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Nucleoside Analog Phosphorylation and Mitochondrial Enzymes

Studies on molecular targets of the anti-leukemic compound 9-ß-D-arabinofuranosylguanine

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By

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In loving memory of my parents

"Try and fail, but don't fail to try."
Stephen Kaggwa

"The process of scientific discovery is, in effect, a continual flight from wonder."
Albert Einstein

Publications on which this thesis is based

I. **Sophie Curbo**, Chaoyong Zhu, Magnus Johansson, Jan Balzarini and Anna Karlsson (2001) Dual mechanisms of 9-β-D-arabinofuranosylguanine resistance in CEM T-lymphoblast leukemia cells, Biochem. Biophys. Res. Commun. 285, 40-45.

II. **Sophie Curbo**, Boris Zhivotovsky, Magnus Johansson and Anna Karlsson (2003) Effects of 9-β-D-arabinofuranosylguanine on mitochondria in CEM T-lymphoblast leukemia cells. Biochem. Biophys. Res. Commun. 307, 942-947.

III. **Sophie Curbo**, Magnus Johansson and Anna Karlsson (2004) Screening for differences in gene expression in 9-β-D-arabinofuranosylguanine resistant cell lines using microarray profiling. Manuscript.

IV. **Sophie Curbo**, Marjan Amiri, Fariba Foroogh, Magnus Johansson and Anna Karlsson (2003) The *Drosophila melanogaster* UMP-CMP kinase encodes an N-terminal mitochondrial targeting sequence. Biochem. Biophys. Res. Commun. 311, 440-445.

V. **Sophie Curbo**, Leila Kanni, Anna Karlsson and Magnus Johansson (2004). Identification and cDNA cloning of the human mitochondrial inorganic pyrophosphate. Manuscript.

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Abbreviations

Nucleosides and nucleotides

dAdo, dAMP, dADP, dATP	deoxyadenosine, mono-, di- and triphosphate
dCyd, dCMP, dCDP, dCTP	deoxycytidine, mono-, di- and triphosphate
dGuo, dGMP, dGDP, dGTP	deoxyguanosine, mono-, di- and triphosphate
dThd, dTMP, dTDP, dTTP	deoxythymidine, mono-, di- and triphosphate
dUrd, dUMP, dUDP, dUTP	deoxyuridine, mono-, di- and triphosphate
-MP, -DP, -TP	5'mono-, 5'di- and 5'triphosphate of nucleosides
N, NMP, NDP, NTP	any ribonucleoside or nucleotide

Nucleoside analogs

araC	1-β-D-arabinofuranosylcytosine (cytarabine)
araG	9-β-D-arabinofuranosylguanine (nelarabine)
araT	1-β-D-arabinofuranosylthymine
AZT	3'-azido-2',3'-dideoxythymidine (zidovudine)
CdA	2-chloro-2'-deoxyadenosine (cladribine)
ddC	2',3'-dideoxycytidine (zalcitabine)
dFdC	2',2'-difluorodeoxycytidine (gemcitabine)
dFdG	2',2'-difluorodeoxyguanosine
d4T	2',3'-didehydro-3'-deoxythymidine (stavudine)
F-araA	2-fluoro-9-β-D-arabinofuranosyladenine (fludarabine)
FIAU	1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil (fialuridine)

Enzymes

ADA	adenosine deaminase
CDA	cytidine deaminase
dCK	deoxycytidine kinase
dGK	deoxyguanosine kinase
dNK	deoxyribonucleoside kinase
hENT1	equlibrative and nitrobenzylmercaptopurine ribonucleoside sensitive transporter
5'-NT	5'-nucleotidase
PNP	purine nucleoside phosphorylase
PPase-I	inorganic pyrophosphatase 1
PPase-II	inorganic pyrophosphatase 2
RR	ribonucleotide reductase
TK1	thymidine kinase 1
TK2	thymidine kinase 2
UMP-CMPK	UMP-CMP kinase

Other abbreviations

CEM	acute T-lymphoblast cell line
DNA	deoxyribonucleic acid
Dm.	*Drosophila melanogaster*
EtB	ethidium bromide
HeLa	cervical adenocarcinoma cell line
MDS	mitochondrial DNA depletion syndrome
mt	mitochondria/-l
mtDNA	mitochondrial DNA
mtRNA	mitochondrial RNA
P_i	inorganic phosphate
PP_i	inorganic pyrophosphate
RNA	ribonucleic acid

Abstract

Nucleoside analogs are commonly used in treatment of cancer and viral infections. A way to improve the therapies would be to minimize the acquirement of resistance and side effects such as delayed cytotoxicity. Nucleoside analogs are phosphorylated and thereby activated by cellular kinases and to understand more about their phosphorylation by mitochondrial enzymes we have studied the molecular targets of the guanosine analog 9-β-D-arabinofuranosylguanine (araG). This analog is a substrate of both the mitochondrial deoxyguanosine kinase (dGK) and the cytosolic deoxycytidine kinase (dCK). The prodrug of the biologically active araG, nelarabine, has proven highly efficient in particular in patients with T-cell acute lymphoblastic leukemia. Although the mechanism of action of araG is not fully understood, the accumulation of araG triphosphates (araGTP) has been correlated to cytotoxicity both *in vitro* and *in vivo*. AraGTP acts as a structural analog of deoxyguanosine triphosphate (dGTP) and is thereby incorporated into DNA. The accumulation of araGTP is independent of the cell cycle, which is not surprising since both dCK and dGK are expressed throughout the cell cycle. Incorporation of araGMP into nuclear DNA has been suggested as a critical event for cytotoxicity. A recent study has suggested a role of mitochondria in the cell specific toxicity of dGTP with intra-mitochondrial accumulation of dGTP and inhibition of mtDNA repair. The dose-limiting toxicity in the clinical trials with nelarabine has been neurotoxicity, but less pronounced adverse effects include other symptoms similar of drugs causing mitochondrial toxicity. We have shown that araG can be incorporated into mtDNA but the mtDNA incorporation does not, however, cause the acute cytotoxicity of araG and we do presently not know to what extent it contributes to the cytotoxic action of the analog. It cannot be excluded that long-term exposure to araG may cause mtDNA alterations with subsequent delayed mitochondrial toxicity. Several studies on mechanisms of resistance to araG have been performed. These studies have shown partly conflicting results as to the molecular mechanism of resistance. In our studies we found that araG resistance can occur by two separate molecular mechanisms that can occur sequentially. The first mechanism is associated with a decrease of araG incorporation into mtDNA and the second event is associated with loss of dCK activity, whereas the dGK activity remained at the same level as in the control cells. We do not yet know how the decreased incorporation of araG into mtDNA contributes to the resistant phenotype, but we know that araG does not cause mtDNA depletion or altered translation of mtDNA-encoded genes. To study differences in gene expression in the araG resistant cells we have initiated microarray analysis.

In the search for enzymes that could contribute to the activation of nucleoside analogs in the mitochondria we found a UMP-CMP kinase from *Drosophila melanogaster* that localized to the mitochondria. The recombinant enzyme accepted pyrimidine nucleoside monophosphates as substrates. The enzyme contained an N-terminal signal targeting the enzyme to the mitochondria. The identification of a functional mitochondrial import signal in the *Dm.*UMP-CMP kinase suggests that this enzyme and its homologues in other species may be involved in the mitochondrial phosphorylation of pyrimidine nucleoside monophosphates. However, the mitochondrial homologue in human cells remains to be identified.

It was recently shown that mutations in the genes coding for dGK and the mitochondrial thymidine kinase 2 (TK2) are associated with mtDNA depletion in patients. However, for the majority of patients with mtDNA depletion syndromes (MDS) the genetic defect causing the syndrome remains to be identified. It is known that the yeast mitochondrial pyrophosphatase is necessary for maintained mtDNA content in the yeast cells, and thus the human mitochondrial pyrophosphatase would be a candidate gene for MDS. Biochemical properties of mammalian mitochondrial pyrophosphatases have been studied on enzyme

purified from tissues, but cloning of the gene encoding the enzyme has not been reported previously. Based on sequence similarity to other pyrophosphatases we identified the cDNA encoding the human mitochondrial pyrophosphatase. We cloned the enzyme and showed that it encoded a functional N-terminal mitochondrial targeting signal. The recombinant enzyme was active and ubiquitously expressed with highest levels in tissues rich in mitochondria such as muscle, liver and kidney. The ubiquitous expression suggests that the mitochondrial pyrophosphatase, like the cytosolic pyrophosphatase, is involved in "house-keeping" hydrolysis of pyrophosphate, which is generated by different metabolic processes in the cells. To test if the human mitochondrial pyrophosphatase is required for normal mtDNA copy number, material from more than 50 patients in the USA with unknown cause of MDS is presently being screened for alterations in the gene encoding the mitochondrial pyrophosphate.

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Introduction

Nucleoside analogs are structurally modified nucleosides that constitute an important class of compounds used in the treatment of hematological malignancies, viral infections and recently also solid tumors [1-4]. The importance of nucleoside analogs as chemotherapeutic agents has increased in later years as new compounds have entered into clinical use and nucleoside analogs have expanded into the field of solid tumors. The nucleoside analogs available for cancer treatment today are not particularly selective. Although many of them have significant anti-tumor activity their use is limited by toxicity to normal rapidly proliferating cells. There is a need for more selective nucleoside analogs or tailored and risk-adjusted therapies. In order to provide that, more knowledge is needed both about the biology of the diseases and about the molecular targets of the nucleoside analogs. To understand more about the phosphorylation of nucleoside analogs in the mitochondria we have studied the cytotoxic effects of 9-β-D-arabinofuranosylguanine (araG), a nucleoside analog that is phosphorylated both by a mitochondrial enzyme and a cytosolic/nuclear enzyme. We have also searched for new mitochondrial enzymes that could contribute to the activation or toxicity of nucleosides or nucleotides in the mitochondria. We identified, cloned and characterized a mitochondrial UMP-CMP kinase from *Drosophila melanogaster*, but so far no human mitochondrial homologue has been found. We have also identified, cloned and characterized the first human mitochondrial pyrophosphatase. This enzyme hydrolysis inorganic pyrophosphate, which is generated as a byproduct in several metabolic processes that occur inside the cells.

Synthesis of DNA precursors

The deoxyribonucleic acid (DNA) of cells stores the biological information needed for life. DNA is, like ribonucleic acid (RNA), built up by nucleotides. Nucleotides have three characteristic components; they consist of a sugar moiety, a nitrogen-ring structured base and one, two or three phosphate groups. Without phosphate the compound is called a nucleoside. In DNA the sugar moiety has a hydrogen group at the 2'-carbon position and is thus called a deoxyribose, whereas it is called a ribose in RNA since there is a hydroxyl group at the 2'-position. Both DNA and RNA contain two purine bases; adenine and guanine. They also contain two pyrimidines; cytosine is found in both types of nucleic acids whereas thymine is specific for DNA and uracil for RNA (Figure I).

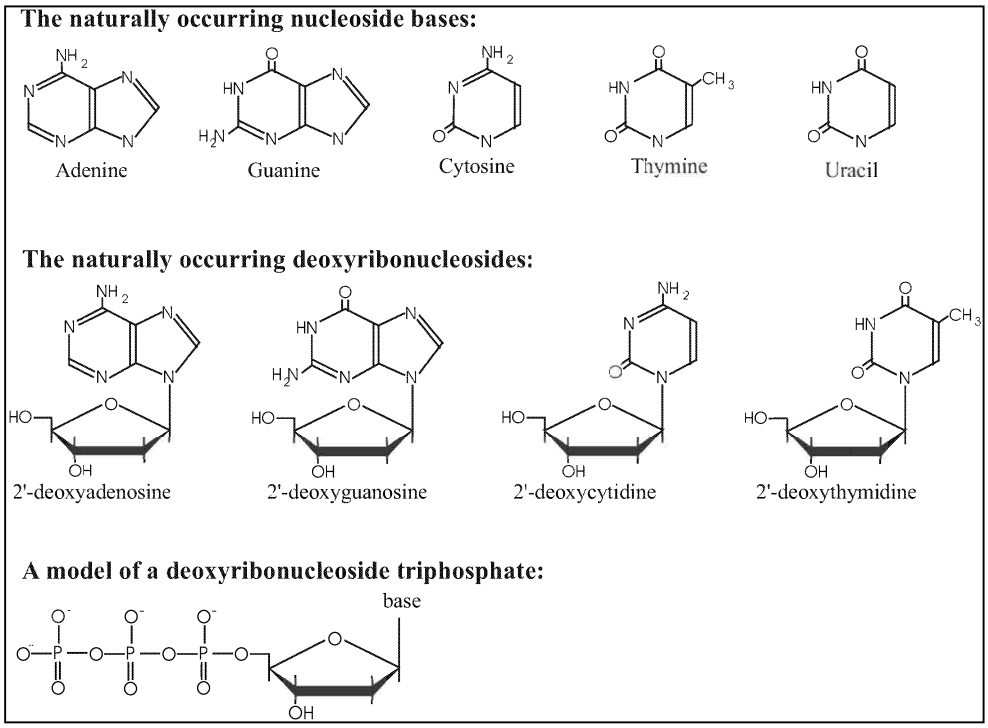

Figure I. The structure of the naturally occurring nucleosides, deoxyribonucleosides and a principal structure of a deoxyribonucleoside triphosphate.

There are two separate pathways for nucleotide synthesis commonly referred to as the *de novo* and the salvage pathways (Fig II). The *de novo* pathway involves initial synthesis of ribonucleotides from small molecules such as ribose-5'-phosphate, amino acids, CO_2 and NH_3. The regulatory step in the *de novo* synthesis is the reduction of the 2'-hydroxyl group of the ribonucleoside diphosphate to the corresponding 2'-deoxyribonucleotide. This reaction is catalyzed by ribonucleotide reductase (RR) and does only occur during the S-phase of the cell cycle [5, 6].

The salvage pathway of deoxyribonucleosides is a complementary route for providing cells with DNA precursors. Deoxyribonucleosides are imported from the extracellular space or derived from dephosphorylation of deoxyribonucleotides. The transport of the deoxyribonucleosides across the cell membrane is the first regulatory step in the salvage pathway. Deoxyribonucleosides can enter and leave through the cell membrane by nucleoside transporter proteins via facilitated diffusion or active transport [7-11]. The next step in the salvage pathway, when nucleosides are phosphorylated to nucleoside monophosphates, is

generally considered to be the rate-limiting step in the salvage pathway. Phosphorylated nucleosides are also trapped inside the cells due to their negative charge. The conversion of deoxyribonucleoside monophosphates to triphosphates occurs in two subsequent freely reversible phosphotransferase reactions catalyzed by nucleoside monophosphate and diphosphate kinases.

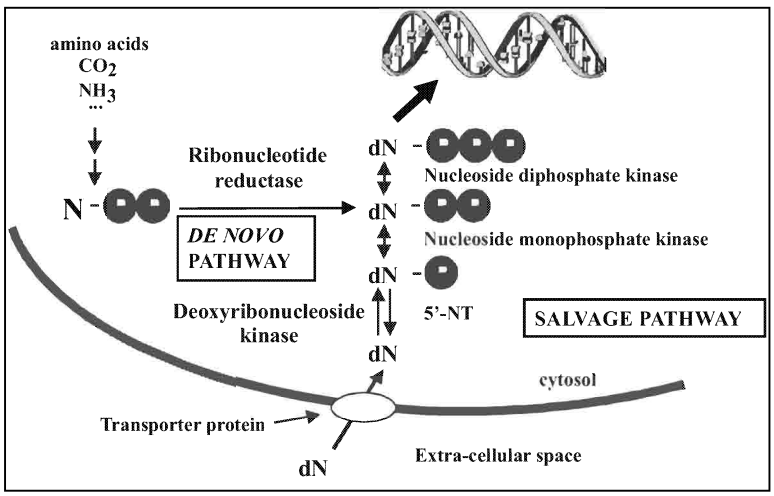

Figure II. The *de novo* and the salvage pathways of deoxyribonucleoside metabolism in human cells.

Purine and pyrimidine metabolism

The 5'-phosphorylation of deoxyribonucleosides to deoxyribonucleoside monophosphates is in mammalian cells mediated mainly by the four major deoxyribonucleoside kinases (dNKs); thymidine kinase 1 and 2 (TK 1 & TK 2), deoxycytidine kinase (dCK) and deoxyguanosine kinase (dGK). These enzymes phosphorylate deoxyribonucleosides and their nomenclature is based on the preferred deoxyribonucleoside substrate, although they have the ability to phosphorylate other substrates (Table I). Although this reaction is irreversible, the deoxyribonucleoside monophosphates can be dephosphorylated by 5'-nucleotidases. The dNKs play an important role in the activation of several nucleoside analogs. These enzymes have been thoroughly studied and a detailed description of their biochemical properties can be found in a review article by Van Rompay et al. [12]. From nucleoside monophosphate the conversion to triphosphate occurs in two reversible steps by nucleotide kinases.

Deoxyribonucleoside kinase	Natural substrates	Subcellular location
Thymidine kinase 1	dThd, dUrd	Cytosol
Deoxycytidine kinase	dCyd, dAdo, dGuo	Cytosol, nucleus
Thymidine kinase 2	dThd, dUrd, dCyd	Mitochondria
Deoxyguanosine kinase	dGuo, dAdo, dIno	Mitochondria

Table I. Natural substrates and subcellular location of the four major human deoxyribonucleoside kinases.

Thymidine kinase 1

Thymidine kinase 1 is a cell cycle regulated enzyme that is virtually only expressed during the S-phase. The transcription of TK1 increases during the S-phase as well as the stability of the TK1 protein [13]. At the end of the S-phase the half-life of the protein decreases and it is rapidly degraded [14]. The natural substrates of the enzyme are deoxythymidine (dThd) and deoxyuridine (dUrd) [15]. The pyrimidine nucleoside analogs AZT, D4T and FIAU can also be phosphorylated by TK1. This enzyme is located in the cytosol of the cells and is widely distributed in all tissues [16, 17].

Deoxycytidine kinase

Deoxycytidine kinase has a broad substrate specificity and phosphorylates both the pyrimidine nucleoside deoxycytidine and the purine nucleosides deoxyadenosine and deoxyguanosine [18]. It also phosphorylates several nucleoside analogs, such as araA, araC, araG, CdA, ddC and dFdC [1, 18-20]. The amino terminus of dCK contains a nuclear signal and fused to the green fluorescent protein (GFP) it localizes to the nucleus [21]. However, the native form of dCK has been shown to be located in the cytosol [22]. The physiological importance of the transport of the protein between subcellular compartments is still unclear. dCK is constitutively expressed throughout the cell cycle. It is found in low amounts in most tissues, except for lymphoid tissues and particularly immature lymphoblasts which express very high levels of dCK [23]. Elevated levels of dCK expression has been detected in several malignant tumors and is implicated as a mechanism of tissue targeted cytotoxicity of dCK phosphorylated nucleoside analogs in lymphoblasts [20, 24, 25].

Thymidine kinase 2

The natural substrates of thymidine kinase 2 are the pyrimidines deoxythymidine, deoxyuridine and deoxycytidine. TK2 also phosphorylates the nucleoside analogs AZT and FIAU, but at lower rates than the natural substrates [15, 26]. In contrast to TK1, TK2 is constitutively expressed throughout the cell cycle. It has been shown that TK2 is located in the mitochondria, but it has also been suggested that an isoform of the enzyme may be present in the cytosol [27, 28].

Deoxyguanosine kinase

Deoxyguanosine kinase has partially overlapping substrate specificity with dCK and phosphorylates deoxyguanosine and deoxyadenosine, but also deoxyinosine [29]. In addition dGK phosphorylates the nucleoside analogs dFdG, araG and CdA, of which the two latter are substrates for dCK as well. dGK is constitutively expressed throughout the cell cycle and the enzyme level is believed to be proportional to the amount of mitochondria in most tissues [1]. There is a mitochondrial targeting sequence in the N-terminal of dGK and the enzyme localizes to the mitochondria [29-31].

Nucleoside monophosphate kinases

Nucleoside monophosphate kinases catalyze the reversible phosphotransferase reaction of nucleoside monophosphates to nucleoside diphosphates. Human cells contain AMPKs, GMPKs, UMP:CMPK and dTMPK. For a review of their substrate specificities and subcellular locations see the paper of Van Rompay *et al.* [32]. This enzymatic step is not rate-limiting for the salvage of deoxyribonucleosides or most of the nucleoside analogs. However, there is evidence that AZT-MP accumulate in cells. AZT is a good substrate for TK1 whereas the cellular dTMPK catalyzes the conversion of AZT-MP to AZT-DP much slower [33, 34]. There are also cytidine and uridine analogs that accumulate as monophosphates [35, 36].

Nucleoside diphosphate kinases

Nucleoside diphosphate kinases catalyze the reversible phosphotransferase reaction of nucleoside diphosphates to triphosphates [37]. These enzymes have broad substrate specificities and show little discrimination between ribo- and deoxyribonucleotides. The conversion to triphosphates is generally not considered to be the rate-limiting step in the

phosphorylation of most nucleoside analogs, but certain anti-viral dideoxyribonucleoside analogs are poor substrates for these enzymes [38].

Catabolism

An important factor in balancing the intracellular deoxyribonucleotide pools is the activity of the enzymes that catalyze the opposing reactions of the phosphorylations [39-45]. The phosphorylation of deoxyribonucleosides by deoxyribonucleoside kinases is balanced by 5'-nucleotidases that dephosphorylate nucleoside monophosphates. Both the nucleoside monophosphate and diphosphate kinase reactions are reversible and thus the nucleoside monophosphate pools equilibrate with the nucleoside di- and triphosphate pools. Nucleosides can be both imported and excreted via the nucleoside transporter proteins in the plasma membrane [7]. Excessive accumulation of deoxyribonucleotides can therefore be prevented through dephosphorylation by 5'-nucleotidases and subsequent excretion of deoxyribonucleosides, whereas deficiencies in the deoxyribonucleotide pools can be adjusted through import and subsequent phosphorylation of deoxyribonucleosides.

A cell can also eliminate the deoxyribonucleosides through purine or pyrimidine catabolism. The discoveries that deficiencies in the purine catabolic enzymes adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP) are associated with immune deficiencies in humans led to intensive studies of the purine catabolism. ADA deaminates deoxyadenosine to deoxyinosine and adenosine to inosine, and a deficiency in this enzyme leads to T-lymphocyte depletion and severe combined immunodeficiency [46]. PNP converts deoxyguanosine to guanine and deoxyinosine and inosine to hypoxantine, and a deficiency in this enzyme does also lead to T-cell depletion and immunodeficiency [47]. The lymphotoxicity in these diseases appears to be mediated by excessive accumulation of dATP and dGTP, respectively, in the target tissues [48-50].

DNA synthesis

An adequate supply of DNA building blocks is a prerequisite for life. Deoxyribonucleotides synthesized by either the *de novo* or the salvage pathway are incorporated into DNA. The *de novo* pathway provides the majority of the dNTPs that are required for DNA replication and cell division. There is, however, also a need for dNTPs in resting and terminally differentiated

cells for processes such as DNA repair and mitochondrial DNA synthesis. The salvage pathway provides the deoxyribonucleotides used in these processes (Figure III).

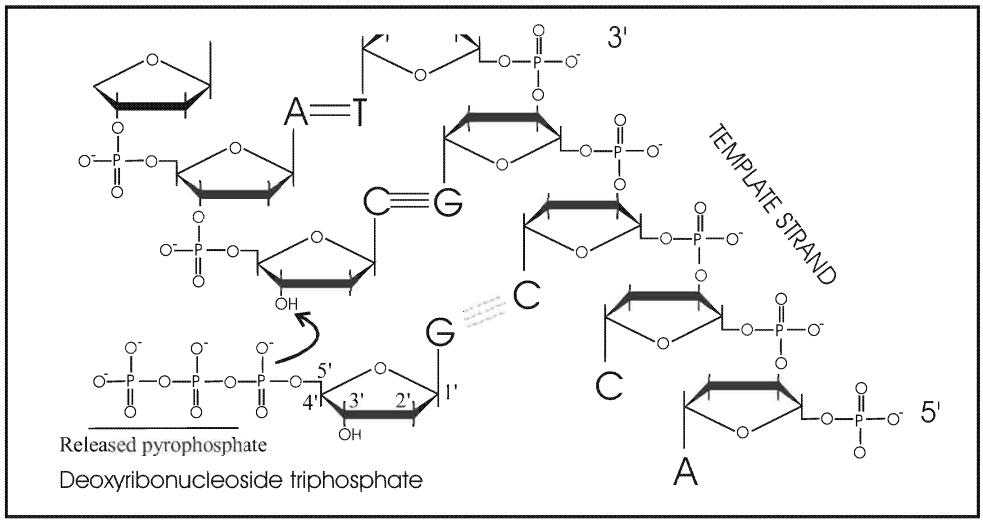

Figure III. Deoxyribonucleoside triphosphates are the substrates of DNA polymerase catalyzed replication of DNA.

Nuclear DNA

Nuclear DNA replication occurs during the S-phase of the cell cycle and the absolute majority of dNTPs needed for this biological process are derived from the *de novo* synthesis [6, 51]. In mammalian cells the DNA replication is catalyzed by several DNA polymerases such as α, β, δ and ε. New dNTPs are added the growing DNA strand to the 3'-hydroxyl end of the polynucleotide chain. The nuclear DNA of the cells is organized in chromosomes; large DNA molecules that contain many genes and are associated with protective proteins such as histones.

Mitochondrial DNA

The replication, transcription and translation of mtDNA are separated from the genetic system of the nuclear DNA and occur in the mitochondrial matrix. In contrast to the nuclear DNA mtDNA can be replicated more than once during the cell cycle [52]. The replication is catalyzed by the mitochondrial DNA polymerase γ. So far no mitochondrial RR has been identified and the salvage pathway is probably crucial for mtDNA replication. However, a recent study has suggested the presence of a deoxynucleotide carrier in the mitochondrial

inner membrane with a substrate specificity for GTP, dCTP, ATP, dGTP and dTTP, in the order of their respective efficiency [53]. Thus it is possible that dNTPs are imported from the cytosol into the mitochondrial matrix for subsequent use in mtDNA synthesis. Accordingly, it is also possible that nucleoside analog triphosphates are transported into the mitochondria. All of the proteins of mtDNA replication, transcription and translation machineries are nucleus-encoded, transcribed and translated in the cytoplasm, and imported into the mitochondria. Since the replication of mtDNA requires a synthesized RNA primer, any defect in mtRNA synthesis will affect mtDNA replication as well.

The frequency of mtDNA mutations is more than ten times higher compared to the mutation rate in nuclear DNA [54]. The reasons for this might be that although the mitochondrial genome is highly packed and lacks introns it is not associated with protective histones and the DNA repair systems within the mitochondria have also been suggested to be less efficient than the repair systems in the nucleus [55].

Nucleoside analogs

In the treatment of leukemia, nucleoside analogs generally act by affecting the growth of the tumor cells with only a moderate selectivity. The surrounding tissues and organs are often affected in an action of the nucleoside analog that is called toxicity, whereas a decrease or eradication of the tumor is called a response. Both the toxicity and the anti-tumor effect are examples of the pharmacodynamic actions of the drug. In order to optimize treatment in patients a relationship between the toxicity and the dose of the administered drug is evaluated under so called therapeutic drug monitoring. Increased knowledge about the mechanism of action of the analogs provide a basis for therapy with minimized toxicity.

Mechanisms of action

Nucleoside analogs need to be phosphorylated intracellularly for pharmacological activity [1, 2, 4]. The accumulation of the analogs is therefore higher in cells that contain high levels of activating enzymes. As mentioned earlier, the initial phosphorylation of the nucleoside analog to its monophosphate form is often the rate-limiting step in the activation. The principal mode of action of nucleoside analogs is through inhibition of DNA synthesis after incorporation of its triphosphate form into the replicating DNA strand. This is the scenario for nucleoside analogs that lack the 3'-OH group which is required for addition of the next nucleotide into

the growing DNA chain. AZT and ddC are two drugs that work according to this principle (Figure III). The cytotoxicity of this type of nucleoside analogs has been suggested to be dependent on rapid DNA replication for efficient incorporation into nuclear DNA [56, 57]. Nucleoside analogs with an intact 3'-OH group are not absolute chain terminators, but there is evidence that incorporation of adjacent such nucleoside analogs severely impair chain elongation [58, 59]. The presence of nucleoside analogs in the DNA is believed to induce repair processes leading to chain breaks and eventually cell death. Examples of this type of analogs are araC, araG, CdA, dFdC, F-araA and FIAU (Figure IV). There are also nucleoside

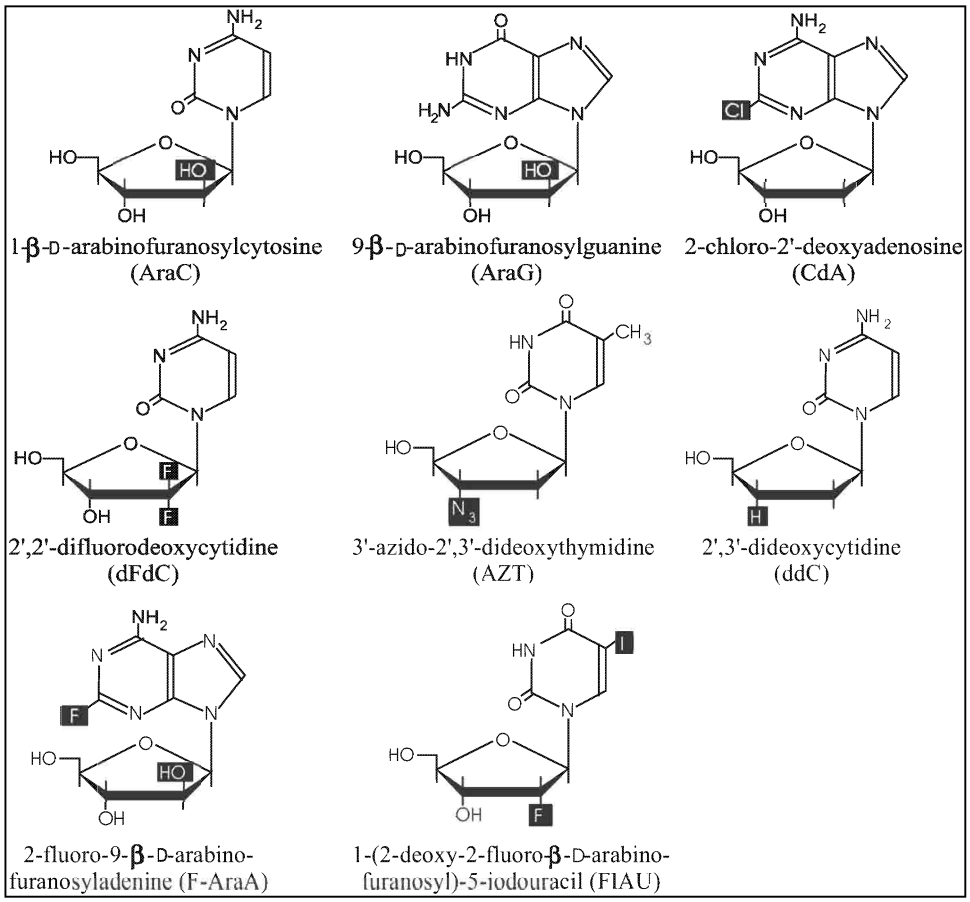

Figure IV. The structure of a selection of nucleoside analogs that are discussed in this thesis.

analogs that can interfere with key enzymes of the deoxyribonucleotide synthesis, such as DNA polymerases, DNA ligase, DNA primase and RR. The latter leads to perturbations of the deoxyribonucleotide pools and consequently impair proper DNA replication. In all cases the accumulation of nucleoside analog triphosphates is counteracted by 5'-nucleotidases and

the dephosphorylation of nucleoside analogs by enzymes involved in the nucleoside and nucleotide catabolism.

Clinical use

There are several nucleoside analogs that are used in the clinic as anti-cancer or anti-viral compounds. In Table II a selection of commonly used nucleoside analogs and their activating deoxyribonucleoside kinases are presented. Below follows a brief description of araC, CdA, dFdC and AZT, four important nucleoside analogs.

Nucleoside analog	dN kinase	Clinical use
araC (cytarabine)	dCK	acute leukemia
araG (nelarabine)	dGK, dCK	acute leukemia, CLL
CdA (cladribine)	dCK, dGK	hairy cell leukemia, CLL
dFdC (gemcitabine)	dCK, TK2	pancreatic, breast & lungcancer
AZT (zidovudine)	TK1, TK2	HIV infection
ddC (zalcitabine)	dCK	HIV infection
F-araA (fludarabine)	dCK	CLL
FIAU (fialuridine)	TK1, TK2	HBV infection

Table II. Nucleoside analogs used in anti-cancer and anti-viral therapy. CLL, chronic lymphatic leukemia; HIV, human immunodeficiency virus; HBV, hepatitis B virus.

AraC

1-β-D-arabinofuranosylcytosine, araC, is one of the most efficient drugs in the treatment of acute myeloid leukemia and it is also commonly used in combination with other anti-cancer drugs in the treatment of diverse types of leukemias and lymphomas [60]. The major active metabolite is araCTP, which is a good substrate for incorporation into DNA by polymerases active in both replication and repair [61]. Once incorporated, araCMP is a poor substrate for the addition of another deoxynucleotide and, in particular, another arabinosyl nucleotide [61, 62]. Once inside the cells, araC is a good substrate for dCK but it can also be catabolized through deamination by cytidine deaminase to the non-toxic metabolite araU.

CdA

2-chloro-2'-deoxyadenosine, CdA, is effective in patients with hairy cell leukemia where it often produces complete remissions, but it is also used in treatment of other indolent types of lymphoid malignancies [63, 64]. CdA is a deoxyadenosine analog resistant to deamination by adenosine deaminase. Inside the cells CdA is phosphorylated to CdAMP by either dCK or dGK and it can then be dephosphorylated by 5'-NT [65]. CdATP is incorporated into the DNA of dividing cells and causes termination of DNA chain elongation with subsequent S-

phase specific apoptosis [3, 63, 66]. The effects of this compound in cells may also be due to inhibition of DNA polymerases with resulting inhibition of DNA repair. It has also been shown that CdA inhibits RR causing a subsequent reduction of the dNTP pools required for DNA synthesis [63]. Recently it was suggested that CdA, when incorporated into gene-regulatory sequences such as the TATA box, can act as a transcriptional antagonist [67]. Different mechanisms have been suggested as to why CdA is effective also in non-dividing cells. Inhibition of DNA repair processes, altered gene transcription with depletion of proteins needed for cell survival as a consequence and alterations in mitochondrial functions and integrity are among the proposed mechanisms. Intracellular phosphorylation of CdA is needed for toxicity and the level of phosphorylation is correlated to the response to CdA. Although the phosphorylation is not the only factor determining the response both dCK and dGK are present throughout the cell cycle rendering resting as well as dividing lymphoid cells susceptible to the effects of CdA [63].

dFdC

2',2'-difluorodeoxycytidine, dFdC, is a deoxycytidine analog with promising activity in solid tumors such as pancreatic, breast and non-small cell lung cancer. dFdC is a deoxycytidine analog that is phosphorylated to its monophosphate form primarily by dCK but to some extent also by TK2. It is a substrate of cytidine deaminase that can deaminate it into the inactive metabolite dFdU and the monophosphate is a substrate of 5'-NT that can dephosphorylate it. dFdCTP is incorporated into the growing DNA strand and then a natural nucleotide is added which masks the incorporated dFdC preventing DNA repair by base pair excision. The DNA polymerases are thereafter unable to proceed in a process called "masked DNA chain termination" [3, 68, 69]. Indirectly, the diphosphate derivative of dFdC also inhibits DNA synthesis and self-potentiates its own activity through inhibition of RR [68]. Reduction of cellular dCTP levels causes a high level of dCK activity which further improves the formation of active dFdC derivatives and incorporation of dFdC into the DNA. Another mechanism that increases the formation of active dFdC in the cells is through direct inhibition of cytidine deaminase [3, 69]. In contrast to other related substances such as araC, dFdC can be incorporated into RNA as well as DNA. The metabolic characteristics distinguishes it from related compounds and may explain part of its effect in slowly dividing solid tumors.

AZT

3'-azido-2',3'-dideoxythymidine, AZT, is a thymidine analog mainly phosphorylated by TK1. However, TK2 has been suggested to be important for the activation of AZT in non-dividing cells that supposedly lack TK1 [70]. Several *in vitro* studies have shown that AZT can be incorporated into DNA. Besides direct inhibition of reverse transcriptase, DNA chain termination is also a mechanism that has been implicated in the therapeutic action of AZT against HIV [71, 72].

Resistance

Acquirement of resistance and side effects such as delayed cytotoxicity are major problems in the treatment of cancer and viral infections with nucleoside analogs. Mechanisms underlying resistance in viral treatments will not be discussed here since my research mainly addresses nucleoside analogs in cancer treatment. According to Galmarini *et al.* [3] there are three general mechanisms of resistance to nucleoside analogs that have been described in cell lines and clinical samples:

1. Insufficient intracellular concentrations of nucleoside analog triphosphates, which might be due to inefficient cellular uptake, decreased levels of activating enzymes, increased catabolism by elevated levels of 5'-NT or deaminases, or expansion of dNTP pools
2. Inability to achieve sufficient alterations in DNA strands or dNTP pools, which might result from altered interactions with DNA polymerases, lack of inhibition of RR or inadequate p53 exonuclease activity
3. Defective induction of apoptosis

In isolated leukemic blasts as well as in *in vitro* studies it has been shown that the sensitivity to araC and CdA is dependent on the cellular abundance of hENT1, the equilibrative and nitrobenzylmercaptopurine ribonucleoside sensitive transporter in the plasma membrane [73, 74]. In patients with acute myeloid leukemia it has also been shown that low transport rates are correlated with poor prognosis to araC therapy [75].

The dCK activity is high in quiescent cells of many cell types, in which dCK phosphorylates nucleosides necessary for DNA repair, but the activity can often get even higher during the S-phase [3, 76]. dCK activity has been shown to be decreased or absent in many cell lines that are resistant to nucleoside analogs such as araC, dFdC and CdA [63, 77]. Bone marrow and

lymphoid tissues normally exhibit very high activities of dCK which together with their high dependence of the salvage pathway might explain the clinical success of several nucleoside analogs in the treatment of hematological malignancies [24, 78]. However, decreased expression of the dCK gene or decreased dCK activity has in some studies been correlated with resistance to araC, CdA and dFdC whereas other studies have reported no significant relationship between dCK activity and the effect of the nucleoside analogs [79-86]. CdA is also a substrate of the mitochondrial dGK and the sensitivity to this drug could also be dependent on the dGK activity and subsequent interference with mitochondrial or nuclear DNA. It is, however, presently not known to what extent dGK is involved in resistance to nucleoside analogs. dFdC can be phosphorylated by the mitochondrial TK2 as well as dCK. Similar to CdA, there is also a possibility that dFdC exert part of its action through interference with mitochondrial or nuclear DNA after being activated in the mitochondrial matrix. Thus, a decreased activity of TK2 could be involved in the acquirement of resistance to dFdC. It is presently not known if downregulation or inactivation of TK2 activity is a cause of resistance to dFdC.

5'-NTs comprise a large and complex group of enzymes that differ in subcellular localization, pH sensitivity, substrate specificity and dependency of ATP [87-89]. In diverse types of leukemia, the levels of cytosolic 5'-NT have been found to correlate with the response to nucleoside analogs. As an example patients with acute myeloid leukemia expressing high levels of high K_m 5'-NT mRNA have been reported to have shorter time-to relapse and overall survival when treated with araC, than patients with no expression of this enzyme [90]. Increased activity of 5'-NT has also been implicated in the resistance to CdA in chronic lymphatic leukemia and hairy cell leukemia [63, 82].

Cytidine deaminase converts araC to araU and dFdC to dFdU. There are several studies reporting a connection between elevated activity of this enzyme and resistance to araC [91, 92]. In studies where cytidine deaminase has been transfected into hematopoietic cells or murine fibroblasts, resistance to dFdC and araC have been reported [93-96]. However, it is still unclear if elevated cytidine deaminase activity can cause resistance to araC *in vivo*.

Both cell lines and AML blasts have been shown to exhibit a high level of resistance to araC when they contain high levels of dCTP [23, 97, 98]. The dCTP competes with araCTP for incorporation into DNA, it decreases the dCK activity through feedback inhibition and

activates the cytidine deaminase allosterically [83, 97, 99]. dFdCTP also competes with dCTP for incorporation into DNA, and cells expressing high levels of dCTP are thus likely less sensitive to both araC and dFdC.

DNA damage caused by nucleoside analogs often induces expression of p53 with subsequent apoptosis as a consequence [100, 101]. Patients with leukemia that also have mutations in their p53 gene have in some studies been connected with a poor prognosis and quite often also resistance to CdA and araC, while other studies have failed to show a relationship between defective p53 and resistance to nucleoside analogs [102-105].

A conclusion from the text above about resistance mechanisms, although it is far from covering the entire field of resistance, is that it is a matter of complex nature. The hematological diseases are heterogeneous in terms of resistance and one should also bear in mind that patients are different and e.g. express different levels of activating enzymes. How each patient responds to treatment is therefore individual and ideally studies about drug actions and mechanisms of resistance performed both *in vitro* and during clinical therapy should be a tool in the individualization of patient therapy in the future.

Mitochondrial toxicity

Several nucleoside analogs interfere with mtDNA and in this section I therefore give a brief discussion about mitochondrial toxicity due to nucleoside analogs. Recently, it was also shown that mutations in dGK and TK2 can cause mtDNA depletion syndrome (MDS) in patients, and this heterogeneous group of diseases is described below. We recently cloned and characterized the first human mitochondrial inorganic pyrophosphatase [106- Paper V]. This gene is a candidate gene for MDS and it is therefore described at the end of this chapter.

Mitochondria

Mitochondria are organelles with double membranes with the major responsibility to make ATP via oxidative phosphorylation. They have various shapes, but are often spherical to elongated and they are most likely connected in an internal network. The outer membrane is permeable to small molecules and ions that can easily reach the intermembrane space, separating the outer and the inner membrane (Figure V). The inner membrane is impermeable and transporter proteins are required for passage of most molecules into and out of the matrix.

The matrix contains tRNAs, ribosomes, a variety of enzymes required for mitochondrial functions and the 16 569 bp circular mitochondrial DNA (mtDNA) in two to ten copies. In all cells, except for erythrocytes that lack mitochondria, there are between 1000 and 10000 copies of mtDNA. In total, mtDNA accounts for approximately 1 % of a cell's DNA. MtDNA has been referred to as the 24th chromosome in humans, and it was the first to be completely sequenced [107, 108]. The mtDNA encodes 13 proteins that are part of four enzyme complexes of the respiratory chain (complex I, III, IV and ATP synthase). It also encodes ribosomal RNAs and transfer RNAs, components of the mitochondrial protein synthesizing machinery [109].

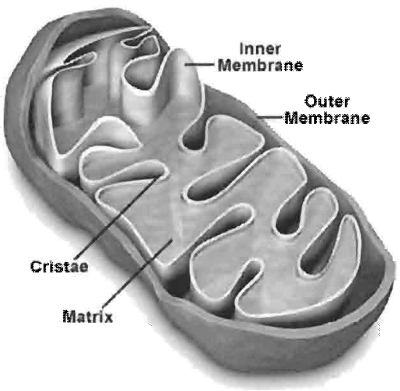

Figure V. Illustration of a mitochondrion with its double membranes.

Several anti-viral nucleoside analogs interfere with mtDNA replication, and adverse effects of these compounds are correlated to mitochondrial dysfunction [110]. Clinical forms of toxicity often occur in anti-viral treatment and spans from mild reversible neuropathy, myopathy and pancreatitis to severe multi-organ failure [110, 111]. For example ddC, ddI and d4T have been shown to induce mitochondrial impairment by reducing the amount of mtDNA. Tissues that are affected by AZT- and FIAU-toxicity consist mainly of non-dividing cells. These cells contain undetectable levels of TK1 and it is likely that TK2 is responsible for phosphorylation of the analogs in these tissues. The importance of nucleoside analog phosphorylation in the mitochondrial matrix for mitochondrial DNA damage is still unresolved. The mitochondrial location of TK2 has been suggested to be important for the mitochondrial toxicity of AZT and FIAU [110]. It was recently shown that the human equilibrative nucleoside transporter 1 (hENT1) is expressed also in the mitochondrial membrane and that the expression of hENT1 in the mt membrane enhances the toxicity of nucleoside analogs such as FIAU [112]. This

strengthens the hypothesis that TK2 would be important for mt toxicity of compounds such as FIAU and AZT. However, ddC which is initially phosphorylated by dCK outside the mitochondria, does also cause mtDNA damage [113]. The recent identification of a carrier in the mitochondrial membrane where the triphosphates of ddC, ddA and ddG are relatively good inhibitors of [^{35}S]-dATP provides a possible entering mechanism for these phosphorylated analogs into the mitochondrial matrix [53]. Purine nucleoside analogs that are substrates of mitochondrial dGK have not, as yet, been shown to induce mitochondrial toxicity. CdA as well as araG, which is discussed further in the next section, are both substrates of dGK and have in the clinical setting shown side effects that are similar to mitochondrial toxicity, such as neuropathy and myelosuppression [3, 114-116]. CdA has also been reported to cause an early effect on cellular mitochondrial function measured as a temporary increase in lactate production [116]. In the same study it was shown that CdA does not cause any decrease in mtDNA content. Taken together, it is thus possible that the subcellular location of the nucleoside analog is of minor importance for mitochondrial toxicity whereas other determinants, such as e.g. the affinity for mitochondrial polymerase γ, are more important.

Mitochondrial DNA depletion syndrome

In 1991, Moraes and colleagues reported a quantitative form of mtDNA defects called mtDNA depletion syndrome (MDS), characterized by depletion of mtDNA [117]. This group of heterogeneous mtDNA diseases is transmitted as an autosomal recessive trait. MDS is phenotypically heterogeneous and consistently the mtDNA depletion may affect specific tissue (commonly muscle or liver) or multiple organs, including heart, brain and kidney. Affected tissues show paucity of mtDNA-encoded translation products, multiple respiratory chain defects and deficient COX activity [118]. MDS normally presents in infancy with hepathocerebral failure, myopathy, cardiomyopathy, or liver failure, or later in life with leukodystrophy and gastroenterological disease [117, 119-123]. In the affected tissues of severe cases the mtDNA levels may be less than 5% of control levels and less than 30% in more mildly affected patients. Infants with severe mtDNA depletion usually die within 12 month [117, 120, 122, 124-126]. The human mtDNA encodes 13 proteins that are involved in the oxidative phosphorylation system [127]. The biochemical basis of organ failure in mtDNA depletion is therefore thought to be insufficient ATP production for cellular requirements. The underlying molecular pathology has only been identified for a minority of

the patients and involves the maintenance of balanced nucleotide pools within the mitochondria; mutation in the thymidine phosphorylase gene has been shown to cause peripheral neuropathy and gastrointestinal symptoms [128], mutations in the gene encoding dGK has been detected in infants with fatal liver failure and encephalopathy [121, 129, 130], and isolated myopathy has been associated with mutations in the gene encoding TK2 [131, 132]. The affected tissues of these patients exhibited mtDNA depletion and respiratory chain dysfunction. However, the mechanism of tissue specificity in these disorders is still unclear and puzzling. Unless the nuclear DNA-encoded factors involved in mtDNA depletion are tissue specific, all tissues, or at least all tissues highly dependent on oxidative phosphorylation such as brain, retina and muscle, ought to be affected. A possible explanation to the variability among patients carrying the same mutations is that the nuclear DNA defect may impair resumption of mtDNA replication in early embryogenesis, leading to variable levels of mtDNA depletion in different stem cells [117]. When the stem cells differentiate into different organs, tissue-specific depletion would occur.

For most of the patients with MDS the genetic defect causing mtDNA depletion remains to be identified. Genetic linkage studies and candidate gene screening are useful for the identification of the molecular basis of yet unknown causes of MDS. One such candidate gene is the gene encoding the human mitochondrial inorganic pyrophosphatase described below.

Inorganic pyrophosphatase

Inorganic pyrophosphate (PP_i) is produced as a byproduct in many biosynthetic reactions that occur in living cells, such as the synthesis of protein, DNA and RNA. PP_i would accumulate quickly into significant concentrations that could stop or even reverse the synthetic reactions and eventually prevent growth, unless it was hydrolyzed by the phosphotransferase inorganic pyrophosphatase (PPase) [133]. The PPase catalyzed hydrolysis of PP_i into inorganic phosphate (P_i) plays an important role in cell anabolism, providing a “thermodynamic pull” for reversible biosynthetic reactions [133]. According to Peller [134] nucleic acid synthesis would be energetically impossible *in vivo* if there was no PPase catalyzed hydrolysis of PP_i. The cytosolic PPase is a ubiquitous enzyme that has been shown to be essential for growth in many organisms including animals, plants and bacteria [135]. It has also been suggested that PPase could play an important role in evolution since the concentration of PP_i has been shown to be important for high-fidelity of DNA synthesis [136-138]. In 1991 Lundin et al

showed that the yeast mitochondrial PPase was important for the mtDNA maintenance of the yeast cells [139]. They suggested that the yeast mitochondrial PPase had a similar function as the cytoplasmic PPase, *i.e.* to facilitate biosynthetic reactions that generate PP_i. In mammalian cells, both a soluble cytosolic and a mitochondrial PPase have been detected in biochemical studies [140-144]. Yang and co-workers purified a cytosolic PPase from bovine retina and cloned the cDNA encoding the enzyme [140, 145]. It was shown to be sequence related to the family of soluble PPases of yeast and bacteria. Recently, the human homologue of the bovine cytosolic PPase was cloned and recombinantly expressed [146, 147]. Less was known about the molecular properties of the mammalian mitochondrial PPase. This enzyme is located in the mitochondrial matrix and biochemical studies suggest that it may be associated to mitochondrial inner membrane proteins such as components of the respiratory chain [141, 142, 148]. Several different PPase containing protein complexes with different molecular mass have been purified from mammalian mitochondria, but it appears that the catalytically active subunit in these complexes has a similar size as the yeast mitochondrial PPase [141, 142]. Yeast cells lacking the mitochondrial PPase exhibit a ρ^0 phenotype with no detectable mtDNA and require growth media that supports anaerobic metabolism [139]. This suggests that deficiency in the expression of the human mitochondrial PPase could result in mtDNA depletion. To investigate the role of the mt PPase in mt pyrophosphate metabolism and mtDNA maintenance, we decided to clone the cDNA and gene encoding the human mt PPase [106]. It is possible that the human mt PPase could be implicated in mt DNA depletion syndromes and screening for mutations in the gene encoding the mt PPase in patients with unknown cause of MDS is presently being performed[1].

AraG

In 1975 it was discovered that patients suffering from purine nucleoside phosphorylase (PNP) deficiency accumulated high levels of dGTP in their T-cells causing T-cell lymphopenia [47]. The greater accumulation of dGTP in T-cells as compared to B-cells was later suggested to be due to a higher phosphorylation rate of deoxyguanosine (dGuo) in T-cells than in B-cells. These findings suggested that dGuo analogs would be selectively toxic to T-cells and thereafter araG, which was synthesized already in 1964, obtained more attention. However,

[1] In a joint collaboration with Dr. Rosalba Carrozzo, Unit of Molecular Medicine, Children's hospital, Rome, Italy, and Dr. Michio Hirano, Department of Neurology, Colombia University College of Physicians and Surgeons, New York, USA.

because of its difficult chemical synthesis and its poor solubility properties it took until 1995 before a prodrug of araG, nelarabine, entered clinical trials.

Mechanism of action

9-β-D-arabinofuranosylguanine, araG, is a deoxyguanosine analog that is a substrate of both dGK and dCK. Although araG is a very poor substrate of dCK as compared to dGK [19], the relative importance of dGK and dCK for araG phosphorylation *in vivo* is not known. However, it was recently shown *in vitro* that dGK was the predominate enzyme at low araG concentrations, whereas dCK was the preferred enzyme at higher araG concentrations [149]. T-lymphoblasts have high expression of dCK and therefore also a high capacity for phosphorylation of dGuo [150, 151]. The accumulation of dGTP results in inhibition of DNA synthesis and subsequent cell death [152, 153]. Deoxyguanosine itself is not suitable for selective treatment since it is a good substrate for PNP. Its analog araG was found to be relatively resistant to hydrolyses by PNP and the accumulation of araGTP independent of the degradation of araG, which raised the question if this analog would be selectively toxic to T-cells [154].

Studies have shown that araG has higher toxicity to T-lymphoblasts than to B-lymphoblasts [155, 156]. The prominent efficiency of araG in T-lymphoblasts as compared to B-lymphoblasts has been attributed both to decreased catabolism of araGTP and to a higher expression of dCK in T-lymphoblasts compared to B-lymphoblasts [155, 156]. Recently, Rodriguez and colleagues published a study in which differences of effect of araG were analyzed in T-lymphoblast (CEM), B-lymphoblast (Raji) and myeloid (ML-1) cell lines [157]. The pharmacokinetics of araGTP accumulation was shown to be favorable but not the only determinant for T-lineage specific cytotoxicity. The incorporation of araGMP into replicating DNA initiated S-phase specific cell death and not S-phase arrest, with possible repair of the damage caused by araG, as in the other lineages. It has been shown before that araGTP can substitute for dATP in the apoptosome [158] and therefore the signal for apoptosis was suggested to be enhanced by cytosolic araGTP by binding to the apoptosome complexes, and finally transcriptional and translational up-regulation of soluble FasL was only seen in the T-lineage and was also suggested to account for the S-phase independent cell death. However, the clinical effect of the prodrug of araG, nelarabine, has demonstrated anti-cancer effect also in a small number of patients with indolent leukemia and recent *in vitro*

studies have also shown sensitivity to araG in B-chronic lymphocytic leukemia [159, 160]. Once phosphorylated to its triphosphate derivative, araGTP acts as a structural analog of dGTP and is thereby incorporated into DNA [161]. The mechanism of action of araG is not fully understood. It is hypothesized that a high accumulation of araGTP in circulating leukemia cells corresponds to a good prognostic value, and it is likely that araGTP is the only cytotoxic metabolite [115, 161]. It has been demonstrated that the accumulation of araGTP is independent of the cell cycle [57], which is not surprising since araG's key enzymes dCK and dGK are expressed throughout the cell cycle. Furthermore, it has been suggested that araG exerts its cytotoxic action by inducing apoptosis and that incorporation of araGMP into nuclear DNA is a critical event for triggering apoptosis [57]. We have seen in several studies that araG can be incorporated into mtDNA [162-164] but we do presently not know to what extent the mitochondrial incorporation contributes to the cytotoxic action of the analog. A recent study by Arpaia *et al.* has suggested a role of mitochondria in the cell specific toxicity of dGTP with intra-mitochondrial accumulation of dGTP and inhibition of mitochondrial DNA repair [165]. We have shown that the acute cytotoxicity of araG is not caused by mitochondrial DNA damage [163- paper II] which supports the hypothesis that incorporation of araG into nuclear DNA is necessary for toxicity or that araGTP has other effects on cellular functions.

Clinical use

In 1991, it was reported that araG was an effective agent for chemoseparation of malignant T-lymphoblasts from human bone marrow [166]. AraG has poor water solubility and its clinical use was for a long time hampered due to lack of a more soluble prodrug. In 1995 it was shown that the prodrug 2-amino-6-methoxypurine arabinoside or 506U that could be synthesized enzymatically from diaminopurine arabinoside [167] was 8-fold more soluble than araG making administration of high drug concentrations possible at physiological pH and much lower volume than would be required for araG [168]. 506U and araG had the same selectivity *in vitro* to T-cells and did not inhibit B-cell growth in the concentrations tested (IC_{50}>100μM). 506U was shown not to be a substrate for dCK or PNP but for dGK that phosphorylated it at a rate of 4% that of araG phosphorylation. Furthermore, 506U was shown to be rapidly converted to araG through demethoxylation by ADA [168]. Based on the accumulated data of 506U a phase I trial was initiated in patients with relapsed and refractory hematologic malignancies [115]. 54% of the patients in the trial achieved a partial or

complete remission after one or two cycles of drug treatment, with major efficacy in T-cell diseases. In patients who achieved a complete or partial remission the peak levels of araGTP was 3-fold higher in comparison to patients who failed therapy [115]. One patient with B-cell chronic leukemia achieved a partial remission from the treatment. The result encouraged further studies with nelarbine, the registered name for 506U, also in indolent leukemia and so far one strategy to potentiate the effect of nelarabine through increase of cellular araGTP has been tested. Clinically achievable levels of F-araATP (fludarabine) can mediate a decrease in deoxynucleotides and especially of the dCTP and dGTP pools, which may result in a decreased feedback inhibition of dCK and dGK, respectively [169, 170]. These enzymes are involved in the key activating step of araG and F-araA and therefore a trial was conducted with the combination of nelarabine and fludarabine against indolent leukemia as well as other types of leukemia. The therapy proved to be effective and well tolerated [171]. A conclusion from this trial was also that there is a need for further evaluation of nelarabine in the treatment of patients that are refractory to fludarabine, since response was seen in some of the fludarabine refractory patients. Nelarabine has also been evaluated as a single agent and has demonstrated clinical efficacy as such in the setting of indolent leukemia [172].

Toxicity and resistance

The dose-limiting toxicity in the clinical trials with nelarabine has been neurotoxicity [173]. Although not as pronounced, adverse effects also include myopathy, myelosuppression and loss of peripheral sensitivity, similar to the symptoms of drugs causing mitochondrial toxicity [110, 174]. We show in Paper II that the acute cytotoxicity of araG is not caused by mtDNA damage. However, it cannot be excluded that long-term exposure to araG may cause mtDNA alterations with subsequent delayed mitochondrial toxicity. Recently published results indicate that mutations in the genes coding for dGK or TK2 are associated with mitochondrial DNA depletion [175, 176]. Since the main supply of deoxyribonucleotides for mitochondrial synthesis comes from the salvage pathway and the rate-limiting step in this pathway is the phosphorylation by dGK or TK2, it is likely that dGK and TK2 are involved in the maintenance of balanced mitochondrial deoxyribonucleotide pools. Although we have shown that araG is predominantly incorporated into mtDNA the contribution of dGK for the cytotoxicity of araG *in vivo* is not known.

So far nelarabine has only been tested in patients that have been refractory to other treatments. The spectrum of different responses to nelarabine reflects the intrapatient variability and also the treatments they have been exposed to before nelarabine was introduced. To my knowledge, no study to reveal the mechanism of resistance to nelarabine has been performed with clinical samples yet. However, several studies on mechanisms of resistance to araG have been performed in cell lines *in vitro.* These studies have shown partly conflicting results as to the molecular mechanism of resistance. Fridland *et al.* reported araG resistance associated with loss of dCK activity [155], whereas araG resistance has been reported to occur with retained dCK activity in other cell lines [161, 177, 178]. In our studies we found that araG resistance can occur by two separate molecular mechanisms that can occur sequentially. The first mechanism is associated with a decrease of araG incorporation into mitochondrial DNA and the second event is associated with loss of dCK activity [162]. In a recent study by Lofti et al. it was also suggested that loss of dCK activity is associated with a higher level of resistance to araG [110, 178].We do not yet know how the decreased incorporation of araG into mtDNA contributes to the resistant phenotype, but from a recent study we know that araG does not cause mtDNA depletion or altered translation of mtDNA encoded genes [163]. Since resistance to nucleoside analogs most often is due to several molecular mechanisms there is a need for further exploration in this area.

The present investigation

Aim of the project

This study was conducted to increase the knowledge about the phosphorylation of nucleoside analogs by mitochondrial kinases with a focus on how the kinases contribute to the cytotoxicity of the nucleoside analogs. We have primarily studied the molecular targets of araG, a substrate of both dGK and dCK. Secondary objectives were therefore to increase the knowledge of the mechanism of action of araG and how resistance to araG is acquired *in vitro*. We have also studied the human mitochondrial inorganic pyrophosphatase with an aim to find out if it is essential for maintained mtDNA integrity.

Specific investigations

- Generation of araG resistant cells and determination of their sensitivity to different nucleoside analogs
- Determination of the phosphorylating activity in crude cell extracts in the wild type and the araG resistant cells for araG, CdA, CdA+dCyd, araT and dThd
- Measurement of the extracellular uptake of araG in wild type and araG resistant cells
- Qualitative determination of the incorporation of araG, araC, araT and dThd into mitochondrial and nuclear DNA in CEM cells
- Quantification of mtDNA in CEM cells after exposure to araG, EtB or ddC
- Determination of the level of expression of the mtDNA encoded cytochrome c oxidase subunit II in CEM cells after exposure to araG, EtB or ddC
- Determination of the sensitivity to araG, araC or vinblastine in CEM cells pretreated with either EtB or ddC to reduce the mtDNA in the cells, using FACS analysis
- Determination of activation of caspases 2, 3 and 9 in CEM cells after exposure to high and low concentrations of araG
- mRNA expression measurements in the three araG resistant cell lines and the wild type CEM cell line by microarray studies
- Cloning and expression of the *Dm.*UMP-CMPK and PPase-I and -II
- Determination of enzyme activity and substrate specificity of *Dm.*UMP-CMPK by high performance liquid chromatography and thin layer chromatography

- Determination of subcellular localization of the enzymes *Dm.*UMP-CMPK and PPase-I and -II fused to the green fluorescent protein, by transfection and subsequent microscopy after staining with mitotracker red and DAPI
- Determination of expression pattern of *Dm.*UMP-CMPK mRNA during different stages of development by using the life cycle microarray data by Arbeitman *et al.* [179]
- Determination of hydrolysis of pyrophosphate and inhibition of hydrolysis of pyrophosphate by addition of Ca^{2+} ions, using a malachite green colorimetric assay
- Determination of expression pattern of PPase-I and -II by northern blot analysis

Comments on methods

The methods used in paper I to V are described in the materials and methods section of each paper. Basic biochemical and molecular biology techniques have been used as described in standard textbooks. The methods of autoradiography, depletion of mtDNA and measurement of PPase hydrolysis of PP_i are, however, described below since these methods have been modified and developed for the presented applications.

Autoradiography

Cells can incorporate radiolabeled nucleosides or nucleoside analogs into DNA. To discriminate between incorporation into nuclear or mitochondrial DNA two methods can be used. One is to measure the relative amount of radioactive compound in separated nuclear and mitochondrial DNA. This method involves a complicated separation procedure. The other method is autoradiography, by which isotope incorporation in nuclear or mitochondrial DNA can be determined qualitatively by specific localization of grains in the nuclear area or in the cytoplasm. The only organelles outside the nucleus of mammalian cells that contain DNA are the mitochondria and thus the grains located in the cytoplasm corresponds to the incorporation of radiolabeled nucleoside or nucleoside analog in mitochondrial DNA. This method is useful to determine the location of nucleoside analog incorporation but it is not possible to use this method to quantify the incorporation. This is mainly due to that [^{3}H]-β-particles have very low energy and some of the β-particles will therefore not reach the emulsion during the development. Thus, the grain densities do not represent the amount of radioactivity incorporated into DNA.

Sensitivity to nucleoside analogs in mtDNA depleted cells

We first tried to create rho0- CEM cells according to the procedures that have previously been used for creating rho0- cells [180]. Although we grew our lymphoblastoid cells in the presence of necessary additives for cells lacking mtDNA (sodium pyruvate, uridine and high glucose levels) the cells died after four to five weeks in the presence of EtB or ddC. We discussed this problem with other researchers and found that no one had succeeded in making a rho0- T-lymphoblastoid cell line. It is possible that the mtDNA is necessary for life in these cells. Instead of using cells completely devoid of mtDNA we chose to decrease the amount of mtDNA with EtB or ddC before testing the sensitivity to araG, araC and vinblastine. After four days of exposure to subtoxic concentrations of ddC or EtB the amount of mtDNA in the cells was less than 20 % compared to the wild-type cells, as determined by slot-blot experiments. We therefore chose to preincubate the CEM cells with EtB or ddC for four days before testing the sensitivity to other drugs. During the experiment the cells were added EtB or ddC together with the drug of interest and also sodium pyruvate, uridine and extra glucose every other day. Since these cells did not have functional mtDNA it was not advisable to use MTT or XTT assays, which depend on mitochondrial dehydrogenase, to determine the IC50-values. Instead we performed FACS analysis to distinguish live from dead cells. We added a nucleic acid stain (SYTOX green) that stains the nucleic acids of dead cells with fluorescence bright green when excited with 488 nm spectral line of the argon laser. Debris was gated out of the forward scatter versus side scatter dot plot and for each drug concentration 10 000 events were counted. The cells that were SYTOX green negative within the gate were regarded as living cells.

PPase catalyzed hydrolysis of pyrophosphate

Pyrophosphate is generated as a byproduct of many metabolic processes that occur inside the cells. Pyrophosphatases hydrolysis PP_i into inorganic phosphate, and we measured the hydrolysis by the recombinant human PPase-I and -II *in vitro* by using a malachite green colorimetric assay, as previously described by Baykov *et al.* [181]. The procedure is based on the finding that the malachite green dye is easily soluble and stable in the presence of 6N acid. In our assay we used 2M H_2SO_4 to immediately stop the enzymatic activity during of the PPases in our assays. All necessary reagents are combined in one concentrated solution of malachite green, H_2SO_4, ammonium molybdate and tween 20, making the assay sensitive and convenient. The addition of Tween 20 is required to stabilize the dye-phosphomolybdate complex at phosphate concentrations above 10μM. The time of color development at 25°C is

about 3 min and thereafter the absorbance can be measured at 620 nm and compared to a NaH_2PO_4 concentration standard. The data was then analyzed and fitted to Michaelis-Menten plots using Statistica (StatSoft).

Summary of papers I-V

The results on which this thesis is based are presented and discussed in detail in papers I through V. Here are only short summaries of the papers presented.

Paper I

Dual mechanisms of 9-B-D-arabinofuranosylguanine resistance in CEM T-lymphoblast leukemia cells, Sophie Curbo, Magnus Johansson, Chaoyong Zhu, Jan Balzarini and Anna Karlsson, Biochem. Biophys. Res. Commun. 285: 40-45, 2001.

In the past years there have been several reports showing partly conflicting results as to the molecular mechanism of resistance to araG. In this study we were able to provide a possible explanation to the somewhat divergent results when we showed that araG resistance can occur by dual mechanisms. We created araG resistant CEM cell lines and characterized them. The sensitivity of two araG resistant cell lines, CEM/araG-1 and CEM/araG-2, to several nucleoside analogs was determined and compared to the wild type CEM cells. The two araG resistant cell lines were both cross-resistant to the guanosine analog dFdG and the thymidine analog araT, but only the CEM/araG-2 cells were also cross-resistant to the cytosine analogs araC and dFdC. We also measured the total nucleoside kinase activity in crude protein cell extracts and found that the araG-, dThd- and CdA+dCyd- phosphorylating activities were similar in the wild-type and the resistant cell lines. However, the CdA phosphorylating activity, without the dCK substrate dCyd, was markedly decreased in the CEM/araG-2 cell line. Since this cell line also exhibited cross-resistance to the cytosine analogs, it strongly suggested that these cells were deficient in dCK activity. In order to find out what caused the resistance in the CEM/araG-1 cells we measured the uptake of radiolabeled araG into the cells, but there was no difference between the CEM/araG-1 cells and the wild type cells. We performed autoradiographs with radiolabeled compounds. The radiolabeled dThd and araC were efficiently incorporated into the nuclear DNA in both the wild type cells and in the CEM/araG-1 cells. AraG and araT, both substrates of mitochondrial enzymes, showed a dotted autoradiography pattern distributed throughout the entire cells, indicating incorporation

of the nucleoside analogs into the mtDNA. However, the level of incorporation of these analogs was markedly lower in the CEM/araG-1 cells than in the wild type cells. Since the CEM/araG-2 cell line that exhibited decreased sensitivity to cytosine analogs and decreased dCK activity also exhibited a higher level of resistance to araG and dFdG, we hypothesized that both araG resistant cell lines had acquired resistance to araT and araG by a single molecular mechanism and that the CEM/araG-2 cells in addition had acquired araC and dFdC resistance by another mechanism. We tested this hypothesis through continued culturing of the CEM/araG-1 cells in an increased concentration of araG and found that the CEM/araG-1 cell line obtained a higher level of resistance to araG and dFdG and also that the cells had acquired cross-resistance to the cytosine analogs araC and dFdC. In summary, we found through autoradiography that the first level of resistance to araG was associated with a decreased incorporation of araG into the mitochondrial DNA (mtDNA) and that the second level of resistance to araG was associated with decreased sensitivity to cytosine analogs and decreased dCK activity.

Paper II

Effects of 9-β-D-arabinofuranosylguanine on mitochondria in CEM T-lymphoblast leukemia cells, Sophie Curbo, Boris Zhivotovsky, Magnus Johansson and Anna Karlsson, Biochem. Biophys. Res. Commun. 307: 942-947, 2003.

We have shown that araG is predominantly incorporated into mtDNA. Because *in vitro* data suggested that mtDNA could be a target of araG and the side effects in patients treated with nelarabine are similar to the symptoms of mitochondrial toxicity, we investigated the role of mitochondria in araG toxicity. Several nucleoside analogs incorporated into mtDNA such as AZT and ddC cause depletion of mtDNA and we therefore quantified mtDNA after exposure to subtoxic concentrations of araG, ddC or ethidium bromide (EtB). The mtDNA content was not altered after araG treatment, but similar to previous reports the mtDNA content was markedly decreased when the cells had been exposed to ddC or EtB. We determined the expression levels of the mtDNA encoded cytochrome c oxidase subunit II (COX II). However, no decrease in the COX II expression could be detected in the cells incubated with araG whereas the cells incubated with ddC and EtB had distinctly lower expression of COX II. We tried to create rho0- CEM cells according to previously used protocols, but the cells could not live without the mtDNA and died after about four weeks in the presence of EtB and

the additional uridine, sodium pyruvate and extra glucose. In an attempt to elucidate the role of mtDNA for araG we preincubated the cells with EtB for four days, which made the cells lose approximately 80% mtDNA, and subsequently tested their sensitivity to araG. The cells' sensitivity to araG was markedly decreased, but these cells were also insensitive to araC, known to exert its action via nuclear DNA, why this phenomenon could not be explained by the decreased mtDNA content. To investigate if the mitochondria-dependent apoptotic pathway was activated upstream of the effector caspase-3 when exposed to high and low concentrations of araG the activities of the initiator caspases-2 and -9 as well as the execution caspase-3-like enzymes were measured. The sequence of caspase activation could not be determined in our experimental system, but it was clear that araG induces apoptosis in a caspase-dependent manner. In summary, we demonstrated that the acute cytotoxicity caused by araG in CEM cells is not mediated through mtDNA damage.

Paper III

Screening for differences in gene expression in 9-β-D-arabinofuranosylguanine resistant cell lines using microarray profiling, Sophie Curbo, Magnus Johansson and Anna Karlsson, Manuscript.

We have previously shown that araG gets incorporated into mtDNA and that one mechanism of resistance to araG is associated with decreased incorporation of araG into mtDNA, but we still do not know the pharmacological importance of the mtDNA incorporation. In paper I we demonstrated three araG resistant cell lines (CEM/araG-1, -2 and -3) generated from acute T-lymphoblastic CEM cells (CEM/0). CEM/araG-1 exhibited decreased incorporation of araG into mtDNA and when this cell line was selected further with increasing concentrations of araG into CEM/araG-3, it became resistant also to deoxycytidine analogs such as araC and dFdC. Although selected in the same way as CEM/araG-1, the CEM/araG-2 cells were cross-resistant to araC and dFdC. The acquired data suggested that araG resistance had occurred with different molecular mechanisms in CEM/araG-1 and -2, and that CEM/araG-3 besides from having acquired resistance to dCyd analogs also exhibited the same molecular alterations as the CEM/araG-1 cells that it was generated from. To investigate the changes in the cell lines at a transcriptional level microarray analysis was performed. The CEM/0 cell line was used as baseline. In the search for changes in gene expression correlated to the decrease of araG incorporation into mtDNA only genes that were up-or down-regulated at the

same time in both CEM/araG-1 and -3 but not in CEM/araG-2 were studied. Nine genes were picked up that followed the criteria and were up-or down-regulated by at least a 2-fold. The genes that were down-regulated have different biological functions such as proteolysis, peptidolysis, peptidase activity, hydrolyzing of tRNAs and aldehyde metabolism. The genes that were up-regulated are also involved in different biological functions such as DNA repair, transcription regulation, cell signaling, development, and intermediate filament assembly. It cannot be excluded that these genes were picked up just by chance. However, since genes that were altered in all cell lines were disregarded and only genes that were up-or down-regulated at the same time in CEM/araG-1 and -3 but not in CEM/araG-2 were considered, the genes picked up by chance are likely fewer than if only one comparison had been made. To be able to tell how and if these genes are involved in araG resistance the data need to be confirmed and investigated further, but these data provide a platform for continued research.

Paper IV

The *Drosophila melanogaster* UMP-CMP kinase encodes and N-terminal mitochondrial targeting sequence, Sophie Curbo, Marjan Amiri, Fariba Foroogh, Magnus Johansson and Anna Karlsson, Biochem. Biophys. Res. Commun. 311: 440-445, 2003.

Cells of *Drosophila melanogaster* express a multi-substrate deoxyribonucleside kinase with broad substrate specificity accepting both purines and pyrimidines. The subsequent phosphorylation step is catalyzed by nucleoside monophosphate kinases, however, little is known about these enzymes in *Drosophila*. We identified, cloned and characterized the *D.melanogaster* homolog of the human UMP-CMPK (*Dm.*UMP-CPMK). The enzyme was recombinantely expressed and its activity was tested. With ATP as phosphate donor *Dm.*UMP-CPMK phosphorylated CMP, dCMP, UMP, dUMP and AMP. However, CMP and dCMP were better substrates than UMP and dUMP and the catalysis of AMP phosphorylation was very low compared to the other substrates. The kinase is encoded by the *dak1* gene and it is approximately 60% identical to the human UMP-CMPK. Expression analysis showed that the *Dm.*UMP-CMPK mRNA was constitutively expressed throughout the *Drosophila* development. The 1124 bp cDNA was completely sequenced and contained an open reading frame (ORF) of 196 amino acid residues corresponding to a protein with a predicted molecular weight of 21.8 kDa. The ORF contained an extended 5'-region compared to the UMP-CMP kinases of other species. This extended region encoded an N-terminal sequence

with properties characteristic of a mitochondrial import signal. When fused to the green fluorescent protein the *Dm.*UMP-CPMK localized to the mitochondria. This is the first time a mitochondrial pyrimidine nucleoside monophosphate kinase has been cloned from any organism, raising the question if there are more mitochondrial pyrimidine monophosphate kinases to be discovered.

Paper V

Identification and cDNA cloning of the human mitochondrial inorganic pyrophosphate, Sophie Curbo, Leila Kanni, Anna Karlsson and Magnus Johansson, Manuscript.

Pyrophosphatases (PPase) catalyze the hydrolysis of inorganic pyrophosphate generated in several enzymatic reactions inside the cells. The human cytosolic PPase has been cloned and shown to be essential for growth. Although biochemical properties of mammalian mitochondrial PPase have been studied on enzyme purified from tissues the gene encoding the enzyme has not been cloned before. Since the yeast mitochondrial PPase has been shown to be necessary for maintained mtDNA integrity it is possible that the human mitochondrial PPase is necessary for maintained mtDNA integrity as well. As a first step to study the importance of the mitochondrial PPase for maintained mtDNA integrity and involvement in PP_i hydrolysis, the gene was cloned. Based on sequence similarity to other pyrophosphatases the cDNA of the mitochondrial PPase (PPase-II) was identified, cloned and characterized. It encoded a 334 amino acid protein ˜60% identical to the human cytosolic PPase (PPase-I). PPase-II was enzymatically active and catalyzed hydrolysis of pyrophosphate with a similar rate as PPase-I. A functional N-terminal mitochondrial presequence was identified in the N-terminus of PPase-II, targeting the enzyme fused to the green fluorescent protein to the mitochondrial matrix. The human PPase-II gene was mapped to chromosome 4q25 and its 1.4kb mRNA was ubiquitously expressed in human tissues with highest levels in tissues rich in mitochondria such as muscle, liver and kidney. We have not found any disease with features typical of mitochondrial abnormalities mapped to this location. However, since the majority of gene defects causing mtDNA depletion syndrome, a heterogenous group of diseases characterized by reduction in mtDNA copy number, are unknown PPase-II is an interesting candidate gene for this

type of syndrome. Material from patients with unknown cause of mitochondrial DNA depletion syndrome is presently being screened for mutations in the PPase-II gene.

Conclusions

- AraG is predominantly incorporated into mtDNA. However, araG does not induce acute mtDNA damage although it can not be excluded that long-term exposure to araG may cause mtDNA alterations with subsequent delayed mitochondrial toxicity.

- AraG resistance can occur by two separate molecular mechanisms that can occur sequentially. The first mechanism is associated with a decrease of araG incorporation into mitochondrial DNA and the second event is associated with loss of dCK activity. We do not yet know how the decreased incorporation of araG into mtDNA contributes to the resistant phenotype, but from a recent study we know that araG does not cause mtDNA depletion or altered translation of mtDNA encoded genes. Taken together with the results from previous studies, it is thus likely that incorporation of araG into nuclear DNA is necessary for acute cytotoxicity.

- The *Drosophila* fly contains a multisubstrate enzyme for the conversion of nucleosides to nucleoside monophosphates, however, it does contain several monophosphate kinases with different substrate specificities for the next phosphorylation step. We identified and cloned the first *Dm*.UMP-CMPK of mitochondrial localization. This finding raises the question if there is also a human mitochondrial UMP-CMPK.

- The human mitochondrial enzyme PPase-II is expressed in the mitochondrial matrix where it hydrolysis inorganic pyrophosphate. PPase-II might be necessary for maintained mtDNA copy number in human cells. Further studies are now being conducted where material from patients with unknown cause of MDS is screened for alterations in the PPase-II gene.

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Appendix: Papers I-V