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Accumulation of γ-globin mRNA and induction of irreversible erythroid differentiation after treatment of CML cell line K562 with new doxorubicin derivatives

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ABSTRACT

Human chronic myelogenous leukemia (CML) cell line K562 can be chemically induced to differentiate and express embryonic and fetal globin genes. In this study, the effects of doxorubicin (DOX), an inducer of K562 cell erythroid differentiation, with those of epidoxorubicin (EDOX) as well as newly synthesized derivatives of both drugs (DOXM, DOXH, and EDOXM) on cell growth and differentiation were compared. Our results revealed that DOX, EDOX and their derivatives caused irreversible differentiation of K562 cells into more mature hemoglobin-containing cells. This phenomenon was linked to time-dependent inhibition of cell proliferation. Considering the impact of the structure of newly synthesized anthracyclines on their cellular activity, our data clearly indicated that among tested anthracyclines DOXM, a morpholine derivative of DOX exerted the highest antiproliferative and differentiating activity. An increase of γ-globin mRNA level caused both by high transcription rate and by mRNA stabilization, as well as an enhancement of expression but not activity of erythroid transcription factor GATA-1 were observed. Therefore, a high level of hemoglobin-containing cells in the presence of DOXM resulted from transcriptional and post-transcriptional events on γ-globin gene regulation. The same morpholine modification introduced to EDOX did not cause, however, similar effects on cellular level. Characterization of new powerful inducers of erythroid differentiation may contribute to the development of novel compounds for pharmacological approach by differentiation therapy to leukemia or to β-globin disorder, β-thalassemia.

1. Introduction

Differentiation of multipotential hematopoietic cells into lineage-committed progenitors requires appropriate programs of gene expression, regulated by specific transcription factors. GATA-1, a zinc finger transcription factor, recognizes the DNA consensus sequence (A/T)GATA(A/G), present in regulatory regions of all erythroid expressed genes studied [1]. It is essential for erythrocyte, megakaryocyte, mast cell and eosinophil differentiation [2–6]. The critical role of GATA-1 for erythroid development has been shown in gene knockout experiments [7,8]. Furthermore, it has been proven that GATA-1 is involved in proliferation arrest during erythroid maturation and that this process is dependent upon its concentration

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Abbreviations: ACLA, aclarubicin; CML, chronic myelogenous leukemia; DRB, daunorubicin; DOX, doxorubicin; EDOX, epidoxorubicin; DOXM, morpholine derivative of doxorubicin; EDOXM, morpholine derivative of epidoxorubicin; DOXH, hexamethyleneimine derivative of doxorubicin

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[9,10]. K562 cell line expresses several erythroid markers and low levels of GATA-1 but can be further differentiated along the erythroid pathway by a variety of chemical compounds, as diverse as sodium butyrate, cytosine arabinoside (ara-C), hydroxyurea, cisplatin, tallimustine, cyclopentenyl cytosine (CPEC), imatinib (STI571) or apicidin [11–18]. No adult hemoglobin can be detected since the β-globin gene is not functional in the K562 cell line [19]. Instead, embryonic and fetal globin expression can be stimulated by treatment with those chemically so diverse differentiation inducers. Drug-induced differentiation of K562 cells was found to be associated with an increase in the expression of γ-globin gene. For those reasons, K562 cells provide a good model not only for studying the potential of chemical inducers in differentiation therapy of leukemia but also for evaluating the γ-globin chain-inducing activity of those compounds which can be used in treatment of diseases with insufficient production of adult β-globin chain, e.g. thalassemia [20]. Among differentiation inducers, the anthracyclines, aclarubicin (ACLA) and DOX have been studied previously, and optimal concentrations of these compounds to specifically induce globin and heme synthesis in K562 cells were established [21]. For ACLA, the optimal differentiating effect was obtained at the concentration, which did not cause a total arrest of growth whereas in the case of DOX-treated K562 cells differentiation correlated with inhibition of proliferation [22,23].

In a previous study [22], we have reported that K562 cells respond to the treatment with newly synthesized anthracyclines. 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 [24] inhibited cell proliferation but only some of them induced differentiation when employed at subtoxic concentrations. Increasing number of hemoglobinized cells indicated that new anthracycline derivatives might trigger erythroid differentiation program.

As an extension of this work, to gain further evidence that K562 cells differentiate towards erythroid lineage, in the current study we examined γ-globin expression, and GATA-1 expression and its DNA-binding activity. Since the cytoplasmic level of mRNA, and hence protein level and its activity, depends not only upon the rate of the synthesis, but also on the mRNA degradation rate, we explored the possibility that anthracycline derivatives might increase mRNA stability of erythroid genes. The results clearly indicate that anthracycline derivatives closely related in their structure might induce differentiation process by distinct mechanisms. Small changes in structure of DOX enhanced its differentiating activity by strengthening its ability to stabilize γ-globin mRNA and stimulation of erythroid gene expression.

2. Materials and methods

2.1. Drugs

Anthracyclines, parent drugs (DOX, and EDOX), and their morpholine derivatives (DOXM, and EDOXM) and hexamethyleneimine derivative (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 [24].

2.2. Cell culture conditions and proliferation assay

Human K562 cells were grown 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 gentamycin (Polfa, Tarchomin, PL). For experiments, K562 cells were seeded at the density 4×10^4/ml and 24 h later parent anthracyclines or their derivatives were added. Cell proliferation and viability were determined by using Trypan blue dye exclusion test as reported previously [22]. All 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 with anthracycline derivatives was carried out up to 6 days and aliquots were removed daily for determination of cell number. For determination of γ-globin mRNA stability, cells were treated with 5 μg/ml actinomycin D (Sigma Chemical Co) for the indicated period of time following 4 days incubation with anthracycline derivatives.

2.3. Benzidine staining

Cells were incubated with 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, and 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. In addition, to monitor changes in the amount and size of hemoglobin containing cells, benzidine stained cells were centrifuged onto a slide and then analyzed using a light microscope BX 41 (Olympus). To find out whether anthracycline derivatives are capable to activate K562 cell differentiation in an irreversible manner, cells were incubated with the compounds for 4 days. 96×10^3 cells were then transferred into fresh medium either with or without the anthracycline derivatives. After additional 4 days of culturing, the proliferation rates and the levels of benzidine-positive cells were assayed as described above.

2.4. Real-time reverse transcription PCR

Quantification of mRNA expression of γ-globin and GATA-1 and stability of γ-globin mRNA was performed by real-time reverse transcription (RT)-PCR. Total RNA was isolated from 5×10^5 K562 cells after treatment with anthracycline derivatives using Total RNA kit (A&A BIOTECHNOLOGY, Gdynia, PL) and was quantitated by A_{260} absorbance. For studying γ-globin mRNA stability, isolations were performed from K562 cells

pretreated with anthracycline derivatives for 4 days, after additional 0, 6, 20, 28, and 48 h of transcriptional inhibition with 5 μg/ml actinomycin D. For assessing the commitment to irreversible differentiation unstimulated and anthracycline-treated cells (1.2×10^5) were cultured without drugs for additional 2 days prior RNA isolation. In all experiments, 0.5–1 μg of total RNA was 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:

Gene	Sequence of primers (5′–3′)	Size of PCR product (bp)	Annealing temperature (°C)
GATA-1	CAGGTACTCAGTGCACCAAC CCATAAAGCACCTGGATGATC	334	60
γ-Globin	CCATAAAGCACCTGGATGATC ATCTGGAGGACAGGGCACTG	212	60
β-Microglobulin	TGAGTGCTGTCTCCATGTTTGA TCTGCTCCCCACCTCTAAGTTG	88	50

All PCR reactions were performed using qPCR™ Core Kit for Sybr™ Green I w/o dUTP (EUROGENTEC, Seraing, B) according to the manufacturer's suggested protocol. Gene expression levels of GATA-1 and γ-globin were assayed by quantitative real-time, fluorescence-based PCR using ABI Prism 7000 Sequence Detection System (Applied Biosystem, Warrington, UK). Their relative expression was normalized to the house-keeping gene β-microglobulin and was calculated using the formula Rel Exp = $2^{-(\Delta\Delta CT)}$ [25]. Results from anthracycline-treated cells were expressed relative to untreated control cells.

2.5. Flow cytometry

Cell cycle analysis was performed on propidium iodide-stained cells as described previously [22]. K562 cells were treated with increasing concentrations of DOXM for 24 h and then analyzed for DNA content. The percentages of the cell population in G_1, S or G_2/M phases were calculated from histograms using the CellQuest software (BD Sciences, San Jose, CA).

2.6. Electrophoretic mobility shift assay

Nuclear extract from either untreated or anthracycline-treated K562 cells were prepared as previously described [26]. They were frozen as aliquots and stored at −80 °C. The double stranded oligonucleotide used in EMSA was from human γ-globin promoter, from −199 to −182 (bold): 5′-AA**TTCCCCACA**CTATCT**CAA**CCC-3′. GATA-1 binding site is underlined. It was labeled by filling in the overhangs with Sequenase 2.0 and [α-^{32}P]dATP, and purified by 7% polyacrylamide gel electrophoresis. DNA–protein complexes were formed by incubating 8 μg of nuclear proteins with 80,000 cpm of labeled oligonucleotide probe in 20 μl of binding buffer (20 mM Hepes-KOH, pH 7.9, 1 mM EDTA, 5 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol, 10% glycerol) in the presence of 2 μg of non-specific competitor poly[dI-dC] poly[dIdC] for 20 min at room temperature. Competition was performed in the presence of a 100-fold excess of non-radioactive probe or oligonucleotide with unrelated sequence: 5′-AATTCCCCGCCCCCGCCCCC-3′ (Sp1 binding site). DNA–protein complexes were resolved on a 5% polyacrylamide gel using 0.5 × TBE buffer. Gels were vacuum-dried and exposed with intensifying screens at −20 °C.

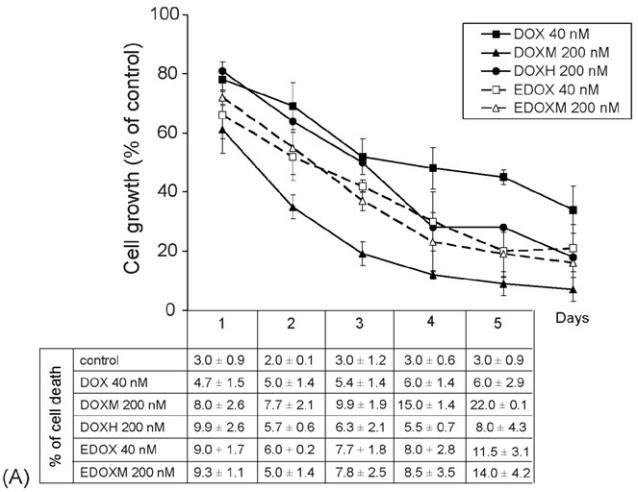
2.7. Statistical analysis

Results from at least three independent experiments were given as means ± S.D. Subsequent analysis was done using ANOVA test. Results were considered as significant when $p < 0.05$.

3. Results

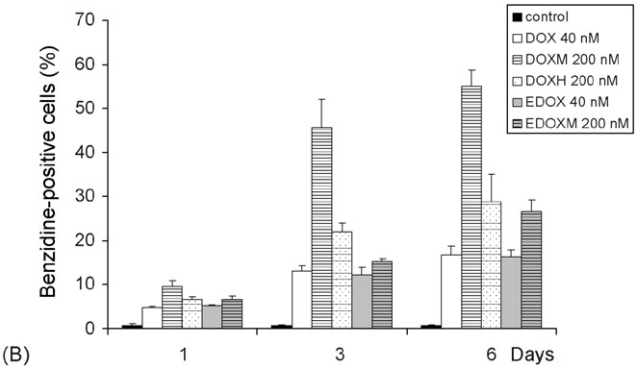
3.1. Induction of K562 cells with new DOX derivatives

Anthracycline derivatives used at subtoxic concentrations, which were selected previously [22] affected cell proliferation in vitro. Depending on the compound, K562 cells ceased division in 2 or in 3 days after treatment but did not undergo massive cell death even after 5 days of culturing (Fig. 1A). The results shown in Fig. 1 were drawn from seven independent induction experiments. The highest reduction of the cell proliferative capacity (Fig. 1A) and maximum efficiency in induction of erythroid differentiation (Fig. 1B) were observed in K562 cells cultured in the presence of 200 nM DOXM. As detected by benzidine staining, more than 50% of K562 cells were heme-producing cells. Since the viability of the cells cultured in the presence of 200 nM DOXM was 90% on day 3, which was the lowest value obtained for tested compounds (Fig. 1A), proliferation and differentiation of K562 cells were also tested at lower concentrations of DOXM on day 3. Both growth rate and the percentage of benzidine-positive cells were affected in a dose dependent manner (Fig. 2A). A high level of inhibition of proliferation and induction of differentiation was already observed at 160 nM and the influence of DOXM at this concentration on growth rate and differentiation was higher than those observed for other DOX derivatives tested in parallel at subtoxic concentrations (compare Fig. 2A with Fig. 1). To further reduce cell death, in some experiments



(A)

% of cell death	1	2	3	4	5
control	3.0 ± 0.9	2.0 ± 0.1	3.0 ± 1.2	3.0 ± 0.6	3.0 ± 0.9
DOX 40 nM	4.7 ± 1.5	5.0 ± 1.4	5.4 ± 1.4	6.0 ± 1.4	6.0 ± 2.9
DOXM 200 nM	8.0 ± 2.6	7.7 ± 2.1	9.9 ± 1.9	15.0 ± 1.4	22.0 ± 0.1
DOXH 200 nM	9.9 ± 2.6	5.7 ± 0.6	6.3 ± 2.1	5.5 ± 0.7	8.0 ± 4.3
EDOX 40 nM	9.0 ± 1.7	6.0 ± 0.2	7.7 ± 1.8	8.0 ± 2.8	11.5 ± 3.1
EDOXM 200 nM	9.3 ± 1.1	5.0 ± 1.4	7.8 ± 2.5	8.5 ± 3.5	14.0 ± 4.2



(B)

Fig. 1 – Time-dependent effects of DOX derivatives on cell proliferation, viability (A) and differentiation (B). Cell cultures were treated with anthracycline derivatives at the selected subtoxic concentrations (40 nM of DOX or EDOX and 200 nM of their derivatives) for up to 6 days. Aliquots were removed daily for a determination of the number of viable, differentiated and dead cells. Growth rates expressed as the percent of control and cell death expressed as the percent of all counted cells were evaluated by trypan blue exclusion. Erythroid differentiation is expressed as percentage of benzidine-positive (hemoglobin-containing) cells out of all viable cells. Data represent the mean (±S.D.) of seven independent experiments.

also 160 nM instead of 200 nM DOXM was used. Similarly, a direct correlation was observed on day 1 between the concentration of DOXM and growth arrest when the cell cycle was analyzed (Fig. 2B). Exposure of exponentially growing K562 cultures to DOXM resulted in a reduction of cell number in G_0/G_1 phase and an accumulation in G_2/M phase. When a higher concentration of DOXM (200 nM) was used, increased accumulation in S phase was also observed [22]. The sub-G_1 fraction was not increased upon treatment with different concentrations of DOXM. K562 cell line is refractory to apoptosis and this seemed to be the major reason for absence of the apoptotic cells in the cultures incubated in the presence of nanomolar concentrations of DOXM. Only a minor percentage of apoptotic cells in DOXM-treated cultures, similar to that obtained in untreated culture, was visualized after annexin V staining (data not shown).

3.2. *Expression levels of erythroid specific genes are changed after stimulation with DOX derivatives*

Since the hemoglobin-inducing activity of anthracycline derivatives is expected to be connected to increased transcription of erythroid genes [27], we monitored changes in the mRNA levels of GATA-1 and γ-globin by quantitative real time RT-PCR (Fig. 3). As previously reported for several other inducers of erythroid differentiation [11,28,29], we found that a growing number of hemoglobinized cells observed in DOX derivatives treated K562 cultures was accompanied by an increased level of γ-globin expression. Treatment for 5 days with DOXM or DOXH at concentrations of 200 nM enhanced γ-globin mRNA levels 6.2 ± 1.0 ($P < 0.01$)-fold and almost 3-fold ($P < 0.001$), respectively. Other compounds were less efficient in the stimulation of expression of this erythroid specific gene. In our hands the reference drug, 40 nM DOX, induced only a 2-fold ($P < 0.001$) increase in γ-globin expression. One additional day of treatment caused a decrease in relative expression of γ-globin gene (not shown). EDOX and EDOXM did not significantly influence the level of γ-globin expression. Similar results were obtained when K562 cells were examined for GATA-1 expression except that the peak values were lower and noted already after 2–3 days of treatment. A 200 nM DOXM caused the highest increase in GATA-1 mRNA level after induction for 3 days (3.6 ± 1.1 ($P < 0.01$)-fold). In summary, our results indicated that expression values for both, γ-globin and GATA-1 were significantly higher after treatment with DOXM as compared with DOX.

3.3. *GATA-1 binding activity is slightly increased during differentiation of K562 cells stimulated with DOX derivatives*

Taking into account the role of GATA-1 transcription factor in erythroid differentiation, we analyzed its DNA-binding activity. Previous studies have shown that some anthracyclines, like ACLA, increased complex formation between GATA-1 and its binding site [21,30]. Using DOX as reference, which was not efficient in stimulation of GATA-1 binding activity [23], we investigated by electrophoretic mobility shift assay (EMSA) the levels of GATA-1 DNA-binding activity in nuclear extracts prepared from K562 cells after 2 and 5 days of treatment with DOX derivatives. EMSA using oligonucleotide derived from the γ-globin promoter and equal amounts of protein from nuclear extracts revealed that the binding of GATA-1 to DNA was slightly increased when cells were cultured in the presence of DOX and its derivatives (Fig. 4). The specificity of the protein–DNA complexes was confirmed by competition with homologous and non-homologous ds-oligonucleotides. Relatively to the control, an increase in the formation of specific nucleoprotein complex was observed on day 2 in the presence of nuclear extracts from DOX-, DOXM-, and DOXH-treated cells but there was no difference between these extracts in the efficiency of complex formation. In contrast, EDOX treatment of K562 cells produced no changes in binding of GATA-1 to the

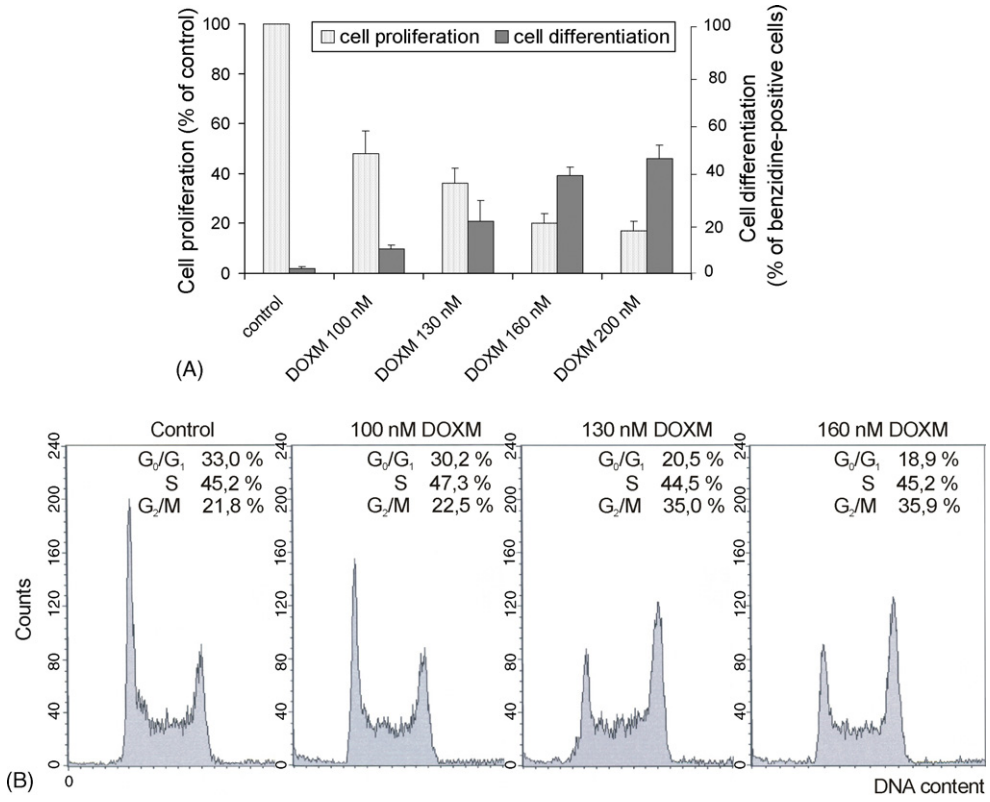


Fig. 2 – Concentration-dependent effects of DOXM on cell proliferation, differentiation and cell cycle arrest in G_2/M phase. (A) Cytostatic effect and erythroid differentiation caused by different concentrations of DOXM was assayed as in Fig.1 but only on day 3. Data represent the mean (±S.D.) of three independent experiments. (B) Flow cytometric analysis showing cell cycle arrest in G_2/M phase. Exponentially growing K562 cultures were incubated with increasing concentrations of DOXM for 1 day before cell cycle fractions were determined using the propidium iodide staining method. The percentages of the cell population in G_1, S or G_2/M phases were calculated from histograms using the CellQuest software.

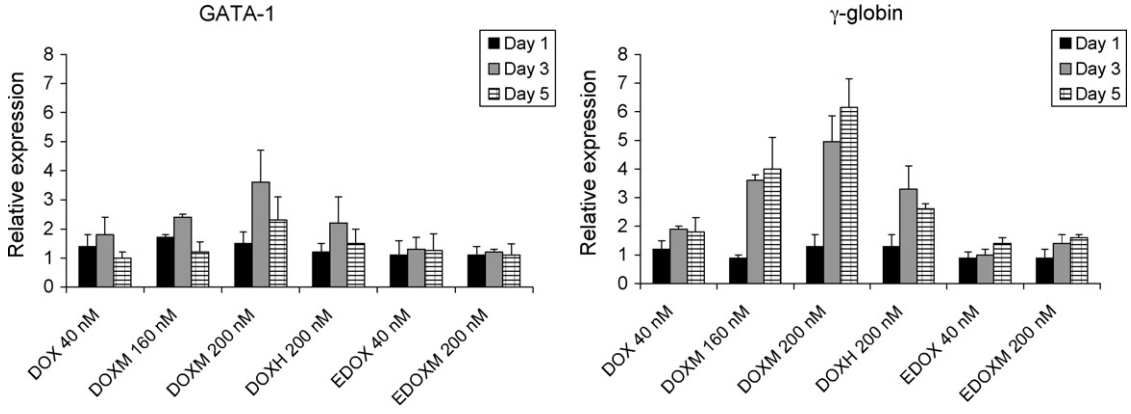


Fig. 3 – Quantitative real time RT-PCR analysis of γ-globin and GATA-1 mRNA from control and DOX derivative-stimulated K562 cells. RNA isolated from untreated cells or cells treated with anthracycline derivatives was reverse transcribed and gene expression levels of GATA-1 and γ-globin were assayed by quantitative real-time PCR using ABI Prism 7000 sequence detection system. A fold increase of γ-globin and GATA-1 mRNA in cultures treated with DOX and its derivatives compared with untreated cultures was calculated. Data were derived from quantitative RT-PCR plots using house-keeping gene β-microglobulin as reference and the formula Rel Exp = $2^{-(\Delta\Delta CT)}$. Each result represents the mean (±S.D.) of three independent experiments.

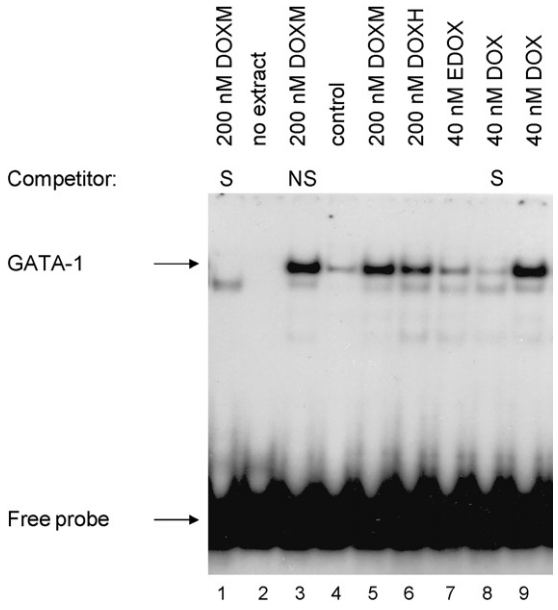


Fig. 4 – EMSA of K562 cell nuclear extracts, comparing the level of GATA-1 binding activity in untreated and DOX derivative-treated cells. EMSA was performed after the incubation of ^{32}P-labeled GATA-1 ds-oligonucleotides with nuclear extracts from untreated (control, lane 4) or DOX derivative-treated cells (details in Section 2). Competition experiment showing the specificity of the protein–DNA complexes is included: lanes 1 and 8 contain specific competitor (an unlabeled GATA-1 ds-oligonucleotide) denoted S and lane 3 contains unrelated ds-oligonucleotide denoted NS. Lane 2 contains only DNA probe. The position of DNA-GATA-1 complex and the free DNA probe are designated by arrows.

DNA probe. The augmentation in GATA-1 protein binding activity in DOX-, DOXM-, and DOXH-treated K562 cells was transient. Its activity was decreased back to the control level on day 5 (not shown). This is in agreement with the results previously obtained for other inducers [23,28].

3.4. *DOX and its derivatives increase stability of γ-globin mRNA*

Besides transcription rate, degradation is another factor determining the steady-state amount of mRNA, and the amount of the protein available to the cell. mRNA stabilization of erythroid genes is known to contribute to maintain physiological functions of the red blood cells and was already observed in chemically induced differentiation of K562 cells [31]. In order to assess γ-globin mRNA stability, our approach was to treat K562 cells for 4 days with anthracyclines and then continue culture in fresh medium without anthracyclines but in the presence and absence of a non-cytotoxic concentration of RNA polymerase inhibitor, actinomycin D (5 μg/ml) for 48 h. At the certain time points aliquots were removed to check γ-globin mRNA levels. The approach was similar to that published previously [28], except that Real time RT PCR instead of RT PCR was used. Total RNAs were extracted at 0, 6, 20, and 28 h after actinomycin D addition to untreated and anthracycline-treated K562 cells. Control experiments showed that actinomycin D had no effect on differentiation of K562 cells (not shown). The half-life of γ-globin mRNA in untreated cells was about 22 h (Fig. 5), similar to that previously observed in K562 cells [28]. In DOX-treated cells the half-life was about 32 h. In cells treated with DOX derivatives, the relative half-life was further increased. It was above 40 h for DOXM- and DOXH-treated cells. The highest level of message stabilization was achieved in cells cultured in the presence of EDOXM, with a half-life of 80 h. Therefore, changes in the steady-state levels of γ-globin mRNA in anthracycline-treated cells resulted at least partially from altered mRNA stability.

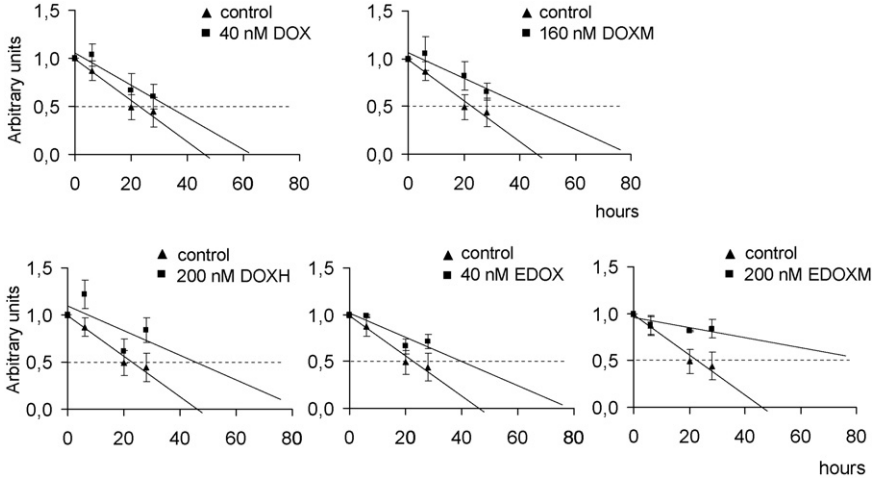


Fig. 5 – γ-Globin mRNA degradation rate is decreased in anthracycline-treated cells. Cells were grown for 4 days in medium alone or in the presence of DOX derivatives. Total RNAs isolated from those cells or cells cultured for additional 6, 20 and 28 h in the presence of actinomycin D (5 μg/ml) were reverse transcribed into cDNA. A decrease in the level of γ-globin mRNA was analyzed by quantitative real-time PCR. In order to evaluate the relative half-life of γ-globin mRNA, gene expression levels were normalized to mRNA in untreated control and linear regression method was used to plot the results.

3.5. *Differentiation of K562 cells in the presence of the new DOX derivatives is irreversible*

To explore whether anthracycline treatment caused irreversible differentiation, K562 cells were cultured in the presence of these compounds for 4 days followed by incubation with or without inducers. The first 4 days of treatment were sufficient for K562 cells to differentiate irreversibly since during additional 4 days of culturing without induction, the population of benzidine-positive cells still increased. The highest increase in the number of benzidine-positive cells cultured for additional 4 days without tested compounds was observed after initial stimulation for 4 days with 160 nM DOXM and 200 nM DOXH. For example, the number of differentiated cells treated with 160 nM DOXM for 4 days was 22×10^3 and increased to 53×10^3 during the subsequent 4 days without DOXM. This more than double increase might indicate that some cells, which already started the differentiation program were still not recognized as benzidine-positive cells on day 4. Confirmatory results were obtained when the level of the γ-globin mRNA was estimated by quantitative RT-PCR (Fig. 6A). DOXM treatment for 4 days caused about four-fold enhancement relatively to the untreated control whereas additional 2 days without DOXM caused further, almost double increase in the level of γ-globin gene expression. When data collected from three independent experiments determining the number of benzidine-positive cells were presented as percentage of total cell population (Fig. 6B), the first 4 days of treatment with 160 nM DOXM or 200 nM DOXH gave 29% and 19% of differentiated cells, respectively. The next 4 days without induction (4/−4) decreased the percentage of benzidine-positive cells to 11% or 10%, respectively. This relative change, however, was due to the proliferation of non-arrested cells that overgrew differentiated ones, which obviously indicated

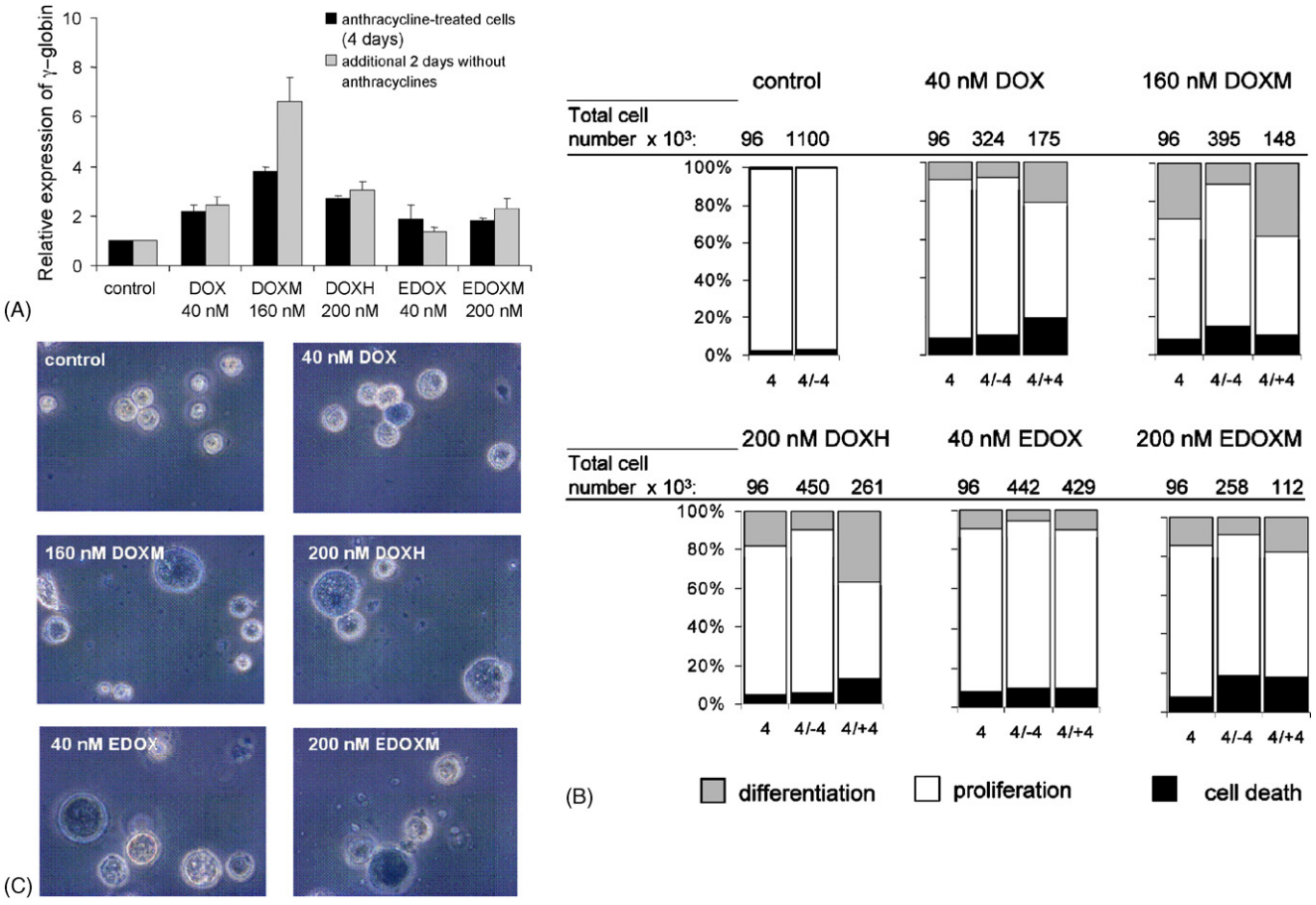


Fig. 6 – Differentiation of K562 cells with DOX and its new derivatives is irreversible. Cells were grown in the absence (control) or in the presence of DOX and its derivatives at indicated concentrations for 4 days (4). After that period, cells were seeded for additional 2 days at density 1.2×10^5/ml in fresh medium without tested compounds for gene expression studies (A) or for additional 4 days at density 96×10^3/ml in fresh medium with (4/+4) or without (4/−4) tested compounds for cell proliferation, viability and differentiation study (B). The white parts of the bars in the part B of the Figure represent the percentage of the viable cells able to proliferate, grey parts are differentiated cells and black parts stand for dead cells. Data represent the mean of 3 independent experiments. (C). Accumulation of hemoglobin in K562 cells treated with DOX and its new derivatives. Control cells and cells after 4 days incubation in the presence of anthracycline derivatives were additionally cultured for 4 days without tested compounds, then stained with benzidine and analyzed using light microscope BX 41 (Olympus) at a magnification of 400×. Hemoglobinized cells developed a blue color with benzidine staining.

that at least two cell populations, differentiated and undifferentiated, were present in cell cultures treated with anthracycline derivatives.

Finally, a progressive increase in the number and the proportion of differentiated cells during the prolonged incubation with DOX and its derivatives (4/+4) was observed. The highest efficiency in this respect was noted again for 160 nM DOXM and 200 nM DOXH. Benzidine-positive cell level exceeded 37% of the population during subsequent 4 days of treatment with those compounds. In contrast to DOXM and DOXH, only a small increase in differentiation during prolonged incubation with 40 nM EDOX or 200 nM EDOXM was observed. Lower cell counts in cultures incubated with or without EDOXM after pretreatment with this compound was mainly caused by cell death.

The irreversibility of differentiation of K562 cells induced by anthracycline derivatives was further investigated by analyzing cells under the light microscope after benzidine staining. There was no qualitative difference between the cultures treated with anthracycline for 4 days or during prolonged incubation (4/+4) or anthracycline-induced cultures incubated subsequently without inducers (4/−4). Benzidine-stained cells were still present in the culture after additional 4 days of incubation without anthracyclines. For clarity only those cells (4/−4) are shown in Fig. 6C. It is interesting to note a difference in size of hemoglobin-containing cells obtained in the presence of DOX, and those obtained in the presence of its derivatives. The difference in size was already observed and analyzed quantitatively after May-Grünwald and Giemsa staining in our previous paper [22]. Now, we showed that mainly cells containing hemoglobin differed in size. DOX-treatment produced rather small benzidine-positive cells whereas DOXM, DOXH, EDOX, and EDOXM-treatment resulted in blue cells that were increased in size.

4. Discussion

It is generally accepted that success of differentiation therapy in treatment of leukemia depends on the ability of the applied drugs to remove the developmental block in leukemia cells, which prevents them from differentiation towards more mature cells. Therefore, our aim was to determine whether structural changes introduced to the parent antibiotic, DOX, could result in more efficient compounds capable of reprogramming CML cells towards a more mature phenotype expressing erythroid genes. Determination of γ-globin production could be used not only to assess the degree of differentiation towards erythrocytes but also to select compounds for further evaluation of their usefulness in the therapy of hematological diseases associated with insufficient expression of normal β-globin genes.

In the previous report [22], we have shown that some of the newly synthesized anthracycline derivatives inhibit human leukemia K562 cell line proliferation and induce erythroid differentiation when used at subtoxic concentrations. In contrast to ACLA, which exerts differentiating effect without requiring a total growth arrest [23], coupling of inhibition of proliferation and induction of differentiation was observed for all tested anthracycline derivatives. As DOXM has the greatest potential to inhibit proliferation and to induce differentiation in vitro, this compound was tested at several concentrations. The lowest concentration of DOXM influencing both growth and differentiation was 100 nM whereas the most pronounced changes in those two processes without high cytotoxic effect were observed at 200 nM. None of the other tested compounds was able to shift the cells from the proliferating to the differentiating pool so efficiently when used at subtoxic concentrations.

The differentiation of K562 cells detected by benzidine staining correlated with an increase in the expression of the erythroid gene γ-globin. A direct correlation was observed on day 3 and 5 after stimulation. This result is in agreement with earlier findings regarding induction of γ-globin gene expression in culture in which an increasing population of hemoglobinized cells was observed after chemical stimulation [11,28,29]. The transcriptional activation of γ-globin gene has been demonstrated for many inducers to be related to the enhancement of the expression of GATA-1 transcription factor. Such results were obtained for ACLA but not for hemin-induced differentiation [21]. The transcriptional up-regulation of GATA-1 is often accompanied by the increase of GATA-1 binding activity. In our experiments, a similar increase in GATA-1 DNA-binding activity was observed in nuclear extracts prepared from K562 cells after induction by DOX, and by DOXM and DOXH whereas the expression level of GATA-1 gene was most significantly increased after DOXM treatment. This difference between the increase in expression and activity of GATA-1 suggested that DOX and its derivatives might influence GATA-1 activity and erythroid gene expression by different mechanisms. It has been shown previously that DOX preferentially increases stability of GATA-1 mRNA whereas another anthracycline, ACLA substantially increases its transcription rate [23]. It has been also shown in ACLA-treated K562 cells that changes in the level of GATA-1 phosphorylation and then the translocation of the protein into the nuclei might contribute to the increased DNA-binding activity of this transcription factor [30]. Similar results were obtained for other inducers of differentiation, sodium butyrate, or *N*-acetylcysteine [32]. Our data for DOX-treated K562 cells showing that DOX rather increased mRNA stability of erythroid genes than their expression confirmed those obtained previously for this drug [27]. Similar results were obtained for EDOX, a 4′-epimer of DOX. New DOX derivatives in turn increased both, the expression level of γ-globin gene and stability of its mRNA. This was paralleled by the appearance of a much higher percentage of benzidine-positive cells than observed after induction with DOX. The results obtained for DOX-derivatives indicate that the amount of γ-globin mRNA at the single cell level as well as in the population was markedly increased [33]. In cultures treated with DOXM and DOXH significantly higher increase in mRNA levels of erythroid genes in comparison to that obtained for DOX was observed whereas an increase in γ-globin mRNA half-life was noted in DOX-treated cells as well as in cells stimulated with DOX derivatives. The differentiation observed in the presence of EDOXM was mainly due to the increased stability of mRNA of erythroid genes, the highest among tested anthracyclines. Altogether, these results suggest that the new DOX-derivatives, DOXM and DOXH acted both at the transcriptional level

and at the post-transcriptional level, in contrast to DOX, EDOX and EDOXM, which might be mainly active at post-transcriptional level.

As shown previously, chemical induction of cell differentiation can be either reversible or irreversible. Indeed, in contrast to butyrate-induced cells which reverted back to untreated control levels after removal of butyrate and similarly as for apicidin induction [18], K562 cells treated with DOX and its new derivatives remained differentiated for at least 4 days after being transferred into the fresh medium which did not contain inducers. This might suggest that differentiation of K562 cells into more mature erythroid cells was irreversible.

As for other inducers of tumor cell differentiation, molecular mechanisms of anthracycline-mediated differentiation are still unclear and need to be further explored [34,35]. Considerable evidence indicates a requirement for p38 MAPKs in the regulation of chemically induced differentiation of K562 cells towards erythrocytes [16,17,36]. We did not study the influence of anthracycline on kinases activated by cellular stress. Further studies are aimed at exploring these possibilities. In the present work, the differences observed between the effects of DOX and its derivatives emphasize the complexity of the mechanisms involved in the chemical induction of tumor cell differentiation and it will be of interest to determine whether our findings can be extended to malignant cells derived from CML patients. Concerning therapy of hematological diseases based on pharmacologically-mediated regulation of the γ-globin expression, several other compounds, including 5-azacytidine, butyrate derivatives, hydroxyurea and erythropoietin were already evaluated in vivo in β-thalassemia patients [20] but still a more efficient drug which can be used at low concentration is in request. New analogues of doxorubicin appeared to be better inducers of γ-globin expression than parent compound but further experiments need to be performed. A first step to verify the inducing activity of the new tested analogues of doxorubicin could be to employ the two-phase liquid culture systems of human erythroid precursors isolated from peripheral blood of normal donors or patients with β-thalassemia [29]. This approach could also be valuable to further analyze the mechanisms of anthracycline-induced differentiation, e.g. the role of caspases or autophagy in cell differentiation [37–39]. In our in vitro study performed on K562 cells, both morphology of differentiated tumor cells as well as growth arrest in G_2/M phase indicated that this process when induced chemically is only to a certain extent similar to the differentiation process of progenitor cells into more mature erythrocytes.

In summary, using the human chronic myelogenous leukemia (CML) cell line K562 we have demonstrated that new derivatives of doxorubicin, DOXM and to a certain extent DOXH proved to be effective inducers of differentiation in vitro, able to commit K562 cells toward erythroid maturation. They induced a higher transcription rate of erythroid genes and stabilized mRNA of those genes. In this respect they were more efficient than DOX.

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REFERENCES

[1] Welch JJ, Watts JA, Vakoc CR, Yao Y, Wang H, Hardison RC, et al. Global expression of erythroid gene expression by transcription factor GATA-1. Blood 2004;104:3136–47.
[2] Labbaye C, Valtieri M, Barberi T, Meccia E, Masella B, Pelosi E, et al. Differential expression and functional role of GATA-2, NF-E2 and GATA-1 in normal adult hematopoiesis. J Clin Invest 1995;95:2346–58.
[3] Migliaccio A, Rana R, Sanchez M, Lorenzini R, Centurione L, Bianchi L, et al. GATA-1 as a regulator of mast cell differentiation revealed by the phenotype of the GATA-1 low mouse mutant. J Exp Med 2003;197:281–96.
[4] Wang X, Crisoino JD, Letting DL, Nakazawa M, Poncz M, Blobel GA. Control of megakaryocyte-specific gene expression by GATA-1 and FOG-1: role of Ets transcription factors. EMBO J 2002;21:5225–34.
[5] Weiss J, Orkin SH. GATA transcription factors: key regulators of hematopoiesis. Exp Hematol 1995;23:99–107.
[6] Yu C, Cantor AB, Yang H, Browne C, Wells RA, Fujiwara Y, et al. Targeted deletion of a high-affinity GATA-binding site in the GATA-1 promoter leads to selective loss of the eosinophil lineage in vivo. J Exp Med 2002;195:1387–95.
[7] Fujiwara Y, Browne C, Cunniff K, Goff SC, Orkin SH. Arrested development of embryonic red cell precursor in mouse embryos lacking transcription factor GATA-1. Proc Natl Acad Sci USA 1996;93:12355–8.
[8] Pevny L, Simon MC, Robertson E, Klein WH, Tsai SF, D'agati V, et al. Erythroid differentiation in chimeric mice blocked by targeted mutation in the gene for transcription factor GATA-1. Nature 1991;349:257–60.
[9] McDevitt MA, Shivdasani RA, Fujiwara Y, Yang H, Orkin SH. A knockdown'' mutation created by *cis*-element gene targeting reveals the dependence of erythroid cell maturation on the level of transcription factor GATA-1. Proc Natl Acad Sci USA 1997;94:6781–5.
[10] Rylski M, Welch JJ, Chen YY, Letting DL, Diehl JA, Chodosh LA, et al. GATA-1-mediated proliferation arrest during erythroid maturation. Mol Cell Biol 2003;23:5031–42.
[11] Bianchi N, Chiarabelli C, Borgatti M, Mischiati C, Fibach E, Gambari R. Accumulation of γ-globin mRNA and induction of erythroid differentiation after treatment of human leukaemic K562 cells with tallimustine. Br J Haematol 2001;113:951–61.
[12] Bianchi N, Ongaro F, Chiarabelli C, Gualandi L, Mischiati C, Bergamini P, et al. Induction of erythroid differentiation of human K562 cells by cisplatin analogs. Biochem Pharmacol 2000;6:31–40.
[13] Cortesi R, Gui V, Gambari R, Nastruzzi C. In vitro effect on human leukemic K562 cells of co-administration of liposome-associated retinoids and cytosine arabinoside (Ara-C). Am J Hematol 1999;62:33–43.
[14] Huang M, Wang Y, Collins M, Graves LM. CPEC induces erythroid differentiation of human myeloid leukemia K562 cells through CTP depletion and p38 MAP kinase. Leukemia 2004;18:1857–63.
[15] Jacquel A, Herrant M, Legros L, Belhacene N, Luciano F, Pages G, et al. Imatinib induces mitochondria-dependent

apoptosis of the Bcr-Abl-positive K562 cell line and its differentiation toward the erythroid lineage. FASEB J 2003;17:2160–3.
[16] Park JI, Choi HS, Jeong JS, Han JY, Kim IH. Involvement of p38 kinase in hydroxyurea-induced differentiation of K562 cells. Cell Growth Differ 2001;12:481–6.
[17] Witt O, Sand K, Pekrun A. Butyrate-induced erythroid differentiation of human K562 leukemia cells involves inhibition of ERK and activation of p38 MAP kinase pathways. Blood 2000;95:2391–6.
[18] Witt O, Mönkemeyer S, Rönndahl G, Erdlenbruch B, Reinhardt D, Kanbach K, et al. Induction of fetal hemoglobin expression by the histone deacetylase inhibitor apicidin. Blood 2003;101:2001–7.
[19] Fordis M, Anagnou NP, Dean A, Nienhuis A, Schechter A. β-globin gene, inactive in the K562 leukemic cells, functions normally in a heterologous expression system. Proc Natl Acad Sci USA 1984;81:4485–8.
[20] Rodgers GP, Saunthararajah Y. Advances in experimental treatment of beta-thalassaemia. Exp Opin Invest Drugs 2001;10:925–34.
[21] Morceau F, Aries A, Lahlil R, Devy L, Jardillier JC, Jeannesson P, et al. Evidence for distinct regulation processes in the aclacinomycin- and doxorubicin- mediated differentiation of human erythroleukemic cells. Biochem Pharmacol 1996;51:839–45.
[22] Czyz M, Szulawska A, Bednarek AK, Düchler M. Effects of anthracycline derivatives on human leukemia K562 cell growth and differentiation. Biochem Pharmacol 2005;70:1431–42.
[23] Jeannesson P, Lahlil R, Chenais B, Devy L, Gillet R, Aries A, et al. Anthracycline as tumor cell differentiating agents: effects on the regulation of erythroid gene expression. Leuk Lymphoma 1997;26:575–87.
[24] Szulawska A, Gniazdowski M, Czyz M. Sequence specificity of formaldehyde-mediated covalent binding of anthracycline derivatives to DNA. Biochem Pharmacol 2005;69:7–17.
[25] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)). Method 2001;25:402–8.
[26] Moll T, Czyz M, Holzmuller H, Hofer-Warbinek R, Wagner E, Winkler H, et al. Regulation of tissue factor promoter in endothelial cells. J Biol Chem 1995;270:3849–57.
[27] Morceau F, Chenais B, Gillet R, Jardillier JC, Jeannesson P, Trentesaux C. Transcriptional and posttranscriptional regulation of erythroid gene expression in anthracycline-induced differentiation of human erythroleukemic cells. Cell Growth Differ 1996;7:1023–9.
[28] Morceau F, Dupont C, Palissot V, Borde-Chiche P, Trentesaux C, Dicato M, et al. GTP-mediated differentiation of the human K562 cell line: transient overexpression of GATA-1 and stabilization of the γ-globin mRNA. Leukemia 2000;14:1589–97.
[29] Lampronti I, Bianchi N, Borgatti M, Fibach E, Prus E, Gambari R. Acumulation of γ-globin mRNA in human erythroid cells treated with angelicin. Eur J Haematol 2003;71:189–95.
[30] Gillet R, Bobichon H, Trentesaux C. Nuclear transcription factor GATA-1 is activated during aclacinomycin-induced erythroid differentiation. Biol Cell 2002;94:267–73.
[31] Schnekenburger M, Morceau F, Duvoix A, Delhalle S, Trentesaux C, Dicato M, et al. Increased glutathione S-transferase P1-1 expression by mRNA stabilization in hemin-induced differentiation of K562 cells. Biochem Pharmacol 2004;68:1269–77.
[32] Partington GA, Patient RK. Phosphorylation of GATA-1 increases its DNA-binding activity and is correlated with induction of human K562 erythroleukaemia cells. Nucleic Acids Res 1999;27:1168–75.
[33] Smith RD, Malley JD, Schechter AN. Quantitative analysis of globin gene induction in single human erythroleukemic cells. Nucleic Acids Res 2000;28:4998–5004.
[34] Tsiftsoglou AS, Pappas IS, Vizirianakis IS. Mechanisms involved in the induced differentiation of leukemia cells. Pharmacol Ther 2003;100:257–90.
[35] Leszczyniecka M, Roberts T, Dent P, Grant S, Fisher PB. Differentiation therapy of human cancer: basic science and clinical applications. Pharmacol Ther 2001;90:105–56.
[36] Sawafuji K, Miyakawa Y, Kizaki M, Ikeda Y, Cyclosporin. A induces erythroid differentiation of K562 cells through p38 MAPK and ERK pathways. Am J Hematol 2003;72:67–9.
[37] Weber GF, Menko AS. The canonical intrinsic mitochondrial death pathway has a non-apoptotic role in signaling lens cell differentiation. J Biol Chem 2005;280:22135–4.
[38] Schwerk C, Schulze-Osthoff K. Non-apoptotic functions of caspases in cellular proliferation and differentiation. Biochem Pharmacol 2003;66:1453–8.
[39] Fader CM, Colombo MI. Multivesicular bodies and autophagy in erythrocyte maturation. Autophagy 2006;2:122–5.