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Effects of anthracycline derivatives on human leukemia K562 cell growth and differentiation

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Abstract

New derivatives of daunorubicin (DRB), doxorubicin (DOX), and epidoxorubicin (EDOX) with an amidine group bonded to C-3′ of daunosamine moiety with either morpholine or hexamethyleneimine ring attached to the amidine group are studied in this paper. We have shown that all of these newly synthesized anthracycline derivatives inhibit human leukemia K562 cell line proliferation but only some of them induce erythroid differentiation when used at subtoxic concentrations. Morpholine derivative of DOX has the greatest potential to inhibit proliferation and to induce differentiation in vitro. The correlation between these two cellular processes was also significant for other tested compounds. In cell cycle analysis, we have demonstrated that those anthracycline derivatives that exert the greatest cytostatic potential caused G_2/M arrest, which in turn, might contribute to the development of a differentiating phenotype. The concentrations of the compounds used in the study are pharmacologically relevant. These new potent inducers of differentiation might be exploited as anticancer drugs for treatment of leukemia by differentiation therapy.

Keywords: Anticancer drugs; Anthracycline derivatives; Erythroid differentiation; Growth arrest; K562 cells; Differentiation therapy

1. Introduction

Chronic myelogenous leukemia (CML) is a myeloproliferative disorder, which is characterized by a chromosomal translocation, resulting in constitutively activated Bcr-Abl tyrosine kinase. The kinase activity of Bcr-Abl maintains a high expression level of the antiapoptotic protein Bcl-xL, which confers resistance to apoptosis induced by anticancer drugs [1,2]. The K562 cell line derived from a human female with chronic myeloid leukemia in blast crisis [3] and represents a widely used in vitro model system for CML, was also shown to be refractory to apoptosis induced by DNA-damaging drugs [4]. Concerning the balance between proliferation and differentiation, the lack of commitment of K562 cells to differentiation can be abrogated with a variety of compounds, both physiological and non-physiological ones, such as anticancer drugs. Several studies indicated that K562 cells could differentiate along erythroid or megakaryocytic lineage depending upon the stimulus used. Phorbol ester (PMA) stimulates megakaryocytic development, whereas many compounds, as diverse as hemin, butyric acid, cytosine arabinoside (ara-C), hydroxyurea, *cis*-platin, tallimustine, cyclopentenyl cytosine (CPEC) or imatinib (STI571) promote erythroid differentiation of these cells [5–13]. Induction of leukemia cell differentiation occurs at low concentrations of drugs, which is not toxic for the cells. Therefore, this less toxic approach referred to as differentiation therapy, which involves compounds that modify the state of differentiation and growth of cancer cells may provide a support to cytotoxic chemotherapy and

Abbreviations: ACLA, aclarubicin; CML, chronic myelogenous leukemia; DOX, doxorubicin; DOXH, hexamethyleneimine derivative of doxorubicin; DOXM, morpholine derivative of doxorubicin; DRB, daunorubicin; DRBH, hexamethyleneimine derivative of daunorubicin; DRBM, morpholine derivative of daunorubicin; EDOX, epidoxorubicin; EDOXM, morpholine derivative of epidoxorubicin; TPA, 12-*O*-tetradecanoyl-phorbol 13-acetate

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radiotherapy [14,15]. Upregulation of the expression of human γ-globin gene and production fetal hemoglobin (HbF), which is also observed in the presence of differentiation inducers is considered to be clinically beneficial in sickle cell disease and β-thalassaemia [16–18].

The anthracycline antibiotics doxorubicin (DOX) and daunorubicin (DRB) have been used for almost 40 years in treatment of solid tumors and leukemias [19]. A number of different mechanisms have been proposed to be responsible for the cytotoxic and antiproliferative effects of these anthracycline antibiotics [20]. Most of them involve direct interaction with DNA and DNA/protein interplay or free radical formation with their consequences for cellular processes. New derivatives of anthracycline antibiotics with a formamidine group bonded to daunosamine moiety with either morpholine (DRBM, DOXM, and EDOXM) or hexamethylene (DRBH and DOXH) rings attached to this group have been synthesized. We have previously shown that these newly synthesized derivatives of DOX, DRB, and EDOX bind DNA and recognize similar but not identical DNA sequences as the parent compounds [21]. The ability of these anthracyclines, except for DOX and DRB antibiotics [22–24] to induce growth arrest and differentiation of leukemia cell lines has not been studied. It is known that DOX and another anthracycline derivative, aclarubicin (ACLA) induce differentiation of K562 cells [23,25] and other leukemia cells [26]. One of the major actions of anthracyclines is inhibition of topoisomerase II [27]. It has been shown that compounds that are able to decrease topoisomerase II activity also permit erythroid differentiation [28,29].

In the present study, we aimed first at evaluating the effect of structurally different anthracycline derivatives on growth rate and viability of K562 cells. The second purpose was to investigate if the continuous exposure of the cells to nanomolar, subtoxic concentrations of these compounds could stimulate differentiation. Our results provide evidence that there is a correlation between the degree of inhibition of proliferation and accumulation of hemoglobinized cells. Interestingly, these effects were accompanied by cell-cycle arrest in G_2/M-phase. The concentration of the compounds used in the study is pharmacologically relevant, and therefore, it may be important to evaluate their usefulness in treating some types of leukemia in monotherapy or in combination with other cytostatic drugs.

2. Materials and methods

2.1. Drugs

Anthracyclines, parent drugs (DRB, DOX, and EDOX), and their morpholine derivatives (DRBM, DOXM, and EDOXM), and two hexamethyleneimine derivatives (DRBH and DOXH), were synthesized in the Institute of Biotechnology and Antibiotics, Warsaw and kindly provided by Dr. Irena Oszczapowicz and Mrs. Malgorzata Wasowska. The drugs were dissolved in water at the concentration 1 mM. Stock solutions of each compound were stored in the dark at −20 °C and diluted immediately before use. The chemical structure of anthracycline derivatives were published elsewhere [21].

2.2. Cells and culture conditions

The human erythroleukemia cell line K562 was cultured in a humidified atmosphere of 5% CO_2 at 37 °C in RPMI 1640 (Gibco, Scotland, UK), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, Scotland, UK) and 50 μg ml^{-1} gentamycin (Polfa, Tarchomin, PL). Cells were passaged three times per week. The cell density of the cultures was maintained between 1×10^5 and 1×10^6 ml^{-1}. For the experiments, cells were seeded at a low density of 4×10^4 ml^{-1}. Exponentially growing cells were used for all experiments described below.

2.3. Proliferation and viability assay

Cell proliferation and viability were determined by Trypan blue exclusion test. K562 cells were seeded in the absence or in the presence of different concentrations of parent anthracyclines or their derivatives. EC_{50} values (concentration of tested agent causing 50% inhibition of cell growth) were estimated after 72 h exposure and only viable cells were counted. In viability assay, the number of cells that did not take up Trypan blue was expressed as the percentage of the total cell number. All other experiments were performed at the concentrations of anthracycline derivatives for which cell viability of $> 90\%$ on day 3 was observed. For determination of growth rate, treatment at the selected concentrations was carried out up to 6 days and aliquots were removed daily for determination of cell number. The medium was not changed during the induction period.

2.4. Benzidine staining

Cells were incubated with the indicated concentrations of anthracycline derivatives up to 6 days. To assess erythroid differentiation in K562 cells we used benzidine/H_2O_2 reaction. Cells were collected at days 1, 3–6, washed twice with ice-cold PBS and resuspended in 0.9% NaCl. Benzidine solution, containing 0.2% (w/v) tetramethylbenzidine (Sigma) in 0.5 M acetic acid, and 0.6% H_2O_2 was used to start the reaction. After 30 min incubation in the darkness at room temperature 200 cells were counted to determine the percentage of benzidine-positive cells (visualized as cells with blue crystals).

2.5. Morphological changes

To determine changes in cell size distribution, 2×10^4 cells were centrifuged (1000 rpm for 5 min, 20 °C) onto a

slide and then stained with May-Grünwald and Giemsa (Sigma Diagnostics, St. Louis, MO, USA). The cells were analyzed using a light microscope BX 41 (Olympus). The size of the cells after treatment with anthracycline derivatives was measured using AnalySIS software (Soft Imaging System, GmbH). Forward scatter and side scatter were measured in FACS analysis to detect changes in size and granularity, respectively.

2.6. Real-time reverse transcription PCR

Anthracycline-treated K562 cells were harvested and washed with PBS. RNA was isolated using total RNA kit (A&A Biotechnology, Gdynia, PL). One microgram of total RNA was then reverse transcribed into cDNA using random hexamers and SuperScript II Reverse Transcriptase (Invitrogen, Life Technologies, Carlsbad, CA, USA). The PCR primer sequences are listed below. All PCR reactions were performed using qPCR^{TM} Core Kit for Sybr^{TM} Green I w/o dUTP (EUROGENTEC, Seraing, B) according to the manufacturer's suggested protocol. Gene expression levels of Ki67 and β-microglobulin were assayed by quantitative real-time PCR using ABI Prism 7000 Sequence Detection System (Applied Biosystem, Warrington, UK). The relative expression of Ki67 gene was normalized to the house-keeping gene β-microglobulin and was calculated using the formula Rel Exp = $2^{-(\Delta\Delta CT)}$ [30].

Gene	Sequence of primers (5′–3′)	Size of PCR product [bp]	Annealing temperature [°C]
Ki67	TCCTTTGGTGGGCACCTAAGACCTG TGATGGTTGAGGCTGTTCCTTGATG	156	56
β-Micro-globulin	TGAGTGCTGTCTCCATGTTTGA TCTGCTCCCCACCTCTAAGTTG	88	50

2.7. Flow cytometry

Flow cytometric analysis was performed to analyze cell cycle progression. A total of 0.3–1 × 10^6 cells was harvested by centrifugation, washed with PBS and fixed in ice-cold 70% ethanol overnight at −20 °C. After two additional washing steps in PBS, cells were stained with propidium iodide/RNAse solution (BD Pharmingen, San Diego, CA, USA) for 30 min at room temperature in the dark. DNA content was measured on a FACS Calibur flow cytometer (Becton-Dickinson, San Diego, CA, USA). Up to 10,000 cells per sample were analyzed. Fluorescence intensity data were acquired using the CellQuestPro program. Cell cycle phase distribution, i.e., the percentages of the analyzed cell population in G_1-, S-, or G_2/M-phases was determined by the ModFit cell cycle analysis program.

Staining of cells for surface CD41a was used to evaluate magakaryocytic differentiation. It employed the PerCP-Cy5.5-conjugated antibody (BD Bioscience, San Jose, CA, USA) at a concentration of 2.5 μg ml^{-1}. About 0.2 × 10^6 cells after stimulation with anthracycline derivatives were washed with PBS, resuspended in PBS/0.3% BSA and incubated for 30 min on ice with antibodies. After washing, cells were analyzed on a FACS Calibur flow cytometer (Becton-Dickinson, San Diego, CA, USA). Fluorescence intensity data were acquired using the CellQuestPro program. Autofluorescence of unstained cells was subtracted from all determinations. As a positive control, K562 cells stimulated with 6 nM TPA (Sigma) were used.

2.8. Statistical analysis

Results from at least three independent experiments were given as mean ± S.D. Subsequent analysis was done using ANOVA test. Relationships between cell proliferation and cell differentiation were analyzed using the non-parametric Spearman rank correlation test. Results were considered as significant when $p < 0.05$.

3. Results

3.1. Anthracycline derivatives reduce proliferation and viability of K562 cell line

Proliferation assay showed some of the newly synthesized anthracycline derivatives to be effective cytostatic agents in the chronic myeloid leukemia cell line K562 (Fig. 1). They inhibited the proliferation in a dose-dependent manner and could be grouped based on EC_{50} values on day 3. The EC_{50} values of parent drugs (DRB, DOX, and EDOX), and some morpholine derivatives (DOXM and EDOXM) were calculated to be about 40 and 80 nM, respectively. Much higher values of EC_{50} were obtained for derivatives of DRB (300 nM for DRBM and DRBH) and for the hexamethyleneimine derivative of DOX (200 nM DOXH). The decrease in cell viability reached significance at different concentrations of compounds (Fig. 2). For parent drugs, 100 nM concentrations reduced cell viability to about 70%, whereas for their derivatives this concentration caused only slight decrease in cell viability (compare black bars in Fig. 2). The highest concentration of anthracycline derivatives resulting in more than 90% viable cells was called subtoxic concentration and was used in all subsequent experiments. In some cases, this subtoxic concentration was similar to the

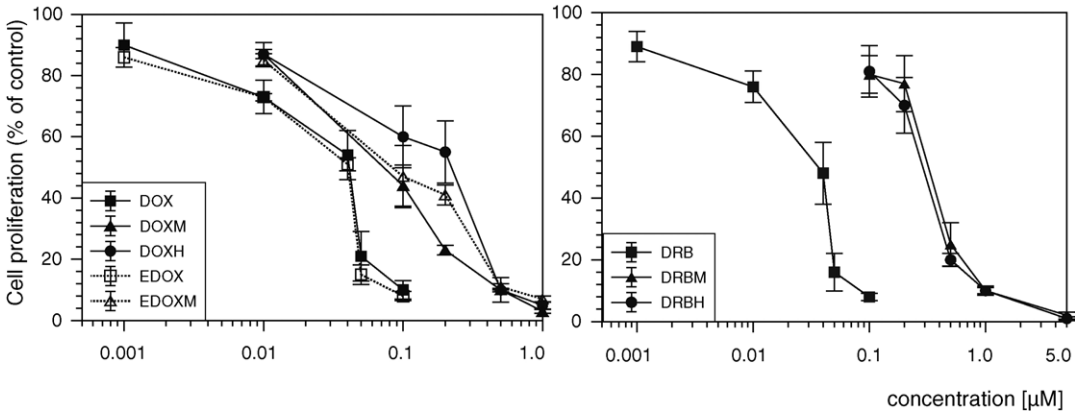

Fig. 1. The inhibitory effect of anthracycline derivatives on K562 cell proliferation. Relative cell number was estimated after 3 days of culturing in the presence of different concentrations of anthracyclines. These data represent the mean ± S.D. from three independent experiments.

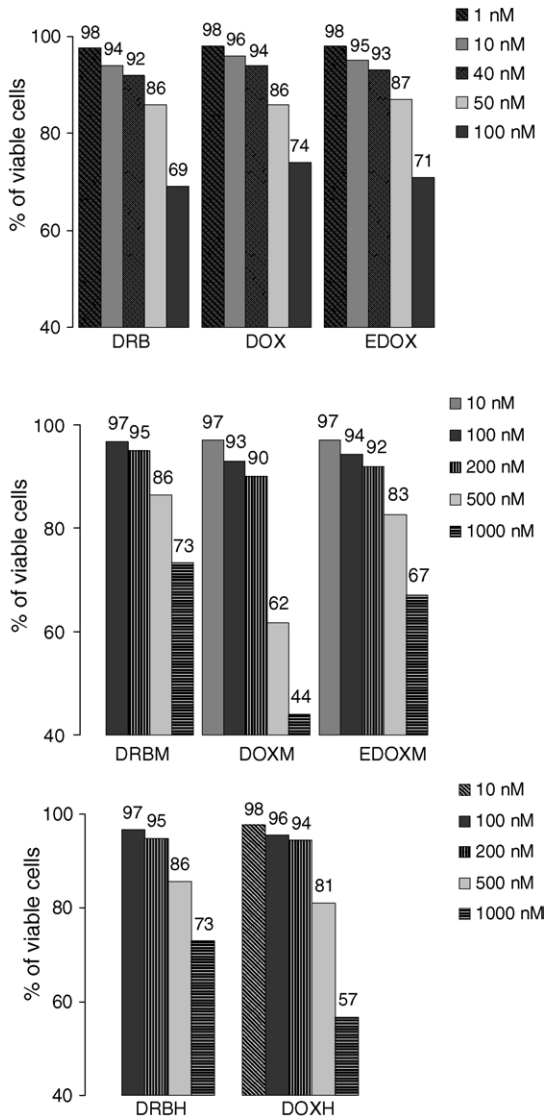

Fig. 2. The effect of anthracycline derivatives on K562 cell viability. Cells were cultured for 3 days in the presence of anthracycline derivatives. Viable cells were identified by their ability to exclude Trypan blue. The number of viable cells is expressed as percentage of total cell number. Data represent the mean ± S.D. of three independent experiments.

concentration necessary to decrease the proliferation rate to about 50% of control (40 nM of parent drugs, 200 nM DOXH). For DOXM and EDOXM, it was much higher (200 nM) than EC_{50} values (80 nM), which indicated higher cytostatic potential of these two derivatives. For DRB derivatives (DRBM and DRBH), the subtoxic concentration was 200 nM, and therefore, was lower than EC_{50} values for these compounds.

3.2. Subtoxic concentrations of anthracycline derivatives differentially reduce growth rate and expression of Ki67

These selected concentrations (40 nM of parent drugs and 200 nM of their derivatives) were used in all subsequent experiments. A statistically significant duration-dependent reduction of the cell proliferative capacity was observed in K562 cells cultured with subtoxic concentrations of anthracyclines continuously for 6 days. Mean results from four independent experiments are shown in Fig. 3. The highest cytostatic response (reduction of the proliferation rate to about 20% of control on day 3) was seen in cell cultures exposed to 200 nM DOXM. A reduction in growth rate by day 3 to less than 50% of control was also observed for parent drugs (40 nM DRB, DOX, and EDOX), and for 200 nM DOXH and EDOXM. In the case of DRB derivatives, the proliferation rate was 70–80% of control culture.

To further study the inhibition of proliferation, we examined the level of expression of Ki67 gene by quantitative real-time RT-PCR analysis. Reduced expres–sion of the proliferation marker Ki67 was observed after 24 h of treatment with anthracyclines. mRNA levels of Ki67 were lowered to 50% or less of the control level (Table 1). The highest decrease was observed in cultures stimulated with 200 nM DOXM (30% of the control level; $p < 0.0005$). No further significant changes were observed after additional 2 or 5 days of culturing (not shown).

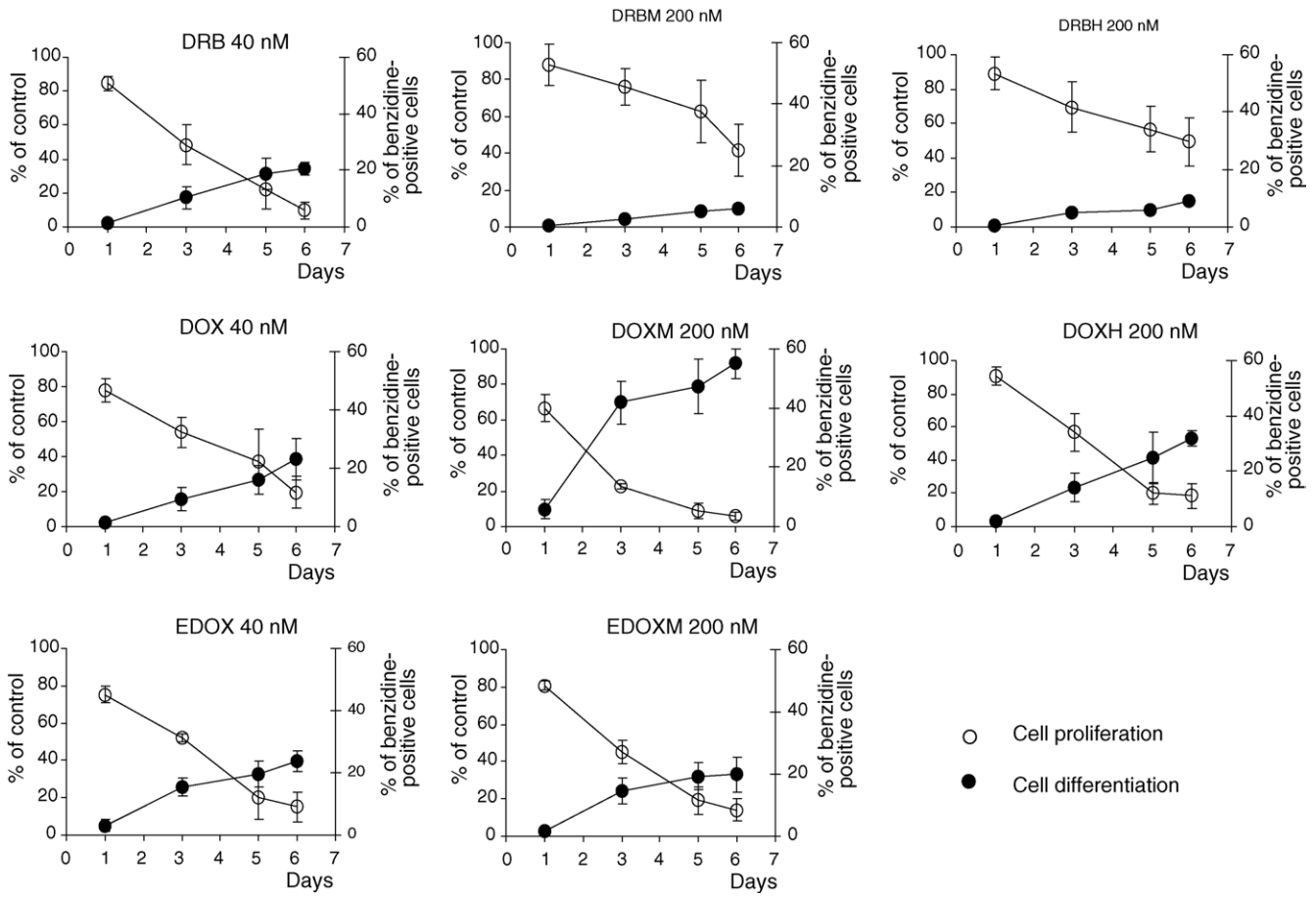

Fig. 3. Time course of anthracycline-induced cell growth inhibition and erythroid differentiation of K562 cells. Cell cultures were treated with anthracycline derivatives at the selected subtoxic concentrations (40 nM of parent drugs and 200 nM of their derivatives) for up to 6 days. Aliquots were removed daily for a determination of the number of viable and benzidine-positive cells. Simultaneously, non-induced cells were cultivated as control. Growth rates are expressed as the percent of control. Erythroid differentiation is expressed as percentage of benzidine-positive (hemoglobin-containing) cells out of all viable cells. Data represent the mean ± S.D. of four independent experiments.

3.3. Anthracycline derivatives affect K562 cell morphology

The effect of anthracycline derivatives on K562 cells was also investigated by morphological analysis using light microscopy. Numerous larger cells were clearly visible in DOXM, DOXH, EDOX, and EDOXM-treated cultures (Fig. 4A). After 4 days in culture, the average cell size of the K562 cells treated with DOXM or EDOX was about 1.5 times bigger than that of control cells, and only 16% of the cells had the same size like the control cells (Fig. 4B). These results were in a good agreement with those obtained by FACS analysis (Fig. 4C). Changes in granularity measured by side scatter (SSC, on the *Y*-axis) were observed for DOX and EDOX and their morpholine derivatives. And again, the most pronounced cell size change measured by forward scatter (FSC, on the *X*-axis) was visible in cultures treated with DOXM and EDOX.

Table 1
Relative Ki67 mRNA expression levels measured by real-time quantitative RT-PCR analysis

Anthracycline derivative	Relative Ki67 expression (mean ± S.D.)	*p*-Value
DOX	0.50 ± 0.28	<0.001
DOXM	0.29 ± 0.20	<0.0005
DOXH	0.48 ± 0.31	<0.001
EDOX	0.43 ± 0.15	<0.0005
EDOXM	0.35 ± 0.12	<0.0001

K562 cells were collected after treatment with anthracycline derivatives for 1 day. DRB and its derivatives were not assessed in this experiment. Control expression level obtained for untreated cells was taken as 1. Mean values from three independent experiments are presented.

3.4. Some anthracycline derivatives induce erythroid differentiation

Since enlarged phenotype might suggest differentiation towards megakaryocytes we evaluated the level of expression of megakaryocytic-specific surface marker, CD41a. In parallel, we used cells stimulated with 6 nM TPA, a well-known inducer of megakaryocytic differentiation. In contrast to TPA, none of anthracycline derivatives induced a detectable expression of CD41a (not shown).

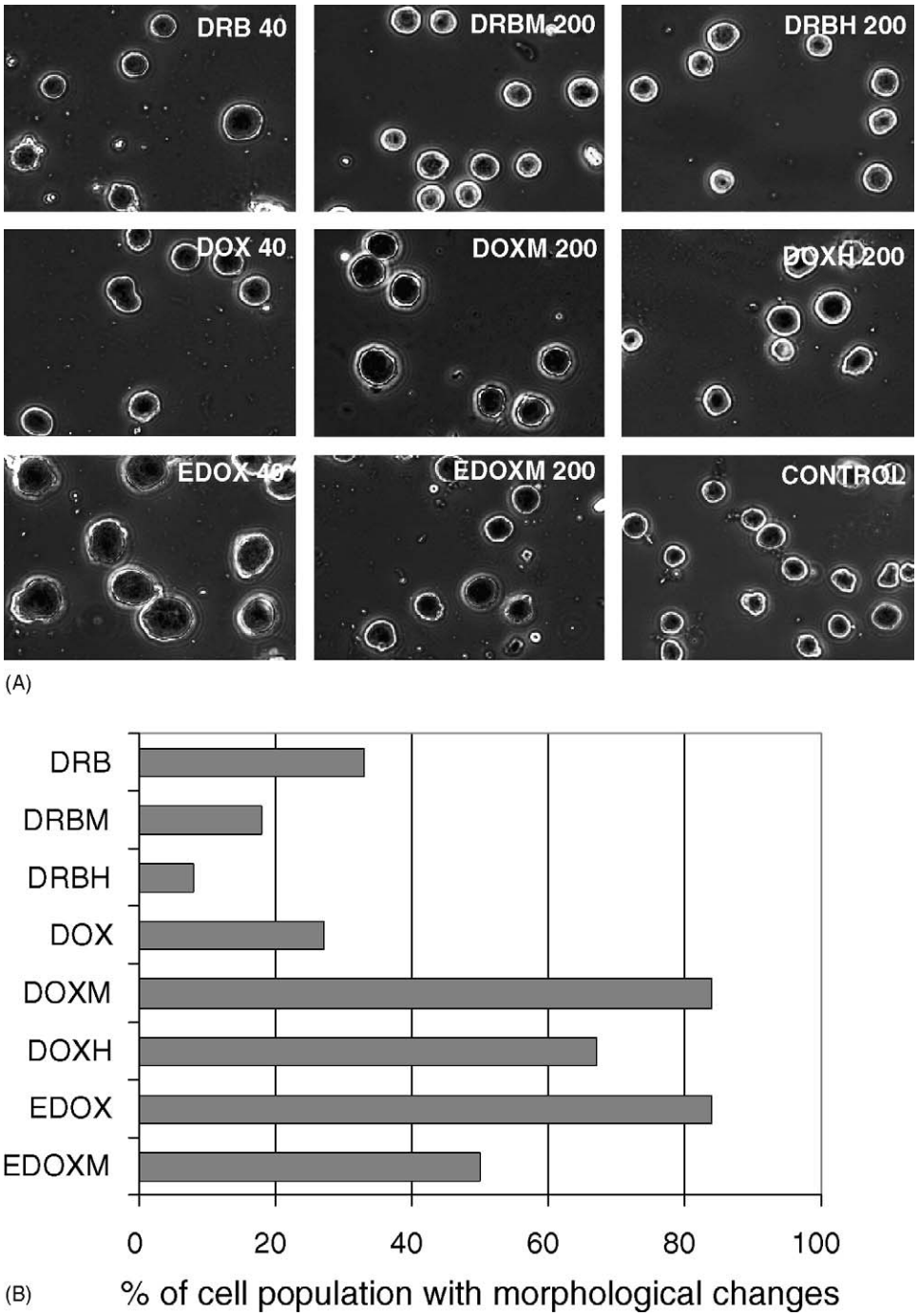

Fig. 4. Morphological changes of K562 cells after treatment with different anthracycline derivatives. (A) Control cells and cells after 4 days incubation in the presence of anthracycline derivatives were stained with May-Grűnwald-Giemsa and analyzed using light microscope BX 41 (Olympus). (B) The size of the cells was measured using AnalySIS software. The percentage of cells with a size exceeding the size of the largest control cell was expressed as % of cell population with morphological changes. (C) Flow cytometry was used to show changes in size (forward scatter on the *X*-axis) and granularity (side scatter on the *Y*-axis) after treatment with anthracycline derivatives.

To assess erythroid differentiation in K562 cells, we used benzidine staining. In untreated cultures, the percentage of benzidine-positive cells was very low (less than 2%). The data plotted in Fig. 3 show that erythroid differentiation was already detectable after 3 days of treatment with all parent compounds (DRB, DOX, and EDOX) reaching 20% of benzidine-positive cells after additional 3 days of incubation. The differentiation on day 3 was accompanied with a marked inhibition of cell proliferation (Fig. 3) but no cytotoxicity (Fig. 2). DRB derivatives did not induce hemoglobin synthesis, whereas derivatives of DOX and EDOX were similar or more potent inducers than parent drugs. The maximal effect was observed in DOXM-treated cells. The percentage of benzidine-positive cells increased as the DOXM incubation period was prolonged. More than 50% of the cells

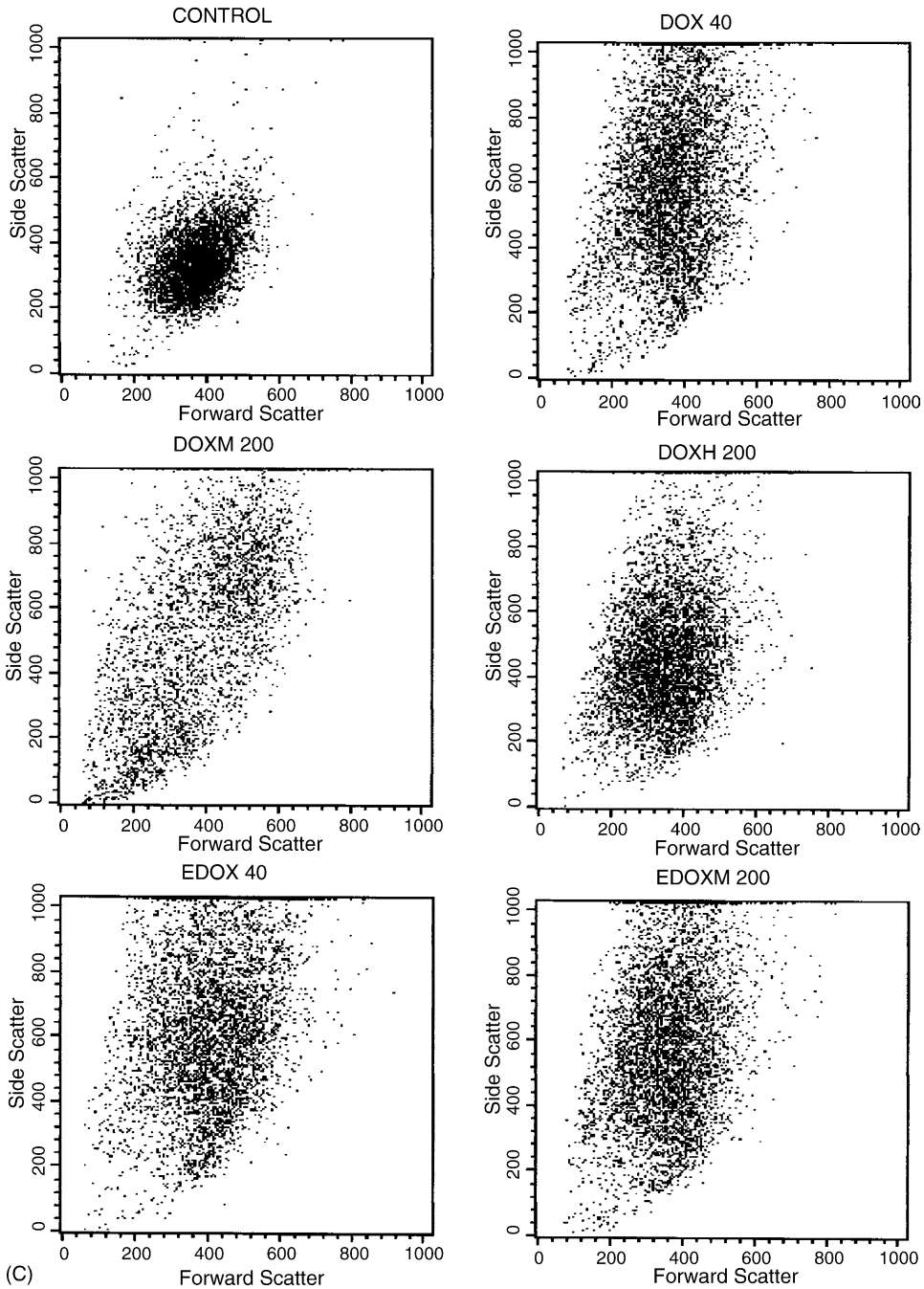

Fig. 4. (*Continued*).

were benzidine-positive on day 6. It appeared that the concentrations of anthracycline derivatives used in the study were optimal to induce differentiation since further increase of concentrations caused rather cell death than an increase in the number of differentiated cells (not shown).

All studied anthracycline derivatives showed a statistically significant negative correlation between cell proliferation and cell differentiation as assessed by Spearman rank correlation test (Fig. 5, Table 2). The strongest negative correlation was observed for DOXM-treated cells with a correlation coefficient reaching the value of $r = -0.9541$ ($p = 0.0001$).

3.5. Induction of differentiation of K562 cells is accompanied by cell-cycle arrest at G_2/M-phase

To further characterize the effect of those anthracycline derivatives, which induced K562 cell differentiation, cell cycle analysis was performed on propidium iodide-stained cells (Fig. 6). Flow cytometric analysis of DNA content revealed that in untreated control K562 cell cultures, G_0/G_1-, S-, and G_2/M-phase cells represented approximately, 34, 54, and 12% of the total cell population, respectively. Exposure of exponentially growing cultures to anthracycline derivatives at concentrations which did not affect cell viability resulted in a marked reduction of cell number in

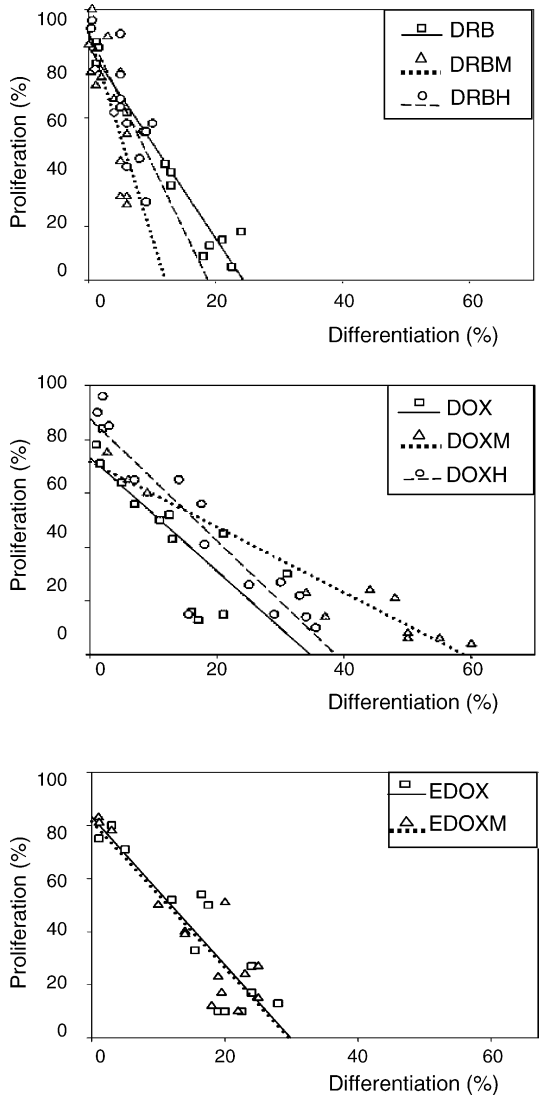

Fig. 5. Indirect correlation of differentiation with proliferation. The data used to plot the time courses for proliferation and for erythroid differentiation (Fig. 3), were correlated using Spearman rank correlation test. Correlation coefficients (r) are shown in Table 2.

G_0/G_1-phase. Decrease in the number of cells in G_0/G_1-phase occurred already after 24 h of treatment with anthracyclines (Fig. 6). The highest reduction was observed for DOXM, from 34% in control cells to 4% in DOXM-treated cells. It was significant also for other derivatives. A marked loss of cells in the G_0/G_1-phase correlated with an accumulation of the K562 cells in S- and/or G_2/M-phase. DOXM and DOXH caused increased cell numbers in S- and G_2/M-phase, whereas EDOX and EDOXM-treated cells accumulated mainly in G_2/M. The strongest shift in cell cycle towards S-phase was observed in DOXM-treated cells, from 53.8% for the control cells to 75.7% and the G_2/M arrest was the highest in the case of EDOXM (from 11.9 to 49.6%).

Table 2
Correlation between inhibition of proliferation and induction of differentiation in K562 cell cultures treated with different anthracycline derivatives expressed as Spearman rank order correlation coefficients (r)

Anthracycline derivatives	Correlation coefficient (r)	p-Value
DRB	−0.8877	0.0001
DRBM	−0.7975	0.0005
DRBH	−0.8282	0.0001
DOX	−0.8803	0.0001
DOXM	−0.9541	0.0001
DOXH	−0.8899	0.0001
EDOX	−0.8105	0.0008
EDOXM	−0.7048	0.0050

When the cell cycle distribution after 3 days of treatment with anthracycline derivatives was compared with day 1 (Table 3), a tendency towards a reduced cell population in G_2/M and an increase in S-phase was evident. These changes were due to the constantly growing number of cycling cells relative to the amount of quiescent differentiated cells in culture and reflected the ability of the compound to reduce cell proliferation. DOXM which decreased cell growth to 20% of control on day 3 (Fig. 3) caused further accumulation of the K562 cells in G_2/M-phase between days 1 and 3 (Table 3), whereas for other compounds, which were less efficient in reducing cell proliferation (Fig. 3) more cells in S-phase were present on day 3 in comparison to day 1 (Table 3).

A small population of cellular fragments was also visible in the FACS analysis which increased with the time of exposure to anthracyclines derivatives (not shown). This subdiploid population on day 3 was, however, not typical for cells in apoptosis. It may rather indicate the slow induction of necrosis in this cell line refractory to apoptosis. After 6 days of continuous exposure to anthracycline derivatives, viability in cell cultures was still in the range between 70 and 90%.

4. Discussion

Dose-dependent regulation of cellular processes is one of the important features of signaling molecules naturally occurring in cells. A similar phenomenon is observed for many compounds tested as potential anticancer drugs. Depending on the concentration used many different cellular processes may be influenced both in studies in vitro and in clinical treatment. DOX, DRB, and EDOX exert their cytotoxic activity against cancer cells mainly by intercalation into DNA and formation of cross-links, inhibition of topoisomerase II and helicase activity, all of these leading to inhibited synthesis of nucleic acids. In the interaction of these drugs with the malignant cells free radical formation, lipid peroxidation and direct membrane effects should be also considered [20]. This study was undertaken to investigate which cellular processes in rapidly growing populations of leukemia K562 cells could be affected by anthracycline antibiotics and their new derivatives at subtoxic concentrations. Some of these newly synthesized anthracycline derivatives used in our

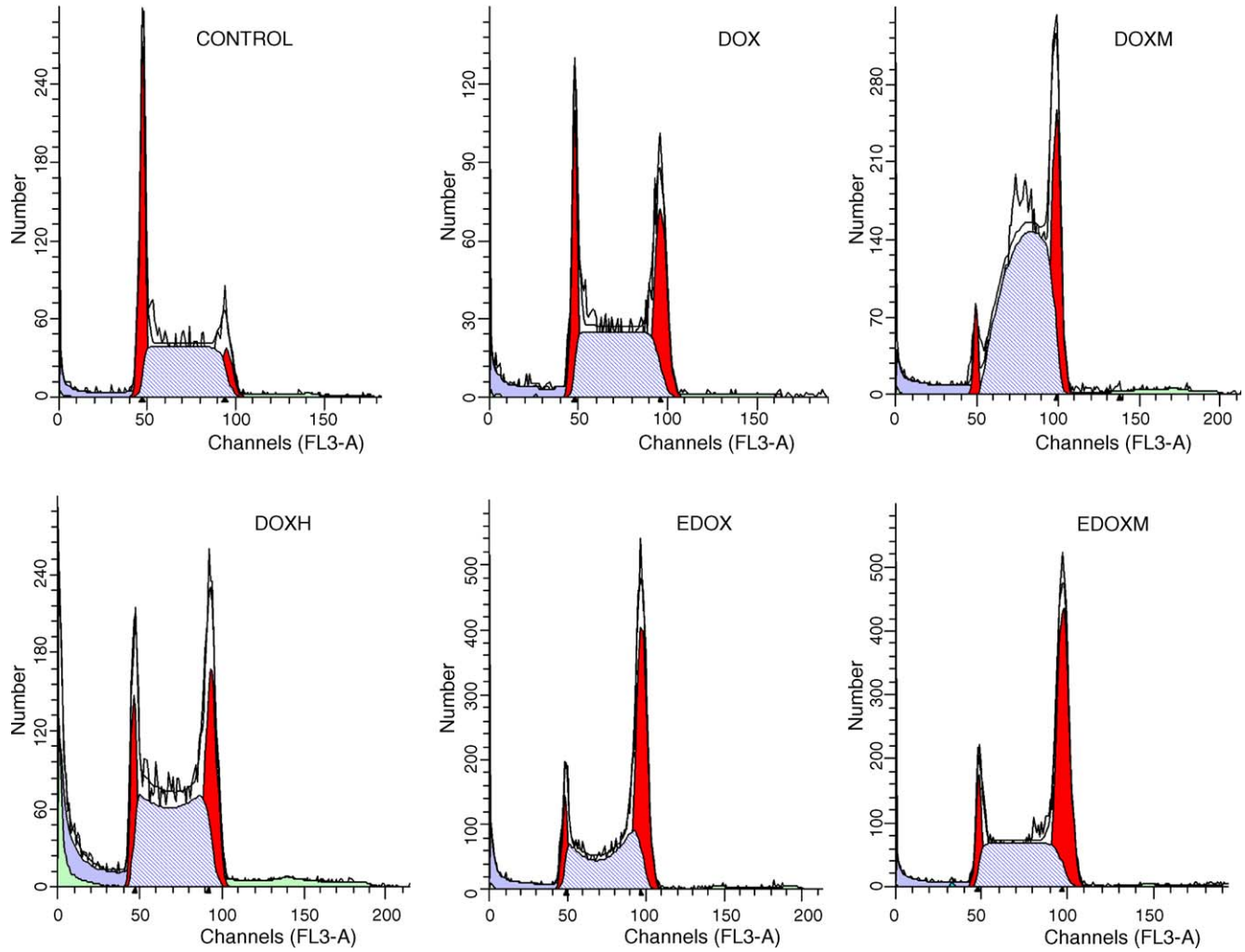

Fig. 6. Cell cycle distribution of K562 cells after treatment with different anthracycline derivatives. The fractions of viable cells in the G_0/G_1-, S-, and G_2/M-phases of the cell cycle were quantified by flow cytometric analysis of propidium iodide-stained cells. Histograms are representative of three independent experiments. Results of ModFit analysis with percentage means attributed to the respective cell cycle stage are included in Table 3.

present study form complexes with DNA at a high concentration (50 μM) and in the presence of CH_2O [21]. Their ability to affect cellular processes at low concentrations, similar to those that reflect the plasma levels in the patient, was not studied previously.

In the first part of our study, to select concentration for each anthracycline derivative which did not affect cell viability but significantly reduced growth rate, we assessed the effect of these compounds on cell viability and proliferation in the CML-derived K562 cell line. Results indicated a clear concentration-dependent decrease in cell viability for all tested compounds. The concentrations, which allowed keeping more than 90% cells alive were, however, different for different derivatives. The highest possible subcytotoxic concentration for each compound was used to investigate its cytostatic effect. The most

Table 3
Flow cytometric analysis of the cell cycle distribution of K562 cells following exposure to different anthracycline derivatives

Anthracycline derivative	Day 1			Day 3		
	G_1	S	G_2/M	G_1	S	G_2/M
Control	34.3 ± 0.4	53.8 ± 2.0	11.9 ± 2.3	39.2 ± 4.4	54.2 ± 1.7	6.6 ± 2.8
DOX	19.6 ± 2.1	48.7 ± 4.4	31.8 ± 6.4	16.1 ± 7.1	58.7 ± 3.8	25.3 ± 11.0
DOXM	4.2 ± 1.1	75.7 ± 6.4	20.2 ± 6.6	5.7 ± 1.6	64.7 ± 9.6	29.7 ± 11.0
DOXH	9.1 ± 2.9	62.7 ± 2.4	28.2 ± 0.9	10.6 ± 4.8	66.7 ± 5.4	22.8 ± 6.5
EDOX	8.9 ± 1.1	42.8 ± 0.5	48.4 ± 0.4	9.3 ± 3.2	55.0 ± 17.0	35.8 ± 13.9
EDOXM	9.3 ± 1.8	41.1 ± 1.7	49.6 ± 0.8	12.5 ± 3.6	54.6 ± 11.0	32.9 ± 14.5

Cells were fixed and stained with propidium iodide, and percentage of cells in each phase of the cell cycle was determined as described in Section 2. Values represent the mean ± S.D. for three experiments.

pronounced growth inhibition was caused by treatment of K562 cells with 200 nM DOXM. Additionally, mRNA levels of Ki67 were used as a marker of cell proliferation. The results confirmed those obtained by counting viable cells. DOXM and to a lesser extent DOX, DOXH, EDOX, and EDOXM decreased Ki67 expression by 50% or more when used at subtoxic concentrations.

It is known that one consequence of in vitro treatment of tumor cells with anthracyclines is the induction of DNA damage, which causes cell-cycle arrest and then promotes apoptosis [31,32]. In most of those in vitro experiments, high concentrations of anthracyclines were used which cannot be reached in cancer therapy. There is clear evidence that the decline in the amount of cancer cells after chemotherapy cannot be explained by apoptosis alone, especially that some of the cells are intrinsically refractory to apoptotic death in response to DNA damage [33,34]. Cell death through mitototic catastrophe, terminal growth arrest through senescence or differentiation has to be also considered [35–38]. In our FACS analysis, there was no indication that anthracycline derivative-induced apoptosis. This observation was expected since: (a) we used low concentrations of anthracyclines which ruled out their cytotoxicity and (b) besides being Bcr/Abl/p210-positive, K562 line is a p53-defective leukemia cell line not able to mediate DNA-damage-induced apoptosis [34,39].

To explore the mechanism responsible for the antiproliferative effects of tested anthracycline derivatives we assessed changes in cell morphology, cell differentiation and cell cycle distribution. Anthracycline-treated cells showed enlarged cell size as observed under the microscope. Loss of proliferative capacity and enlarged cell size might indicate the induction of senescence. This was observed previously for K562 cells treated with hydroxyurea. The induction of the senescence-like changes was associated with cell arrest at G_1-phase of cell cycle [40]. Our results, however, have shown that anthracycline-treated K562 cells were growth arrested in G_2/M-phase, not in G_1. The change in morphology might also suggest differentiation towards megakaryocytes. DNA content analysis, however, of anthracycline-treated K562 cells did not show polyploidization, which accompanies megakaryocytic differentiation. Also, CD41a remained undetectable on K562 cells stimulated with anthracycline derivatives. Changes in morphology might also be related to chromatin reorganization, which in turn by selective gene expression, would induce differentiation program but not towards megakaryocytes [29,41]. This prompted us to investigate if anthracycline derivative-induced differentiation towards erythrocytes. Indeed, a growing population of benzidine-positive cells was observed under the respective experimental conditions. A higher ability of anthracycline derivatives to inhibit proliferation and to increase cell size and granularity was accompanied by stronger differentiating potential. The correlation between inhibition of proliferation and induction of differentiation was significant. In this respect, the chemical induction of differentiation is similar to normal hematopoiesis, where the proliferative capacity of cells decreases as the degree of differentiation increases. Some of the new anthracycline derivatives were more potent differentiation inducers than parent drugs. DOXM-induced differentiation of more than 50% cells after 6 days of stimulation, whereas in DOX-treated cultures, only 20% cells were differentiated. Our results showing that induction of differentiation depends on the chemical structure of the inducers and even small changes in their structure could cause a marked reduction or increase in differentiating potential are consistent with previously published results [23].

Concerning the relationship between the DNA-binding activity and the capacity to induce erythroid differentiation, our results did not confirm a concept that DNA-binding compounds, especially those with GC selectivity are more powerful inducers of erythroid differentiation of human K562 cells [11]. In our previous work [21], DOX, DRB, and EDOX had the highest affinity to DNA in general, and to GC sequences in particular, as assessed by footprinting assay, restriction endonuclease protection analysis, and EMSA for Sp1 and AP-1 transcription factors. DOXM and DRBM with lower affinity to DNA also differed in their sequence preferences. Some increased affinity to TC sequence was observed for those morpholine derivatives. Hexamethyleneimine derivatives (DOXH, DRBH) bound to DNA with affinity undetectable in footprinting assay. Our present results showed that among the most potent differentiation inducers both, the compounds with a low affinity to DNA (DOXM) and no affinity to DNA (DOXH) could be found. And opposite, the parent drugs, which showed high affinity towards DNA appeared to be less potent differentiation inducers. We did not observe the positive correlation between affinity to DNA, especially to GC sequences, and differentiation potential.

Although the exact mechanism of differentiation induced by tested anthracyclines has yet to be fully elucidated, there is some evidence suggesting similar mechanism as observed for Ara-C-induced K562 cell differentiation [42]. It was shown that Ara-C stimulated both phosphorylation of Chk2, which led to cell-cycle arrest at G_1/S and phosphorylation of Chk2, which in turn caused cell-cycle arrest at G_2/M. It has been pointed out that both pathways are important in the chemically induced differentiation of leukemia cells. In our experiments, we observed cell-cycle arrest at G_2/M prior to K562 cell differentiation towards erythrocytes. However, G_2/M arrest seemed to be not sufficient for induction of differentiation. Some of the tested compounds, e.g., EDOX and EDOXM caused accumulation cells in G_2/M-phase to a high extent already during the first 24 h, but the highest level of differentiation was observed for DOXM and DOXH for which accumulation of the cells in S-phase preceded G_2/M arrest. Other studies [43] have shown that the G_2/M-phase arrest caused by DOX is related to the disruption of

$p34^{cdc2}$/cyclin B activity. A study on signal transduction pathway(s) leading to growth arrest and then cell differentiation should be performed to resolve the differences in the mode of action of different anthracycline derivatives. Concerning effects on the regulation of erythroid gene expression, it has been shown that some anthracycline derivatives (ACLA) may activate the regulatory regions of erythroid genes, whereas other derivatives (DOX) may act at the post-transcriptional level by increasing the half-lives of erythroid mRNAs [23,25].

Up to day 3, the percentage of K562 cells that became subdiploid was much lower than the percentage of benzidine-positive cells suggesting that prior differentiation might be necessary to induce cell death. With time when more cells were differentiated the population of cellular fragments was also growing. Similar results have been reported previously. No features of apoptosis have been detected in DOX and ACLA-treated K562 cells [23], however, another anthracycline derivative, DRB was shown to trigger apoptosis in other leukemia cell lines, such as HL-60 and U937 [44]. It have been also reported that DOX might promote apoptosis in K562 cells, but at much higher concentrations than those necessary to induce differentiation [45]. Further studies, however, are necessary to elucidate the precise mechanism of anthracycline-induced K562 cell differentiation and its consequences for the cell survival. Mechanisms involved in differentiation of leukemia cells induced by several other compounds were recently reviewed [46].

In conclusion, we have shown that efficiency of erythroid differentiation of K562 cells depends on the chemical structure of anthracycline derivatives. At subcytotoxic concentration, DOXM has the greatest potential to inhibit proliferation and to induce differentiation in vitro. Differentiation is preceded by cell-cycle arrest in G_2/M-phase. The concentrations of anthracycline derivatives used in this study are pharmacologically relevant. The initial plasma concentration in the patient after bolus administration falls into the range 1–2 μM. It declines rapidly within 1 h to 25–250 nM which is also the concentration maintained by continuous infusion [20,47]. In clinical applications, doses of cytostatic drugs are strictly limited by their toxicity. In the case of anthracyclines, it is cardiotoxicity [27]. Therefore, those of the newly synthesized anthracycline derivatives which when used at low, nanomolar concentrations were not cytotoxic but able to induce differentiation of rapidly dividing cells might be exploited as potential therapeutic agents especially in combination drug therapies.

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