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**ANTHRACYCLINE  
PHARMACODYNAMICS  
AND  
PHARMACOKINETICS  
IN ACUTE MYELOID  
LEUKEMIA**

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*There is a single light of science, and to brighten it anywhere is to brighten it everywhere.*

*Isaac Asimov (1920-1992)*

To my parents, thank you for everything.....



## ABSTRACT

The goal of this thesis has been to study pharmacodynamics and pharmacokinetics of anthracyclines in acute myeloid leukemia (AML) with the ultimate goal to improve chemotherapy-

**1. By studying the relation between daunorubicin (DNR) and idarubicin (IDA) uptake *in vitro* and *in vivo* and apoptosis *in vitro* in leukemic cells isolated from patients with AML.**

We studied the intracellular concentrations of DNR and IDA and apoptosis in leukemic cells after a one hour pulse incubation with increasing concentrations of anthracyclines. A clear concentration-response relationship was found between intracellular anthracycline concentrations and apoptosis although there was a large interindividual variation. Furthermore, the intracellular concentrations of DNR *in vivo*, directly after DNR infusion, were approximately tenfold lower than the concentrations needed to induce effective apoptosis *in vitro*. A significant correlation was found between *in vivo* intracellular concentrations and clinical remission. We also found a significant relation between apoptosis induction by IDA *in vitro* and clinical remission. The results indicate that the intracellular anthracycline levels *in vivo* are suboptimal and treatment protocols that increase the intracellular levels of anthracyclines should be considered.

**2. By studying DNR metabolism in leukemic cells isolated from patients with AML.** The metabolism of DNR in leukemic cell extracts from 25 AML patients was determined and related to the expressions of carbonyl reductase 1 (CR1) and aldo-keto reductase 1A1 (AKR1A1). We found a large interindividual variation (up to 47-fold) in the leukemic cells ability to convert DNR to its main metabolite daunorubicinol (DOL) and the metabolic rate was significantly correlated with CR1 expression. Zeralone analogue-5, a specific inhibitor of CR1, significantly inhibited reduction of DNR. Our results support that CR1 is the most important enzyme for DNR metabolism in leukemic cells.

**3. By studying the effect of the leukemic cell burden on plasma levels of DNR.** Plasma and mononuclear cells were isolated from 40 patients with AML at the end of DNR infusion, after 5 h, and 24 h after the start of the DNR infusion. We found a weak and significant inverse correlation between the white blood cell count (WBC) and plasma levels of DNR. By using a population based pharmacokinetic model we found a significant correlation between the WBC count and volume of distribution (Vd). This study suggests that the leukemic cell burden lowers plasma levels of anthracyclines although further studies are needed to investigate if patients with a high WBC would benefit from higher doses of anthracyclines.

**4. By comparing the uptake mechanisms of different anthracyclines in leukemic cells.** The mechanisms behind anthracycline uptake are not completely understood. In this study we compared the uptake of five anthracyclines; DNR, doxorubicin (DOX), epirubicin (EPI), idarubicin (IDA), and pirarubicin (PIRA) by leukemic cells and investigated the possible involvement of specific carriers. HL-60 cells were incubated for one hour with the anthracyclines under various conditions and then the cellular uptake was determined. DNR, IDA, and PIRA had the highest intracellular accumulation. The uptake of DOX, DNR, and IDA was significantly reduced at 0° C. Suramin, a purinergic-2-receptor inhibitor, strongly inhibited the uptake of all anthracyclines except PIRA and dipyridamole, a nucleoside transport inhibitor, only inhibited the uptake of DNR. The addition of nucleosides reduced the uptake of DNR, IDA and PIRA. The results of this study indicate that anthracyclines may have different uptake mechanisms. Furthermore, our data also suggest that the uptake might be carrier mediated with a possible involvement of the nucleoside transporter family.



## LIST OF PUBLICATIONS

- I. **Bogason A**, Bhuiyan H, Masquelier M, Paul C, Gruber A, and Vitols S. Uptake of anthracyclines *in vitro* and *in vivo* in acute myeloid leukemia cells in relation to apoptosis and clinical response. *Eur J Clin Pharmacol*. 2009 Dec;65(12):1179-86. Epub 2009 Oct 10.
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- III. **Bogason A**, Quartino AL, Lafolie P, Masquelier M, Karlsson MO, Paul C, Gruber A, and Vitols S. Inverse relationship between leukemic cell burden and plasma levels of daunorubicin in patients with acute myeloid leukemia. *Submitted to British journal of clinical pharmacology*.
- IV. **Bogason A**, Karim H, Bhuiyan H, Lafolie P, and Vitols S. Comparison of uptake mechanisms for different anthracyclines in leukemic cells. *Manuscript*.

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## List of abbreviations

AKR1A1	aldoketoreductase 1A1
ALL	acute lymphoid leukemia
AML	acute myeloid leukemia
AUC	area under the curve
CLL	chronic lymphoid leukemia
CML	chronic myeloid leukemia
CR1	carbonyl reductase 1
CR	complete remission
DNR	daunorubicin
DNRol	daunorubicinol
DOX	doxorubicin
EDTA	ethylenediaminetetraacetic acid
FCS	fetal calf serum
HPLC	high performance liquid chromatography
IDA	idarubicin
NADPH	Nicotinamide adenine dinucleotide phosphate
MNC	mononuclear cells
NBMPR	nitrobenzylthioinosine 5'-monophosphate
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PIRA	pirarubicin
PVDF	Polyvinylidene Fluoride
SDS	sodium dodecyl sulfate
TRIS	tris(hydroxymethyl)aminomethane
WBC	white blood cell

## Introduction

### Description

In the early 19<sup>th</sup> century Virchow noticed that a patient had abnormally high levels of white blood cells not dependant on inflammation and termed this affliction “Weisses Blut”, which is German for white blood. Leukemia, the name now used comes from the Greek words leukos (white) and haima (blood) and also means white blood [1]. Leukemia is a disease in which the normal hematopoiesis is altered and it is characterized by a clonal proliferation of hematopoietic precursor cells. Leukemic cells rapidly accumulate in the bone marrow cavity making the bone marrow unable to produce normal hematopoietic cells resulting in symptoms such as easy bruising, anemia and frequent infections.

Clinically, leukemia can be separated into acute and chronic forms and then based on which cell type is affected. Combining these two classification systems results in four major groups of leukemia:

- *Chronic lymphocytic leukemia (CLL)*
- *Chronic myelogenous leukemia (CML)*
- *Acute lymphoblastic leukemia (ALL)*
- *Acute myeloid leukemia (AML)*

Acute leukemias affect children and young adults but is more common in the elderly whereas CLL almost exclusively affect the elderly. CMLs are rarely seen in children but is rather common in young adults. AML is sometimes also defined as ANLL (acute non lymphocytic leukemia) since it also includes acute promyelocytic leukemia (APL) and acute monocytic leukemia. AML is subclassified according to WHO based on morphological and chromosomal differences. Chronic leukemias differ from the acute leukemias in the sense that they have a slower excessive build-up of mature abnormal blood cells whereas acute

leukemias are associated with a rapid clonal expansion of immature blood cells. This is also reflected in the way the different leukemias are treated, acute leukemias must be treated immediately while the chronic forms can often be monitored for some time in order to determine the optimum therapy. CML often end up in blast transformation, where the immature blood cells grow rapidly, which makes the disease similar to the acute ones. Acute myeloid leukemia is a relatively rare disease with about 320 cases per year in Sweden making it the most common form of acute leukemia in Sweden. The incidence for AML increases with age and culminates between 71-80 years of age [2].

### **Therapy and outcome**

Acute myeloid leukemia is a fast progressing disease that generally requires immediate treatment. The goal of AML therapy is to eradicate all leukemic cells and restore normal hematopoiesis. Generally, treatment of AML consists of two phases: induction and consolidation. During the induction phase the patients are given intense myelosuppressive chemotherapy usually consisting of an anthracycline together with cytarabine. The chemotherapy will induce a period of bone marrow aplasia, as the goal is to obtain a complete remission (CR), a state defined as having less than 5 % blasts in the bone marrow and normal peripheral blood counts. Patients achieving CR have a much improved survival rate and it is a prerequisite for becoming cured. The consolidation phase aims to maintain the CR status and may include transplantation of autologous or allogenic stem cells.

Anthracyclines like daunorubicin (DNR) and idarubicin (IDA) have been used to treat leukemias for over 40 years. They are always co-administered together with cytarabine and this has been the golden standard for AML therapy since the 70s [3, 4].

## **Anthracyclines**

Anthracyclines are a group of compounds that have been widely used for chemotherapeutic purposes since the first anthracyclines, DNR and doxorubicin (DOX), were discovered in the early 1960s. Since then new anthracyclines like epirubicin (EPI) and IDA have been developed and are currently in use in treatment protocols of many different cancers including breast cancer, lymphomas, lung cancer and leukemias. Anthracyclines are potent cytotoxic drugs that are derived from the bacteria *streptomyces peucetius* and they are characterised by their strong red colour as apparent from their nomenclature ending with rubicine derived from rubis, the french word for ruby.

## **Anthracycline mechanism of action**

The target for chemotherapy of malignant disease is the tumor cell. An important factor for therapeutic activity is the grade of exposure of the cells to the drug. Once the drug has reached the tumor cells, other factors like duration of exposure, transmembrane uptake, intracellular retention, heterogeneity of the tumor cells, affinity of the drug for the target, amount of the target intracellularly, amount of competing natural substrates, and metabolic transformation (activation or detoxification) will influence the antitumor effect.

Anthracyclines are amongst the most effective cytotoxic drugs ever developed [5]. The most important reason for their effectiveness apart from their ability to accumulate rapidly in cells is that they have so many ways of destroying the tumour cells which is also the reason for anthracyclines being such a broadspectrum ranged cytotoxic drug. The main mechanisms of action for anthracyclines are thought to be:

- DNA binding, anthracyclines bind strongly to DNA and inhibit the synthesis of macromolecules and DNA replication.

- Generation of free oxygen radicals, leading to both DNA damage and lipid peroxidation which damages the cell membranes.
- Inhibition of topoisomerase II, an enzyme vital for the DNA duplication preceding cell division.

DOX and IDA have also been reported to inhibit topoisomerase I [6, 7]. Furthermore, anthracyclines like many other genotoxic substances, can induce apoptosis through activation of p53 [8, 9], but relations between cellular anthracycline uptake, apoptosis induction *in vitro*, and patient outcome are unclear [10, 11]. Unfortunately, the toxic anthracyclines do not only affect tumour cells as they have a dose- limiting bonemarrow toxicity and a characteristic cumulative cardiotoxic effect that irreversibly leads to congestive heart failure [12, 13]. This is a big problem as it severely limits the clinical use of anthracyclines. The mechanisms behind the cardiotoxicity of anthracycline are not completely understood but their C-13 metabolites generate reactive oxygen species which might be particularly harmful for cardiac cells [12].

Anthracyclines can kill cells by inducing either apoptosis (programmed cell death) or necrosis (uncontrolled/spontaneous cell death) [14]. Apoptosis is characterised as an energy consuming process during which the cells DNA and organelles are systematically broken down to apoptotic bodies which will then be consumed by phagocytes. The entire transformation of a cell to apoptotic bodies occurs in a controlled manner while maintaining cell membrane integrity. Necrosis on the other hand is an energy independent process in which cells break down without maintaining membrane integrity. During necrosis the same chemical signals, as during apoptosis, are not sent out to the immune system which results in that phagocytes are unable to consume the dead cells and an inflammation occurs.

There are many morphological and biochemical ways of detecting apoptosis in cells. Morphological changes of apoptotic cells can be seen as membrane blebbing, nuclei condensation and cell shrinkage while biochemical events include caspase activation, DNA fragmentation and mitochondrial changes [15].

### **Cellular uptake of anthracyclines**

The mechanisms behind cellular anthracycline uptake are not completely understood.

Anthracyclines are generally believed to be transported into the cells through passive diffusion [16, 17] but more recent studies indicate that transport proteins might be involved in cellular anthracycline uptake [18, 19]. Furthermore, evidence for such transport mediated uptake was not found in normal mononuclear blood cells [18] opening up the possibility of different uptake mechanisms in normal and malignant cells. Results from previous studies also indicate that different transport mechanisms could be involved in the uptake of DOX and pirarubicin (PIRA) in Ehrlich ascites carcinoma cells [20], but the specific transporters were not identified. This is why studies on cellular anthracycline uptake using protein inhibitors such as suramin, a purinergic-2-receptor blocker, and nucleoside transport inhibitors such as nitrobenzylthioinosine 5'-monophosphate (NBMPR) and dipyridamole are important to conduct.

### **Anthracycline pharmacokinetics and relations to clinical outcome**

Anthracyclines are lipophilic compounds that distribute rapidly in body tissues, binding to plasma proteins and cell membranes. They have a relatively high volume of distribution (Vd) most of them exceeding 500 L/m<sup>2</sup> and IDA, being the most lipophilic, exceeding 1800 L/m<sup>2</sup> while having a plasma protein binding ranging from 50 to 85 %. Anthracyclines are rapidly

cleared from plasma through liver metabolism with a terminal half-life that ranges from 16-48 hours [21-23] and are excreted primarily through the hepatobiliary route [24].

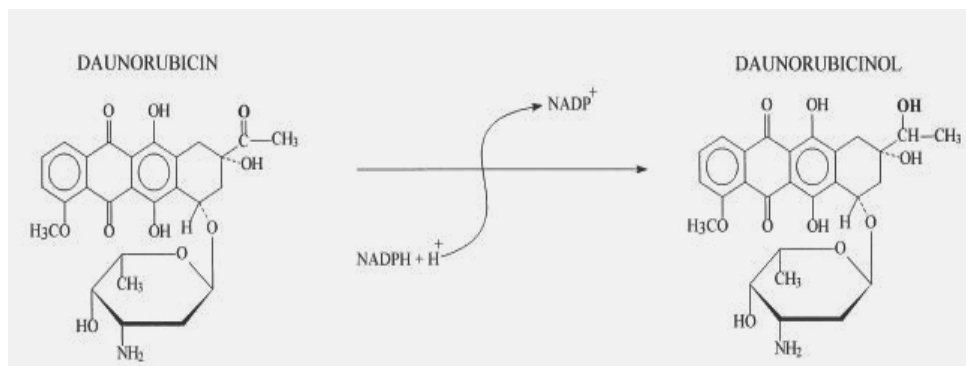
Several pharmacokinetic studies have been performed both comparing different anthracyclines and investigating pharmacokinetic relations to patient outcome [25-29] with varying degrees of success. Palle et al found that children with AML, who entered complete remission, had a significantly higher median plasma concentration of DOX and a lower clearance than those who did not enter complete remission [27]. Kokenberg et al found that the DNR concentration in WBCs correlated with DNR concentrations in bonemarrow nucleated cells and they also found an inverse correlation between cellular AUC of DNR and WBC [28]. Another approach has been to try and find relations between anthracycline induced cell death *in vitro*, cellular anthracycline uptake *in vivo* and patient outcome but despite intensive research only a few investigators have reported correlations between leukemic cell death *in vitro* and patient outcome [30, 31] and reduced cellular DNR uptake and therapeutic failure [32]. There are still several unanswered questions regarding anthracycline pharmacokinetics, cell death, and patient outcome.

Metabolism is an efficient way for the body to defend itself against foreign compounds, e.g. by converting a water insoluble molecule into a more water soluble one and thus facilitating the excretion of the molecule via the urinary or bowel system. Metabolism plays an important role in the elimination of a drug from the human body. Different enzymes are responsible for the metabolism of drugs and sometimes the conversion of a drug into its metabolite can make the metabolite more toxic, as is the case with cyclophosphamide [33]. In AML patients, there is a pronounced inter- and intra individual variation in plasma levels of anthracyclines despite standardized dosing based on body surface area [27, 34]. The variation in pharmacokinetics is most likely due to a variation in systemic metabolism of the drugs [35]. There is still no



consensus on which enzyme is responsible for anthracycline metabolism [12, 36, 37]. There is also no data on whether the AML cells themselves metabolize anthracyclines and possibly contribute to systemic metabolism. Two distinct enzyme superfamilies, aldoketoreductases and short-chain dehydrogenases/reductases, are believed to be responsible for the formation of the major metabolites, the 13-hydroxy derivatives, daunorubicinol (DNRol), idarubicinol, and doxorubicinol [12].

Three enzymes were reported to be capable of anthracycline carbonyl reduction in human liver: aldoketoreductases AKR1A1, AKR1C2, and, the short-chain dehydrogenase/reductase, carbonyl reductase 1 (CR1) [36]. However, AKR1C2 has also been reported not to metabolize DOX or DNR [37]. Currently it is believed that AKR1A1 and CR1 are the major anthracycline metabolizing enzymes and that enzyme specificity might vary with anthracycline type [35, 38-40].



**Fig 1. Daunorubicin carbonyl reduction.**

### **The "inoculum effect"**

The inoculum effect is an expression that is defined as "a significant decrease in the minimum inhibitory concentration of an antibiotic when the number of organisms inoculated is increased" [41] i.e. that the effect of an antibiotic decreases with increasing amounts of bacteria.

AML patients show great differences in WBC at diagnosis ranging from leucopenia to more than 400 million cells per ml blood and if the inoculum effect occurs *in vivo* during anthracycline induction therapy it could have a significant impact on the treatment. Despite great differences in WBC the size of the tumour burden is not taken into account when determining the anthracycline dose for an AML patient, as the dosage is currently standardised based on body surface area. Although WBC has been identified as a prominent risk factor for AML patients in many studies [42] it is not yet completely clear how WBC can affect the pharmacokinetics of DNR [28]. Furthermore, our group has previously shown that DNR cytotoxicity is greatly affected by leukemic cell density *in vitro*, i.e. we have shown that the inoculum effect occurs *in vitro* for DNR in HL 60 cells [43]. The leukemic cells take up so much DNR at higher cell densities that there simply isn't enough DNR for all the cells. This effect also seems to apply for other cytotoxic drugs. In addition to our previous study, other *in vitro* studies have been made comparing the cytotoxicity of different anti-tumoral agents at various cell densities in cell lines [43, 44]. However the inoculum effect does not seem to occur with cisplatin [45] indicating that not all cytotoxic drugs might be susceptible to the inoculum effect. To our knowledge it has not been shown whether or not the inoculum effect occurs *in vivo* in AML patients during DNR treatment.

## **Aims**

The aims of this thesis were to increase the knowledge about the pharmacodynamics and pharmacokinetics of anthracyclines in AML therapy by using both *in vitro* and *in vivo* approaches. The long term goal is to contribute to an optimisation of anthracycline therapy in leukemic patients.

1. To investigate the relation between anthracycline cellular uptake *in vitro/in vivo* and apoptosis *in vitro* in leukemic cells isolated from AML patients and to correlate the findings to patient outcome.
2. To characterize the most important enzymes involved in C-13 reduction of DNR to DNRol in leukemic cells isolated from patients with AML.
3. To study if an “inoculum effect” occurs in AML patients receiving anthracycline induction therapy.
4. To compare cellular uptake mechanisms of five anthracyclines: DOX, DNR, EPI, IDA and PIRA and try to elucidate whether the uptake is protein mediated.

## **Methods**

### **Clinical samples**

Heparinized peripheral blood samples were obtained from altogether 40 patients with newly diagnosed AML treated at the Center for Hematology, in Huddinge and Solna, Karolinska University Hospital. Leukemia was classified according to the French-American-British criteria. Samples were obtained before therapy, directly after, 4h, and 24 h after anthracycline infusion. Post-infusion samples were drawn from a venous line not used for anthracycline infusion. Cells from patients with a high WBC (>30) were saved in portions of 75 million and kept in - 80° C for the metabolic assay and western blot. Plasma and MNCs were isolated at 4°C by centrifugation on 3ml Lymphoprep (d. 1.077 g/ml) (Nycomed, Norway) at 550g for 15 minutes. After 3 washes with PBS the cell number and cell volume was determined using a Coulter counter Z2 (Beckman Coulter, Fullerton, CA, USA) and incubations were carried out directly with 1 ml cells/ml. The studies were approved by the regional ethical committee in Stockholm and informed consent was obtained.

### **Drug analysis**

The mean cell volume was determined with the cell counter as described above which was used when calculating the intracellular concentration. One million cells were lysed in ice-cold water, sonicated for 10 seconds using a ultrasonic processor, VCX 400 (Sonics & Materials, Danbury, CO, USA), and extracted with 60% acetonitrile for DNR and IDA analysis. Prior to the acetonitrile extraction, 75 µl plasma was added to the samples in order to prevent adsorption of IDA and DNR to the plastic tubes. DNR and IDA concentrations in cells and plasma was determined by HPLC using a phenyl-µ-Bondapak column (3.9 x 150 mm, 5 mm. Waters Associates, Milford, MA, USA) eluted with acetonitrile and 0.2% ammonium formate pH 4 (60:40 v/v) at a flow rate of 1.5 ml/min. The drug was quantified using a Shimadzu RF-

551 fluorescence HPLC monitor at  $\lambda_{\text{excitation}}$  485 nm and  $\lambda_{\text{emission}}$  560 nm. The detection limit of the assay is  $5 \cdot 10^{-3} \mu\text{M}$  and the range of quantification is 0,03-20  $\mu\text{M}$ , with a coefficient of variation (CV) of less than 7 %. Cellular drug uptake is expressed in  $\mu\text{M}$ .

### **Metabolic assay**

The carbonyl reduction assay used was previously described by Ax et al [46].

Leukemic cell homogenates were lysed in 50 mM Tris-HCl buffer (pH 7,4, 0,125M KCl, 1,0 mM EDTA) by sonication for 10 seconds using a ultrasonic processor, VCX 400 (Sonics & Materials, Danbury, Co, USA). Protein concentration was determined with the Biorad assay with bovine serum albumine as standard. The experiments were performed in eppendorftubes (1.5 ml) containing 250  $\mu\text{l}$  TRIS-HCl buffer (pH 7,4, 0,125M KCl, 1,0 mM EDTA), 5  $\mu\text{l}$  1 mM DNR and 220  $\mu\text{l}$  of leukemic cell extract diluted to 2.5 mg/ml protein. The tubes were kept in a waterbath at 37°C and the reaction was started by adding 25  $\mu\text{l}$  of NADPH (20 mM in Tris-HCl pH 7.4). The leukemic cell extracts were incubated for 60 minutes after which the samples were extracted in 60 % acetonitrile on ice. This was followed by a HPLC determination of DNR and DNRol.

### **RNA preparation**

Total RNA extraction was performed using Qiagen mini RNA Kit according to manufacturer's protocol. RNA was reverse transcribed into cDNA with a poly(T)<sub>12</sub> protocol. The cDNA was diluted 10 times prior real-time PCR analysis.

### **Quantitative real-time PCR**

Primers for AKR1A1 and carbonyl reductase were ordered from Cybergene® AB, Stockholm, Sweden. Beta-actin (Applied Biosystems, Foster City, USA) was chosen as an endogenous housekeeping control gene. Quantitative real-time PCR was performed using the

ABI 7500 Fast PCR Detection System (Applied Biosystems, Foster City, USA). Reaction mixtures contained 1xPower SYBR® Green PCR master mix (Applied Biosystems, Foster City, USA), 0.25 µM primers, 5 µl cDNA template in a total volume of 25 µl. Thermal cycling conditions included activation at 95° C (10 min) followed by 40 cycles each of denaturation at 95° C (15 sec) and annealing/elongation at 60° C (1 min).

Each reaction was performed in triplicate and no-template controls were included in each experiment. The expression was calculated using the  $\Delta\Delta CT$  formula.

### **Western blot**

We added 250 µl of a protease inhibitor cocktail (Roche diagnostics GMBH) to 75 million cells and lysed them by sonication for 10 seconds using an ultrasonic processor, VCX 400 (Sonics & Materials, Danbury, Co, USA). Protein concentration was determined using the Biorad assay with bovine serum albumine as standard. We loaded 10 µg of protein in each well on a 4-15 % TRIS-glycin gel from Biorad. The gel was run for 1 hour at 130V and the protein transferred to a PVDF-membrane over night at 30V. The membrane was blocked with 5 % dry milk (in washing buffer). The primary antibodies for AKR1A1 and CR1 were obtained from The Abnova corporation®. The secondary antibody used was Goat-anti-Mouse (Dakocytomation®, 1/1000). The primary antibodies used were diluted 1/2000 in washing buffer.

Results

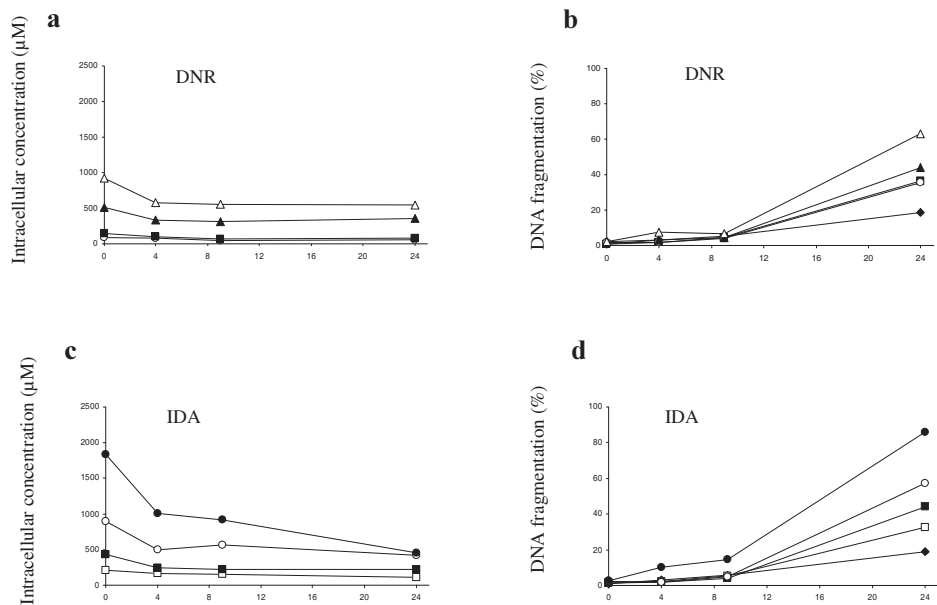
Study 1

This study examined the relationship between intracellular levels of anthracyclines, apoptosis and patient outcome in leukemic cells isolated from AML patients.

Cellular DNR and IDA levels and apoptosis induction

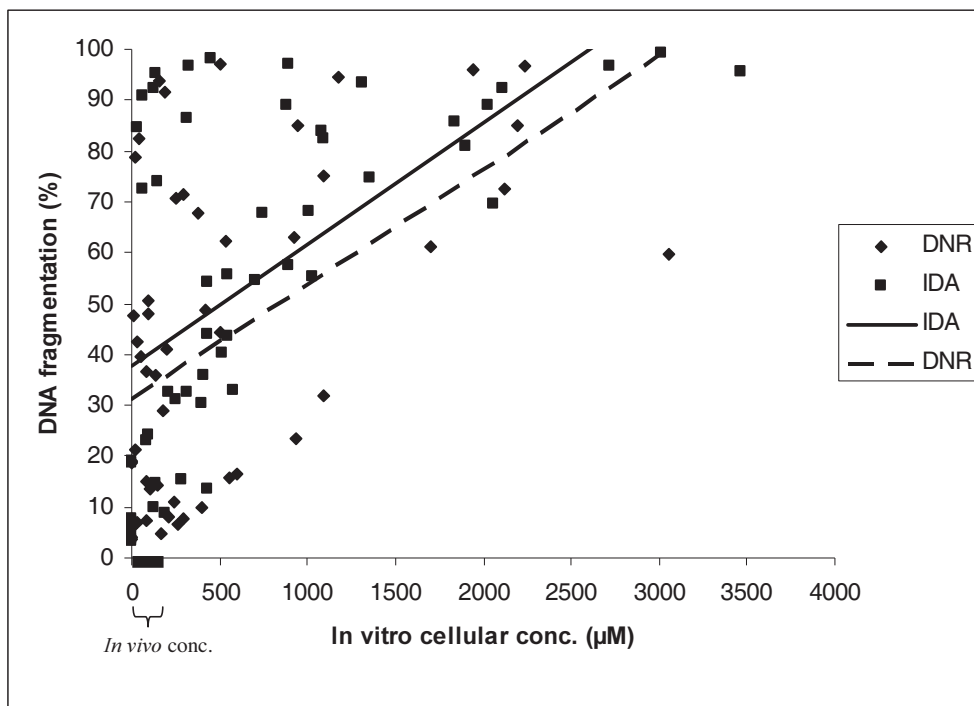
We studied DNR and IDA uptake and apoptosis induction in leukemic cells isolated from 20 patients by incubating the leukemic cells with various DNR and IDA concentrations *in vitro*. There was a great interindividual variation in initial anthracycline uptake and apoptosis induction between patients but a clear concentration-response relationship between drug uptake and apoptosis induction was always present for a given patient (Fig 1).

Fig 1



**Figure 1.** Time course for cellular uptake, retention and apoptosis for DNR (**a, b**) and IDA (**c, d**) in cells from an AML patient after a 1-h pulse incubation with DNR (0(◆) (0.5(■), 1(○), 4(▲) and 8(△)  $\mu$ M) and IDA (0.25 (□), 0.5 (■), 1(○), and 2(●)  $\mu$ M)

Cellular DNR/IDA concentrations  $>1200 \mu\text{M}$  apoptosis, 24 h after the one hour pulse incubation, were always associated with pronounced apoptosis ( $>60\%$ ) (Fig 2). The slopes of the regression lines for DNR ( $n=16$ ) and IDA ( $n=15$ ) were nearly identical, with  $k$ -values of 0.022 and 0.024 respectively. The  $R^2$  values were 0.25 ( $p<0.001$ ) and 0.35 ( $p<0.0001$ ) for DNR and IDA respectively.

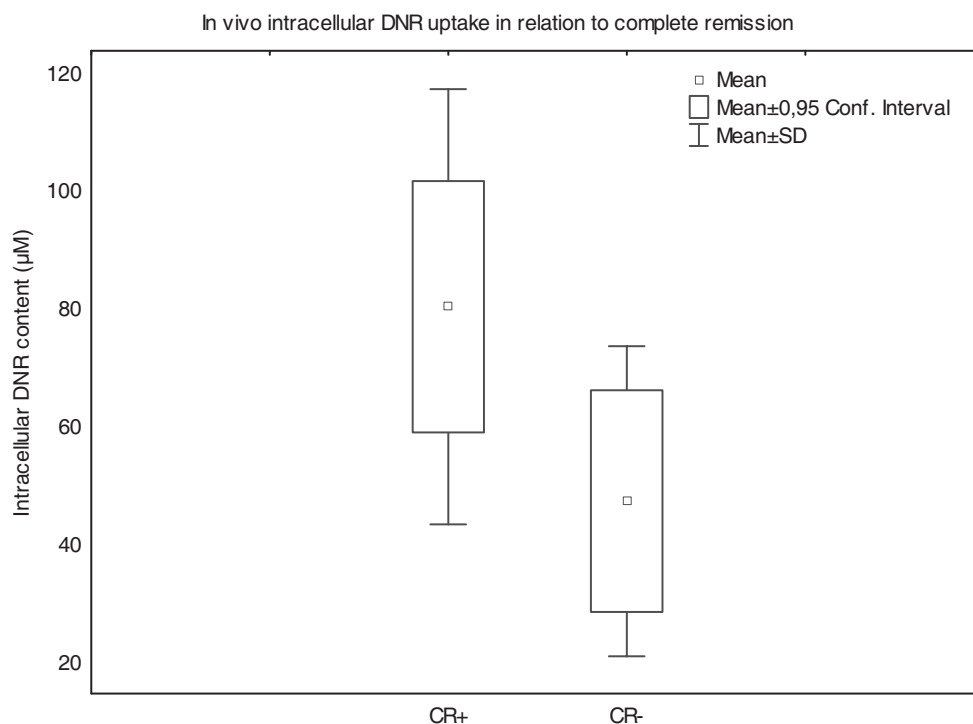




**Figure 2.** Intracellular concentrations of DNR and IDA *in vitro* directly after a 1 h pulse incubation versus apoptosis induction in leukemic cells at 24 h. The cells were isolated from 16 AML patients and pulse incubated for 1 h with 0.5, 1.0, 4.0, and 8.0  $\mu\text{M}$  DNR (n=16) or 0.25, 0.5, 1.0, and 2.0  $\mu\text{M}$  IDA (n=15). The black box covering the range 4-137  $\mu\text{M}$  represents the intracellular anthracycline concentrations found *in vivo* immediately after anthracycline infusion.

### **Cellular anthracycline levels in vivo**

We further analysed the intracellular uptake in leukemic cells isolated from 24 patients undergoing anthracycline induction therapy. We found that the intracellular concentrations, immediately after a 1-hour anthracycline infusion, in all cell samples were roughly > 10 times lower than the intracellular concentration needed to result in a pronounced apoptosis *in vitro*. We also found a significant difference in intracellular levels of DNR between patients who went into complete remission and those who did not (Fig 3). The mean and SD values for *in vivo* intracellular DNR concentrations in CR<sup>+</sup> patients and CR<sup>-</sup> patients were 80,6 +/- 36,9  $\mu\text{M}$  (n=14) and 47,6 +/- 26,3  $\mu\text{M}$  (n=10) respectively (p < 0.05, students t-test).



**Figure 3.** *In vivo* intracellular concentration of DNR in leukemic cells isolated from 24 patients directly after a 1 hour DNR infusion in relation to clinical outcome. CR+, patients achieving complete remission, CR-, patients not achieving complete remission.

#### **Apoptosis induction in vivo in relation to clinical response**

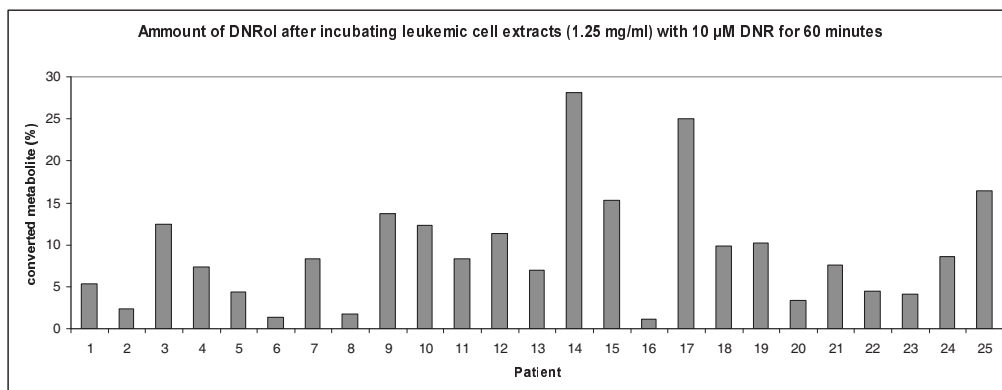
We also investigated whether there was a relation between apoptosis induction *in vitro* and clinical response in 20 AML patients. Apoptosis was measured in leukemic cells 24 h after a 1-h pulse incubation with 0.25 µM IDA and related to clinical response. Patients that received full dose induction treatment and went into complete remission (CR+) were generally more sensitive to IDA *in vitro* compared to cells from patients who did not achieve complete remission (CR-). The mean and SD values for apoptosis in CR+ patients and CR- patients were 43,0 +/- 19,9 % (n=11) and 19,4 +/- 9.5 % (n=5) respectively (p < 0.05, students t-test).

## Study 2

In this paper we determined what enzyme is most important for the carbonyl reduction of DNR in leukemic cells.

### Anthracycline metabolism by leukemic cell extracts

A summary of the metabolic capacity gathered from cell extracts of the 25 AML patients is shown in figure 4. The metabolic capacity showed up to 47-fold interindividual variation and the mean fraction of DNRol formed during one hour was 9,2 % with a SD of 6,8 % (range 0,6-28 %).



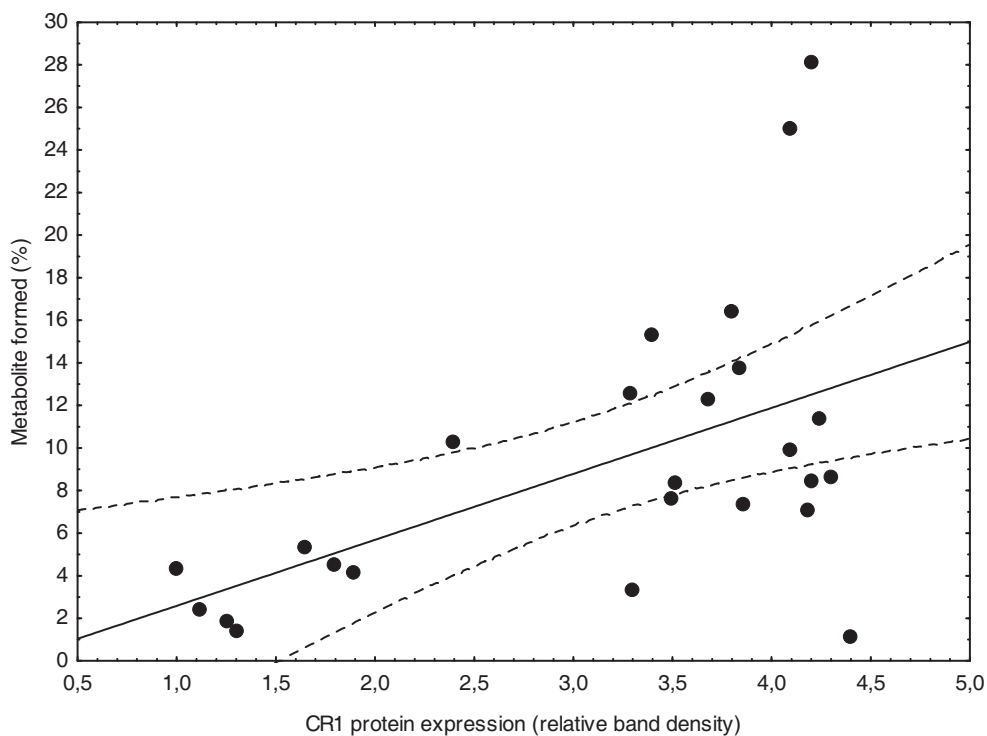
**Figure 4.** Metabolic capacity of leukemic cell extracts (1.1 mg/ml protein) from 25 AML patients measured as % DNRol formed after a 60 minute incubation with 10  $\mu$ M DNR.

### Enzyme mRNA expression in relation to metabolic capacity.

No correlation was found between the mRNA levels for the metabolising enzymes and the metabolic capacity of the cell extracts.

### Protein expression of enzymes in relation to metabolic capacity.

AKR1A1 protein levels showed no correlation with the metabolic capacity of the leukemic cell extracts. However, we found a significant correlation between CR1 expression and DNR metabolism in the leukemic cell extracts,  $p < 0.05$ ,  $R^2 = 0,229$ ,  $n = 25$  (Fig 5).



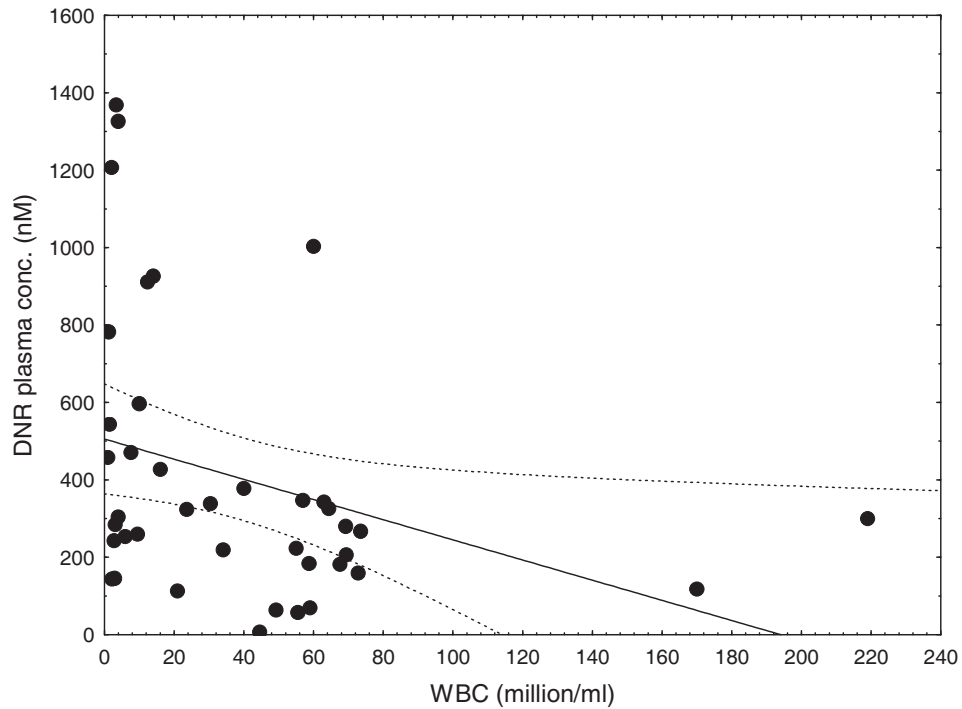
**Figure 5.** Protein expression of carbonyl reductase 1 in relation to % DNRol formed after a 1 hour incubation of leukemic cell extracts (1,1 mg /ml prot) with 10  $\mu$ M DNR. The dotted lines represent the 95 % confidence limits.

### Study 3

In this work we investigated the relationship between WBC and plasma/intracellular concentrations of DNR in patients with AML receiving induction therapy.

### Plasma levels of DNR in relation to WBC

Plasma levels of DNR were studied in 40 patients at 0, 4 h, and 24 h after DNR infusion in relation to the WBC. The plasma levels of DNR decreased in a biphasic fashion showing a pronounced interindividual variability. A weak and significant inverse correlation was found between DNR levels and WBC directly after the DNR infusion,  $R^2 = 0,13$ ,  $p < 0,05$  (Fig 6).



**Figure 6.** DNR plasma concentrations in leukemic patients (n=40) in relation to WBC immediately after a 1 hour DNR infusion. The dotted lines represent the 95 % confidence bands.

### Population pharmacokinetic modelling

We used a population pharmacokinetic model to study the relation between WBC counts and pharmacokinetic parameters. We found a significant correlation between WBC counts and the central volume of distribution (dOFV 4.77,  $p < 0.05$ ), where the central volume of distribution is increased with 1.4% per million cells/ml blood change of WBC count from the mean baseline WBC count (39 million/ml) (Table 1).

Table 1.

Population pharmacokinetic parameter estimates

	Population PK model				Population PK model with baseline WBC			
	Typical estimate (RSE %)		Interindividual variability CV % (RSE %)		Typical estimate (RSE %)		Interindividual variability CV % (RSE %)	
CL (L/h)	115	10	52	21	114	10	51	20
V <sub>c</sub> (L)	373	25	127	26	412	25	120	29
Q (L/h)	252	18			254	20		
V <sub>p</sub> (L)	1120	11			1120	14		
Proportional residual error (%)	52.4	8			51.7	7		
Covariate $\theta_{V-WBC}$					0.0138	47		
Correlation CL-V (%)	70	65*			65	58*		

CV, coefficient of variation; RSE, relative standard error; CL, clearance; V<sub>c</sub>, volume of central compartment; Q, intercompartmental clearance; V<sub>p</sub>, volume of peripheral compartment.

\* =  $p < 0.05$ .

$$V_{c_{WBC,i}} = V_c * (1 + \theta_{Vc-WBC} * (WBC - \text{median}_{WBC})) = 412 * (1 + 0.0138 * (WBC - 39))$$

## Study 4

In this study we investigated and compared cellular uptake mechanisms for different anthracyclines in cultured leukemic cells.

### Dose-uptake studies

Dose-uptake relationships were studied for five anthracyclines; DNR, DOX, EPI, IDA, and PIRA in cultured HL-60 cells. We found that they had similar cellular uptake at extracellular concentrations 0.1-1.0  $\mu\text{M}$  while the uptake of DNR, IDA, and PIRA increased strongly at concentrations  $>1.0 \mu\text{M}$  (Fig 7).

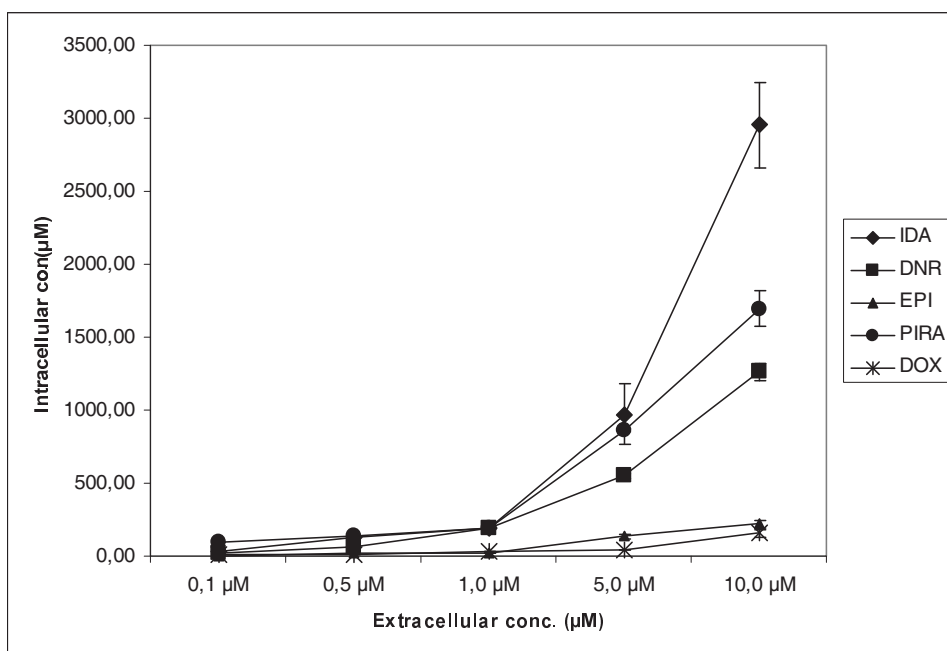


Figure 7. Effect of dose on anthracycline uptake in HL-60 cells after a 1 hour incubation with IDA, DNR, EPI, PIRA or DOX (n=4, mean and SD).

**Effect of temperature and various transport inhibitors on anthracycline uptake.**

We also incubated the cells with anthracyclines under various conditions and found several differences in their ability to enter HL60 cells (Fig 8). PIRA and EPI was not temperature dependent while DNR, DOX and IDA had significantly lower uptake at 0°C. DNR, DOX, EPI, and IDA uptake was inhibited by suramin while dipyridamole inhibited DNR uptake only. NBPMR did not significantly affect the uptake of any anthracyclines studied.



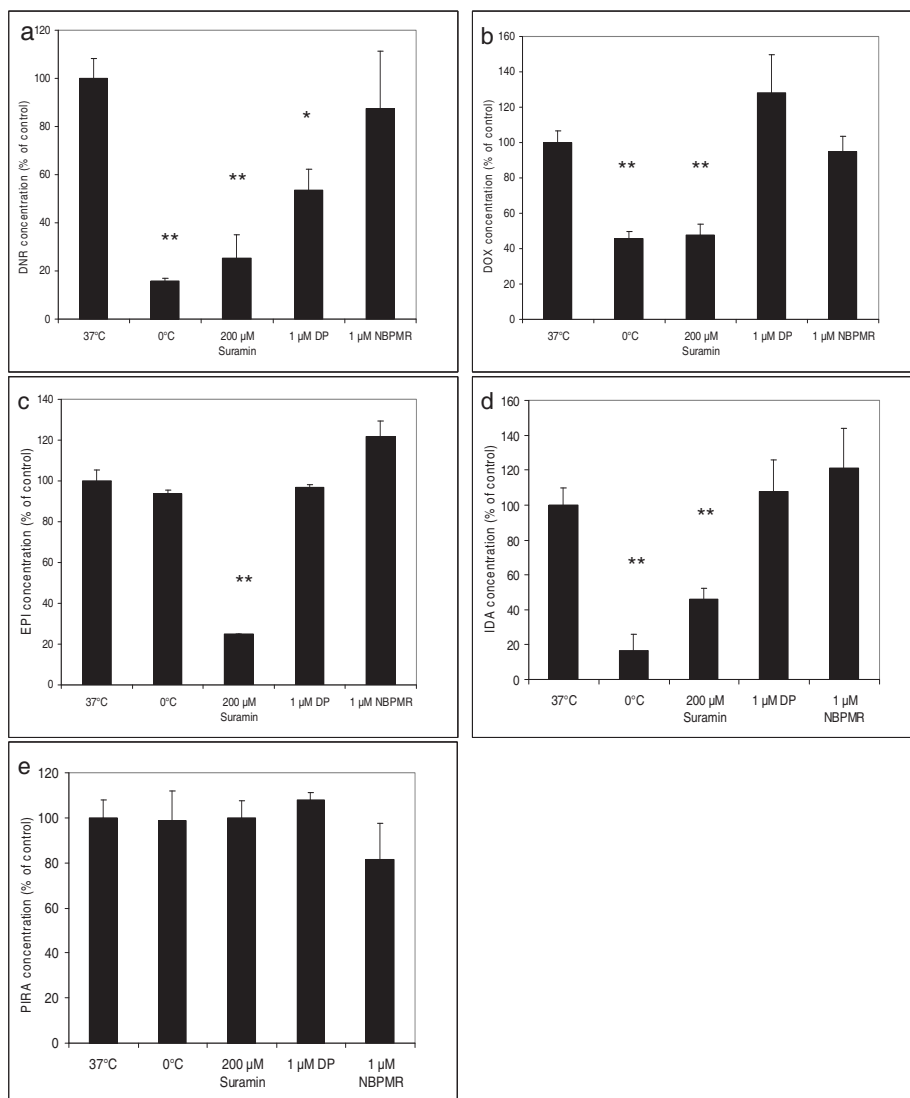


Figure 8: Effect of temperature and various inhibitors on the uptake of 4  $\mu$ M DOX (a), 4  $\mu$ M DNR (b), 4  $\mu$ M EPI (c), 1  $\mu$ M IDA (d), and 1  $\mu$ M PIRA (e) in HL-60 cells after a 1 hour incubation. DP = dipyridamole, NBMPR = nitrobenzylthioinosine 5'-monophosphate (n = 4, mean and SD). \* =  $p < 0.05$  and \*\* =  $p < 0.01$  compared with the control (37°C).

### **Effect of nucleosides on anthracycline uptake.**

The addition of nucleosides inhibited anthracycline uptake to varying degrees. The strongest inhibition was found by adenosine on IDA uptake, by thymidine on PIRA uptake, and by adenosine and cytosine on DNR uptake.

## **Discussion and conclusions**

### **Study 1**

In this study investigated the importance of cellular DNR and IDA uptake in apoptosis induction in leukemic cells isolated from AML patients. We found that apoptosis was generally detectable between 4-9 hours after a 1-hour pulse incubation with DNR/IDA. Although there was a great interindividual difference in cellular uptake we found that there was always a clear concentration response relationship between cellular anthracycline uptake and apoptosis for a given patient. One reason for the interindividual variation in DNR/IDA uptake could be the expression of various protein efflux pumps, e.g. p-glycoprotein and multidrug resistance-associated proteins [47, 48]. Some studies also indicate that nucleoside transport proteins are involved in anthracycline uptake and it can be speculated that a variation in expression of these proteins might also be a reason for the interindividual variation seen in our study (see study 4) [18, 49]. We found that at cellular concentrations above 1200  $\mu\text{M}$ , the leukemic cells always entered pronounced apoptosis. Interestingly, the intracellular concentrations achieved in vivo were roughly 10 times lower than what was needed to induce a pronounced apoptotic response in vitro. Although several studies have investigated a relation between intracellular DNR concentration and patient outcome the results are still inconclusive. In one study Kokenberg et al could not find any relation between intracellular concentrations of DNR and response to treatment [28] while they found a tendency towards higher intracellular DNR concentrations in responders in another study

[50]. In our study, we found that in vivo intracellular concentration correlated with patient outcome which supports the importance of achieving higher intracellular anthracycline concentrations in AML treatment.

## **Study 2**

Anthracycline metabolism can influence therapeutic results in several ways. First and foremost anthracycline metabolism can affect drug exposure leading to great interindividual variation in plasma levels of anthracyclines and thus influence anthracycline therapy directly. Furthermore, it has been implicated in cellular resistance as several of the anthracycline C-13 metabolites are less cytotoxic than their respective mother compound, not including idarubicinol [46, 51]. The C-13 metabolites have also been implicated as the main cause of the characteristic cardiotoxicity of anthracyclines [39, 52, 53]. Several enzymes have been identified which have the ability to convert anthracyclines to their C-13 derivatives [36, 54]. There are studies showing that various anthracyclines have a varying specificity for carbonyl reducing enzymes even though they are structurally similar. In studies on human heart cells it was found that AKR1A1 is the predominant enzyme for DOX metabolism whereas CR1 was found to be the predominant DNR metabolising enzyme [38-40]. Moreover, enzymes responsible for anthracycline metabolism seem to vary with tissue as well since it has been found that AKR1A1 seems to be the predominant DOX metabolising enzyme in human liver [35].

In order to elucidate what enzyme plays the pivotal role in DNR metabolism in leukemic cells we investigated the metabolic capacity of leukemic cell extracts from AML patients and correlated this to protein expression of CR1 and AKR1A1. We found that leukemic cells from AML patients have a pronounced interindividual variation in their ability to metabolise DNR

to its C-13 alcohol metabolite DOL. Furthermore, the metabolic activity of the leukemic cell extracts correlated well with CR1 protein expression but not with AKR1A1 protein expression indicating that CR1 is the enzyme that is most likely to be responsible for DNR metabolism in leukemic cells. This was further supported by the experiment with Zeraleone analogue 5, a specific CR1 inhibitor, that significantly inhibited metabolism in all samples studied. It is noteworthy that several genetic polymorphisms of CR1, that affect the metabolic efficiency, have been identified which could contribute to interindividual variations in DNR metabolism [55, 56].

### Study 3

In this study we investigated if the “inoculum effect” occurs *in vivo* i.e. does WBC affect plasma levels of DNR. We found that WBC indeed affects the plasma levels of DNR in AML patients receiving DNR induction therapy. Other studies have provided evidence that the peripheral blast cell count has an effect on the pharmacokinetics of anthracyclines in ALL and AML. Frost *et al* studied doxorubicin (DOX) pharmacokinetics in children with acute lymphoblastic leukemia and found that patients with WBC counts higher than 50 million cells per ml had significantly lower plasma concentrations of doxorubicin [34]. In other studies Ackland *et al* and Piscitelli *et al* studied DOX pharmacokinetics in patients with various solid cancers. Both investigators found an inverse correlation between the WBC count (in this case not leukemic cells) and the plasma concentration of DOX in patients with normal WBC levels [57, 58]. Although we found an inverse relation between WBC and plasma levels of DNR immediately after DNR infusion we could not find any relationship between cellular DNR levels and WBC. Other investigators have reported a lack of relationship between plasma levels of anthracyclines and cellular drug uptake which could possibly be attributed to a variation in expression of drug efflux pumps and other factors at the cellular level that could

affect cellular uptake of DNR [59-61]. Furthermore, since we could only find a relationship at one time point (immediately after DNR infusion) one could speculate that the inoculum effect diminishes with time due to rapid disappearance of leukemic cells from the blood stream.

#### **Study 4**

In study 4 we compared cellular drug uptake by HL-60 cells and the possible involvement of various transporters/receptors for different anthracyclines. We chose a one-hour incubation at 37° C since previous uptake studies by us and others have shown that a plateau is reached after one hour in leukemic cells [43, 47]. Anthracycline uptake varied markedly and IDA had the highest uptake. One reason for the higher IDA uptake could be its higher lipophilicity but a high uptake could also be explained by the drugs lower affinity for p-gp as compared with other anthracyclines [47, 62, 63]. In a similar study conducted on rat hepatocytes the authors concluded that at low extracellular concentrations the uptake was carrier mediated while the steeper linear increase in IDA uptake at higher extracellular concentrations was due to diffusion. A rationale for why anthracycline uptake would be protein mediated at lower concentrations and diffusion facilitated at higher concentrations was however not given [64]. We found that suramin, a p2-receptor antagonist [65], inhibited the uptake of DNR, DOX, IDA, and EPI, but not that of PIRA, indicating that p2-receptor signaling might somehow be involved in anthracycline uptake. It is noteworthy that IDA, DOX and DNR uptake was temperature dependant in contrast to EPI and PIRA indicating that EPI and PIRA might have an anthracycline uptake mechanism separate from the others. Inhibition of uptake by low temperature supports energy dependent carrier mechanisms. Dipyridamole, an NT-inhibitor, inhibited the uptake of DNR but not the uptake of any of the other anthracyclines supporting that cellular uptake of DNR might be facilitated by a protein belonging to the nucleoside transporter receptor family. That DNR uptake could be mediated by nucleoside transporters

was further reflected by the reduced DNR uptake after addition of adenosine, cytidine, thymidine, and uridine. It has been suggested by others that nucleoside transporters could be involved in DOX and PIRA uptake but carriers for IDA and DNR are yet to be discovered [18]. We found that addition of adenosine inhibited IDA uptake while thymidine and uridine inhibited the uptake of PIRA which was surprising since we could not see any effect of the nucleoside transport inhibitors (DP and NBMRP) on these drugs. The results obtained are complicated to interpret since the anthracyclines studied all had different uptake profiles with the closest common denominator being a reduced uptake by suramin. Nevertheless, the study supports that anthracyclines have different ways of entering leukemic cells and that carriers may be involved regardless of anthracycline. Further studies are needed to more in detail clarify the suggested uptake mechanisms discovered in this study.

## **General conclusions and future perspectives.**

1. A cellular concentration-response relationship for IDA/DNR and apoptosis was always present for a given AML patient.
2. Intracellular concentrations of IDA/DNR above 1200  $\mu\text{M}$  were associated with pronounced apoptosis *in vitro*.
3. Intracellular concentration of DNR directly after infusion correlated with remission induction in AML.
4. The intracellular concentrations of DNR *in vivo* were low as compared to the cellular concentrations needed to induce apoptosis *in vitro*.
5. There was a large interindividual variation in how leukemic cells from AML patients metabolised DNR.
6. CR1 seems to be the major anthracycline metabolising enzyme in leukemic cells.

7. The leukemic cell mass can affect the plasma levels of DNR in AML patients receiving DNR induction therapy.
8. Cellular uptake of anthracyclines by leukemic cells is, at least in part, a protein dependent process.
9. Various nucleoside transporters are likely involved in anthracycline uptake in leukemic cells.

We found that high intracellular concentration of DNR constantly resulted in pronounced apoptosis. It is noteworthy that the intracellular concentrations *in vivo* immediately after DNR infusion were more than 10-fold lower than those required to induce effective apoptosis *in vitro*. Still the majority of patients go into clinical remission. The explanations for this could be that the patients also receive cytosine arabinoside and/or that mechanisms operating *in vivo* modulate the apoptotic response of the leukemic cells. The leukemic cells *in vivo* are also exposed to drug for a longer time period than during our *in vitro* incubation. Nevertheless, infusion protocols that could lead to higher cellular DNR levels should be considered and tested. Possibly infusion times and doses can be altered to result in higher intracellular concentrations with acceptable toxicity. There have been some studies investigating different infusion times for DNR in AML patients but the results are contradictory. In one study it was shown that a 24-hour continuous infusion resulted in a higher accumulation of DNR in the leukemic cells compared to a short time infusion [66] while another study showed that a bolus infusion gave a higher intracellular concentration of DNR than a long term infusion [67]. However, the studies were only performed on a few patients and larger numbers of patients are needed to statistically ascertain whether one infusion protocol gives rise to higher intracellular concentrations as compared to another. Of particular interest is that two recent publications reported that a doubling of the standard dose

of DNR (from 45 mg/m<sup>2</sup> to 90 mg/m<sup>2</sup>) led to a higher response rate without any obvious additional toxicity [68, 69]. It could be speculated that higher intracellular levels were reached but intracellular DNR concentrations were not determined in these studies.

Of interest would be to study how cytosine arabinosid combined with DNR influences the leukemic cells apoptotic response *in vitro* and also to study whether we can detect apoptosis *in vivo* directly after isolation from blood following DNR and cytosine arabinoside infusion. Such studies could help to optimize drug treatment since drug infusions are repeated. A low apoptotic response *in vivo* could advocate a dose increase next cycle. Furthermore, if it would be possible to reliably measure apoptosis *in vivo* it would open up new possibilities to both optimize current chemotherapy and test new chemotherapeutic drugs. Alternatively, if apoptosis is difficult to determine *in vivo*, one could monitor the intracellular concentrations obtained post infusion and use this data to adjust (increase) the following dose. Indeed, in paper 1 we report that patients who went into complete remission had a significantly higher mean intracellular level post infusion than those who did not reach complete remission. Pharmacokinetic studies on a cellular level are important for all types of cancer and a greater knowledge within this area could facilitate the optimisation of other chemotherapeutic regimens.

Anthracycline metabolism is an area under much discussion since the metabolites have been implicated in both drug resistance [46] and cardiotoxicity [12]. Additional studies are needed to investigate the role of cellular metabolism in drug resistance in AML. To our knowledge such studies are lacking. Of particular interest would be to assay cellular metabolism of anthracyclines *in vitro* and relate the results to apoptosis development *in vitro* and also clinical response. Furthermore, since CR1 seems to be an important enzyme for DNR



carbonyl reduction in leukemic cells, and if further studies establish that cellular metabolism of DNR is an important resistance factor, new drugs could be developed that inhibit CR1. It would also be interesting to study whether or not a high metabolic activity in leukemic cells from AML patients with a high WBC count can affect the systemic metabolism of DNR and thus affect the patients exposure for DNR and possibly also contribute to an increased risk for cardiotoxicity. Another approach could be to genotype AML cells which have a high DNR metabolism in an attempt to find polymorphisms responsible for a more efficient CR1. Genotyping could then possibly be used to predict the individual patients clearance and to tailor the dose. Furthermore, their leukemic cells might be more resistant making them good candidates for CR1 inhibition treatment.

That the WBC count could affect pharmacokinetics of anthracyclines has been speculated on for more than 20 years. However, with both old and recent results in mind it would be interesting to see how a DNR dose adjustment for WBC count would affect the treatment outcome for patients with a high WBC count. Indeed it has been shown that AML patients undergoing leukapheresis had significantly better chance for survival the first 21 days post treatment than patients who did not receive leukapheresis [70]. An “inoculum effect” could potentially have more general tumour biological impact if this phenomenon also occurs in the treatment of solid tumours.

The mechanism behind cellular anthracycline uptake has been debated on since they were first introduced and there are still no conclusive evidence as to how anthracyclines are taken up by cells even though a common theory has been that they are taken up through diffusion [17] or through a flip flop mechanism [71]. We found evidence supporting different uptake mechanisms for the anthracyclines and that nucleoside transporters seem to be involved in

anthracycline uptake. This information could be used to further elucidate the proteins that are involved in the uptake mechanisms. Increased knowledge about uptake mechanisms in normal and leukemic cells could in the future be used to selectively increase the uptake in leukemic cells.

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I



## Uptake of anthracyclines in vitro and in vivo in acute myeloid leukemia cells in relation to apoptosis and clinical response

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### Abstract

**Aims** To study anthracycline-induced apoptosis in leukemic cells isolated from patients with acute myelogenous leukemia (AML) in vitro and to compare intracellular anthracycline concentrations causing apoptosis in vitro with those obtained in vivo during anthracycline treatment.

**Methods** Mononuclear blood cells from AML patients were isolated before ( $n=20$ ) and after anthracycline infusion ( $n=24$ ). The pre-treated cells were incubated in vitro with daunorubicin (DNR) and/or idarubicin (IDA). Anthracycline concentrations were determined by high-performance liquid chromatography, and apoptosis was detected by propidium iodine staining using a flow cytometer.

**Results** There was a clear concentration–response relationship between intracellular anthracycline levels and apoptosis albeit with a large interindividual variation. Intracellular levels  $>1200 \mu\text{M}$  always led to high apoptosis development ( $>60\%$ ) in vitro. The intracellular concentrations of DNR in vivo ( $n=24$ ) were more than tenfold lower than the concentrations needed to induce effective apoptosis in vitro, although a significant relation between in vivo concentra-

tions and clinical remission was found. We also found a significant relation between apoptosis induction in leukemic cells by IDA in vitro and clinical remission.

**Conclusions** Our results indicate that intracellular anthracycline levels in vivo are suboptimal and that protocols should be used that increase intracellular anthracycline levels.

**Keywords** Anthracyclines · Apoptosis · Leukemia

### Introduction

Acute myelogenous leukemia (AML) is a blood disorder characterized by the clonal expansion of immature myeloid progenitor cells. Treatment involves intensive chemotherapy to eliminate the leukemic cell population. At the present time, almost all standard induction treatment protocols include anthracyclines, generally daunorubicin (DNR) or idarubicin (IDA) in combination with cytarabine. These achieve a complete remission rate of 50–60% in an unselected population and a long-term survival of only 10–20% [1]. It has been claimed that IDA, which is more potent in vitro, is more efficient than DNR in terms of increasing the complete remission rate, prolonging remission duration, and improving survival in AML [2, 3]. However, the therapeutic superiority of IDA has not yet been established [4].

The target for cancer chemotherapy is the tumor cell. An important factor affecting therapeutic activity is the grade of exposure of the cells to the drug. Once the drug has reached the tumor cells, other factors, such as the duration of exposure, transmembrane uptake, intracellular retention, heterogeneity of the tumor cells, affinity of the drug for the target, amount of the target intracellularly, amount of

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competing natural substrates, and metabolic transformation (activation or detoxification), will influence the antitumor effect.

Previous studies have shown that there is no clear relationship between the concentration of anthracyclines in plasma and those in leukemic cells [5, 6]. Although anthracyclines have been studied for more than four decades, a clear correlation between their pharmacokinetics and therapeutic effect has not been shown, with exception of one study reporting a relationship between plasma concentrations of doxorubicin and the outcome of induction therapy [7].

It has been suggested that anthracycline cytotoxicity involves several mechanisms, with the most important believed to be DNA adduct formation, interference with DNA topoisomerases, and the formation of reactive oxygen species. Anthracyclines induce apoptotic cell death in various cultured cells [8], but the associations between cellular anthracycline uptake, apoptosis induction, and patient outcome remain unclear [9, 10].

In a previous study we found a clear dose response relationship between the extracellular daunorubicin dose and apoptosis induction in leukemic cell lines and leukemic cells isolated from a few patients with AML [11]. We used DNA fragmentation to measure cell death and found that this was preceded by caspase-3 activation, which supports the induction of apoptosis in leukemic cells. No evidence was found for an extracellular concentration range in vitro within which apoptosis induction was optimal. There was, however, a pronounced interindividual variation in DNA fragmentation for a given extracellular concentration of DNR.

In the investigation reported here, we studied the relationship between intracellular anthracycline concentration and apoptosis with the aim of identifying effective intracellular anthracycline levels for apoptosis induction. We also investigated a possible relationship between anthracycline-induced apoptosis in vitro and clinical response. Cellular concentrations of IDA/DNR in relation to apoptosis induction were studied after a 1-h pulse incubation of mononuclear blood cells (MNC) isolated from patients with AML. Intracellular anthracycline concentrations in vitro were compared to those obtained in vivo during anthracycline treatment.

## Materials and methods

### Materials

Zavedos (IDA) and Cerubidin (DNR) were provided by Pfizer (Strängnäs, Sweden) and Sanofi-Aventis (Bromma, Sweden), respectively. Propidium iodide (PI) sodium citrate plus and Triton X-100 was purchased from Sigma-Aldrich

(St. Louis, MO). Polypropylene tubes for DNA fragmentation assays obtained from Becton Dickinson (Lincoln Park, NJ). RPMI 1640 cell culture medium, 10% fetal calf serum, L-glutamine, penicillin, and streptomycin was supplied by PAA laboratories (Pasching, Germany). Tissue culture flasks were from Techno Plastic Products AG (Trasadingen, Switzerland).

### Clinical samples

Heparinized peripheral blood samples were obtained from a total of 33 patients with newly diagnosed AML, classified according to the French–American–British criteria [12]. Samples were obtained before therapy and directly after anthracycline infusion. In vitro cellular uptake and apoptosis concentration–response experiments were performed on samples of cells obtained before therapy from 16 of the 33 patients. Anthracycline-induced apoptosis in vitro with a fixed concentration of IDA (0.25  $\mu$ M) was evaluated on cells isolated before therapy from 20 (Table 1) of the 33 patients. Post-infusion samples were obtained from 24 of the 33 patients and drawn from a venous line not used for anthracycline infusion for the analysis of cellular drug content in vivo. The MNCs were isolated at 4°C by centrifugation on Lymphoprep (1.077 g/ml) (Nycomed, Oslo, Norway) [13]. After three washes with phosphate buffered saline (PBS), the cell number and cell volume were determined using a Coulter counter Z2 (Beckman Coulter, Fullerton, CA), and the cells were incubated as described below. The study was approved by the regional ethical committee in Stockholm, and informed consent from all patients was obtained.

### Treatment

The patients were treated at the center of Hematology at Karolinska University Hospital in Huddinge or Solna, Stockholm, Sweden and received a combination therapy of cytarabine and either IDA or DNR. The induction protocols were: (1) 2-h infusions with 1 g/m<sup>2</sup> cytarabine twice daily on days 1–4 (day 1 represents the start of the treatment), followed by a 1-h infusion with 10 mg/m<sup>2</sup> IDA on days 1 and 2, or (2) a 2-h infusion once daily with 200 mg/m<sup>2</sup> cytarabine for 7 days followed by a 1-h infusion with DNR 60 mg/m<sup>2</sup> on days 1–3. Complete remission was defined as <5% blasts in the bone marrow. Additional therapy was given to some patients (see Table 1 for details).

### Cell incubation procedure

We studied concentration–response relationships in vitro between extracellular/intracellular anthracycline concentrations and apoptosis in isolated cells obtained from 14 of the

**Table 1** Clinical characteristics and in vitro apoptosis data of 20 AML patients

Patient <sup>a</sup>	Diagnosis	Sex	Age (years)	WBC ( $\times 10^9/l$ )	Blasts (%)	Complete remission	Cell death <sup>b</sup> (%)	Treatment <sup>c</sup>
1	M2	F	20	55	50	+	40	DA
2	M1	F	59	73	90	+	59	DA
3	M3	F	42	68	70	+	58	DA
4	M4	F	80	219	40	+	42	D/C/V
5	M1	M	66	40	45	+	35	DA
6	M4	M	29	75	60	+	33	IC
7	M2	M	49	67	60	-	30	ICE
8	M3	F	77	28	25	-	15	DA
9	AML <sup>e</sup>	F	75	44	70	-	14	DA+atra
10	M5	M	36	73	60	-	9	ICE
11	M5	F	59	21	27	+	36	DA
12	M5	F	53	45	65	+	23	DA
13	M4	F	36	69	36	+	16	DA
14	AML <sup>e</sup>	F	51	56	80	+	42	DA
15	M4	F	67	56	70	-	47	DA
16	M1	F	82	270	90	-	97	DA
17	AML <sup>e</sup>	F	82	55	58	-	92	DA
18 <sup>d</sup>	AML <sup>e</sup>	F	78	170	70	-	81	DA
19	AML <sup>e</sup>	F	39	162	90	+	89	DA
20	M5	F	45	117	28	-	29	DA

AML, Acute myelogenous leukemia; WBC, white blood cells; F, female; M, male

<sup>a</sup>Patients 15, 16, 17, and 18 received reduced anthracycline doses<sup>b</sup>Apoptosis in leukemic cells 24 h after a 1-h pulse incubation with 0.25  $\mu M$  idarubicin<sup>c</sup>D, Daunorubicin; C, cytarabine; I, idarubicin; E, etoposide; atra, all-trans retinoic acid<sup>d</sup>Secondary AML<sup>e</sup>Not further subclassified

patients (13 receiving DNR and IDA and one receiving IDA only). This analysis required a large number of isolated cells, and the total number of cells isolated from four patients was not sufficient for these concentration–response studies. Cells from all 20 patients were also incubated in vitro with one fixed IDA concentration (0.25  $\mu M$ ) in order to study apoptosis development and a possible relationship with clinical response (remission). The MNC were incubated in a humidified incubator with 5% CO<sub>2</sub> at 37°C in RPMI 1640 cell culture medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100  $\mu g$ /ml). For the concentration–response analyses of cellular drug uptake/retention and apoptosis, cells were incubated at a concentration of  $10^6$ /ml in 20 ml for 1 h with DNR (0, 0.5, 1.0, 4.0 8.0  $\mu M$ ) and/or IDA (0, 0.25, 0.5, 1.0, 2.0  $\mu M$ ) in 75-cm<sup>2</sup> tissue culture flasks, then washed twice with ice-cold PBS pH 7.4 by centrifugation at 550 *g* for 5 min at 4°C, resuspended in drug-free medium at the same cell concentration, and incubated again at 37°C in 75-cm<sup>2</sup> tissue culture flasks. Aliquots were removed [2 ml for the fluorescence activated cell sorter (FACS) analysis and 2 ×

1 ml for drug determination], at specified time points (0, 4, 9, and 24 h after the pulse), the cells harvested by centrifugation at 550 *g* for 5 min at 4°C, and washed twice with ice-cold PBS. A sample of the cells was used for immediate determination of DNA fragmentation, and cell pellets were frozen at –20°C for later determination of cellular drug content.

#### DNA fragmentation with PI staining and flow cytometry

We used a propidium iodide (PI)-based staining procedure to detect cell death as previously described [14]. In an earlier study we found that DNA fragmentation correlates strongly with caspase-3 activation in leukemic cells and, therefore, we used DNA fragmentation as a measure of apoptosis [11]. The appearance of cells less intensively stained than G1 cells (sub-G1 or A<sub>0</sub> cells) in flow cytometric DNA histograms was used as a marker of apoptosis. Briefly, a cell pellet containing 1 million cells was gently resuspended in 0.5 ml PI staining solution [PI 50  $\mu g$ /ml in 0.1% (w/v) sodium citrate plus 0.1% (v/v)

Triton X-100] in 12×75-mm polypropylene tubes. The tubes were placed at 4°C in the dark for 1–3 days before making the flow cytometry measurements. The PI fluorescence of individual nuclei was measured using a FACScan flow cytometer (Becton and Dickinson, Mountain View, CA) with a single 488-nm Argon laser. The red fluorescence due to PI staining of DNA was detected in the FL-3 channel, and the data were registered on logarithmic and linear scales. The forward scatter and side scatter of particles were measured simultaneously. The flow rate was set at 12 µl/s, and at least 10,000 target events were collected for each sample. Analysis was performed using Cell Quest software (BD Biosciences, San Jose, CA).

#### Drug analysis

The mean cell volume was determined with the cell counter as described above and used to calculate the intracellular concentration. One million cells were lysed in ice-cold water, sonicated for 10 s using an ultrasonic processor (VCX 400; Sonics & Materials, Danbury, CO), and extracted with 60% acetonitrile for the DNR and IDA analysis. Prior to the acetonitrile extraction, 75 µl plasma was added to the samples to prevent the adsorption of IDA and DNR to the plastic tubes. The DNR and IDA concentrations in the cells was determined by high-performance liquid chromatography (HPLC) using a phenyl-µ-Bondapak column (3.9×150 mm, 5 mm; Waters Assoc, Milford, MA) eluted with acetonitrile and 0.2% ammonium formate pH 4 (60:40 v/v) at a flow rate of 1.5 ml/min. The drug was quantified using a Shimadzu RF-551 fluorescence HPLC monitor (Waters Assoc) at  $\lambda_{\text{excitation}}$  485 nm and  $\lambda_{\text{emission}}$  560 nm. The detection limit of the assay is  $5 \times 10^{-3}$  µM, and the range of quantification is 0.03–20 µM, with a coefficient of variation of <7%. Cellular drug uptake is expressed in micromoles.

#### Statistical analysis

Regression lines were calculated according to the method of least squares. Student's *t* test was used for testing significance, and *P* values <0.05 were considered to be significant.

### Results

#### Cellular anthracycline levels and apoptosis induction

We isolated leukemic cells from 20 AML patients before induction therapy, incubated the cells with various extracellular concentrations of anthracyclines, and measured cellular drug uptake and apoptosis at various time points after exposure. Apoptosis usually began 4–9 h after the

pulse incubation and increased the most, depending on the cellular drug concentration, during the first 24–48 h. We chose to systematically study apoptosis development up to 24 h after the end of the pulse incubation since the most pronounced apoptosis occurred during this time period and the treatment protocols included the administration of anthracycline in 24-h cycles.

There was a clear concentration–response relationship between cellular drug uptake and apoptosis for a given patient as well as a large variation in the initial uptake of DNR and apoptosis between cells from different patients. Despite the variation in initial uptake, the retention (4–24 h) was similar between cells from different patients.

We found a large variation in the uptake of DNR and apoptosis between cells from different patients when these cells were incubated with IDA. Apoptosis usually began 4–9 h post-incubation initiation. In accordance with the DNR studies, IDA retention was fairly similar between cells from different patients.

Figure 1 shows the cellular uptake, retention, and apoptosis of IDA or DNR in AML cells obtained from a patient that were incubated with a 1-h pulse of various concentrations of IDA or DNR. IDA was taken up to a greater extent by the AML cells than DNR even though we used fourfold higher concentrations of DNR. At similar intracellular concentrations of IDA and DNR, apoptosis development was almost identical.

Figure 2 shows the intracellular DNR concentrations [mean and standard deviation (SD)  $353 \pm 248$  µM, range 17–933 µM, *n*=16] and IDA ( $871 \pm 530$  µM, range 136–2031 µM, *n*=15) and apoptosis induction (DNR:  $45 \pm 29\%$ , range 10–97%; IDA:  $72 \pm 20\%$ , range 40–97%) when the cells had been pulse incubated with 1 µM DNR or IDA. The interindividual variation for both parameters is pronounced, and no relation is observed ( $r^2=0.014$ ,  $P=0.69$  and  $r^2=0.056$ ,  $P=0.46$  for DNR and IDA, respectively).

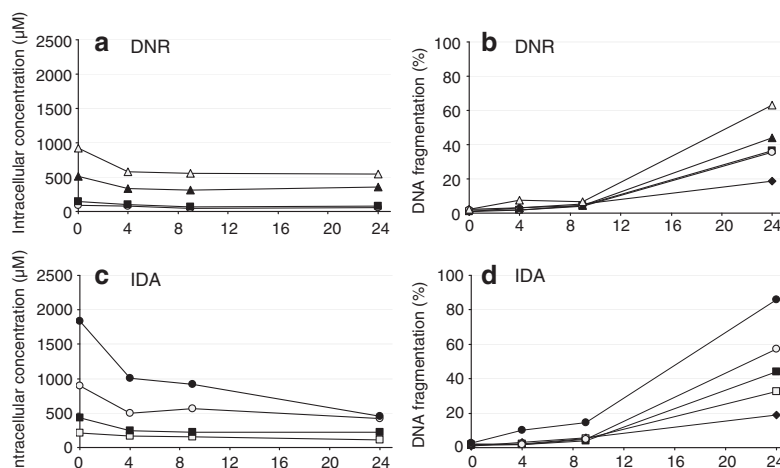
Figure 3 shows the cellular concentrations of IDA and DNR at time 0 in relation to apoptosis development 24 h after the pulse incubation for all patient samples at all treatment concentrations studied. Apoptosis was pronounced (>60%) at concentrations >1200 µM. The slopes of the regression lines for DNR (*n*=16) and IDA (*n*=15) were nearly identical, with *k*-values of 0.022 and 0.024, respectively. The  $R^2$  values were 0.25 ( $P<0.001$ ) and 0.35 ( $P<0.0001$ ) for DNR and IDA, respectively.

#### Cellular anthracycline levels in vivo

We further analyzed the intracellular uptake in leukemic cells isolated from 24 patients undergoing anthracycline induction therapy. The intracellular concentrations in all cell samples (mean ± SD) immediately after a 1-h anthracycline infusion were roughly more than tenfold lower ( $66.9 \pm 36.3$  µM,



**Fig. 1** Time course for cellular uptake, retention, and apoptosis for daunorubicin (DNR; **a, b**) and idarubicin (IDA; **c, d**) in cells from patient no. 6 after a 1-h pulse incubation with DNR [concentration ( $\mu\text{M}$ ): filled diamond 0, filled square 0.5, open circle 1, filled triangle 4, open triangle 8] or IDA [concentration ( $\mu\text{M}$ ): open square 0.25, closed square 0.5, open circle 1, closed circle 2]



range 4–137  $\mu\text{M}$ ,  $n=24$ ) than the intracellular concentration (1200  $\mu\text{M}$ ) needed to result in a pronounced (>60%) apoptosis in vitro (Fig. 3). However, there was a significant difference in the in vivo intracellular levels of DNR between patients who went into complete remission (CR+;  $80.6 \pm 36.9 \mu\text{M}$ ,  $n=14$ ) and those who did not (CR-;  $47.6 \pm 26.3 \mu\text{M}$ ,  $n=10$ ) (Student's  $t$  test  $P < 0.05$ ) (Fig. 4).

#### Apoptosis induction in vitro in relation to clinical response

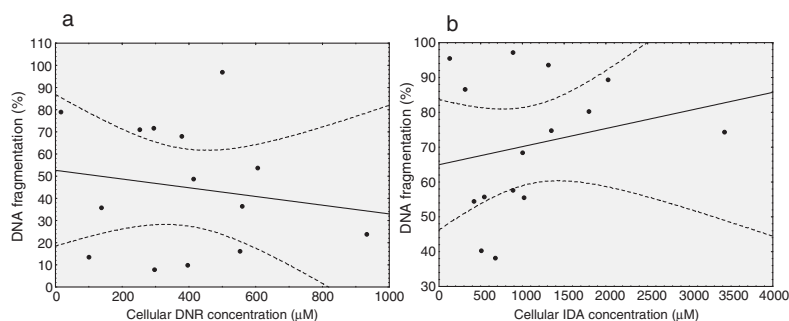
We also investigated whether there was a relation between apoptosis induction in vitro and clinical response in 20 AML patients (Table 1). Apoptosis was measured in leukemic cells 24 h after a 1-h pulse incubation with 0.25  $\mu\text{M}$  IDA and related to clinical response. A 0.25  $\mu\text{M}$  concentration of IDA was chosen since this concentration empirically gave a large scatter of apoptosis values among the patient samples. The cells of patients who received full dose induction treatment and went into complete remission

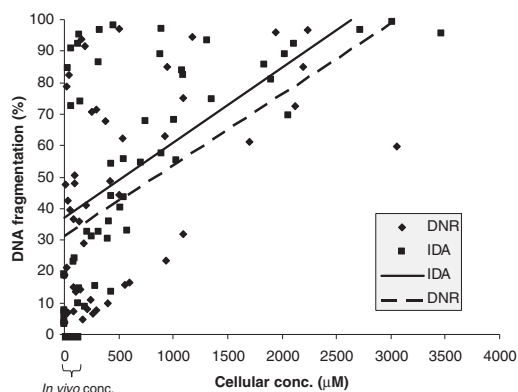
(CR+) were generally more sensitive to IDA in vitro than those obtained from patients who did not achieve complete remission (CR-). The mean and SD values for apoptosis in CR+ patients and CR- patients were  $43.0 \pm 19.9\%$  ( $n=11$ ) and  $19.4 \pm 9.5\%$  ( $n=5$ ), respectively (Student's  $t$  test  $P < 0.05$ ). Patients 15–18 were excluded from the statistical analysis since they received reduced anthracycline doses. No difference in IDA sensitivity was observed when all 20 patients were included in the statistical analysis. The mean and SD values for apoptosis in CR+ and CR- patients were  $43.0 \pm 19.9\%$  ( $n=11$ ) and  $46.0 \pm 35.1\%$  ( $n=9$ ), respectively (Student's  $t$  test  $P > 0.05$ ).

#### Discussion

In order to investigate the importance of DNR/IDA cell uptake in apoptosis induction in AML cells we conducted a series of experiments on MNC isolated from AML patients.

**Fig. 2** In vitro intracellular concentration of DNR (**a**,  $n=16$ ) and IDA (**b**,  $n=15$ ) in leukemic cells obtained from acute myelogenous leukemia (AML) patients at time 0 in relation to apoptosis at 24 h following a 1-h pulse incubation with 1  $\mu\text{M}$  DNR or IDA. The dotted lines represent the 95% confidence limits





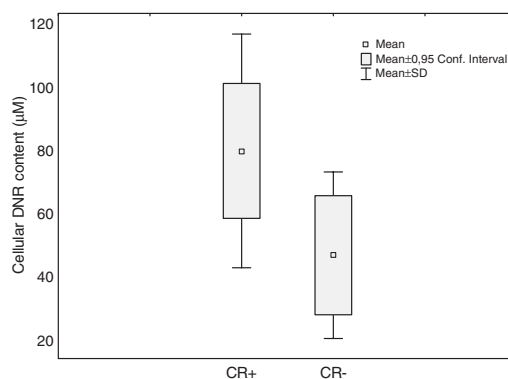
**Fig. 3** Intracellular concentrations of DNR and IDA in vitro at time 0 versus apoptosis induction in leukemic cells at 24 h. The cells were isolated from 16 AML patients and pulse incubated for 1 h with 0.5, 1.0, 4.0, and 8.0  $\mu\text{M}$  DNR ( $n=16$ ) or 0.25, 0.5, 1.0, and 2.0  $\mu\text{M}$  IDA ( $n=15$ ). The black box along the x-axis covering the range 4–137  $\mu\text{M}$  represents the intracellular in vivo anthracycline concentration range immediately after anthracycline infusion

In an attempt to mimic the in vivo pharmacokinetics of anthracyclines, we chose a 1-h pulse incubation. Apoptosis induction was detectable approximately 4–9 h after the pulse incubation, reaching up to 100% apoptosis at the highest concentrations 24 h after the pulse incubation. This time course demonstrates the importance of choosing the correct time-point for evaluating anthracycline-induced apoptosis.

There was a large interindividual variation in vitro in how cells from the AML patients took up DNR/IDA and subsequently underwent apoptosis. However, a clear concentration–response relationship between cellular drug uptake and apoptotic response was always present for any one individual. A prior expectation of our study was that we would be able to demonstrate a certain cellular threshold level for IDA/DNR that induced apoptosis. However, this was not the case. The data in Figs. 2 and 3 clearly demonstrate a pronounced interindividual variation in apoptosis for a given cellular drug concentration. Nevertheless, the data in Fig. 3 also demonstrate that cellular IDA/DNR levels  $>1200 \mu\text{M}$  are associated with strong apoptosis development. One reason for the pronounced interindividual variation in cellular anthracycline uptake could be the expression of various protein efflux pumps, such as members of the ATP-binding cassette (ABC) superfamily (e.g. p-glycoprotein) [15]. The results of several studies have also indicated that nucleoside transport proteins are involved in cellular anthracycline uptake. It is therefore possible that alterations in the expression of these proteins could contribute to variations in cellular drug uptake [16–18].

We found that the in vivo intracellular concentrations required to trigger a strong apoptotic response in vitro were more than tenfold lower than the intracellular concentrations required to trigger the same response. However, we also found that these intracellular post-infusion levels were significantly higher in patients achieving complete remission than in those who did not. The possible existence of a relation between intracellular concentrations of DNR and clinical outcome in AML patients has been studied by a number of investigators. In one study, Kokenberg et al. did not find any difference in in vivo cellular DNR concentrations between responders and non-responders [19], while in another study they found a tendency towards higher concentrations in responders [20]. In accordance with our results, Galettis et al. found a significant difference in the cellular DNR levels (area under the curve) between responders and non-responders, but they found no correlation between cellular DNR levels in vivo and cellular p-glycoprotein expression [21].

Our results support that modifications to treatment protocols aimed at increasing the intracellular concentration of anthracyclines could be beneficial by triggering a stronger apoptotic response, thereby leading to a better treatment outcome. How anthracycline infusion times may be altered in order to increase the intracellular concentration in vivo needs further investigation. There have been contradictory results from studies investigating how intracellular concentrations vary with anthracycline infusion time. One study on AML patients showed that a 24-h continuous infusion resulted in a higher leukemic cell accumulation of DNR compared to a short time infusion [22]. In this study, eight patients were analyzed and one patient was used as his/her own control. Another study on AML patients, a four-arm parallel group



**Fig. 4** In vivo intracellular concentration of DNR in leukemic cells isolated from 24 patients directly after a 1-h DNR infusion in relation to clinical outcome. CR+ Complete remission ( $n=14$ ), CR- Not complete remission ( $n=10$ )

study including 18 patients, showed that a bolus infusion achieved a higher intracellular concentration of DNR than a long-term infusion [23]. Both studies were based on very few patients. One study on DNR treatment of 77 patients with acute lymphoblastic leukemia showed that a 24-h continuous infusion resulted in lower relapse rates than a 30-min infusion of DNR [24]. Alternatively, another study on 178 children with ALL was unable to find any difference in patient outcome between a 1-h and a 24-h DNR infusion protocol [25]. Most studies have compared anthracycline plasma concentrations in patients receiving different infusion protocols, even though it has been shown that there is no clear correlation between plasma concentration and the intracellular concentration of anthracyclines in patients undergoing treatment [5, 6]. A more accurate approach to determining the efficacy of different DNR infusion protocols would be to compare the intracellular levels of DNR together with patient outcome.

Why then is the response to therapy relatively good despite the low cellular *in vivo* concentrations? There are several possible explanations. Firstly, there are mechanisms *in vivo* facilitating apoptosis at low intracellular anthracycline levels. Secondly, the patient receives combination therapy with cytarabine, and the contribution by this drug leads to apoptosis induction despite the low intracellular anthracycline levels. Thirdly, the low intracellular anthracycline levels *in vivo* do not induce apoptosis, and other mechanisms, such as growth inhibition, are more important for the treatment effect.

The results of our *in vitro* apoptosis induction assay with anthracyclines showed that IDA-induced apoptosis was significantly higher among responders than among non-responders. Several *in vitro* tests have been developed with the aim of predicting outcome, with varying degrees of success. A number of research groups have reported a significant correlation between AML patient response and *in vitro* drug sensitivity using a variety of methods, such as differential staining cytotoxicity (DISC) assay [26–28] and the methylthiazol tetrazolium colorimetric assay [29, 30]. The DISC assay is known to be labor intensive and time consuming, taking about 4–5 days to complete [26, 28]. With our *in vitro* apoptosis determination method, it is possible to obtain a result within 48 h after blood sampling. Interestingly, in carrying out a study with a 1-h DNR and IDA pulse incubation of isolated MNC from patients with AML and acute non-myeloid leukemia, the researchers found that Fas (APO-1, CD95) induction and anthracycline-induced apoptosis were significantly higher when complete remission was achieved [31]. Apoptosis, which was evaluated after 18 h, was determined from caspase 3 activation and phosphatidylserine exposure using flow cytometry. Attempts have also been made to find correlations between *in vitro* drug uptake, P-gp expression, and clinical outcome [32–34].

Guerci et al. found relations between reduced intracellular DNR uptake, P-gp expression, and therapeutic failure [35].

We conclude that a high initial cellular anthracycline uptake is of importance for inducing apoptosis *in vitro* in AML cells. Our observations that post-infusion *in vivo* intracellular concentrations were low but still significantly related to clinical response suggest that treatment protocols achieving higher initial intracellular anthracycline levels may improve the clinical outcome of AML patients.

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II



# **Daunorubicin metabolism in leukemic cells isolated from patients with acute myeloid leukemia**

by

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## Abstract

**Background:** Anthracyclines like daunorubicin (DNR) are important drugs in the treatment of acute myeloid leukaemia (AML). *In vitro* studies have shown that cellular metabolism of anthracyclines could play a role in drug resistance. Currently, it is not known what enzyme is responsible for anthracycline metabolism in leukemic cells.

**Aims:** To study C-13 reduction of DNR to daunorubicinol (DOL) in leukemic cells isolated from patients with AML and to determine the most important enzyme involved.

**Methods:** Mononuclear blood cells from 25 AML patients were isolated at diagnosis and used in a metabolic assay to determine the fraction DOL formed. mRNA and western blot analysis were performed on the 2 most likely candidates for anthracycline metabolism; carbonyl reductase 1 (CR1) and aldoketoreductase 1A1 (AKR1A1). DNR and DOL concentrations were determined by HPLC.

**Results:** We found a large interindividual variation (up to 47-fold) in leukemic cell DNR metabolism. No correlation between mRNA levels of the enzymes and metabolism were found. Cellular DNR metabolism correlated significantly with CR1 protein expression, determined by western blot, ( $p < 0.05$ ,  $R^2 = 0,229$ ) while no significant correlation was found with AKR1A1 protein expression.

**Conclusions:** DNR metabolism in AML cells shows a pronounced interindividual variability. Our results support that CR1 is the most important enzyme for conversion of DNR to DOL in AML cells. This information could in the future be used to genotype CR1 and possibly help to individualise dosing.



## Introduction

Anthracyclines, like daunorubicin (DNR) and idarubicin are together with cytarabine the most important cytostatics for treatment of acute myeloid leukaemia (AML) [1]. There is a pronounced inter- and intraindividual variation in plasma levels of anthracyclines despite standardized dosing based on body surface area [2, 3]. The variation in pharmacokinetics is largely due to variation in systemic metabolism of the drugs [4]. Despite their long clinical use there is no consensus on which enzyme is responsible for anthracycline metabolism [5-7]. There is also no data on whether the AML cells themselves metabolize anthracyclines and possibly contribute to systemic metabolism.

Two distinct enzyme superfamilies, aldoketoreductases and short-chain dehydrogenases/reductases, are believed to be responsible for the formation of the major metabolites, the 13-hydroxy derivatives, daunorubicinol (DOL), idarubicinol, and doxorubicinol [5]. DOL and doxorubicinol are significantly less cytotoxic than the parent drugs while it seems that idarubicinol and idarubicin are equipotent [8, 9]. The metabolism of anthracyclines has been shown to be up-regulated in cells following exposure to the drugs *in vitro* leading to drug resistance [10].

Three enzymes were reported to be capable of anthracycline carbonyl reduction in human liver: aldoketoreductases AKR1A1, AKR1C2, and, the short-chain dehydrogenase/reductase, carbonyl reductase 1 (CR1) [6]. AKR1C2 has, however, also been reported not to metabolise doxorubicin or DNR [7]. Currently it is believed that AKR1A1 and CR1 are the major anthracycline metabolizing enzymes and that enzyme specificity might vary with anthracycline type [4, 11-13].

To our knowledge, anthracycline metabolism in leukemic cells from AML patients has not been specifically studied previously. Consequently, it is not known which enzyme is the most important for anthracycline-C13 metabolism in leukemic cells and may contribute to systemic metabolism and cellular resistance towards anthracyclines. In this study we determined metabolism of DNR in leukemic cells isolated from AML patients and analyzed the relation with mRNA and protein levels of AKR1A1 and CR1.

## **Materials and Methods**

### *Clinical samples*

Heparinized peripheral blood was obtained from 25 patients with newly diagnosed AML. There were 17 females and 8 males with a mean age of 61 years (range 36-84 years) and a mean white blood cell count of 82 million cells/ml (range 14-200 million/ml). Mononuclear cells were isolated by centrifuging 5 ml blood on 3 ml Lymphoprep (Nycomed, Norway) at 550g for 15 minutes at 4<sup>0</sup>C. After two washes with PBS the cell number was determined using a Coulter counter Z2 (Beckman Coulter, Fullerton, CA, USA). The cells were frozen as pellets containing 75 million cells and kept at -80°C until the day of the experiment. The study was approved by the regional ethical committee in Stockholm and informed consent was obtained.

### *DNR metabolism in cell homogenates*

The carbonyl reduction assay used was previously described by Wolfram et al [14]. Leukemic cells were sonicated in 50 mM Tris-HCl buffer (pH 7,4, 0,125 M KCl, 1,0 mM EDTA) for 10 seconds using an ultrasonic processor, VCX 400 (Sonics & Materials, Danbury, Co, USA). Protein concentration was determined with the Biorad assay (Biorad – Sundbyberg, Sweden).

The experiments were performed in eppendorf tubes (1.5 ml) containing 250 µl TRIS-HCl buffer (pH 7.4, 0.125M KCl, 1.0 mM EDTA), 5 µl 1 mM DNR and 220 µl of leukemic cell extract diluted to 2.5 mg/ml protein. The tubes were kept in a waterbath at 37°C and the reaction was started by adding 25 µl of NADPH (20 mM in Tris-HCl pH 7.4). The leukemic cell extracts were incubated for 60 minutes after which the samples were extracted in 60 % acetonitrile on ice. This was followed by HPLC determination of DNR and DOL. The coefficient of variation (CV) for the metabolic assay was < 11 % (n=5).

#### *RNA preparation*

Total RNA extraction was performed using Qiagen mini RNA Kit (Qiagen AB, Solna, Sweden) according to manufacturer's protocol. RNA was reverse transcribed into cDNA with a poly(T)<sub>12</sub> protocol. The cDNA was diluted 10 times prior real-time PCR analysis.

#### *Quantitative real-time PCR*

Primers for AKR1A1 and carbonyl reductase were ordered from Cybergene® AB, Stockholm, Sweden. Beta-actin (Applied Biosystems, Foster City, USA) was chosen as an endogenous housekeeping control gene. The forward primer sequence for CR1 was:

5'- TGCCTCTGGAACACGCTGCGGGGCTCC, reverse primer,

5'- GACCAGCACGTCCAGGCCCCCGTACTC. The forward primer sequence for AKR1A1

was: 5'- CATTGATTGTGCTGCTATCTACGG, reverse primer,

5'- GCCTTCCAAGTCTCCTTGCTAGTGG. Quantitative real-time PCR was performed using the ABI 7500 Fast PCR Detection System (Applied Biosystems, Foster City, USA). Reaction mixtures contained 1xPower SYBR® Green PCR master mix (Applied Biosystems, Foster City, USA), 0.25 µM primers, 5 µl cDNA template in a total volume of 25 µl. Thermal cycling

conditions included activation at 95° C (10 min) followed by 40 cycles each of denaturation at 95° C (15 sec) and annealing/elongation at 60° C (1 min).

Each reaction was performed in triplicate and no-template controls were included in each experiment. The expression was calculated using the  $\Delta\Delta CT$  formula.

#### *Drug analysis*

DNR and DOL concentrations in samples were determined by HPLC using a phenyl- $\mu$ -Bondapak column (3.9 x 150 mm, 5 mm. Waters Associates, Milford, MA) eluted with acetonitrile and 0.2% ammonium formate pH 4 (60:40 v/v) at a flow rate of 1.5 ml/min. The samples were extracted with 60% acetonitrile. The drug was quantified using a Shimadzu RF-551 fluorescence HPLC monitor at  $\lambda_{\text{excitation}}$  485 nm and  $\lambda_{\text{emission}}$  560 nm. The detection limit of the assay is  $5 \times 10^{-3}$   $\mu$ M and the range of quantification is 0.03-20  $\mu$ M, with a CV < 7 %.

#### *Western blot*

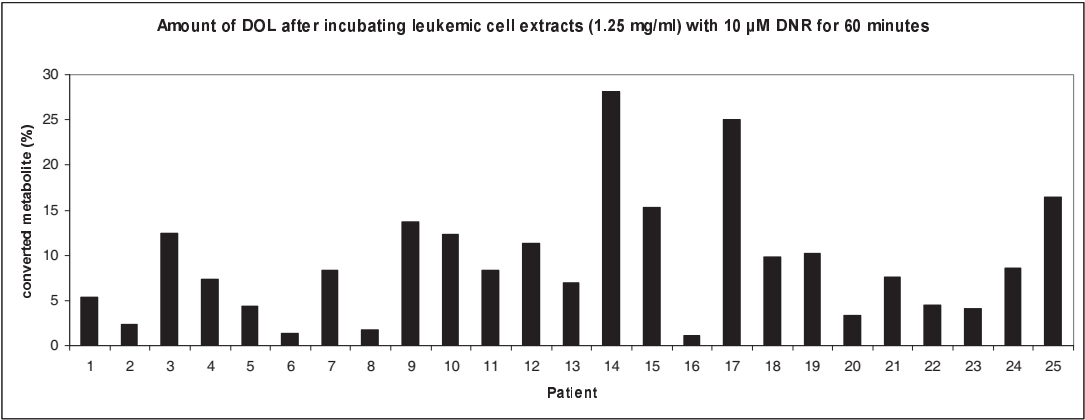
The leukemic cells were isolated as described above and lysed by the addition of 250  $\mu$ l of a protease inhibitor cocktail (Roche diagnostics GMBH) and sonication for 10 seconds using an ultrasonic processor, VCX 400 (Sonics & Materials, Danbury, Co, USA). Protein concentration was determined using the Biorad assay. 10  $\mu$ g of protein was loaded in each well on a 4-15 % TRIS-glycin gel from Biorad. The gel was run for 1 hour at 130V and the protein transferred to a PVDF-membrane over night at 30V. The membrane was blocked in 5 % dry milk (in washing buffer). The primary antibodies for AKR1A1 and CBR1 were obtained from the Abnova Corporation, Taipei City, Taiwan. The secondary antibodies used was Goat-anti-Mouse (Dakocytomation®, 1/1000). The primary antibody used were diluted 1/2000 in washing buffer. The protein bands on the membranes were visualized using enhanced chemiluminescence

reagents containing horseradish peroxidase substrate (Bio-Rad, Sundbyberg, Sweden). Images were scanned and quantified by Quantity One software (Bio-Rad).

## Results

### DNR metabolism by leukemic cell extracts

The metabolism of DNR by cell extracts of the 25 AML patients is shown in figure 1. The cellular metabolism of DNR showed an up to 47-fold interindividual variation and the mean fraction of DOL formed during one hour was 9,2 % with a SD of 6,8 % (range 0,6-28 %).



*Fig 1. Metabolism of DNR by leukemic cell extracts from 25 AML patients expressed as % DOL formed after a 60 minute incubation with 10 µM DNR.*

### Metabolism of DNR in relation mRNA levels in leukemic cells.

Figure 2 shows the relation between the relative mRNA levels of CR1 ( $R^2 = 0,0011$ ,  $p > 0,05$ ) and AKR1A1 ( $R^2 = 0,0044$ ,  $p > 0,05$ ) and the metabolism of DNR by leukemic cell extracts from 14 of the patients. We found no significant correlation between the mRNA levels and the metabolism of DNR and hence chose not to pursue mRNA quantification for the remaining patients.

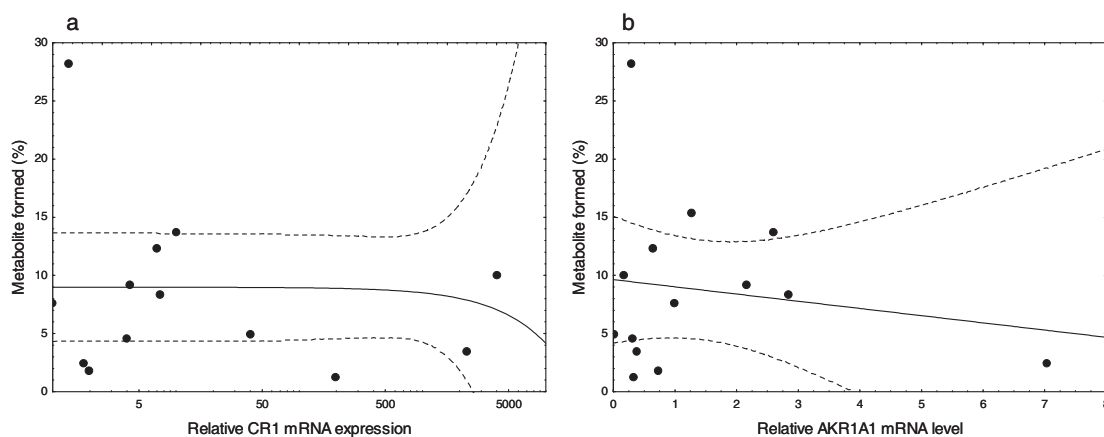
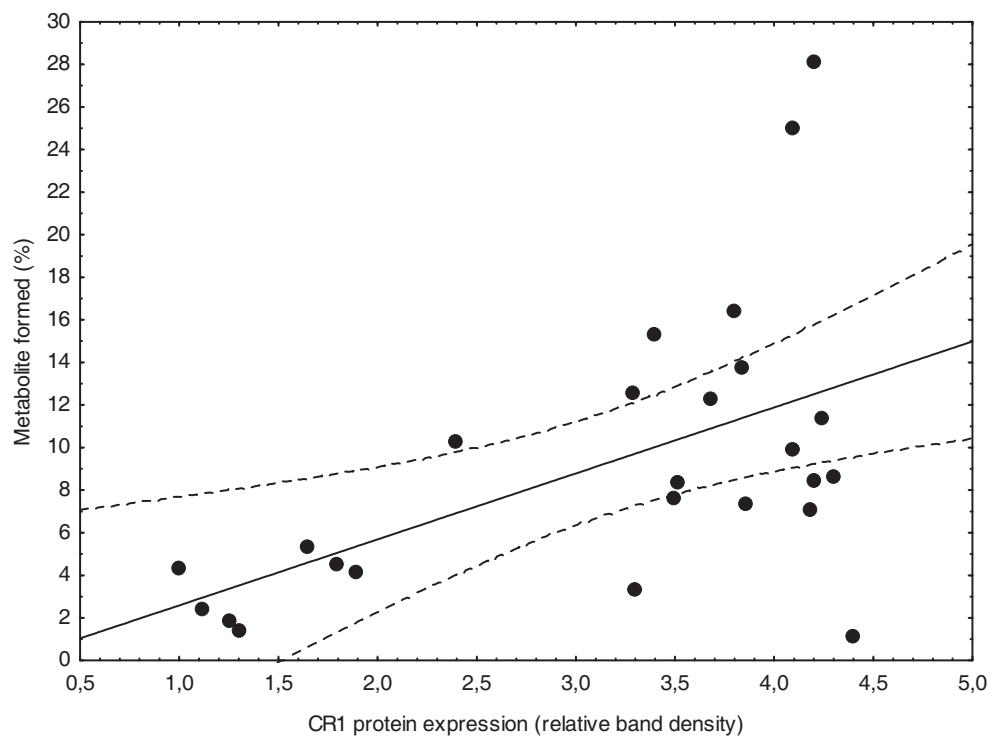


Fig 2. Relative mRNA levels for CR1 (A) and AKR1A1 (B), in relation to the metabolic capacity of the leukemic cell extracts. The dotted lines represent the 95 % confidence limits.

### Protein expression of enzymes in relation to metabolism of DNR.

We found a good correlation between CR1 protein levels and the metabolism of DNR by the leukemic cell extracts ( $R^2 = 0,29$ ,  $p < 0.01$ ,  $n = 25$ , figure 4). However, we could not find a significant correlation between AKR1A1 expression and DNR metabolism in the leukemic cell extracts ( $R^2 = 0,0044$ ,  $p > 0.05$ ,  $n = 25$ , figure 3).



*Fig 3. Protein expression of carbonyl reductase 1 in relation to % DOL formed after a 1 hour incubation of leukemic cell extracts with 10  $\mu$ M DNR. The dotted lines represent the 95 % confidence limits.*



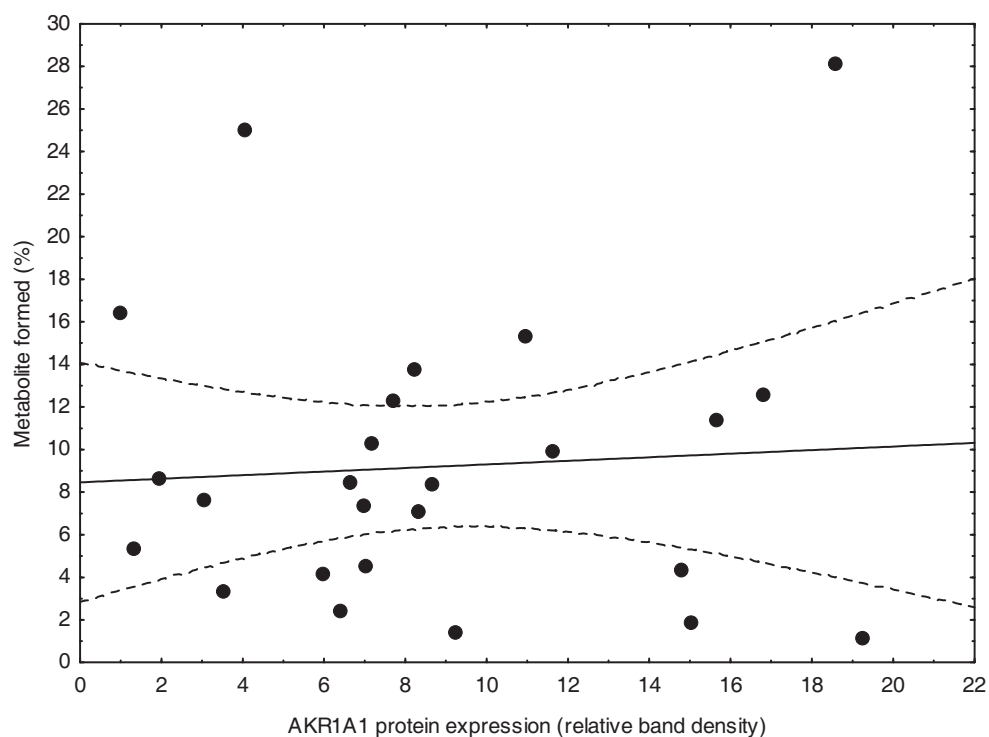
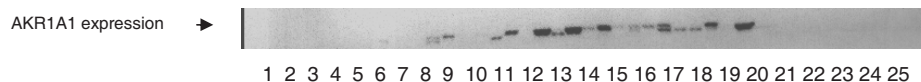


Fig 4. Protein expression of aldoketo reductase 1A1 in relation to % DOL formed after a 1 hour incubation of leukemic cell extracts with 10  $\mu$ M DNR. The dotted lines represent the 95 % confidence limits.



## Discussion

There are many studies concerning the variation in plasma levels of anthracyclines and their metabolites both in AML patients and in patients with solid tumours [15, 16]. The reason for the



variation in anthracycline plasma levels is not completely understood although variation in liver metabolism plays an obvious role [3].

In this study we analysed the metabolism of DNR in leukemic cell homogenates from AML patients in order to investigate interindividual variation and to identify which enzyme is responsible for DNR metabolism in the cells. We found that leukemic cell samples showed a large variation in their ability to metabolize DNR. The metabolic capacity showed no correlation with mRNA levels of the enzymes considered to be the most important for anthracycline metabolism. However, we found a significant correlation between the metabolic capacity of the leukemic cell extracts and the protein levels of CR1. The absence of a correlation between mRNA levels and metabolism and a significant correlation between protein levels and metabolism supports post transcriptional regulation of the enzyme. Various anthracyclines seem to have different carbonyl reducing enzyme specificity. In the human heart doxorubicin and DNR are predominantly converted to their C-13 alcohol metabolites by AKR1A1 and CR1 respectively [11-13]. There are studies supporting that enzymes responsible for the anthracycline carbonyl reduction might vary in different tissues. For example, it has been shown that CR1 is the predominant enzyme for doxorubicin metabolism in human liver [4] while other studies provide evidence that AKR1A1 is the predominant doxorubicin metabolising enzyme in human heart [11, 17].

The role of CR1 in drug metabolism is not completely understood since other enzymes may catalyze the same reactions, mainly the cytochrome P-450 enzymes. For example, haloperidol is a known substrate for CR1 but it can also be metabolised by aldo-ketoreductases 1C1 and 1C2 and by cytochrome P-450 enzymes [18]. Similarly, anthracyclines can also be metabolised by the

cytochrome P-450 pathway but the formation of the C-13 alcohol metabolites of anthracyclines are still attributed to enzymes belonging to the short chain dehydrogenases/reductases (CR1) and aldo-ketoreductase families [5].

Treatment with anthracyclines is not only limited by bone marrow depression but also by the onset of a dose and time-dependent cardiotoxicity. Acute and sub acute anthracycline cardiotoxicity are usually reversible within several days while chronic administration causes a cumulative dose-dependent and life threatening cardiomyopathy. The mechanisms for cardiac toxicity are still debated but strong evidence suggests the involvement of secondary alcohol metabolites. C-13 reduction of anthracyclines may therefore also contribute to cardiotoxicity [12, 19, 20].

Studies performed on cell lines which over-express several carbonyl reducing enzymes show that carbonyl reduction of anthracyclines is a resistance factor to [14, 21]. Another study showed that selective inhibition of CR1 in the A549 cancer cell line with 3-(7-isopropyl-4-(methylamino)-7H-pyrrolo[2,3-d]pyrimidin-5yl)phenol (hydroxyl-PP-Me) significantly increased sensitivity towards DNR [22]. It is therefore possible that anthracycline metabolism in leukemic cells may contribute to drug resistance and possibly also to cardiac toxicity since the metabolites have associated with cardiotoxic effects [12, 20, 23].

An important question that remains to answer is whether the metabolism in leukemic cells correlates with liver metabolism of the drugs. If this would be the fact, it could in the future be possible to assay metabolism in leukemic cells or to genotype the enzyme and predict systemic metabolism. Such information could be of value to individualize the dose of anthracyclines. It

cannot be ruled out that metabolism in the leukemic cells might play a significant role in the systemic metabolism of DNR in patients with a large tumour burden and hence contribute to clearance and cardiotoxicity. Recent studies also indicate that there are functional genetic polymorphisms of CR1 with different anthracycline metabolic rates [24, 25], indicating that in the future genotyping might be used for better individualisation of anthracycline therapy.

We conclude that CR1 but not AKR1A1 is an important enzyme for DOL formation in leukemic cells. Further studies are needed to elucidate if the metabolism in leukemic cells reflect liver metabolism and also contribute to the systemic metabolism of DNR.

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# **Inverse relationship between leukemic cell burden and plasma levels of daunorubicin in patients with acute myeloid leukemia.**

by

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What is already known about the subject

- In vitro studies show that daunorubicin (DNR) cytotoxicity decreases with increasing cell density due to a high cellular uptake and depletion of drug in the medium.
- It is not known whether such an effect also occurs in vivo.

What this study adds

- We show that a large leukemic cell burden lowers the plasma levels of daunorubicin in patients with acute myeloid leukemia (AML).
- Our analysis supports that a large leukemic cell burden increases the central volume of distribution for DNR.
- Our study indicates that a dose adjustment of DNR may be of importance in AML patients with high white blood cell counts.

## Abstract

**Aims:** It has been shown that the cellular uptake and cytotoxicity of anthracyclines decrease with increasing cell density *in vitro*, an event termed “*the inoculum effect*”. It is not known whether such an effect occurs *in vivo*. In this study the relationships between white blood cell (WBC) count, plasma, and cellular levels of daunorubicin (DNR) in patients with acute myeloid leukemia (AML) were investigated.

**Methods:** Plasma and mononuclear blood cells were isolated from peripheral blood from 40 patients with AML at end of infusion (time 1 h), 5, and 24 h following the first DNR infusion. DNR concentrations were determined with HPLC and related to the WBC count at diagnosis. A population pharmacokinetic model was used to estimate the correlations between baseline WBC count, volume of distribution, and clearance of DNR.

**Results:** A clear but weak inverse relationship between the baseline WBC count and plasma levels of daunorubicin ( $R^2 = 0,11$ ;  $p < 0,05$ ) at time 1 was found. Furthermore, a clear relationship between baseline WBC count and DNR central volume of distribution using population pharmacokinetic modelling (dOFV 4.77,  $p < 0,05$ ) was also noted. Analysis of plasma DNR and the metabolite daunorubicinol (DOL) levels in patients with a high WBC count support that the low DNR/DOL levels are due a distribution effect.

**Conclusion:** This study supports that the leukemic cell burden influences the plasma levels of anthracyclines. Further studies are needed to explore if patients with high a WBC count may require higher doses of anthracyclines.

**Abbreviations:**

ALL – acute lymphoblastic leukemia

AML – acute myeloid leukemia

DNR – daunorubicin

DOL – daunorubicinol

DOX – doxorubicin

HPLC – high pressure liquid chromatography

WBC – white blood cell

OFV - Objective function value

RSE - Relative standard error

VPC - Visual predictive check

## Introduction

Anthracyclines, mainly daunorubicin (DNR), are important drugs in the treatment of acute myeloid leukemia (AML) but the optimal dosage of DNR is still unclear. A major problem with anthracycline treatment is the severe toxicity of the compounds, like bone marrow depression and a cumulative cardiotoxicity. The clinician has to weigh the treatment effect against potential toxicity, administering a DNR dose that is too low will lead to a weak antileukemic effect while too much could lead to severe side effects. Dose individualisation is therefore important [1].

In this work we investigated if the tumour burden may affect the pharmacokinetics of DNR, which ultimately may give support for dose adjustments in the treatment of AML. The importance of establishing DNR pharmacokinetic - pharmacodynamic relationships in AML patients is supported by a study that showed that children with AML who reached complete remission had a lower doxorubicin clearance and a higher median doxorubicin plasma concentration than those who did not reach complete remission [2].

It has been shown *in vitro* that the cellular uptake and cytotoxicity of anticancer agents decreases with increasing cell density, an event termed “*the inoculum effect*”.

In a previous study we reported how daunorubicin and cytosine arabinoside cytotoxicity was affected by increasing cell density in HL-60 cells and in leukemic cells isolated from patients with AML [3]. We found an inverse relation between cytotoxicity and cell density as a consequence of a lower cellular daunorubicin uptake at higher cell densities. In fact, the cells took up so much DNR over the course of 2 hours that the medium was depleted of drug. In addition to our study, other *in vitro* studies have been made comparing the cytotoxicity of different anti-tumoral agents at various cell densities in cell lines. Kobayashi *et al.* found a

negative correlation between the cell density of Molt-3 cells, an acute lymphoblastic leukemia (ALL) cell line, and the cytotoxicity of doxorubicin and vincristine [4]. We hypothesised that the situation might be the same *in vivo* in AML patients treated with DNR. We therefore determined if a high white blood cell (WBC) count was associated with lower levels of DNR in plasma and leukemic cells.

## **Methods:**

### *Patients*

40 patients with AML were studied, classified according to the French-American-British criteria (Table 1) [5]. The mean age was 61,3 years (range 33-83 years) with a male/female ratio of 19/21. The WBC count was measured at diagnosis and the mean WBC count was  $39 \times 10^6$  cells/ml blood (range  $1-219 \times 10^6$ ). There were 33 de novo AML and 7 secondary AML (prior myelodysplastic syndrome, prior chemotherapy, or radiation therapy). The study was approved by the Ethics committee at Karolinska institute and informed consent was obtained.

### *Blood sampling and isolation of plasma and cells*

Venous blood samples (10 ml) were collected in heparinised tubes at 1 h (end of infusion), 5 h, and 24 h after DNR infusion. Plasma and leukemic cells were isolated at 4°C by centrifugation on Lymphoprep (d. 1.077 g/ml) (Nycomed, Norway) [6]. Intracellular DNR concentration could be determined for 24/40 patients. The intracellular concentrations of DNR in leukemic cells in relation to clinical outcome has been reported previously by us [7].

### *Treatment*

The patients were treated at the center of Hematology at Karolinska university hospital in Huddinge or Solna, Stockholm, Sweden, and received an induction combination therapy with

cytarabine (a 2-hour infusion 200 mg/m<sup>2</sup> days 1-7) and DNR (a 1-hour infusion 60 mg/m<sup>2</sup> days 1-3 immediately after cytarabine infusion). Complete remission was defined as < 5 % blasts in the bone marrow.

#### *HPLC analysis of daunorubicin and daunorubicinol*

The mean cell volume was determined with the cell counter as described above which was used when calculating the intracellular concentration. One million cells were lysed in ice-cold water, sonicated for 10 seconds using a ultrasonic processor, VCX 400 (Sonics & Materials, Danbury, CO, USA), and extracted with 60% acetonitrile for DNR. Prior to the acetonitrile extraction, 75 µl plasma was added to the cellsamples in order to prevent adsorption of DNR to the plastic tubes. 150 µl plasma and cell suspension was extracted with 240 µl acetonitrile together with 10 µl of 40 µM idarubicin as internal standard for DNR and the active metabolite daunorubicinol (DOL) analysis. The DNR and DOL concentrations were determined by HPLC using a phenyl-µ-Bondapak column (3.9 x 150 mm, 5 mm. Waters Associates, Milford, MA, USA) eluted with acetonitrile and 0.2% ammonium formate pH 4 (50:50 v/v) at a flow rate of 1.5 ml/min [3]. DNR was quantified by a fluorescence detector model Shimadzu RF-551 fluorescence HPLC monitor at  $\lambda_{\text{excitation}}$  485 nm and  $\lambda_{\text{emission}}$  560 nm. The detection limit of the assay is  $5 \cdot 10^{-3}$  µM and the range of quantification is 0,03-20 µM (for both DNR and DOL), with a coefficient of variation (CV) of less than 7 % for DNR and less than 13 % for DOL. Cellular drug concentration is expressed in µM.

#### *Statistical analysis*

Regression lines were calculated according to the method of least squares. Student's t-test was used for test of significance and P values <0.05 were considered significant.

### *Population pharmacokinetic modelling*

The data was analyzed using nonlinear mixed effects modelling and the first-order conditional estimation (FOCE) implemented in NONMEM 7 (Icon Development Solutions, MD, USA). The plasma concentrations were log-transformed as this approach resulted in a more symmetrical distribution of residuals around zero compared with untransformed data. Model development was guided by the objective function value (OFV) and precision in parameter estimates (relative standard error RSE %) obtained by NONMEM as well as graphical assessment and scientific plausibility. The OFV obtained from NONMEM, was used to differentiate between two nested models, using the log-likelihood ratio test. The difference in OFV between two nested models is approximately  $\chi^2$ -distributed. A decrease in OFV of 3.84 was required for the addition of one parameter to the model to be considered significant, which corresponds to  $p < 0.05$ . The R-based program Xpose 4 (<http://xpose.sourceforge.net/>) was used for graphical visualization of the model fit and PsN (<http://psn.sourceforge.net/>) was used for executing simulations and calculations for visual predictive checks (VPC). The predictive performance of the model was assessed by visual predictive check. Using the original dataset as a template 500 data sets were simulated from the model and the prediction intervals with 95% confidence interval were computed and superimposed on the observed data. The model is considered to perform well if the confidence interval included the corresponding percentiles of the observed data.

The model development was based on a previously developed population pharmacokinetic model of DNR and DOL in AML patients by Callies *et al.* [8]. The model was applied to the data without re-estimating the parameters and the predictability of the model was assessed by VPC. The model was then refined using only the DNR plasma concentrations. A linear two



and three compartment model was fitted to the data. The interindividual variability was explored on all pharmacokinetic parameters of the model and was described by a log-normal variance model. The covariance matrix for random effects was evaluated for correlations between the parameters. The residual variability was described with a proportional error model.

Once the basic pharmacokinetic model was established the covariate model was developed in a step-wise manner. In the first step the covariates of interest (baseline WBC, age, gender, body weight, body surface area), were each tested on each of the pharmacokinetic parameters at a time. The relationship that resulted in the largest drop in OFV was kept in the model and the covariates were each re-tested on each of the parameters one at the time. This process continued until the addition to the model did not meet the statistical significant criteria of 0.05. The process was followed by a backward deletion step where one covariate at the time was omitted from the model. The omitted relationship that resulted in the smallest increase in OFV was removed from the model and each covariate relationship was once more omitted from the model until the statistical criteria of 0.05 was met. The continuous covariates were included on each of the pharmacokinetic parameters in the basic model according to:

$$P_{COV_i} = \theta_p \bullet (1 + \theta_{p-COV} \bullet (COV_i - median_{cov}))$$

where  $P_{COV_i}$  is the typical population parameter estimate given the covariate in patient i,  $\theta_p$  is the typical parameter for an individual with the median covariate, and  $\theta_{p-COV}$  is the fractional deviation in  $\theta_p$  with each unit change in the covariate from the median covariate value.

Gender was included in the model as the percentage change ( $\theta_{p-COV}$ ) of the typical parameter  $\theta_p$  for women compared to men according to:

$$P_{men} = \theta_p$$

$$P_{women} = \theta_p \bullet (1 + \theta_{p-cov})$$

## Results

### Statistical analysis

The plasma levels of DNR and DOL showed a pronounced interindividual variation and the largest interindividual variation in DNR and DOL plasma concentrations was seen immediately after the DNR infusion (at time 1 h) with DNR and DOL mean and SD levels of 403,8 +/- 349,2 nM, range 60-1370 nM and 181,1 +/- 162,6 nM, range 0-670, respectively. We found a significant inverse correlation between DNR plasma levels and the baseline WBC count at 1 hour (Fig 1) but not at 5, and 24 hours after DNR infusion. At 1 h:  $R^2=0,11$ ,  $p < 0,05$ . At 5 h:  $R^2 = 0,05$ ,  $p = 0,22$ . At 24 h:  $R^2 < 0,01$ ,  $p = 0,9$ . There were no significant differences in mean WBC count and mean plasma level of DNR/DOL at any time point between patients who entered or did not enter complete remission (data not shown). No relation was found between the baseline WBC count and plasma DOL levels at any time point (data not shown). At 1 h DNR concentrations correlated with DOL concentrations in plasma ( $n = 40$ ,  $R^2 = 0.18$ ),  $p < 0.01$  (Fig 2). Although we found an inverse correlation between baseline WBC count and plasma concentrations of DNR we could not find any relation between the baseline WBC count and intracellular DNR concentration ( $n=24$ ,  $R^2 = 0,02$ ),  $p = 0,55$  (Fig 3).

### Population pharmacokinetic modelling

There was a clear discrepancy between the observed DNR and DOL data in our study and the predictions by the previously developed model by Callies *et al.* as seen in the VPC (Fig 4).

The model predicts a more rapid fall in plasma levels than the observed data which is also evident when comparing the observed data from the two studies.

The three compartment model for DNR by Callies *et al.* was fitted to the data, however due to the sparsity of the data the model was reduced to a two compartment model. The model captured the plasma concentrations time-profile of DNR well, as assessed by VPC (Fig 5a) and the parameters were estimated with good precision (Table 2). The typical values were estimated to 115 L/h for clearance and 373 L for the central volume of distribution. The interindividual variability of clearance (52%) and central volume of distribution (127%) was high and the two parameters were highly correlated, which means that a patient with high clearance also has a high central volume of distribution.

Using the basic population pharmacokinetic model the influence of patient demographics and baseline WBC count on the pharmacokinetic parameters were explored. A significant positive correlation between baseline WBC count and DNR central volume of distribution was estimated (dOFV 4.77,  $p < 0.05$ ), where the central volume of distribution is increased with 1.4% per million cells/ml blood change of WBC count from the mean baseline WBC count (39 million/ml). However, the uncertainty in estimated correlation parameter ( $\theta_{V-WBC}$ ) needs to be considered as well, as just a minor decrease in the unexplained variability between patients was observed (Table 2) and no difference was seen in the VPC after inclusion of baseline WBC count in the model (Fig 5b). The addition of baseline WBC count on clearance did not further improve the model fit (dOFV 1.97,  $p = 0.16$ ). Inclusion of baseline WBC on clearance alone resulted in no change in OFV. Hence, no correlation between baseline WBC count and clearance could be established. None of the patient demographic factors were found

to significantly explain any of the variability in clearance or volume of distribution between the patients and thus not included in the final model.

## **Discussion**

Age and peripheral WBC count have been identified as the most important pre-treatment risk factors in AML [9]. The WBC count in AML patients at diagnosis shows a great interindividual variation ranging from leucopenia to more than 400 million cells per ml blood. The main reason for the WBC count being such an important prognostic risk factor is probably the fact that most patients with a high WBC count have genetic mutations leading to an increased proliferation rate and a more aggressive leukemia [10]. In the present study we however found no significant difference in mean WBC count between patient who entered or did not enter complete remission possibly due to the fact that our study was underdimensioned to detect this difference.

Patients with acute leukemia have a large interindividual variation in plasma levels of anthracyclines [11-14]. The reason for this variation is not completely understood but variation in liver metabolism certainly plays a major role. Furthermore, the importance of anthracycline plasma level for patient outcome is also not clear [2, 15, 16]. We investigated if an inverse relation between the baseline WBC count and plasma concentration of DNR could be observed in patients with AML during induction therapy. We found a large interindividual variation in plasma levels of DNR (~ 100 times) in spite of the fact that the AML patients received the same dose of DNR per m<sup>2</sup> body surface. This large interindividual variation in plasma levels demonstrates the need for better dose individualisation.

A population pharmacokinetic model was developed and adequately described the time-course of DNR plasma levels. Compared to the previous developed model by Callies *et al.* the population estimate was approximately 46% higher for clearance and 33% lower for the distribution volume at steady state than in our study. Since the mode of DNR infusion might affect the intracellular accumulation of DNR [17, 18] we speculate that this difference may be due to the fact that patients in Callies study received a 10 minute infusion while the patients in the current study received a one hour infusion [8] since it has been shown, at least for doxorubicin, that infusion times can affect PK parameters [19].

We found a significant inverse correlation at the end of the DNR infusion, between the baseline WBC count and the plasma concentration of DNR that diminished at later sampling times. The diminished correlation could possibly be explained by the fact that the peripheral blood leukemic cells decrease rapidly during induction therapy.

The population analysis identified that the inverse correlation seen, at sampling immediately after infusion of DNR, is due to an increased central volume of distribution most likely as a result of a rapid uptake of drug into the large leukemic cell mass. This is supported by the fact that patients with very high WBC count ( $>50 \times 10^6/\text{ml}$ ) and low plasma DNR levels also had low plasma DOL levels which supports that the low DNR plasma concentration is not due to an increased systemic metabolism but more likely due to drug distribution. Furthermore, it has been speculated that the metabolism in the leukemic cells might play a significant role in the systemic metabolism of DNR [8] and consequently an even bigger role in patients with a large tumour burden but we found no significant evidence of this in our study as clearance was unaffected by WBC count. Even though the pronounced interindividual variation in plasma concentration was partly explained by WBC count a large extent of the interindividual

variability in central volume as well as in clearance remained unexplained. The large variability in clearance is most likely due to variation in liver metabolism [2, 19].

Previous studies have provided evidence that the peripheral blast cell count has an effect on the pharmacokinetics of anthracyclines in ALL and AML. Frost *et al.* studied doxorubicin (DOX) pharmacokinetics in children with acute lymphoblastic leukemia and found that patients with a WBC count higher than 50 million cells per ml had significantly lower plasma concentrations of DOX [14]. In other studies Ackland *et al.* and Piscitelli *et al.* studied DOX pharmacokinetics in patients with various solid cancers. Both investigators found an inverse correlation between the WBC count (in this case not leukemic cells) and the plasma concentration of DOX in patients with normal WBC levels [20, 21]. Kokenberg *et al.* investigated the pharmacokinetics of DNR in AML and they could not find an inverse correlation between the plasma levels of DNR and the WBC count as we did [16]. When compared to the current study, the AML patients in Kokenbergs study received different DNR doses during a bolus infusion whereas the patients in our study all received the same dose (except 3 patients who received reduced dose) during a 1 hour infusion. To our knowledge it has not been shown before that WBC count at diagnosis can influence the volume of distribution for DNR in AML patients.

The results indicate that patients with a high WBC count may require larger anthracycline doses, since plasma concentrations of DNR were lower in patients with a high WBC count due to high volume of distribution although this requires further studies. Furthermore, this finding also implies that the cellular concentrations would be lower, given that the underlying mechanism is the uptake of DNR to a large mass of leukemic cells as the same amount of drug is divided on more cells. However, in spite of a trend towards lower mean intracellular DNR content in patients with a high WBC count though this did not reach statistical

significance. This could possibly be explained by the fact that the study was underdimensioned to show this difference. Intracellular concentrations could only be measured in 24/40 patients due to insufficient number of isolated cells in patients with a low WBC count. Difficulties to isolate cells after treatment due to rapid elimination of cells from blood was observed. However, other investigators have also reported a lack of relationship between plasma levels of anthracyclines and cellular drug uptake which possibly could be explained by variation in expression of drug efflux pumps and other factors at the cellular level that could affect cellular uptake of DNR [22-24]. Kokenberg, however, found an inverse correlation between the plasma AUC of DNR and intracellular AUC of DNR and a negative correlation between intracellular DNR concentration and peripheral blast cells count at diagnosis [16].

Of particular interest is, in this connection, our recent results that AML patients entering complete remission had a higher intracellular concentration of DNR than patients who did not enter complete remission [7]. Noteworthy, in two recent studies it was found that a doubling of the standard dose of DNR (from 45 mg/m<sup>2</sup> to 90 mg/m<sup>2</sup>) led to a higher response rate without any obvious additional toxicity [25, 26]. It could be speculated that higher intracellular levels were reached but intracellular DNR concentrations were not determined in these studies. These studies support that there is room for a DNR dose increase in treatment of AML patients.

This study could be of a broader tumour biologic and clinical interest. It is possible, and even likely, that solid tumours may influence the pharmacokinetics of anticancer agents thereby influencing dosage requirements. Further studies are needed to investigate the influence of large tumour burdens on the pharmacokinetics of cytotoxic drugs.

**Conclusion**

An inverse correlation between the WBC count at diagnosis and plasma levels of DNR immediately after DNR infusion was found in patients with AML receiving induction therapy. This correlation was identified by population modelling to be caused by an increased central volume of distribution with higher levels of WBC. The results indicate that patients with high WBC count may require higher DNR doses although this needs further investigation.

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**Conflict of interest**

There were no conflicts of interest declared in this study.



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**Figure legends.**

Figure 1. Plasma DNR concentrations in relation to WBC count in AML patients (n=40) directly after a 1 hour DNR infusion. The dotted lines represent the 95 % confidence limits.

Figure 2. Plasma concentration of DNR in relation to plasma concentration of DOL in AML patients (n=40) directly after a 1 hour DNR infusion. The dotted lines represent the 95 % confidence limits.

Figure 3. Intracellular concentration of DNR in relation to WBC count in AML patients (n=24) directly after a 1 hour DNR infusion.

Figure 4. Visual predictive checks (80% prediction interval) based on the population pharmacokinetic model by Callies *et al.* superimposed on the observed DNR and DOL plasma concentrations. Observed data (dots) and the 95% confidence intervals around the simulated median (dark grey), 10th and 90th percentiles (light grey) are shown. Solid and dotted lines are the corresponding median and percentiles, respectively, of the observed data.

Figure 5. Visual predictive checks (80% prediction interval) for (a) the basic population pharmacokinetic model of DNR in plasma and (b) for the model including WBC counts. Observed data (dots) and the 95% confidence intervals around the simulated median (dark grey), 10th and 90th percentiles (light grey) are shown. Solid and dotted lines are the corresponding median and percentiles, respectively, of the observed data.



Figures

Fig 1

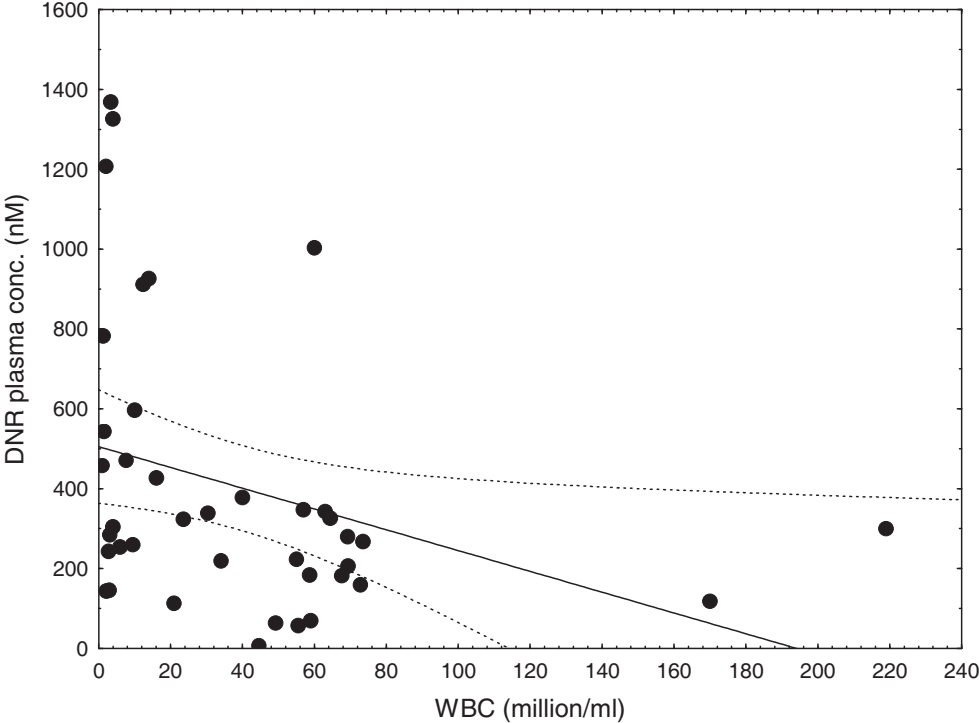


Fig 2

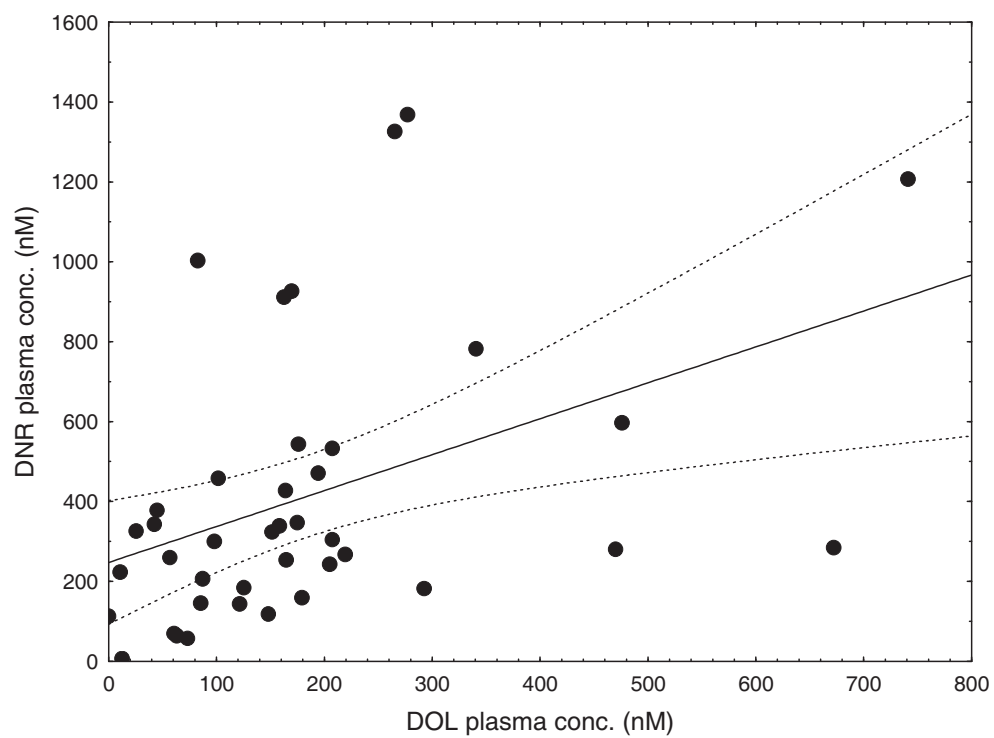


Fig 3

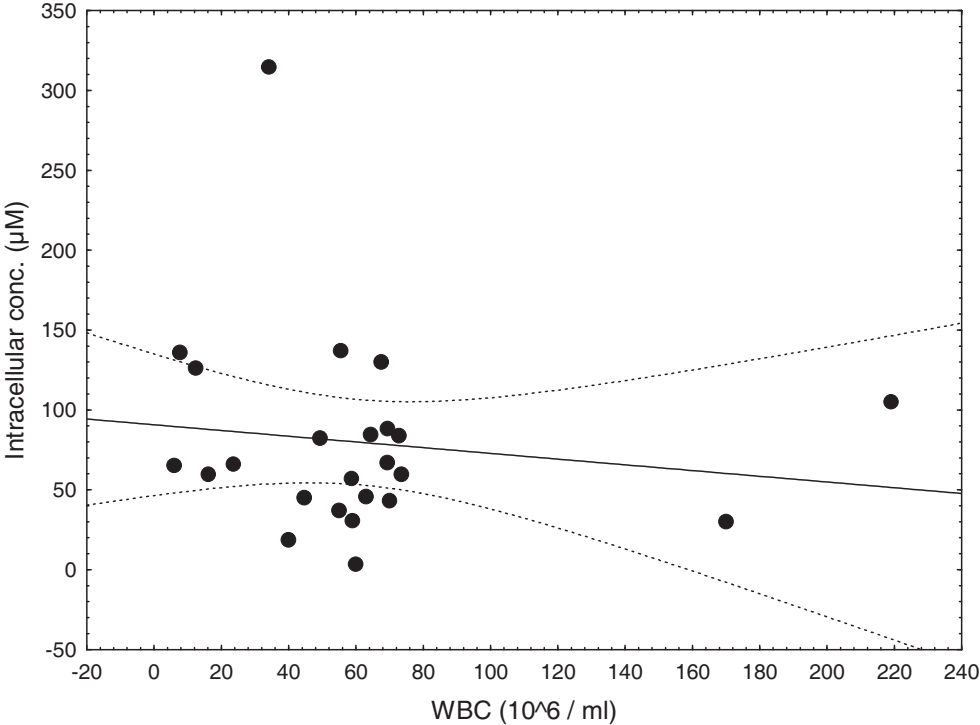


Fig 4

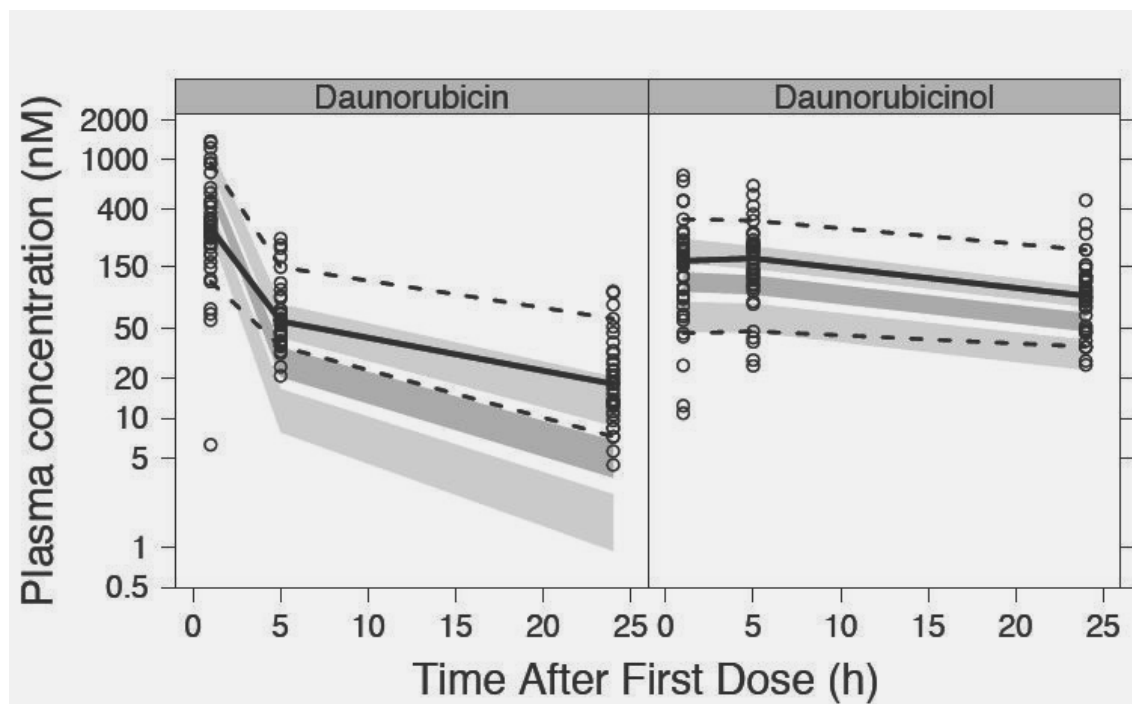




Fig 5

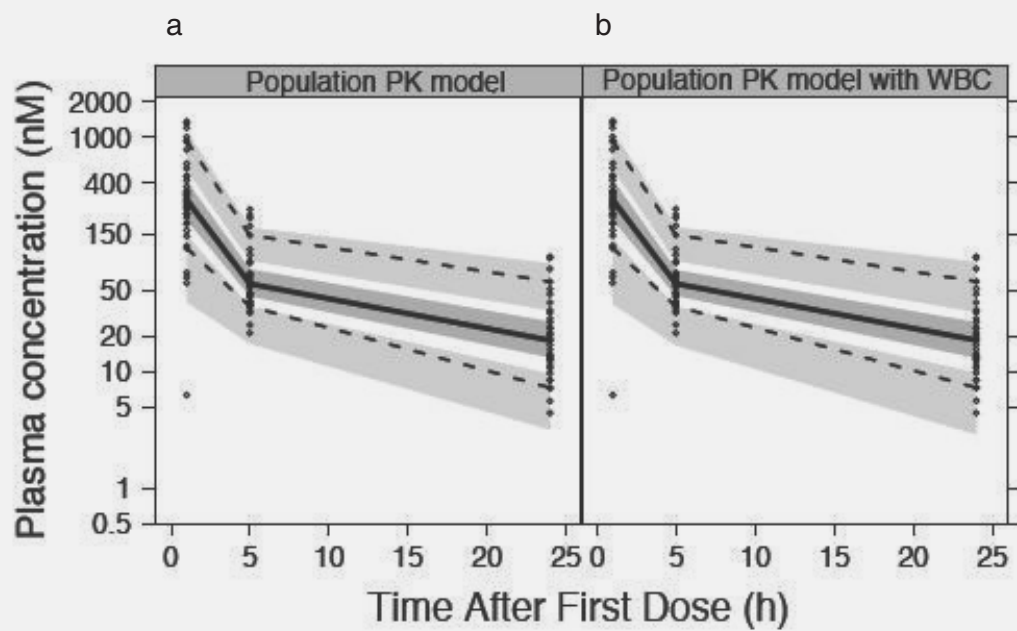


Table 1.

Patient characteristics and outcome

Patient No.	Sex	Age	Diagnosis	DNR dose (mg/m <sup>2</sup> )	WBC	Outcome
1	M	33	M5	60 mg/m <sup>2</sup>	12.4	+
2	M	52	M2	60 mg/m <sup>2</sup>	61.7	-
3	M	36	M5	60 mg/m <sup>2</sup>	58.7	-
4	F	79	M6	60 mg/m <sup>2</sup>	1.5	+
5	M	45	AML*	60 mg/m <sup>2</sup>	14	+
6	M	69	M5	60 mg/m <sup>2</sup>	23.6	+
7	F	83	M4	60 mg/m <sup>2</sup>	219	+
8	F	52	AML*	60 mg/m <sup>2</sup>	34.1	+
9	F	45	M3	60 mg/m <sup>2</sup>	67.6	+
10	M	54	M2	60 mg/m <sup>2</sup>	9.5	-
11	F	61	AML*	60 mg/m <sup>2</sup>	64.4	+
12	F	52	M1	60 mg/m <sup>2</sup>	72.8	+
13	M	64	M1	60 mg/m <sup>2</sup>	4	+
14	F	59	M4	60 mg/m <sup>2</sup>	69.4	+
15	M	63	AML*	60 mg/m <sup>2</sup>	49.3	-
16	M	41	M3	60 mg/m <sup>2</sup>	59	+
17	F	44	AML*	60 mg/m <sup>2</sup>	2.2	+
18	M	73	M2	60 mg/m <sup>2</sup>	3	+
19	M	75	AML*	60 mg/m <sup>2</sup>	1	+
20	M	71	AML	60 mg/m <sup>2</sup>	73.5	-
21	M	54	M2	60 mg/m <sup>2</sup>	3.4	+
22	F	61	AML*	60 mg/m <sup>2</sup>	16.3	+
23	F	68	AML*	60 mg/m <sup>2</sup>	55.5	+
24	M	52	M4	45 mg/m <sup>2</sup>	60	-
25	F	60	M5	60 mg/m <sup>2</sup>	21	+
26	M	76	M5	60 mg/m <sup>2</sup>	63	+
27	M	79	AML*	60 mg/m <sup>2</sup>	170	-
28	F	80	AML*	60 mg/m <sup>2</sup>	2.1	+
29	F	37	M4	60 mg/m <sup>2</sup>	69.3	+
30	F	54	M5	60 mg/m <sup>2</sup>	44.6	+
31	F	83	AML*	60 mg/m <sup>2</sup>	55	-
32	F	67	AML*	60 mg/m <sup>2</sup>	4	-
33	F	65	M2	60 mg/m <sup>2</sup>	30.4	+
34	F	80	AML*	30 mg/m <sup>2</sup>	2.8	-
35	M	68	AML*	54 mg/m <sup>2</sup>	56	+
36	F	64	AML*	60 mg/m <sup>2</sup>	3.7	-
37	F	54	AML*	60 mg/m <sup>2</sup>	10	-
38	M	39	M5	60 mg/m <sup>2</sup>	57	+
39	F	61	AML*	60 mg/m <sup>2</sup>	1.3	-
40	F	61	AML*	60 mg/m <sup>2</sup>	3.1	+

\* Not further subclassified

+ remission

- absence of remission

Table 2

Population pharmacokinetic parameter estimates

	Population PK model				Population PK model with baseline WBC			
	Typical estimate (RSE %)		Interindividual variability CV % (RSE %)		Typical estimate (RSE %)		Interindividual variability CV % (RSE %)	
CL ( L/h )	115	10	52	21	114	10	51	20
V <sub>c</sub> (L)	373	25	127	26	412	25	120	29
Q (L/h)	252	18			254	20		
V <sub>p</sub> (L)	1120	11			1120	14		
Proportional residual error (%)	52.4	8			51.7	7		
Covariate $\theta_{V-WBC}$					0.0138	47		
Correlation CL-V (%)	70	65*			65	58*		

CV, coefficient of variation; RSE, relative standard error; CL, clearance; V<sub>c</sub>, volume of central compartment; Q, intercompartmental clearance; V<sub>p</sub>, volume of peripheral compartment.

\* = p < 0.05.

$$V_{c_{WBC,i}} = V_c * (1 + \theta_{Vc-WBC} * (WBC - median_{WBC})) = 412 * (1 + 0.0138 * (WBC - 39))$$



IV



# **Comparison of uptake mechanisms for different anthracyclines in leukemic cells**

by

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Keywords: anthracyclines, leukemic cells, nucleoside transporters, suramin.

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## **Abstract**

**Aims:** The mechanisms behind cellular anthracycline uptake are not completely understood. It is of great interest to gain more insight into cellular uptake mechanisms since this could possibly be used to increase the selectivity of the drugs. We therefore compared the uptake of 5 different anthracyclines, daunorubicin (DNR), doxorubicin (DOX), epirubicin (EPI), idarubicin (IDA), and pirarubicin (PIRA) by leukemic cells and also investigated the possible involvement of specific carriers.

**Methods:** HL-60 cells were incubated with increasing concentrations of anthracyclines for 1 hour and cellular drug uptake was determined. The cells were also preincubated with various protein inhibitors or nucleosides and then with the anthracyclines after which drug uptake was determined with HPLC. Apoptosis was determined with propidium iodine staining and flow cytometry.

**Results:** Of all anthracyclines studied DNR, IDA and PIRA had the highest cellular uptake with a sharp increase in uptake at extracellular concentrations  $> 1 \mu\text{M}$ . Uptake of DOX, DNR and IDA was greatly reduced at  $0^\circ\text{C}$ . Suramin, a purinergic-2-receptor inhibitor, strongly inhibited the uptake of all anthracyclines except PIRA and dipyridamole, a nucleoside transport inhibitor, only inhibited the uptake of DNR. The addition of nucleosides reduced the uptake of DNR, IDA and PIRA.

**Conclusion:** Our results suggest different uptake mechanisms for various anthracyclines. We found evidence for carrier mediated uptake mechanisms, the data support the involvement of the nucleoside transporter family. Furthermore, our results also indicate an involvement of purinergic-2-receptor signalling in anthracycline uptake.



## Introduction

Anthracyclines like daunorubicin (DNR), doxorubicin (DOX), epirubicin (EPI), pirarubicin (PIRA) and idarubicin (IDA) are important anticancer agents. They are highly effective against a wide variety of malignancies including leukemias, stomach and breast cancer [1-3]. The adverse affects of these agents include a dose related acute bone marrow suppression, alopecia, and cardiomyopathy [4, 5]. De novo and acquired resistance to these drugs are major clinical problems that restrict their usefulness [6, 7]. The adverse effects of the drugs are caused by their uptake in normal cells. It is therefore of interest to gain more insight into the cellular uptake mechanisms since this could possibly be used to increase the selectivity of the drugs.

There are still several controversies regarding the mechanism of cellular anthracycline uptake. Anthracyclines are generally believed to be transported into the cells through passive diffusion [8, 9].

Previous studies have however also indicated that DOX and PIRA can be taken up by nucleoside transporters (NTs), including equilibrative NT (ENT) and concentrative NT (CNT) in HL-60 cells [10, 11]. Evidence for such transport mediated uptake was however not found in normal mononuclear blood cells [10]. This opens up the possibility of different uptake mechanisms in normal and malignant cells. Moreover, higher expression of nucleoside transporters has been reported in tumour cells as compared with normal cells [12]. Subsequently it has been found that different transport mechanisms are involved in the uptake of DOX and PIRA in Ehrlich ascites carcinoma cells [13], but the specific transporters were not identified. Data in this study also supported that the uptake mechanisms for anthracycline might be tumour type specific [13]. Further studies have shown that uridine-transportable CNT were involved in the uptake of PIRA into Ehrlich ascites carcinoma cells [14].

As some anthracyclines seem to be taken up into the cells via nucleoside transporters, it prompted us to compare the uptake mechanisms for different anthracyclines in leukemic cells using different protein inhibitors like dipyridamole (a NT antagonist), nitrobenzylthioinosine 5'-monophosphate (a NT antagonist) and suramin.

Suramin is an interesting molecule with diverse biological effects. It has been shown to inhibit the binding of growth factors to receptors, inhibit cellular energy metabolism and it is also a purinergic receptor antagonist [15-17]. Since suramin has also been seen to modulate the cytotoxic effect of DOX *in vitro* [18] we wanted to see whether or not suramin could affect DOX uptake in leukemic cells. Identification of transport proteins and mechanisms for anthracycline uptake could lead to increased uptake of anthracyclines to leukemic cells through modification of the transport process or the drug itself and thereby improve leukemia treatment.

## Methods

### *Cell incubation procedure*

Human leukemic HL 60 cells were incubated in a humidified incubator with 5% CO<sub>2</sub> at 37°C in RPMI 1640 cell culture medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 µg/ml). For dose-uptake analyses cells were incubated at a concentration of 10<sup>6</sup>/ml in 8 ml growth medium for 1 h with 0,1, 0,5, 1, 5 and 10 µM DOX, EPI, PIRA, DNR or IDA. HL-60 cells were also preincubated for 1 h with suramin (2-500 µM), nucleosides (100 µM), dipyridamole (1µM), nitrobenzylthioinosine 5'-monophosphate (NBMPR, 1µM) (Sigma Aldrich AB, Stockholm, Sweden) or kept at 0°C (for the duration of the experiment) before being incubated with anthracycline in 15 cm<sup>2</sup> tissue culture flasks. Directly after the anthracycline incubation, aliquots were removed (2x1

ml for drug determination), cells harvested by centrifugation at 550 g for 5 minutes at 4°C, and washed twice with ice cold PBS (pH 7.4). In some experiments, the remaining cells were washed twice with PBS and then resuspended in drugfree medium for 24 h after which an aliquot of the cells was used for determination of DNA fragmentation.

#### *Drug analysis*

Cells were lysed in ice-cold water, sonicated for 10 seconds using a ultrasonic processor, VCX 400 (Sonics & Materials, Danbury, CO, USA), and precipitated with 60% acetonitrile for anthracycline analysis. Anthracycline concentrations in cells and medium was determined by HPLC using a phenyl- $\mu$ -Bondapak column (3.9 x 150 mm, 5 mm. Waters Associates, Milford, MA, USA) eluted with acetonitrile and 0.2% ammonium formate pH 4 (60:40 v/v) at a flow rate of 1.5 ml/min. PIRA was used as internal standard for DOX and DNR while DOX was used as an internal standard for IDA, PIRA and EPI. The anthracyclines were quantified by a fluorescence detector model Shimadzu RF-551 fluorescence HPLC monitor at  $\lambda_{\text{excitation}}$  485 nm and  $\lambda_{\text{emission}}$  560 nm. The detection limit of the assay is  $5 \cdot 10^{-3} \mu\text{M}$  and the range of quantification is 0.03-20  $\mu\text{M}$ , with a coefficient of variation (CV) of less than 7 %. Cellular drug concentration is expressed in  $\mu\text{M}$ .

#### *Determination of apoptosis*

We used a propidium iodide (PI)-based staining procedure as previously described [19]. The appearance of cells less intensively stained than G1 cells (sub-G1 or  $A_0$  cells) in flow cytometric DNA histograms was used as a marker of apoptosis. Briefly, a cell pellet containing  $10^6$  cells was gently resuspended in 0.5 ml PI staining solution (PI 50 $\mu\text{g/ml}$  in

0.1% (w/v) sodium citrate plus 0.1% (v/v) Triton X-100) in 12 x 75 mm polypropylene tubes. The tubes were placed at 4°C in the dark for 1 to 3 days before flow cytometry measurements. The PI fluorescence of individual nuclei was measured using a FACScan flow cytometer (Becton and Dickinson, Mountain View, CA, USA) with a single 488-nm Argon laser. The red fluorescence due to PI staining of DNA was detected in the FL-3 channel and the data was registered on logarithmic and linear scales. The forward scatter and side scatter of particles were measured simultaneously. The flow rate was set at 12  $\mu$ l /second and at least 10000 target events were collected for each sample. Analysis was performed using Cell Quest<sup>TM</sup> software.

#### *Statistical analyses*

Student's t-test was used for test of significance and P values <0.05 were considered significant.

## **Results**

### **Dose-uptake studies**

We studied dose-uptake relationships for the different anthracyclines (fig 1). All anthracyclines had similar cellular uptake at extracellular concentrations 0,1-1,0  $\mu$ M (fig 1). However, IDA, DNR and PIRA uptake increased markedly at extracellular concentrations > 1.0  $\mu$ M.

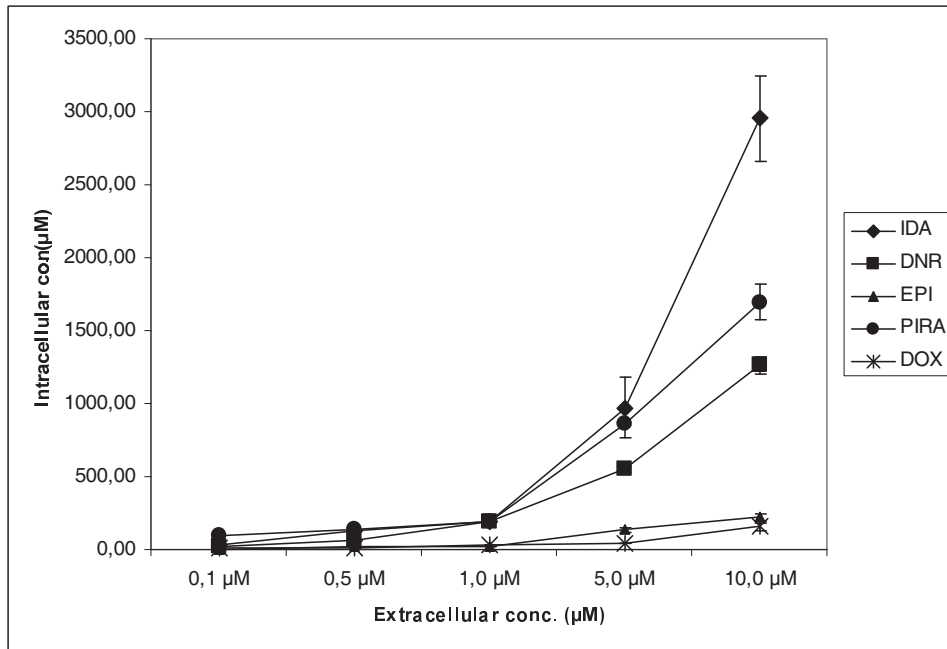


Figure 1. Effect of dose on anthracycline uptake in HL-60 cells after a 1 hour incubation with IDA, DNR, EPI, PIRA or DOX (n=4, mean and SD).

#### Effect of suramin on DOX uptake

Suramin inhibited DOX uptake in a concentration-response dependent manner (fig 2). The highest suramin concentration inhibited DOX uptake by 95 %. There was a parallel decrease in DOX induced cell death in cells exposed to suramin. Virtually no cell death could be detected in cells exposed to 200 and 500 µM suramin (Fig 3.).

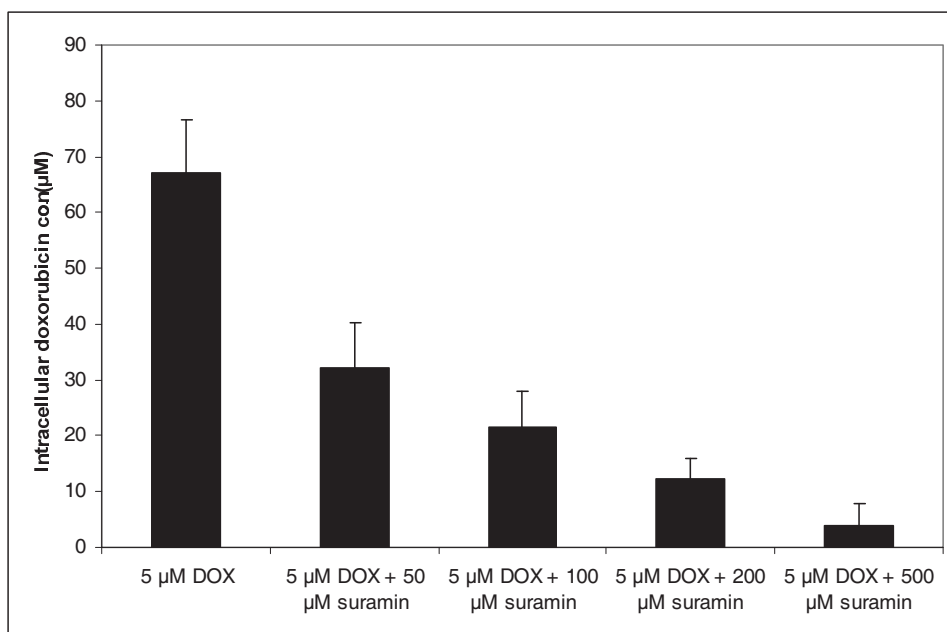


Figure 2. Effect of suramin on DOX uptake in HL-60 cells after a 1 hour incubation with 5 μM DOX (n = 4, mean and SD).

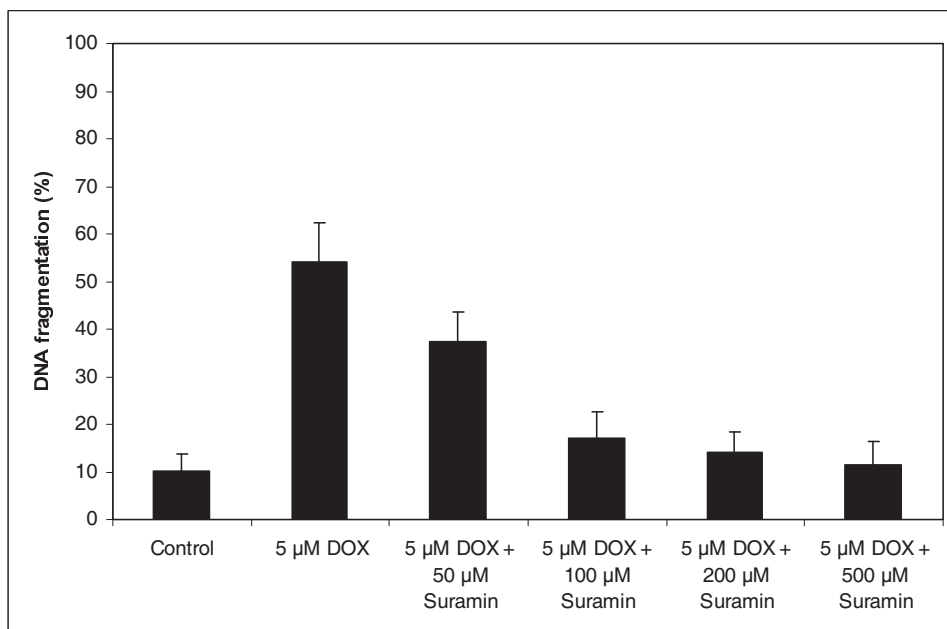


Figure 3. Effect of suramin on DOX induced cell death in HL-60 cells after a 1 hour incubation with 5  $\mu$ M DOX (n = 4, mean and SD).

#### **Effect of temperature and various transport inhibitors on anthracycline uptake.**

We next compared the cellular uptake of the various anthracyclines at 0°C, 37°C, and also studied the effects of suramin, DP, a nucleoside transport (NT) inhibitor, or NBMPR, a equilibrative nucleoside transport (eNT) inhibitor (fig 4). DOX, DNR and IDA uptake was highly temperature dependant and greatly inhibited by suramin. DP inhibited the uptake of DNR only. EPI and PIRA uptake was unaffected by temperature and EPI uptake was also strongly inhibited by suramin. None of the transport inhibitors affected PIRA uptake (fig 4).

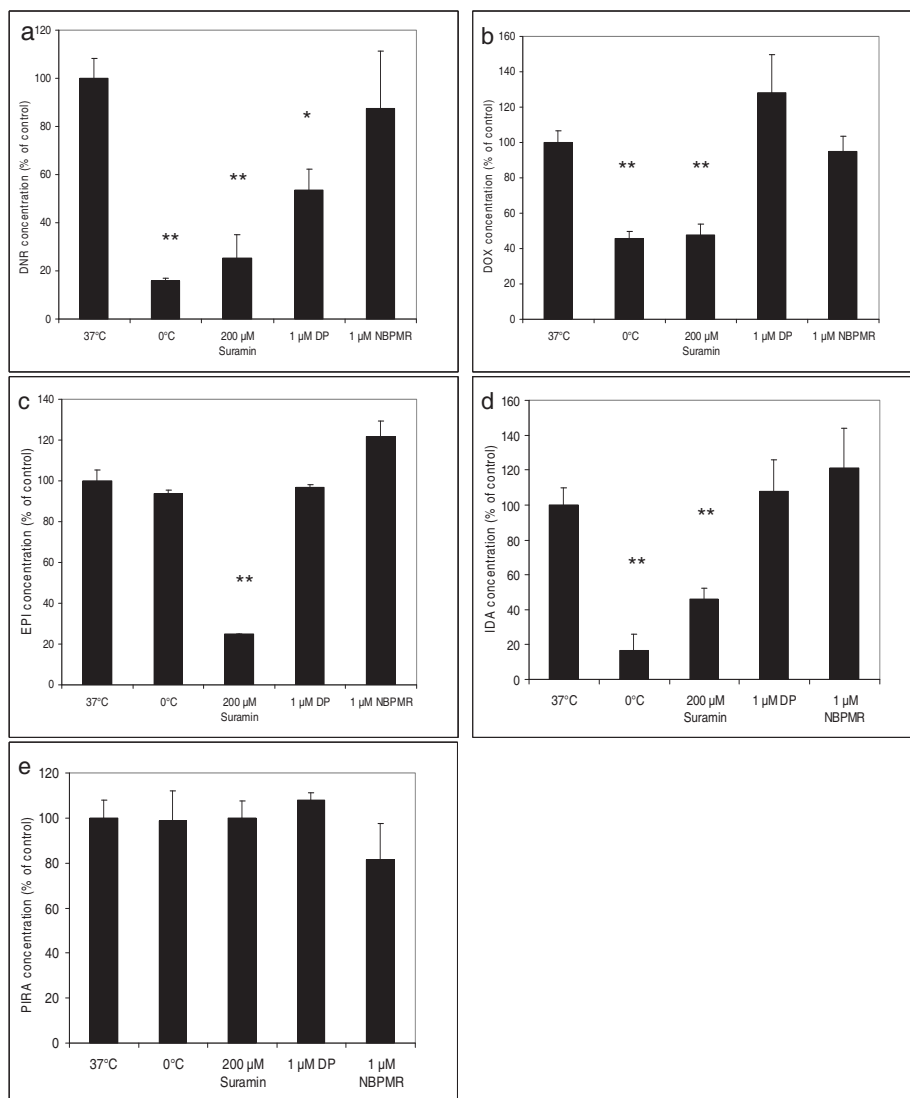


Figure 4. Effect of temperature and various inhibitors on the uptake of 4  $\mu$ M DOX (a), 4  $\mu$ M DNR (b), 4  $\mu$ M EPI (c), 1  $\mu$ M IDA (d), and 1  $\mu$ M Pira (e) in HL-60 cells after a 1 hour incubation. DP = dipyridamole, NBMPR = nitrobenzylthioinosine 5'-monophosphate (n = 4, mean and SD). \* = p<0.05 and \*\* = p<0.01 compared with the control (37°C).



**Effect of nucleosides on anthracycline uptake.**

We also investigated the effect of direct addition of nucleosides on anthracycline uptake. No clear effect of nucleosides on DOX or EPI uptake was found. However, DNR, IDA and PIRA uptake was inhibited to some degree by nucleosides. DNR uptake was inhibited by adenosine, cytidine, thymidine and uridine while PIRA was inhibited by 60 % after exposure to thymidine and by 40 % by uridine (fig 5). IDA uptake was inhibited by 65 % after addition of adenosine (fig 5).

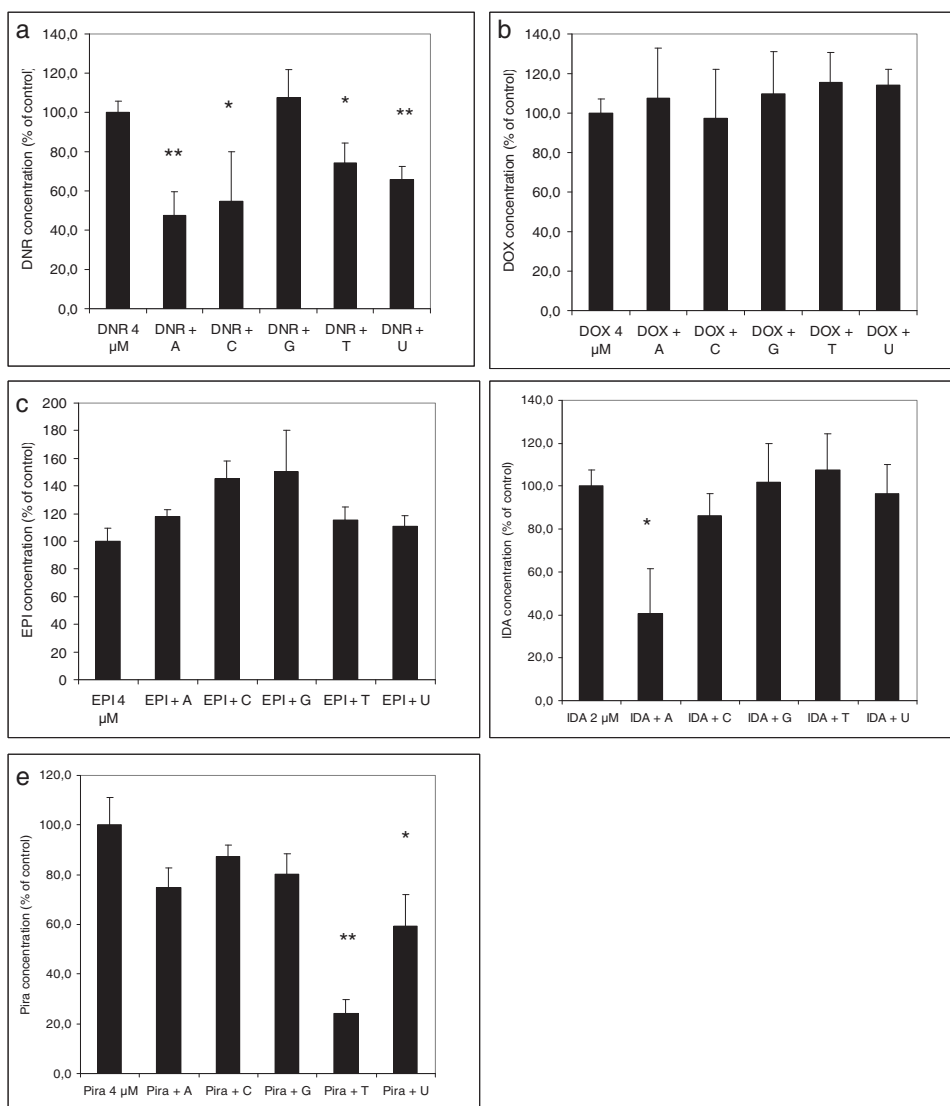


Figure 5. Effect of nucleosides on the uptake of 4  $\mu$ M DNR (a), 4  $\mu$ M Dox (b), 4  $\mu$ M EPI (c), 1  $\mu$ M IDA (d  $\mu$ M), and 1  $\mu$ M Pira (e) in HL-60 cells after a 1 hour incubation measured a % uptake of the control in the presence of nucleosides, A = adenosin, C = cytidine, G = guanosin, T = thymidine, U = uridine (n = 4, mean and SD). \* = p<0.05 and \*\* = p<0.01

## Discussion

Anthracyclines are believed to be taken up by passive or facilitated diffusion [20, 21] and/or by a flip flop mechanism [22] and then pumped out in an energy dependant manner via drug transporters like p-glycoprotein (p-gp) [21, 23]. In this study we compared cellular drug uptake and the possible involvement of various transporters/receptors for different anthracyclines. We chose a one hour incubation at 37° C since previous uptake studies by us and others have shown that a plateau was reached in one hour in leukemic cells [24, 25].

Anthracycline uptake varied markedly in HL-60 cells. DNR, IDA and PIRA accumulated in a biphasic manner while the uptake of DOX and EPI increased linearly over the entire concentration range studied. At concentrations >1  $\mu$ M, the cellular uptake of DNR, IDA and PIRA increased rapidly. A reason for the higher uptake of IDA could be that it is more a lipophilic drug but it could also be explained by it's lower affinity for p-gp as compared with other anthracyclines [25-27]. It could be speculated that at low extracellular concentrations efflux pumps, like p-gp, could be able to cope with the intracellular concentrations and pump out IDA leading to low intracellular concentrations. In contrast, at higher extracellular concentrations the efflux pumps are saturated and hence disrupt the influx/outflux equilibrium of IDA thereby leading to a dramatic increase in intracellular concentration. In a similar study conducted on rat-hepatocytes the authors concluded that at low extracellular concentrations the uptake was carrier mediated while the steeper linear increase in IDA uptake at higher extracellular concentrations was due to diffusion. A rationale for why anthracycline uptake would be protein mediated at lower concentrations and diffusion facilitated at higher concentrations was however not given [28].

We found that suramin, a p2-receptor antagonist [29], inhibited the uptake of DOX in a dose dependant manner to such an extent that HL-60 cells were rescued from apoptosis. This indicates that p2-receptor signaling could be involved in the cellular uptake of DOX. The

inhibitory effect of suramin on DOX uptake and apoptosis is surprising considering that previous studies have shown that suramin enhances the cytotoxic effects of DOX in prostate cancer cells [30, 31]. However, since there are indications that the mechanisms behind anthracycline uptake might be tissue and tumour specific [12, 13], suramin might not affect DOX uptake in prostate cancer cells in the same way as in leukemic cells. Furthermore, we also found that suramin inhibited the uptake of DNR, IDA, and EPI, but not that of PIRA.

It is noteworthy that IDA, DOX and DNR uptake was temperature dependant in contrast to EPI and PIRA indicating that EPI and PIRA might have an anthracycline uptake mechanism separate from the others. Inhibition of uptake by low temperature supports energy dependent carrier mechanisms. Dipyridamole, an NT-inhibitor, inhibited the uptake of DNR but not the uptake of any of the other anthracyclines supporting that cellular uptake of DNR might be facilitated by a protein belonging to the nucleoside transporter receptor family. It has been suggested by others that nucleoside transporters could be involved in DOX and PIRA uptake but carriers for IDA and DNR are yet to be discovered [10]. We found that addition of adenosine inhibited IDA uptake while thymidine and uridine inhibited the uptake of PIRA which was surprising since we could not see any effect of the nucleoside inhibitors (DP and NBMRP) on these drugs. That DNR uptake could be mediated by nucleoside transporters was further reflected by the reduced DNR uptake after addition of adenosine, cytidine, thymidine, and uridine. However, we could not see any effect of nucleosides on DOX or EPI uptake. We are aware that the inhibitors used in this study, suramin, DP, and NBMPR are agents with diverse biological effects and their proposed specificity may be questioned. The results obtained in this study are therefore complicated to interpret since the anthracyclines studied all have different uptake profiles with the closest common denominator being a reduced anthracycline uptake in the presence of suramin. Nonetheless, our study supports that anthracyclines have different ways of entering leukemic cells and that some form of transport

carrier seems to be involved regardless of anthracycline. Further studies are needed to more in detail clarify the suggested uptake mechanisms discovered in this study.

## Conclusion

Our study provides evidence for carrier mediated uptake mechanisms for anthracyclines and that they might vary depending on anthracycline. The data support the involvement of the nucleoside transporter family and also indicate the involvement of purinergic-2-receptor signaling in anthracycline uptake.

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