Pharmacokinetic studies of daunorubicin in patients with acute myeloid leukemia

(Farmacokinetische studies van daunorubicine in patiënten met acute myeloide leukemie)

PROEFSCHRIFT

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Aan Hans
Aan mijn ouders

Contents

Abbreviations

AML	- acute myeloid leukemia
ALL	- acute lymphocytic leukemia
ara-C	- cytosine arabinoside
a.u.	- arbitrary units
AUC	- area under the concentration-time curve
BN	- brown Norway rat
BNML	- acute myeloid leukemia in the brown Norway rat
Cl	- plasma clearance
CR	- complete remission
DNA	- deoxyribonucleic acid
DNR	- daunorubicin
DOL	- daunorubicinol
DOX	- doxorubicin
EDTA	- ethylene-dinitro-tetra-acid
EORTC	- European Organization for Research and Treatment of Cancer
FAB	- French-American-British working party
FACS	- fluorescence activated cell sorter
HPLC	- high-performance liquid chromatography
IV	- intravenous
k_{el}	- elimination rate constant
MDR	- multiple drug resistance
NADP	- nicotinamide adenine dinucleotide phosphate
NADPH	- nicotinamide adenine dinucleotide phosphate, reduced
PBS	- phosphate-buffered saline
PR	- partial remission
R	- Spearman correlation coefficient
RD	- resistant disease
RNA	- ribonucleic acid
SD	- standard deviation
$t_{1/2}$	- halving time
V_1	- volume of central (plasma) compartment
Vd_{area}	- apparent distribution volume
WBC	- white blood cells

CHAPTER 1

General introduction

1.1. Clinical studies with daunorubicin

Since the introduction of daunorubicin (DNR) as an antitumor agent into clinical studies in the late 1960s, its use and study of its activity have been confined almost exclusively to the acute leukemias. In fact, there have been no systematic studies of the efficacy of DNR in patients with solid tumors of various types (1-3), despite the fact that the drug is closely related to doxorubicin (DOX), an anticancer drug with a broad clinical spectrum (4). The possibility that DNR might be at least as active as DOX in solid tumors remains to be determined (5,6).

With respect to the acute leukemias, it has been shown that both children and adults with acute lymphocytic leukemia (ALL) show significant clinical responses to chemotherapy programs that include DNR (7,8). In addition, DNR is effective in the treatment of pediatric acute myeloid leukemia (AML) (9,10). DNR was introduced into clinical trials in adult AML at a time when treatment of this type of leukemia was beginning to receive greater attention as a potential target for specific therapy (11,12). Until that time AML had a very poor prognosis. Untreated AML was rapidly fatal with a median survival of 2-6 months. Death frequently resulted from infection or haemorrhage, caused by the granulocytopenia and thrombocytopenia that was the consequence of the bone marrow suppression resulting from the infiltration of the bone marrow by leukemic cells. In those days hematologists questioned whether patients with AML should be treated at all (13), since most of the available cytotoxic drugs were relatively ineffective in patients with AML and only showed significant activity in the treatment of ALL. In AML these drugs (e.g., methotrexate, vincristine, prednisone, 6-mercaptopurine, cyclophosphamide, L-asparaginase and cytosine arabinoside) resulted in complete remission (CR) rates of less than 25%. It then appeared that DNR as a single agent produced CR rates of 20-50% (14-17). When DNR was subsequently combined with cytosine arabinoside (ara-C) during remission induction (RI) therapy, survival of all treated AML patients was significantly better than when DNR was used as a single agent. Studies were then performed that identified the combination regimen of DNR and ara-C (e.g., DNR on days 1 through 3 associated with a seven-day treatment of ara-C) with slight variations as probably being the most efficacious RI regimen used to date (18-20). Since then, complete remissions in 60-80% of patients with AML have been reported repeatedly (19-22).

Despite the advances in the treatment of AML, CR rates and their durations vary significantly among patients and the biological basis of this is only partly understood (23). Patients who do not enter remission are a heterogeneous group including individuals with persistent leukemia due to inadequate reduction of drug

sensitive leukemic cells or the early reappearance of leukemic cells following initial marrow hypoplasia after chemotherapy. In these cases, the leukemia appears resistant to the drugs used. Other individuals die from infections or hemorrhagic complications during marrow hypoplasia during or after RI therapy. Outcome of RI therapy is determined by a multiplicity of interactive factors (listed in Table 1). Understanding of these factors and their role in the response to therapy is essential for further progress of therapy of AML.

Table 1:
Factors which determine the outcome of remission induction therapy in AML

Patients characteristics (pretreatment)	
1. age:	determines tolerance to side effects of chemotherapy
2. past medical history of prior cytotoxic therapy, preleukemic syndrome or the presence of specific chromosomal aberrations:	determines bone marrow repopulating capacity after chemotherapy or prognosis
3. medical complications (e.g., infection, bleeding, organ failure), concomitant disease and the use of other drugs:	determine early death from intercurrent disease
AML cell properties	
4. sensitivity to chemotherapeutic agents:	determines response to therapy
5. maturation stage of leukemic stem cell:	determines repopulation and differentiation capacity of AML
Pharmacological factors	
6. chemotherapeutic agents used and ability of patient to metabolize and excrete these drugs:	determine toxicity of regimen
7. access to pharmacologically sanctuary sites and ability of uptake of drugs by AML cells:	determine delivery of chemotherapeutic agents to target tissue in vivo

Apart from the above mentioned factors, there are many other clinical, hematological, cytological, and cytochemical parameters that are of prognostic value in predicting the likelihood of achieving CR. Favourable factors may include: a) female sex, b) the presence of Auer rods (24), c) more differentiated FAB classification subtypes (M2, M3, M4, M5b) (25,26), and d) translocation (8;21) and inversion-16 patterns on the pretreatment cytogenetic analysis (27). Unfavourable factors may include: a) high tumor load (28, 29), b) decreased levels of serum albumin and calcium, and elevated levels of blood glucose, blood urea nitrogen, and serum creatinine (24), and c) the presence of MY4 and MY7 myeloid surface antigens on the AML cells (30).

Besides optimization of supportive care to prevent death from intercurrent complications (e.g., transfusion policy, antibiotics, in vivo application of hematopoietic growth factors or other biological response modifiers), further improvements in CR rate may be expected from a) the development of more effective and b) less toxic agents, c) more effective/less toxic time scheduling of available agents in combination therapy or d) a more rational approach to treatment through individualization of therapy. Particularly, individual differences of drug handling may play an important role in determining the clinical response to therapy.

The aim of the present study was to identify pharmacologic variables of DNR that may give insight into the mechanisms which are responsible for individual differences of clinical response of AML patients to RI therapy. The phases of these investigations are outlined in more detail in paragraph 1.3. As a background for those investigations the pharmacology of DNR will be introduced briefly (paragraph 1.2.).

1.2. Daunorubicin

1.2.1. Physical-chemistry

In 1959 the microorganism isolated from a soil sample collected in Apulia, Italia, was recognized as a new species and named Streptomyces peucetius (31). The first clinically effective compound was discovered independently in Italy and in France in 1963. The group of Di Marco and coworkers performed its isolation from S. peucetius and designated the compound daunomycin (32). Dubost and co-workers gave the name rubidomycin to their product isolated from S. coeruleoribidus (33). When the identity of these two antibiotics was established, the name daunorubicin was chosen to reflect the dual origin (in France the name rubidomycin is still used). A different strain of S. peucetius, known as the caesius variety, produced the 14-hydroxy analog of DNR, doxorubicin (34).

DNR and DOX belong to the group of anthracycline antitumor antibiotics. The name anthracycline is based upon structural features of compounds in this family: they have anthraquinone chromophores related to those of the tetracyclines (35). Besides antitumor activity, DNR shows moderate inhibiting effects on some gram-positive and gram-negative bacteria, and fungi (36), and on bacteriophages and double-stranded DNA viruses (37,38).

Fig.1 shows the chemical structures of DNR and DOX. The DNR molecule is constituted of a hydrophobic part, the aglycone (daunomycinone; red visible color), which is linked to the aminosugar by a glycoside band, involving the 7-hydroxyl group of the aglycone. The aminosugar (daunosamine) forms the hydrophilic part of the drug molecule (Fig.1). The intact aminosugar residue is essential for biological activity (39). DNR, as well as DOX, contain both acidic and basic functions, such as the amino group in the sugar moiety and the two phenolic groups in the aglycone part of the molecule which can donate a proton, whereas in concentrated acid one of the quinone carbonyl groups can accept a proton (40,41). Alterations in the chemical structure in either the aglycone part or the aminosugar part of the molecule influence its polarity and the charge, which both determine its affinity to cell structures like membranes and DNA (42). Most anthracyclines, having identical fluorophores, exhibit the same fluorescent properties. On excitation of the DNR

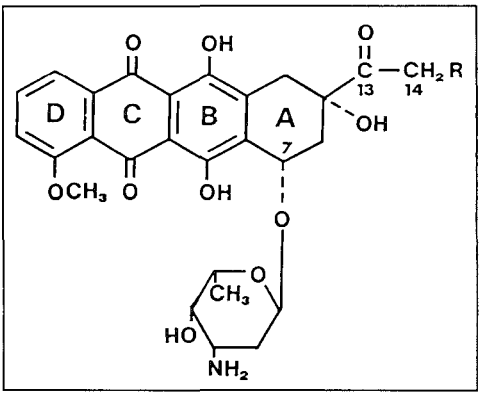


Fig. 1:
Chemical structure of daunorubicin and doxorubicin

R = H (daunorubicin)
R = OH (doxorubicin)

molecule at 470 nm in ethanol emission maxima could be detected at 554 and 585 nm (43) (Fig.2). Fluorescence analysis of the anthracyclines can be used for bioanalytical purposes, such as the analysis in tissues and body fluids, and for pharmacokinetic studies.

1.2.2. Mechanism of action

Three potential mechanisms of action by which DNR kills tumor cells and causes its characteristic toxicities have been described:

A. Interaction with DNA and RNA synthesis
DNA has generally been assumed to be the primary target of the biological action of DNR. The binding of DNR to DNA involves more than one class of sites (44). On one hand DNR may intercalate with DNA and on the other hand it may bind weakly to DNA. As regards the 'strong' intercalation complex with DNA (45,46), the B and C rings of the chromophore of the molecule (Fig.1) intercalate between adjacent base pairs into the double helix of DNA (Fig.3) (47). Whether DNR preferentially binds to certain base compositions or base sequences is somewhat controversial (48). Some have suggested preference for G-C- (49,50) others for A-T-sequences (51).

The 'weakly' bound DNR molecules are thought to be attached to DNA by means of electrostatic interaction involving the DNA phosphate groups and the DNR amino group (44). Upon binding of the drug to DNA the properties of the template DNA are modified, leading to inhibition of the processes of DNA replication and RNA transcription (52,53). Intercalation of DNR together with inhibition of topoisomerase II (54,55) leads to enhanced DNA fragmentation, DNA breaks and chromosomal aberrations with inhibition of repair. In addition, the mechanism of action of DNR involves the inhibition of DNA- and RNA-polymerases (56,57). Among the various species of RNA, synthesis of ribosomal RNA appears to be the most sensitive to DNR (58).

B. Free radical formation
DNR can be enzymatically reduced to semiquinone free radicals (catalysed by the microsomal P450 reductase) (59-61), which in turn can generate reactive oxygen radicals such as superoxide, hydrogen peroxide or hydroxyl radical (Fig.4). These reactive oxygen species may cause DNA strand scission (62-64) and peroxidation of lipids causing membrane damage of cells and of mitochondria (64).

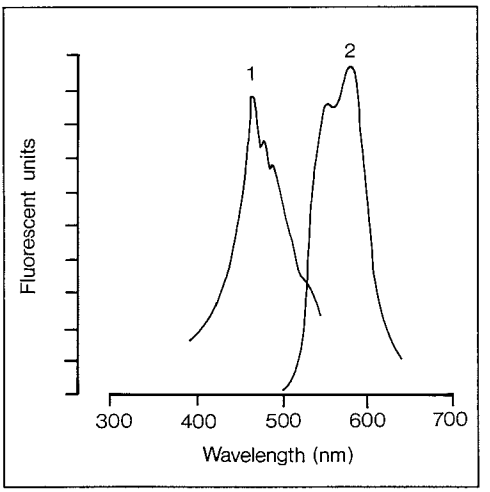


Fig. 2:
Fluorescence spectra of daunorubicin

1 = excitation spectrum
2 = fluorescence spectrum

C. Membrane effects

Cellular targets other than the nucleus may be of importance in the DNR action. This is suggested by the complex formation of anthracyclines with negatively charged phospholipids like cardiolipin (65-67). This phospholipid is a major component of the inner membranes of mitochondria which are abundant in the cardiac muscles. The binding of anthracyclines to cardiolipin together with the formation of toxic oxygen and hydroxyl radicals have been proposed as mechanisms for the cardiac toxicity (63,65,68). Furthermore, it appears that anthracyclines immobilized by a polymer linkage can be actively cytotoxic without entering the cell, merely by interaction at the plasma membrane (69,70).

Other mechanisms of action of DNR have been proposed as well. These include bioreductive alkylation of DNA by anthracyclines (71-73), inhibition of the mitochodrial respiratory chain (74,75), and metal ion chelation (76).

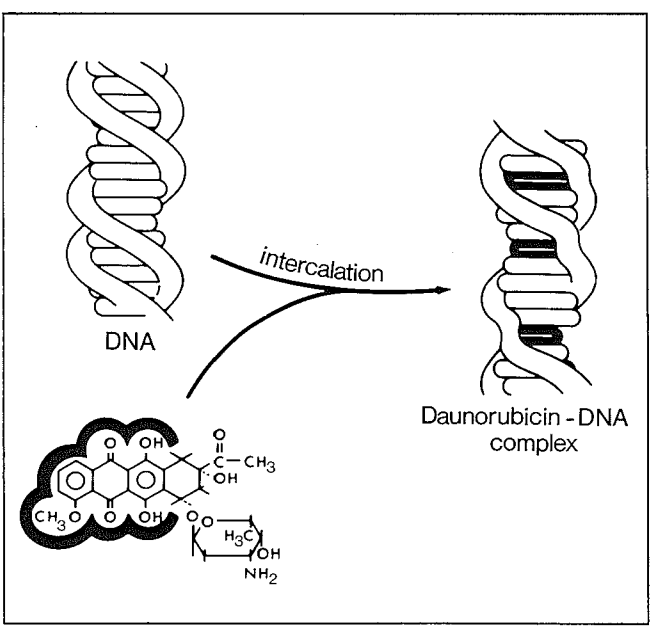
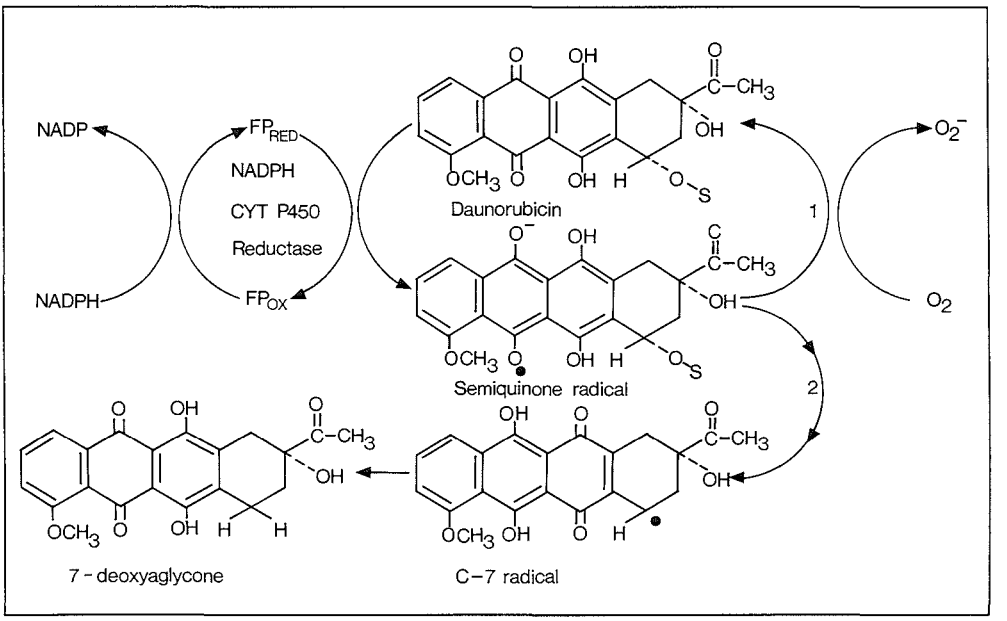


Fig. 3:
Intercalation of daunorubicin into DNA

Fig. 4:

Mechanism for enzymatic microsomal (NADPH cytochrome P-450 reductase) catalysis of daunorubicin free radical formation with subsequent nonenzymatic rearrangement to 7-deoxyaglycone product

FPred = flavoprotein reduced
FPox = flavoprotein oxidized
S = the aminosugar daunosamine.



1.2.3. Metabolism

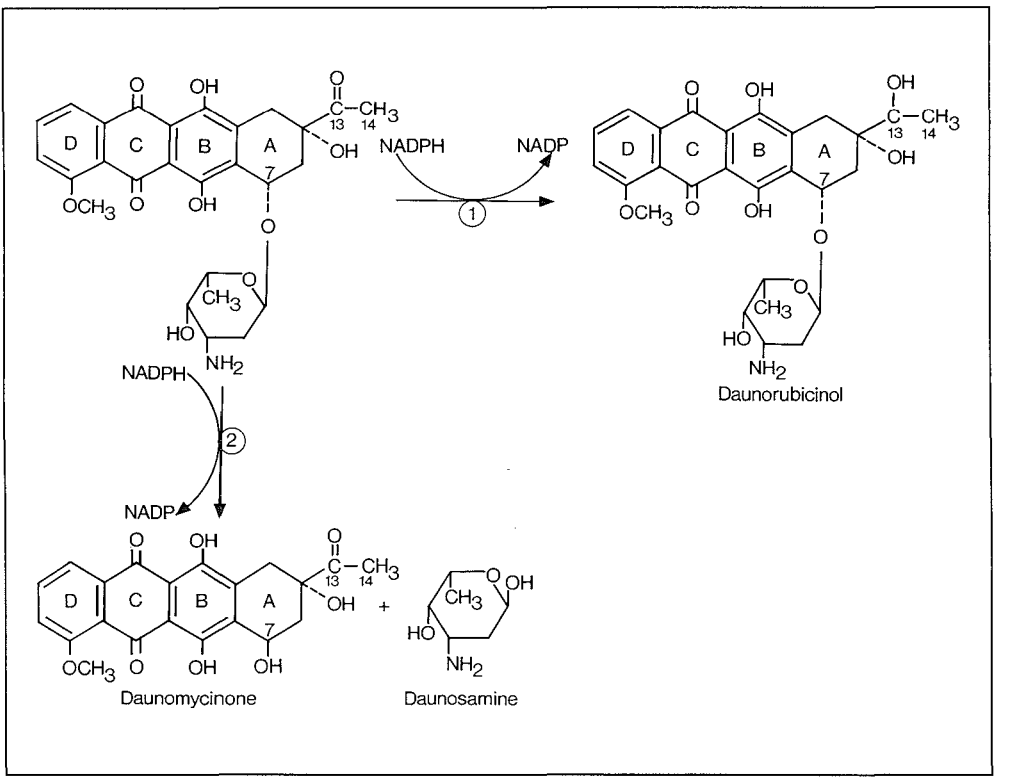
Following in vivo administration DNR is degraded by two processes occuring simultaneously (Fig. 5).

A. Reduction of the side-chain carbonyl group to produce the main metabolite daunorubicinol (DOL) occurs via a ubiquitous, cytoplasmatic NADPH-requiring aldo-keto reductase (77), an enzyme which is native to all tissues. In blood aldo-keto reductase is present in erythrocytes and leukocytes, including leukemic blast cells (78), but not in platelets or plasma (79). The presence of the active enzyme in blood cells implicates that DNR metabolism can occur in blood samples after they are drawn (80). The metabolite DOL has high specific inhibitory activity on DNA and RNA synthesis once it is in the cells (81). DOL is more polar than the parent drug DNR, which limits its rate of uptake by the cells and thus the resulting intracellular concentration (42). However, since the antitumor effect of DOL approaches that of DNR, DOL can contribute to the antitumor and pharmacological activity of DNR in man.

B. Reductive cleavage of the daunosamine sugar from DNR and DOL to form aglycones is mediated by microsomal, NADPH-dependent cytochrome P450 reductase. This reaction is exquisitely sensitive to molecular oxygen and takes place

Fig. 5:

Metabolism of daunorubicin by 1) carbonyl reduction to daunorubicinol and 2) reductive glycoside cleavage to daunomycinone and daunosamine



especiallly in the liver (82). The aglycones appears to be biologically inactive (83), although it is possible that the aglycone products have biological activity when liberated intracellularly (84).

Various other metabolites are detected in human urine and in bile (85), but their biological activities have not yet been reported.

1.2.4. Side effects

The use of DNR is limited by its toxicity to normal tissues. The major acute dose-limiting toxicities are bone marrow depression and stomatitis. Irreversible cardiomyopathy represents another important side effect that limits the cumulative dose used over time (86). The incidence of cardiomyopathy increases significantly above a cumulative dose of 450 mg/m^2 and may attend in 2.2% of the patients that have received total dosages of DNR of more than 650 mg/m^2 (87).

Other side effects include nausea, vomiting and (reversible) alopecia (86). Anthracyclines also cause serious local tissue necrosis after extravasation due to long persistence of the drug in the skin (88,89). Finally, some drug interactions of the anthracyclines have been reported i.e., interactions with barbiturates (90), with heparin (91) and with amphotericin B (92).

1.2.5. Resistance

The development of resistance to the anthracycline drugs represents one of the major obstacles to effective therapy with these agents. Preclinical studies aimed at the elucidation of mechanisms of anticancer drug resistance have shown that mammalian cell lines can develop so-called multiple drug resistance (MDR) after in vitro challenging. When these cell lines are continuously exposed to gradually increasing doses of naturally occuring anticancer agents, such as anthracycline antibiotics and vinca alkaloids, drug resistant sublines can be obtained which are not only resistant to the selecting agent, but also cross-resistant to a variety of structurally unrelated drugs (93,94). This MDR phenotype is characterized by:

a) decreased drug accumulation (95) due to active drug efflux (96,97),
b) increased intracellular drug accumulation and subsequent restoration of drug sensitivity by exposure to a variety of agents (such as calcium channel blockers and calmodulin antagonists) (98), and
c) gene amplification (99) and overexpression of a 170-kDa transmembrane glycoprotein (P-glycoprotein) (100).

It is generally accepted now that MDR is due to overexpression of P-glycoprotein, which functions in the plasma membrane as a rapid energy-dependent unidirectional drug efflux pump (101-104). In human two P-glycoprotein genes have been identified (mdr1 and mdr3) (104-106). Until now only human mdr1 gene has been shown to induce MDR (105). A number of agents, including calcium antagonists such as verapamil and calmodulin inhibitors such as cyclosporin-A, interfere with outward drug transport and thereby restore chemosensitivity (98,107-110).
There is accumulating evidence that MDR can also occur in human hematological malignancies, including AML (111-116). Normal bone marrow cells, spleen cells and lymphocytes have a very low level of mdr1 expression. Both in untreated as well as in chemotherapy treated AML patients overexpression of the mdr1 gene can be detected. However, the overexpression of mdr1 occurs more frequently in leukemic cells from treated AML patients than in those from untreated AML patients (113,114,116). In addition, mdr1 overexpression in AML cells is associated with a decreased drug accumulation in vitro that can be restored by cyclosporin-A (116). This indicates that drug-resistant leukemia patients whose tumor cells show the MDR phenotype could theoretically benefit from treatment with cytotoxic drugs in combination with inhibitors of the P-glycoprotein drug pump.

The data presented above appear to form a cohesive and logical explanation for the emergence of anthracycline resistance. However, other mechanisms for resistance are possible and probable (117). These include:

a) alterations in drug binding, either to the cell membrane or to the nucleus: changes in drug efflux which occur following manupilation of cellular metabolism might result from an alteration in intracellular binding sites, possibly by phosphorylation and a change in configuration, thus causing a change in the 'releasable' fraction of drug (118);
b) differences in reduction to the semiquinone radicals: cells differ in their ability to reduce anthracyclines and it may be possible that anthracycline-resistant cells have defective reductase enzyme systems, whereby the drug is inadequately activated to form free radicals (119,120).

1.2.6. Pharmacokinetics and pharmacodynamics

The area of clinical pharmacology that describes the concentration-time course of the drug, which is related to drug absorption, distribution, metabolism and excretion, is generally referred to as pharmacokinetics. On the basis of these data, parameters can be defined which describe the fate of the drug and its metabolites in the body (121,122). Pharmacodynamics is the discipline that deals with the relationship between the drug concentration and the specific biological effect (123), which in the case of AML can be considered as the relationship between DNR pharmacokinetics and killing of leukemic blast cells.

A. Assays

Drug concentration measurements represent the core of any pharmacokinetic study. Methods developed to determine anthracyclines in biological fluids are multifold and include the following:

1. Total extractable plasma fluorescence (spectrophotofluorimetry) (43,124,125); thin layer chromatography (TLC) followed by elution and fluorescent measurement (126);
2. TLC with quantitative fluorescence scanning of the thin-layer plate by radioimmunoassay (127);
3. Isotopic methods (128).

However, with the introduction of reliable extraction procedures and high-performance liquid chromatographic (HPLC) detection methods (129), sensitive and selective methods became available that appear of value for comparative pharmacokinetic studies in vitro (cell lines) and in vivo (animal/man). More recently, flow cytometric techniques have opened possibilities for further research with regard to anthracycline concentration measurements in individual cells (130-132).

B. In vitro studies

In vitro studies have shown that, at the cellular level, the anthracycline drug accumulates rapidly intracellularly and reaches a steady state concentration which exceeds the extracellular level by a factor of 450-800 (133,134). The cellular drug uptake is directly proportional to the extracellular drug concentration (42), increases at higher temperature (135) and at higher pH (136), and is inversely related to the number of incubated cells (137). Fluorescent microscopy (138) and subcellular fractionation studies (133) of cells have shown that the drug is highly concentrated in the nuclei and in the lysosomes.

The final intracellular drug concentration is a complex function of:

a) the passive (non-energy dependent) influx of drug involving a carrier-mediated transport across the cell membrane,
b) intracellular drug transport and nuclear accumulation of DNR and binding to DNA, RNA and cellular proteins, as well as
c) active and/or passive drug efflux from the cytoplasm to the extracellular environment (139,140).

C. In vivo studies: experimental animals

The in vivo pharmacokinetics of DNR and DOX have been studied extensively in a variety of animal species (141-144). These studies demonstrated that the drugs are cleared from the plasma rapidly and that they are incorporated in tissues, in particular in kidneys, heart and lungs. The DNR and DOX concentrations in tissues

and the tissue-plasma ratios differ significantly in various organs. In addition, the drugs are extensively metabolized in most animals. However, in rats no metabolism of DOX could be demonstrated (145). The drugs are slowly excreted into the bile (which is the primary route of excretion) and into the urine.

Comparative pharmacokinetic studies of the anthracyclines in vivo in animal-tumor models is useful in order to obtain information which cannot be obtained in man. Studies with the brown Norway acute myeloid leukemia (BNML), a rat model which resembles AML in humans (146), have provided information as regards:

a) the distribution kinetics of leukemic cells in the body (147),
b) comparative distribution kinetics of DOX (148) and DNR (149) between normal tissues and leukemia-infiltrated tissues in vivo and,
c) pharmacokinetics of DNR in normal tissues and bone marrow toxicity (150,151).

For instance, results of these studies have shown that in the presence of a high leukemic cell load the intravenously injected DNR is rapidly taken up and retained by the leukemic tumor mass in e.g., spleen, liver and lungs, and that, as a consequence, the bone marrow functions as a pharmacological sanctuary (149).

However, it has also been shown, that after infusion of BNML cells in the rat about 50% of leukemic cells lodge in the bone marrow (147). It is not known to what extent DNR carried by cells in vivo can add to the transport of DNR to target tissues in AML.

D. In vivo studies: man

Initial reports of DNR pharmacokinetics in man have been concerned with DNR given as intravenous (IV) bolus injections in patients with solid tumors or leukemia (152,153). Following IV administration of DNR, a rapid fall in plasma levels of the drug was observed, associated with substantial extravascular accumulation in most tissues, except those of the central nervous system (142). The disappearance of DNR from plasma follows first-order elimination of a biphasic nature, with a rapid initial (= distribution) phase followed by a long elimination phase (153). The rapid decline in plasma DNR levels is associated with a fast appearance of the main metabolite DOL in the plasma (153). The excretory pathway of DNR and DOL is primarily biliary (90%) with urinary excretion occuring to a lesser degree (10%) (154,155). Since the main excretory pathway for DNR is through the bile, it has been recommended that the dose be reduced in the presence of hepatic dysfunction (156).

Extensive tissue determinations of anthracycline uptake in man have never been made, except for rare 'postmortem' examinations of DOX in cancer patients (157) and some cellular pharmacokinetic studies of DNR in AML patients (154,158-160). In AML patients it was shown, that after DNR administration the drug accumulated in the leukemic cells up to concentrations which exceeded the plasma concentrations 400-4000 times, although with great interindividual differences among patients. Although the numbers of patients investigated in these studies were small, the combined results suggest that plasma concentrations of DNR provide little information on the drug concentrations in the leukemic cells and in the tissues.

Until now, limited data have been reported on a relationship between plasma pharmacokinetic parameters of anthracyclines and clinical response. For instance, it has been demonstrated that high plasma levels of DOX are associated on one hand with death during RI therapy, and on the other hand in patients attaining remission,

long remissions (161). However, these results could not be confirmed for DNR (instead of DOX) plasma levels (162). In addition, high plasma levels of aglycones (which are inactivated metabolites of DNR) are associated with RI failure mainly due to toxicity, while low levels are associated with a greater probability to attain CR. Plasma concentrations of the active compounds (DNR and DOL) were not associated with response (162). However, since DNR exerts its effect mainly intracellularly, measurements of leukemic cell content of DNR in AML patients during treatment with DNR, rather than plasma DNR concentrations, may be prognostic of clinical (i.e., therapeutical and/or toxicological) outcome. As yet, studies dealing with intracellular drug concentrations and clinical correlations have not been performed.

1.3. Introduction to the experimental work

Although progress has been made in pharmacokinetic areas, incomplete knowledge of the relationship between cytotoxic drug concentration-time profiles and drug effects has remained an obstacle for optimization of therapy. We assumed that estimation of drug concentrations in plasma and tissue may help to explain why individuals have different responses to treatment with the drug. Since the targets of the cytostatic therapy in AML patients, i.e., the circulating leukemic cells and the leukemic cells in the bone marrow are easily accessible, these patients offer a unique opportunity to study the pharmacokinetics in different pharmacological compartments. Therefore, it was our objective to simultaneously monitor the DNR concentrations attained in vivo in blood and bone marrow of patients with AML during the RI treatment. In an attempt to estimate the predictive value of cellular DNR concentrations in AML, we tried to elucidate possible relationships between the cellular pharmacokinetic parameters of DNR and:

a) the plasma distribution kinetics of the drug,
b) the tumor load of the patient and,
c) the clinical response to treatment.

In addition, a preclinical study was planned in the rat with the purpose of investigating certain aspects of cellular DNR pharmacokinetics in pharmacological compartments that are not accessible in man.

In **chapter 2** experimental determinants of the quantification of cellular DNR using HPLC were analysed: i.e., the quality of isolation and separation of nucleated cells from other cells and the recovery of DNR from these cells using a specific extraction method. The parameters that were established were applied in the subsequent clinical studies dealing with monitoring of the in vivo cellular and plasma pharmacokinetics of DNR in patients with AML.

In **chapter 3** laser flow cytometry was applied to measure DNR concentrations in individual cells and in isolated nuclei and the applicability of flow cytometry for measuring cellular DNR concentrations after in vivo uptake was evaluated. The data were compared with the results obtained with HPLC.

In **chapter 4** the in vivo distribution kinetics of DNR entrapped in cells (normal nucleated cells or leukemia cells) were determined in normal and leukemic rats, using a leukemia model which in many ways closely resembles AML in humans. These kinetics were then compared to the distribution kinetics of DNR after an IV injection of free DNR of the same dosage. The possible role of cells to transport DNR

to different pharmacological compartments (i.e., bone marrow, spleen, liver and heart) was investigated.

In **chapter 5** the in vivo cellular DNR concentrations in blood and bone marrow were determined in 37 patients with AML during first RI treatment and compared to the simultaneously observed plasma distribution kinetics of the drug. In addition, the data were correlated to the tumor load and the clinical response to treatment.

In **chapter 6** DNR concentrations in blood and bone marrow cells of 17 patients with AML were determined after in vivo uptake of the drug, and compared with the results obtained with HPLC. In addition, the usefulness of flow cytometry to determine the concentrations of DNR in subpopulations of hemopoietic cells, i.e., in particular in the leukemic blast cell fraction, was evaluated.

The results of the investigations are discussed in **Chapter 7.**

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CHAPTER 2

Quantitative evaluation of intracellular uptake of daunorubicin in acute myeloid leukemia: a method analysis

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Summary

Critical technical parameters to establish a reliable method for quantifying the intracellular content of anthracyclines were evaluated in patients with acute myeloid leukemia (AML); two methods were used for the isolation of leukocytes from the peripheral blood and two methods, for the extraction of daunorubicin (DNR), daunorubicinol (DOL), and doxorubicin (DOX) from these cells, followed by drug analysis using high-performance liquid chromatography (HPLC). At 0-4 °C the recovery of leukocytes after methylcellulose separation was low (64%). Cold hypotonic lysis gave better recovery (100%) when performed at the same temperature. After low-volume (2 ml extraction mixture) drug extraction from isolated leukocytes, the recoveries of DNR, DOL, and DOX from the cells were low, and they were inversely related to the cellularity of the sample, irrespective of the amount of drug in the cells. With high-volume extraction (5 ml extraction mixture) the recoveries were better (up to 95%), but they remained dependent on the cellularity. A correction factor accounting for these cellularity-related recoveries was applied to calculate the DNR and DOL contents of the leukocytes. Finally, using this information, plasma and cellular DNR and DOL levels were measured in seven patients with AML during their first course of remission induction therapy. The cellular DNR levels appeared to vary over a broad range and did not correlate with plasma pharmacokinetics.

Introduction

Daunorubicin (DNR) is a major drug in the treatment of acute myeloid leukemia (AML). In current treatment protocols the drug is frequently administered during remission induction (RI), consolidation, and maintenance therapy. The clinical response of patients with AML treated with anthracycline-containing chemotherapy varies considerably. Although the complete remission rate of AML is high (60-70%) (8), a significant proportion of cases is primarily refractory. In the responding

patients the relapse rate of AML is high, owing to incomplete eradication of leukemic cells. The duration of response may vary from 2 months to several years. The reasons for this considerable variation in therapeutic effect may relate to different biological properties of the leukemic cells per se and their susceptibility to the cytoreductive effect of the drug, or to interindividual differences in drug penetration into the leukemic cell. Monitoring of daunorubicin and doxorubicin (DOX) plasma levels in AML patients during RI has yielded little information to add to our understanding of the reasons for the interindividual variation in initial response and eventual relapse (13). Moreover, the plasma concentrations of DNR and its major metabolite daunorubicinol (DOL) were not correlated with the concentrations of the drug in leukemic cells obtained from AML patients (6,11). Since DNR apparently exerts its major effect by intercalation into DNA (1,7,19), the nucleus of leukemic cells can be regarded as the major target of DNR therapy. Determinations of DNR and its metabolite in leukemic cells, rather than in plasma, are likely to provide more important parameters for estimation of the response to the drug. However, little is known about the methodological factors of the assay, and therefore the results of the studies may suffer from inadequate technical performance or reproducibility. We report an analysis of certain factors which directly affect the outcome of the intracellular drug measurements. Different methods of sampling AML cells from the peripheral blood, followed by high-performance liquid chromatography for evaluation of the DNR content, were examined. The quality of isolation and separation of the leukemic blasts from other cells and the extraction method were investigated for maximal recovery and for avoidance of artefacts. The data obtained were then applied to a clinical study in seven newly diagnosed AML patients treated with daunorubicin as part of the remission induction chemotherapy regimen.

Materials and methods

Drugs and chemicals

Daunorubicin, daunorubicinol and doxorubicin were a gift from Farmitalia Carlo Erba (Milan, Italy). The drugs were freshly dissolved in methanol and diluted in water to give a final concentration of 200 μg/ml, and stored at -20 °C in the dark. The HPLC solvents were HPLC grade reagents (J.T. Baker Chemicals B.V., Deventer, Holland). Dulbecco's phosphate-buffered saline (PBS) at pH 7.3 had the following composition in deionized water: NaCl 136.8 mM, KCl 2.7 mM, Na_2HPO_4 8.1 mM and KH_2P0_4 1.5 mM. Lysis buffer (290 mosmol) had the following composition in deionized water: NH_4Cl 155.1 mM, $NaHCO_3$ 11.9 mM and EDTA 0.1 mM. Methylcellulose (Dow Methocel, viscosity 4000 cps) was dissolved in deionized water and Dulbecco's medium to prepare a final 2% solution (w/v).

Collection and isolation of human peripheral blood leukocytes

Human leukocyte suspensions were prepared from buffy coats, which were obtained as a byproduct following single donor platelet pheresis (Aminco, Celtrifuge 1), with ACD-A as an anticoagulant. We wished to achieve maximal leukocyte recovery from the blood for measurement of drug uptake and compared two separation methods:

A. Sedimentation of the cells in methylcellulose. One part of the blood was mixed with one part of ice-cold PBS and methylcellulose 40:1 (v/v) in a siliconized tube and placed on ice. After 20 min the leukocytes were harvested. The same procedure was repeated for the residual cells of the pellet. Immediately after collection, the cells were placed on ice. Then, the cells were washed with PBS at

4 °C in order to remove the remaining methylcellulose (10 min, 450 g). Finally, the leukocytes were resuspended in PBS.

B. Separation by hypotonic lysis. After washing the cell suspension with PBS to remove the plasma, the cells were resuspended in a small volume of PBS. One part of the cell suspension was mixed with four parts of lysis buffer and placed on melting ice. After 10 min the suspension was centrifuged, the supernatant was removed, the leukocytes were washed with PBS to remove the remaining lysis buffer, and finally the leukocytes were resuspended in PBS.

Then the cell suspension was diluted with PBS to final concentrations of 1, 3, 10 and 30 x 10^9 cells/l. Total cell counts following separation were compared with the initial count of the blood sample before separation. DNR, DOL, and DOX (50 µl of each) were added to 2 ml of each leukocyte suspension to give final concentrations of 0.1, 0.5, and 2.0 µg DNR, DOL and, DOX per ml suspension. After 5 min the samples were stored at -20 °C until further processing.

Collection and isolation of whole blood, plasma and leukocytes from AML patients

The criteria of the French-American-British working party were used to diagnose AML (4). The patients were treated according to the EORTC LAM-6 protocol. Remission induction chemotherapy consisted in DNR 45 mg/m^2 body surface on days 1, 2 and 3 by IV bolus, cytosine arabinoside (ara-C) 200 mg/m^2 IV on days 1-7 and vincristine 1 mg/m^2 IV on day 2.

Blood samples (10 ml) were drawn into tubes with EDTA as the anticoagulant, immediately before and at +5, +10, +20, +30 min and at +1, +4, +8, and +24 h, after the first dose of DNR was administered during the first course of RI treatment. The samples were cooled to 4 °C, the cells were counted, and 2 ml whole blood was taken from each sample and frozen. Another part of each of these samples was centrifuged at 4 °C, after which the plasma was collected and frozen. The buffy coat was depleted of erythrocytes by cold hypotonic lysis. The remaining leukocytes, usually myeloblasts, as determined by microscopic examination of stained cells, were resuspended in PBS, counted, and also frozen.

Therapeutic end-points of remission induction regiments

The criteria for evaluating response were those established by the Cancer and Acute Leukemia Group B (14). In brief, complete remission (CR) or partial remission (PR) is achieved when after one or two RI courses the proportion of leukemic cells has to < 5.0% (CR) or to 5.1%-25.0% (PR) while hematopoiesis and peripheral blood counts have returned to normal.

Failures of the induction treatment were characterized according to Preisler (12): Type 1: absolute drug resistance; type 2: relative drug resistance; type 3: regeneration failure; type 4: hypoplastic death; type 5: early death; type 6: extramedullary leukemic persistence.

Extraction

A. Low-volume extraction. Borate buffer (0.2 ml; 0.5 M, pH 9.8) and 2 ml extraction mixture (chloroform: methanol 4:1) were added to 2.0 ml cell suspension in a siliconized tube. The tubes were closed with silicone corks and vortexed for 2 min. After centrifugation at 1000 g for 10 min, 100 µl of the lower organic layer were injected into the column.

B. High-volume extraction. Borate buffer (0.2 ml; 0.5 M, pH 9.8) and 5 ml extraction mixture were added to 2.0 ml leukocyte suspension in a 30-ml glass bottle and

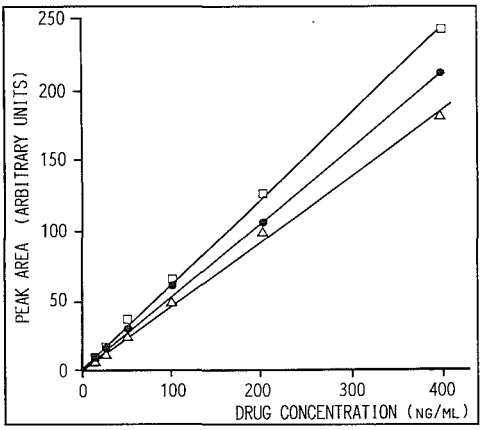

Fig. 1:
Calibration of peak heigth versus concentration of DNR (●), DOL (□), and DOX (▲) in chloroform:methanol (4:1)

Sample analyzed in duplicate.

shaken for 20 min at 300 rpm on a Mini-shaker (MSR, Salm and Kipp, Breukelen, Holland) in the dark. After this, 1 ml of the organic phase was dried with a gentle stream of nitrogen. Dried samples were reconstituted with 300 µl chloroform: methanol (4:1) and 100-µl aliquots were then analyzed by HPLC.

The patient plasma samples were extracted using the low-volume extraction technique, and the whole blood and leukocyte samples were extracted using the high-volume extraction method, with DOX added as internal standard.

High-performance liquid chromatography (HPLC)
HPLC separation (adapted from Baurain et al. (2)) was accomplished using a Waters Associates M-510 pump and a Waters Associates automatic sample injector (Model 710B). The stationary phase consisted of 7-µm silica gel particles prepacked into a 250 x 4.6 mm stainless steel column (Lichrosorb Si-60-7, Chrompack). The mobile phase consisted of chloroform, methanol, acetic acid, water and 3 mM $MgCl_2$ solution in water (720:210:40:24:6 by volume), filtered (Millipore FH 0.5 µm) and used at a flow rate of 0.8 ml/min. Fluorescent detection was accomplished using a Gilson Spectra-Glo fluorometer at 480 and 560 nm for the excitation and emission wavelengths, respectively, and equipped with a 45-µl flow cell. The integration of the peak areas was performed by a Shimadzu Model CR3A integrator (United Technologies Packard, Delft, Holland).

The linearity and sensitivity of the method were determined from serial concentrations of DNR, DOL and DOX in chloroform: methanol (4:1). Linear calibrations were obtained for all three compounds in the concentration range of 12.5 - 400 ng/ml (Fig. 1) (coefficient of variation 5%-7% at 50 ng/ml). The lower detection limit of DNR in plasma was 5 ng per injection of 100 µl and after extraction from biological fluids, 25 ng/ml biological fluid or 5 µg/10^9 leukocytes.

Mathematical modeling
The data from the seven AML patients were analyzed by fitting a two-compartment model to the observed plasma and intracellular DNR concentrations (15). The first (central) compartment represents the plasma distribution of DNR. The second (peripheral) compartment represents the concentration-time curves of DNR in tissues, including the cellular elements in blood. The plasma and intracellular concentration-time disappearance of the parent drug (DNR) are described by the following equations, respectively:

$$C(t) = A \cdot e^{-\alpha.t} + B \cdot e^{-\beta.t} \quad (1)$$

$$C(t) = A \cdot (e^{-\beta.t} - e^{-\alpha.t}) \quad (2)$$

where C(t) is the plasma (Eq. 1) or intracellular (Eq. 2) level of DNR at each time t after the injection, A and B are constants, and α and β are first-order elimination rate constants.

The DNR area under the concentration-time curves (AUC) in plasma and leukocytes, the volume of the central (plasma) compartment (V_1), the apparent distribution volume (Vd_{area}) and the plasma clearance (Cl) were calculated from this model.

Results

Separation of human leukocytes

For determination of DNR and DOL concentrations attained in vivo in leukemic cells of AML patients, it is necessary to use a separation method which provides pure leukocyte fractions without contamination of erythrocytes or selective loss of leukocytes. The separation was performed at 0-4 °C, to prevent metabolism of DNR (16). Other possible degradation of DNR was avoided by working in reduced daylight and using siliconized tubes (17,18). We compared two procedures for cell separation. In the first place, methylcellulose was used for sedimentation of leukocytes. Secondly, using the lysis buffer, the leukocytes were isolated following hypotonic lysis of the erythrocytes. The recoveries of the leukocytes and erythrocytes were expressed relative to the total number of leukocytes and erythrocytes before separation (set at 100%). The leukocyte purification factor designates the ratio of the percentage recovery of leukocytes in the leukocyte pellet and in the whole blood sample.

Table 1:

Recoveries of leukocytes and erythrocytes in human leukocyte pellets after separation[a]

Separation method	N[b]	Recovery of leukocytes (%) (mean ± SD)	Recovery of erythrocytes (%) (mean ± SD)	Leukocyte purification factor (mean ± SE)[c]
Methylcellulose	86	65 ± 24	3 ± 3	66 ± 15
Lysis buffer	67	106 ± 16	2 ± 3	490 ± 102

a: Before separation the leukocyte concentration of the blood samples varied from 0.25 to 170×10^9 cells/l (set at 100%)

b: Number of experiments

c: The leukocyte purification factor designates the ratio of the percentage recovery of leukocytes in the leukocyte pellet and in the whole blood sample

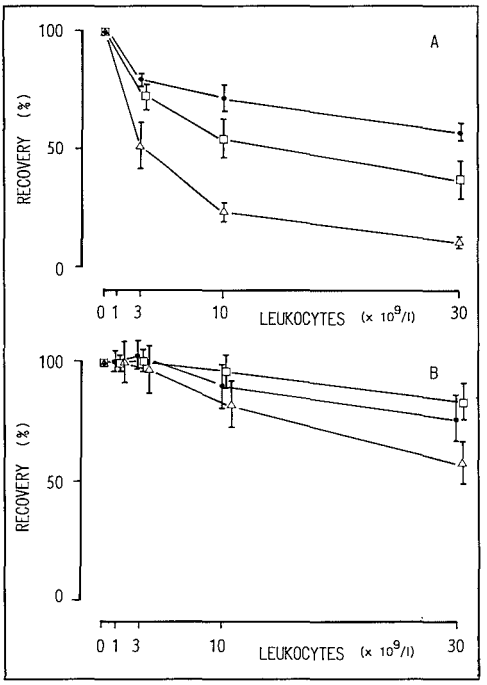

Fig. 2:
Recovery of DNR (●), DOL (□), and DOX (▲) after extraction from different concentrations of leukocytes, using low-volume extraction (A) and high-volume extraction (B) methods.

Mean ±SD of 6 (A) and 12 (B) experiments.

As is shown in Table 1, the recovery of leukocytes after hypotonic lysis was complete (106%) and superior to that after methylcellulose separation (65%). The recovery of erythrocytes was equivalent with both methods (2%-3%), which indicates almost complete removal of erythrocytes from the leukocyte pellets after separation with either method. In further experiments we used hypotonic lysis for cell separation.

Table 2:
Patient characteristics

Patient	Sex	Age	FAB classification	WBC (x 10^9/l) at initial diagnosis	% Blasts in peripheral blood at initial diagnosis	Dose of DNR (mg/m^2)	Dose of DNR (mg)	Number of induction courses	Outcome of remission induction therapy
1	M	42	M1	1.44	7	45	75	2	CR
2	M	73	M1	13.0	75	45	80	1	CR
3	F	65	M1	1.406	0	45	70	2	CR
4	M	45	M4	4.1	84	45	90	2	PR
5	M	37	M4	107.3	76	45	90	2	PR
6	M	26	M4	170.0	99	45	80	2	PR
7	M	17	M2	6.5	79	45	80	2	Type I failure

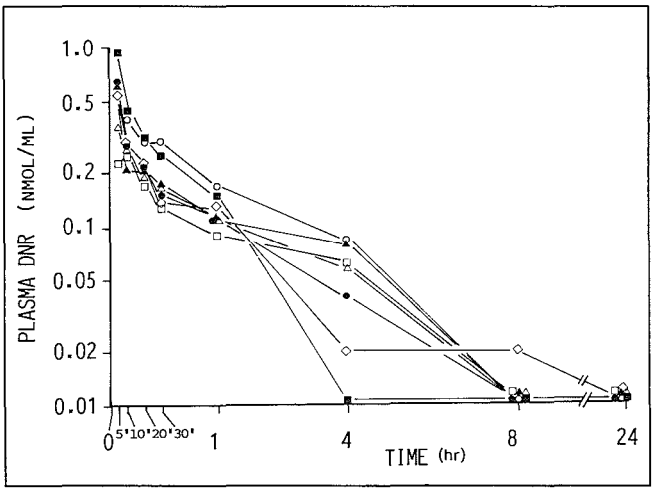

Fig. 3:
Plasma concentration-time curves of DNR after first IV bolus injection of DNR in seven AML patients during first RI treatment.

Patients (see Table 2) are indicated by the following symbols: 1, O–O; 2, ●–●; 3, ✧–✧; 4, ❑–❑; 5, ■–■; 6, Δ–Δ; 7, ▲–▲.

Recovery of DNR, DOL, and DOX after extraction from leukocytes

Low-volume extraction of DNR, DOL and DOX from biological samples, such as plasma and urine, yields recoveries of approximately 100% (2,3). When this extraction method is applied to biological samples containing variable concentrations of cells, it appears that the recoveries of DNR, DOL, and DOX depend on the cellularity of the extracted sample, regardless of the amount of drug added to the cells. As shown in Fig. 2A, the recovery of DOX is very low compared with that of DNR and DOL, especially in samples with large amounts of cells. Since DOX has been added as an internal standard for the quantification of DNR and DOL, better recovery is necessary.

Large-volume extraction with a long period of extraction gives better recoveries of DNR, DOL, and DOX (Fig. 2B). Nevertheless, again the recovery of DOX is lower than that of DNR and DOL. The recovery of all drugs depends on the cellularity of the sample. These results indicate that determination of the DNR content of human leukocytes may be subject to variation, depending on the method of isolation, the volume and duration of extraction, and the cell concentration of the suspension.

Pharmacokinetics of DNR in AML patients

Table 2 shows certain characteristics of seven previously untreated patients with AML. At diagnosis a large variation in the number of peripheral white blood cells (WBC) was apparent (range 1.4-170 x 10^9 cells/l). For calculation of DNR in whole blood and in leukocytes, correction factors were used to account for the cellularity of the samples. As shown in Fig. 3, the plasma concentrations of DNR rapidly declined to minimal or undetectable levels within +4 h to +8 h after administration. This rapid decline was associated with the appearance of the major metabolite DOL in the plasma. Generally, DOL plasma concentrations exceeded DNR levels within +1 h after administration, and at +4 h after administration the DOL concentration was 80% or more of the total amount of detectable anthracycline (data not shown). With our method of drug determination no metabolites other than DOL were found.

In Fig. 4 the whole blood concentration-time curves of DNR are shown. When they are compared with those for plasma, it is obvious that a more variable decline can be observed. At +4 h after administration of the drug two patients had high DNR (60%) and DOL (40%) blood levels, while the other patients had low or undetectable levels.

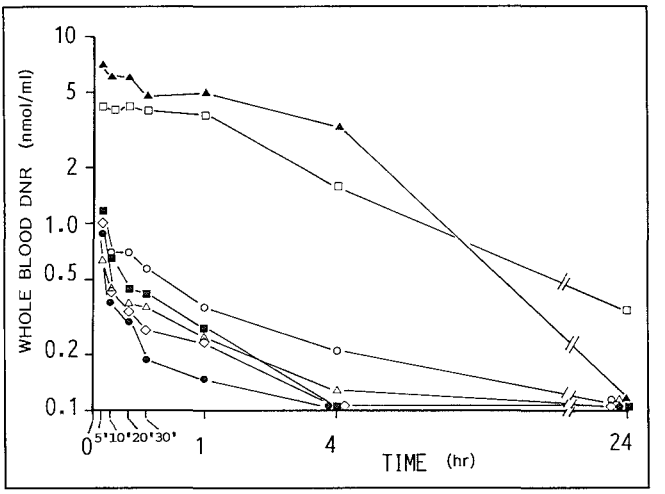

Fig. 4:
Whole blood concentration-time curves of DNR after first IV bolus injection of DNR in seven AML patients during first RI treatment.

Patients (see Table 2) are indicated by the following symbols: 1, ○–○; 2, ●–●; 3, ✧–✧; 4, ❑–❑; 5, ■–■; 6, Δ–Δ; 7, ▲–▲.

DNR accumulated extensively in the leukocytes, at concentrations which were even more variable than those in plasma and blood (Fig. 5). Maximum cellular DNR concentrations were reached almost immediately after administration of the drug in all patients. Intracellular DOL was present at much lower concentrations than in plasma (20%).

Tables 3 and 4 show the calculated plasma and cellular pharmacokinetic parameters of DNR in these seven AML patients. Clearly, the wide interindividual variation in the AUCs for DNR in leukocytes is not reflected by the AUCs in plasma of the same patients. Thus, the data suggest that the leukocytes should be regarded as a separate compartment.

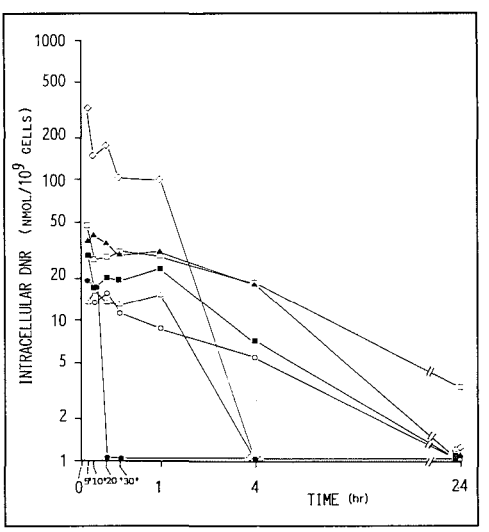

Fig. 5:
Leukocytes concentration-time curves of DNR after first IV bolus injection of DNR in seven AML patients during first RI treatment.

Patients (see Table 2) are indicated by the following symbols: 1, ○–○; 2, ●–●; 3, ✧–✧; 4, ❑–❑; 5, ■–■; 6, Δ–Δ; 7, ▲–▲.

Table 3:

Plasma pharmacokinetic parameters of daunorubicin in AML patients after IV bolus injection at t=0

Patient	$t_{1/2\alpha}$ (h)	$t_{1/2\beta}$ (h)	V_1[a] (x 1000 l)	Vd_{area}[b] (x 1000 l)	Cls[c] (x 1000 l/h)
1	0.07	1.04	0.12	0.57	0.38
2	0.07	0.77	0.23	0.34	0.31
3	0.07	2.17	0.13	0.64	0.20
4	0.10	0.48	0.12	0.32	0.47
5	0.18	0.81	0.60	0.74	0.46
6	0.02	0.61	0.01	0.25	0.28
7	0.01	0.76	0.01	0.33	0.30

a: Volume of the central (plasma) compartment
b: Apparent distribution volume
c: Plasma clearance

Table 4:

Area under the plasma and leukocyte concentration-time curves (AUC) for daunorubicin in AML patients after IV bolus injection at t=0

Patient	0-4 h	0-8 h	0-24 h	0-∞ h
Plasma AUC (nmol x h/ml)				
1	0.35	0.37	0.37	0.37
2	0.47	0.49	0.49	0.49
3	0.49	0.61	0.65	0.65
4	0.36	0.36	0.36	0.36
5	0.27	0.27	0.27	0.27
6	0.54	0.54	0.54	0.54
7	0.50	0.51	0.51	0.51
Leukocyte AUC (nmol x h/10^9 cells)				
1	4.61	4.61	4.61	4.61
2	33.61	44.72	50.02	50.07
3	175.20	175.43	175.43	175.43
4	60.83	82.92	95.28	95.48
5	104.04	158.67	214.41	218.95
6	106.88	157.34	199.94	202.10
7	24.81	26.14	26.21	26.21

Discussion

For better understanding of the wide variation in individual response to DNR in AML, individual drug monitoring in the leukemic cells is of interest. Therefore, we decided to develop an efficient and reliable method that would be useful for measuring concentrations of DNR and DOL in the cells of AML patients. Methylcellulose or Ficoll-Isopaque are frequently used for separation of leukocytes. We found that the recovery of leukocytes after methylcellulose separation when applied at 0–4 °C is low (64%), which is most likely due to the relatively low temperature used for the procedure. At 0–4 °C methylcellulose is a gel with increased viscosity, which may interfere with proper separation of blood cells (6). At 20 °C methylcellulose forms a colloid solution, and at this temperature the recovery of leukocytes is better (95%). Obviously, this temperature, although permitting satisfactory cell recoveries, has the disadvantage of being associated with the metabolic degradation of DNR to DOL by aldoketoreductase, an enzyme which is present in leukocytes and in myeloblasts (9,10). Cold hypotonic lysis gave clearly superior results. It could be performed at 0–4 °C, with excellent separation of white and red blood cells and 100% recovery of all nucleated cells.

The extraction method is shown also to be critical. The recovery of DNR depends on the volume of extraction and the duration of extraction (Fig. 2A, B). Owing to the use of large volumes in bottles, the surface exposures of the two liquid phases are increased and a better transfer of the drugs into the organic phase is obtained. Extraction times up to 20 min improved drug recoveries, but extractions beyond 20 min did not contribute further.

Even under conditions of optimal cell isolation and drug extraction the recoveries of DNR and DOX remain dependent on the cellularity of the sample. This is the case irrespective of the amount of drug in the cells. Thus, the number of cells submitted to extraction is inversely related to the fraction of intracellular DNR and added DOX to enter the organic phase. In other words, the number of leukocytes correlates negatively with the partition coefficients of DNR and DOX. It remains to be determined whether this is caused by fractional binding of the drugs to cell fragments, or whether the cell fragments mechanically prevent a proper exchange of the drug molecules between the two liquid phases. In calculations of the DNR content of leukocytes, it is therefore still necessary to correct for cellularity of the sample. This correction factor is variable and is derived from the concentration of cells in the sample. It might be that excessive denaturation of DNA, for example by pretreatment with silver nitrate, improves the extraction ratio of DNR from DNA binding sites. To avoid heterogenous recoveries from different samples, we standardized the patient blood samples and leukocytes with PBS at final concentrations between 1 and 10×10^9 cells/l before starting on the extraction procedure.

With the above-defined criteria it is possible to monitor the in vivo plasma and cellular pharmacokinetics of DNR and DOL in patients in a reproducible way during treatment. In the small series of patients with AML treated with DNR during remission induction and with variable numbers of circulating leukemic cells, it was found that:

1) plasma levels of DNR and DOL do not correlate with blood and leukocyte concentrations of DNR and DOL;

2) plasma pharmacokinetic parameters do not correlate with cellular pharmacokinetic parameters;
3) in vivo cellular levels of DNR are considerably more variable than in vivo DNR levels in plasma or whole blood;
4) the pattern of DNR metabolism is different in the leukocytes and the plasma.

Although these conclusions are based on a small number of patients, they demonstrate that the plasma concentration of DNR and DOL provides little information on the drug concentrations attained in the leukocytes. These findings are in agreement with the results of DeGregorio et al. (6), who indicated that plasma concentrations of DNR and DOL were not useful for estimation of the in vitro inhibition of DNA synthesis. Our findings are also consistent with the conclusion of Preisler et al. (13), who showed that there was no significant correlation between plasma levels of anthracyclines and clinical response to therapy in patients with AML. Therefore, to analyze the clinical effectiveness of DNR it may be useful to pursue the pharmacokinetics of DNR and metabolites not only in plasma but particularly in leukocytes in blood and bone marrow.

It remains to be investigated whether the determinations of drug concentrations in the leukocytes will indeed provide prognostic indicators of the variable outcome of remission induction therapy and the duration of complete remission in acute leukemia patients. As yet, our studies described here may be of use to set a methodological baseline for these approaches.

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CHAPTER 3

Comparative studies of the in vitro uptake of daunorubicin by human hemopoietic cells and nuclei measured by flow cytometry and HPLC

Parts of this chapter (in vitro data) combined with **Chapter 6** (in vivo data) are based on investigations published in:

Leukemia 2:511-517, 1988

Esther Kokenberg, Pieter Sonneveld, Ruud Delwel, Willemijn Sizoo, Anton Hagenbeek and Bob Löwenberg
In vivo uptake of daunorubicin by acute myeloid leukemia (AML) cells measured by flow cytometry

Abstract

The major white cell subpopulations present in bone marrow and blood can be discriminated by forward and perpendicular light scatter two parameter flow cytometry. Fluorescent properties of daunorubicin (DNR) allow measurement of the concentration of this drug in hematopoietic cells by flow cytometry as a third parameter. We evaluated the applicability of flow cytometry for measuring DNR uptake in direct comparison with high performance liquid chromatography (HPLC). In vitro studies revealed good correlations between the mean cellular and nuclear fluorescence measured by flow cytometry and the cellular and nuclear DNR concentrations determined with HPLC. In addition, it was shown that the nuclear DNR associated fluorescence involved DNR bound to the nucleus rather than free DNR.

Introduction

The pharmacokinetics of anthracyclines in plasma of AML patients studied with thin layer chromatography (1,2) and high-performance liquid chromatography (HPLC) (3,4), show that the anthracyclines accumulate rapidly in the tissues outside the plasma compartment. Studies with cell lines have shown that most anthracyclines, including daunorubicin (DNR), accumulate almost exclusively in the nucleus (5), as was to be expected from the high binding constants of these agents for DNA (6).

However, data on cellular anthracycline concentrations in human cells after in vivo administration are scarce (7,8). With HPLC this information can be obtained. However, the HPLC method is laborious and does not provide insight into anthracycline concentrations present in different subpopulations of cells, e.g., normal hematopoietic cells versus leukemic cells. With flow cytometry the cellular drug fluorescence can be determined as a function of subpopulations of cells. On the other hand a disadvantage of flow cytometric measurements is the apparently reduced fluorescence of DNR, once the drug is bound to DNA of intact cells. This effect is called quenching of fluorescence emission (9,10). Therefore, in the present report we have analyzed whether the DNR associated fluorescence as measured with flow cytometry is:

a) linearly related to the DNR content of cells and
b) represents the total cellular (i.e., cytoplasm and nuclear) DNR content of cells.

After flow cytometric measurements, quantitation of the DNR content of the same cells was done by HPLC analysis. Following this approach we wanted to evaluate the applicability of flow cytometry for measuring DNR concentrations in leukemic cells in vivo.

Materials and methods

Anthracyclines

Daunorubicin and doxorubicin were obtained as the commercial preparations Cerubidine (Rhône-Poulenc, Paris, France) and Adriablastina (Farmitalia Carlo Erba, Milan, Italy), respectively. The drugs were dissolved in methanol, diluted in water and stored at -20 °C as 200 μg/ml stock solutions. Aliquots were thawed as required and diluted appropriately with water.

Collection and isolation of normal WBC

White blood cells (WBC) suspensions were prepared from buffy coats from healthy donors, which were obtained as a side product following single donor platelet pheresis (Aminco, Celtrifuge 1), with ACD-A as anticoagulant. The buffy coat cells were washed with phosphate buffered saline (PBS) (450 g, 10 min) to remove the plasma, and depleted of erythrocytes and normoblasts following cold hypotonic lysis (11). Then, the WBC were harvested, washed two times with PBS, counted and finally the WBC were resuspended at a concentration of 10×10^9 cells/l RPMI medium (pH 7.2).

Incubation of WBC with DNR

For the measurements of DNR in whole cells, DNR was added to 1.5 ml of the WBC suspension at final concentrations ranging from 8 up to 150 nmol/10^9 cells. For the measurements of DNR in nuclei, WBC (24×10^6 cells in 3 ml) were incubated in duplicate with two different DNR concentrations (i.e., 120 nmol/10^9 cells and 60 nmol/10^9 cells). In addition, in order to determine the DNR leakage from nuclei, WBC (200×10^6 cells in 20 ml) were incubated with DNR at a final concentration of 190 nmol/10^9 cells. A blank sample (unstained cells) was used in parallel incubations in order to measure background fluorescence. The cells were incubated in a shaking water bath at 37 °C for 30 min. After incubation the cells were put on melting ice and washed two times by centrifugation (450 g, 10 min, 4 °C) and resuspension of the pellets in ice cold RPMI. Finally, the cells were resuspended in PBS and counted. Parts of the cell suspensions were stored at -20 °C until analysis with HPLC, other

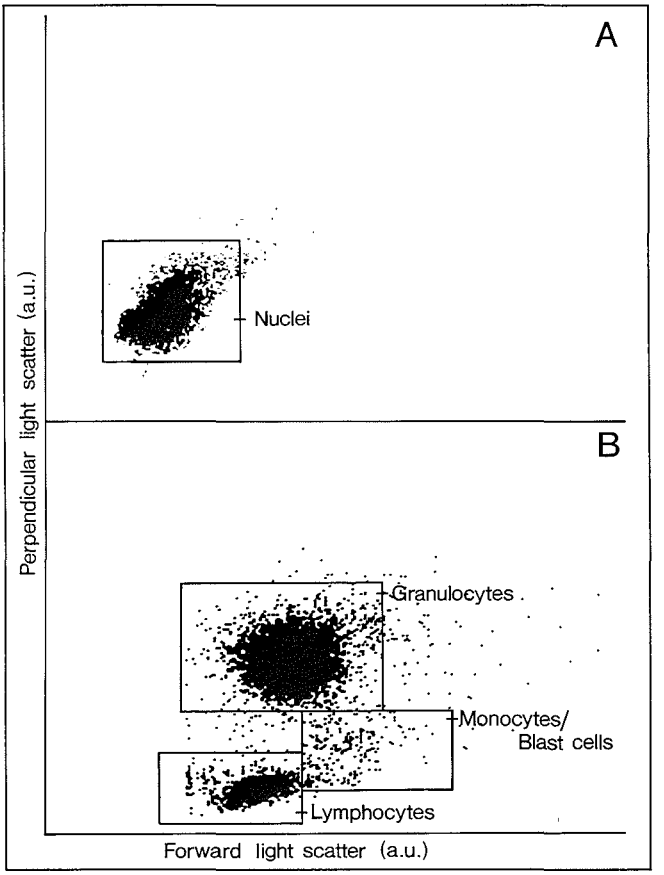

Fig. 1:
Dot display of forward light scatter versus perpendicular light scatter of nuclei (panel A) isolated from normal human leukocytes (panel B)

The erythrocytes and normoblasts had been lysed. Each dot represents one nucleus or one cell.

parts were kept on ice and used for determination of the cellular DNR concentration with flow cytometry, while the rest of the cells was used for isolation of nuclei.

Isolation of nuclei
The cells were centrifuged, resuspended in buffer (3.0 mM $MgCl_2$, 1.0 mM monothioglycerol, 250 mM sucrose, 10 mM Tris-HCl, pH 7.7, 0.2% (v/v) Triton X-100) and fractionated by Dounce homogenization (12). The nuclei were washed two times (600 g, 15 min, 4 °C), resuspended and counted. Microscopic examination showed that most nuclei were in tact. Part of the suspension was stored at -20 °C for HPLC measurement and part was used for flow cytometric determination of the nuclear DNR concentration. The cytosol fractions were also stored at -20 °C for HPLC measurements.

Measurement of DNR leakage from nuclei
The nuclei were resuspended in 30 ml buffer (3.0 mM $MgCl_2$, 1.0 mM monothioglycerol, 250 mM sucrose, 10 mM Tris-HCl, pH 7.7) and dialyzed against a great volume (1 liter) of the same buffer. At several time intervals (at 0, +10, +20, +30, +45, +60 min as well as at +1.5, +2, +2.5 and +3h after start of dialysis) a sample of the suspension of nuclei was analysed for DNR associated fluorescence by flow cytometry and HPLC.

Measurement of cellular and nuclear DNR content by flow cytometry
The fluorescent properties of DNR permitted measurement of DNR content of

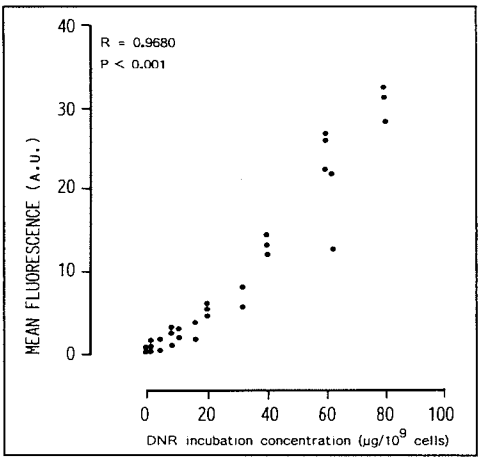

Fig. 2:
Correlation of DNR incubation concentrations with mean cellular fluorescence

Data of four incubation experiments with identical incubation conditions (i.e., 15×10^6 cells/1.5 ml RPMI, incubated at 37 °C for 30 min) (n = 29).

individual cells/nuclei with a Becton-Dickinson (Mountain View, CA) fluorescence activated cell sorter (FACS 440). In the FACS 440 system the cells/nuclei traverse the light beam of a Spectra-Physics 5 W argon laser tuned at 488 nm, 0.4 W, which is close to the absorption maximum of DNR. The fluorescence emitted by the cells/nuclei upon excitation by the laser light is registered on a photomultiplier. The scatter light was blocked with a 550 nm long-pass glass filter. The signals from the photomultiplier were linearly amplified. The instrument was calibrated with 1.0 and 2.83 µ diameter fluorescent standard beads (Polysciences Inc., Warrington, PA). The forward and perpendicular light scatter from the cells/nuclei were measured on separate detectors. The intensities of these signals, which are determined by cell size and cell structure respectively, were used to distinguish the different cell types in WBC or in bone marrow (13). For each sample, at least 10×10^3 cells/nuclei were analysed at a flow rate of $1\text{-}2 \times 10^3$ cells/sec. Cell debris were excluded from analysis by elevating the threshold of the forward light scatter. The scatter signals from the nuclei were quite homogeneous so that no subpopulations of nuclei could be distinguished (Fig. 1).

Data analysis was performed using a Hewlett Packard 68B system. Fluorescent profiles were recorded as histograms of fluorescence intensity versus cell/nuclei frequency. The relative value of DNR fluorescence was obtained for each population by calculating the mean of the fluorescence distribution. Mean fluorescence was expressed in arbitrary units, taking into account the blank samples that were used to correct for background fluorescence (subtraction of blank values).

Analysis of cellular and nuclear DNR and daunorubicinol by HPLC
Concentrations of DNR and its major metabolite daunorubicinol (DOL) were determined using straight-phase HPLC analysis as described previously (11,14,15). For drug quantification, doxorubicin was used as an internal standard.

Statistics
The Spearman test was used for calculating correlation coefficients.

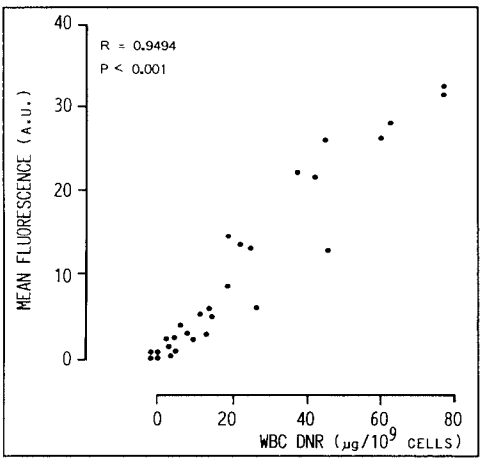

Fig. 3:
Correlation between the cellular DNR concentrations as measured with HPLC (x-axis) and the mean cellular fluorescence as measured with flow cytometry (y-axis).

Data are shown from four incubation experiments with identical incubation conditions (i.e., 15×10^6 cells/1.5 ml RPMI, incubated at 37 °C for 30 min) (n=29).

Results

Recovery of nuclei and DNR after isolation of nuclei from WBC
The recoveries of nuclei isolated from the cells were expressed relative to the total number of nucleated cells before isolation and appeared almost complete (mean 94%; Table 1). The recoveries of DNR in nuclei and cytosol after cellular fractionation, as shown in Table 1, were almost complete as well (mean 97%), which ruled out loss of nuclei or DNR during the isolation procedure.

Correlation of flow cytometry with HPLC
Measurements in whole cells (white blood cells).
Normal WBC were incubated (at 37 °C, 30 min) with varying DNR concentrations ranging from 8 to 150 nmol/10^9 cells. After incubation the cellular fluorescence of

Table 1:
Recoveries of nuclei and nuclear and cytosolic DNR in human leukocyte pellets after cellular fractionation

Sample number	Incubation concentration (nmol DNR/10^9 cells)	Recovery of nuclei (%)[a]	Recovery of DNR in nuclei and cytosol (%)[b]
1	120	92	95
2	120	100	84
3	60	88	128
4	60	83	81

a: Nuclei before isolation represents 100%.
b: Values relative to cellular concentrations of DNR, as determined with HPLC (100%).

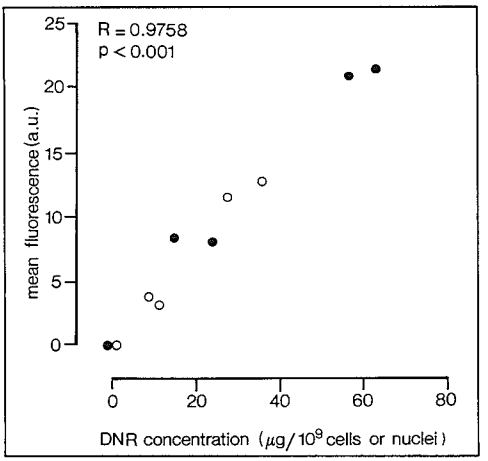

Fig. 4:
Relationship between the cellular (●) and nuclear (○) DNR concentrations as measured with HPLC (x-axis) and the mean fluorescence as measured with flow cytometry (y-axis)

10 x 10 ^{3}cells was measured by flow cytometry. A positive relationship was observed between the extracellular DNR concentrations in the incubation medium and the mean cellular fluorescence ($p < 0.001$, Fig. 2). A portion of the cells were used for determination of the cellular DNR and DOL contents by HPLC. Approximately 70% of DNR had been taken up by the cells during the 30 min drug exposure time from the incubation medium. Flow cytometry does not distinguish between the parent drug (DNR) and the metabolite DOL. However, with HPLC analysis no detectable amounts of cellular DOL were found. This indicates that cellular metabolites had contributed a negligible amount to the total fluorescence (assessed with flow cytometry) at the concentrations applied.

The mean cellular fluorescence as measured with flow cytometry was compared with the amount of DNR measured following extraction and HPLC. These two measurements correlated well ($p < 0.001$, Fig. 3), indicating that flow cytometric data

Table 2:
Nuclear drug concentration in normal human leukocytes determined with HPLC and flow cytometry

Sample number	Incubation concentration (nmol DNR/10^9 cells)	HPLC nuclear amount of DNR (%) [a]	Flow cytometry nuclear amount of DNR (%) [a]
1	120	56	60
2	120	49	55
3	60	59	45
4	60	45	38

a: Values relative to total cellular DNR concentration (100%).

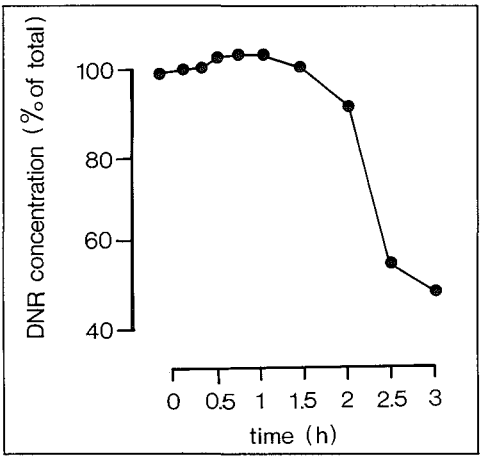

Fig. 5:
Concentration-time course of DNR in nuclei isolated from normal human leukocytes after in vitro incubation of the cells with DNR

The nuclei were dialyzed against a DNR free buffer. The DNR concentration is expressed relative to the concentration at start of the dialysis which was set at 100%.

provide a good quantitative measure of cellular drug content. The DNR related fluorescence signals obtained from the cells incubated with a range of DNR concentrations in vitro were intense and easily detectable with flow cytometry. From Figs. 2 and 3 it appears that the fluorescence intensity after incubation with the lowest DNR concentration (8 nmol DNR/10^9 cells) approached the detection limit. When concentrations of DNR in these cells were subsequently determined with HPLC, only small peaks (about 6 nmol DNR/10^9 cells) were observed in the chromatograms.

Measurements in nuclei.
Isolated WBC were incubated with two different DNR concentrations: 60 nmol and 120 nmol/10^9 cells. After isolation of the nuclei, cellular and nuclear fluorescence were measured by flow cytometry and HPLC. With flow cytometry the fluorescence signals from the cells as well as from the nuclei were easily detectable. The nucleus/whole cell ratios of DNR as determined with flow cytometry or HPLC were concordant (Table 2). Under these experimental conditions about 50% of DNR was associated with the nucleus, regardless the incubation concentration used.

In addition, the DNR related fluorescence signals obtained from the cells and nuclei correlated well ($p < 0.001$) with the absolute amount of DNR measured after chemical extraction and chromatography (Fig. 4). Thus, flow cytometry does not only provide a good quantitative measure of cellular but also of nuclear DNR content.

Leakage of DNR from nuclei
We first determined whether the nuclear DNR measured with flow cytometry was free DNR in the nucleus or DNR really bound to DNA. Therefore, DNR containing nuclei were dialyzed against DNR free buffer. The concentration of DNR in the nuclei at start of the dialysis was determined with flow cytometry (set at 100%). The nuclear concentration of DNR determined at several time intervals after start of dialysis is shown in Fig. 5 and is expressed relative to the concentration before dialysis. These data indicate that during the first 1.5 h after start of dialysis the nuclear drug level is stable, but after that time the nuclear drug level rapidly falls to a low level. The same samples were also analysed with HPLC showing identical concentration-time data. In addition, the fall in the DNR level was associated with an increase in nuclear fragmentation as was evident from microscopic examination. Thus, after a stable period of 1.5 h of dialysis, DNR associated fluorescence per nucleus had declined and the nuclei began to desintegrate.

Discussion

The main advantage of flow cytometry compared with the traditional methods of estimation of cellular levels of anthracyclines relates to the possibility to identify subpopulations of (hematopoietic) cells. Also it is possible to identify and therefore exclude, any dead cells or cell debris which may contaminate the cell mixture and interfere with the estimation (16). However, when flow cytometry is used for quantifying the cellular DNR concentrations, the cellular fluorescence caused by DNR is said to be influenced by quenching when the drug is intercalated in DNA (9,10). In such instances conventional methods such as HPLC analysis are needed to validate the flow cytometry methodology. Therefore we compared the cellular DNR associated fluorescence with the DNR content of human nucleated cells as measured with HPLC.

The incubation concentrations of DNR were low in comparison to the concentrations used by others (8,16-20) but were deliberately chosen in order that resulting cellular concentrations in vitro correspond to those attained in vivo in WBC and bone marrow cells in patients on treatment with DNR (11,14). Like others (16,21-23) we found a positive correlation between the extracellular DNR concentrations and the resulting mean fluorescence following incubation in vitro. In addition, we showed good correlations between the mean cellular fluorescence and the amount of DNR taken up by the cells in vitro. Further, the detection level of flow cytometry appeared comparable to that of HPLC. Our data are in agreement with results of previous reports indicating concordances between flow cytometric and fluorometric analysis to quantitate cellular anthracyclines (18,20,24) and between fluorescence and cellular content of radiolabeled DNR (16).

The results of the DNR concentrations determined in nuclei versus whole cells indicate that flow cytometry data provide a measure of total cellular DNR content: DNR fluorescence originating from the cytoplasm and from the nucleus. However, since it was not known to what extent the nuclear DNR measured with flow cytometry was free or nuclear bound, we determined the leakage of DNR from the nuclei. The leakage experiment showed that DNR remained tightly bound in the nuclei for a stable period of 1.5 h. After that time, the nuclei began to desintegrate and the DNR was quickly released into the DNR free buffer. These results indicate that the nuclear DNR associated fluorescence as measured with flow cytometry involves DNR bound to the nucleus rather than free DNR. The available evidence thus supports the conclusion that flow cytometric measurements of cellular DNR fluorescence can be considered as a good quantitative measure of total cellular drug content, including nuclear bound DNR.

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CHAPTER 4

Kinetics of daunomycin in leukemia cells and leukocytes in vivo in the rat

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Abstract

The capability of nucleated blood cells and leukemic cells to transport daunomycin (DNR) to target tissues in the body was investigated in the rat. The in vivo distribution kinetics of DNR entrapped in leukemia cells (brown Norway acute myeloid leukemia, BNML) or in nucleated bone marrow cells, which had been exposed to DNR (0.2 mg/5 x 10^7 cells) in vitro, were determined. It was found that BNML leukemia cells and normal bone marrow cells take up DNR according to a linear pattern up to 400 μg/5 x 10^7 cells. When these DNR loaded cells are infused into the rat, dose dependent distribution kinetics are observed. Compared to IV injection of the same dosage, cell-bound DNR leads to a higher concentration and a higher tissue area under the curve of DNR in the liver ($p < 0.05$) and the spleen ($p < 0.05$), while equal levels are attained in bone marrow. Lower concentrations and area under the curve of DNR are observed in cardiac tissue of normal rats ($p < 0.001$) and leukemic rats ($p < 0.05$). It is concluded that DNR entrapped into marrow and leukemia cells follows different kinetics from free DNR in plasma.

Introduction

The anthracycline antibiotic daunomycin (DNR) is a prominent drug for the remission-induction and consolidation therapy of acute myelocytic leukemia. The toxicity of this drug is considerable and includes bone marrow depression, alopecia and cardiotoxicity (1). It has been known for some time that the rapid decline of plasma concentrations may be attributed to the conversion of DNR to its major metabolite daunorubicinol (DOL) and to uptake of the drug by many tissues, including leukemic cells (2-6). We have previously reported the distribution kinetics of DNR in the rat following different dosages and schedules of administration (7-9). From these data it appears that intracellular levels of DNR exceed plasma levels after the initial distribution phase. Thus, intracellular DNR levels may better correlate with the target tissue effect.

In the present study we have examined the distribution of DNR carried by leukemic cells in vivo, and compared it with DNR carried in normal bone marrow nucleated cells and with the normal plasma distribution of DNR. The purpose of this study is to

determine to what extent the nucleated cells add to the transport of DNR to target tissues in acute leukemia.

Materials and methods

Chemicals

Daunomycin, daunomycinol and Adriamycin were supplied by Farmitalia (Milan, Italy). Chromatographic assays were performed with HPLC quality reagents, purchased from Baker Chemicals (Holland).

Animals

Twelve-week-old female barrier derived inbred brown Norway (BN/BiRij) rats raised in the inbred Rijswijk colony were used. At the time of the experiments their body weight averaged 150-200 grams.

Leukemia model

The brown Norway myeloid leukemia (BNML) was chosen as a model. This acute myelocytic leukemia originated in 1971 in a female rat of the inbred brown Norway rat strain in the Rijswijk colony (BN/BiRij) following three intravenous (IV) injections of 2 mg of 9,10-dimethyl-1,2-benzanthracene 100 days earlier. The leukemia has since been maintained by transplantation of leukemic cells directly or by cryopreserved batches. The characteristics of BNML have been described extensively (10) and can be summarized as follows:

1) the growth fraction is low, with an increased cell loss rate as the terminal stage of the disease approaches. This leads to a relatively low net cell production rate;
2) it is cytochemically and cytologically identical to human acute myeloid leukemia (AML);
3) the mean survival time after IV inoculation of 10^7 leukemic spleen cells is 22 days;
4) the response to DNR and other cytostatic drugs is comparable with that of human AML patients.

The experiments with leukemic animals were performed in BN rats inoculated with leukemia, at a stage of the disease comparable with that of human AML patients at clinical admission (day 14 after IV transplantation of 10^7 leukemic spleen cells). At this stage, many organs such as the spleen, liver, lungs and bone marrow are heavily infiltrated by leukemic cells.

Incubation of cells

Normal rat nucleated bone marrow cells were harvested from the femurs of untreated rats as described previously (11). Fifty million nucleated bone marrow cells in 1 ml RPMI medium + 5% fetal calf serum (pH 7.4) were incubated for 20 min at 37 °C with DNR concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml.

After incubation, the cells were put on melting ice and washed twice by centrifugation (400 g at 4 °C) and the pellets resuspended in ice cold RPMI. Finally, the cells were resuspended in 1 ml RPMI. The concentration of DNR in the cells was determined by chemical extraction and liquid chromatography as described below. No measurable loss of intracellular drug from the cells to the extracellular drug-free medium could be observed in cells which had been incubated with DNR and stored at 4 °C for maximally 1 hr.
Subsequently the DNR-containing cells were injected into a tail vein of a rat at a final volume of 1 ml (5×10^7 cells/ml).

BNML leukemia cells were obtained from the spleen of a rat which had been inoculated with leukemia cells 17 days before, as described earlier (12). Leukemic cells were incubated with DNR in the same way as normal bone marrow cells. After the incubation and the washing procedures had been completed, 5 x 10^7 leukemic cells in 1 ml of RPMI buffer were injected into the tail vein of leukemic rats at day 14 after they had been inoculated with BNML leukemia.

In addition, one group of normal BN/BiRij rats and one group of leukemic BN/BiRij rats at day 14 of the disease were treated IV with a single bolus injection of DNR at a dosage which was identical to the amount of drug administered when entrapped in cells. After administration of DNR rats were killed by exsanguination at + 30 min, +1 h, +4 h and +24 h. Each treatment group consisted of three rats at each time interval. To obtain bone marrow cells, a femur was cut into two parts and each part was repeatedly flushed with 2 ml of saline. Femoral bone marrow, the liver, the spleen and the heart were taken out, washed and immediately frozen at -20 °C until further processing. Plasma was obtained by adding citrate to aortic artery blood samples.

Drug assay

Daunomycin and daunomycinol (DOL) concentrations were determined by straight phase high pressure liquid chromatography. Plasma samples adjusted to pH 9.8 were extracted with a chloroform-methanol (4:1) mixture. After centrifugation, 1 ml of the organic phase was evaporated to dryness under nitrogen at 30 °C. The residue was redissolved in 300 µl mobile phase. Ten percent homogenates of tissues in NaCl (0.9%), were adjusted to pH 9.8 and extracted with a chloroform-methanol (4:1) mixture. One hundred microliter aliquots of the organic phases were injected directly on the column. Two milliliters of the bone marrow suspension were adjusted to pH 9.8 and thereafter extracted with chloroform-methanol (4:1). For plasma, the recovery of the extraction procedure was 90-95%. For tissues, the recovery was 85-90%. Adriamycin was used as an internal standard for drug quantification.

HPLC separation (adapted from Baurain et al. (13)) was accomplished using a Waters Associates M-510 pump and a Waters Associates automatic sample injector (Model 710B). The stationary phase consisted of 7-µm silica gel particles prepacked into a 250 x 4.6 mm stainless steel column (Lichrosorb Si-60-7, Chrompack). The mobile phase consisting of chloroform, methanol, acetic acid, water and 3 mM $MgCl_2$ solution in water (720:210:40:24:6 by volume) was filtered (Millipore FH 0.5 µm) and used at a flow rate of 0.8 ml/min. Fluorescence detection was accomplished using a Gilson Spectra-Glo fluorometer at 480 and 550 nm (cut-off filter) for the excitation and emission wavelengths, respectively, equipped with a 15 µl flow cell.

The lower limit of detection of DNR and DOL was 10 ng/ml (plasma) and 10-20 ng/g (tissue).

Calculations

The trapezoidal method was used for the calculation of the tissue area under the curve (AUC) from zero to +4 h after drug administration. The Spearman correlation test, linear regression and the Wilcoxon test were used for statistical analysis.

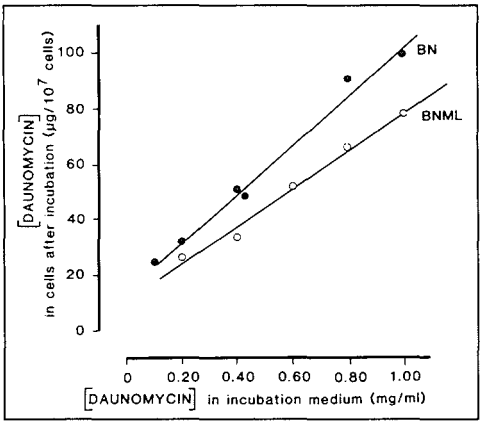

Fig. 1:
Intracellular concentration of DNR in normal rat BN bone marrow cells (●–●) and BNML rat spleen leukemia cells (○–○) following an incubation period of 20 min with different extracellular concentrations of DNR

Spearman correlation coefficients:
BN: 0.98 ($p < 0.001$);
BNML: 0.99 ($p < 0.001$).

Results

Upon incubation of rat bone marrow nucleated cells with DNR the uptake of the drug depends on the extracellular drug concentration ($p < 0.001$) (Fig. 1). Also, when BNML leukemia cells instead of bone marrow nucleated cells are incubated, a linear relationship is observed between the extracellular concentration of DNR and the resulting intracellular drug concentration ($p < 0.001$) (Fig.1).

However, the uptake of DNR in BNML cells is less when they are incubated with comparable extracellular concentrations. The loss of DNR from the cells during the incubation and the washing procedure was always less than 5% if the cells were kept at 4 °C in the dark for no longer than 1 h before they were injected into the animals. The viability of DNR incubated nucleated bone marrow cells and BNML leukemia cells immediately after the incubation procedure depended strictly on the extracellular drug concentration. At all concentrations used (0.2-1.0 mg/ml), less than 30% dead cells were observed, except at the highest concentration (52%), as determined by trypan blue exclusion.

Following IV injection of the same number of nucleated bone marrow cells incubated with different concentrations of DNR, the in vivo recovery of DNR was tested by determining the drug concentration in the spleen at +1 h after drug administration (Fig. 2). A linear correlation can be observed between the administered drug amount and the spleen tissue concentration of DNR ($p < 0.001$).

In subsequent experiments nucleated bone marrow cells containing DNR at different concentrations were injected IV into normal BN rats. The total amounts of administered DNR entrapped in these cells were 125, 275 and 425 μg, respectively. Following this way of administration, the disposition of DNR was determined in bone marrow, liver, spleen, heart and plasma. In plasma no DNR at concentrations above the detection limit (10 ng/ml) is present at any time. The organ concentrations of DNR depend on the administered dose. (Fig. 3). Considerable quantitative differences are observed between these organs, i.e. the concentration in the spleen is at least 5-fold the concentration in other tissues at the same time. In Table 1 the tissue area under the curves from zero to +4 h are given. The main finding is that with cellular DNR a considerably lower AUC is achieved in heart tissue, compared with higher AUCs in spleen and liver. In bone marrow no difference is observed.

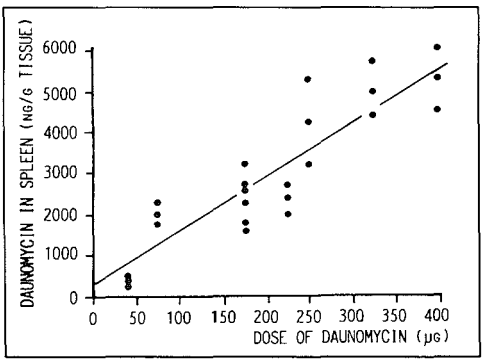

Fig. 2:
In vivo concentrations of DNR in spleen tissue of BN rats following IV administration of different dosages of the drug entrapped in 5 x 10^7 nucleated bone marrow cells

The concentrations are expressed as nanogram per gram of wet tissue. The Spearman correlation was 0.89.

We also investigated how far leukemic cells are capable of transporting DNR to the leukemia-infiltrated tissues. For this purpose BNML leukemic cells were obtained from the spleen of terminal leukemic rats and incubated with various concentrations of DNR. The final intracellular amounts of DNR were 75, 175 and 250 µg per 5 x 10^7 cells. After IV administration of these cells the disposition of DNR is different in each organ studied, with the highest concentrations attained in liver and spleen (Fig. 4).

Finally, the distribution of DNR entrapped in cells was compared with IV bolus injection, both after a dose of 200 µg per animal (approx. 1.0 mg/kg).
In normal rats, DNR administered as an IV bolus injection reaches a concentration in the heart which is significantly higher than when the drug is given entrapped in cells ($p < 0.001$) (Fig. 5). The amount of DOL in heart tissue with either way of administration is proportional to the concentration of parent drug. The DNR concentration attained in the spleen and the liver is higher following administration of the drug entrapped in cells than after IV bolus (spleen: $p < 0.05$; liver: $p < 0.05$).

In bone marrow no significant difference is observed, except at +4 h, when the concentrations are significantly higher following the IV bolus gift.

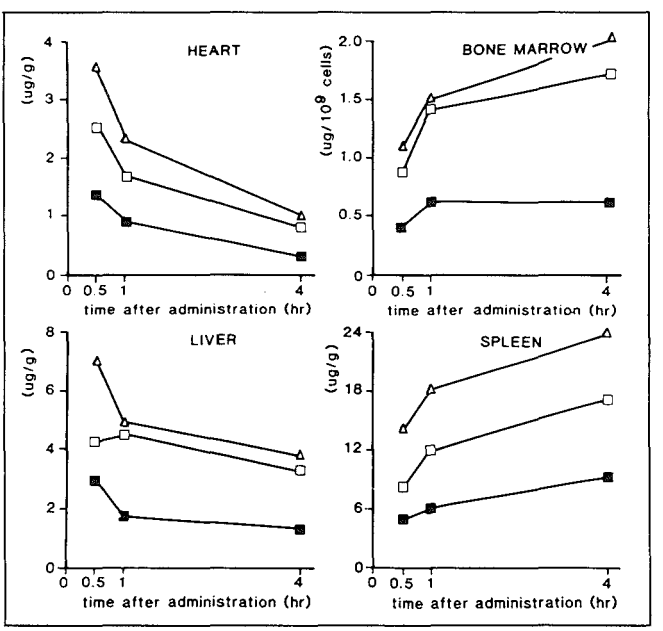

Fig. 3:
In vivo concentration of DNR in heart, bone marrow, liver and spleen in the normal BN rat following IV administration of 5 x 10^7 nucleated bone marrow cells with a final intracellular DNR content of 125 µg (■–■), 275 µg (□–□) and 425 µg (Δ–Δ).

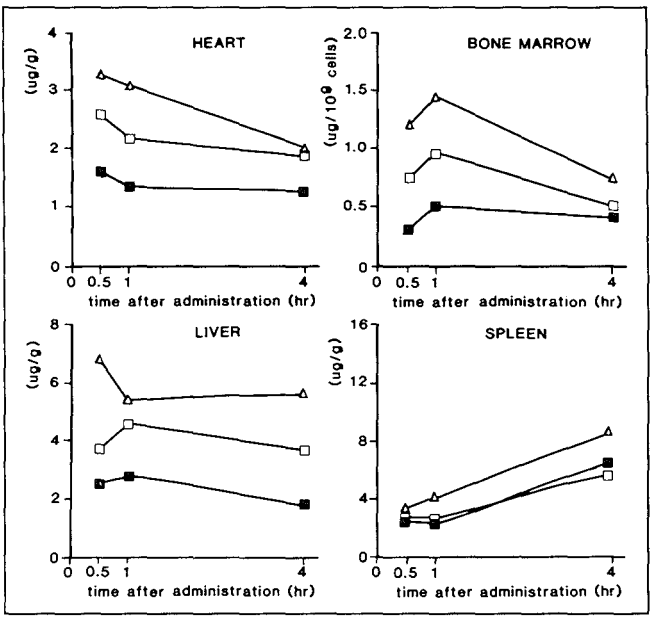

Fig. 4:
In vivo concentration of DNR in heart, bone marrow, liver and spleen in the BNML rat following IV administration of 5 x 10^7 BNML leukemia cells with a final intracellular DNR content of 75 µg (■–■), 175 µg (□–□) and 250 µg (Δ–Δ).

In BNML leukemic rats a similar difference is observed between the two ways of administration (Fig. 6). DNR entrapped in BNML cells leads to lower concentrations in the heart than with IV bolus administration ($p < 0.05$). In spleen and liver the opposite phenomena is observed. Here at all time points the concentrations of DNR are significantly higher when the drug is administered in cells compared with IV administration (spleen: $p < 0.05$; liver: $p < 0.05$). In bone marrow again no difference is observed.

Table I:
Area under the curve (0-4 h) of daunorubicin in tissues of the non-leukemic rat (BN)[a]

	AUC of DNR in tissue (mean ± SD)			
Dose of DNR	Heart (µg.h/g)	Spleen (µg.h/g)	Liver (µg.h/g)	Bone marrow (µg.h/10^9 cells)
125 µg/5.10^7 cells	2.89 ± 0.36	26.13 ± 2.21	6.32 ± 0.58	2.45 ± 0.63
275 µg/5.10^7 cells	5.59 ± 0.44	50.67 ± 2.48	14.96 ± 0.90	5.46 ± 0.54
425 µg/5.10^7 cells	7.56 ± 0.81	75.12 ± 8.17	17.75 ± 4.34	6.10 ± 0.64
200 µg/5.10^7 cells	4.11 ± 0.68	38.26 ± 4.86	9.82 ± 1.60	3.61 ± 0.80
200 µg IV	8.50 ± 0.39	18.85 ± 1.55	6.84 ± 0.46	5.02 ± 1.23

a: Abbreviations: DNR: daunorubicin; AUC: area under the curve in tissue; BN: brown Norway rat

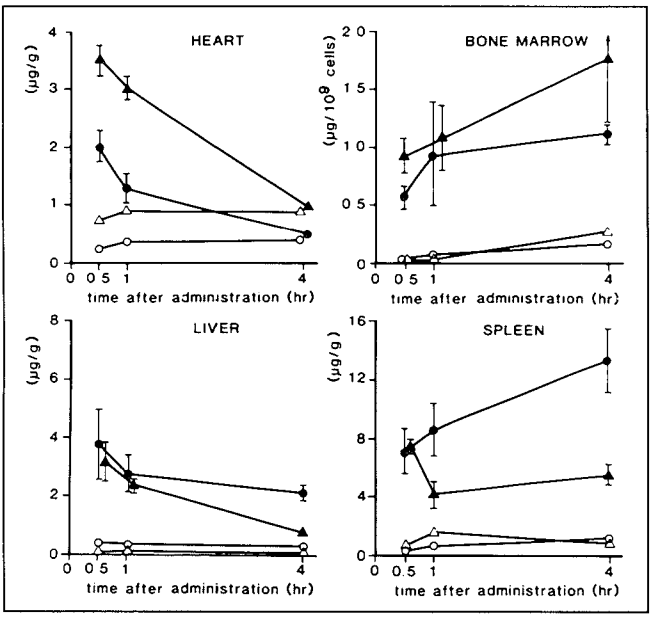

Fig. 5:
In vivo concentration of DNR and DOL in heart, bone marrow, liver and spleen in the BN rat following a bolus injection of 200 µg DNR (DNR: ▲–▲; DOL: Δ–Δ) or IV administration of 200 µg of DNR entrapped in 5 x 10^7 nucleated bone marrow cells (DNR: ●–●; DOL: ○–○) (mean ± SD)

These findings are summarized in Table 2, using the tissue AUC as calculated from the observed concentrations at each dosage and/or way of administration.

Discussion

The pharmacokinetics of daunorubicin are characterized by a short initial plasma half-life, rapid accumulation of the drug in tissues and a long elimination phase (4,5). The in vivo cellular levels of DNR and DOL in AML patients are quite variable, and they cannot be predicted from plasma concentrations of DNR and DOL (2,4).

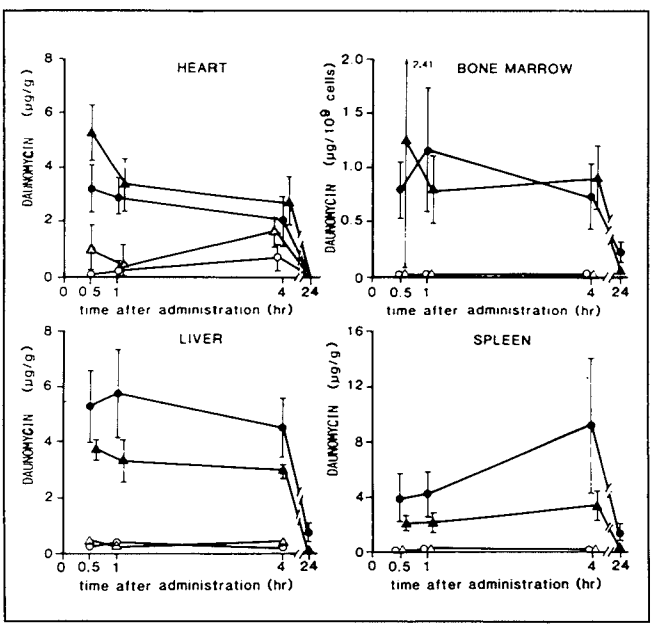

Fig. 6:
In vivo concentration of DNR and DOL in heart, bone marrow, liver and spleen in the BNML rat after an IV bolus injection of 200 µg DNR (DNR: ▲–▲; DOL: Δ–Δ) or IV administration of 200 µg of DNR entrapped in 5 x 10^7 BNML cells (DNR: ●–●; DOL: ○–○) (mean ± SD)

Table 2:

Area under the curve (0-4 h) of daunorubicin in tissues of the leukemic rat (BNML)[a]

Dose of DNR	AUC of DNR in tissue (mean ± SD) Heart (μg.h/g)	Spleen (μg.h/g)	Liver (μg.h/g)	Bone marrow (μg.h/10^9 cells)
75 μg/5.10^7 cells	5.22 ± 0.11	14.96 ± 1.61	8.94 ± 1.52	1.96 ± 0.51
175 μg/5.10^7 cells	8.09 ± 0.78	14.78 ± 1.28	15.52 ± 1.80	2.53 ± 0.80
250 μg/5.10^7 cells	9.99 ± 0.69	22.25 ± 4.71	21.54 ± 3.03	4.19 ± 1.85
200 μg/5.10^7 cells	9.91 ± 1.92	23.27 ± 9.38	19.43 ± 3.81	3.58 ± 1.11
200 μg IV	11.13 ± 2.76	10.02 ± 0.92	12.00 ± 1.44	3.44 ± 1.26

a: Abbreviations: DNR: daunorubicin; AUC: area under the curve in tissue; BNML: brown Norway myeloid leukemia

Following IV bolus injection of DNR, a rapid fall of plasma levels is associated with increasing levels of DNR and DOL in leukemic cells, which are capable of acquiring DOL by cellular uptake as well as by intracellular conversion of DNR (3,4,14). DOL by itself possesses considerable antitumor activity, although the relative activity towards leukemia as compared to DNR has not been thoroughly investigated. From these arguments, it is clear that pharmacokinetic analysis of DNR plasma concentrations only is probably not adequate for the interpretation of its anti-leukemic effect. However, because of the complicated pharmacokinetic picture, it is difficult to assess the role of intracellular DNR as regards the distribution kinetics of DNR and DOL.

In previous reports we have approached the study of DNR pharmacokinetics in the rat (7-9), and we have demonstrated specific accumulation of DNR and to a lesser extent of DOL in spleen, liver and bone marrow. However, the net amount of DNR and DOL attained in these organs was shown to depend on the tumor load in leukemic animals (9). It can be appreciated that differences of the transport of DNR/DOL carried by leukemic cells and/or normal white cells are responsible for the observed differences.

In case of acute myelocytic leukemia it has been suggested that proliferating leukemic cells can return from the blood to the spleen and the bone marrow (15,16) and that they can be released into the circulation again (17). The permeability of the capillaries in these organs is a major reason for the infiltration of leukemic cells during the development of the disease (18). Thus, one might expect that DNR-containing leukemic cells return to and settle in the liver, the bone marrow and possibly the spleen. Because of the observed accumulation of DNR in white cells in peripheral blood following IV administration (5), we decided to investigate to what

extent DNR-containing cells contribute to effective concentrations of DNR and DOL in these organs after IV administration. The study was performed with DNR-loaded nucleated bone marrow cells infused in normal rats as well as with DNR-loaded leukemic cells infused in leukemic rats. From Fig. 1 it is clear that exposure of BN normal rat bone marrow to DNR results in dose-dependent intracellular uptake of the drug. By incubating the cells with high drug concentrations, up to 400 μg of DNR is contained in 2×10^7 cells. Following administration of these cells into the rat the concentrations of DNR attained in liver and spleen are larger than with a regular IV administration of the same dose, while in bone marrow the concentrations are comparable with both ways of administration.

After administration of DNR-carrying cells, no detectable drug concentrations were present in the plasma of any sample. However, it is not known whether the efflux of drug from the cells during circulatory transport was minor. It is also possible that low but substantial levels of DNR in plasma were rapidly eliminated. Administration of DNR-loaded cells results in lower cardiac levels of this drug when compared with IV bolus administration, suggesting that intracellular DNR does not have a major role with respect to cardiotoxicity. A major difference with IV bolus is the selective accumulation of the drug in liver and spleen. These data indicate that DNR present in cells is preferentially distributed to hemopoietic tissues. It is, however, more relevant to investigate this phenomena in leukemic rats, because the kinetics of IV DNR are significantly different between normal and leukemic rats (9).

The distribution kinetics of leukemia cells in the BNML acute leukemia in the rat have been thoroughly investigated by Hagenbeek and Martens (10). When untreated BNML cells are infused into leukemic animals at a mature stage of the disease, 42% of the cells lodge in the liver, 8% in the spleen and 50% in the bone marrow. It was demonstrated that the majority (± 99%) of the leukemic cells are present in these slowly exchangeable tissues.
The present study shows that the distribution of DNR/DOL contained in BNML leukemia cells does not exactly follow this pattern. Taking into account a mean liver weight of 8.5 g, a mean spleen weight of 2 g and a mean femoral cellularity of 0.33×10^8 cells (representing 1.2% of the total bone marrow) the recovery of DNR from these organs at +4 h after IV administration of BNML cells loaded with 200 μg DNR, is 16.6 μg in the spleen (8.3%), 38 μg in the liver (19%) and 1.8 μg in the bone marrow (0.89%).
Thus, the uptake of DNR in the spleen is exactly as can be expected from the distribution kinetics of leukemic cells, but in liver and especially in bone marrow substantially less uptake of DNR is observed. This discrepancy may be due to a partial loss of the homing characteristics of the BNML cells as a result of the high (intra)cellular drug concentration.

The concentrations attained in bone marrow are of the same order as after IV bolus administration of the same dose. In liver and in spleen, significantly larger organ concentrations are observed. It remains therefore to be determined to what extent DNR is released in vivo from the infused cells once they have arrived in tissues and whether the drug is subsequently taken up by adjacent leukemia cells and/or normal tissue. We could not demonstrate any loss of DNR from the cells during circulatory transport, as judged from the lack of detectable plasma concentrations. In vitro incubation of DNR-containing cells at 37 °C, however, leads to leakage of DNR from the cells (6).

From the present study we conclude that DNR contained in leukemia leads to significantly higher levels in spleen and liver, but not in heart and in bone marrow when compared to IV bolus DNR. In view of the reported large levels of DNR in peripheral white cells of AML patients after IV administration, these data may indicate that DNR contained in leukemic cells may add significantly to establishing high tissues levels. Although these findings have no direct therapeutic implications, they may add to our understanding of the complicated association between the DNR concentration and its antileukemic effect.

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CHAPTER 5

Cellular pharmacokinetics of daunorubicin: relationships with the response to treatment in patients with acute myeloid leukemia

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Abstract

In an attempt to identify pharmacokinetic factors that determine the response of acute myeloid leukemia (AML) patients to induction chemotherapy, we determined the concentrations of daunorubicin (DNR) and the main metabolite daunorubicinol (DOL) in vivo and particularly evaluated the concentrations in blood and bone marrow nucleated cells. Cell measurements were obtained in 37 evaluable patients during their first remission induction treatment with DNR and cytarabine (ara-C) and directly compared with the plasma distribution kinetics of DNR. We show that

1) plasma DNR concentrations do not correlate with DNR concentrations in bone marrow nucleated cells; but
2) plasma area under the curve (AUC) values of DNR correlate inversely ($p < 0.01$) with AUC values of DNR in white blood cells (WBC);
3) concentrations of DNR in WBC correlate positively ($p < 0.01$) with DNR concentrations in bone marrow nucleated cells; and
4) the concentrations of DNR in WBC show a negative correlation ($p < 0.01$) with the numbers of peripheral blast cells at diagnosis.

We then tested whether the pharmacokinetic parameters had predictive value for the clinical outcome of therapy, but none of the plasma levels or WBC and bone marrow concentrations of DNR predicted treatment outcome. The inverse correlaton between the concentrations of DNR in WBC and the numbers of peripheral blast cells suggests that the effective DNR concentrations achieved intracellularly are mainly a function of the tumor load so that lesser amounts of DNR accumulate intracellularly when the AML cell numbers in blood are higher.

Introduction

Daunorubicin (DNR) is a major drug in the treatment of acute myeloid leukemia (AML). Therapy with DNR in combination with cytarabine (ara-C) is commonly used for induction of complete remission (CR) in AML (1,2). Although the CR rate is high (3), 20% to 40% of patients with AML do not enter CR upon treatment with schedules of DNR and ara-C. Patients not attaining CR are either those individuals whose leukemia is resistant to the therapy or those with early death during remission induction (RI) therapy (4). Further improvements in the CR rate depend mainly on the development of more effective or less toxic agents and individualization of RI therapy.

Monitoring the pharmacokinetics of antineoplastic drugs may show interindividual variations between patients. In recent years, studies have been performed to identify pharmacologic variables that determine the response of AML patients to chemotherapy consisting of ara-C and an anthracycline. At present, studies concerning cellular cytidine kinase and deaminase have failed to predict clinical outcome (5), as have studies of cellular ability to form activated ara-C (ara-CTP) (6-8). However, the ability of blast cells to retain intracellular ara-CTP following in vitro exposure to ara-C has been reported to correlate with remission duration in those patients who subsequently achieved a CR (7,9). More recently, the cellular pharmacokinetic studies of Plunkett et al suggest that retention of ara-CTP may be a determinant of therapy response. However, these relationships have been established for treatment with high-dose ara-C, not for conventional dosages of the drug (10,11). In case of Adriamycin (doxorubicin (DOX); Adria Laboraties, Columbus, OH), plasma levels do not correlate with the clinical response to the drug in patients with AML (12). DNR, like DOX, exerts its cytotoxic effect mainly through intercalation into DNA (13,14), and the nuclei of AML cells are the principal targets of DNR therapy. Therefore, determinations of cellular DNR and its pharmacologically active metabolite daunorubicinol (DOL), rather than the plasma parameters, would be expected to give prognostic information with regard to treatment response (15,16). Moreover, the results from a recent study have indicated that the number of tumor cells (tumor load) play a significant role, as far as the distribution of anthracyclines in tissues is concerned (17). The objective of the investigations presented here was to determine the in vivo cellular DNR concentrations in blood and bone marrow in patients during RI treatment, and to compare these with the simultaneously observed plasma distribution kinetics of this drug. In addition, the possible effect of the tumor load on the pharmacokinetic parameters of DNR was studied. We also examined whether the in vivo cellular DNR concentrations had predictive value for the clinical outcome of therapy.

Materials and methods

Patients

Forty patients with previously untreated AML were enrolled in this study. The French-American-British (FAB) classification system was used to diagnose AML (18,19). Patients were treated according to the AML-6 protocol of the European Organization for Research and Treatment of Cancer (EORTC). After October 1983, elderly patients (i.e., > 65 years) with AML were treated according to the EORTC AML-7 protocol, and after April 1986, according to the newly activated AML-9

protocol. RI chemotherapy contained DNR, 45 mg/m^2 (AML-6 protocol) or 30 mg/m^2 (AML-7 and AML-9 protocol) on days 1, 2, and 3 by intravenous (IV) bolus; and ara-C, 200 mg/m^2 (AML-6 and AML-7 protocol) or 100 mg/m^2 (AML-9 protocol) IV on days 1 through 7. Protocol AML-6 also included vincristine, 1 mg/m^2 IV on day 2.

The criteria for evaluating response were those established by the Cancer and Acute Leukemia Group B (20). A CR was defined as a reduction in the percentage of AML blast cells to < 5.0%, in association with a recovery of hematopoiesis and peripheral blood counts to normal for at least 4 weeks. Partial remission (PR) was defined by a decrease in the percentage of AML blast cell infiltrate of the bone marrow to 5.1% to 25.0%. When two courses of RI therapy had been applied and no CR reached, the outcome of therapy was considered as failure. The spectrum of failures of the induction treatment was classified according to Preisler (21): type 1, absolute drug resistance; type 2, relative drug resistance; type 3, regeneration failure; type 4, hypoplastic death; type 5, early death; and type 6, extramedullary leukemic persistance.

Collection and isolation of plasma, WBC, and bone marrow from AML patients
The optimal conditions for the collection of white blood cells (WBC), bone marrow nucleated cells and plasma, as well as the methodology of drug analysis, have been described in detail (22). In brief, blood samples (10 ml) were drawn into tubes with EDTA as anticoagulant, immediately before (zero minutes), and at +5, +10, +20 and, +30 min as well as at +1, +2, +4, +6, +8 and +24 h after the first dose of DNR during the first cycle of RI treatment. The samples were cooled to 4 °C and the cells were counted. After centrifugation, the plasma was collected and frozen, and the buffy coat was obtained. The buffy coat was depleted of erythrocytes by cold hypotonic lysis (22) and the WBC were resuspended in phosphate-buffered saline (PBS), counted, and also frozen. Complete plasma and WBC data were successfully obtained in 35 and 33 cases, respectively.

Bone marrow cells were collected from the patients at two time points, ie, at +1 h (n = 31 patients) and at +24 h (n = 26 patients) after DNR treatment. Bone marrow (2 to 10 ml) was aspirated from the posterior iliac crest under local anesthesia into a syringe and immediately put into tubes containing EDTA for anticoagulation. The samples were cooled to 4 °C and the cells were counted. The quality of the bone marrow aspirates was assessed by calculating the admixture of peripheral nucleated cells in the samples according to Hodrinet et al. (23). After the erythroid cells had been lysed by exposure to hypotonic NH_4Cl at 4 °C, the nucleated cells were counted and then stored at -20 °C until further processing by high-performance liquid chromatograpy (HPLC). All patients had given informed consent to donate blood and bone marrow.

Analysis of plasma and intracellular DNR and DOL contents by HPLC
Concentrations of DNR and DOL were determined using HPCL drug analysis as described previously (17,22,24). Briefly, 0.5 ml of a thawed plasma sample was vortexed with 0.2 ml borate buffer and 2 ml chloroform: methanol (4:1) in a siliconized tube. After centrifugation at 1000 x g for 10 min, 1 ml of the organic phase was evaporated to dryness under nitrogen at 35 °C.

To avoid heterogeneous drug recoveries from patient samples with different cell concentrations, we standardized the WBC and bone marrow samples with PBS at final concentrations between 10^9 to 10 x 10^9 cells/l before entering the extraction

procedure. Borate buffer (0.2 ml) and 5 ml chloroform: methanol (4:1) were added to 2 ml of the cell suspension and shaken for 20 min. After centrifugation, 1 ml of the organic phase was dried under nitrogen.

Dried samples were reconstituted in 300 µl chloroform: methanol (4:1) and 100 µl aliquots were then injected onto an HPLC column containing 7 µm silicagel particles (Lichrosorb Si-60-7; Chrompack, Middelburg, The Netherlands). The mobile phase consisted of chloroform, methanol, acetic acid, water, and 3 mmol/l $MgCl_2$ solution in water (720:210:40:24:6 by volume); the flow rate was 1.3 ml/min. Detection of anthracycline was accomplished with a fluorometer (Gilson Spectra-Glo; Meyvis en Co BV, Bergen op Zoom, The Netherlands) using excitation at 480 nm and emission at 550 nm. Retention times and areas of the peaks were recorded by a Shimadzu Model CR3A integrator (Chrompack Packard BV, Delft The Netherlands). Concentrations of DNR and DOL were quantified by using doxorubicin as an internal standard. The lower detection limit of DNR was 5 ng/ml plasma and 2 $\mu g/10^9$ nucleated cells.

Pharmacokinetic analysis

The data from the 40 AML patients were analyzed by fitting a two-compartment open model to the observed plasma DNR concentrations (25,26). The plasma concentration-time curve following IV bolus injection of DNR was described by the following equation:

$$C(t) = A.e^{-\alpha.t} + B.e^{-\beta.t} \qquad (1)$$

where C(t) is the plasma level of DNR at each time (t) after injection, A and B are constants, and α and β are first-order decay rate constants. The plasma area under the concentration-time curve (AUC), the apparent distribution volume (Vd_{area}) and the elimination rate constant (k_{el}) were calculated from this model. The plasma clearance (Cl) during 24 h following drug administration was derived from:

$$Cl = \frac{\text{dose}}{AUC\ 0-24\,h} \qquad (2)$$

The plasma AUC of DOL and the intracellular AUC of DNR were determined by the trapezoidal method (27).

Statistics

The Spearman test was used for calculating correlation coefficients and the Wilcoxon test was used for comparison of groups (28,29).

Results

Clinical data

During the study period, 40 new patients with AML were entered of whom 3 (75, 69, and 67 years) died within the first week following completion of therapy or during the hypoplastic phase (failures type 4 or 5). Table 1 lists the clinical and treatment characteristics of the remaining 37 AML patients who could be evaluated for response.

The results of RI therapy were as follows: CR, 20 of 37 (54%); PR, ten of 37 (27%); and resistant disease (RD) (failure type 1), seven of 37 (19%). The group of aged AML patients among these received a somewhat lower dose of DNR (30 mg/m^2); the

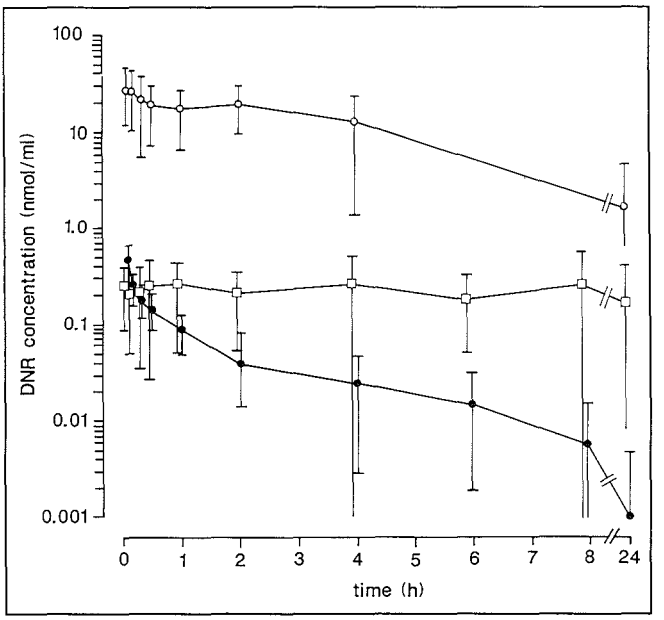

Fig. 1:
Cellular and plasma concentration-time curves of DNR and DOL after IV bolus injection of DNR

Data from 37 AML patients during first RI treatment. (Mean ± SD; ○, DNR in WBC; ●, plasma DNR; □, plasma DOL).

outcome of RI therapy of those seven evaluable elderly patients was not different (CR, five of seven; RD, two of seven).
WBC counts (range, 0.417 to 239 x 10^9 cells/l), percentages of peripheral blast cells (range, 1% to 100%), and absolute numbers of peripheral blast cells (range, 0.009 to 210 x 10^9 cells/l) at diagnosis showed significant interindividual variations (Table 1).

Pharmacokinetic parameters of DNR in plasma, WBC, and bone marrow
The pharmacokinetics of DNR were studied in all patients during the first remission induction therapy. The plasma concentrations of DNR fell to minimal levels at +8 h after IV injection (Fig. 1). The rapid decline was associated with a fast appearance of the major metabolite DOL in the plasma. Generally, DOL plasma concentrations exceeded DNR plasma levels as early as at +0.5 h to +1 h after administration and at +4 h the DOL concentrations accounted for > 85% of the total amount of measurable anthracycline. With our method of analysis no metabolites other than DOL were found.

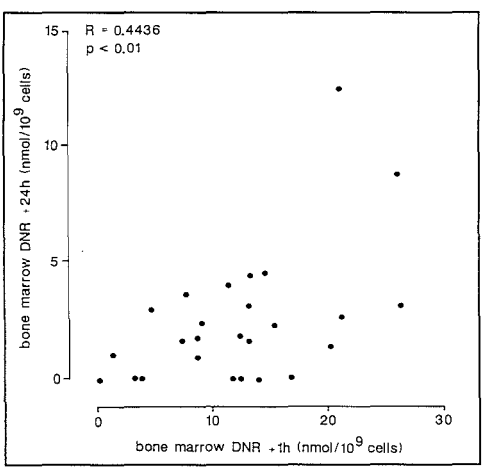

Fig. 2:
Daunorubicin concentrations in the bone marrow nucleated cells

Relationship between +1-hour v +24-hours measurements (n = 26 cases).

Table I:

Patient characteristics

Patient	Sex	Age	FAB classification	WBC[a] ($\times 10^9$/l)	Blasts[a] in peripheral blood (%)	Blasts in[a] peripheral blood ($\times 10^9$/l)	Blasts[a] in bone marrow (%)	Dose of DNR (mg/m^2)	Dose of DNR (mg)	Number of RI courses	Outcome of RI therapy
1	M	32	M1	3.2	90	2.88	90	45	100	1	CR
2	M	74	M4	3.8	1	0.04	68	30	60	1	CR
3d	M	73	M1	13.0	70	9.10	49	45	80	1	CR
4	M	40	M2	17.6	61	10.7	84	45	75	1	CR
5	F	56	M2	3.3	5	0.17	36	45	75	1	CR
6d	M	23	M2	15.0	39	5.85	57	45	80	1	CR
7	M	58	M2	0.417	1	0.004	32	45	90	1	CR
8f	F	67	M5	0.590	10	0.06	94	30	50	1	CR
9	F	34	M2	1.493	34	0.51	86	45	70	2	CR
10	M	60	M4	40.1	26	10.4	74	45	90	2b	CR
11	M	62	M2	1.545	1	0.02	53	45	80	2b	CR
12	M	39	M1	0.9	1	0.009	62	45	85	2b	CR
13	F	65	M1	0.937	1	0.009	83	45	70	2	CR
14	M	56	M4	1.736	4	0.07	70	45	85	2b	CR
15	F	62	M5	13.4	6	0.80	80	45	90	2b	CR
16	M	58	M1	20.0	62	12.4	85	45	75	2	CR
17	M	37	M1	0.7	1	0.007	93	45	100	2	CR
18f	M	61	M1	140.4	94	132	85	30	55	2	CR
19f	F	66	M2	0.538	2	0.01	47	30	45	2	CR
20f	F	64	M2	0.799	6	0.05	80	30	50	2	CR
21c+e	M	66	M5	48.5	66	32.0	73	45	70	1	PR
22e	M	47	M4	102	83	84.7	67	45	90	1	PR
23e	M	56	M1	150	100	150	96	45	80	1	PR
24	M	42	M1	1.44	18	0.26	66	45	75	2	PR
25	M	47	M4	5.0	65	3.25	85	45	90	2	PR
26	M	37	M4	115	76	87.4	81	45	90	2	PR
27	M	26	M4	239	88	210	86	45	80	2b	PR
28	M	33	M5	17.6	41	7.22	94	45	80	2b	PR
29	M	46	M5	45.0	40	18.0	64	45	80	2	PR
30	M	43	M5	60.0	20	12.0	77	45	95	2	PR
31d	M	70	M1	58.5	95	55.6	97	45	80	1	Failure type I
32	M	44	M4	2.0	5	0.10	78	45	95	1	Failure type I
33	M	70	M4	33.0	1.5	0.50	43	30	60	1	Failure type I
34	F	73	M4	1.32	22	0.29	70	30	50	1	Failure type I
35d	F	42	M4	4.2	8	0.34	33	45	80	1	Failure type I
36	M	17	M2	6.5	79	5.14	70	45	80	2	Failure type I
37c	M	66	M5	16.4	36	5.90	84	45	80	2	Failure type I

a) At diagnosis (before therapy).

b) Patients who were not only analyzed for DNR pharmacokinetics during the first, but also during the second course of RI therapy.

c) Patients (> 65 years) who were treated according to the AML-6 protocol of the EORTC, before the AML-7 protocol for elderly patients was activated (October 1983).

d) Protocol variations: patients who were classified as having an antecedent hematologic disorder and who were not treated according to the AML-6, AML-7 or AML-9 *protocols, but who received lower amounts of* DNR: patients no. 6 and 35: DNR, 45 mg/m^2 days 1, 2; patients no. 3 and 31: DNR, 45 mg/m^2 day 1.

e) Patients who achieved PR after one course of RI therapy, but who did not receive a second course of RI therapy, because of patient refusal of further chemotherapy (patient no. 21) or death from pneumonia (patients no. 22 and 23).

f) Patients (> 60 years) who were treated according to the AML-9 protocol for elderly patients, activated April 1986.

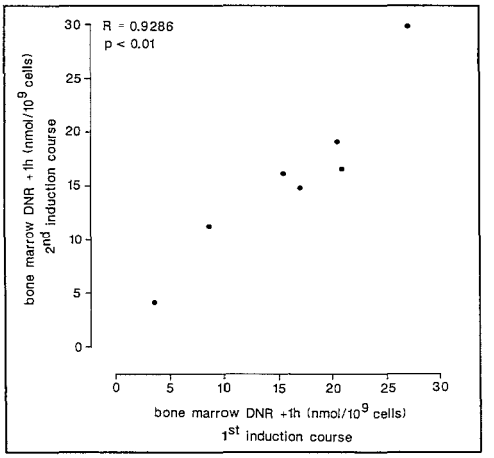

Fig. 3:
Daunorubicin concentrations in the bone marrow nucleated cells (at +1 h)

Direct comparison between first v second cycle of RI treatment (n = 7 cases).

DNR accumulated heavily in the WBC. Assuming that 10^9 cells represent a volume of about 1 ml, we determined the ratio of WBC to plasma concentration of DNR in the blood. This WBC to plasma DNR ratio increased from 55 at +5 min, to 200 at +1 h, and subsequently towards 470 at +4 h after IV injection. In all patients, maximum WBC concentratons of DNR were reached almost immediately upon administration of the drug. At +24 h, the cellular DNR concentrations had decreased to low levels in all 37 patients. In 18 of 37 patients, no DOL metabolite was detectable in the WBC. In the other patients (19 of 37), the WBC concentrations of DOL ranged from 3% (at +5 min) to 15% (at +4 h) of the total amount of measurable anthracycline, and at +24 h the WBC concentrations of DOL had decreased to low or undetectable levels (data not shown).

The DNR concentrations in the bone marrow nucleated cells at +1 h as well as at +24 h showed great variations among the patients (range, 0 to 27 nmol/10^9 nucleated bone marrow cells (+1 h); 0 to 13 nmol/10^9 nucleated bone marrow cells (+24 h)). The +24-hour bone marrow DNR concentrations were always lower than the comparative +1-hour values (Fig. 2). As for the WBC determinations, the DOL concentrations in bone marrow nucleated cells (+1 h, +24 h) were low, as compared with DNR, and accounted for 6% and 40% of the total amount of measurable anthracycline, respectively.

Twenty-one patients received identical chemotherapy in a second effort to attain CR. Seven of these patients were studied again during this repeat course of RI therapy. Keeping in mind the considerable interindividual differences in the DNR concentrations in the bone marrow, we compared the uptake of DNR in the bone marrow nucleated cells (at +1 h) for those seven patients during the two successive chemotherapy cycles. It appeared that the DNR values in bone marrow cells were equivalent during both chemotherapy cycles (Fig. 3).

The plasma concentration-time curves of DNR could be described by equation 1, with a goodness of fit > 0.99 in all cases. Table 2 shows the calculated plasma pharmacokinetic parameters of DNR, the calculated plasma AUC at zero to 24 h of DOL, and the calculated WBC AUC at zero to 24 h of DNR. A wide interindividual range of all parameters is apparent from these data. No correlation appeared between the age of the patients and the plasma clearance derived from equation 2.

Table 2:
Pharmacokinetic parameters

Patient	$t_{1/2\alpha}$ (min)	$t_{1/2\beta}$ (min)	k_{el} (h^{-1})	Vd_{area}[a] (l)	Plasma clearance (l/h)	Plasma DNR AUC 0-24 h (nmol.h/ml)	Plasma DOL[b] AUC 0-24 h (nmol.h/ml)	WBC DNR[b] AUC 0-24 h (nmol.h/10^9 cells)
1	1.16	45	14.28	452	416	0.46	5.88	128
2	1.73	52	6.92	466	374	0.31	6.28	148
3	1.24	85	14.63	247	121	1.26	5.79	93
4	1.06	22	12.06	443	847	0.17	5.92	164
5	5.67	116	1.51	595	213	0.67	5.10	370
6	1.10	32	22.68	176	230	0.66	4.10	206
7	2.71	123	1.52	1318	447	0.38	2.33	396
8	4.70	113	1.03	632	232	0.41	3.08	68
9	4.50	717	1.11	1636	95	1.09	8.51	9
10	2.52	113	5.58	504	186	0.92	19.03	34
11	0.75	31	44.48	154	204	0.74	2.49	19
12	0.97	22	5.64	730	1353	0.12	2.56	ND
13	4.04	130	1.61	639	204	0.65	1.01	ND
14	3.19	76	3.22	400	220	0.73	4.00	380
15	1.80	74	5.60	844	473	0.36	2.27	449
16	2.34	48	3.64	474	411	0.35	5.91	138
17	1.94	100	5.08	949	393	0.48	2.17	84
18	NDc	ND	ND	ND	ND	ND	ND	ND
19	ND	ND	ND	ND	ND	ND	ND	ND
20	2.20	118	3.74	710	251	0.38	1.55	62
21	1.19	36	15.76	341	394	0.34	0.89	29
22	1.19	128	8.49	631	204	0.83	36.29	28
23	1.89	52	6.94	974	782	0.19	3.36	549
24	1.16	78	13.02	403	214	0.67	2.54	4
25	1.15	33	15.78	202	256	0.67	2.69	151
26	6.10	185	0.77	1180	266	0.64	6.77	333
27	1.18	123	9.93	546	184	0.82	2.97	309
28	8.44	217	0.91	1347	258	0.58	2.35	300
29	2.52	79	3.96	898	475	0.32	1.26	152
30	6.05	87	1.31	1246	598	0.30	3.49	241
31	1.07	14	15.88	224	650	0.23	3.96	86
32	1.15	18	21.79	217	494	0.36	3.01	63
33	1.21	26	9.61	429	685	0.17	4.89	96
34	7.37	67	1.40	801	496	0.19	2.01	629
35	4.55	92	1.33	734	331	0.46	15.52	71
36	6.19	138	0.87	864	260	0.58	2.64	36
37	6.41	113	1.48	940	346	0.44	2.02	396

a) Apparent distribution volume.
b) The AUC was determined by the trapezoidal method.
c) ND, not done (blood sampling or isolation of WBC was not possible at all time points).

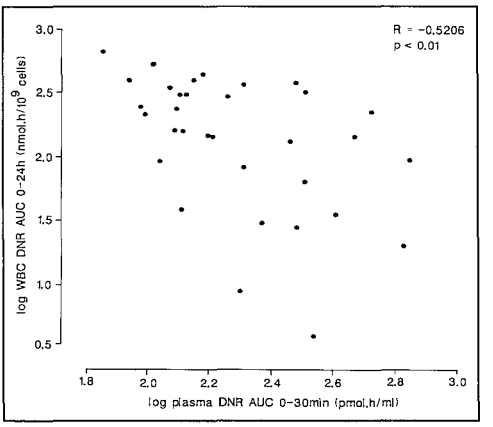

Fig. 4:
Daunorubicin AUC values in plasma and in WBC

Relation between plasma AUC at zero to +30 min and WBC AUC at zero to +24 h (n = 33 cases).

Interrelations between cellular and plasma pharmacokinetic parameters
The plasma pharmacokinetic parameters of DNR were compared with those in WBC. It appeared that the concentrations of DNR in plasma (at any of the time points) did not correlate with DNR concentratios in WBC. However, the integrals of the concentrations (C) of DNR in plasma over t, where C x t indicates AUC, showed negative relationships with AUC values of DNR in WBC. This suggests that C x t may be an important parameter in describing the pharmacokinetics of DNR. The plasma AUC values (zero to 30 min and zero to 60 min) of DNR were inversely correlated with the WBC AUC values at zero to +4 h, and with the WBC AUC values at zero to +24 h of DNR. An example of the inverse relationship between the plasma AUC of DNR and the WBC AUC of DNR is shown in Fig. 4. In accordance with the WBC data, the bone marrow concentrations of DNR (+1 h, +24 h) did not show any correlation with the plasma DNR concentrations either. However, AUC values of DNR in bone marrow nucleated cells could not be determined.

Concerning the interrelations between DNR concentrations in nucleated cells derived from blood (WBC) and bone marrow, a positive correlation between the DNR concentrations in WBC and the DNR concentrations in bone marrow nucleated cells was apparent at +1 h ($p < 0.02$, Fig. 5).

Correlations of pharmacokinetic parameters and tumor load
The DNR levels attained in WBC shortly after administration of the drug showed inverse correlations with the peripheral blast cell numbers at diagnosis. As an example, the inverse relationship between DNR levels in WBC at +10 min and the

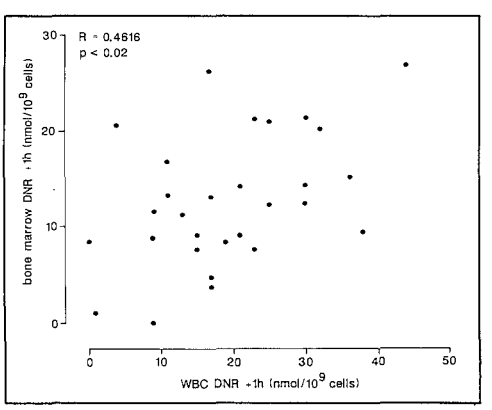

Fig. 5:
Intracellular concentrations of DNR

Relation between DNR concentrations in WBC and bone marrow nucleated cells at +1 h (n = 29 cases).

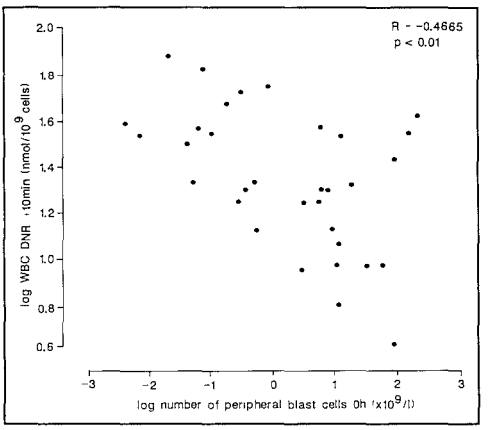

Fig. 6:
Intracellular DNR concentrations in WBC

Relation with the absolute number of peripheral blast cells (n = 33 cases). Peripheral blast cell values were obtained at diagnosis (ie, before treatment). DNR data were obtained at +10 min after IV administration of the drug.

numbers of peripheral blasts (at diagnosis) is shown in Fig. 6 ($p < 0.01$). Thus, the uptake of DNR by WBC was significantly reduced when blast cell numbers in the blood were high.
The percentages of blast cells in the pretreatment bone marrow did not correlate with any of the pharmacokinetic parameters determined. Absolute blast cell numbers in the pretreatment bone marrow were not determined.

Correlations of pharmacokinetic parameters and response to therapy
The relevance of pharmacokinetics of DNR in relation to the outcome of treatment was investigated by comparing the above-mentioned pharmacokinetic parameters among the three response categories of patients, ie, CR, PR, or RD patients.
The amount of DNR present in WBC at any time after administration (illustrated as an example for the +10-minute values in Fig. 7A) had no predictive value for the clinical response to therapy. A lack of relationship was also apparent between the DNR levels in bone marrow nucleated cells at +1 h and at +24 h (Fig.7B) and therapeutic response. Neither did any of the plasma DNR levels (determined day 1) predict for the outcome of treatment. As an example, the +4-hour plasma DNR levels

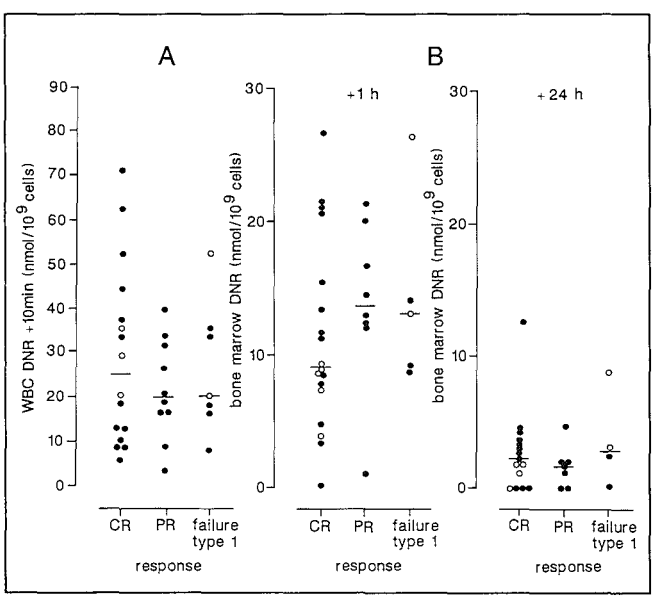

Fig. 7:
Lack of predictive value of the intracellular DNR concentrations in (A) WBC (at +10 min) or (B) bone marrow nucleated cells (at +1 h or at +24 h) for the probability to attain CR or PR.

●, DNR, 45 mg/m^2;
○, DNR, 30 mg/m^2.
Bars indicate median values.

on day 1 are shown in Fig. 8. Although the patients having RD showed a tendency of lower plasma levels of DNR as compared with CR or PR patients, the differences were not statistically significant.
Figure 7 and 8 also show that the patients who received the lower dose schedule of DNR (30 mg/m^2 instead of 45 mg/m^2) generally did not have reduced cellular and plasma DNR levels. The absence of a relationship between DNR measurements and DNR dosages among these patients may be explained by the high interindividual range of the cellular and plasma DNR levels observed in vivo.
Neither in plasma nor in cells were the levels of the metabolite DOL associated with clinical response to therapy (data not shown).
Since several patients (six of 20) underwent a bone marrow transplantation in CR, duration of remission could be determined in 14 nontransplant patients only. In this small group of patients the plasma or cellular pharmacokinetic parameters of DNR had no predicitive value for remission duration.

Discussion

The responsiveness of human AML to cytotoxic drugs is determined by many variables. To better understand the response of individual patients to chemotherapy, individual monitoring of drug levels in AML cells may be useful. Prior investigations that dealt with the cellular metabolism of the other active drug in AML, ara-C at conventional dosages, had failed to demonstrate a clear relation with the clinical response (5-11). Likewise, the analysis of the plasma kinetics of DOX, an anthracycline, in a multicenter study did not provide pharmacokinetic variables indicative for clinical response (12). The major goal of the present study was to investigate whether the in vivo cellular uptake and metabolism of DNR in the target tissue, ie, the bone marrow nucleated cells and WBC, had any relation with the clinical outcome of treatment.
In a group of 37 patients with previously untreated AML who were evaluable for response, the pharmacokinetic parameters of DNR showed considerable interindividual variability. These differences were unlikely to be due to

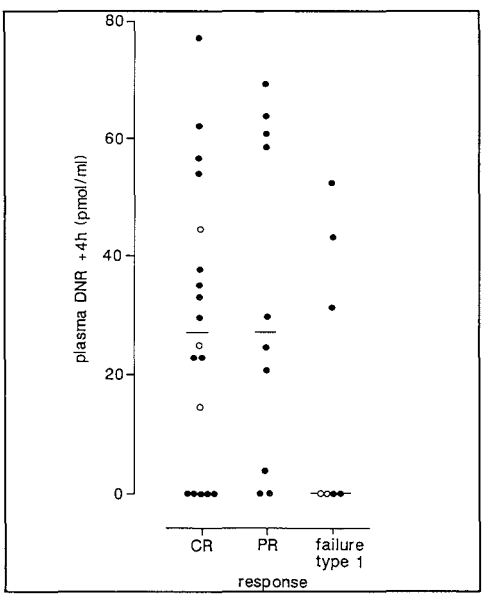

Fig. 8:
Lack of prognostic significance of the plasma DNR levels (at +4 h) for the probability to attain CR or PR

●, DNR, 45 mg/m^2; ○, DNR, 30 mg/m^2. Bars indicate median values.

methodological variability, rather they were indicative of patient heterogeneity. In seven patients in whom we were able to perform pharmacokinetic measurements during two successive chemotherapy cycles (within a time interval of 4 to 6 weeks), the DNR contents of bone marrow nucleated cells (at +1 h) were similar on the two occasions. Certain predetermined pharmacokinetic parameters were analyzed prospectively in the patients, ie,

1) plasma kinetics of DNR,
2) uptake of DNR in WBC at sequential time points, and
3) levels of DNR in bone marrow nucleated cells at +1 h and +24 h after injection of the drug.

The analysis also included the determination of a major metabolite of DNR, ie, DOL. Of 37 patients, 20 achieved CR, ten PR, and seven had RD. The older AML patients who received a somewhat lower dose of DNR (30 mg/m^2) were also included in the analysis. It appeared that the response rate among these patients was comparable to that of younger subjects. In this study, the patients did not receive DNR as a single drug. They were treated with combination chemotherapy containing DNR and ara-C. When interpreting the experimental data from this study, we assumed that

1) the AML cells of the refractory patients were not sensitive to DNR, and
2) the cells of the 20 CRs were most probably sensitive to DNR (we realize that a limited fraction of these patients might have attained CR following treatment with ara-C alone (30)).

Quantification of drug effects in AML on the basis of its plasma pharmacokinetics is of clinical value, in particular when plasma drug levels reflect those in target tissues. However, the biological features of human AML are heterogeneous. Not only the grade of differentiation of the cells and their clonogenic and metabolic activity, but also the sites of the disease in the body may vary considerably among patients. These differences complicate the relationships between plasma drug concentrations and the therapeutic effects. Measurements of the anthracycline DOX and its metabolite doxorubicinol in plasma at +3 h had failed to disclose different drug levels for therapy responsive and resistant patients (12). In our study, plasma DNR levels at a comparable time after administration (+4 h) did not predict for the outcome of RI therapy either. In addition, neither did any of the plasma DOL levels predict for the outcome of treatment, but it is possible that the plasma levels of other metabolites would give more valuable information as has been suggested for the DNR aglycones (31).

It is noteworthy that plasma AUC values of DNR were inversely correlated with AUC values of DNR in WBC, ie, the higher the AUC values of DNR in WBC, the lower the plasma AUC values of DNR. This suggests that the DNR is readily absorbed from plasma by WBC, but that the exchange between WBC and plasma is slow. Our data indicate that DNR levels in WBC and bone marrow nucleated cells are comparable. This is in agreement with the concept of exchangeable pools of AML blast cells, traversing between blood and bone marrow. Therefore, it cannot be expected that plasma concentrations of DNR respresent the levels attained in AML blast cells in tissues. Monitoring of cellular DNR concentratons in blood, rather than plasma DNR concentrations, may yield an indication of the bone marrow concentration of DNR instead. In analogy with this, DeGregorio et al. (16) found that plasma concentrations of DNR and DOL did not correlate with the inhibition of DNA synthesis of myeloblasts.

Theoretically, the plasma pharmacokinetics of DNR and DOL may not only have prognostic meaning for antileukemic response, but also for predicting toxicity. Therefore, we also evaluated a possible relationship between plasma pharmacokinetics of DNR and DOL and the duration of bone marrow depression (expressed as the length of the nadirs [in days] of granulocytes and thrombocytes), but no positive relationship appeared (data not shown). This paper mainly addressed the issue of pharmacokinetic relevance and response. The probability to obtain CR was not associated with high concentrations of DNR in WBC nor in bone marrow nucleated cells. With respect to the cellular DNR pharmacokinetic parameters, no differences between the cytological subgroups of leukemia (M1, M2, M4 and M5) were observed. Thus, no relation was evident between the cellular DNR levels attained in vivo and the clinical response to therapy. On the other hand, the data revealed an inverse relationship between the numbers of circulating blast cells and the DNR levels in WBC. This suggests that the WBC concentrations of DNR attained in vivo are a function of the tumor load, and that lesser amounts of DNR accumulate in the WBC when the circulating blast cell numbers in blood are higher. Our observations would be in agreement with the results of the preclinical investigations of Nooter et al. (17), which indicated that the presence of a high leukemic cell load plays a role in the different pharmacokinetics of DNR in normal and leukemic rats. Previous studies have established that AML patients presenting with higher tumor loads generally have inferior responses to treatment as compared to patients presenting with lower tumor loads (32,33). Therefore, we hypothesize that the effect of the tumor burden on the cellular uptake of DNR in AML patients is a determinant of the clinical responsiveness to treatment.

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In vivo uptake of daunorubicin by acute myeloid leukemia cells measured by flow cytometry

This chapter (in vivo data) combined with parts of **Chapter 3** (in vitro data) are based on investigations published in:

Leukemia 2:511-517

Esther Kokenberg, Pieter Sonneveld, Ruud Delwel, Willemijn Sizoo, Anton Hagenbeek and Bob Löwenberg

In vivo uptake of daunorubicin by acute myeloid leukemia cells (AML) measured by flow cytometry.

Abstract

Monitoring of daunorubicin (DNR) concentrations in leukemic cells in blood and bone marrow in vivo of patients with acute myeloid leukemia may yield insight into the interindividual variations of the clinical response to treatment. DNR concentrations of leukemic cells in blood and bone marrow were measured with flow cytometry in 17 evaluable patients during their first remission induction treatment with DNR and cytosine arabinoside. The results indicate that:

a) DNR fluorescence of leukemic blast cells is intermediate between the smaller lymphocytes and the approximately equally large granulocytes;
b) DNR fluorescence of peripheral blast cells and bone marrow blast cells correlate well ($p<0.001$); and
c) patients reaching complete remission show a tendency of higher DNR fluorescence of leukemic blast cells than do partial responders.

Introduction

Daunorubicin (DNR) is an anthracycline antibiotic effective in the treatment of acute myeloid leukemia (AML) (1-5). Differences in the responsiveness of AML to the cytotoxic effects of DNR among patients are not well understood, and may be related at least in part to differences in metabolism and distribution of the drug. The pharmacokinetics of anthracyclines in plasma have been studied extensively with high-performance liquid chromatography (HPLC) in AML patients (6-8) and have shown that plasma anthracycline levels do not correlate with the clinical response to

the drug (9,10). Since the cytotoxic effect of DNR is correlated with an interaction with DNA (11-13), it is likely that monitoring of the cellular DNR levels will give insight into the factors determining the differences in the responses.

In a previous study, that was based on HPLC measurements, we could not establish a relation between the DNR levels attained in vivo in white blood cells (WBC) or bone marrow cells and the clinical response to therapy (10). However, using HPLC, the DNR concentrations were measured in total WBC or whole bone marrow nucleated cells only and separate measurements of DNR concentrations in subpopulations of cells (i.e., leukemic cells) were not carried out. In vitro studies with cell lines (14-22) employing animal (23) or human (leukemic) cells (24-27) and the data presented in Chap. 3 have shown that the fluorescent property of the anthracyclines allows for measuring of the cellular drug concentrations with flow cytometry. However, data on the anthracycline concentrations attained *in vivo* in human leukemic cells during treatment are scarce (28).

The objective of the investigations presented here was to evaluate the applicability and potential usefulness of flow cytometry for measuring DNR concentrations in leukemic cells in peripheral blood and bone marrow *in vivo*.

Materials and methods

Patients

Seventeen patients with previously untreated AML were enrolled in the study. The criteria of the French-American-British working party were used to classify the cytological diagnosis of AML (29,30). Chromosome banding analysis of nucleated bone marrow cells was performed according to standard techniques (A. Hagemeijer, Dept. of Cell Biology and Genetics, Erasmus University Hospital Dijkzigt, Rotterdam) (31). Patients were treated according to the AML-6 protocol of the EORTC. Elderly patients with AML (i.e., over 65 years) were treated according to the EORTC AML-7 protocol, and after April 1986 according to the AML-9 protocol. Remission induction (RI) chemotherapy included DNR, 45 mg/m^2 (AML-6 protocol) or 30 mg/m^2 (AML-7 and AML-9 protocols) on days 1, 2 and 3 by intravenous (IV) bolus; and ara-C, 200 mg/m^2 (AML-6 and AML-7 protocols) or 100 mg/m^2 (AML-9 protocol) IV on days 1-7. Protocol AML-6 also included vincristine, 1 mg/m^2 IV on day 2. The criteria for evaluating response were those established by the Cancer and Acute Leukemia Group B (32). The spectrum of failures of the induction treatment were classified according to Preisler (33).

Collection and isolation of WBC and bone marrow from AML patients

The optimal conditions for the collection of WBC and bone marrow nucleated cells, as well as the methodology of drug analysis by HPLC have been described in detail before (34). In brief, blood samples (10 ml) were drawn into tubes with EDTA immediately before (blank sample), at +1 h (n = 17 patients) and at +24 h (n = 14 patients) after the first dose of DNR during the first cycle of RI treatment. The samples were cooled to 4 °C, and after centrifugation (450 x g, 10 min, 4 °C) the plasma was removed. The buffy coat was depleted of erythrocytes by cold hypotonic lysis (34) and the WBC were resuspended in PBS, counted and kept at 4 °C until flow cytometric analysis. Part of the WBC suspension was stored at -20 °C until further processing by HPLC.

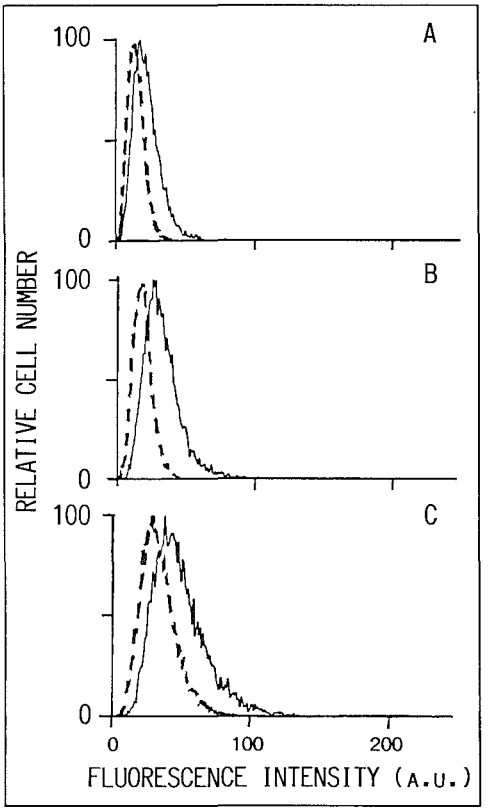

Fig. 1:
Frequency distribution of the fluorescence intensity of human nucleated blood cells in vivo +1 h after administration of DNR

The cell types were identified by forward and perpendicular light scatter;
panel A: lymphocytes;
panel B: granulocytes;
panel C: leukemic blast cells.
The dotted lines (blanks) represent unstained cells, specific for each cell type.

Bone marrow cells were collected from the patients at the same time intervals as well i.e., immediately before, at +1 h (n = 17 patients) and at +24 h (n = 14 patients) after the onset of DNR infusion. Bone marrow (2-10 ml) was aspirated from the posterior iliac crest under local anesthesia and put into tubes containing EDTA. The samples were cooled to 4 °C and after lysis of erythroid cells the nucleated cells were resuspended, counted and stored or used for flow cytometry.
All patients had given informed consent to donate blood or bone marrow.

Measurement of cellular DNR content by flow cytometry
The DNR content of individual cells was measured with flow cytometry as described in Chapter 3. Briefly, cells from the cell suspension passed the 488 nm laser beam running at 0.4 W of a Becton-Dickinson (Mountain View, CA) fluorescence activated cell sorter (FACS 440). The forward and perpendicular light scatters from the cells were used to distinguish the different cell types in WBC or in bone marrow (35). Dead cells among the WBC and bone marrow populations were excluded from analysis by elevating the threshold of the forward light scatter. For each sample, at least 10×10^3 cells were analysed at a flow rate of $1\text{-}2 \times 10^3$ cells/s.
Data analysis was performed using a Hewlett Packard 68B system. Fluorescent profiles were recorded as histograms of fluorescence intensity versus cell frequency. A representative example is shown in Fig. 1. The relative value of DNR fluorescence was obtained for each population by calculating the mean of the fluorescence distribution. Mean fluorescence was expressed in arbitrary units, taking into account the blank samples that were used to correct for background fluorescence (subtraction of blank values).

Table 1:

Clinical characteristics of patients

Patient	Sex	Age	Cytogenetic Karyotype[a]	Prognostic category[f]	FAB classif-ication	WBC ($\times 10^9$ /l)[a]	Blasts in blood (%)[a]	Blasts in blood ($\times 10^9$ /l)a	Blasts in bone marrow (%)[a]	Dose of DNR (mg /m^2)	Dose of DNR (mg)	Number of RI courses	Outcome RI therapy
1	F	56	NN[e]	I	M2	3.3	5	0.17	36	45	75	1	CR
2[b]	M	23	AN t(8;21)	F	M2	15.0	39	5.85	57	45	80	1	CR
3	M	58	AA +8	U	M2	0.417	0	0	32	45	90	1	CR
4[d]	F	67	AN +8	U	M5	0.590	10	0.06	94	30	50	1	CR
5	M	17	AN t(9;11)	U	M4	4.1	39	1.60	63	45	90	1	CR
6	M	56	AN -5,7q-	U	M4	1.736	4	0.07	77	45	85	2	CR
7	F	62	NN	I	M5	13.4	44	5.90	84	45	90	2	CR
8	M	58	NN	I	M1	20.0	62	12.4	85	45	75	2	CR
9	M	37	AN +4	U	M1	0.7	0	0	93	45	100	2	CR
10[d]	F	64	NN	I	M2	0.799	6	0.05	80	30	50	2	CR
11	M	33	AA 16q-	U	M5	17.6	54	9.50	94	45	80	2	PR
12	M	46	NN	I	M5	45.0	40	18.0	71	45	80	2	PR
13	M	43	AA inv(16)	F	M5	60.0	70	42.0	84	45	95	2	PR
14	M	44	AN 7q-	U	M4	2.0	5	0.10	78	45	95	1	NR[g]
15[c]	M	70	AN hypodiploid	U	M4	33.0	15	0.50	43	30	60	1	NR
16[b]	F	42	AA pseudodiploid	U	M4	4.0	73	2.92	69	45	80	1	NR
17[d]	M	68	AN +8	U	M2	1.49	3	0.04	56	30	60	1	NR

a: At diagnosis (before therapy).

b: Protocol variations: patients who were suspected of having an antecedent hematologic disorder were treated according to the AML-6 protocol, but received DNR during two instead of three days.

c: Patient (> 65 years) who was treated according to the AML-7 protocol for elderly patients (see: Materials and methods).

d: Patients (> 60 years) who were treated according to the AML-9 protocol for elderly patients (see: Materials and methods).

e: NN: diploid karyotype (100% normal cells); AN: mixture of abnormal and normal cells; AA: 100% abnormal cells.

f: F: favorable; I: intermediate; U: unfavorable (adapted from MJ Keating et al., 1987, Leuk Res 11, 119-133).

g: NR: no response patients, failure type 1.

Analysis of cellular DNR and daunorubicinol by HPLC

Concentrations of DNR and its major metabolite daunorubicinol (DOL) were determined using straight-phase HPLC analysis as described previously (10,34,36). To avoid heterogeneous drug recoveries from patient samples with different cell concentrations, we standardized the leukocyte and bone marrow samples with PBS at final concentrations between 10^9 to 10×10^9 cells/l before entering the extraction procedure. For drug quantification, doxorubicin was used as an internal standard.

Statistics

The Spearman test was used for calculating correlation coefficients.

Results

Clincal data

Table 1 lists the clinical and treatment characteristics of 17 patients with AML, who were included in the clinical study. The results of RI therapy were as follows: CR 10/17 (59%), PR 3/17 (18%), and resistant disease (NR) (failure type 1) in 4/17 (23%). Four aged AML patients among these individuals received the relatively lower dose of DNR (30 mg/m^2). The outcome of RI therapy of those 4 elderly patients was: CR 2/4 and NR 2/4.

The cytogenetic data have been listed in Table 1. An euploid karyotype (NN) was found in 5/17 patients. Eight of the 12 patients with an abnormal karyotype had a mozaicism of abnormal and normal cells (AN), while 4 patients had 100% abnormal cells (AA). The cytogenetic findings were classified in prognostic cytogenetic categories according to Keating et al. (37). [He analysed the influence of karyotype on response rate, remission duration and survival. He concluded that inv(16) and t(8;21) were favorable prognostic categories (more common in young patients), diploid, t(15;17), and 45,X,-Y had intermediate prognosis, and all other categories were unfavorable prognostic groups (more common in older patients)]. As indicated in Table 1 favorable karyotypes were found in 2/17 patients, intermediate karyotypes in 5/17 patients and unfavorable karyotypes in 10/17 patients. Correlation of the prognostic categories with response show that 4/4 patients with resistant disease had unfavorable karyotypes. The CR and PR patients did not show any correlation with the cytogenetic prognostic categories.

Cellular DNR concentrations in vivo

As flow cytometry does not distinguish between the parent drug (DNR) and the metabolite DOL but measures overall fluorescence, the patient WBC and bone marrow samples were also assayed with HPLC for cellular content of DNR and DOL. HPLC measured no demonstrable DOL metabolite in the cells in 9/17 patients. In the other patients the metabolite was never more than 15% (at +1 h) of the total amount of measurable anthracycline and at +24 h the cellular concentrations of DOL had decreased to minimal or undetectable levels. Therefore, the contribution of the metabolite DOL was not taken into consideration in the following analyses.

The *in vivo* relationships between fluorescence and HPLC values correlated somewhat less well than those for the *in vitro* incubations (Chapter 3), but again a positive relationship was obvious between the cellular DNR concentrations in WBC and bone marrow nucleated cells at +1 h and at +24 h as determined by HPLC versus flow cytometry ($n = 55$, $R = 0.5695$, $p < 0.001$) (data not shown). The DNR fluorescent signals obtained from WBC and bone marrow cells at +1 h were easily detectable with flow cytometry. At +24 h the cellular DNR fluorescent signals had decreased to low levels in all patients and approached the detection limit, which was in concordance with the DNR concentrations derived from HPLC analysis.

Cellular DNR concentrations in subpopulations of cells

All the above reported measurements were done with whole WBC or bone marrow nucleated cells. We then investigated the DNR concentrations in subpopulations of

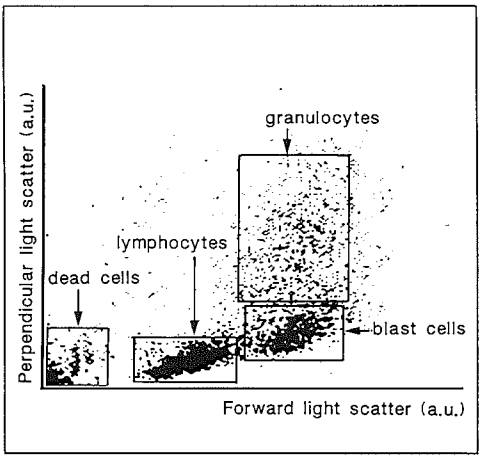

Fig.2:
Dot display of forward light scatter (FLS) versus perpendicular light scatter (PLS) of nucleated bone marrow cells from a patient with AML

The erythrocytes and normoblasts were lysed. Each dot represents one cell. The combination of FLS and PLS reveals distinct cell types which were identified by sorting the cells followed by cytospin preparation, staining and microscopic examination.

cells, i.e., in particular in the blast cell fraction. Since prior treatment of a blood or bone marrow sample with lysis buffer had removed the erythrocytes and the normoblasts almost completely, the lymphocytic, myeloid and blast cell populations could be clearly distinguished on the basis of forward and perpendicular light scatter properties (34) as indicated in Fig. 2. The cell types were identified by sorting the cells followed by cytospin preparation, staining and microscopic examination. By placing windows in the forward-perpendicular light scatter plane, the mean fluorescence of these distinguishable subpopulations was measured.

Separate measurements of DNR in subpopulations of cells showed a good correlation between DNR concentrations in peripheral blast cells and in bone marrow blast cells at +1 h ($p < 0.001$, Fig. 3). However, the blast cell DNR concentrations in the bone marrow were lower than the comparative values of blast cells in the blood. In several cases blood and bone marrow samples of the same patient contained enough non-leukemic cells for measurement of DNR concentrations. Good correlations were found between DNR concentrations in normal blood and bone marrow cell types at +1 h (lymphocytes: n=9, R=0.6958, $p < 0.05$; granulocytes: n=8, R=0.9286, $p < 0.01$). Again, the cellular DNR concentrations in the bone marrow were lower than the comparative values in the blood cells (data not shown).

Flow cytometric measurements showed that different cell types expressed different levels of DNR-associated fluorescence. As is shown in Fig. 4 (at +1 h, left panels), DNR fluorescence of blast cells exceeded that of lymphocytes, while granulocytes

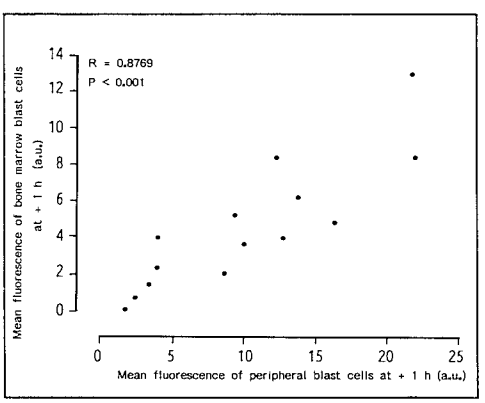

Fig. 3:
Relationship between DNR fluorescence of peripheral blast cells and bone marrow blast cells at +1 h (n = 14 patients on chemotherapy)

DNR fluorescence was assessed for cells in the forward-perpendicular blast window.

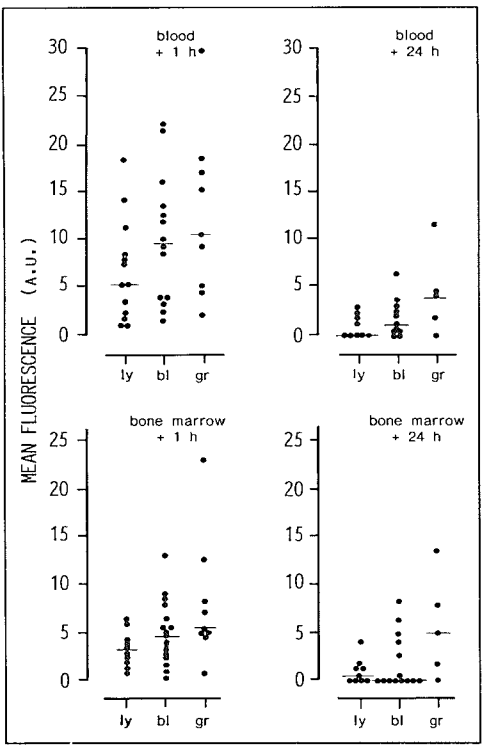

Fig. 4:
Cellular DNR fluorescence in subpopulations of hematopoietic cells in vivo

The subpopulations were distinguished according to their light scatter properties and separately analysed. Upper panels, blood at +1 h and at +24 h after start of DNR infusion. Lower panels, bone marrow at +1 h and at +24 h.
ly = lymphocytes;
bl = blast cells;
gr = granulocytes.
Bars indicate median values.

concentrated relatively more drug than the other cell types. At +24 h (Fig. 4, right panels) the same trends in DNR content were observed. In eight patients, in whom sufficient numbers of these three subpopulations of cells (i.e., lymphocytes, granulocytes and blast cells) were distinguishable in the blood at +1 h, the value of the mean DNR fluorescence of granulocytes was set at 100%. The relative mean DNR fluorescence of lymphocytic and blast cells were 53% and 88%, respectively (Table 2). Thus, the uptake of DNR by leukemic blast cells was intermediate between the smaller lymphocytes and the approximately equally large granulocytes.

Table 2:
Relative DNR fluorescence in subpopulations of hematopoietic cells in vivo

Fluorescence	Lymphocytes	Blast cells	Granulocytes
arbitrary units	7.6 ± 5.63	11.0 ± 6.29	13.5 ± 8.84
relative %	53 ± 16.1	88 ± 15.2	100

Cellular DNR fluorescence was measured in three cell types in blood samples of AML patients at +1 h after start of DNR chemotherapy (n = 8 cases). Cell types were distinguished according to their light scatter properties. Fluorescence of the granulocytes was set at 100%. Values are means ± SD.

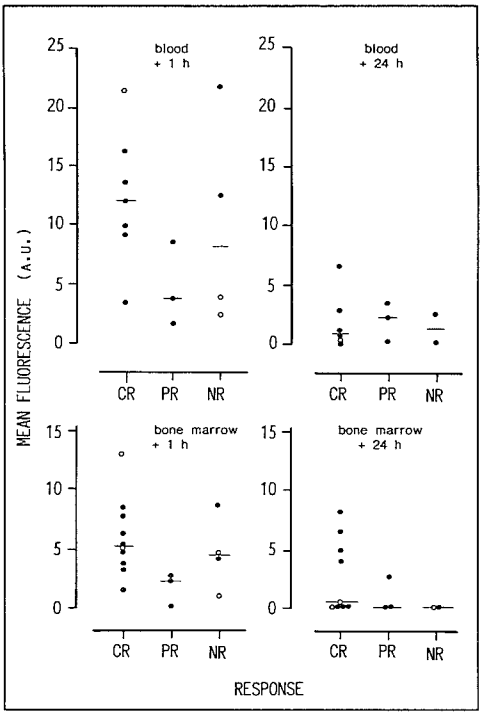

Fig. 5:
DNR fluorescence of leukemic blast cells in blood (upper panels, at +1 h and at +24 h) or bone marrow (lower panels, at +1 h and at +24 h) in relation to the response to treatment

● = DNR 45 mg/m^2;
○ = DNR 30 mg/m^2.
Bars indicate median values.

Relevance of DNR concentrations in blast cells for the response to treatment
The relevance of DNR concentrations in blast cells in relation to the outcome of treatment was investigated by comparing the DNR concentrations in blast cells among the three response categories of patients, i.e., CR, PR or NR patients. The amount of DNR present in the bone marrow and the circulating blast cells at +1 h showed large interindividual variations (Fig. 5, left panels). At +24 h (Fig. 5, right panels) the DNR concentrations in blast cells had decreased to low or undetectable levels in most of the patients. Although the CR patients showed a tendency of higher DNR levels in blast cells at +1 h as compared to PR patients, no statistically significant relationship between successful RI and blast cell DNR concentrations could be derived from the limited number of data available thus far. It is noteworthy that, in analogy with previous reports (38,39), the tumor load parameter (i.e., numbers of circulating blast cells at diagnosis) appeared again to correlate with the probability to obtain CR, although patient numbers are small. Thus CR patients had

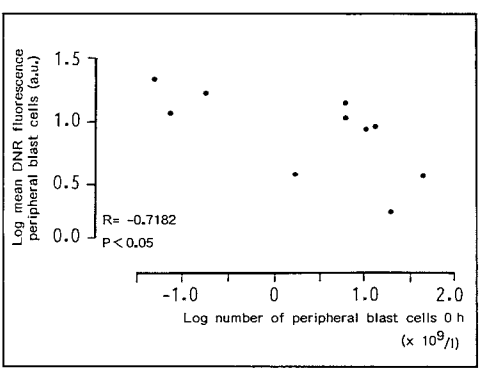

Fig. 6:
Daunorubicin fluorescence of peripheral blast cells: negative relation with the absolute number of peripheral blast cells (CR + PR patients, n = 10 cases)

Peripheral blast cell values are those at diagnosis (i.e., prior to treatment). DNR data were obtained at +1 hr after IV administration of the drug.

somewhat lower blast cell counts as compared to the PR patients (Table 1), and the DNR levels attained in peripheral blast cells at +1 h in CR patients were greater than in PR cases. It is not surprising therefore that the peripheral blast cell numbers at diagnosis showed an inverse relationship with the DNR concentrations in peripheral blast cells at +1 h in CR and PR patients ($p < 0.05$, Fig. 6). This suggests that the uptake of DNR by peripheral blast cells is progressively reduced as a function of rising blast cell numbers in the blood.

The group of NR patients could not be distinguished from the CR patients when DNR blast cell levels (Fig. 5) or numbers of peripheral blast cells at diagnosis (Table 1) were considered.

Discussion

The responsiveness of human AML to cytotoxic drugs is determined by many variables. Monitoring of drug levels in AML cells offers another potentially useful approach for better understanding the response of individual patients to chemotherapy.

Since the hydroxy metabolite of DNR (DOL) has the same fluorescent properties as the parent drug (40,41), flow cytometry detects both compounds and cannot discriminate between the parent drug and the metabolite. We used the HPLC technique to measure the DOL concentrations of the cell samples. Only minimal amounts of cellular DOL were detected by HPLC at +1 h and at +24 h, so that DOL contaminated the DNR measurements to a limited extent only. This is in accordance with the results from our previous studies dealing with DNR and DOL uptakes by the cells (10,34). Those had indicated that the cellular DOL concentrations were consistently lower than the cellular DNR concentrations.

The detection limit of flow cytometry for minimal DNR concentrations is comparable to that of HPLC. Although the fluorescent signals obtained from cells incubated in vitro with various concentrations of DNR were strong and easily detectable (Chapter 3), the fluorescence of cells exposed in vivo to DNR was relatively weak. However, HPLC measurements confirmed the relatively low in vivo cellular DNR concentrations, so that generally the power of both cellular DNR assays was similar.

The data of these investigations show that the DNR levels in peripheral blast cells and bone marrow blast cells at +1 h correlated well. This is in agreement with the concept of exchangeable pools of AML blast cells, traversing between blood and bone marrow. In analogy with this, Sahar et al. (27) had reported identical DNR uptakes in vitro by leukemic blast cells from peripheral blood and bone marrow, based upon flow cytometric analyses.

It appeared that different types of human hematopoietic cells expressed different levels of cellular DNR fluorescence. A progressively greater DNR-associated fluorescence was evident for lymphocytes, leukemic blast cells and granulocytes, respectively. This trend is suggested, at least in part, to correlate with increased cell size. The fact that a part of intracellular DNR is bound to the nucleus (16,42) and the rest remains in the cytoplasm, could explain why cells with a large volume (more cytoplasm) would generally show a greater DNR fluorescence than those of small size. This relationship is not likely to be strict as it appeared that granulocytes, being of approximately equivalent size as compared to blasts, concentrated more

drug. This is most probably related to the lysosomal granules present in the cytoplasm of granulocytes (43) that have been shown to bind DNR (14).

In a previous study (10) we reported that the probability to obtain a CR was not associated with the cellular DNR levels attained in vivo. This study, however, was done with total WBC or whole bone marrow nucleated cells. We now examined with flow cytometry as to whether the DNR concentrations in the blast cell subset selectively could be more predictive for response. It appeared that the CR patients could be distinguished from PR patients on the basis of a trend of higher DNR levels in blast cells. These observations are of interest in view of the fact that the CR and PR patients could also be distinguished by a previously described tumor load parameter (38,39) i.e., blast cell counts at diagnosis with high circulating blast cell numbers being associated with poor treatment results. Taking these data together an inverse relationship became apparent between the numbers of circulating blast cells and the DNR levels in peripheral blast cells in the group of CR and PR patients. Assuming then that blast cell concentrations of DNR attained in vivo are likely to be a function of the tumor load, we propose that lesser amounts of DNR accumulate in the blast cells when the circulating blast cell numbers in patients are higher, and thus these lower concentrations are less effective in inducing CR. This may represent one biological variable of the clinical response to cytotoxic treatment. Accordingly we hypothesize that CR patients and PR patients may belong to a similar spectrum of varying drug sensitivities, but the fact that PR cases apparently present with a higher tumor load may sometimes explain their inferior response to treatment as compared to the complete responders. On the other hand, the fact that the NR patients could not be distinguished from the CR patients with respect to the DNR levels in blast cells or the number of circulating blast cells would be consistent with the concept of individual drug sensitivity. In the NR cases an intrinsic factor, i.e., intrinsic drug resistance, would most likely be responsible for the poor treatment results (44).

It is also possible that other variables, such as certain non random cytogenetic abnormalities, have a strong role in the clinical response of AML patients to cytotoxic drugs. The small seize of our series does not lend itself for an adequate analysis of clinical, hematological or cytogenetic prognostic variables. The cytogenetic findings listed in our study and their relationships with age, sex and CR rate have been considered in view of the results of Keating et al.(37). The latter investigations have indicated that abnormalities in chromosomes have been noted in approximately half of the AML patients. Somewhat more frequent cytogenetic abnormalities were found in patients less than 50 years of age and in male patients. The resistant disease patients in our series all demonstrated the unfavorable karyotypes according to Keating et al. A similar correlation was not apparent for the CR and PR patients, but this may be due to the small number of patients in our study.

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CHAPTER 7

General discussion

Introduction

Knowledge of pharmacokinetics has become important for the effective use of several classes of drugs, such as antibiotics, anticonvultants, and cardiovascular agents. In the field of cancer chemotherapy, the approach of optimizing treatment in clinical situations by using pharmacokinetic information is not generally established. This has been due, in part, to the lack of assay methods for detecting low concentrations of these extremely chemically reactive compounds which may express biological activity at low levels. However, during the last few years the application of e.g., high-performance liquid chromatography (HPLC) techniques to the study of these drugs has yielded specific and rapid assay methods. Large interindividual variations have been found in the absorption, distribution and elimination of many antineoplastic agents. So far, in the case of daunorubicin (DNR) treatment of patients with acute myeloid leukemia (AML) no attention has been paid to the variability of the cellular pharmacokinetics of DNR among subjects and the influence of the disease state, which may vary significantly among patients. Therefore, in this study an attempt has been made to measure cellular DNR concentrations in AML patients during treatment and to interpret the significance of the observed drug concentrations in the context of the clinical response to treatment. Thus, the results of this study may answer the question whether there is a place for pharmacokinetics in the treatment of patients with AML.

Major conclusions

Major conclusions from this study were:

1. Flow cytometry has been shown to be an equally reliable method for measuring cellular anthracycline concentrations as HPLC. An advantage of HPLC is the possibility to determine the concentrations of the parent drug (DNR) and its main metabolite daunorubicinol (DOL) separately. The attractivity of flow cytometry is the possibility to relate measurements of anthracycline concentrations to subpopulations of hematopoietic cells (i.e., leukemic cells vs normal cells).
2. The study of cellular pharmacokinetics of DNR in AML patients has shown, that the white blood cells (WBC) accumulated the drug. Thereafter, the WBC-plasma DNR ratio increased due to the shorter half-time of DNR in plasma than in WBC. In addition, the DNR concentrations in WBC and in bone marrow cells correlated well at +1 h after DNR injection, although the DNR concentrations in the bone marrow cells were consistently lower than the comparative values of DNR in WBC.
3. With respect to the influence of the pharmacokinetics of DNR on the therapeutic effects, the HPLC study revealed that none of the plasma DNR and DOL pharmacokinetic parameters (i.e., concentration and area under the curve)

predicted for outcome of treatment. In addition, the probability to obtain a complete remission (CR) was not associated with the cellular DNR levels attained in vivo.

4. Using flow cytometry, however, it appeared that the CR patients could be distinguished from PR patients on the basis of higher DNR levels in leukemic blast cells. This suggests that the measurements of DNR concentrations in the *leukemic blast cell subset* could be more predictive for clinical response than measurements of total WBC or whole bone marrow cells.
5. It has already been established that CR and partial response (PR) patients could be distinguished by a patient characteristic, i.e., a tumor load parameter (1,2), defined by the blast cell counts at diagnosis: high circulating blast cell numbers being associated with poor treatment results. As explanation for high tumor load determining an unfavourable treatment result one may consider altered cell kinetics or pharmacokinetic differences leading to a deviate pattern of drug handling and subsequently less cell kill. The results of our study showed an influence of the tumor load on the pharmacokinetics of DNR: an inverse relationship became apparent between the numbers of circulating blast cells and the DNR levels in these cells in the group of CR and PR patients. Thus, these data appear to shed light on the mechanisms of poor response to treatment in patients with high tumor load as they attain generally significantly lower cellular drug concentrations. These lower concentrations can be regarded as representing one of the biological variables responsible for the poor treatment results attained in the group of PR patients. On the other hand, the resistant disease (RD) patients could not be distinguished from the CR patients with respect to the DNR levels in blast cells or the number of circulating blast cells.

Major discrepancies

The following issues need further discussion:

1. The observed relationship between the cellular DNR concentrations in WBC and bone marrow cells at +1 h after DNR injection may indicate a rapid penetration of free DNR into both cell compartments. Another explanation is the possibility of AML blast cells loaded with DNR traversing from blood to bone marrow and reversely as has been demonstrated by studying the distribution kinetics of untreated leukemia cells in the BNML acute leukemia rat (3). Our study of the distribution kinetics of the DNR-carrying BNML cells in the rat (Chapter 4) showed that the DNR concentration attained in bone marrow following administration of these cells to the rat was of the same order as that after IV bolus administration (free DNR) of the same dose. In addition, we could not demonstrate any loss of DNR from these cells during circulation. This was evident from the lack of detectable plasma DNR concentrations. These data support the hypothesis that after IV administration of DNR the drug is taken up by the AML blast cells (and other WBC) and transported to the bone marrow. The study of cellular pharmacokinetics of DNR in AML patients showed that the cellular DNR concentrations in peripheral blast cells and in bone marrow cells correlated well but the cellular DNR concentrations in the bone marrow were consistently lower than those in peripheral blast cells.
2. The quantification of plasma concentrations of DNR and its active metabolite DOL does not predict their antitumor activity. This situation is contrary to what has been observed from traditional drug level monitoring, where blood or plasma levels of drugs are representative for their therapeutic efficiency and thus can be

used for optimization of the dose and treatment schedules, e.g., in the treatment of epilepsy and infectious diseases. However, the cytotoxic effects of DNR, the metabolism of DNR, and the mechanisms of resistance to DNR are mainly intracellular processes. Differences in drug handling between leukemic blast cells may explain the difficulties in using plasma DNR concentrations as a means of predicting therapeutic efficiency.

In contrast to the differences in therapeutic effect of DNR in AML patients, the toxic effects of a given dose of DNR to normal tissues show less variability. Nausea, vomiting, alopecia and myelosuppression occur in all patients to some degree. Theoretically, one might therefore expect a better correlation between blood or plasma levels of DNR and toxicity to DNR. The results of our study, however, did not disclose a correlation between plasma pharmacokinetics of DNR and the duration of the myelosuppression, which was regarded as a parameter of toxicity. This lack of correlation suggests more complicated relationships between plasma DNR levels and toxicity. For this purpose the routine plasma DNR level monitoring in patients with AML does not appear useful.

3. Although it appeared possible to distinguish CR patients from PR patients on the basis of a trend of higher DNR levels in leukemic blast cells, this difference was not statistically significant. One reason for this may be the limited number of cases on which this study was based. The lack of a strong correlation between response and cellular DNR concentrations may reflect the complex relationship between tissue drug concentrations and therapeutic effect in vivo. In vitro studies, testing the sensitivity of AML cells to DNR, have shown that the survival of blast cells following exposure to DNR decreases with increasing cellular drug concentrations (6-9), but relatively little is known about the factors that influence the relationship between drug concentrations and therapeutic effect in man. It must be kept in mind that in determining drug efficacy, the drug (or active metabolite) tissue concentrations are only one factor of importance. Another important determinant is for instance the sensitivity of a given cell to that particular agent. The sensitivity depends on several factors, including cell kinetics and intrinsic resistance mechanisms. Thirdly, a complicating factor in the interpretation of the complex relationship between tissue drug concentrations and therapeutic effect in vivo may be the time that passed between the drug concentrations determined during the first 24 hours of the remission induction treatment and the therapeutic DNR effect assessed about two to three weeks later. It is likely that other approaches will be needed to define the relationship between tissue drug concentrations and therapeutic response, including cell kinetic phenomena and cell kill studies.

   A fourth factor which must be considered is the common practice of employing combinations of antineoplastic agents in AML treatment (i.e., DNR and ara-C). This implies that clinical outcome also depends on the efficacy of ara-C which in turn will obscure direct relationships between DNR pharmacokinetics and treatment results. Simultaneously determined pharmacokinetics of ara-C in AML patients during treatment with combination therapy may be helpful in providing insight into these effects.
4. The RD patients could not be distinguished from the CR patients with respect to the DNR levels in the blast cells. An explanation is that during a course with antineoplastic drugs, a large fraction of the blast cells may be killed, but the surviving cells can regrow. The DNR concentration of this subpopulation of

biologically important blasts with self-renewal capacity may not be reflected in the blast population as a whole and this might explain the lack of a discriminant effect in CR versus RD patients. Thus, it may be useful to determine the DNR concentrations in subpopulations of leukemic blast cells and to identify the occurrence of resistant blast cells, i.e., cells which display the so-called multidrug resistant phenotype (10-13). Such cells have to be subject of studies directed to the mechanism of resistance to DNR treatment, with the possible result of circumvention of DNR resistance in AML patients.

Future prospects

If therapy is totally ineffective or so successful that all patients are cured, then the analysis of prognostic variables is a waste of time. Considering the current state of leukemia treatment, namely that therapy is curative in certain patients but it fails in others, the analysis of possible prognostic factors, including the clinical pharmacology of DNR, is important. With respect to the question whether in the future there may be a place for pharmacokinetics in the treatment of patients with AML, the answer is probably yes. However, pharmacokinetic studies should not be performed in isolation, but be integrated with other techniques. For instance, a valuable interpretation of pharmacokinetics might be done in association with studies on intrinsic cell resistance (14-16). In these latter studies a biochemical marker (mdr1 mRNA) was compared with in vitro pharmacokinetic studies of DNR, in order to demonstrate the presence of MDR in AML cells. It was shown that mdr1 overexpression in AML cells was associated with a decreased drug accumulation in vitro that could be restored by cyclosporin-A. In addition, with the use of a clonogenic assay (17) combined with drug uptake studies, it was shown that cyclosporin-A not only restored the intracellular DNR accumulation but also the DNR sensitivity. These in vitro data indicate that clinical trials with cytotoxic drugs in combination with inhibitors of the P-glycoprotein drug pump are warranted. In the future therefore, it is likely that DNR pharmacokinetic studies in AML patients will support such clinical trials aimed at the treatment of drug resistance in AML patients.

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Summary

Since its introduction in the late 1960s daunorubicin (DNR), an anthracycline antibiotic, has become the major drug effective in the treatment of acute myeloid leukemia (AML). Combination therapy with cytarabine (ara-C) is commonly used for induction of complete remission (CR) in AML, with CR rates up to 60-80%. Despite the advances in the treatment of AML, CR rates and their duration vary significantly among patients. Certain patients may be cured, others do not respond to therapy and have an extremely grave prognosis. These differences may be related at least in part to differences in metabolism and distribution of the drug. The investigations described in this thesis deal with the identification of pharmacokinetic variables of DNR that may give insight into the mechanisms which are responsible for individual differences of clinical response of AML patients to therapy.

In **chapter 1** a short overview is given of the pharmacology of DNR and of what is known about DNR plasma pharmacokinetic parameters in relation to response. This discussion results in the suggestion that instead of plasma DNR levels, measurements of cellular DNR concentrations may be more useful for clinical correlations.

In **chapter 2** the evaluation of certain factors which directly affect the quantification of cellular DNR using high-performance liquid chromatography (HPLC) is described. It appeared, that the best recovery of leukocytes from the blood is attained when cold hypotonic lysis is used. It could be performed at 0-4 °C, with excellent separation of white and red blood cells and 100% recovery of all nucleated cells. The best extraction recoveries of DNR, daunorubicinol (DOL), and doxorubicin from human leukocytes is attained with high-volume extraction (5 ml extraction mixture). However, the recoveries remained dependent on the cellularity of the cell sample. To account for these cellularity-related recoveries a correction factor was needed to calculate the DNR and DOL contents of the leukocytes. In addition, the WBC and bone marrow samples of AML patients were standardized with PBS at final concentrations between 10^9 to 10×10^9 cells/l before entering the extraction procedure. The obtained data were applied to measure plasma and cellular DNR and DOL levels in seven patients with AML during their first course of remission induaction (RI) therapy. The cellular DNR levels varied over a broad range and did not correlate with plasma pharmacokinetics.

In **chapter 3** the applicability of flow cytometry for measuring cellular DNR concentrations was validated with the use of a conventional HPLC method. It appeared, that the DNR related fluorescence signals obtained from cells and nuclei measured with flow cytometry correlated well with the absolute amount of DNR (assessed with HPLC) in the cells as well as the nuclei. In addition, it was shown that the nuclear DNR associated fluorescence involves DNR bound to the nucleus rather than free DNR. These data support the conclusion that flow cytometric measurements of cellular DNR represent the total (i.e., cytoplasm and nuclear bound)

DNR content of cells. Flow cytometry was subsequently applied in the study of the in vivo cellular pharmacokinetics of DNR in AML, described in chapter 6.

In **chapter 4** the capability of nucleated bone marrow cells and leukemic cells (Brown Norway acute myeloid leukemia, BNML) to transport DNR to target tissues in the body was investigated in the rat. The cells in vitro took up DNR according to a linear pattern. When these DNR loaded cells were infused in the rat, dose dependent distribution kinetics were observed. Compared to IV injection of the same dosage, cell-bound DNR leads to higher DNR concentrations in the liver and in the spleen, while equal DNR levels were attained in bone marrow. Lower DNR concentrations were observed in cardiac tissue. Hence, DNR entrapped into bone marrow and leukemia cells follows different kinetics than does free DNR in plasma.

In **chapter 5** the study of plasma and cellular pharmacokinetics of DNR measured with HPLC was applied to and evaluated in 37 AML patients during their first RI treatment with DNR and ara-C. Plasma DNR concentrations did not correlate with DNR concentrations in bone marrow nucleated cells. However, plasma area under the curve (AUC) values of DNR correlated inversely with AUC values of DNR in white blood cells (WBC). In addition, concentrations of DNR in WBC correlated positively with DNR concentrations in bone marrow nucleated cells. None of the plasma levels or WBC and bone marrow concentrations had predictive value for clinical response to therapy. Notably, the concentrations of DNR in WBC showed a negative correlation with the numbers of peripheral blast cells at diagnosis, suggesting that the effective DNR concentrations achieved intracellularly are mainly a function of the tumor load. This suggests that lesser amounts of DNR accumulate intracellularly when AML cell numbers in blood are higher.

In **chapter 6** cellular DNR concentrations of leukemic cells in blood and bone marrow were measured with flow cytometry. The data were obtained in 17 AML patients during their first RI treatment with DNR and ara-C. The results indicate that DNR fluorescence of leukemic blast cells is intermediate between the smaller lymphocytes and the approximately equally large granulocytes. It was also shown, that the DNR fluorescence of peripheral blast cells and bone marrow blast cells correlated well. With respect to the response to treatment, it appeared that the CR patients could be distinguished from partial response (PR) patients on the basis of a trend of higher DNR levels in blast cells. The CR and PR patients could also be distinguished by the number of circulating blast cells and an inverse relationship became apparent between the numbers of circulating blast cells and the DNR levels in these cells. On the other hand, resistant disease patients could not be distinguished from the CR patients on the basis of DNR pharmacokinetics or tumor load parameters, suggesting that most likely intrinsic drug resistance is responsible for the poor treatment results in these cases.

Finally, in **chapter 7** the results of this thesis are discussed against the background of the question whether there is a place for pharmacokinetics in the treatment of patients with AML.

Samenvatting

Sinds eind jaren zestig neemt daunorubicine (DNR), een cytostaticum uit de anthracycline antibioticum groep, een belangrijke plaats in bij de behandeling van patiënten met acute myeloide leukemie (AML). Bij het induceren van een complete remissie wordt meestal gebruik gemaakt van een combinatietherapie bestaande uit DNR en cytarabine (ara-C) waarmee remissie percentages van 60% tot 80% bereikt kunnen worden. Ondanks de geboekte vooruitgang in de behandeling van patiënten met AML, zijn er toch nog grote verschillen in response waar te nemen tussen de patiënten onderling. Deze verschillen betreffen zowel het al dan niet bereiken van een complete remissie als ook de variabele duur van een eventueel bereikte remissie. Dit betekent dat de behandeling voor bepaalde patiënten leidt tot genezing van hun leukemie, maar dit betekent ook dat er patiënten zijn die niet goed op de therapie reageren en waarbij de leukemie cellen niet uit het lichaam verdwijnen. Voor deze laatste groep van patiënten is de kans op genezing gering en de prognose erg somber. Het is mogelijk dat de verschillen in individuele response gedeeltelijk terug te voeren zijn op verschillen in metabolisme en verdeling van DNR in het lichaam. In dit proefschrift is een aanzet gegeven tot het bestuderen en identificeren van farmacokinetische variabelen van DNR in patiënten met AML, met als doel het vergroten van ons inzicht in de factoren die bepalend zijn voor de therapie resultaten.

In **hoofdstuk 1** wordt een overzicht gegeven van de farmacologie van DNR en van de plasma farmacokinetische parameters van DNR in relatie tot de response in patiënten met AML. De resultaten van de beschreven studies hebben geleid tot de vraag of het bepalen van cellulaire DNR concentraties, in plaats van plasma DNR concentraties, niet zinvoller is bij het bestuderen van de relatie tussen farmacokinetiek en response.

In **hoofdstuk 2** wordt de hoge druk vloeistof chromatografie (HPLC) methode beschreven die in dit onderzoek gebruikt is om de concentraties van DNR in plasma en in de cellen te meten. Met name bij het kwantificeren van de hoeveelheid cellulair DNR zijn een aantal factoren van belang gebleken. Zo bleek dat met behulp van koude (0-4 °C) hypotone lysis een maximaal rendement van de isolatie van leukocyten uit bloed te verkrijgen was: de rode en de witte bloedcellen werden goed van elkaar gescheiden waarbij 100% van de kernhoudende cellen werd teruggevonden. Met betrekking tot de extractie van DNR, daunorubicinol (DOL) en doxorubicine uit menselijke leukocyten gaf de methode van groot-volume extractie (5 ml extractie mengsel) de beste resultaten. Ondanks het gebruik van een groot extractie volume, bleef het rendement van de extractie van DNR en DOL uit cellen afhankelijk van het aantal cellen aanwezig in het te bepalen monster. Het was daarom noodzakelijk om bij het berekenen van de cellulaire concentraties van DNR en DOL gebruik te maken van een correctie factor. Tevens werd het celaantal van de te extraheren bloed en beenmerg monsters gestandaardiseerd door de monsters te

verdunnen met PBS tot eindconcentraties van 1 tot 10 x 10^9 cellen per liter. Met behulp van de beschreven HPLC methode werden DNR en DOL concentraties gemeten in plasma en cellen afkomstig van zeven patiënten met AML tijdens hun eerste remissie inductie kuur. De gemeten cellulaire DNR concentraties lieten grote verschillen zien tussen de patiënten onderling. Er waren geen relaties waarneembaar tussen de plasma DNR concentraties en de cellulaire DNR concentraties.

In **hoofdstuk 3** wordt de toepasbaarheid van de laser flow cytometriemethode beschreven bij het bepalen van de cellulaire DNR concentraties, waarbij de resultaten vergeleken worden met de eerder beschreven HPLC methode. Het bleek dat de fluorescentie signalen afkomstig van DNR in cellen en kernen (gemeten met de flow cytometer) goed overeen kwamen met de absolute hoeveelheid DNR in de cellen en kernen (gemeten met HPLC). Dialyse experimenten toonden aan dat de kern fluorescentie van DNR voor het grootste deel afkomstig is van DNR gebonden in de kern en in mindere mate afkomstig is van vrij in de kern aanwezig DNR. Deze resultaten ondersteunen de conclusie dat met behulp van flow cytometrie de totale cellulaire DNR concentratie (DNR in cytoplasma en DNR gebonden in de kern) aangetoond wordt. De flow cytometrie methode is aansluitend gebruikt voor het bepalen van de cellulaire DNR concentraties in AML patiënten (beschreven in hoofdstuk 6).

In **hoofdstuk 4** wordt het mogelijke aandeel in het transport van DNR naar de weefsels door normale en leukemische kernhoudende cellen beschreven. Het opladen van de cellen met DNR in vitro verliep volgens een lineair patroon. Na het inspuiten van deze cellen in de bloedbaan van de rat werden dosis afhankelijke DNR concentraties in de weefsels gemeten. De DNR weefsel concentraties na toediening van met DNR opgeladen cellen werden vergeleken met DNR weefsel concentraties verkregen na toediening van eenzelfde hoeveelheid DNR intraveneus. Verschillen in DNR weefselverdeling werden gevonden: na toediening van celgebonden DNR waren de DNR concentraties in lever en milt hoger en in hartweefsel lager dan na intraveneus toegediend DNR. Opvallend was de bevinding dat de DNR concentraties in beenmerg vergelijkbaar waren.

In **hoofdstuk 5** wordt beschreven hoe de HPLC methode gebruikt werd voor het meten van DNR concentraties in plasma en in cellen (bloed en beenmerg) afkomstig van 37 patiënten met AML tijdens hun eerste kuur met DNR en ara-C. De plasma DNR concentraties correleerden niet met de DNR concentraties gemeten in de kernhoudende cellen van het beenmerg. Er bleek wel een verband te bestaan tussen de DNR concentraties in plasma en in de witte bloedcellen: de oppervlakte onder de concentratie-tijd-curve (AUC) van DNR in plasma was negatief gecorreleerd met de AUC van DNR in de witte bloedcellen. Tevens correleerden de DNR concentraties in witte bloedcellen met de DNR concentraties van de kernhoudende cellen van het beenmerg. De gemeten DNR concentraties in plasma, witte bloedcellen of beenmergcellen hadden geen voorspellende waarde met betrekking tot de klinische respone op de ingestelde behandeling met DNR.
Opvallend was de relatie tussen de DNR concentraties in de witte bloedcellen en het aantal blasten die in het bloed aanwezig waren op het moment dat de diagnose AML werd gesteld. Naarmate er meer blasten in het bloed aanwezig waren werden er lagere cellulaire DNR concentraties gemeten. Hieruit blijkt dat de te bereiken cellulaire DNR concentratie mogelijk afhankelijk is van het aantal aanwezige tumorcellen.

In **hoofdstuk 6** wordt beschreven hoe de DNR concentraties in blasten, afkomstig uit bloed en beenmerg van 17 patiënten met AML, gemeten werden met behulp van de flow cytometer. De hoeveelheid DNR in blasten was hoger dan de DNR concentraties gemeten in de kleinere lymfocyten, maar lager dan de DNR concentraties in de qua grootte vergelijkbare granulocyten. De DNR concentraties in blasten uit het bloed correleerden positief met de DNR concentraties in beenmerg blasten. De DNR concentraties in blasten werden ook vergeleken met de klinische response op de behandeling met DNR. Het bleek dat de patiënten die in complete remissie kwamen werden gekenmerkt door hogere DNR concentraties dan de patiënten die slechts een partiële response op de therapie vertoonden. Deze twee groepen van patiënten konden ook van elkaar onderscheiden worden op grond van het aantal blasten in het bloed. Tevens bleek dat de hoeveelheid DNR in de blasten negatief gecorreleerd was met het aantal blasten in het bloed. Opvallend was de bevinding dat de patiënten die niet goed op de therapie hadden gereageerd (refractaire leukemie patiënten) op basis van de gemeten DNR concentraties in blasten en op basis van het aantal blasten in het bloed niet konden worden onderscheiden van de complete remissie patiënten. Bij de refractaire patiënten zijn waarschijnlijk andere factoren, zoals het multidrug resistentie fenotype, verantwoordelijk voor de slechte therapie resultaten.

Tenslotte worden in **hoofdstuk 7** de resultaten van dit onderzoek besproken in het kader van de vraag of het toepassen van farmacokinetische studies van DNR, nu en in de toekomst, van belang is bij de behandeling van AML patiënten met DNR.

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Curriculum vitae

De schrijfster van dit proefschrift werd geboren op 27 december 1958 te 's-Gravenhage.

Na het behalen van het diploma Atheneum B in 1977 aan het Thomas More College te 's-Gravenhage werd in datzelfde jaar begonnen met de studie geneeskunde aan de Erasmus Universiteit te Rotterdam. In maart 1984 werd deze studie voltooid met het artsexamen.

Van maart 1984 tot januari 1988 was zij verbonden aan de afdeling Klinische Farmacologie van de Dr. Daniel den Hoed Kliniek/Rotterdamsch Radio-Therapeutisch Instituut. In deze periode werkte zij, onder supervisie van Dr. B. Löwenberg en Dr. P. Sonneveld, aan klinisch farmacologisch onderzoek van cytostatica bij leukemie patiënten. Het onderzoek, financieel mogelijk gemaakt door het Koningin Wilhelmina Fonds, heeft geresulteerd in dit proefschrift.

Vanaf januari 1988 is zij in opleiding tot Medisch Microbioloog op de afdeling Klinische Microbiologie en Ziekenhuishygiëne (Hoofd: Prof. dr. D.M. MacLaren) van het Academisch Ziekenhuis der Vrije Universiteit te Amsterdam.