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Effect of Cyclophosphamide Pretreatment on Daunorubicin in Rat Acute Leukaemia Model

Kees Nooter, Arjan de Vries, Anton C.M. Martens and Anton Hagenbeek

The total number of leukaemic cells at the time of therapy may affect the tissue and target cell distribution and antitumour efficacy of cytotoxic drugs. The effects of low dose cyclophosphamide pretreatment on daunorubicin concentrations in leukaemic bone marrow were investigated in rats. At day 12 after transplantation of the leukaemia, rats were injected intraperitoneally with cyclophosphamide (30 mg/kg). 2 days later the leukaemic rats received daunorubicin intravenously (7.5 mg/kg). Cyclophosphamide pretreatment led to a significant increase in daunorubicin concentration in the femoral bone marrow, by a factor of about 7. The log leukaemic stem cell kill (LCK) values, as estimated by a survival assay, were 1.8, 0.7, and 5.4 for the leukaemic rats injected with cyclophosphamide (day 12), with daunorubicin (day 14), or with cyclophosphamide (day 12) plus daunorubicin (day 14), respectively). The simultaneous administration of cyclophosphamide and daunorubicin at day 14, induced a LCK of 2.7, a value that was the sum of the LCKs of cyclophosphamide and daunorubicin alone. Low-dose cyclophosphamide pretreatment led to an increased daunorubicin accumulation in femoral bone marrow of leukaemic rats, and was synergistic with daunorubicin.
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INTRODUCTION

IN MOST leukaemic patients some leukaemic stem cells survive current chemotherapies [1]. Theoretically, the surviving tumour cells can endure higher drug concentrations and resist treatment. However, no unifying biochemical mechanism has been identified yet, in leukaemias, that makes the cells drug resistant. The high relapse rate in patients with acute myelocytic leukaemia (AML) may also be explained by the surviving tumour cells being exposed to inappropriate drug concentrations. Indeed, in the Brown Norway rat (BNML) model, we found that femoral bone marrow accumulated less daunorubicin than normal bone marrow, especially at the stage of high leukaemic cell burden [2]. Daunorubicin, with its high affinity for cellular DNA, may be rapidly taken up and retained by the easily accessible leukaemic cells. Removal of a large mass (about 1 g) of leukaemic cells by splenectomy resulted in partial restoration of the reduced drug uptake by femoral bone marrow. Using the same animal model for human AML, we have been working on chemoreduction of tumour load before the start of the 'real' chemotherapy.

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MATERIALS AND METHODS

Chemicals

Daunorubicin, daunorubicinol, and doxorubicin were donated by Farmitalia. Cyclophosphamide was obtained from

Asta Werke (Bielefeld, F.R.G.) All reagents were of high-performance liquid chromatography (HPLC) quality and were purchased from Baker Chemicals (Deventer, Holland).

Animals

12-week-old, barrier-derived, inbred male brown Norway (BN/BiRij) rats raised in the Rijswijk colony and weighing 140–160 g were used.

Leukaemia model

We used the BNML model [3, 4]. The pharmacokinetics of daunorubicin were investigated in BNML rats at a stage of the disease comparable with that of AML patients at clinical admission (days 12–14 after intravenous transplantation of 10^7 leukaemic spleen cells). At this stage, organs such as the spleen, liver, and bone marrow are heavily infiltrated by leukaemic cells [3].

Normal haemopoietic and leukaemic stem cell assays

In a modified colony forming unit-spleen (CFU-S) assay [5], the number of CFU-S was assessed by removing the femoral bone marrow and intravenous inoculation of marrow cells into lethally irradiated mice. Between 8 and 12 days later colonies are visible on the surface of the spleen. Each colony is assumed to have been derived from a pluripotent stem cell. Stem cells producing colonies around day 8 differ from those that produce colonies around day 12, which belong to a more immature stem cell population [6]. For one stem cell assay the pooled bone marrow from eight femurs of 4 rats were used.

The number of leukaemic stem cells was measured by the leukaemic colony forming unit-spleen (LCFU-S) assay [3], which enables determination up to an 8-log leukaemic cell kill. By repeat CFU-S and LCFU-S assays at different times after drug administration, we have found a nadir at 24 h, which was thus the time we measured.

HPLC

Daunorubicin and daunorubicinol concentrations were measured by straight-phase high performance liquid chromatography (HPLC) [2]. Liver and spleen were extracted as 10% homogenates in phosphate buffer (0.05 mol/l; pH 8.5) with chloroform–methanol (4:1). 100 µl of the organic phase was injected directly into 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 per ml. 2 ml suspension was adjusted to pH 9.8 and extracted with chloroform–methanol. Extraction recovery was 85–90%. The coefficient of variation was 1–10%. Doxorubicin was used as internal standard. Each point in the results represents the mean (S.D.) of 6–8 animals.

Experimental procedures

Daunorubicin 7.5 mg/kg was administered as a single bolus in the tail vein of normal and leukaemic rats. Cyclophosphamide 15 or 30 mg/kg was administered as an intraperitoneal bolus. These drug dosages in rats are comparable to clinical doses of 40 mg/m^2 daunorubicin and 80 and 160 mg/m^2 cyclophosphamide in man [7]. The injections were done under light ether anaesthesia. At specific time (0.5, 1, 2, 4, 6, and 24 h) after drug injection, the animals were killed by exsanguination. Organs were removed, and either used immediately or rapidly cooled in liquid nitrogen and stored at −20°C.

Table 1. Effect of cyclophosphamide (CY) treatment on survival of BNML rats treated with daunorubicin (DAU) (7.5 mg/kg)

Treatment		MST*		
Day 12	Day 14	(days)		LCK†
—	—	20.8	(0.35)	—
CY (15 mg/kg)	—	24.0	(0.71)	0.80
—	CY (15 mg/kg)	24.0	(1.41)	0.80
—	DAU	23.5	(0.71)	0.68
CY (15 mg/kg)	DAU	32.0	(1.41)	2.80
—	CY (15 mg/kg) + DAU	28.0	(1.41)	1.80
CY (30 mg/kg)	—	28.0	(1.41)	1.80
—	CY (30 mg/kg)	28.5	(0.71)	1.93
CY (30 mg/kg)	DAU	42.5	(4.95)	5.43
—	CY (30 mg/kg) + DAU	31.5	(2.12)	2.68

*Mean (S.D.) median survival time (two experiments, 8 rats per group).
†Log leukaemic stem cell kill (4 days increase in lifespan = 1 LCK).

RESULTS

Tissue distribution of daunorubicin

The femoral bone marrow obtained from leukaemic rats contained less drug than that from normal rats (Fig. 1). This difference was significant ($P = 0.05$, Wilcoxon's signed-rank test) over the whole time studied, for daunorubicin as well as for daunorubicinol. Differences were also found in net drug-

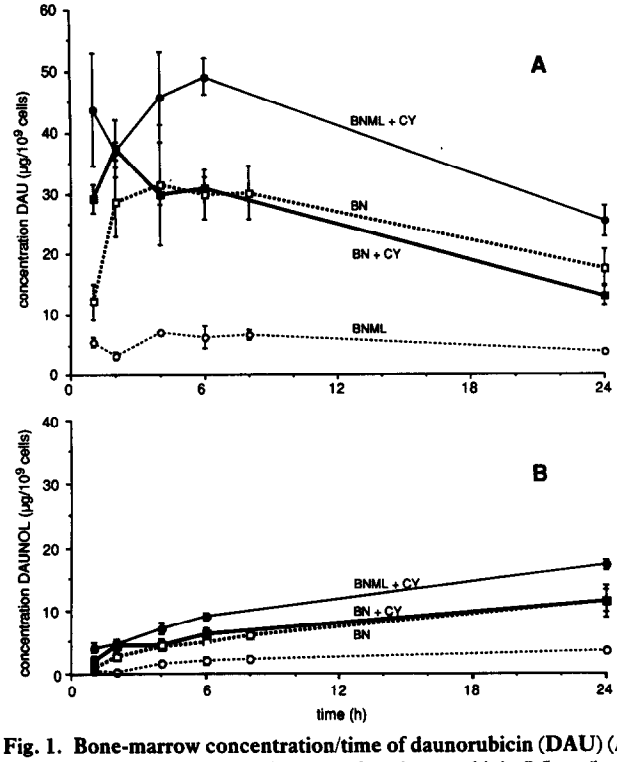

Fig. 1. Bone-marrow concentration/time of daunorubicin (DAU) (A) and daunorubicinol (DAUNOL) (B) after daunorubicin 7.5 mg/kg in normal (BN) (□– – –□) and leukaemic (BNML) rats (○– – –○) or after same dose combined with cyclophosphamide (CY) 30 mg/kg 2 days earlier in BN rats (■–■) and BNML rats (●–●). Mean and S.D.

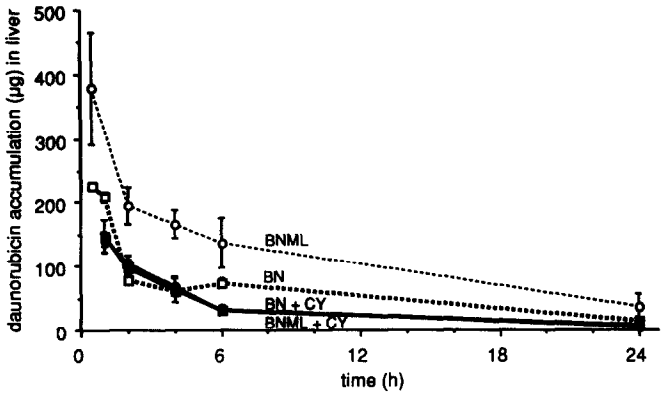

Fig. 2. Daunorubicin accumulated by liver in normal (□– – –□) and leukaemic rats (○– – –○) treated with daunorubicin or in BN rats (■–■) and BNML rats (●–●) treated with same dose after cyclophosphamide 2 days earlier.

uptake between the leukaemia-infiltrated organs (liver and spleen) and the control organs from normal rats (Figs. 2 and 3). The total amount of daunorubicin taken up and retained by the leukaemic liver and spleen was significantly increased compared with controls ($P = 0.05$, for both organs). We then investigated the effects of cyclophosphamide pretreatment on daunorubicin distribution in normal and leukaemic rats. At day 12 after transplantation of 10^7 leukaemic cells, rats were injected with cyclophosphamide 30 mg/kg. This dose resulted in a tumour load reduction of about 2 log, as estimated by prolongation in survival time (Table 1). 2 days later the leukaemic rats were injected with daunorubicin 7.5 mg/kg, and drug concentrations were measured in the bone marrow, the spleen and the liver. Cyclophosphamide pretreatment of the leukaemic rats led to a significant ($P = 0.05$) increase in daunorubicin concentrations in femoral bone marrow, by a factor of about seven (Fig. 1A). In normal rats cyclophosphamide pretreatment had no effect on daunorubicin concentrations in bone marrow. Cyclophosphamide pretreatment also had an effect on the daunorubicin content of the leukaemic liver and spleen (Figs. 2 and 3). The elevated amounts of daunorubicin accumulated by the leukaemic spleen and liver were reduced to normal levels.

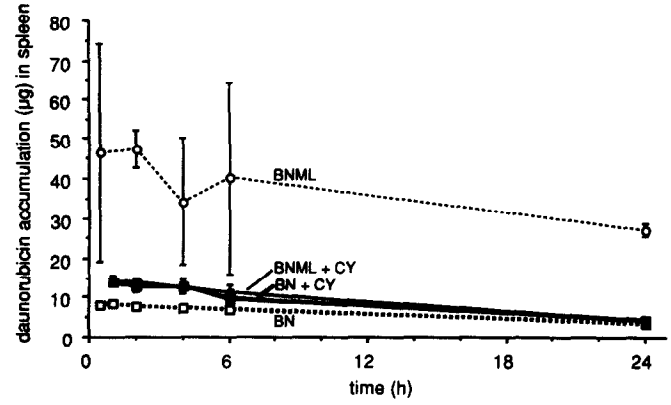

Fig. 3. Daunorubicin accumulated by spleen in normal (□– – –□) and leukaemic rats (○– – –○). Solid symbols = after cyclophosphamide pretreatment.

Cyclophosphamide pretreatment and survival

Given the relation that an increase in life-span of 4 days equals about 1 log leukaemic stem cell kill (LCK) [3], the therapeutic effects of the different chemotherapeutic treatments were calculated. Cyclophosphamide 15 or 30 mg/kg alone induced an LCK of 0.80 and 1.80 log, respectively (Table 1). Daunorubicin 7.5 mg/kg had only a marginal effect (0.68 LCK). However, cyclophosphamide pretreatment had a strong synergistic effect with daunorubicin (2.80 and 5.43 LCK, respectively). When cyclophosphamide and daunorubicin were given simultaneously on day 14, there was no synergistic effect.

The synergism of cyclophosphamide and daunorubicin was confirmed by LCFU-S assay (Fig. 4). Cyclophosphamide alone (30 mg/kg at day 12 after transplantation) reduced the number of LCFU-S per femur by 3.5 log. 1 day later, probably due to regrowth [8], the reduction in LCFU-S by cyclophosphamide was about 2 log. A subsequent injection of daunorubicin 7.5 mg/kg caused an additional 3.8 log LCFU-S kill per femur. In contrast, daunorubicin alone only led to a 0.5 log reduction in the total number of femoral LCFU-S.

Improved therapeutic index

The haematotoxic effects of the various chemotherapeutic treatments were also assessed by measuring the survival of the day 8 and day 12 CFU-S (Fig. 4B and C). Cyclophosphamide alone had hardly any effect. Daunorubicin alone induced a 1.8 LCK on the day 8 CFU-S and a 0.3 LCK on day 12. However, the combination of cyclophosphamide followed 2 days later by daunorubicin had a synergistic effect on day 12 with a lesser effect on day 8. The total LCK induced by the combination treatment on the LCFU-S, day 8 CFU-S and day 12 CFU-S was 5.7, 3.0 and 1.7, respectively. The therapeutic index (TI), as defined by LCFU-S kill divided by CFU-S kill, was increased by pretreatment with cyclophosphamide compared with daunorubicin treatment alone. The TI for daunorubicin alone was 0.3 for the day 8 CFU-S and 1.7 for the day 12 CFU-S. However, the TI for the combined treatment was 1.9 for the day 8 CFU-S and 3.4 for the day 12 CFU-S.

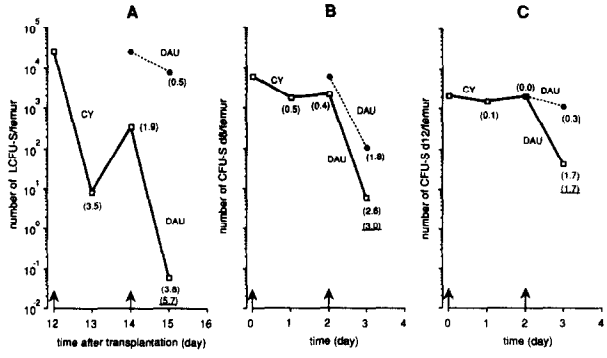

Fig. 4. Effects of daunorubicin (●– – –●), cyclophosphamide (□–□) or cyclophosphamide followed 2 days later by daunorubicin on survival of leukaemic stem cells (LCFU-S) (A), and normal stem cells (day 8 CFU-S [B], day 12 CFU-S [C]). BNML rats received cyclophosphamide at day 12 after transplantation of the leukaemic cells, daunorubicin at day 14, or combined treatment as indicated. Normal rats received cyclophosphamide at day 0 or daunorubicin at day 2, or cyclophosphamide at day 0 followed by daunorubicin at day 2. Numbers in parentheses indicate log stem cell kill. Underlined numbers in parentheses = combined treatment.

DISCUSSION

Pharmacological variables that affect the response of AML patients to chemotherapy have been studied [9, 10]. Inter-individual variability in anthracycline pharmacokinetics is considerable. However, in one study [10] it was found that the daunorubicin levels in white blood cells after intravenous administration were inversely correlated with the peripheral blast cell numbers at diagnosis: uptake by leukaemic cells was reduced when blast cell numbers were high. For uptake of daunorubicin into bone marrow, a wide heterogeneity has been observed. In patients with acute leukaemia, a high leukaemic cell load, as estimated by peripheral white cell counts, has an unfavourable prognosis [11–14]. We therefore hypothesized that the total number of malignant cells at the start of chemotherapy had an effect on the tissue and target cell distribution of the anticancer drugs. Due to the inaccessibility of most leukaemia infiltrated organs in man the number of leukaemic cells in the body is difficult to assess. In bone marrow aspirates, the lower limit of detection of leukaemic blasts is 5%, which implies that in the marrow compartment about 5×10^{10} leukaemic cells remains undetectable, if the total leukaemic cell burden at diagnosis is estimated at 10^{12}.

We used the BNML model. After bolus administration of the daunorubicin, the leukaemia-infiltrated liver and spleen accumulated much larger amounts of the drug than normal liver and spleen, while the drug concentrations in the leukaemic femoral bone marrow were reduced, which confirmed our previous report [2]. At least in rats, the bone marrow has slow net drug-uptake kinetics compared with, for example, heart tissue [15]. This finding, in combination with the rapid and increased drug accumulation by the extramedullary leukaemia-infiltrated organs, could explain low anthracycline concentrations found in leukaemic bone marrow. Reduced anthracycline drug accumulation in the bone marrow of leukaemic patients could be one of the causes for the large inter-individual variations in remission induction rates and duration.

Our data showed that decreased daunorubicin accumulation in the bone marrow could be circumvented by pretreatment aimed at the reduction of leukaemic cell load with anthracycline therapy. The pharmacokinetic data were in agreement with the survival assays, estimating overall therapeutic effect, and for leukaemic stem cell kill, which measures the effect in the femoral bone marrow compartment. With cyclophosphamide pretreatment daunorubicin bone marrow concentrations in the leukaemic rats were significantly higher than in normal rats. The most likely explanation is that leukaemic cells *in vitro* accumulate more daunorubicin than normal haemopoietic cells do [2], and *in vivo* the leukaemia-infiltrated femoral marrow can also take up larger amounts of drug; and by killing off leukaemia cells in organs such as spleen and liver and to a lesser extent in the femoral bone marrow by cyclophosphamide, more daunorubicin is available for the residual leukaemia cells in bone marrow.

Cyclophosphamide pretreatment at day 12 led to an increase in daunorubicin concentration in the femoral bone marrow (by a factor of about 7) and to strong potentiation of LCK by daunorubicin. However, simultaneous administration of cyclophosphamide and daunorubicin at day 14 had only an additive effect on LCK, as estimated by survival assay. These observations suggested that the increased daunorubicin concentrations in the femoral bone marrow after cyclophosphamide pretreatment were responsible for the increased therapeutic effect of daunorubicin. The anti-leukaemic effect of the combination of both drugs was more than additive and, due to the low toxicity to normal stem cells (especially the more primitive day 12 CFU-S), the therapeutic index was improved.

Extrapolation to man indicates that pretreatment of full-blown leukaemia at diagnosis with low-dose effective chemotherapy may increase the efficacy of subsequent anthracycline-containing remission induction chemotherapy.

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