 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 K_m 5´-NT activity, inosine monophosphate was used as substrate as previously described (Spychala & Mitchell, 1994) and 3 mM ATP was added to activate the enzyme. Separation of inosine monophosphate and the products from dephosphorylation (inosine and hypoxhantine) were separated on a thin-layer chromatography plate (PEI-cellulose) and developed in 1-butanol:H_2O:methanol:NH_4 (60:20:20:1 vol/vol). The enzyme activity was expressed as pmol/million cells/min or pmol/mg cellular protein/min.

CDP reduction assay

Activity of RR was measured utilizing the CDP assay method earlier described by Steeper and Steuart (Steeper & Steuart, 1970). Cells were extracted with a hypotonic buffer and 200 µg protein was used for the reaction. CDP was used as substrate and is reduced to dCDP during the reaction. Dithiothreitol was used as reducing agent and ATP as effector. The reactions were boiled and dCMP was separated from CMP using a Dowex-50 column. The amount of CDP reduction was calculated from the radioactivity in the dCMP fraction and expressed as nM.

Amount of deoxyribonucleoside triphosphate pools

The intracellular amounts of dNTP pools were determined using a DNA-polymerase-based method (Sherman & 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 (Sherman & Fyfe, 1989). One of the four dNTPs was radiaolabelled and its degree of incorporation into newly synthetised DNA was detected. The elution of ^{3}H-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 also used. The dNTPs were extracted with 0.4 M perchloric acid and the ribonucleotides were removed by peroxidate 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.

Western blotting

The Western blot technique was used to determine protein levels of dCK, dGK, the R1 and R2 subunits, p53R2, cytochrome c, members of the Bcl-2 family, P-gp, multi-drug resistance associated protein (MRP 1-6) and topoisomerase II α and β. The protein mixtures were electrophoretically separated in SDS-polyacrylamide gels and transferred to polyvinylidene difluoride or a 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

The mRNA levels of various genes were determined using semi-quantitative PCR or real time quantitative PCR (RQ-PCR). For semi-quantitative PCR β2-microglobulin was used as internal control. The PCR product for the gene of interest was electrophoresed in 1.5% agarose gels and visualized as ethidium bromide fluorescent bands. For RQ-PCR two primers and in between them a dual labeled 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 fluourescence increases proportionally 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).

Cloning of cDNA standards

Plasmid cDNA standards for β2-microglobulin, dCK, dGK and 5´-NT were used for the standard curves for RQ-PCR. To construct clones containing these genes, they were amplified by PCR, purified and ligated into the pBluescript vector pMON (Borovkov & Rivkin, 1997). The vectors were then transformed into ***E.coli*** cells (***E.coli*** XL-1) by electroporation. Bacteria carrying the recombinant plasmids were screened for white colonies onLB agar plates containing ampicillin, X-gal and IPTG. To determine which plasmids contained the inserts of interest single colonies were PCR amplified and sized by electrophoresis. The plasmids were further identified by capillary DNA sequence analysis (ABI Prism 310 Genetic Analyzer, Applied Biosystems). Plasmids with the insert of interest were grown and purified and used as standards in RQ-PCR.

DNA fragmentation

DNA fragmentation was detected by propidium iodide staining and fluorescence –activated cell sorting (FACS) analysis. 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 (excited at 568 nm) by FACS analysis.

Caspase activities

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 their preferred substrate and the fluorochrome AMC is liberated and fluorescence is emitted. Cells were added to a microtiterplate and lysed and thereafter diluted in the reaction buffer containing the respective substrate (50

µM). 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.

Cytochrome c release

To investigate the release of cytochrome c from mitochondra upon treatment with CdA, cells were extracted and fractionated into cytosolic and mitochondrial fractions. Briefly, cells were lysed in a STE-buffer containing sucrose, Tris and ethylenediaminetetraacetic acid and were then centrifuged. The supernatant (cytosolic fraction) was subjected to immunoblotting for cytochrome c. The cytosolic fraction was also used as a cell-free model for caspase activation. To determine the capability of cells to activate caspases the cytosols were incubated in the presence of 1 mM dATP or 1 mM CdATP and 40 nM cytochrome c. Then caspase-3 activity was measured as described above.

Mitochondrial transmembrane potential ($\Delta\Psi_{mito}$)

Changes in $\Delta\Psi_{mito}$ were quantified using the potential-sensitive fluorochrome tetramethylrhodamine ethyl ester (TMRE). TMRE is a cell permeable fluorophore that loses its fluorescence upon dissipation of the mitochondrial inner membrane potential. Cells were incubated with 25 nM TMRE for 30 min at 37°C. Cells were analyzed using FACS (FL-2 channel).

$Ca2^+$

Changes in intracellular Ca^{2+} concentration were analyzed using the Indo-1 AM (1H-Indole-6-carboxylic acid, 2-[4-[bis[2-[(acetyloxy)methoxy]-2-oxoethyl]amino]-3- [2-[2-[bis[2-[(acetyloxy)methoxy]-2-oxoetyl]amino]-5-methylphenoxy]ethoxy]phenyl]-, (acetyloxy)methyl ester) fluorophore. Cells were incubated with 1 µM Indo-1 AM for 30 min at 37°C, washed and resuspended in Ca^{2+}-free PBS and subjected to FACS analysis (FL-4 channel). The amount of Ca^{2+} accumulated in the mitochondria needed to induce MPT was monitored using a Ca^{2+}-sensitive electrode. Briefly, cells were washed in Ca^{2+}-free PBS, resuspended in a buffer containing 0.15 M KCl, 5 mM KH_2P0_4, 1 mM $MgSO_4$, 5 mM succinate, 5 mM Tris pH 7.4 and permeabilized with digitonin. The ability of the mitochondria to accumulate Ca^{2+} was determined by sequential additions of Ca^{2+} until the accumulated Ca^{2+} was released upon induction of MPT. The Ca^{2+} fluxes were monitored with the Ca^{2+}-sensitive electrode.

Spectral karyotyping (SKY)

The principle of SKY is the use of combinations of chromosome painting probes, as previously described (Schrock et al., 1996). One fluorochrome or a combination of different fluorochromes is used to identify each chromosome with a unique colour. The image of the emission spectra for the chromosomes is captured with a CCD camera and the display colours are obtained. Then the classification colours are applied to the chromosomes. Briefly, slides with metaphase spreads were freshly prepared and fixed prior to hybridization. The 24 differentially labeled chromosome-specific painting probes (SKY kit from Applied Spectral Imaging (ASI) and Cot-1 DNA, were denatured and hybridized to denatured tumor metaphase chromosomes according to the protocol recommended by ASI.

Comparative genomic hybridization (CGH)

Using CGH the entire genome of the tumor cell is compared to a reference DNA and gene amplifications or deletions are detected (Kallioniemi et al., 1992). The tumor DNA and the reference DNA were labelled with fluorochromes with different colours (green: fluorescein isothiocyanate-deoxy-UTP and red: Texas Red) by nick translation. The slides were hybridized in equal amounts to a normal metaphase slide. The amount of genetic material from tumor cells in relation to the reference DNA will be reflected by the intensity of the green versus red colour along the chromosomal regions.

Statistical analysis

Statistics were calculated using StatView software. The data were expressed as mean and standard deviation. A p-value of ≤ 0.05 was considered as statistically significant.

RESULTS AND DISCUSSION

Study I

Biochemical pharmacology and resistance to 2-chloro-2-arabino-fluoro-2-deoxyadenosine, a novel analogue of cladribine in human leukemic cells.

The aim of the study was to compare the cellular metabolism of CAFdA to that of the structurally similar NA CdA. We studied this in human leukemic cells and peripheral leukemic cells isolated from CLL and AML patients.

Incubation of cells isolated from the blood of CLL and AML patients with various concentrations of CdA or CAFdA led to accumulation of CdA and CAFdA nucleotides in a dose-dependent manner (Figure 10). A significantly higher rate of phosphorylation to monophosphates was observed for CAFdA than for CdA in cells from CLL patients (n=14, P=0.04). The differences in the phosphorylation were even more pronounced for the respective triphosphates in both CLL (n=14, P=0.001) and AML (n=4, P=0.04) cells. The retention time for CAFdA 5´-triphosphate was longer than that for CdA 5´-triphosphate in cells from leukemic patients. Using recombinant dCK, CAFdA was also shown to be a better substrate for dCK than CdA and the natural substrate deoxycytidine.

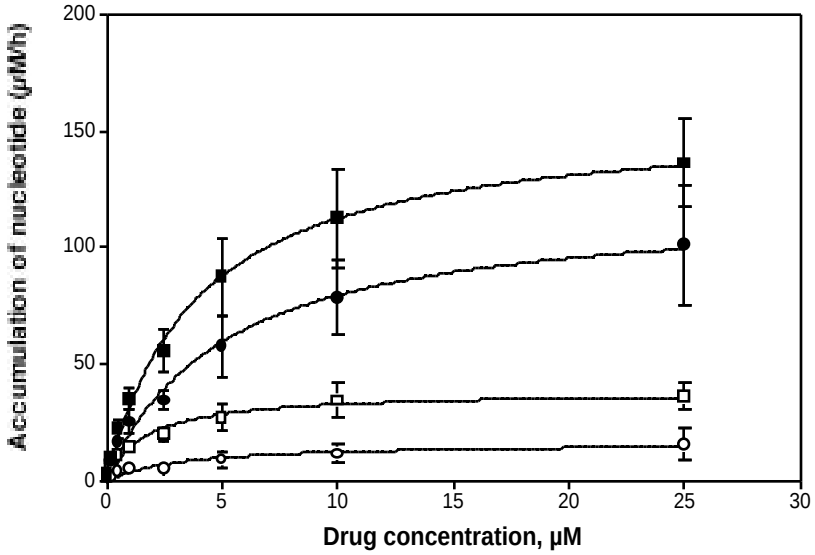

Figure 10. Accumulation of CdAMP(●), CdATP(❍), CAFdAMP(■) and CAFdATP(□) in samples from patients with CLL (n=14) patients. Peripheral

blood leukemic cells were incubated with the indicated concentrations of CAFdA and CdA for 2 hours. CAFdAMP and CAFdATP was accumulated to a higher extent in the patient samples than were CdAMP and CdATP.

One human lymphoid (CCRF-CEM) cell line and one myeloid (HL60) cell line were selected for resistance to CAFdA. The two resistant clones were characterized with respect to growth inhibition, cross-resistance to other anti-metabolites and levels of the activating enzyme dCK were investigated. We found that resistance was correlated with a lower accumulation of CAFdA nucleotides and reduced dCK activity for the CAFdA resistant cell lines.

Taken together, we demonstrated that CAFdA is intracellularly accumulated to a higher extent than is CdA, due to that CAFdA is more efficiently phosphorylated by dCK and eliminated more slowly than CdA. Based on these data one would expect that CAFdA is a more potent drug for hematological malignancies than CdA. We also conclude that resistance to CAFdA was due to lack of dCK activity.

Study II

Molecular and biochemical mechanisms of fludarabine and cladribine resistance in a human promyelocytic cell line.

The aim of this study was to elucidate the resistance mechanisms for CdA and Fara-A. Earlier studies have shown responses to CdA in CLL patients resistant to fludarabine. (Juliusson et al., 1992; Rondelli et al., 1997). In order to investigate this, we developed the myeloid cell line HL60 resistant to CdA and Fara-A, respectively, as models of acquired resistance.

Both CdA and Fara-A resistant cells displayed cross-resistance to other anti-metabolites CAFdA, Ara-C and dFdC activated by dCK. The CdA resistant cell line had a much higher degree of cross-resistance to the analogues than did the Fara-A resistant cell line. Consistent with this, the formation of CdA and Fara-A nucleotides was very low for CdA resistant cells and decreased approximately 50% for Fara-A resistant cells. Further characterization revealed that dCK activity was decreased to 10% for CdA resistant cells and to 60% for Fara-A resistant cells compared to the wild type (Figure 11).

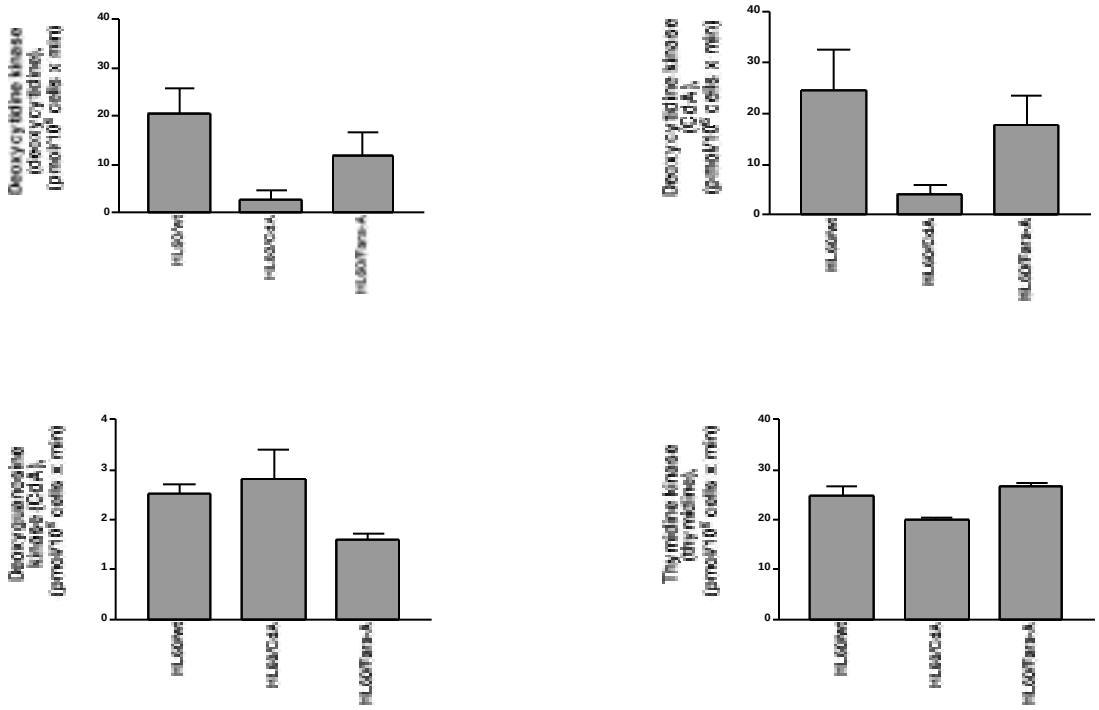

Figure 11. Enzyme activities; dCK was measured with deoxycytidine and CdA as substrates. The dGK activity was measured using CdA as substrate and an excess of deoxycytidine and TK1 was measured using thymidine as substrate.

The dGK and TK1 activities did not differ considerably in any of the cell lines. Western blot analysis revealed no detectable dCK protein in CdA resistant cells, while in Fara-A resistant cells dCK was expressed at the same level as in the parental cells. dCK mRNA levels were similar to enzyme activity and protein levels. The high K_m 5´-NT mRNA levels were not significantly elevated in the resistant cells.

RR maintains a balanced supply of dNTP pools in the cell and may also be a major cellular target for CdA and Fara-A nucleotides. The dATP and dGTP pools were increased more than 7-fold, while TTP pools were unchanged and the dCTP pool was decreased 4-fold or more in Fara-A resistant cells compared to the parental cell line (Figure 12).

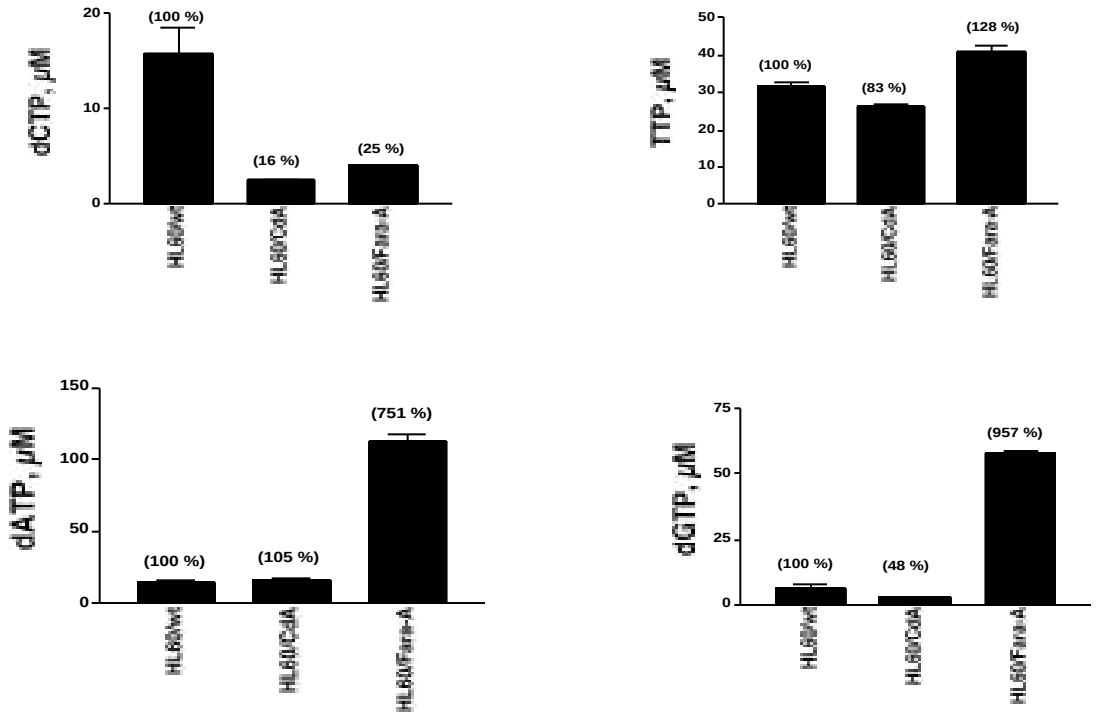

Figure 12. dNTP pools for parental HL60 cell lines, CdA and Fara-A resistant sublines. The dCTP, TTP, dATP and dGTP pools were measured using a DNA-polymerase based method.

There were no indications for alterations in nucleoside transport and there were no difference in toxicity of multidrug resistance-related drugs such as daunorubicine, vincristine and paclitaxel. However, there was a low degree of cross-resistance to the topoisomerase interactive agent etoposide, the reason for this being unknown, but this cross-resistance has been reported earlier (Lotfi et al., 2001).

In conclusion, CdA resistance is mainly due to dCK deficiency. The Fara-A resistant cells might have another contributing resistance factor other than dCK deficiency. Since the Fara-A resistant cells had larger amounts of dATP and dGTP pools, the regulation of RR may be altered. The large amount of dATP pools for the Fara-A resistant cells may also compete with Fara-A for incorporation into DNA, contributing to resistance.

Study III

Real-time quantitative PCR assays for deoxycytidine kinase, deoxyguanosine kinase and 5´-nucleotidase mRNA measurement in cell lines and in patients with leukemia.

The objective of the study was to develop a quantitative method to rapidly and from small amounts of sample, measure mRNA expression of enzymes important for the metabolism of NAs . We established a real time quantitative method to measure dCK, dGK and high K_m 5-NT. Techniques which have earlier been employed for analysis of these enzymes are enzyme activity measurements, immunoblotting, Northern blot and semi-quantitative PCR. One disadvantage with enzyme activity measurements for dCK and dGK is a problem with overlapping substrate specificity between enzymes. RT-PCR allows more accurate quantitative determination, but the method requires post-PCR handling and the quantification of the intensity of bands is performed manually.

The real time PCR method is based on cleaveage of the Taqman probe by the exonuclease activity of the Taq polymerase, separating the fluorogenic reporter dye from the quencher. The fluorescence increases proportionally to the amount of PCR product. For an absolute quantification a standard curve is needed, cDNA standards being constructed for each gene by cloning the gene into a vector which was then amplified in an ***E.coli*** system. A serial dilution of the dCK cDNA standards gives a plot of total fluorescence versus the cycle number (Figure 13). A standard curve is made from the amplification plot, with the cycle number plotted against the copy number.

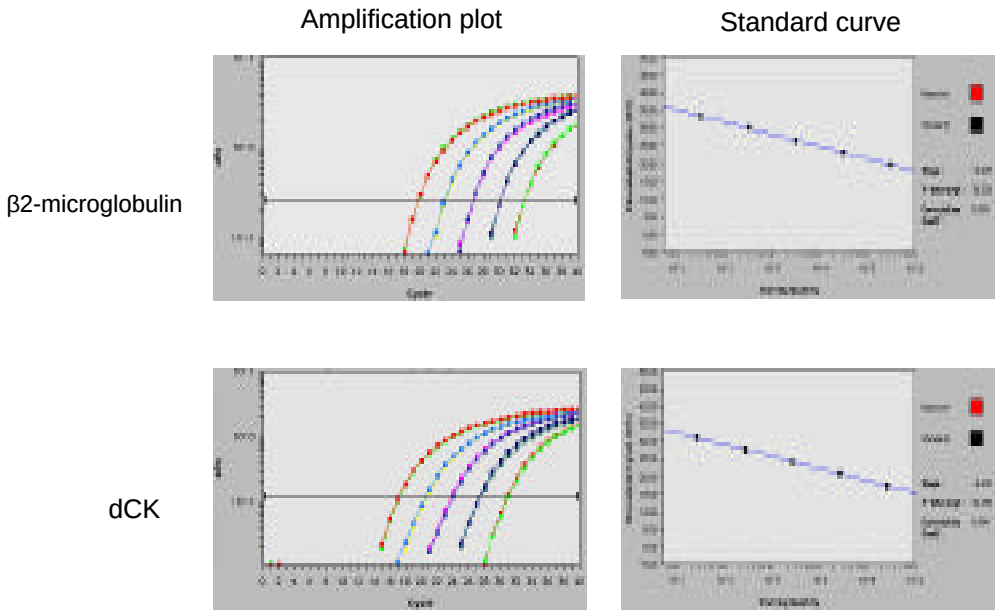

Figure 13. Amplification plot and standard curve for β2-microglobulin and dCK from a serial dilution of the cDNA standards. The amplification curve shifts to the right as the input amount of template decreases. The dynamic range was wide with dilutions from 10 to 10^5 copies.

A MOLT-4 wild type and its Ara-G resistant subline was used for the methods comparison. We determined that the levels of dCK and high K_m 5´-NT had similar levels as determined by RQ-PCR, semi-quantitative PCR and enzyme activity measurements. The mRNA levels of dGK using RQ-PCR and semi-quantitative PCR yielded similar results. However, the enzyme measurements showed a higher degree of dGK reduction compared to the mRNA expression. The between-run coefficients of variation of the ratio of the copy number for dCK, high K_m 5´-NT and β2-microglobulin were less than 11% and for dGK it was less than 22%. The mRNA expression for dCK, dGK and high K_m 5´-NT in pediatric ALL and AML patients showed a large interindividual variability from 0.06 to 2.34, non-detectable to 0.06 and 0.04 to 0.30, respectively (expressed as the ratio of the respective gene and the reference gene).

The present method is a valuable tool in the determination of dCK, dGK and high K_m 5´-NT mRNA gene expressions in cell lines and in clinical samples. This convenient and specific method is a tool in the effort to identify genetic markers serving as prognostic factors to optimize the treatment of hematological malignancies with NAs.

Study IV

Resistance of leukemic cells to 2-chlorodeoxyadenosine is due to a lack of calcium-dependent cytochrome c release.

Recent reports have indicated that the triphosphate form of CdA can substitute for dATP in binding to Apaf-1 together with cytochrome c and induce apoptosis (Leoni et al., 1998; Genini et al., 2000b). The purpose of this study was to delineate possible defects in the induction of apoptosis in lymphoblastic CEM leukemia cells resistant to CdA. Three cell lines resistant to CdA were included in the study, with varying levels of dCK activity and accumulation of nucleotides. All CdA resistant sublines showed absence of DNA-fragmentation, caspase-3 and caspase-9 activity after treatment with CdA. We demonstrated that when CdATP and cytochrome c were added to cell-free extracts, caspase-3 activity increased in all cell lines. This suggests that the resistant cell lines are capable of activating caspases (Figure 14).

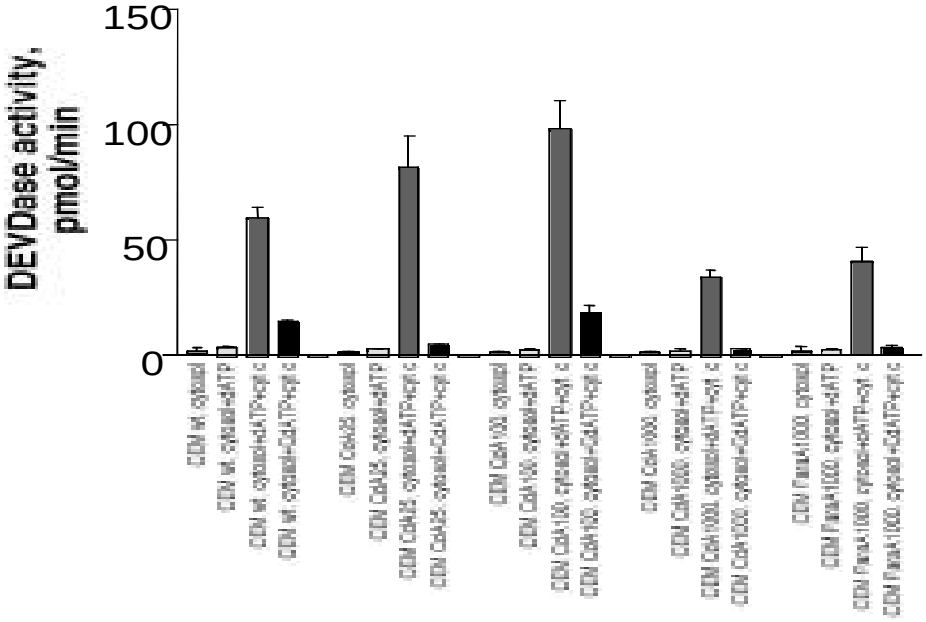

Figure 14. DEVDase activity in cell-free extracts in the CCRF-CEM parental cells, CdA25, CdA100, CdA1000 and Fara-A resistant sublines. The cytosolic extract is assayed with addition of dATP, dATP and cytochrome c and CdATP and cytochrome c. The resistant cell lines were capable of inducing apoptosis.

The drop of the $\Delta\Psi_{mito}$ and subsequent cytochrome c release into the cytosol are two events preceding apoptosis. We did not detect either of these events in the resistant cells after CdA treatment. Conversely, the parental cell line was positive for both the drop in $\Delta\Psi_{mito}$ and cytochrome c release after incubation with CdA.

Bcl-2 proteins act as regulators of mitochondrial integrity. We did not record upregulation of the anti-apoptotic Bcl-2 proteins, Bcl-2 or Bcl-xL or downregulation of the pro-apoptotic Bcl-2 proteins, Bax, Bak, Bid or Bad in the resistant cell lines.

Mitochondria protect the cell against excessive Ca^{2+} levels through their buffering capacity. Increased Ca^{2+} levels may induce changes of $\Delta\Psi_{mito}$. We observed that larger amounts of Ca^{2+} were required to alter the $\Delta\Psi_{mito}$ in the resistant cells. We also found that the DNA-fragmentation and caspase-3 activity induced upon CdA treatment was inhibited by BAPTA-AM, which is a Ca^{2+} chelator. Thapsigargin releases stores of Ca^{2+} from the endoplasmic reticulum. Upon treatment with thapsigargin the parental cells underwent apoptosis but the resistant cells did not exhibit an increase in DNA-fragmentation or caspase-3 activity (Figure 15).

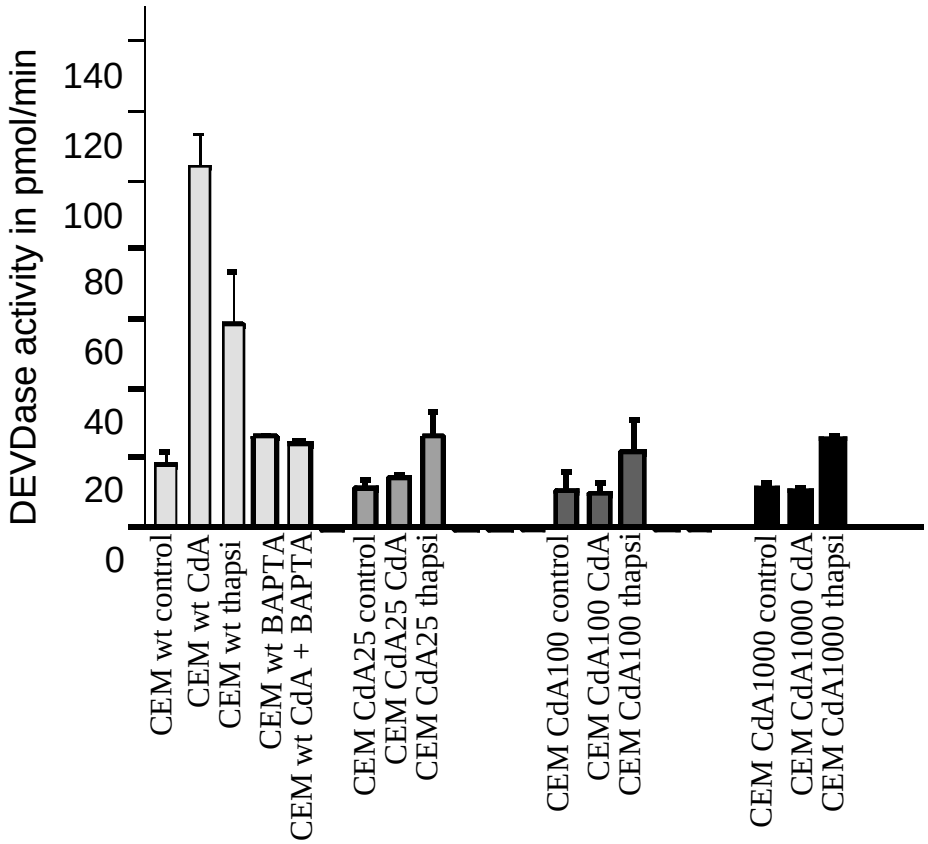

Figure 15. DEVDase activity upon incubation of parental and resistant CCRF-CEM cells with the Ca^{2+} chelator BAPTA-AM and thapsigargin, that releases stores of Ca^{2+} from the endoplasmic reticulum. Cells were treated with 1 µM thapsigargin or 1 µM CdA for 12 hours prior to analysis of caspase-3 activity. Pre-incubation with 50 µM BAPTA-AM abrogated the CdA-induced caspase-3 activity.

The possibility that resistance was also connected to the Fas-mediated pathway was investigated by incubation of cells with anti-Fas monoclonal antibody. This induced apoptosis in both parental and the resistant cells.

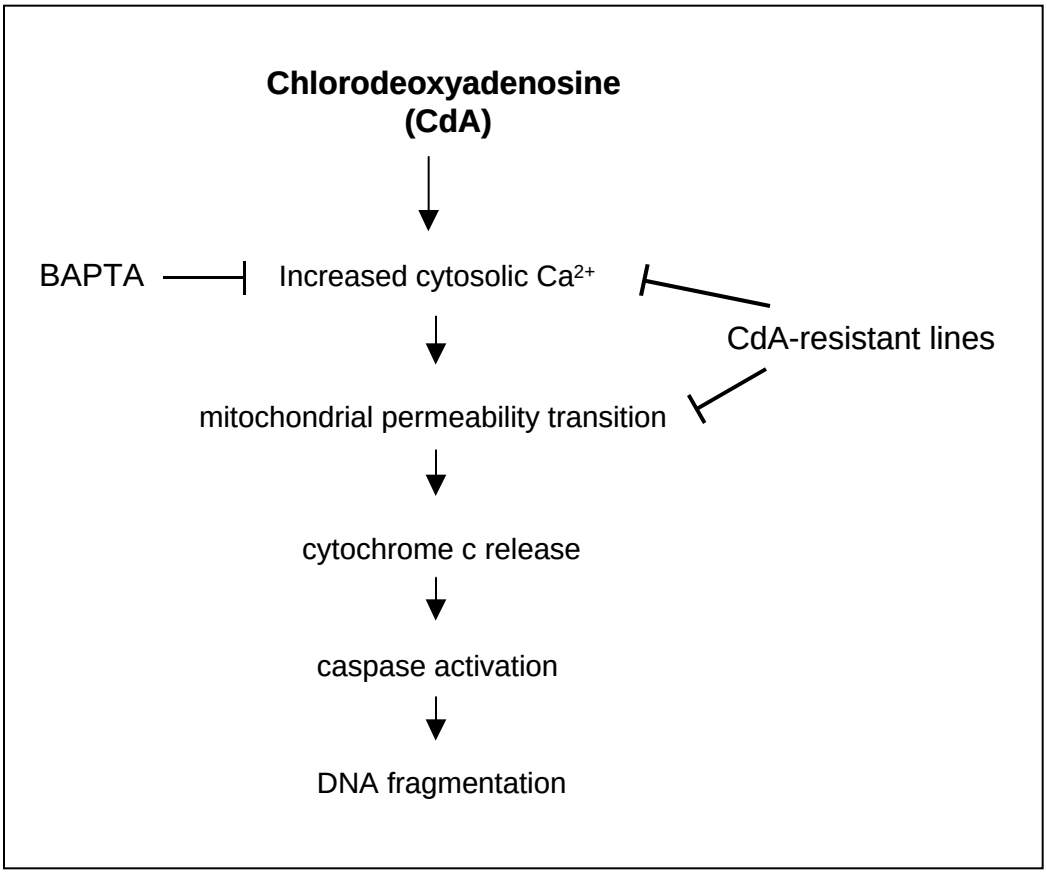

Figure 16. Suggested mechanism for CdA-induced apoptosis. The CdA resistant cell lines are characterized by a lack of caspase activation, cytochrome c release and a drop in the $\Delta\Psi_{mito}$. These events are preceded by increased cytosolic Ca^{2+} levels. Treatment with CdA causes a decreased Ca^{2+} buffering capacity in the CEM wild type cells but not in any of the resistant cells. The Ca^{2+} chelator, BAPTA-AM blocks CdA-induced caspase-3 activity and DNA fragmentation in the CEM wild type cells. Thapsigargin releases Ca^{2+} stores from the endoplasmic reticulum induces caspase activation and DNA fragmentation in wild type cells but not in the resistant cells. These results suggests that mitochondria may be a target for Ca^{2+}-dependent apoptotic events initiated by CdA.

We conclude that the increase of Ca^{2+} levels in the cytosol contributes to CdA-induced apoptosis. Resistance to CdA is dependent on altered sensitivity to the Ca^{2+} levels, which are essential for the induction of mitochondrial changes that ultimately lead to apoptosis.

Study V

Downregulation of deoxycytidine kinase in human leukemic cell lines resistant to cladribine and clofarabine and increased ribonucleotide reductase activity contributes to fludarabine resistance.

The chemical structures of the NAs CdA, CAFdA and Fara-A are very similar. The purpose of this study was to investigate the mechanisms of acquired resistance to CdA and Fara-A, and to compare them to that of the recently developed analogue CAFdA, in a lymphoid leukemia cell line.

The three resistant cell lines displayed cross-resistance to other anti-metabolites phosphorylated by dCK. An exception was the Fara-A resistant cell line that did not show cross-resistance to CAFdA, Ara-G or difluorodeoxyguanosine. No cross-resistance to tubercidine or daunorubicine was detected, indicating that resistance was not correlated to nucleoside transport or multi-drug resistance. The CdA and CAFdA resistant cell lines did not form detectable levels of CdATP, CAFdATP, Ara-CTP or Fara-ATP. For these cell lines we detected a deficiency of dCK at the level of enzymatic activity, protein level and mRNA expression (Figure 17). Also important for the amount of active drug in the cell is the dephosphorylating enzyme high K_m 5´-NT, and the three resistant cell lines expresed approximately 2-fold elevated levels of high K_m 5´-NT activity.

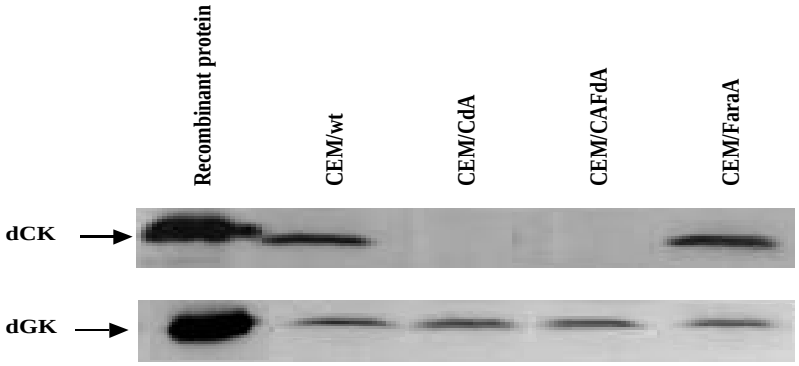

Figure 17. Protein levels of dCK and dGK for the wild type cell lines and the resistant sublines. Recombinant dCK and dGK was used as control.

As for the Fara-A resistant cell lines, nucleotides were only formed to a somewhat lower extent than in the parental cell line and dCK activity was not significantly lowered. Protein levels of R1 were not changed in the resistant

cell lines. The amount of the small subunit R2 of RR was higher in the Fara-A resistant cells, which translated into a higher RR activity as compared to the parental cells (Figure 18). The R2 levels of the CAFdA resistant cells were very low, whereupon the question arises of how the cells are supplied with dNTP pools since dCK was also downregulated. The recently identified RR subunit, p53R2, which is regulated by p53 was analyzed by Western blotting. The CAFdA resistant cell line showed no expression of p53R2 full-size protein. A protein of lower molecular weight was also detected by the p53R2 antibody, which was highly expressed in these cells. As a strategy to overcome resistance caused by altered activity of RR, the use of RR inhibitors has been successfully used. Co-incubation of Fara-A with the RR inhibitor didox enhanced cytotoxicity to the Fara-A resistant cells by a factor of 20.

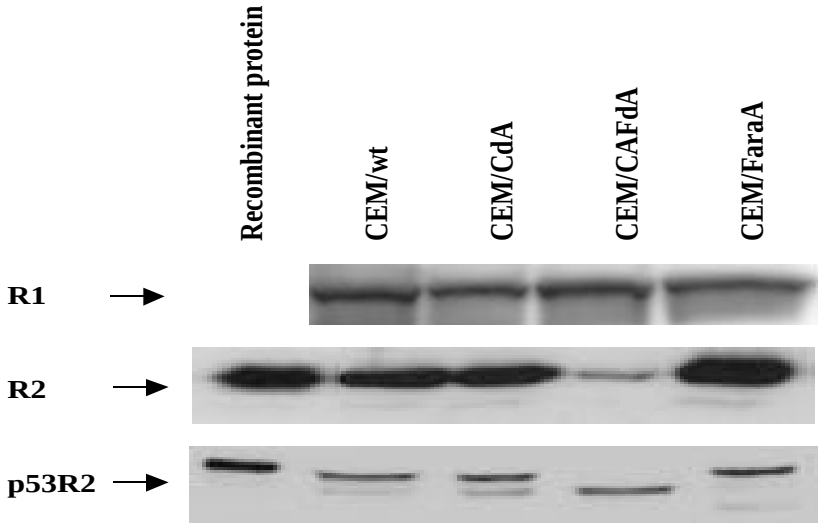

Figure 18. Protein levels of R1, R2 and p53R2. Demonstrating similar levels for R1. The R2 subunit was increased in the CEM/Fara-A and was much lower in the CEM/CAFdA, compared to wild type. The CAFdA resistant cell line had no detectable p53R2 band, but a shorter protein was also detected by the antibody, and was highly expressed in these cells.

Using SKY- and CGH analysis, the structural and numerical instability of the chromosomes were studied. Exposure of the cells to the NAs apparently induced chromosomal aberrations, not detected in the parental cell line, the most profound changes being apparent for the CAFdA cell line.

In summary, CdA and CAFdA resistance is due to the deficiency of dCK. Our results suggest that dCK deficiency does not play a major role in resistance to

Fara-A, for these cells the increased activity of RR contributing to resistance. Fara-A resistance was reversed by a combination of didox with Fara-A, probably because of inhibition of RR. Since no dCK activity and low RR activity were evident in the CAFdA resistant cells, we suggest that p53R2 may play a role in the synthesis of precursors for DNA synthesis and repair in these cells.

Study VI

Resistance to mitochondrial- and Fas-mediated apoptosis in human leukemic cells with acquired resistance to 9-β-D-arabinofuranosylguanosine.

The purpose of this study was to elucidate whether apoptotic pathways were affected in human T-lymphoblastic MOLT-4 cell lines with acquired resistance to Ara-G. We have previously shown that Ara-G resistant cell lines possess low levels of dGK and slightly lowered dCK levels (Lotfi et al., 2002).

In the parental cell line, Ara-G (100 µM) treatment for 36 hours resulted in both caspase-3 activity and DNA fragmentation. The Ara-G resistant cells showed no increased caspase-3 activity or DNA-fragmentation upon the same treatment. Other apoptotic changes such as activation of caspase-9, dissipation of $\Delta\Psi_{mito}$ and release of cytochrome c were also observed in the MOLT-4 wild type cells upon Ara-G treatment. These changes were not detected for the Ara-G resistant sublines.

A drop in the $\Delta\Psi_{mito}$ was observed in the wild type cells after treatment with tributyltin (TBT), an inducer of mitochondrial permeability transition (Stridh et al., 1999a; Stridh et al., 1999b), and carbonyl cyanide ***m***-chlorophenylhydrazone (CCCP), an uncoupling agent that reduces the $\Delta\Psi_{mito}$ (Figure 19). However, $\Delta\Psi_{mito}$ was not affected by these agents in the Ara-G resistant cells.

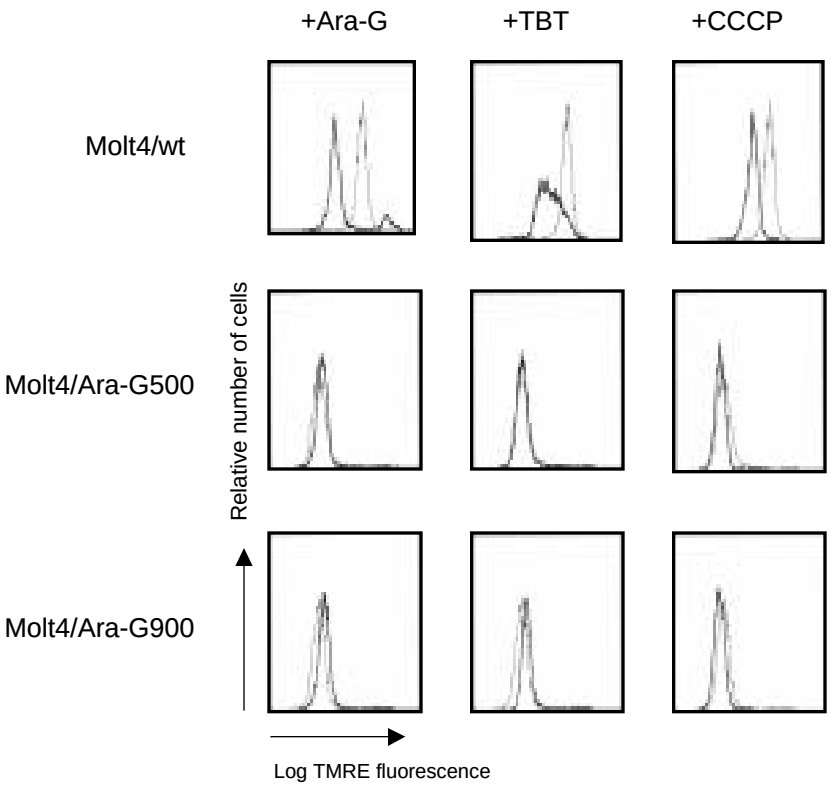

Figure 19. Effect on $\Delta\Psi_{mito}$ after treatment with Ara-G alone, TBT or CCCP. The MOLT-4 cell lines were incubated in the presence of 100 µM Ara-G for 36 hours. The membrane potential was measured by staining cells with the mitochondrial probe TMRE for 20 minutes followed by flow cytometry analysis.

To further characterize the mechanism of apoptosis-resistance in Ara-G resistant cells, we studied the protein expression of Bcl-2 and Bcl-xL by western blotting. There was no difference in the Bcl-2 levels between wild type and Ara-G resistant cells but an increased expression of Bcl-xL was observed in Ara-G resistant cells (Figure 20) suggesting that the mechanism of apoptosis-resistance may be located at the mitochondrial level.

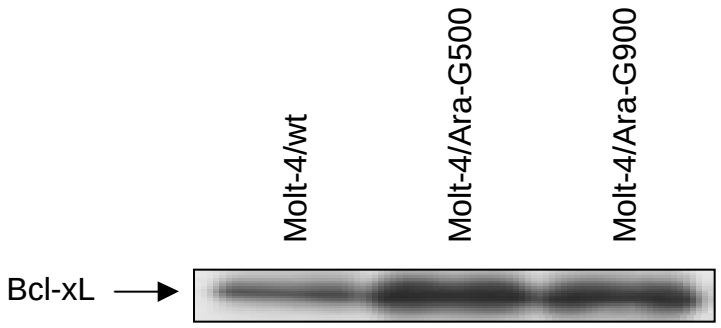

Figure 20. Immunoblotting of the anti-apoptotic Bcl-2 family protein Bcl-xL in wild type and Ara-G resistant cell lines.

To further investigate the induction of apoptosis, cells were treated with anti-Fas antibody. The Ara-G resistant cells did not exhibit caspase-3-like activity but the wild type cells did. Semi-quantitative PCR revealed that there was no detectable expression of Fas in the resistant cells. The reduced Fas mRNA expression was further confirmed by the protein analysis of Fas, analyzed by flow cytometry. The MFI for the Fas-resistant cells were 2.2 for MOLT-4/Ara-G500 and 4.3 for MOLT-4/Ara-G900 as compared to 13.0 for the wild type.

In conclusion, this study suggests that the Fas receptor pathway and mitochondria are involved in Ara-G-induced apoptosis. The $\Delta\Psi_{mito}$ could not be reduced by either TBT or CCCP in Ara-G resistant cells, suggesting that the mechanism for resistance is located at the mitochondrial level. In conclusion, we show that the mechansim of resistance to Ara-G correlates with both the lack of Fas and increased levels of Bcl-xL.

CONCLUSIONS

The focus of these studies was understanding the mechanism of resistance to CdA, CAFdA, Fara-A and Ara-G.

Some general conclusions from this thesis are drawn:

- The CAFdA nucleotides were accumulated to a higher extent than CdA nucleotides in samples from CLL and AML patients. This is due to slower elimination of CAFdA nucleotides and more efficient phosphorylation of CAFdA by anabolic enzymes. Acquired resistance to CAFdA in leukemic cell lines is caused by dCK deficiency.

- The mechanism for resistance to CdA and Fara-A differ in the myeloid HL60 leukemia cell line. Resistance to CdA is caused by dCK deficiency. Fara-A resistance is due to somewhat lower dCK activity and larger amounts of dNTP pools.

- A real time quantitative PCR method was established in order to quantitatively measure the mRNA expression of dCK, dGK and high K_m 5´-NT. The RQ-PCR is a specific and convenient method to measure gene expressions in leukemic cell lines and in samples from patients with hematological malignancies.

- Resistance to CdA is caused by changes in Ca^{2+}-sensitive mitochondrial changes. An elevated level of Ca^{2+} contributes to CdA-induced apoptosis.

- CdA, CAFdA and Fara-A are NAs with very similar chemical structures. Despite this they have different patterns of resistance. Acquired resistance to CdA and CAFdA in CCRF-CEM cells is due to dCK deficiency. Altered activity of RR contributes to Fara-A-resistance.

- Resistance to Ara-G is associated with perturbations in apoptotic events. Beyond the downregulation of dGK activity, the resistance to Ara-G corresponds with an upregulation of Bcl-xL and downregulation of Fas.

FUTURE PERSPECTIVES

NAs are pro-drugs that need to be activated by phosphorylation in order to have their effects. The importance of the levels of the activating and deactivating enzymes dCK and 5´-NTs has been shown in several earlier studies (Tattersall et al., 1974; Stammler et al., 1997; Galmarini et al., 2001). In the clinical setting, however, there are other factors which contribute to the response to treatment with NAs. More important than dCK or 5´-NTs to consider separately, might be the ratio of dCK/5´-NT (Kawasaki et al., 1993). There are several 5´-NTs and it remains to be determined which one has the largest importance for clinical resistance to NAs. After the first phosphorylation step NAs are further phosphorylated by NMPK and NDPK. Cellular metabolism studies showed that CdAMP was the major metabolite, accumulating to a higher extent than CdATP (Avery et al., 1989). In light of this, there is a needs to further analyse NMPK. The role of these two enzymes in resistance to NAs is not presently known and need to be further investigated.

The mitochondrial enzyme dGK also phosphorylates NAs and is able to redistribute into the cytosol during the early phase of apoptosis (Jullig & Eriksson, 2001). This may be of importance for the activation of co-factors for induction of apoptosis such as dATP or triphosphates of NAs. The role of dGK in response to treatment with NAs and whether inactivation of dGK is involved in resistance to NAs are not yet clarified.

CdA induces apoptosis via several pathways, via the mitochondrial and Apaf-1 pathway and via the Fas/FasL pathway (Leoni et al., 1998; Nomura et al., 2000; Marzo et al., 2001). Future projects should involve the investigation of the relative importance of different ways for induction of apoptosis, and also whether clinical resistance to NAs is connected to defects in the induction of apoptosis.

In order to overcome resistance to NAs, a number of strategies have been utilized and studied. One is the inhibition of RR, CdA, Fara-A and RR inhibitors such as HU and didox, which have been used and shown to have effect (Howell et al., 1982; Schilsky et al., 1987). The use of DNA damaging agents to start DNA repair and the opportunity for the NA to be inserted into DNA has been shown to be therapeutically effective in CLL patients (Robertson et al., 1995; Keating, 1997; Van Den Neste et al.,

1999). The use of new NAs with different mechanisms of action and resistance may also be a strategy to overcome resistance.

ACKNOWLEDGEMENTS

This work was carried out at the Department of Medicine, Division of Clinical Pharmacology, at the Karolinska Hospital. I wish to express my gratitude to those who have contributed to this thesis. Especially I want to thank:

Docent **Freidoun Albertioni**, my main supervisor. Thank you for guiding and inspiring me through my research project. For a tremendous interest and engagement in every single experiment, and for letting me develop and giving me free hands to develop my own ideas. I will not forget the sending back and forth of manuscripts during the process of writing.

Professor **Staffan Eriksson**, my co-supervisor. Thank you for letting me share some of your great knowledge of the metabolism of NAs and always helping improve manuscripts.

Professor **Jan Liliemark**, my co-supervisor. For sharing your knowledge of hematology and especially NAs, and for valuable criticism and support.

Professor **Paul Hjemdahl**, Head of the Division of Clinical Pharmacology. For letting me start as a Ph.D student and providing a good atmosphere and scientific environment at Clinical Pharmacology.

My co-authors, thank you for great collaboration, without your contribution this work would not have been possible. **Kourosh Lotfi**, **Tatiana Spasokoukotskaja**, for invaluable guidance and help with biochemical methods, **Birgitta Pettersson**, for company at the AACR conference in Philadelphia, **Curt Peterson**, for having created a stimulating scientific atmosphere in the oncology group at the Division of Clinical Pharmacology and for valuable advice, support and stimulating discussions, **Jan Sällström**, **Eva Liliemark**, **Stefan Söderhäll**, **Göran Gustafsson**, for help with the statistical calculations, **Joya Chandra**, **Vladimir Gogvadze**, **Scott Kaufmann**, **Sten Orrenius**, **Howard Elford**, **Emma Flordal** and **Svetlana Lagercrantz,** for help in studying the “chromosomes” and **Helene Stridh**, for invaluable discussions on apoptosis. I would also like to thank **Klas Jönsson** for sharing his knowledge in cloning and sequencing.

All the persons in the lab, especially **Helena Nordgren** for friendship and support, for sharing good and bad moments during these years. In the oncology group, **Michéle Koitsalu**, **Loukas Tatidis**, **Sigurd Vitols**, **Kerstin Palmquist-Munck**, **Jacek Gregorczyk**, **Linn Hjortsberg** and **Åsa Konradsson** for stimulating scientific and other discussions and laughs in the

lab. In the cardiovascular group, **Maud Daleskog**, **Maj-Christina Johansson**, **Pia Hillelson**, **Lillemor Melander**, **Nailin Li** and **Hu Hu**, being the lab “next door” and making the office a nice place. The rest of the staff at the Division of Clinical Pharmacology for all the good times in the lab and elsewhere.

Docent **Robert Harris** for linguistic revision and advice for this thesis.

To **Anna Berg** and **Ebba Bråkenhielm** for being kind and taking some of your valuable time to revise and comment on this thesis.

To all my friends outside the lab, for friendship, support, so much fun and reminding me about the important things in life ☺.

Finally, I would like to thank my family, my parents **Inger** and **Bo** and my dear sisters **Sara** and **Lisa** with their families **Magnus**, **Johan** and **Tove** for endless support and always being there. Most of all I would like to thank **Markus**, my love, for your endless support and encouragement especially during the last months. This work would not have been accomplished without you. You are very special to me .

Financial support for this thesis was obtained from the **Swedish Children Cancer Foundation** and the **Swedish Cancer Society**.

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