ABSTRACT

We compared the pharmacokinetics of daunomycin (7.5 mg/kg i.v. bolus injection) in normal and leukemic rats using a leukemia model which resembles acute myeloid leukemia in humans. Due to a more rapid decrease in plasma concentration, the area under the plasma concentration/time curve (AUC) for up to 2 h after drug injection was smaller (2.2 times) in the leukemic rats than that for normals. However, due to higher plasma levels during the drug elimination phase, the total AUC was somewhat larger (1.3 times) in the leukemic rats. In the leukemia-infiltrated organs (spleen, liver, and lungs), significantly higher daunomycin concentrations (per gram wet weight) were found than in those obtained from normal rats. In contrast, femoral bone marrow from leukemic rats contained less daunomycin (per 10^9 nucleated cells) than did normal marrow. Quantification of the daunomycin uptake in vitro by flow cytometry showed that leukemic cells from bone marrow and spleen have an equal net drug uptake. Our data suggest that, in the presence of a high leukemic cell load, the intravenously injected daunomycin is rapidly taken up and retained by the leukemic tumor mass in, e.g., spleen, liver, and lungs, and that, as a consequence of this, the femoral marrow functions as a kind of pharmacological sanctuary.

INTRODUCTION

The anthracycline drugs Adriamycin and daunomycin are major agents in the remission-induction and consolidation therapy of acute leukemia in humans. After an i.v. bolus injection, these drugs are rapidly distributed over the tissues. The plasma distribution phase is relatively short and, due to a high affinity of anthracycline drugs for the cellular DNA (1), the drug elimination phase is much longer. Although the pharmacokinetics of anthracyclines in humans and in animals have been described extensively in the literature (1-6), knowledge about a possible effect of the tumor load on the pharmacokinetic parameters of these drugs is not very much in evidence (7-10). In view of the unpredictable variations in clinical responsiveness to chemotherapy in cancer patients and the inter-individual differences in the ratio of anthracycline dose and AUC (3), it is an attractive hypothesis that the total number of tumor cells has an effect on the tissue and target cell distribution of anthracycline drugs in humans. Especially in the case of acute leukemia, a widely disseminated malignant disease in which large numbers of easily accessible tumor cells are present in the body, one can imagine that the injected anthracycline drugs with their high affinity for cellular DNA are rapidly taken up and retained by the tumor cells, leading to changes in the pharmacokinetic profiles of the drugs. Therefore, the present studies were undertaken to compare the comparative pharmacokinetics of daunomycin in normal and leukemic rats using an experimental leukemia model which has been proven to resemble acute myeloid leukemia in humans as far as growth characteristics (11) and responses to chemotherapy (12) are concerned.

MATERIALS AND METHODS

Chemicals. Daunomycin, daunomycinol, and Adriamycin were kindly supplied by Farmitalia (Milan, Italy). For the chromatographic drug determinations, all reagents were of high-performance liquid chromatography quality and were purchased from Baker Chemicals (Deventer, Holland).

Animals. Twelve-week-old barrier-derived inbred female Brown Norway (BN/Birij) rats raised in the inbred Rijswijk colony and weighing 140-160 g were used in the experiments.

Leukemia Model. The Brown Norway acute myeloid leukemia 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 i.v. 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 the Brown Norway acute myeloid leukemia have been described extensively (11, 12) and can be summarized as follows: (a) 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; (b) it is cytochemically and cytologically identical to human acute myeloid (promyelocytic) leukemia; (c) the mean survival time after i.v. inoculation of 10^7 leukemic spleen cells is 22 days; and (d) the chemotherapeutic responses to acute myeloid leukemia treatment schedules (including daunomycin and cytarabine) are comparable with those obtained in humans (12).

The distribution and elimination kinetics of daunomycin were investigated in Brown Norway acute myeloid leukemia rats at a stage of the disease comparable with that of human acute myeloid leukemia patients at clinical admission (day 13 after i.v. transplantation of 10^7 leukemic spleen cells). At this stage, organs such as the spleen, liver, lungs, and bone marrow are heavily infiltrated by leukemic cells (11).

Experimental Procedures. Daunomycin (7.5 mg/kg body weight) was administered as a single i.v. bolus injection in the tail vein of normal and leukemic rats. This dosage in rats is comparable to a clinical dose of 40 mg/m^2 in humans (13). The injection of drug was done under light ether anesthesia, and it was dissolved in 0.5 ml physiological saline. In one experiment, leukemic rats were splenectomized prior to drug injection. At specific time intervals (2, 4, 6, 8, 10, and 30 min and 1, 2, 3, 4, 6, and 24 h) after drug injection, the animals were sacrificed by exsanguination under ether anesthesia. Plasma was obtained by anticoagulation of aortic blood samples by EDTA. Organs of interest were removed and rapidly cooled in liquid nitrogen and then stored at −20°C until further processing. Urine and bile were collected in 2-h aliquots and stored at −20°C in two separate experiments. Bile was obtained by cannulation of the bile duct.

1 Supported by a grant from the Netherlands Organization for the Fight against Cancer, the "Koningin Wilhelmina Fonds."
2 To whom requests for reprints should be addressed.
3 The abbreviations used are: AUC, area under the plasma concentration/time curve; AU, arbitrary units.

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Drug Determination by High-Performance Liquid Chromatography.

Daunomycin and daunomycinol concentrations were determined by straight-phase high-performance liquid chromatography as described previously (14). Plasma, urine, and bile samples adjusted to pH 9.8 were extracted with a chloroform-methanol (4:1) mixture. After centrifugation of the urine and bile samples, 100-μl aliquots of the organic phases were injected directly onto the column. After centrifugation of the plasma samples, 1 ml of the organic phase was evaporated to dryness under nitrogen at 30°. The residue was redissolved in 150 μl mobile phase. Tissues were extracted as 10% homogenates in phosphate buffer (0.05 M; pH 8.5) with a chloroform-methanol (4:1) mixture. One hundred-μl aliquots of the organic phases were injected directly onto the column. To obtain bone marrow cells, a femur was cut into two parts, and each part was flushed with 2 ml physiological saline. The bone marrow suspension was then adjusted to 10^7 nucleated cells/ml. Two ml of the bone marrow suspension were adjusted to pH 9.8 and thereafter extracted with chloroform-methanol (4:1). For plasma, urine, and bile, the recoveries from the extraction procedures were 90–95%. For tissues, the recovery was 85–90%. The coefficient of variation of the analytical method varies between 1 and 10%. Adriamycin was used as an internal standard for drug quantification. Each point in the plasma and tissue concentration/time curves represents the mean ± SD of 6–8 animals. The results are expressed as ng/ml biological fluid for plasma, urine, and bile, as μg/g wet weight for the tissues, and as μg/10^7 nucleated bone marrow cells. For the pharmacokinetic modeling of the drug, the equation describing an open three-compartment model with excretion from the central compartment only was used (15). The coefficients, exponents, and compartmental volumes in the integrated equations were estimated by fitting the triexponential function A·e^−αt + B·e^−βt + C·e^−γt to the observed plasma concentrations. The concentration/time curves were generated by iterative numerical analysis. The half-life time of plasma drug concentrations during the α-, β-, and γ-phases (t½ = ln(2)/α, β, or γ) and the AUC (A = C(t)/β) were also calculated.

Determination of Intracellular Anthracycline Content by Laser Flow Cytometry. The quantification of the intracellular anthracycline content by means of a FACS II flow cytometer (Becton-Dickinson, Mountain View, CA) has been described earlier in detail (16). In the FACS II, the cells are contained in a liquid jet. They traverse the light beam of a 5-W argon ion laser (Spectra Physics Model 164-05). The laser is tuned at 488 nm (0.8 W). This wavelength is close to the absorption maximum of the anthracyclines. The fluorescence which is emitted by the cells upon excitation by the laser light is registered on a photomultiplier. The scatter light is blocked by two 520-nm long-pass glass filters. The signals from the photomultiplier are amplified and classified by a pulse height analyzer (Nuclear Data Model ND 100). The forward and perpendicular light scatter signals from the cells are measured on separate detectors and analyzed in a similar manner. The intensity of the signals is related to cell size and cell structure, respectively (17).

One million nucleated bone marrow or spleen cells obtained from normal or leukemic rats in 1 ml 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered Hanks’ balanced salt solution (pH 7.4) were incubated (30 min at 37°) with 3 μg daunomycin per ml incubation medium. After incubation, the cells were put on melting ice and washed 4 times by centrifugation (300 × g at 4°) and resuspension of the pellets with ice-cold phosphate-buffered saline (KC1: 0.003 m; KH2PO4, 0.003 m; NaCl, 0.15 m; NaHPO4, 0.01 m, pH 7.4). Finally, the cells were resuspended in 1 ml buffer and kept at 4° until further processing. This incubation procedure did not lead to a substantial increase in dead cells. When cells which had been incubated in the presence of daunomycin for 30 min were washed and resuspended in drug-free medium at 37°, a portion of the drug which had been taken up was released by the cells. However, storage of the incubated cells at 4° for a maximum of 1 h did not result in a measurable loss of intracellular drug into the drug-free medium. The incubated cells were used subsequently to determine the intracellular anthracycline content on the basis of fluorescence intensity.

RESULTS

Plasma Disappearance of Daunomycin in Normal and Leukemic Rats. After a single i.v. bolus injection of daunomycin (7.5 mg/kg), the plasma concentration/time relationship could be very well described by a triexponential equation for normal and leukemic rats (correlation coefficients of 0.99599 and 0.99883, respectively) (Chart 1, A and B). The major fluorescent metabolite was daunomycinol. The half-life of plasma elimination of daunomycin during the α-phase was 0.8 min in the normal rats and 0.7 min in the leukemic animals. A shorter β-phase and a somewhat longer γ-phase were found in leukemic rats as compared with normal rats (15.7 min and 13.1 h versus 29.6 min and 9.9 h). Due to increased plasma concentrations during the β- and γ-phases in leukemic rats, the AUC0–∞ was increased from 3.73 μg/ml·h in normal rats to 4.92 μg/ml·h in leukemic rats. However, the daunomycin AUC0–∞ was much larger in normal rats as compared with that for leukemic rats (2.74 μg/ml·h versus 1.25 μg/ml·h).

Tissue Distribution of Daunomycin in Normal and Leukemic Rats. Differences were found in the amount of drug taken up by the tissues between normal and leukemic rats (Table 1). For example, heart tissue in leukemic animals contained less daunomycin than that found in normal rats. However, in the leukemic liver and lungs (organs that are infiltrated with leukemic cells), elevated levels of daunomycin were observed. On microscopic examination, the kidneys showed a minimal amount of leukemic cell infiltration. Yet, elevated drug levels were found. In all cases, daunomycinol was the major fluorescent metabolite. The highest levels of daunomycinol were found in the kidneys of the leukemic rats. Also in livers and lungs obtained from leukemic animals, the daunomycinol concentrations were increased as compared with normal rats. Graphical representations of tissue drug concentration/time courses are shown in Charts 2 and 3. In Chart 2, the daunomycin and daunomycinol contents expressed as μg/g wet weight of tissue in a normal spleen [maximum concentration, 18.2 ± 1.8 (SD) μg/g] and in a leukemic spleen (maximum concentration, 24.9 ± 2.5 μg/g) are compared. At 2 and 24 h after drug injection the daunomycin concentrations in normal and leukemic spleens differ significantly (Wilcoxon’s signed-rank test, α = 0.05). However, the femoral bone marrow obtained from leukemic animals contained less daunomycin (expressed as μg/10^7 nucleated cells) than that found in normal rats (Chart 3). This difference was statistically significant (α = 0.05) over the whole time course studied, as determined by the Wilcoxon’s signed-rank test. Splenectomy of the leukemic animals prior to drug injection resulted in a partial restoration of the daunomycin uptake by the leukemic bone marrow (Chart 3). At 4, 6, and 24 h after drug injection, the daunomycin concentrations in the bone marrow of leukemic and splenectomized leukemic rats differed significantly (Wilcoxon’s signed-rank test, α = 0.05). Sham operation had no significant effect on the daunomycin concentrations in the leukemic bone marrow (data not shown). Splenectomy of normal rats resulted in a slight (not statistically significant) increase in the daunomycin concentration in the femoral bone marrow (data not shown).

In Vitro Daunomycin Uptake of Leukemic and Normal Hemopoietic Cells. Using the FACS II, rat bone marrow subpopulations (erythrocytes, lymphocytes, granulocytes, and blast cells)
DAUNOMYCIN DISTRIBUTION IN NORMAL AND LEUKEMIC RATS

Chart 1. Plasma disappearance curves in rats treated with daunomycin (7.5 mg/kg) as an i.v. bolus injection. A, daunomycin: O, normal rats; △, leukemic rats; B, daunomycinol: O, normal rats; △, leukemic rats. Bars, SD. The lines were generated by iterative numerical analysis using equations describing a tricompartmental distribution of daunomycin and daunomycinol.

Table 1: Tissue concentrations of daunomycin and daunomycinol in organs of normal and leukemic rats

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<td>4 h</td>
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Daunomycin concentrations in leukemic tissues indicated differ significantly from the controls (Wilcoxon's signed-rank test, α = 0.05).

can be identified on the basis of forward and perpendicular light scatter (17). When fluorescence measurements are combined with light scatter measurements and when specific areas are selected in the forward perpendicular light scatter plane, the anthracycline content of the different cell types in bone marrow can be studied separately (16). We showed previously that for the different bone marrow subpopulations there is a linear relationship between the intracellular daunomycin content and drug dose in the incubation medium (16). We compare here the daunomycin net cellular uptake in rat femoral bone marrow obtained from normal or leukemic rats after incubation with 3 µg daunomycin per ml culture medium. Chart 4A shows the fluorescence distribution over the different hemopoietic cell types. The abscissas represent the relative fluorescence intensity on a logarithmic scale. The ordinates represent the number of cells. Unstained controls have the same amount of nonspecific fluorescence irrespective of their cell type. The order of the fluorescence intensity expressed in AU of the different populations in bone marrow is lymphocytes (168 AU) < granulocytes (275 AU) < leukemic blast cells (480 AU) < normal blasts (635 AU). In Chart 4, B and C, are compared the frequency distributions of the fluorescence intensity of the total nucleated cell populations obtained from normal or leukemic bone marrow (Chart 4B) and from normal or leukemic spleen (Chart 4C). For leukemic rats, no differences were observed in the fluorescence intensity of the nucleated cells obtained from either bone marrow or spleen.
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Chart 3. Bone marrow concentration/time course of daunomycin after an i.v. bolus injection (7.5 mg daunomycin/kg). Bars, SD. Nucleated bone marrow cells were obtained from normal rats, leukemic rats, and leukemic rats which had been splenectomized prior to drug injection.

Urine and Bile Excretion in Normal and Leukemic Rats. The cumulative 24-h urine and bile excretions expressed as a percentage of the total administered dose in normal and leukemic rats are shown in Table 2. After a daunomycin bolus injection in normal rats, 9.0% of the total administered dose is excreted via the urine and 27.5% is excreted via the bile route as daunomycin and daunomycinol within 24 h. For leukemic rats, these figures are somewhat higher: 12.2% for the urine and 36.2% for the bile.

DISCUSSION

A wide variation in chemotherapeutic responses is found in the treatment of cancer patients. Sensitivity of the tumor cells to the drugs (18) and the amount of tumor mass at the start of therapy (19) can thereby play a role. As far as the tumor mass is concerned, it has been postulated (19) that chemotherapy fails because cells develop resistance to anticancer drugs and that due to mutational events this resistance is mass related. Another phenomenon that could also be of importance in the variation in chemotherapeutic responses is an altered drug distribution induced by the tumor load. Indeed, great variations in, e.g., plasma levels of anthracycline-treated patients have been observed (3, 20). It is unknown as to what extent these variations in plasma concentrations can be attributed to differences in tumor mass. Our results indicate significant differences in daunomycin plasma and tissue distribution between normal and leukemic rats. Differences were also found in the urine and bile excretions of anthracyclines between normal and leukemic rats. Especially the daunomycin excretion via the bile route was increased.

In our leukemia model, the spleen is heavily infiltrated with leukemic cells at day 13 (the increase in weight was a factor of 3.6). Thus, the daunomycin increase per gram wet weight in the spleens in leukemic rats suggests that the leukemic cells take

Table 2

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<th>Normal rats</th>
<th>Leukemic rats</th>
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<td></td>
<td>DAU</td>
<td>DAUNOL</td>
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<tr>
<td>Urine</td>
<td>4.3 ± 0.5</td>
<td>4.7 ± 1.1</td>
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<tr>
<td>Bile</td>
<td>12.7 ± 6.3</td>
<td>14.8 ± 5.1</td>
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*DAU, daunomycin; DAUNOL, daunomycinol.

*Mean ± SD.
up more drug than do normal spleen cells. Indeed, increased drug levels were found in liver and lungs, organs which were also heavily infiltrated by leukemic cells. At day 13 after the leukemia transfer, the normal hemopoiesis in the femoral marrow is about 70% replaced by infiltrating leukemic cells (11). Thus, the most striking observation in our study is that, in the leukemic rats, the femoral bone marrow contained significantly less drug than was found in normal marrow. The results of the in vitro experiments showed that leukemic cells obtained from either the femoral bone marrow or the spleen can take up equal amounts of daunomycin. Thus, the lower daunomycin concentrations in the femoral marrow of leukemic rats seem to be due to specific characteristics of the femur. Since in leukemic rats the total AUC for daunomycin is increased as compared with normals, one would expect continued accumulation of daunomycin in the leukemic bone marrow. However, an explanation might be found if one considers two observations: (a) In normal rats, the femoral bone marrow requires a longer time (at least 2 h) to reach a maximum in daunomycin accumulation than does the spleen; and (b) in vitro, the maximum amount of daunomycin that can be taken up by a cell at optimal incubation time is determined by the drug concentration in the incubation medium (16, 21). If this last phenomenon is also true in vivo, then the reduced femoral drug content in the leukemic rats can be explained by the initially lower plasma concentrations in the leukemic rats.

In leukemic rats, the AUC_{0-\infty} is about 50% of that found in normal rats. An explanation for this lower AUC_{0-\infty} in leukemic rats could just be the presence of easily accessible leukemic cells elsewhere in the body (e.g., spleen and liver) which rapidly take up the i.v. injected daunomycin. The reduced daunomycin concentration in, e.g., heart tissue as compared with normal rats is in support of this explanation. The increase in total AUC_{0-\infty} in the leukemic animals as compared with normal ones is caused by the higher plasma daunomycin concentrations in the leukemic rats during the drug elimination phase. The latter can probably be ascribed to an increased daunomycin elimination from the leukemia infiltrated organs.

A strong suggestion for a role of the leukemic cell load in daunomycin distribution is provided by the experiment in which removal of a large mass (about 1 g) of leukemic cells by splenectomy resulted in a partial restoration of the reduced drug uptake by the leukemic femoral bone marrow. The increased daunomycin content per gram wet weight found in the kidneys of leukemic animals cannot be explained by leukemic cell infiltration. Microscopic examination of these organs does not reveal abundant leukemic cell infiltration. On the other hand, we can imagine that dead (leukemic) cells are trapped in the glomeruli of the kidneys. Indeed, increased numbers of dead cells with pyknotic nuclei were found in the glomeruli microscopically. As we have shown previously (16) that dead cells take up several times more anthracyclines than do living ones, the increased daunomycin concentration in the kidneys could be explained by the presence of dead (leukemic) cells in this organ. It is obvious that this phenomenon of increased drug uptake by dead leukemic cells also can be of importance in the elevated daunomycin content of, e.g., leukemic spleen, liver, and lungs.

Our observations suggest that some tissue compartments in vivo, among which is the femoral marrow, might receive less drug due to the leukemic cell load elsewhere in the body and that leukemic cells which have infiltrated these compartments might be exposed to ineffective drug concentrations. Further study will be required to evaluate the role of drug levels in leukemia-infiltrated tissues with regard to leukemic stem cell kill. Preliminary results on leukemic stem cell kill after an i.v. bolus injection of daunomycin in leukemic rats are in agreement with the pharmacokinetic data presented here. After a single daunomycin dosage of 7.5 mg/kg body weight, about 1% of the leukemic stem cells in the spleen survived treatment, while this figure was about 30% for the femoral bone marrow.

Earlier work of one of us (P. S.) on the pharmacokinetics of Adriamycin using the same transplantable rat leukemia model (10) showed reduced anthracycline levels not only in the femoral bone marrow but also in the spleen. In that study, it was concluded that leukemic cells in vitro take up less drug than do normal hemopoietic cells. However, we recently carried out flow cytometry studies and found that the Adriamycin uptake by leukemic cells in vitro is high as compared with other hemopoietic cells. Apparently, the leukemic status in the BN rat also has effects on the pharmacokinetic behavior of Adriamycin. In addition, we found that after in vitro incubation, daunomycin accumulates in the cells to a greater extent than is found for Adriamycin. This has also been observed in other cell types and is thought to be due to a higher lysosomal accumulation of daunomycin as compared with Adriamycin (22). In the above-mentioned study (10), the technique used for determining anthracyclines could not discriminate between parent drug and metabolites. Using high-performance liquid chromatography, we found that Adriamycin is very poorly metabolized in the BN rat. Thus, besides physicochemical differences (e.g., lipophility, affinity to macromolecules) between Adriamycin and daunomycin, the difference in metabolism can play a role in the different pharmacokinetic behavior of the two drugs observed in the leukemic BN rats.

ACKNOWLEDGMENTS

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DAUNOMYCIN DISTRIBUTION IN NORMAL AND LEUKEMIC RATS


Differences in the Pharmacokinetics of Daunomycin in Normal and Leukemic Rats

Kees Nooter, Pieter Sonneveld and Anton Martens