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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 × 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 × 10^7 *cells. When these DNR loaded cells are infused into the rat, dose dependent distribution kinetics are observed. Compared to i.v. 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.

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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 g.

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 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 leukemia 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 an i.v. 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 i.v. 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 of the washing procedures had been completed, 5×10^7 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 i.v. 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 hr, 4 hr and 24 hr. 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 per cent 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 95–90%. 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 silic agel particles prepacked into a 250 × 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 0 to 4 hr after drug administration. The Spearman correlation test, linear regression and the Wilcoxon test were used for statistical analysis.

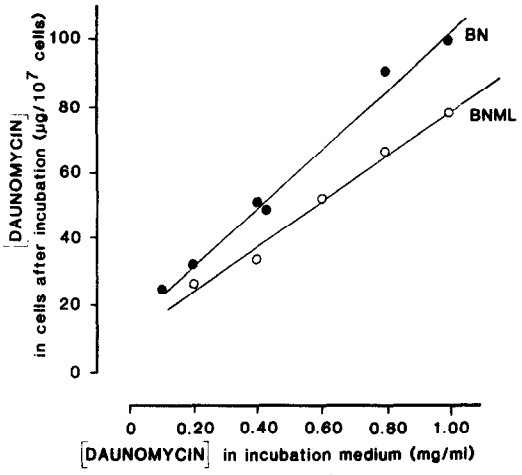

Fig. 1. Intracellular concentration of DNR in normal rat BN bone marrow cells (●—●) and BNML rat spleen leukemia cells (○—○) following an incubation period of 20 min *with different extracellular concentrations of DNR. Spearman correlation coefficients: BN:* 0.98 ($P < 0.001$); *BNML:* 0.99 ($P < 0.001$).

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 hr 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 i.v. 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 hr 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 i.v. 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 0 to 4 hr 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.

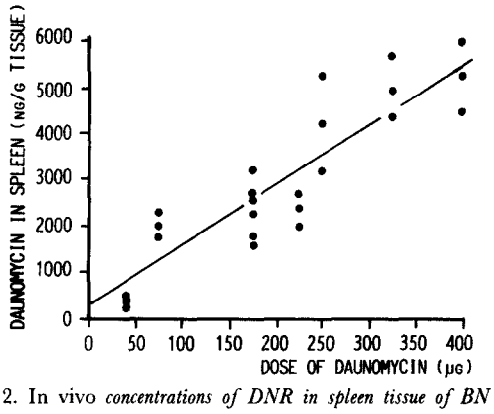

Fig. 2. In vivo *concentrations of DNR in spleen tissue of BN rats following i.v. administration of different dosages of the drug entrapped in* 5×10^7 *nucleated bone marrow cells. The concentrations are expressed as nanogram per gram of wet tissue. The Spearman correlation was* 0.89.

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 5×10^7 cells. After i.v. 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 i.v. bolus injection, both after a dose of 200 μg per animal (approx. 1.0 mg/kg).

In normal rats, DNR administered as an i.v. 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 adminstration of the drug entrapped in cells than after i.v. bolus (spleen: $P < 0.05$; liver: $P < 0.05$).

In bone marrow no significant difference is observed, except at 4 hr, when the concentrations are significantly higher following the i.v. bolus gift.

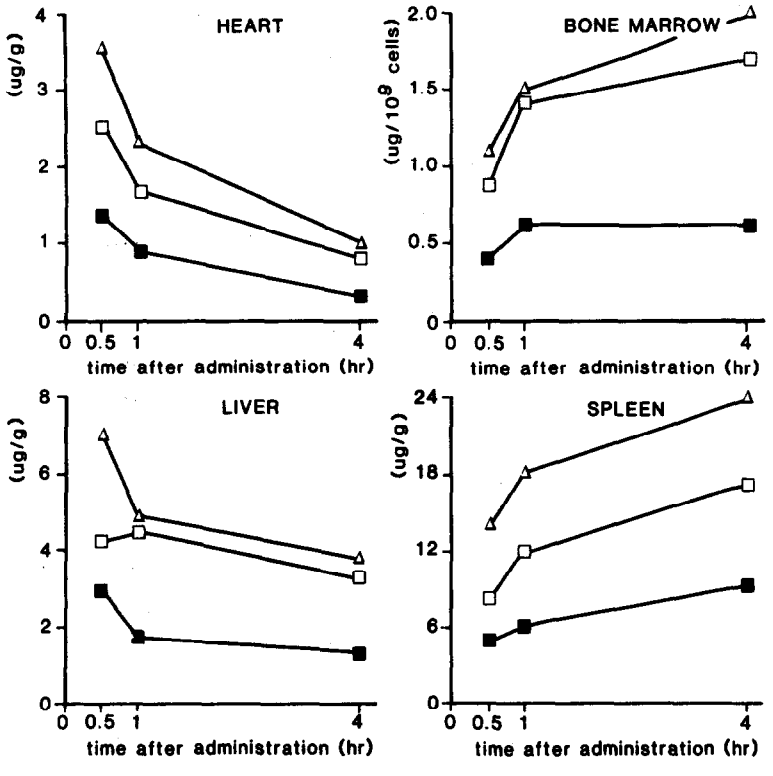

Fig. 3. In vivo *concentration of DNR in heart, bone marrow, liver and spleen in the normal BN rat following i.v. administration of* 5 × 10^7 *nucleated bone marrow cells with a final intracellular DNR content of* 125 μg (■—■), 275 μg (□—□) *and* 425 μg (△—△).

Table 1. *Area under the curve* (0–4 hr) *of daunorubicin in tissues of the non-leukemic rat (BN)**

Dose of DNR	AUC of DNR in tissue ($\bar{X}$ ± S.D.) Heart (μg/hr/g)	Spleen (μg/hr/g)	Liver (μg/hr/g/)	Bone marrow (μg/hr/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 i.v.	8.50 ± 0.39	18.85 ± 1.55	6.84 ± 0.46	5.02 ± 1.23

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

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 i.v. 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 i.v. administration (spleen: $P < 0.05$; liver: $P < 0.05$). In bone marrow again no difference is observed. These findings are summarized in Table 2, using the tissue AUC as calculated from the observed concentrations at each dosage and/or way of adminstration.

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]. Following i.v. 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,

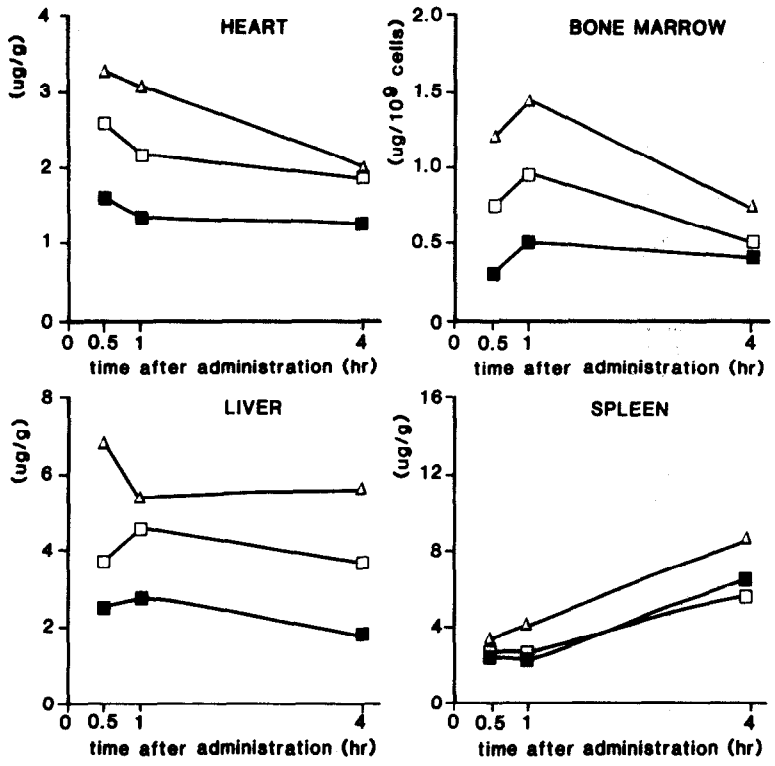

Fig. 4. In vivo *concentration of DNR in heart, bone marrow, liver and spleen in the BNML rat following i.v. administration of* 5×10^7 *BNML leukemia cells with a final intracellular DNR content of* 75 μg (■—■), 175 μg (□—□) *and* 250 μg (△–△).

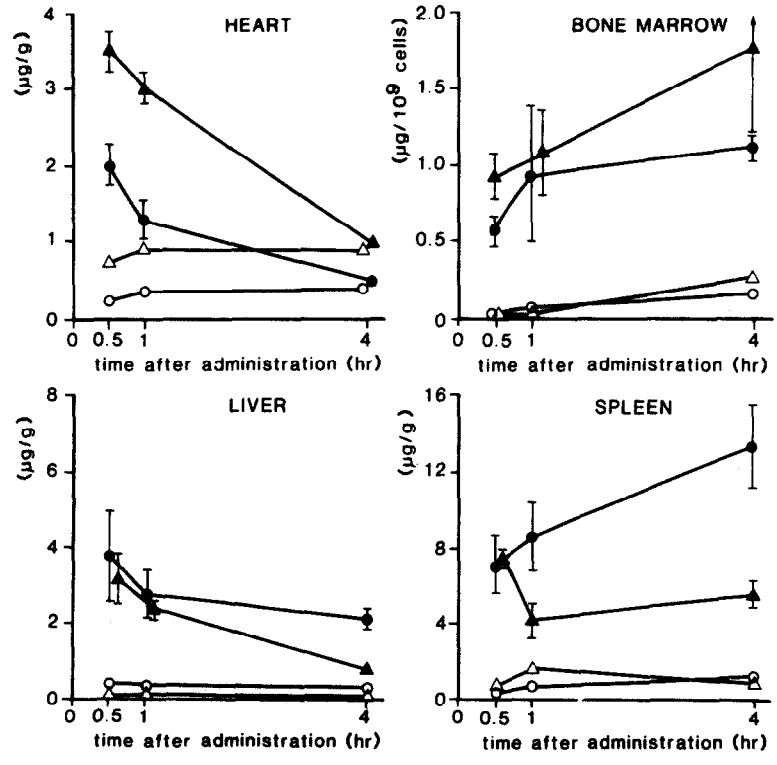

Fig. 5. In vivo *concentration of DNR and DOL in heart, bone marrow, liver and spleen in the BN rat following a bolus injection of* 200 μg *DNR* (*DNR*: ▲—▲; *DOL*: △—△) *or i.v. administration of* 200 μg *of DNR entrapped in* 5×10^7 *nucleated bone marrow cells* (*DNR*: ●—●; *DOL*: ○—○) (*M* ± *S.D.*).

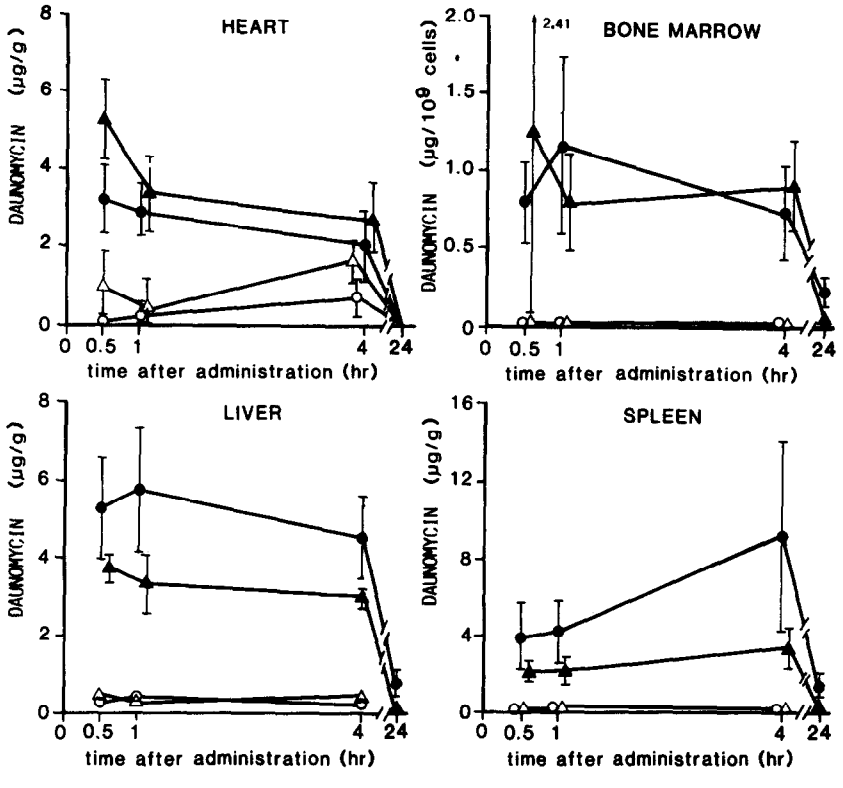

Fig. 6. In vivo *concentration of DNR and DOL in heart, bone marrow, liver and spleen in the BNML rat after an i.v. bolus injection of* 200 μg *DNR (DNR:* ▲—▲; *DOL:* △—△) *or i.v. administration of* 200 μg *of DNR entrapped in* 5×10^7 *BNML cells (DNR:* ●—●; *DOL:* ○—○) *(M ± S.D.).*

Table 2. Area under the curve (0–4 hr) *of daunorubicin in tissues of the leukemic rat (BNML)**

Dose of DNR	AUC of DNR in tissue ($\bar{X}$ ± S.D.) Heart (μg/hr/g)	Spleen (μg/hr/g)	Liver (μg/hr/g)	Bone marrow (μg/hr/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 i.v.	11.13 ± 2.76	10.02 ± 0.92	12.00 ± 1.44	3.44 ± 1.26

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

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 i.v. administration [5], we decided to investigate to what extent DNR-containing cells contribute to effective concentrations of DNR and DOL in these organs after i.v. 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 a 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 i.v. 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 i.v. bolus administration, suggesting that intracellular DNR does not have a major role with respect to cardiotoxicity. A major difference with i.v. 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 i.v. 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 hr after i.v. 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 i.v. 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 i.v. bolus DNR. In view of the reported large levels of DNR in peripheral white cells of AML patients after i.v. 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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