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Drastic effect of cell density on the cytotoxicity of daunorubicin and cytosine arabinoside

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Abstract

White blood cell count (WBC) is generally accepted as a prognostic risk factor in acute myeloid leukemia (AML) outcome and displays a marked interindividual variation. The dose regimen currently used ignores the size of the tumor burden and the standardization of the dose is generally based on body surface area. In this study we have investigated the effect of cell density on the cytotoxic activity of daunorubicin (DNR) and cytosine arabinoside (AraC) towards HL60 cells and leukemic cells isolated from patients with AML. We demonstrate that drug cytotoxicity decreased with cell density and that apoptosis induction by DNR in isolated leukemic cells was greatly reduced at higher cell density. A marked reduction of the uptake of DNR and AraC in HL60 parental and mitoxantrone resistant cells was observed with increasing cell density. Such a drug depleting effect by cells at high density has been previously described for vincristine, doxorubicin and paclitaxel. By extrapolating the in vitro results to the in vivo situation, one could hypothesize that a high WBC can lower the plasma concentration through high uptake in the tumor burden, leading to a shortage of drug in leukemic blasts. Patients with high WBC might therefore benefit from a dose increase of DNR and/or AraC.

Keywords: Daunorubicin (DNR); Cytarabine (AraC); Acute myeloid leukemia; Cell count

1. Introduction

Over the last two decades new strategies such as better supportive measures, variation in agents, dose intensity and bone marrow transplantation have significantly improved the outcome of acute leukemia. However, the benefits of high dose induction therapy should be balanced against the increase in toxicity and the risk of treatment-related death [1,2]. In an attempt to identify the patients who represent the best target for a more intensive therapy, several groups have studied important prognostic factors associated with treatment success or failure. Cytogenetic data has gained more and more support in allowing the assignment of patients in different prognostic groups. Age and peripheral blast count have been identified as the most significant pre-treatment risk factors in many studies [3]. From these data it can be concluded that an elevated blast count at treatment induction results in a worse outcome. This is most probably due to the fact that patients with a high WBC have genetic mutations leading to a high cell proliferation rate. For instance, fms-like tyrosine kinase 3 (FLT3) internal tandem duplications (FLT3-ITDs) that are present in nearly 25% of patients with acute myeloid leukemia (AML) and have been associated with poor outcome are also associated with high WBC [4]. If an elevated WBC reflects a proliferative state that has escaped the normal apoptosis mechanism, it would by itself hinder a successful treatment.

The interindividual differences in WBC at diagnosis are marked, ranging from leukopenia to more than 400 million cells/ml blood. Two important questions arise: Has the blast cell count an effect on the cytotoxicity of common treatment agents used in acute leukemia chemotherapy? Secondly, should the drug dose be adjusted to compensate for increase in tumor burden size?

The role of cell density in the cytotoxicity of some antitumoral agents used in cancer chemotherapy has been studied previously by Kobayashi et al. who named this effect the ''inoculum effect'' [5]. A negative correlation between the cell density of Molt-3 cells, an ALL cell line, and the cytotoxic activity of doxorubicin (Dox) and vincristine (Vcr) was observed. A mechanism was proposed

Abbreviations: AraC, cytosine arabinoside; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; DNR, daunorubicin; WBC, white blood cell count.

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where the inoculum effect is the result of the unavailability of the drug [6]. Such an in vitro reduction of uptake at high cell density was not observed for cisplatin in Molt-3 cells [7]. A negative effect of cell density on paclitaxel uptake was also observed by Kuh et al.; they proposed a mathematical model for computing intracellular pharmacokinetics of paclitaxel, where predicted and experimental data were in good agreement [8].

Daunorubicin (DNR) and cytosine arabinoside (AraC) have been included for the past three decades in most effective regimens to treat adults and children with AML and the drugs are still first-line agents. In this study we have investigated the effect of cell density on their cytotoxic activities towards HL60 cells, a promyelomonocytic leukemia and leukemic cells isolated from patients with AML. We demonstrate that their cytotoxicity decreased with cell density and that apoptosis induction by DNR in isolated leukemic cells was greatly reduced at higher cell density. Finally, a marked effect of cell density was observed on the uptake of DNR and AraC in HL60 cells.

2. Materials and methods

2.1. Materials

AraC was purchased from Sigma (St. Louis, MO, USA) and DNR was from Rhône Poulenc. Tritium labeled AraC ([5-^{3}H]AraC, 1.2 TBq/mmol) was from Amersham, UK.

Roswell Park Memorial Institute (RPMI)-1640 medium, fetal calf serum, L-glutamine and penicillin-streptomycin were from Gibco (Life Technologies, Paisley, UK).

2.2. Clinical samples

Heparinized peripheral blood was obtained from patients with AML. Mononuclear cells were isolated by centrifuging 5 ml blood on 3 ml Lymphoprep (Nycomed, Norway) at 550 × *g* for 15 min at 4 °C. After two washes with PBS the cell number was determined using a Coulter counter Z2 (Beckman Coulter, Fullerton, CA, USA) and the cells were incubated with drugs for cytotoxicity determination or drug uptake at various cell densities. The study was approved by the ethical committee at Karolinska Institute.

2.3. Cell lines and drug uptake studies

The human promyelocytic leukemia cell line HL60 and the mitoxantrone resistant subline HL60/Nov were kind gifts from Dr. Astrid Gruber (Department of Hematology and Infectious disease, Karolinska Hospital, Sweden). Drug resistance was developed by continuous exposure to increasing concentrations of mitoxantrone and cells were maintained in medium containing 100 ng/ml mitoxantrone (Wyeth, Lederle, USA). The cells were cultured twice a week in growth medium consisting of RPMI 1640 cell culture medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml).

In order to study the cellular uptake of DNR and AraC, exponentially growing cells in growth medium containing HEPES were plated at various densities and incubated with 1.2 μM DNR for 2 h or 1 μM tritiated AraC for 24 h. Aliquots of medium were removed after various time intervals. Cells were centrifuged and the medium was kept on ice or frozen until drug analysis. The cell pellets were washed twice with ice-cold PBS and frozen until drug analysis.

2.4. Drug analysis

Cells were lysed in ice-cold water, sonicated for 5 s using a ultrasonic processor, VCX 400 (Sonics & Materials, Danbury, CO, USA), and extracted with 50% acetonitrile for DNR analysis. DNR concentration in cells and medium was determined by HPLC using a phenyl-μ-Bondapak column (3.9 mm × 150 mm, 5 mm, Waters Associates, Milford, MA) eluted with acetonitrile and 0.2% ammonium formate pH 4 (50:50 v/v) at a flow rate of 1 ml/min. The drug was quantified using a Gilson Model FL-1B fluorescence spectrophotometer. AraC concentration in cells and medium was obtained by measuring tritium radioactivity in a liquid scintillation counter (RackBeta, LKB Wallac, Turku, Finland) in 10 ml Ecoscint scintillation fluid.

2.5. Cytotoxicity assay

Cytotoxicity was assessed using a modified MTT-microcultured tetrazolium colorimetric dye reduction assay described by Mosmann [9]. Briefly, cell lines in exponential growth or isolated leukemic cells from AML patients were collected and 50 μl aliquots of the cell suspension were dispensed at various cell densities in 96-well round-bottomed microplates containing 50 μl of DNR or AraC dilutions in sex replicate rows. For pulse incubations the cells were washed twice with 200 μl ice-cold PBS after a 2 h incubation at 37 °C, and incubated further in 100 μl growth medium. Plates were incubated in a humidified incubator in 5% CO_2 for 3 days at 37 °C. Then 10 μl of MTT (3-[4,5-dimethylthiazol-2-y1]-2,5-diphenyl tetrazoliumbromide) (Sigma-Aldrich Corp.) solution (5 mg/ml in PBS) was added to each well and the plates were incubated for 4 h at 37 °C. Formazan crystals were dissolved with 100 μl 10% SDS in 100 mM HCl followed by overnight incubation at 37 °C. Absorbance of the formazan product was measured at 540 nm with reference at 650 nm in an ELISA plate reader (Labsystems Multiscan RC, Helsinki, Finland). The optical density (OD) is linearly related to the number of viable cells. A survival index was calculated at each drug concentration as a mean of six values by the

following equation: (OD treated well/mean OD control wells) × 100%, after correction for the background OD of the wells with culture medium alone. The $IC_{50,}$ which is the drug concentration that kills 50% of the cells, was determined graphically and used as a measure of resistance.

2.6. DNA fragmentation analysis

Estimation of the number of cells in G1, S and G2/1 phase and the amount of DNA fragmentation was performed by propidium iodide (PI) staining and flow cytometry analysis as described [10]. Following incubation of leukemic cells with DNR, cells were pelleted and resuspended in PBS containing propidium iodide (50 μg/ml), 0.1% Triton X-100 and 0.1% sodium citrate. Samples were analyzed using a FACScan flow cytometer (Becton and Dickinson, Mountain View, CA, USA) with a single 488 nm Argon laser. The red fluorescence due to PI staining of DNA was detected in the FL-3 channel and the data were registered on a logarithmic scale. The flow rate was set at 12 μl/s and 10^4 events were collected for each sample. Analysis was performed using the Cell Quest™ software.

3. Results

3.1. Cell density DNR apoptosis induction and cytotoxicity

The influence of cell density on the cytotoxic effect of DNR was studied 3 days after a 2-h pulse incubation of HL60 cells and leukemic cells isolated from a patient with AML.

As illustrated in Fig. 1a, a pronounced reduction in DNR cytotoxicity towards HL60 cells and AML cells was observed with increasing cell density. Indeed under these conditions the IC_{50} of DNR increased linearly with cell density for both types of cells and was most pronounced for the AML cells. Similar effects of cell density were obtained when DNA fragmentation, induced by DNR in isolated leukemic cells from a patient with AML, was measured. As shown in Fig. 1b, the induction of apoptosis in cells at a density of 10 million cells/ml required much higher drug concentrations than cells at a density of 1 million/ml.

3.2. Cell density and DNR uptake

In order to investigate the mechanism behind the effect of cell density on DNR toxicity we studied the uptake of DNR in HL60 cells at different cell densities. The high binding of DNR by the cells caused a depletion of DNR in the medium. As illustrated in Fig. 2a, the concentration of DNR in the cells at the end of the 2 h incubation decreased markedly with cell density. It amounted to 520 μM at a cell density of 0.5 million/ml and was about five times lower at a cell density of 8 million/ml. In other words, at low cell density the drug was able to concentrate 400 times in the cells in contrast to 80 times at a cell density of 8 million cells/ml. At low cell density DNR cellular uptake represented 14% of the DNR added to the medium, and at 4 million cells/ml it amounted to nearly 50%. In the medium, the drug concentration decreased drastically with cell density, amounting to close to 30% of the initial DNR concentration at a density of 8 million cells/ml (Fig. 2b).

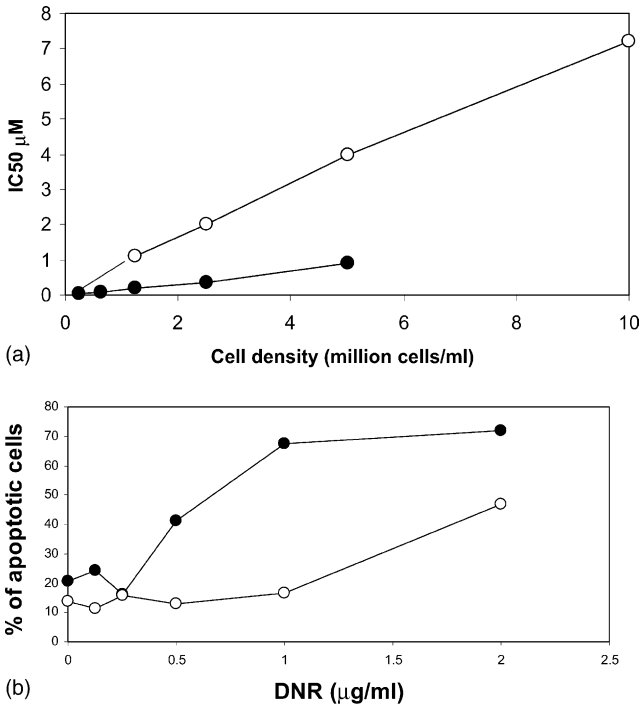

Fig. 1. Relationship between cell density and DNR cytotoxicity/apoptosis induction in HL60 cells and isolated AML cells. (a) IC_{50} values were determined after a 2 h pulse incubation of HL60 cells (filled circles) and isolated AML cells (open circles) with DNR followed by a 3-day incubation in drug free medium. Graphic determination from survival index data calculated as a mean of six determinations. (b) DNA fragmentation (in percent of initial value) was determined in AML cells at two cell densities, 1 million cells/ml (filled circles) and 10 million cells/ml (open circles), after a 2 h pulse incubation with various DNR concentrations followed by a 22 h incubation in drug free medium.

In order to clarify whether the results could be explained by DNR distribution effects alone we calculated, in analogy to a lipid–water partition coefficient, a drug distribution coefficient, defined as the ratio between DNR concentration in cells and in medium. This coefficient, determined with the 2 h uptake data and presented in Fig. 3, was close to 500 and similar at different cell densities.

3.3. Cytotoxicity of DNR and AraC towards parental and resistant HL60 cells at different cell densities

The cytotoxicity of DNR and AraC was determined in HL60 cells and the mitoxantrone resistant subline HL60/Nov in order to investigate if a cell density effect was also

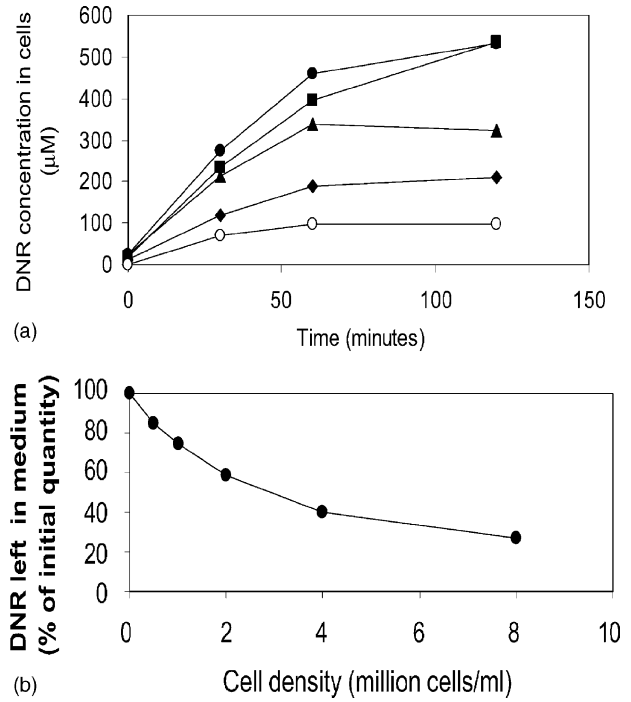

Fig. 2. Effect of cell density on DNR uptake in HL60 cells. (a) Time course for DNR concentration during a 2-h incubation of HL60 cells with 1.2 μM drug at different cell densities, 0.5 million cells/ml (filled circles), 1 million cells/ml (filled squares), 2 million cells/ml (filled triangles), 4 million cells/ml (filled losanges), and 8 million cells/ml (open circles). (b) Percentage of DNR left in the medium at different cell densities after 2 h.

applicable to a resistant cell line expressing the breast cancer resistance protein BCRP [11].

The cytotoxic effect of DNR and AraC towards HL60 and HL60/Nov cells was markedly reduced when the cell density increased as depicted in Fig. 4 which relates the IC_{50} to cell density. For DNR, the IC_{50} increased similarly with cell density in the parental and the resistant cells; in contrast, the IC_{50} of AraC increased more with cell density in the resistant HL60/Nov subline than in the parental cell line.

3.4. Uptake of AraC in HL60 and HL60/Nov cells at different cell densities

To investigate the mechanism behind the effect of cell density on AraC toxicity and to understand the marked effect of cell density on AraC toxicity towards HL60/Nov, we compared the uptake of 1 μM ^{3}H-AraC in HL60 parental and resistant cells (Fig. 5a and b). In both cell lines the uptake pattern was fairly similar with a rapid initial uptake phase that was slower at higher cell densities. Cellular drug uptake culminated at a value that was lower at higher cell densities; the uptake of AraC in resistant cells was slower and culminated at a lower level than in the parental cells. The initial uptake phase slowed down to a plateau and finally the radioactivity levels decreased in the cells. After 24 h the radioactivity levels decreased in the parental and in the resistant cells to 67 and 58%, respectively, of the maximal uptake value in low-density cells and to 10% for both cell lines at high density. The radioactivity leaking from the cells was recovered in the medium, as illustrated in Fig. 5c and d. At high cell density the radioactivity in the medium decreased and only 37 and 47% of the initial quantity were detected after 2 h incubation in the parental and resistant cells, respectively. Thereafter, the radioactivity increased gradually in the medium and reached 79 and 82%, respectively, after 24 h. As for DNR, we calculated a drug distribution coefficient for AraC in HL60 and HL60/Nov cells at 2, 6 and 24 h (Fig. 6). The 2 h coefficient was fairly constant for all the three densities; however after 6 and 24 h it was markedly reduced with increasing densities. The pattern

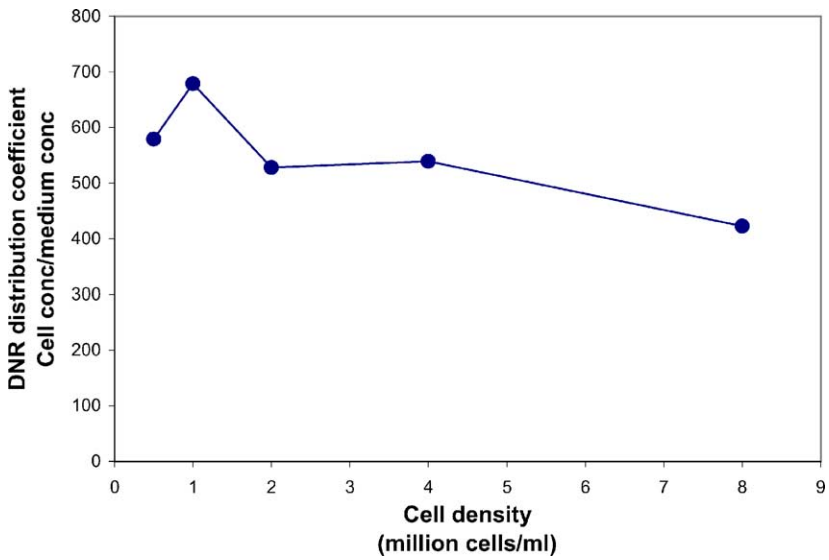

Fig. 3. DNR distribution coefficient in HL60 cells. The ratio between DNR concentration in the cells and medium was determined after a 2-h incubation of HL60 cells at different cell densities with 1.2 μM DNR. Calculated from values of Fig. 2 experiment.

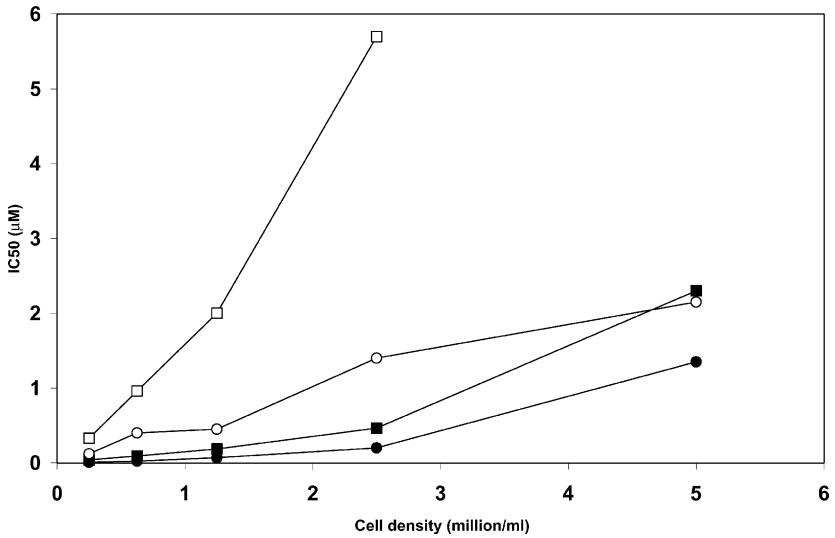

Fig. 4. DNR and AraC cytotoxicity towards HL60 and HL60/Nov cells. Effect of cell density (HL60 cells, filled symbols and HL60/Nov cells, open symbols) on the IC_{50} values of DNR (circles) and AraC (squares) after a 3-day continuous incubation. Graphic determination from survival index data calculated as a mean of six determinations.

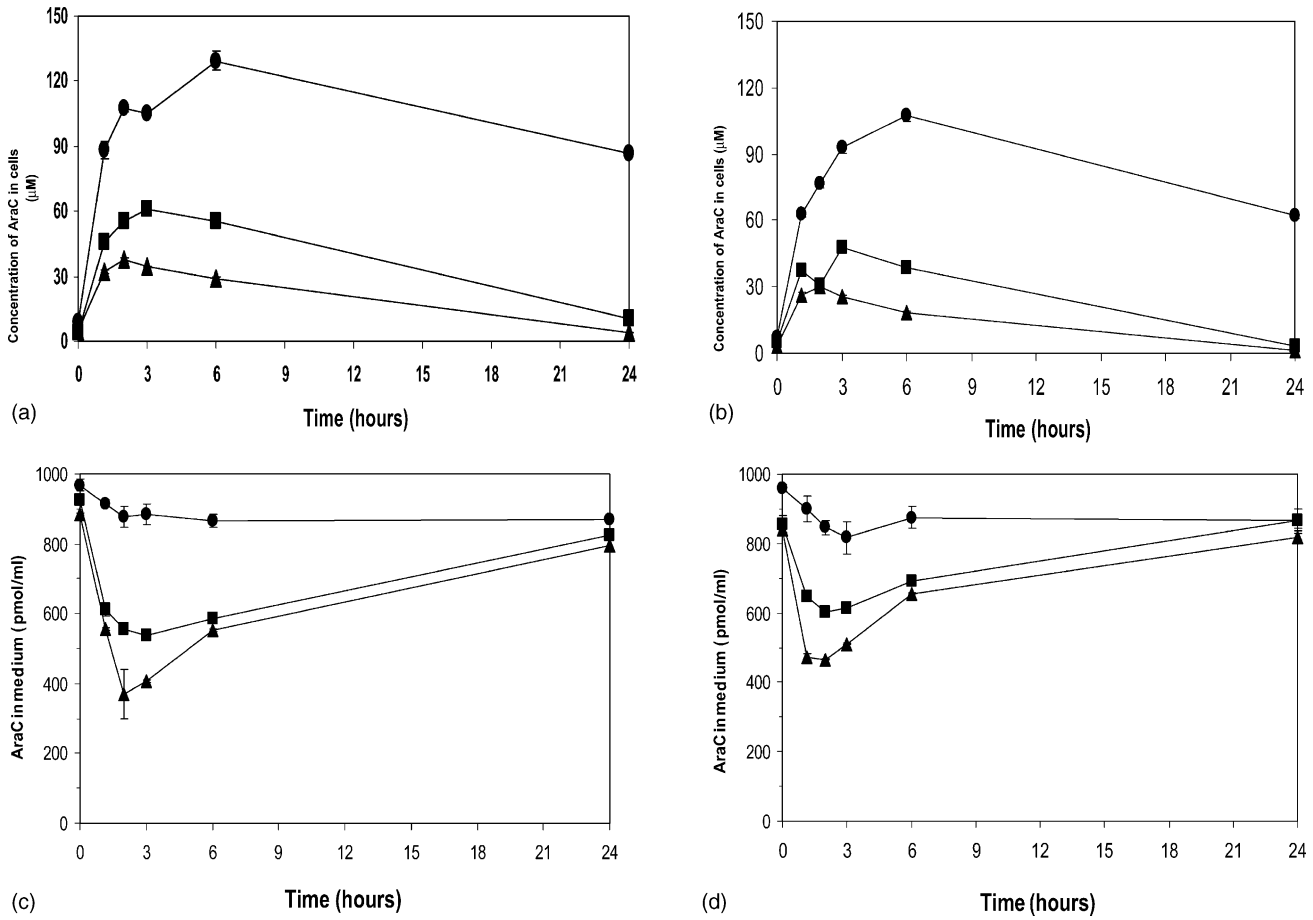

Fig. 5. Effect of cell density on AraC uptake in HL60 and HL60/Nov cells. Time course for cellular drug uptake (a and b) and drug concentration in medium (c and d) during a 24 h incubation of HL60 cells (a and c) and HL60/Nov cells (b and d) with 1 μM ^{3}H-AraC at three different cell densities, 1 million cells/ml (circles), 10 million cells/ml (squares), and 20 million cells/ml (triangles). Mean and S.D. determination of triplicate.

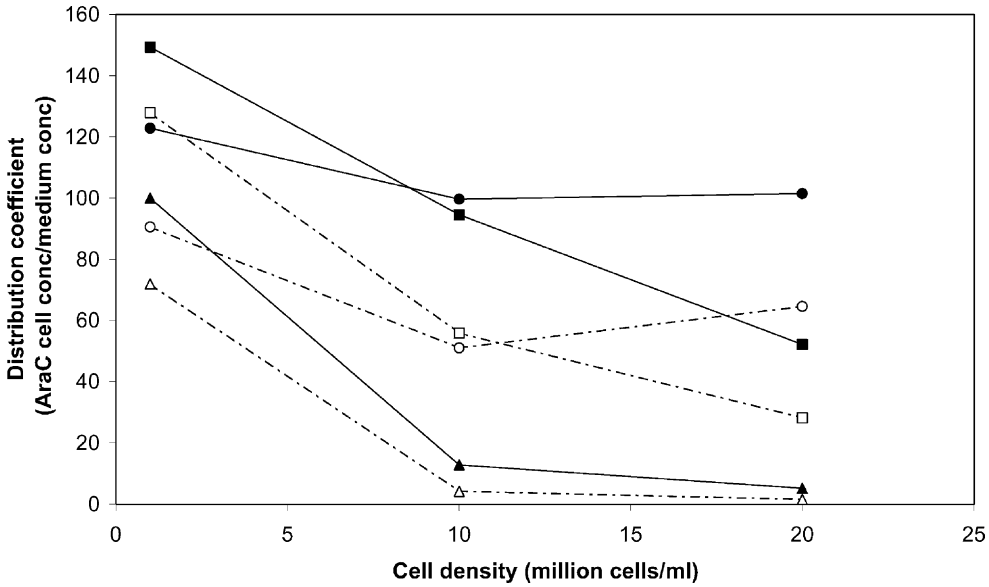

Fig. 6. AraC distribution coefficient in HL60 and HL60/Nov cells. The ratio between radioactivity concentration in the cells and in the medium was determined after 2 h (circles), 6 h (squares) and 24 h (triangles) of incubation of HL60 cells (filled symbols) and HL60/Nov cells (open symbols and dotted lines) with 1 μM ^{3}H-AraC at three different cell densities. Calculated from mean values of Fig. 5 experiment.

was fairly similar in the parental and the resistant cell lines, except for a lower distribution coefficient in the resistant cell line.

4. Discussion

In leukemia, several pharmacokinetic studies have been designed with the hope of correlating drug plasma concentrations to treatment outcome and the results have been quite disappointing [12–14]. The high variability in drug plasma concentration is certainly due to interindividual variations in drug metabolism but could possibly also partly reflect variation in WBC at treatment induction.

The impact of WBC on leukemia outcome has been described in many studies and is generally accepted as a significant prognostic risk factor in ALL and AML [15]. In AML patients carrying the t(8;21) translocation, one of the most recurrent chromosomal abnormalities, WBC was the only identified prognostic factor [16]. Moreover, the WBC index, defined as the product of WBC by the ratio of marrow blasts, was an even more powerful factor. Using multivariate analysis, the WBC index was the only prognostic factor for disease-free survival, complete remission duration, and overall survival.

The current study highlights the large capacity of leukemic cells to take up cytotoxic drugs, such as DNR and AraC, to such an extent that they deplete the medium of the drugs. The marked decrease in cellular uptake of DNR is most likely the main reason underlying the clear reduction in cytotoxic effect and apoptosis induction at high cell density. The drug distribution coefficient data showing a constant ratio between DNR medium and cellular concentration support the notion that the reduced cell uptake observed at high density is mainly caused by drug distribution effects. The high cellular volume of distribution of DNR is due to extensive intracellular binding to DNA and sequestration in acid vesicles. However, drug distribution effects for AraC did not seem to be the sole mechanism and drug was also depleted by deamination. The deamination kinetics are dependent on the total number of cells/ml and an increase in cell number results in increased cellular deaminating capacity [17]. This is illustrated by our uptake studies of AraC in HL60 parental and HL60/Nov cells: the drug distribution coefficients of tritiated AraC were not constant with cell density for incubation longer than 2 h. The decreased cellular AraC uptake associated with high-density cell incubations was most likely caused by its transformation into the inactive metabolite AraU that subsequently diffused back to the medium. Moreover as the cellular deaminating capacity is saturable, the AraC concentration also has an impact on its deamination [17], and a reduction in AraC concentration could therefore result in an increased deamination rate. In this study we have also determined that the relation between cell density and DNR/AraC cytotoxicity also applied to the resistant cell line HL60/Nov. The lower cytotoxicity of AraC in these cells, as compared with the parental line, was not caused by an increased deamination activity but was rather related to a decreased cellular uptake. The relationship between cytotoxicity and cell density is an interesting observation to keep in mind for in vitro studies. It is relevant for relating experiments using different cell densities to each other, as well as to the understanding of different concentration ranges between in vitro and in vivo settings.

On the basis of our results we hypothesize that a high WBC at diagnosis could affect the success of induction treatment by causing a reduction of drug concentration in plasma through a high uptake in the tumor burden, lowering the concentration of drug in the leukemic blasts and thus reducing the chances for the patient to enter a remission. For AraC an increased burden of leukemic cells may also accelerate the deamination of the drug. Leukemic blasts have been noted to possess significant deaminase activity [18] but in contrast to cell lines freshly isolated leukemic blasts featured a low AraC deamination rate in model system [17]. We are aware that it is difficult to extrapolate in vitro data to the in vivo situation. The apparent volume of distribution of anthracyclines, such as DNR, is very high (20–40 l/kg) so even if the tumor cells are well accessible and represent up to several hundred grams, the major part of DNR should distribute rapidly to peripheral compartments such as liver, lungs, kidney, spleen and heart. Consequently drug uptake in leukemic cells should have a minor effect on DNR plasma concentration. Nevertheless, estimations based on in vitro and in vivo data support that leukemic cells in blood take up a significant amount of DNR. Indeed, HL60 cells and isolated AML cells incubated for 1 h with 1 μg/ml DNR take up approximately 0.2 and 0.1 μg/million cells, respectively. Considering an AML patient with a WBC of $100 \times 10^9\ l^{-1}$ receiving a DNR dose of 1.5 mg/kg (total dose: 105 mg for a 70 kg patient), assuming a plasma DNR concentration of 0.25 μg/ml [19] and a blood volume of 5 liters (0.5×10^{12} cells in total), it can be calculated that the uptake of DNR in circulating leukemic cells would lie in the range of 12.5–25 mg. This is quantitatively important compared to the total dose administered. Moreover, this estimation does not take into account DNR taken up by the leukemic cell population in the bone marrow.

Similar figures are also obtained by using in vivo cellular uptake data. Pharmacokinetic studies of DNR in AML patients treated with 60 mg/m^2 [20] reported a cellular DNR peak concentration between 0.2 and 0.5 nmol/mg protein, which corresponds to 10–30 mg DNR in the circulating leukemic cells assuming a WBC of $100 \times 10^9\ l^{-1}$ and 0.2 mg protein/million cells.

Such an effect of the WBC on anthracycline cellular uptake was observed in an in vivo study in leukemic patients [21]. However, the study was too small to be conclusive and compared patients with different types of leukemia. In a large pharmacokinetic study of doxorubicin in children with ALL, the patients with WBC $>50\times10^9\ l^{-1}$ at diagnosis had significantly lower doxorubicin concentrations (median 55.3 ng/ml) than those with WBC $<10\times10^9\ l^{-1}$ (median 64.4 ng/ml, $P = 0.015$) [22]. The importance of a WBC reduction to optimize induction treatment is supported by studies describing an improvement in leukemia treatment due to dose intensification. High dose AraC/DNR induction treatment led to high complete remission rates for adult patients with AML and this high dose schedule appeared to overcome poor risk features such as high WBC at presentation [23]. In adult ALL the outcome was improved by a dose intensification treatment including a pre-induction course of AraC and etoposide for patients presenting a WBC equivalent to or superior to $30 \times 10^9\ l^{-1}$ at diagnosis [24]. For patients with WBC $>100\times10^9\ l^{-1}$, this approach effectively reduced the WBC and the majority of patients attained a complete remission. Further dose intensification seemed necessary however in view of the high relapse rate in this group. Pre-treatment with AraC and teniposide of children with ALL with WBC $>100\times10^9\ l^{-1}$ [25], or with methotrexate also [26] improved the outcome.

These observations indicate that patients with high WBC might benefit from a dose increase and that an optimal administration schedule should be evaluated properly for treatment with DNR and/or AraC. The relationship between WBC and DNR and AraC plasma and cell concentrations deserves further study aiming to improve and individualize the current dosing regimen.

Acknowledgments

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References

[1] Burnett AK, Goldstone AH, Stevens RM, Hann IM, Rees JK, Gray RG, et al. Randomised comparison of addition of autologous bone-marrow transplantation to intensive chemotherapy for acute myeloid leukaemia in first remission: results of MRC AML 10 trial. UK Medical Research Council Adult and Children's Leukaemia Working Parties. Lancet 1998;351(9104):700–8.

[2] Weick JK, Kopecky KJ, Appelbaum FR, Head DR, Kingsbury LL, Balcerzak SP, et al. A randomized investigation of high-dose versus standard-dose cytosine arabinoside with daunorubicin in patients with previously untreated acute myeloid leukemia: a Southwest Oncology Group Study. Blood 1996;88(8):2841–51.

[3] Matthews JP, Bishop JF, Young GA, Juneja SK, Lowenthal RM, Garson OM, et al. Patterns of failure with increasing intensification of induction chemotherapy for acute myeloid leukaemia. Br J Haematol 2001;113(3):727–36.

[4] Boissel N, Cayuela JM, Preudhomme C, Thomas X, Grardel N, Fund X, et al. Prognostic significance of FLT3 internal tandem repeat in patients with de novo acute myeloid leukemia treated with reinforced courses of chemotherapy. Leukemia 2002;16(9):1699–704.

[5] Kobayashi H, Takemura Y, Ohnuma T. Relationship between tumor cell density and drug concentration and the cytotoxic effects of doxorubicin or vincristine: mechanism of inoculum effects. Cancer Chemother Pharmacol 1992;31(1):6–10.

[6] Kobayashi H, Takemura Y, Holl JF, Ohnuma T. Vincristine saturation of cellular binding sites and its cytotoxic activity in human lymphoblastic leukemia cells: mechanism of inoculum effect. Biochem Pharmacol 1998;55(8):1229–34.

[7] Takemura Y, Kobayashi H, Miyachi H, Hayashi K, Sekiguchi S, Ohnuma T. The influence of tumor cell density on cellular accumula-

tion of doxorubicin or cisplatin in vitro. Cancer Chemother Pharmacol 1991;27(6):417–22.
[8] Kuh HJ, Jang SH, Wientjes MG, Au JL. Computational model of intracellular pharmacokinetics of paclitaxel. J Pharmacol Exp Ther 2000;293(3):761–70.
[9] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65(1/2):55–63.
[10] Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods 1991;139(2):271–9.
[11] Austin Doyle L, Ross DD. Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2). Oncogene 2003;22(47): 7340–58.
[12] Speth PA, van Hoesel QG, Haanen C. Clinical pharmacokinetics of doxorubicin. Clin Pharmacokinet 1988;15(1):15–31.
[13] Balis FM, Holcenberg JS, Poplack DG, Ge J, Sather HN, Murphy RF, et al. Pharmacokinetics and pharmacodynamics of oral methotrexate and mercaptopurine in children with lower risk acute lymphoblastic leukemia: a joint children's cancer group and pediatric oncology branch study. Blood 1998;92(10):3569–77.
[14] Fleming RA, Capizzi RL, Rosner GL, Oliver LK, Smith SJ, Schiffer CA, et al. Clinical pharmacology of cytarabine in patients with acute myeloid leukemia: a cancer and leukemia group B study. Cancer Chemother Pharmacol 1995;36(5):425–30.
[15] Friedmann AM, Weinstein HJ. The role of prognostic features in the treatment of childhood acute lymphoblastic leukemia. Oncologist 2000;5(4):321–8.
[16] Nguyen S, Leblanc T, Fenaux P, Witz F, Blaise D, Pigneux A, et al. A white blood cell index as the main prognostic factor in t(8;21) acute myeloid leukemia (AML): a survey of 161 cases from the French AML Intergroup. Blood 2002;99(10):3517–23.
[17] Braess J, Pfortner J, Kern W, Hiddemann W, Schleyer E. Cytidine deaminase—the methodological relevance of AraC deamination for ex vivo experiments using cultured cell lines, fresh leukemic blasts, and normal bone marrow cells. Ann Hematol 1999;78(11): 514–20.
[18] Mancini WR. The role of deoxycytidine-metabolizing enzymes in the cytotoxicity induced by 3′-amino-2′,3′-dideoxycytidine and cytosine arabinoside. Cancer Chemother Pharmacol 1992;30(2):139–44.
[19] Gewirtz DA. A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. Biochem Pharmacol 1999;57(7):727–41.
[20] Paul C, Liliemark J, Tidefelt U, Gahrton G, Peterson C. Pharmacokinetics of daunorubicin and doxorubicin in plasma and leukemic cells from patients with acute nonlymphoblastic leukemia. Ther Drug Monit 1989;11(2):140–8.
[21] Tapiero H, Munck JN, Fourcade A. Relationship between the intracellular accumulation of anthracyclines and effectiveness in vitro and in vivo. Drugs Exp Clin Res 1986;12(1–3):257–64.
[22] Frost BM, Eksborg S, Bjork O, Abrahamsson J, Behrendtz M, Castor A, et al. Pharmacokinetics of doxorubicin in children with acute lymphoblastic leukemia: multi-institutional collaborative study. Med Pediatr Oncol 2002;38(5):329–37.
[23] Stein AS, O'Donnell MR, Slovak ML, Nademanee A, Dagis A, Schmidt GM, et al. High-dose cytosine arabinoside and daunorubicin induction therapy for adult patients with de novo non M3 acute myelogenous leukemia: impact of cytogenetics on achieving a complete remission. Leukemia 2000;14(7):1191–6.
[24] Daenen S, van Imhoff GW, van den Berg E, de Kam PJ, Haaxma-Reiche H, Vellenga E, et al. Improved outcome of adult acute lymphoblastic leukaemia by moderately intensified chemotherapy which includes a 'pre-induction' course for rapid tumour reduction: preliminary results on 66 patients. Br J Haematol 1998;100(2): 273–82.
[25] Dahl GV, Rivera GK, Look AT, Hustu HO, Kalwinsky DK, Abromowitch M, et al. Teniposide plus cytarabine improves outcome in childhood acute lymphoblastic leukemia presenting with a leukocyte count greater than or equal to 100×10^9/l. J Clin Oncol 1987;5(7): 1015–21.
[26] Niemeyer CM, Gelber RD, Tarbell NJ, Donnelly M, Clavell LA, Blattner SR, et al. Low-dose versus high-dose methotrexate during remission induction in childhood acute lymphoblastic leukemia (protocol 81-01 update). Blood 1991;78(10):2514–9.