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ORIGINAL ARTICLE

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Increased myelotoxicity of idarubicin: is there a pharmacological basis?

Results of a pharmacokinetic and an in vitro cytotoxicity study

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Abstract *Background*: Clinical trials evaluating idarubicin (IDA) in acute myeloid leukemia, multiple myeloma and non-Hodgkin's lymphoma (NHL) have provided some evidence for an increased myelotoxicity of IDA compared to other anthracyclines. IDA is known to be less sensitive towards multidrug resistance mediated by P-glycoprotein (P-gp). This phenotype is a major impediment to successful antineoplastic treatment, but P-gp is also expressed on hematopoietic stem cells (HSC). *Methods*: We investigated the pharmacokinetics of IDA and etoposide (ETO) in seven previously untreated patients with aggressive NHL. The patients received a CHOP-derived protocol (CIVEP) in which doxorubicin (DOX) was substituted by IDA 11–16 mg/m^2 and ETO 3×100 mg/m^2 was added. Furthermore, we evaluated in vitro the impact of P-gp expression on the cytotoxicity of DOX and IDA in cells from three parental chemosensitive leukemia and lymphoma cell lines (HL60, U937, CCRF) and their resistant sublines, as well as in CD34-positive HSC. *Results*: The peak plasma levels (C_{max}), terminal elimination half-life ($t_{1/2}$) and area under the concentration curve (AUC) both for IDA and for ETO did not differ from published data. In cell line models the numbers of viable cells in a P-gp-expressing resistant CCRF-VCR100 subline were significantly more reduced by IDA ($P<0.001$), but there was no difference in the cytotoxicities of IDA and DOX in chemosensitive CCRF cells and in the (non-P-gp-expressing) resistant U937 and HL60 sublines. Cytotoxicity against HSC was more pronounced after incubation with IDA than after treatment with DOX ($P=0.014$), even when a tenfold higher concentration of DOX than of IDA was used. The addition of cyclosporin A increased the cytotoxic effect of DOX but not that of IDA in HSC. *Conclusions*: The pharmacokinetics of IDA and its main metabolite idarubicinol in CHOP-derived protocols were not different from data obtained with other combinations or monotherapy. The increased myelotoxicity of IDA may be a consequence of P-gp expression in CD34-positive HSC.

Keywords Idarubicin myelotoxicity · Pharmacokinetics · P-glycoprotein · Hematopoietic stem cells

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Introduction

Idarubicin (IDA) is a 4-demethoxy-anthracycline analogue of daunorubicin (DNR). In contrast to other anthracyclines, the main metabolite idarubicinol (IDAol) exerts a high antitumor activity and has a very long half-life. IDA has demonstrated greater cytotoxicity than DNR or doxorubicin (DOX) in various cell line models [3, 22, 29]. At a dose ratios of approximately 1:3 to 1:4, the clinical efficacy of IDA has been reported to exceed that of DNR in clinical trials in patients with acute myeloid leukemia (AML) [38]. Although IDA alone or in combination regimens also induces high response rates in patients with multiple myeloma [10, 17] and non-Hodgkin's lymphomas (NHL) [5, 8, 13, 15, 47], the use of this drug is not well established in these diseases.

Whereas IDA compared to other anthracyclines has shown reduced cardiac toxicity in clinical studies [4, 24, 42], there is some evidence for increased myelotoxicity. Prolonged myelosuppression and a higher rate of infections after IDA than after DNR treatment was observed in the above-mentioned AML trials [43, 44]. Similar results have recently been reported for myeloma

patients who received IDA instead of DOX in the VAD regimen [18], and for patients with aggressive NHL [40]. In the latter study, a CHOP-derived protocol was evaluated in which DOX was substituted by IDA, and etoposide (ETO) was added (CIVEP). Since IDA and ETO are both metabolized and eliminated by the liver, show a high protein binding and act as topoisomerase II inhibitors, a pharmacological interaction could be assumed. Furthermore, the described higher activity of IDA in the treatment of AML is thought to be based on the reduced susceptibility to P-glycoprotein (P-gp) encoded by the mdr1 gene. Since P-gp expression is more frequent in AML than in NHL [37, 41], this advantage in efficacy could be diminished in lymphoma patients. Nevertheless, IDA substitution might have an impact on hematotoxicity, because P-pg is expressed in almost all hematopoietic progenitor cells [7, 9, 21]. Consequently, we investigated the pharmacokinetics of IDA and ETO in seven patients receiving the CIVEP regimen, and examined the effects of IDA and DOX in different cell models with P-gp-dependent as well as P-gp-independent resistance mechanisms.

Patients and methods

Pharmacokinetic studies

In a phase I/II dose-finding trial the German High Grade NHL study group evaluated a CHOP-derived protocol in which DOX 50 mg/m^2 was substituted by IDA 14 to 16 mg/m^2, and ETO 100 mg/m^2 for three consecutive days was added (CIVEP). Seven patients aged between 23 and 61 years, who were treated with CIVEP chemotherapy because of newly diagnosed aggressive NHL, were enrolled in the pharmacokinetic study after written informed consent had been obtained. All patients had normal renal and liver function. The patients received cyclophosphamide 750 mg/m^2 and vincristine 2 mg on day 1, ETO 100 mg/m^2 for three consecutive days, and prednisone for five consecutive days. ETO was given as an intravenous infusion for 60 min in five patients, and as a 24-h continuous infusion in two patients. IDA (14, 15 or 16 mg/m^2) was administered intravenously over 5 min on day 1. In one patient who had WHO grade 4 neutropenia with WHO grade 3 infection after receiving IDA 16 mg/m^2, the IDA dose was reduced to 11 mg/m^2 in the second cycle.

During the first and the second treatment course 5-ml heparinized blood samples (Sarstedt, Nurnberg, Germany) were collected before IDA administration, and 5, 10, 15, 20, 30, 40, 60, 90, 120 and 150 min, and 4, 6, 8, 12, 24, 48, 96, 120 and 144 h after infusion. The samples were centrifuged immediately at 5°C for 10 min, and the plasma was aliquoted into Cryo vials and stored at −20°C until assayed. However, no significant degradation of IDA and IDAol was found at room temperature over a period of 24 h when tested with spiked plasma samples.

Measurement of ETO, IDA and IDAol concentrations in plasma

Sample preparation and measurement of IDA and IDAol plasma levels by high-performance liquid chromatography (HPLC) were done as described previously [33]. In brief, for online enrichment, pure water (HPLC quality) at a flow rate of 2 ml/min and a 30×4 mm 5 µ C18 column (Machery & Nagel, Dueren, Germany) were used. After an enrichment time of 3 min, the enrichment column was switched by a rheodyne valve into the analytical flow and IDA/IDAol were eluted retrogradely onto a 250×4.6 mm 5 µ C18 analytical column. Detection was performed by a fluorescence detector adjusted to 250 nm excitation and 570 nm emission. For quantification, the external standard method was used by regression analysis of seven spiked plasma samples with 0.1, 0.5, 1, 5, 10, 25 and 50 ng/ml IDA and IDAol. This system had a detection limit of 100 pg/ml for IDA and IDAol using 2 ml plasma. Within-day variation was 8% for IDA and 7% for IDAol at a concentration of 5 ng/ml. Between-day variation determined on ten consecutive days with plasma spiked with 5 ng/ml of IDA/IDAol was 9% and 7%, respectively. At the detection limit, the coefficient of variation was 14% for IDA and 16% for IDAol as demonstrated by ten measurements with spiked plasma samples.

ETO in plasma was determined by HPLC using liquid-liquid extraction with 2 ml chloroform for sample preparation and online enrichment with a mixture comprising 12% v/v acetonitrile and 88% v/v water/triethylamine/acetic acid (97.7/1.5/0.7625% v/v, pH 5.6) using a 8×3 mm 5 µ 120 C18 nucleosil guard column (Machery & Nagel). The flow rate for the enrichment system was adjusted to 3 ml/min. An enrichment time of 3 min provided clean samples for the HPLC analysis which was performed on a 250×4.6 mm 3 µ C18 Nucleosil analytical column. The analytical eluent contained 33% acetonitrile and 67% triethylamine acetate. The flow rate in the analytical system was 0.4 ml/min. A fluorescence detector adjusted to 285 nm excitation and 320 nm emission was used for detection.

For quantification, the external standard method was used by linear regression analysis of five spiked plasma samples with 0.005, 0.05, 0.5, 5 and 10 µg/ml. This system has a detection limit of 5 ng/ml for ETO using 1 ml plasma. Within-day variation was 9% for the lowest, and 2% for the highest concentration. At the detection limit, the coefficient of variation was 9% for plasma, as demonstrated by ten measurements with spiked plasma. The between-day variation determined on ten consecutive days with plasma spiked with 5 µg/ml of ETO was 10%.

Pharmacokinetic parameters

Elimination half-life ($t_{1/2}$), area under the concentration curve from zero to infinity (AUC) and maximum concentration (C_{max}) of IDA and IDAol in plasma were calculated using a three-compartment model based on the TOPFIT computer program providing an optimized adaptation of coefficients of variation between the observed and calculated data [19]. An optimal regression coefficient of >0.89 was found in all patients using a linear three-compartment model described by the equation:

$$Cp = A'1e^{-\alpha t} + A'2e^{-\beta t} + A'3e^{-\gamma t}$$

where Cp is the plasma concentration at a specific time point, t is time, A1–3 are dimensionless coefficients required to describe the time-course in a specified compartment, and α, ß and γ are elimination rate constants.

For ETO a two-compartment model was used described by the equation:

$$Cp = A'1e^{-\alpha t} + A'2e^{-\beta t}$$

The regression coefficient for all applied ETO curves was >0.91. As a weighting function for the measured data $1/y$ was used for ETO, IDA and IDAol. The plasma decay curve of IDAol was determined independently of the respective data of the parent compound assuming a first-order rate constant of metabolism.

In vitro cytotoxicity

In vitro cytotoxicity was measured using the colorimetric methylthiazol-tetrazolium (MTT) assay. Cells from three parental chemosensitive leukemia and lymphoma cell lines (HL60—acute myeloid leukemia, CCRF—T-lymphoblastic leukemia, U937—histiocytic

lymphoma) and their chemoresistant sublines (HL60-res, CCRF-VCR100, U937-res) were cultured with RPMI medium and 75 ng/ml DOX or 20 mg/ml IDA. The resistant phenotype was induced by continuous exposure of cells to ETO (HL60-res and U937-res) or vincristine (HL60-VCR100). Cell viability was monitored with trypan blue staining. Depending on the viability rate, the concentration of cytotoxic drugs was stepwise increased to a final concentration of 1 μ*M* ETO and 100 ng/ml vincristine, respectively.

Resistance of CCRF-VCR100 cells to chemotherapy is mediated by P-gp expression, while the mechanisms of resistance in U937-res and HL60-res cells are non-P-gp-dependent. Polymerase chain reaction (PCR) was used for the amplification of P-gp messenger RNA in CCRF-VCR100 cells (data not shown, for the technique see references 20 and 31). From each line, 1×10^6 of sensitive cells and 1×10^6 resistant cells were incubated with IDA or DOX in triplicate for 48 h in wells of microculture plates at 37°C in humidified air containing 5% CO_2. After 48 h, MTT salt was added which was cleaved to purple formazan crystals by metabolically active cells. After 4 h, the crystals were dissolved in 100 μl acidified isopropranolol, and formazan production was quantified using a spectrophotometer at 562 nm. The optical density (OD) is linearly related to the number of viable cells. Cell survival was calculated by the equation (OD treated well/mean OD control well)×100%. Furthermore, fresh hematopoietic stem cells (HSC) from healthy donors after CD34-positive selection using CliniMACS (Miltenyi Biotec, Bergisch Gladbach, Germany) were incubated with 200 ng/ml DOX or 20 ng/ml IDA with or without the addition of the mdr1 modulator cyclosporin A (CSA). Six wells of leukemic cells as well as HSC were cultured in drug-free medium as control.

The results of the cytotoxicity assays were analyzed for significance using the Chi-squared test. *P* values less than 0.05 were considered significant.

Results

Pharmacokinetic studies

Pharmacokinetic data were available from 13 treatment cycles. IDA doses ranged from 11 to 16 mg/m^2 (one cycle 11 mg/m^2, two cycles 14 mg/m^2, seven cycles 15 mg/m^2, three cycles 16 mg/m^2). The dose-dependent pharmacokinetic parameters determined after administration of IDA 11, 14 and 16 mg/m^2 were linearly calculated in relation to an IDA dose of 15 mg/m^2 to achieve better comparability. ETO was applied as short-term infusion in nine cycles, whereas four cycles with continuous infusion were analyzed.

Figure 1 shows typical plasma decay curves of IDA and IDAol after treatment with 15 mg/m^2. Peak levels (C_{max}) of IDA were reached immediately after injection. The maximum concentrations at this point ranged from 24.3 to 98.4 ng/ml with an average of 61.2 ng/ml. Plasma levels of the unchanged drug declined rapidly with an average terminal elimination half-life ($t_{1/2\gamma}$) of 22 h (range 10.4 to 34.6 h). The plasma concentration of the metabolite IDAol exceeded the levels of the parent compound after 1 h. Maximum IDAol levels were detected approximately 9 h after IDA administration and ranged from 6.9 to 15.2 ng/ml (average 10.4 ng/ml). IDAol concentrations then decreased very slowly for a terminal half-life of about 47 h (range 30.3 to 68.7 h), which was more than twice as long as the $t_{1/2\gamma}$ of IDA.

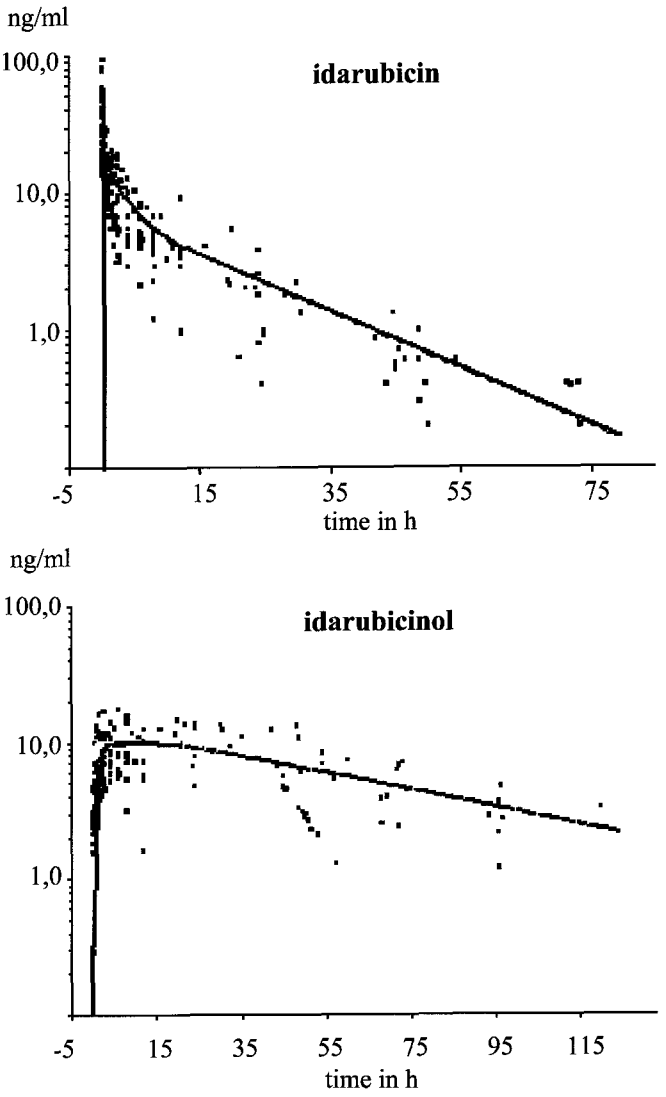

Fig. 1 Mean plasma decay curves of IDA and IDAol after intravenous administration of 15 mg/m^2 IDA

The AUC for IDA and IDAol showed high intraindividual variation and a tendency to increase at higher dose levels. The AUC for IDAol was on average 4.3 (range 1.4 to 7.5) times higher than that for IDA.

The mean ETO plasma decay curves after short-term or continuous infusion are shown in Fig. 2. Maximum ETO levels ranged from 7.6 to 22.7 μg/ml after short-term administration and 1.4 to 1.8 μg/ml (steady state) during continuous administration. Peak levels were reached at the end of the 1-h infusion and after 15 h of continuous infusion (steady state), respectively. Whereas C_{max} during continuous infusion was only 10% of that after short-term infusion, the AUCs after both administration modalities were similar. The mean pharmacokinetic parameters for IDA, IDAol and ETO are summarized in Table 1.

In six out of seven patients in the pharmacokinetic study, the IDA doses had to be reduced due to hematological toxicity between the second and fourth cycles

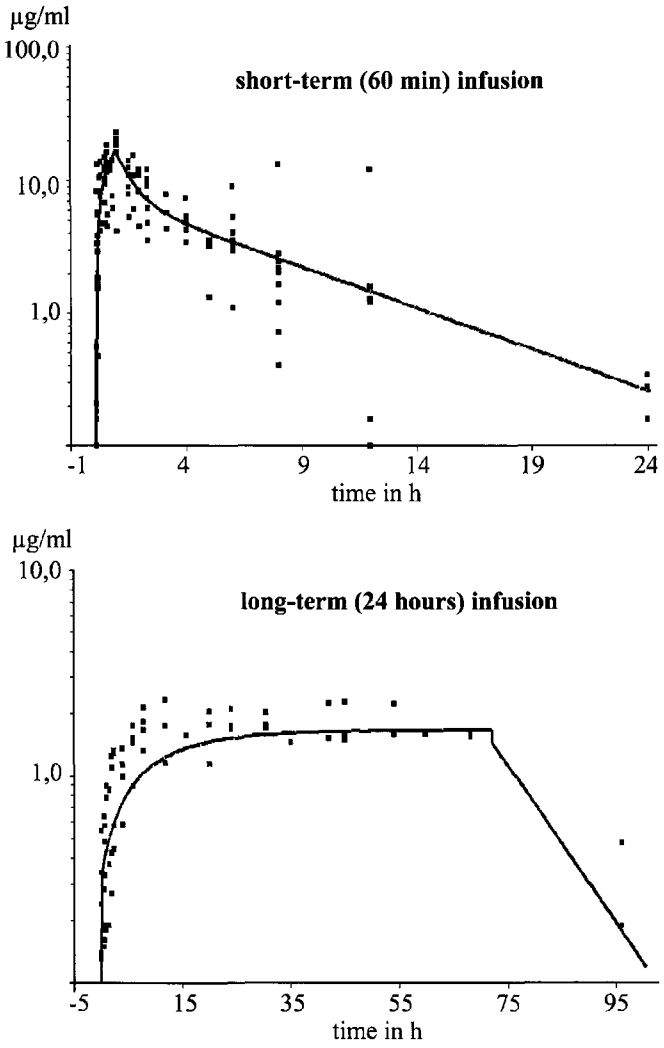

Fig. 2 Mean plasma decay curves of ETO after intravenous short-term (60-min) infusion and after long-term (24-h) infusion of 100 mg/m^2

for the following cycles. Three patients experienced an early relapse and received salvage chemotherapy or radiotherapy.

Cytotoxicity assay

The results of the MTT cytotoxicity assays are presented in Figs. 3 and 4. In the chemosensitive CCRF cells, no difference in antileukemic efficacy of IDA (20 ng/ml) and DOX (75 ng/ml) was found, but the number of viable CCRF-VCR100 cells (P-gp/mdr1-expressing) was significantly more reduced by IDA ($P < 0.001$). In contrast, IDA exerted greater cytotoxicity than DOX in chemosensitive U937 and HL60 cells ($P \leq 0.001$), but there was no significant difference in cell killing in the resistant cells (U937-res, HL60-res). The cytotoxicity against HSC was more pronounced after incubation with IDA (20 ng/ml) than after incubation with DOX (200 ng/ml; $P = 0.014$), even when a tenfold higher concentration of DOX than IDA was used. The addition

Table 1 Pharmacokinetic parameters of idarubicin (IDA), idarubicinol (IDAol) and etoposide (ETO) (IDA 15 mg/m^2 i.v. bolus; ETO 100 mg/m^2 i.v. 1-h or 24-h infusion) (*n.d.a.* no data available)

	IDA			IDAol			Ratio AUC IDA/IDAol	ETO					
								1-h infusion			24-h infusion		
	C_{max} (ng/ml)	$t_{1/2\gamma}$ (h)	AUC (ng·h/ml)	C_{max} (ng/ml)	$t_{1/2\gamma}$ (h)	AUC (ng·h/ml)		C_{max} (µg/ml)	$t_{1/2\beta}$ (h)	AUC (µg·h/ml)	C_{max} (µg/ml)	$t_{1/2\beta}$ (h)	AUC (µg·h/ml)
Mean	61.2	21.8	173.1	10.4	47.1	747.0	1:4.31	17.0	2.9	57.5	1.6	3.4	41.5
SD	23.3	7.6	59.4	2.8	11.7	316.3	1:1.75	5.1	1.2	17.3	0.2	1.9	5.74
Coefficient of variation	38%	35%	34%	27%	25%	42%	41%	30%	39%	30%	12%	57%	14%
Published data[a]	45–256	12.7–34.7	190–489	8.2–33.7	52.1–63.1	597–840	1:2.3–1:3.0	12.8–63.3	2.18–11.1	47.4–201	n.d.a.	n.d.a.	n.d.a.

[a]IDA/IDAol, references 16, 35 and 46; ETO infusions, references 6, 25, 28 and 36

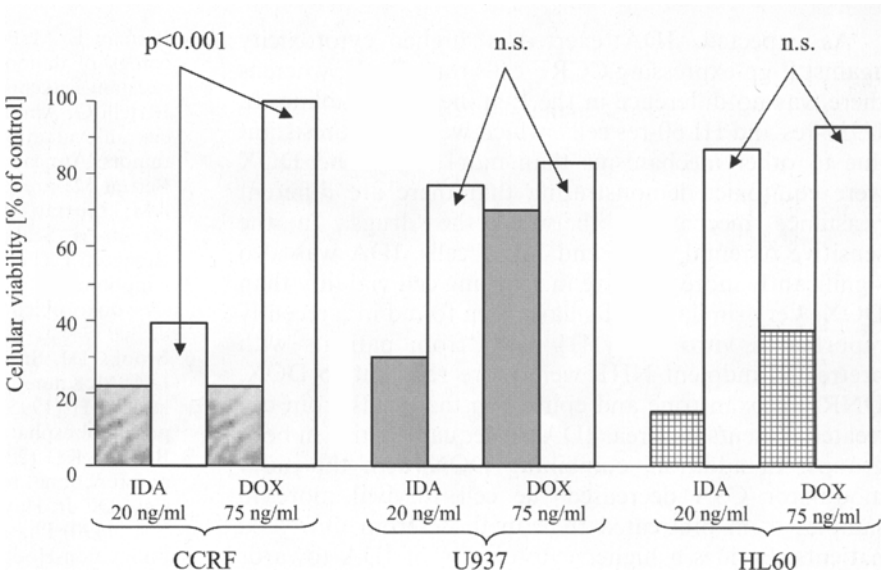

Fig. 3 Results of in vitro cytotoxicity assays (MTT) with leukemic and lymphoma cell lines. The *shaded columns* represent the chemosensitive lines, the *white columns* the chemoresistant sublines (*n.s.* not significant)

of cyclosporin as an mdr1 modulator to anthracycline-treated HSC increased (but not significantly) the cytotoxic effect of DOX but not that of IDA.

Discussion

While IDA is well established in the management of patients with AML, the agent has failed to replace DOX as standard anthracycline for the treatment of aggressive NHL. This is due to unsatisfactory response rates and survival in some NHL trials [2, 11, 12, 23]. Furthermore, IDA has shown greater hematotoxicity than DOX and DNR in patients with AML, myeloma and lymphoma [18, 40, 43, 44].

As part of the German CIVEP trial [40], we investigated the pharmacokinetics of IDA and IDAol in 13 treatment cycles. The AUC of IDAol in plasma was about four times higher than the AUC of IDA. In consideration of its high cytotoxicity and the prolonged half-life, an important contribution of the metabolite IDAol to the therapeutic efficacy and side effects of IDA treatment is likely. In a recently published study, Fukushima et al. [14] demonstrated a significant correlation between the $t_{1/2}$ of IDAol in plasma and the nadir of neutrophils. Even though there were high inter- and intraindividual variations of C_{max}, $t_{1/2}$ and AUC in our study (data not shown), the findings did not differ from the results published by others (Table 1). This was also true for the kinetics of ETO short-term infusion. Pharmacokinetic data for a continuous infusion of ETO in the literature are limited. Nevertheless, with regard to the AUC of ETO, which is the main determinant of hematological toxicity in continuous-infusion schedules [25], we found similar results for daily 1-h infusion and prolonged administration. Taken together, we conclude that a combination of IDA and ETO does not alter the pharmacokinetics of either drug, and therefore an impact on hematotoxicity can be excluded.

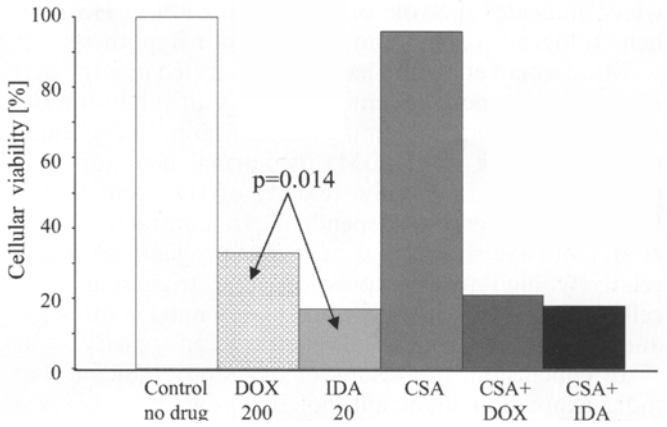

Fig. 4 Results of in vitro cytotoxicity assays (MTT) with CD34-positive hematopoietic stem cells

Therefore, in a consecutive in vitro study we addressed the question as to whether there is an association between the frequency of mdr1 expression in different hematological malignancies and the cytotoxicity of IDA and DOX. While the levels of expression are variable and appear to be assay-dependent, most studies have shown a P-gp phenotype in fewer than 20% of previously untreated lymphoma patients [26, 30, 32, 45]. In contrast, mdr1 is frequently present in blast cells at diagnosis of de novo AML with a reported incidence of up to 75% among untreated patients [37]. Furthermore, P-gp is also expressed in nonmalignant cells of the liver, kidneys and intestine [39], and in hematopoietic progenitor cells [7, 9, 21]. The highest levels of P-gp among the progenitors have been found in pluripotent stem cells [9]. The increased stem cell toxicity of IDA treatment could therefore be due to an effect of P-gp, which protects the hematopoietic progenitors against damage caused by DNR or DOX, but not against that caused by IDA.

As expected, IDA exerted a higher cytotoxicity against P-gp-expressing CCRF cells than DOX, whereas there was no difference in the P-gp-negative subline. In U937-res and HL60-res cells, which were chemoresistant due to other mechanisms than mdr1, IDA and DOX were equitoxic, demonstrating that there are different resistance mechanisms between the drugs. In the sensitive parental U937 and HL60 cells, IDA was also significantly more effective in reducing cell viability than DOX. Very similar results have been found in a recently reported in vitro study [1]. Cells from patients with pretreated indolent NHL were more resistant to DOX, DNR, mitoxantrone and epirubicin than cells from untreated patients, whereas IDA was equally active in both groups. In addition, combining DOX with the mdr1 modulator CSA decreased the cell survival more in samples from pretreated than in those from untreated patients. Besides a higher cytotoxicity of IDA towards CD34-positive HSC, we demonstrated that the addition of CSA significantly increased the toxicity of DOX, which indicates the role of P-gp expression in HSC for hematological toxicity and confirms our hypothesis.

Minderman et al. [27] have demonstrated in vitro that IDA is much more potent than DOX in inhibiting the formation of colony-forming units of granulocyte-macrophage lineage (CFU-GM) by normal hematopoietic progenitors. In this study, toxicity of IDA and IDAol was not proliferation-dependent. In contrast, Smeets et al. [34] have shown that quiescent progenitors have a relatively high mdr1 expression, and triggering these cells into proliferation downregulates mdr1 expression and increases anthracycline retention and toxicity.

In conclusion, the results of our study indicate that mdr1-expression in hematopoietic progenitors plays a role in the hematological toxicity induced by anthracycline treatment. The impairment of quiescent progenitors could explain the clinical observation of increased cumulative hematotoxicity after the use of IDA-containing chemotherapy regimens in patients with aggressive NHL and the necessity of dose reductions in advanced treatment cycles. The pharmacokinetics of IDA and IDAol in CHOP-derived protocols are not different from those obtained with other combinations or monotherapy.

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