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PHARMACODYNAMICS

Uptake of anthracyclines in vitro and in vivo in acute myeloid leukemia cells in relation to apoptosis and clinical response

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Abstract
Aims To study anthracycline-induced apoptosis in leukemic cells isolated from patients with acute myelogenous leukemia (AML) in vitro and to compare intracellular anthracycline concentrations causing apoptosis in vitro with those obtained in vivo during anthracycline treatment.
Methods Mononuclear blood cells from AML patients were isolated before (n=20) and after anthracycline infusion (n=24). The pre-treated cells were incubated in vitro with daunorubicin (DNR) and/or idarubicin (IDA). Anthracycline concentrations were determined by high-performance liquid chromatography, and apoptosis was detected by propidium iodine staining using a flow cytometer.
Results There was a clear concentration–response relationship between intracellular anthracycline levels and apoptosis albeit with a large interindividual variation. Intracellular levels >1200 μM always led to high apoptosis development (>60%) in vitro. The intracellular concentrations of DNR in vivo (n=24) were more than tenfold lower than the concentrations needed to induce effective apoptosis in vitro, although a significant relation between in vivo concentrations and clinical remission was found. We also found a significant relation between apoptosis induction in leukemic cells by IDA in vitro and clinical remission.
Conclusions Our results indicate that intracellular anthracycline levels in vivo are suboptimal and that protocols should be used that increase intracellular anthracycline levels.

Keywords Anthracyclines · Apoptosis · Leukemia

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Introduction

Acute myelogenous leukemia (AML) is a blood disorder characterized by the clonal expansion of immature myeloid progenitor cells. Treatment involves intensive chemotherapy to eliminate the leukemic cell population. At the present time, almost all standard induction treatment protocols include anthracyclines, generally daunorubicin (DNR) or idarubicin (IDA) in combination with cytarabine. These achieve a complete remission rate of 50–60% in an unselected population and a long-term survival of only 10–20% [1]. It has been claimed that IDA, which is more potent in vitro, is more efficient than DNR in terms of increasing the complete remission rate, prolonging remission duration, and improving survival in AML [2, 3]. However, the therapeutic superiority of IDA has not yet been established [4].

The target for cancer chemotherapy is the tumor cell. An important factor affecting therapeutic activity is the grade of exposure of the cells to the drug. Once the drug has reached the tumor cells, other factors, such as the duration of exposure, transmembrane uptake, intracellular retention, heterogeneity of the tumor cells, affinity of the drug for the target, amount of the target intracellularly, amount of

competing natural substrates, and metabolic transformation (activation or detoxification), will influence the antitumor effect.

Previous studies have shown that there is no clear relationship between the concentration of anthracyclines in plasma and those in leukemic cells [5, 6]. Although anthracyclines have been studied for more than four decades, a clear correlation between their pharmacokinetics and therapeutic effect has not been shown, with exception of one study reporting a relationship between plasma concentrations of doxorubicin and the outcome of induction therapy [7].

It has been suggested that anthracycline cytotoxicity involves several mechanisms, with the most important believed to be DNA adduct formation, interference with DNA topoisomerases, and the formation of reactive oxygen species. Anthracyclines induce apoptotic cell death in various cultured cells [8], but the associations between cellular anthracycline uptake, apoptosis induction, and patient outcome remain unclear [9, 10].

In a previous study we found a clear dose response relationship between the extracellular daunorubicin dose and apoptosis induction in leukemic cell lines and leukemic cells isolated from a few patients with AML [11]. We used DNA fragmentation to measure cell death and found that this was preceded by caspase-3 activation, which supports the induction of apoptosis in leukemic cells. No evidence was found for an extracellular concentration range in vitro within which apoptosis induction was optimal. There was, however, a pronounced interindividual variation in DNA fragmentation for a given extracellular concentration of DNR.

In the investigation reported here, we studied the relationship between intracellular anthracycline concentration and apoptosis with the aim of identifying effective intracellular anthracycline levels for apoptosis induction. We also investigated a possible relationship between anthracycline-induced apoptosis in vitro and clinical response. Cellular concentrations of IDA/DNR in relation to apoptosis induction were studied after a 1-h pulse incubation of mononuclear blood cells (MNC) isolated from patients with AML. Intracellular anthracycline concentrations in vitro were compared to those obtained in vivo during anthracycline treatment.

Materials and methods

Materials

Zavedos (IDA) and Cerubidin (DNR) were provided by Pfizer (Strangnas, Sweden) and Sanofi-Aventis (Bromma, Sweden), respectively. Propidium iodide (PI) sodium citrate plus and Triton X-100 was purchased from Sigma-Aldrich (St. Louis, MO). Polypropylene tubes for DNA fragmentation assays obtained from Becton Dickinson (Lincoln Park, NJ). RPMI 1640 cell culture medium, 10% fetal calf serum, L-glutamine, penicillin, and streptomycin was supplied by PAA laboratories (Pasching, Germany). Tissue culture flasks were from Techno Plastic Products AG (Trasadingen, Switzerland).

Clinical samples

Heparinized peripheral blood samples were obtained from a total of 33 patients with newly diagnosed AML, classified according to the French–American-British criteria [12]. Samples were obtained before therapy and directly after anthracycline infusion. In vitro cellular uptake and apoptosis concentration–response experiments were performed on samples of cells obtained before therapy from 16 of the 33 patients. Anthracycline-induced apoptosis in vitro with a fixed concentration of IDA ($0.25\,\mu M$) was evaluated on cells isolated before therapy from 20 (Table 1) of the 33 patients. Post-infusion samples were obtained from 24 of the 33 patients and drawn from a venous line not used for anthracycline infusion for the analysis of cellular drug content in vivo. The MNCs were isolated at 4°C by centrifugation on Lymphoprep (1.077 g/ml) (Nycomed, Oslo, Norway) [13]. After three washes with phosphate buffered saline (PBS), the cell number and cell volume were determined using a Coulter counter Z2 (Beckman Coulter, Fullerton, CA), and the cells were incubated as described below. The study was approved by the regional ethical committee in Stockholm, and informed consent from all patients was obtained.

Treatment

The patients were treated at the center of Hematology at Karolinska University Hospital in Huddinge or Solna, Stockholm, Sweden and received a combination therapy of cytarabine and either IDA or DNR. The induction protocols were: (1) 2-h infusions with 1 g/m^2 cytarabin twice daily on days 1–4 (day 1 represents the start of the treatment), followed by a 1-h infusion with 10 mg/m^2 IDA on days 1 and 2, or (2) a 2-h infusion once daily with 200 mg/m^2 cytarabin for 7 days followed by a 1-h infusion with DNR 60 mg/m^2 on days 1–3. Complete remission was defined as <5% blasts in the bone marrow. Additional therapy was given to some patients (see Table 1 for details).

Cell incubation procedure

We studied concentration–response relationships in vitro between extracellular/intracellular anthracycline concentrations and apoptosis in isolated cells obtained from 14 of the

Table 1 Clinical characteristics and in vitro apoptosis data of 20 AML patients

Patient[a]	Diagnosis	Sex	Age (years)	WBC ($\times 10^9$/l)	Blasts (%)	Complete remission	Cell death[b] (%)	Treatment[c]
1	M2	F	20	55	50	+	40	DA
2	M1	F	59	73	90	+	59	DA
3	M3	F	42	68	70	+	58	DA
4	M4	F	80	219	40	+	42	D/C/V
5	M1	M	66	40	45	+	35	DA
6	M4	M	29	75	60	+	33	IC
7	M2	M	49	67	60	-	30	ICE
8	M3	F	77	28	25	-	15	DA
9	AML[e]	F	75	44	70	-	14	DA+atra
10	M5	M	36	73	60	-	9	ICE
11	M5	F	59	21	27	+	36	DA
12	M5	F	53	45	65	+	23	DA
13	M4	F	36	69	36	+	16	DA
14	AML[e]	F	51	56	80	+	42	DA
15	M4	F	67	56	70	-	47	DA
16	M1	F	82	270	90	-	97	DA
17	AML[e]	F	82	55	58	-	92	DA
18[d]	AML[e]	F	78	170	70	-	81	DA
19	AML[e]	F	39	162	90	+	89	DA
20	M5	F	45	117	28	-	29	DA

AML, Acute myelogenous leukemia; WBC, white blood cells; F, female; M, male

[a] Patients 15, 16, 17, and 18 received reduced anthracycline doses

[b] Apoptosis in leukemic cells 24 h after a 1-h pulse incubation with 0.25 μ*M* idarubicin

[c] D, Daunorubicin; C, cytarabine; I, idarubicin; E, etoposide; atra, all-trans retinoic acid

[d] Secondary AML

[e] Not further subclassified

patients (13 receiving DNR and IDA and one receiving IDA only). This analysis required a large number of isolated cells, and the total number of cells isolated from four patients was not sufficient for these concentration–response studies. Cells from all 20 patients were also incubated in vitro with one fixed IDA concentration (0.25 μ*M*) in order to study apoptosis development and a possible relationship with clinical response (remission). The MNC were incubated in a humidified incubator with 5% CO_2 at 37°C in RPMI 1640 cell culture medium supplemented with 10% fetal calf serum, 2 m*M* L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml). For the concentration–response analyses of cellular drug uptake/retention and apoptosis, cells were incubated at a concentration of 10^6/ml in 20 ml for 1 h with DNR (0, 0.5, 1.0, 4.0 8.0 μ*M*) and/or IDA (0, 0.25, 0.5, 1.0, 2.0 μ*M*) in 75-cm^2 tissue culture flasks, then washed twice with ice-cold PBS pH 7.4 by centrifugation at 550 *g* for 5 min at 4°C, resuspended in drug-free medium at the same cell concentration, and incubated again at 37°C in 75-cm^2 tissue culture flasks. Aliquots were removed [2 ml for the fluorescence activated cell sorter (FACS) analysis and 2× 1 ml for drug determination], at specified time points (0, 4, 9, and 24 h after the pulse), the cells harvested by centrifugation at 550 *g* for 5 min at 4°C, and washed twice with ice-cold PBS. A sample of the cells was used for immediate determination of DNA fragmentation, and cell pellets were frozen at –20°C for later determination of cellular drug content.

DNA fragmentation with PI staining and flow cytometry

We used a propidium iodide (PI)-based staining procedure to detect cell death as previously described [14]. In an earlier study we found that DNA fragmentation correlates strongly with caspase-3 activation in leukemic cells and, therefore, we used DNA fragmentation as a measure of apoptosis [11]. The appearance of cells less intensively stained than G1 cells (sub-G1 or A_o cells) in flow cytometric DNA histograms was used as a marker of apoptosis. Briefly, a cell pellet containing 1 million cells was gently resuspended in 0.5 ml PI staining solution [PI 50 μg/ml in 0.1% (w/v) sodium citrate plus 0.1% (v/v)

Triton X-100] in 12×75-mm polypropylene tubes. The tubes were placed at 4°C in the dark for 1–3 days before making the flow cytometry measurements. The PI fluorescence of individual nuclei was measured using a FACScan flow cytometer (Becton and Dickinson, Mountain View, CA) 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 logarithmic and linear scales. The forward scatter and side scatter of particles were measured simultaneously. The flow rate was set at 12 µl/s, and at least 10,000 target events were collected for each sample. Analysis was performed using Cell Quest software (BD Biosciences, San Jose, CA).

Drug analysis

The mean cell volume was determined with the cell counter as described above and used to calculate the intracellular concentration. One million cells were lysed in ice-cold water, sonicated for 10 s using a ultrasonic processor (VCX 400; Sonics & Materials, Danbury, CO), and extracted with 60% acetonitrile for the DNR and IDA analysis. Prior to the acetonitrile extraction, 75 µl plasma was added to the samples to prevent the adsorption of IDA and DNR to the plastic tubes. The DNR and IDA concentrations in the cells was determined by high-performance liquid chromatography (HPLC) using a phenyl-µ-Bondapak column (3.9×150 mm, 5 mm; Waters Assoc, Milford, MA) eluted with acetonitrile and 0.2% ammonium formate pH 4 (60:40 v/v) at a flow rate of 1.5 ml/min. The drug was quantified using a Shimadzu RF-551 fluorescence HPLC monitor (Waters Assoc) at $\lambda_{excitation}$ 485 nm and $\lambda_{emission}$ 560 nm. The detection limit of the assay is 5×10^{-3} µ*M*, and the range of quantification is 0.03–20 µ*M*, with a coefficient of variation of <7%. Cellular drug uptake is expressed in micromoles.

Statistical analysis

Regression lines were calculated according to the method of least squares. Student's *t* test was used for testing significance, and *P* values <0.05 were considered to be significant.

Results

Cellular anthracycline levels and apoptosis induction

We isolated leukemic cells from 20 AML patients before induction therapy, incubated the cells with various extracellular concentrations of anthracyclines, and measured cellular drug uptake and apoptosis at various time points after exposure. Apoptosis usually began 4–9 h after the pulse incubation and increased the most, depending on the cellular drug concentration, during the first 24–48 h. We chose to systematically study apoptosis development up to 24 h after the end of the pulse incubation since the most pronounced apoptosis occurred during this time period and the treatment protocols included the administration of anthracycline in 24-h cycles.

There was a clear concentration–response relationship between cellular drug uptake and apoptosis for a given patient as well as a large variation in the initial uptake of DNR and apoptosis between cells from different patients. Despite the variation in initial uptake, the retention (4–24 h) was similar between cells from different patients.

We found a large variation in the uptake of DNR and apoptosis between cells from different patients when these cells were incubated with IDA. Apoptosis usually began 4–9 h post-incubation initiation. In accordance with the DNR studies, IDA retention was fairly similar between cells from different patients.

Figure 1 shows the cellular uptake, retention, and apoptosis of IDA or DNR in AML cells obtained from a patient that were incubated with a 1-h pulse of various concentrations of IDA or DNR. IDA was taken up to a greater extent by the AML cells than DNR even though we used fourfold higher concentrations of DNR. At similar intracellular concentrations of IDA and DNR, apoptosis development was almost identical.

Figure 2 shows the intracellular DNR concentrations [mean and standard deviation (SD) 353 ± 248 µ*M*, range 17–933 µ*M*, *n*=16] and IDA (871 ± 530 µ*M*, range 136–2031 µ*M*, *n*=15) and apoptosis induction (DNR: 45 ± 29%, range 10–97%; IDA: 72 ± 20%, range 40–97%) when the cells had been pulse incubated with 1 µ*M* DNR or IDA. The interindividual variation for both parameters is pronounced, and no relation is observed (r^2=0.014, *P*=0.69 and r^2=0.056, *P*=0.46 for DNR and IDA, respectively).

Figure 3 shows the cellular concentrations of IDA and DNR at time 0 in relation to apoptosis development 24 h after the pulse incubation for all patient samples at all treatment concentrations studied. Apoptosis was pronounced (>60%) at concentrations >1200 µ*M*. The slopes of the regression lines for DNR (*n*=16) and IDA (*n* =15) were nearly identical, with k-values of 0.022 and 0.024, respectively. The R^2 values were 0.25 (*P*<0.001) and 0.35 (*P*<0.0001) for DNR and IDA, respectively.

Cellular anthracycline levels in vivo

We further analyzed the intracellular uptake in leukemic cells isolated from 24 patients undergoing anthracycline induction therapy. The intracellular concentrations in all cell samples (mean ± SD) immediately after a 1-h anthracycline infusion were roughly more than tenfold lower (66.9 ± 36.3 µ*M*,

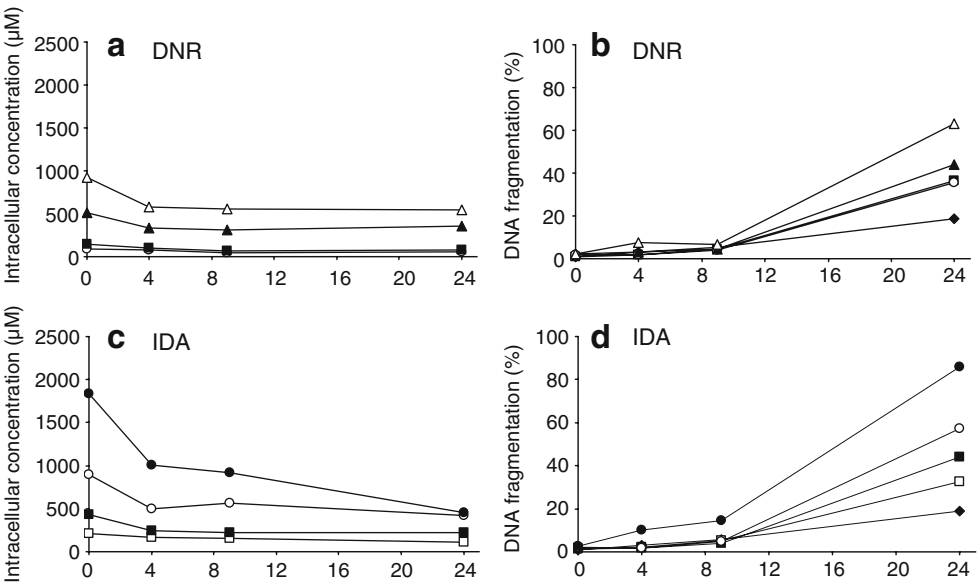

Fig. 1 Time course for cellular uptake, retention, and apoptosis for daunorubicin (*DNR*; **a**, **b**) and idarubicin (*IDA*; **c**, **d**) in cells from patient no. 6 after a 1-h pulse incubation with DNR [concentration (μ*M*): *filled diamond* 0, *filled square* 0.5, *open circle* 1, *filled triangle* 4, *open triangle* 8] or IDA [concentration (μ*M*): *open square* 0.25, *closed square* 0.5, *open circle* 1, *closed circle* 2]

range 4–137 μ*M*, *n*=24) than the intracellular concentration (1200 μ*M*) needed to result in a pronounced (>60%) apoptosis in vitro (Fig. 3). However, there was a significant difference in the in vivo intracellular levels of DNR between patients who went into complete remission (CR+; 80.6 ± 36.9 μ*M*, *n*=14) and those who did not (CR−; 47.6 ± 26.3 μ*M*, *n*=10) (Student's *t* test $P < 0.05$) (Fig. 4).

Apoptosis induction in vitro in relation to clinical response

We also investigated whether there was a relation between apoptosis induction in vitro and clinical response in 20 AML patients (Table 1). Apoptosis was measured in leukemic cells 24 h after a 1-h pulse incubation with 0.25 μ*M* IDA and related to clinical response. A 0.25 μ*M* concentration of IDA was chosen since this concentration empirically gave a large scatter of apoptosis values among the patient samples. The cells of patients who received full dose induction treatment and went into complete remission (CR+) were generally more sensitive to IDA in vitro than those obtained from patients who did not achieve complete remission (CR−). The mean and SD values for apoptosis in CR+ patients and CR− patients were 43.0 ± 19.9% (*n*=11) and 19.4 ± 9.5% (*n*=5), respectively (Student's *t* test $P < 0.05$). Patients 15–18 were excluded from the statistical analysis since they received reduced anthracycline doses. No difference in IDA sensitivity was observed when all 20 patients were included in the statistical analysis. The mean and SD values for apoptosis in CR+ and CR− patients were 43.0 ± 19.9% (*n* =11) and 46.0 ± 35.1% (*n*=9), respectively (Student's *t* test $P > 0.05$).

Discussion

In order to investigate the importance of DNR/IDA cell uptake in apoptosis induction in AML cells we conducted a series of experiments on MNC isolated from AML patients.

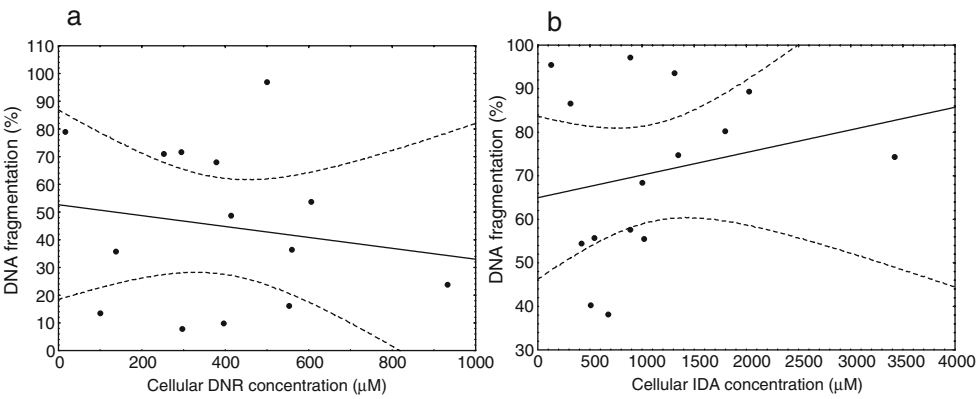

Fig. 2 In vitro intracellular concentration of DNR (**a**, *n* =16) and IDA (**b**, *n*=15) in leukemic cells obtained from acute myelogenous leukemia (AML) patients at time 0 in relation to apoptosis at 24 h following a 1-h pulse incubation with 1 μ*M* DNR or IDA. The *dotted lines* represent the 95% confidence limits

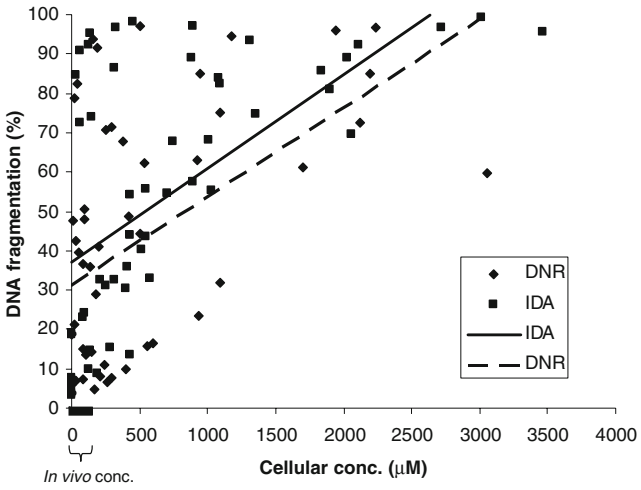

Fig. 3 Intracellular concentrations of DNR and IDA in vitro at time 0 versus apoptosis induction in leukemic cells at 24 h. The cells were isolated from 16 AML patients and pulse incubated for 1 h with 0.5, 1.0, 4.0, and 8.0 μ*M* DNR (*n*=16) or 0.25, 0.5, 1.0, and 2.0 μ*M* IDA (*n*=15). The *black box along the x-axis* covering the range 4–137 μ*M* represents the intracellular in vivo anthracycline concentration range immediately after anthracycline infusion

In an attempt to mimic the in vivo pharmacokinetics of anthracyclines, we chose a 1-h pulse incubation. Apoptosis induction was detectable approximately 4–9 h after the pulse incubation, reaching up to 100% apoptosis at the highest concentrations 24 h after the pulse incubation. This time course demonstrates the importance of choosing the correct time-point for evaluating anthracycline-induced apoptosis.

There was a large interindividual variation in vitro in how cells from the AML patients took up DNR/IDA and subsequently underwent apoptosis. However, a clear concentration–response relationship between cellular drug uptake and apoptotic response was always present for any one individual. A prior expectation of our study was that we would be able to demonstrate a certain cellular threshold level for IDA/DNR that induced apoptosis. However, this was not the case. The data in Figs. 2 and 3 clearly demonstrate a pronounced interindividual variation in apoptosis for a given cellular drug concentration. Nevertheless, the data in Fig. 3 also demonstrate that cellular IDA/DNR levels >1200 μ*M* are associated with strong apoptosis development. One reason for the pronounced interindividual variation in cellular anthracycline uptake could be the expression of various protein efflux pumps, such as members of the ATP-binding cassette (ABC) superfamily (e.g. p-glycoprotein) [15]. The results of several studies have also indicated that nucleoside transport proteins are involved in cellular anthracycline uptake. It is therefore possible that alterations in the expression of these proteins could contribute to variations in cellular drug uptake [16–18].

We found that the in vivo intracellular concentrations required to trigger a strong apoptotic response in vitro were more than tenfold lower than the intracellular concentrations required to trigger the same response. However, we also found that these intracellular post-infusion levels were significantly higher in patients achieving complete remission than in those who did not. The possible existence of a relation between intracellular concentrations of DNR and clinical outcome in AML patients has been studied by a number of investigators. In one study, Kokenberg et al. did not find any difference in in vivo cellular DNR concentrations between responders and non-responders [19], while in another study they found a tendency towards higher concentrations in responders [20]. In accordance with our results, Galettis et al. found a significant difference in the cellular DNR levels (area under the curve) between responders and non-responders, but they found no correlation between cellular DNR levels in vivo and cellular p-glycoprotein expression [21].

Our results support that modifications to treatment protocols aimed at increasing the intracellular concentration of anthracyclines could be beneficial by triggering a stronger apoptotic response, thereby leading to a better treatment outcome. How anthracycline infusion times may be altered in order to increase the intracellular concentration in vivo needs further investigation. There have been contradictory results from studies investigating how intracellular concentrations vary with anthracycline infusion time. One study on AML patients showed that a 24-h continuous infusion resulted in a higher leukemic cell accumulation of DNR compared to a short time infusion [22]. In this study, eight patients were analyzed and one patient was used as his/her own control. Another study on AML patients, a four-arm parallel group

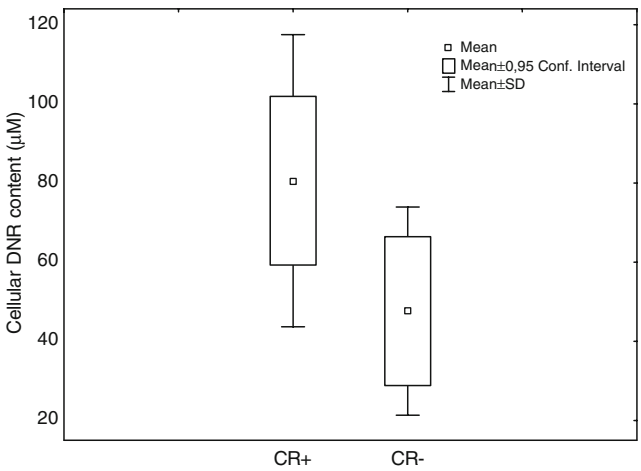

Fig. 4 In vivo intracellular concentration of DNR in leukemic cells isolated from 24 patients directly after a 1-h DNR infusion in relation to clinical outcome. *CR+* Complete remission (*n* =14), *CR−* Not complete remission (*n* =10)

study including 18 patients, showed that a bolus infusion achieved a higher intracellular concentration of DNR than a long-term infusion [23]. Both studies were based on very few patients. One study on DNR treatment of 77 patients with acute lymphoblastic leukemia showed that a 24-h continuous infusion resulted in lower relapse rates than a 30-min infusion of DNR [24]. Alternatively, another study on 178 children with ALL was unable to find any difference in patient outcome between a 1-h and a 24-h DNR infusion protocol [25]. Most studies have compared anthracycline plasma concentrations in patients receiving different infusion protocols, even though it has been shown that there is no clear correlation between plasma concentration and the intracellular concentration of anthracyclines in patients undergoing treatment [5, 6]. A more accurate approach to determining the efficacy of different DNR infusion protocols would be to compare the intracellular levels of DNR together with patient outcome.

Why then is the response to therapy relatively good despite the low cellular in vivo concentrations? There are several possible explanations. Firstly, there are mechanisms in vivo facilitating apoptosis at low intracellular anthracycline levels. Secondly, the patient receives combination therapy with cytarabine, and the contribution by this drug leads to apoptosis induction despite the low intracellular anthracycline levels. Thirdly, the low intracellular anthracycline levels in vivo do not induce apoptosis, and other mechanisms, such as growth inhibition, are more important for the treatment effect.

The results of our in vitro apoptosis induction assay with anthracyclines showed that IDA-induced apoptosis was significantly higher among responders than among non-responders. Several in vitro tests have been developed with the aim of predicting outcome, with varying degrees of success. A number of research groups have reported a significant correlation between AML patient response and in vitro drug sensitivity using a variety of methods, such as differential staining cytotoxicity (DISC) assay [26–28] and the methylthiazol tetrazolium colorimetric assay [29, 30]. The DISC assay is known to be labor intensive and time consuming, taking about 4–5 days to complete [26, 28]. With our in vitro apoptosis determination method, it is possible to obtain a result within 48 h after blood sampling. Interestingly, in carrying out a study with a 1-h DNR and IDA pulse incubation of isolated MNC from patients with AML and acute non-myeloid leukemia, the researchers found that Fas (APO-1, CD95) induction and anthracycline-induced apoptosis were significantly higher when complete remission was achieved [31]. Apoptosis, which was evaluated after 18 h, was determined from caspase 3 activation and phosphatidylserine exposure using flow cytometry. Attempts have also been made to find correlations between in vitro drug uptake, P-gp expression, and clinical outcome [32–34]. Guerci et al. found relations between reduced intracellular DNR uptake, P-gp expression, and therapeutic failure [35].

We conclude that a high initial cellular anthracycline uptake is of importance for inducing apoptosis in vitro in AML cells. Our observations that post-infusion in vivo intracellular concentrations were low but still significantly related to clinical response suggest that treatment protocols achieving higher initial intracellular anthracycline levels may improve the clinical outcome of AML patients.

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