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ORIGINAL ARTICLE

The α1-adrenergic receptor antagonists, benoxathian and prazosin, induce apoptosis and a switch towards megakaryocytic differentiation in human erythroleukemia cells

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Abstract The erythroleukemia cell lines K562 and human erythroleukemia (HEL) are established models to study erythroid and megakaryocytic differentiation *in vitro*. In this study, we show that the α1-adrenergic antagonists, benoxathian and prazosin, inhibit the proliferation and induce apoptosis in K562 and HEL cells. Furthermore, both tested substances induced the expression of the megakaryocytic marker CD41a, whereas the expression of the erythroid marker glycophorin-a was decreased or unchanged. Even though the expression of differentiation markers was similar after benoxathian and prazosin treatment in both cell lines, endomitosis of erythroleukemia cells was observed only after prazosin treatment. So far, benoxathian and prazosin are the first described extracellular ligands, which cause megakaryocytic differentiation in K562 and HEL cells. In summary, these results indicate a possible role of α1-adrenergic receptor signaling in the regulation of erythroid and megakaryocytic differentiation, even though the receptor dependence of the observed effects needs further investigation.

Keywords Erythroleukemia cells · α1-adrenergic antagonists · Apoptosis · Erythroid differentiation · Megakaryocytic differentiation

Konrad Schauenstein deceased at May 22, 2007.

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Introduction

An increasing number of data indicate that hematopoiesis in bone marrow is not only modulated by various cytokines but also by neuroendocrine mediators including the major neurotransmitter of the sympathetic nervous system, norepinephrine [1, 2]. Muthu et al. [3] have shown that murine hematopoietic stem cells express adrenergic receptors. Further evidence for a potential sympathetic modulation of hematopoiesis through adrenergic signaling came from observations that the bone marrow is innervated by sympathetic nerve fibers, which secrete catecholamines in a daily rhythm [4, 5]. In addition, besides sympathetic nerves, also bone marrow cells themselves could be identified as a source of catecholamines [6].

An observation in our lab was that, in colony forming assays, the α1-specific adrenergic antagonist benoxathian inhibits the generation of glycophorin-a (GPA) expressing cells from erythroid progenitor cells derived from human umbilical cord blood (R.F., 2006, unpublished results). This observation is in line with

previous studies, which revealed that the *in vitro* erythropoiesis can be influenced by norepinephrine treatment [7, 8]. In order to elucidate the underlying mechanisms of this inhibitory effect of benoxathian on the erythroid lineage, the human erythroleukemia cell lines K562 [9] and human erythroleukemia (HEL) [10] were used as a model system. Common features of K562 and HEL are an erythroblast-like phenotype, expression of GPA, and cell growth and survival independence from erythropoietin and cytokines. In K562 cells, this cytokine-independent growth is derived from the existence of the Philadelphia chromosome [9] and in HEL cells through the V617F mutation of *JAK2* [11], previously reported as the cause of polycythemia vera [12]. So far, little is known about the influence of adrenergic agonists and antagonists on the growth of erythroleukemia cells and erythropoiesis. Gauwerky and Golde [13] have demonstrated that β-adrenergic stimulation induced enhanced growth in K562 cells, whereas responses to α1-adrenergic stimulation could be seen just under hormone-depleted conditions. Previously, He and He revealed that prazosin, an α1-adrenergic antagonist, induced apoptosis in the K562 cell line [14].

The aim of our study is to compare the effects of two α1-adrenergic antagonists, prazosin and benoxathian, on growth and differentiation of human erythroleukemia cells in comparison to the α2-adrenergic antagonist yohimbine. In summary, this study shall provide new insights into the adrenergic regulation of erythroid differentiation and disclose a new mechanism to control cytokine-independent growth of transformed progenitor cells.

Study design

Cells and cell culture

HEL cells (obtained from DSMZ, Braunschweig, Germany) and K562 cells were maintained in RPMI 1640 medium (Cambrex, East Rutherford, NJ, USA) supplemented with glutamine, penicillin/streptomycin, and 10% fetal calf serum (PAA, Pasching, Austria). Cell cultures were kept in an incubator at 37°C, 5% CO_2 in a fully humified atmosphere.

Both HEL and K562 cells, with a start cell number of 1×E4 cells/ml, were incubated in 24-well plates 2 or 3 days with different concentrations of the receptor subtype-specific adrenergic antagonists, prazosin HCl (α1), benoxathian HCl (α1), or yohimbine HCl (α2), in triplicates. All antagonists were obtained from Sigma Austria and were added to the culture medium dissolved in Aqua bidest. After the incubation period, proliferation and viability of the cells were measured with a CASY-1® Cell Counter and Analyzer (Schaerfe, Reutlingen, Germany).

Caspase 3 activity

In order to detect the induction of apoptosis in erythroleukemia cells by adrenergic antagonists, the activation of caspase 3 was measured by flow cytometric analyses using the FITC Active Caspase 3 Apoptosis Kit of Becton Dickinson (BD, San Diego, CA, USA). The assay was performed according to the protocol of the producer after cells were incubated with/without adrenergic antagonists in 12-well plates with a start cell number of 1×E4 cells/ml for 48 h at standard cell culture conditions. After cell preparation and antibody-staining for active caspase 3, cells were analyzed by a BD-FACScan flowcytometer using CELLQuest software (BD).

Analysis of CD235a and CD41a expression by flow cytometry

Phenotypic differentiation of the cells was assessed by flow cytometry after 48 h cultivation with benoxathian (50 μM), prazosin (15 μM), or yohimbine (150 μM). A PE-Cy5-labeled mouse anti-human GPA (CD235a) antibody was used to analyze the erythroid phenotype and a PE-labeled mouse anti human GPIIb (CD41a) antibody to determine megakaryocytic differentiation. Both antibodies were purchased from BD Austria. Flow cytometric analyses were performed on a BD-FACScan using CELLQuest software (BD) and WinMDI. For dual-labeling, compensation was set by using single-labeled positive cells before acquisition of experimental data.

Electron microscopy

For ultrastructure analysis, untreated and prazosin treated (K562, 10 μM; HEL, 15 μM) erythroleukemia cells were cultivated 72 h in 75 cm^2 tissue culture flasks, washed one time in phosphate buffer and fixed in a fixative (2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer/pH 7.4) for 1 h at room temperature. After washing the cells with 0.1 M cacodylate buffer/pH 7.4 overnight, cells were postfixed in a 1% osmium tetraoxide solution in the same buffer for 30 min at room temperature. After cells were rinsed for 30 min in 0.1 M cacodylate buffer, cells were dehydrated in a series of acetones and embedded in TAAB epoxy resin (TAAB Laboratories, Aldermaston, UK) that was cured for 3 days at 60°C. Ultra-thin sections of 70 nm were cut using a Leica Ultracut UCT ultramicrotome, collected on copper grids and were then stained with uranyl acetate and lead citrate. These sections were examined with a Zeiss EM 902 transmission electron microscope.

Statistical analysis

Results are expressed as mean values plus or minus standard deviation. Data were analyzed with the unpaired Student's *t* test using Sigma Stat 3.5. *p* values less than 0.05 were considered as significant.

Results

The α1-adrenergic antagonists, prazosin and benoxathian, inhibit the proliferation and induce apoptosis in human erythroleukemia cells

The addition of the adrenergic antagonists to cultures of K562 and HEL cells yielded in an inhibition of proliferation in both cell lines at different concentrations (Fig. 1). Of the three tested substances, prazosin exhibited the strongest effect. After treating the cells with 10 μM prazosin, the number of living cell was about 20% in comparison to untreated controls. Benoxathian and yohimbine also inhibited proliferation of erythroleukemia cells, but they were less potent than prazosin (Fig. 1). Flow cytometric analyses of active caspase 3—an indicator of apoptosis—revealed that the growth inhibition of prazosin and benoxathian was accompanied by a significantly increased rate of apoptosis, whereas yohimbine only marginally activated caspase 3 (Fig. 2). Fifty micromolar benoxathian induced caspase 3 activity more effectively in K562 cells than in HEL cells. On the contrary, prazosin was more potent to induce apoptosis in the HEL cell line.

Prazosin induces an increase in cell size and endomitosis in K562 and HEL cells

Besides inhibition of proliferation and apoptosis, morphological changes of the cells could be observed in prazosin-treated HEL (Fig. 3) as well as in K562 cells (Fig. 4a). Cells from both cell lines treated with concentrations between 10 and 20 μM of prazosin showed an increase in cell size and polylobulated nuclei. After 48 h incubation with 10 μM prazosin K562 cells showed a significant ($p<0.01$) increase of cells with polylobulated nucleus, as assessed by light-microscopic analysis of May/Grünwald–Giemsa-stained cells (Fig. 4a, b). In the HEL cell line, 15 μM of prazosin was required to induce a significant ($p<0.001$) increase of cells with polylobulated nucleus. No significant increase of cells with polylobulated nucleus could be observed after treatment with benoxathian or yohimbine in both tested cell lines. In addition, HEL, but not K562 cells, showed the tendency of increased adherence to the cell culture plate after benoxathian and prazosin treatment (Fig. 3). Furthermore, HEL cells formed apical spicular structures after prazosin treatment (Fig. 3c). Ultrastructure analysis of prazosin-treated K562 and HEL cells showed a heterogeneous composition of the analyzed cell samples. Some cells showed several characteristics of megakaryocytes, an increase in size, the appearance of polylobulated nuclei, and a complex cytoplasmatic structure (Fig. 5b, e), while other cells were not distinguishable from untreated cells. Some of the huge cells with polylobulated nuclei exhibited a system of vacuoles reminiscent of a demarcation membrane system, a typical sign of megakaryocyte differentiation (Fig. 5b, c, and f) [15, 16]. These

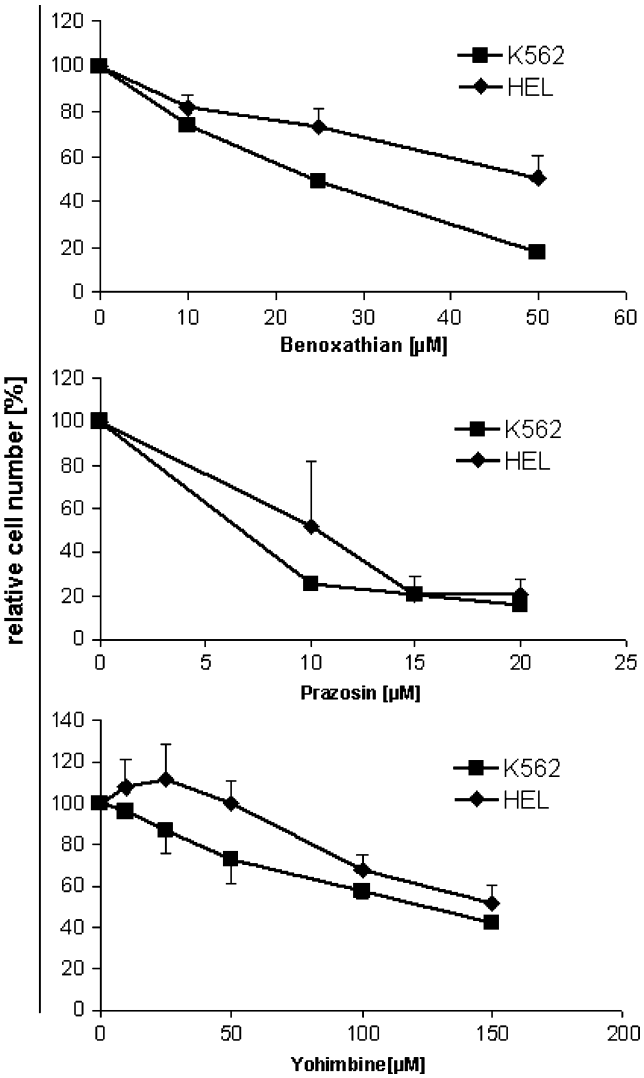

Fig. 1 α1-Adrenergic antagonist treatment inhibits the proliferation of human erythroleukemia cells. Number of living cells of K562 and HEL cell lines in response to a 3-day treatment with 10–50 μM benoxathian ($n=4$), 10–20 μM prazosin ($n=5$), and 10–150 μM yohimbine ($n=4$), calculated as a percentage compared to untreated controls. Results are expressed as mean values plus or minus standard deviation

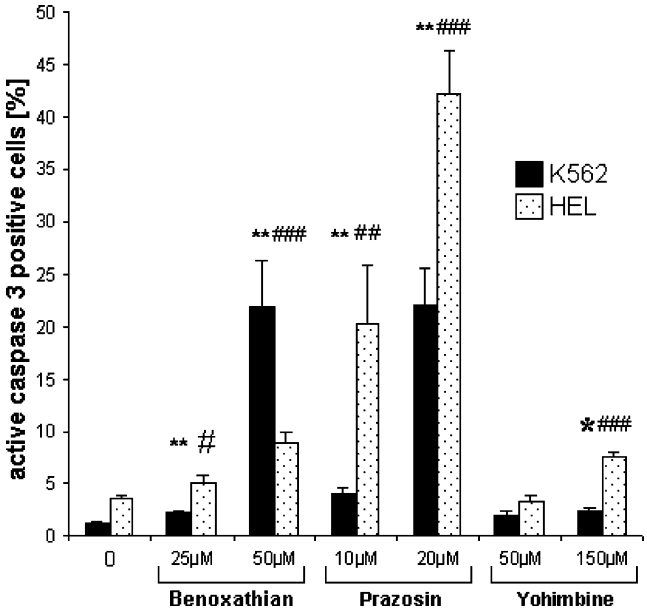

Fig. 2 α1-Adrenergic antagonist treatment of K562 and HEL cells induces apoptosis through activation of caspase 3. Flow cytometric analyses reveal the percentage of active caspase-3-positive cells after 48 h treatment with 25–50 µM benoxathian (n=4), 10–20 µM prazosin (n=4), and 50–150 µM yohimbine (n=3). Results are expressed as mean values plus or minus standard deviation. *p<0.01, **p<0.001 versus control of K562; $^{\#}p$<0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 versus control of HEL according to unpaired Student's t test

vacuoles were only seen in a minority of the cells and were confined to parts of the cytoplasm. α-Granules, a further characteristic hallmark of megakaryocytes [15, 16], were missing. In addition, the examination of the cells by electron microscopy confirmed the induction of apoptosis by prazosin, as prazosin-treated erythroleukemia cells partially showed chromatin condensation and nuclear fragmentation (Fig. 5b).

α1-Antagonistic treatment changes the expression of lineage specific differentiation markers on erythroleukemia cells

In order to confirm that α1-adrenergic treatment has an effect on differentiation of erythroleukemia cells, the expression of GPA, as specific erythroid marker, and CD41a (GPIIb), as megakaryocyte marker, were examined (Fig. 6). Fluorescent-activated cell sorting analyses showed that, in the K562 cell line, 15 µM prazosin and 150 µM yohimbine significantly (p<0.05) decreased the expression of GPA measured by the mean fluorescence intensity ratio (MFI ratio) of anti-GPA-PECy5 stained cells normalized to unstained control (Fig. 6b). Fifty micromolar benoxathian did not significantly change the MFI ratio in comparison to the untreated control. In the HEL cell line, 15 µM prazosin, 50 µM benoxathian, and 150 µM yohimbine significantly (p<0.05) decreased the MFI ratio in comparison to the untreated control (Fig. 6b).

The percentage of cells expressing GPA in K562 cells (Fig. 6c, I) was decreased by 15 µM prazosin (p<0.01), 50 µM benoxathian (p<0.001), and also by 150 µM yohimbine (p<0.05). In the HEL cell line, only prazosin (p<0.01), but not benoxathian or yohimbine, reduced the percentage of GPA-expressing cells (Fig. 6c, I). On the contrary, adrenergic antagonists increased the rate of cells expressing CD41a in both cell lines in a different manner (Fig. 6c, II). The percentage of CD41a-expressing K562 cells was elevated by adrenergic antagonists in ascendenting order by yohimbine (p<0.05), benoxathian (p<0.001), and prazosin (p<0.001). In HEL cells, which co-express GPA and CD41a, a new population, expressing neither GPA nor CD41a, or just CD41a alone appeared after treatment with prazosin (Fig. 6a). The percentage of CD41a-expressing HEL cells was increased by benoxathian (p<0.01) but not by prazosin or yohimbine (Fig. 6c, II).

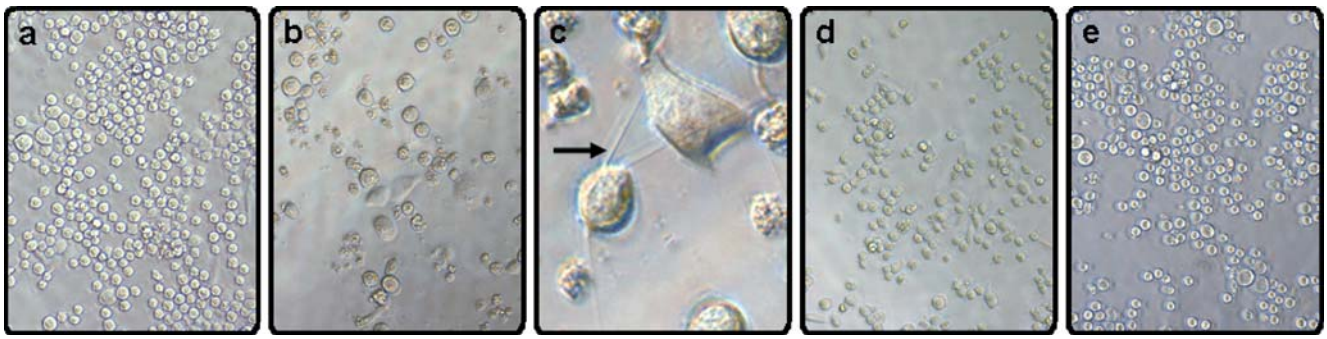

Fig. 3 Microscopic analysis of α-antagonist treated HEL cells. **a–e** HEL cells after 72 h without treatment (**a**) and addition of 15 µM prazosin (**b**, **c**), 50 µM benoxathian (**d**), or 150 µM yohimbine (**e**), viewed by an inverted microscope. Prazosin treatment resulted in increased cell size and apoptosis in HEL cells. At higher magnification (**c**) spicular structures of the cells could be detected (*arrow*). Original magnification of all pictures except **c**, ×200, **c** ×400

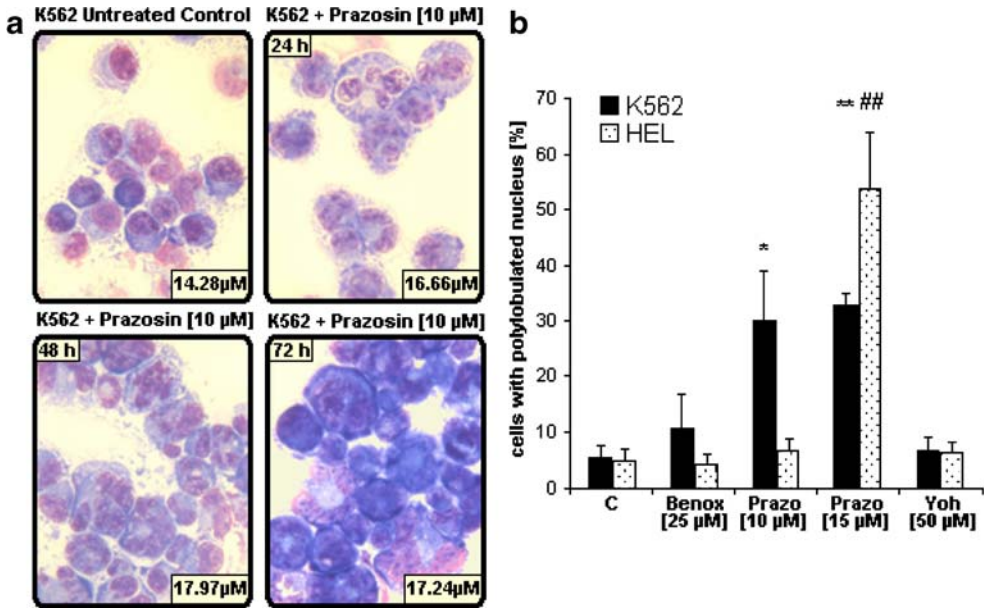

Fig. 4 Prazosin induces endomitosis and an increase in cell size in human erythroleukemia cells. **a** Micrographs of May/Grünwald–Giemsa-stained K562 cells treated with 10 µM prazosin for different periods are shown. After treatment with 10 µM prazosin, an increase in cell size and a time-dependent change in morphology could be observed over a period of 72 h. The *right corner* of the respective picture refers to the mean diameter of the cells. Original magnification, ×630. **b** The graph shows the percentage of K562 and HEL cells with polylobulated nuclei after 48 h treatment with either 25 µM benoxathian (*Benox*, n=4), prazosin (*Prazo*, 10 µM, n=4; 15 µM, K562, n=3; HEL, n=4), or 50 µM yohimbine (*Yoh*, n=4) in comparison to untreated controls (*C*, n=4). Cells were harvested after cultivation, spinned on slides, and stained with May/Grünwald–Giemsa. Two hundred cells per each condition were analyzed for appearance of a polylobulated nucleus. Results are expressed as mean values plus or minus standard deviation. *$p<0.01$, **$p<0.001$ versus control of K562; $^{\#\#}p<0.001$ versus control of HEL according to unpaired Student's t test

In addition to the increase in size, induction of endomitosis and the concomitant upregulation of CD41a and downregulation of GPA provides evidence that prazosin induces megakaryocytic differentiation in erythroleukemia cells. On the contrary, benoxathian is able to suppress effectively the expression of GPA but induces just a low level of CD41a expression.

Discussion

In summary, the α1-adrenergic antagonists, prazosin and benoxathian, inhibit proliferation, induce apoptosis through activation of caspase 3, and modulate differentiation properties of K562 and HEL cells.

The induction of apoptosis in K562 cells through prazosin treatment is in line with the results of He and He [14] even though they did not describe the differentiation effect observed in our study. An explanation why He and He have not seen the same effects may be the specific concentrations used in their study and the different start cell number of the culture. The capacity of prazosin to induce megakaryocytic differentiation appears to be strictly dose-dependent with an optimal concentration of 10 µM in K562 cells. Ten micromolar prazosin also induced the expression of CD41a and endomitosis in K562 cells but only marginally activated caspase 3. On the contrary, in HEL cells, a highly elevated level of active caspase 3 could be detected following the same 10 µM prazosin treatment. As to the induction of apoptosis, the HEL cell line was more sensitive to prazosin treatment, whereas K562 cells were more effectively affected by benoxathian. To summarize, benoxathian and prazosin influence differentiation at lower drug concentrations but subsequent apoptosis at higher drug concentrations, indicating that the compounds interfere with signaling pathways controlling both differentiation as well as survival of the cells.

The different expression of differentiation markers and morphological changes of both cell lines suggest an inhibition of the erythroid phenotype and a shift toward megakaryocytic differentiation after prazosin treatment. Transmission electron microscopy revealed that a minority of the cells displayed vacuoles reminiscent of a demarcation membrane system, whereas α-granules could not be detected. We conclude that prazosin treatment can induce some features of megakaryocytic differentiation, including increasing cell size, polyploidization of the nucleus, and weak CD41a expression, but does not generate cells with the whole spectrum of characteristics of the megakaryocytic lineage. These results are in line with the proposal of

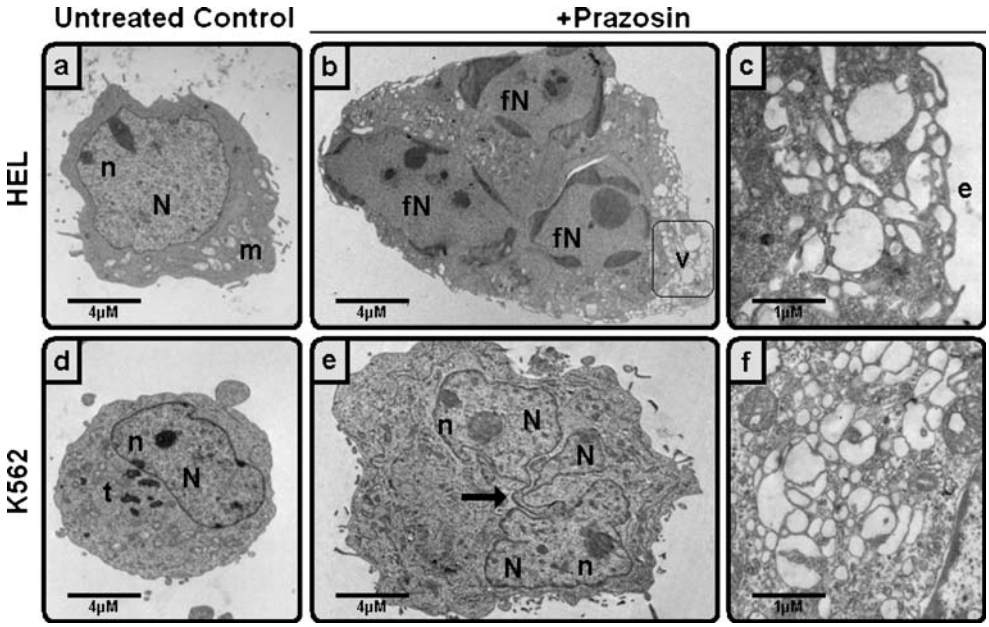

Fig. 5 Ultrastructure analysis of untreated and prazosin-treated erythroleukemia cells. K562 and HEL cells were incubated with 10 μM (*K562*) or 15 μM (*HEL*) prazosin for 72 h and analyzed by transmission electron microscopy (TME). TME analysis revealed that prazosin-treatment of both K562 and HEL cells results in an increase in cell size, the appearance of polylobulated nuclei and also signs of apoptosis as chromatin condensation and nuclear fragmentation (**b**, **e**). In a minority of cells of both cell lines, vacuoles reminiscent of a demarcation membrane system could be seen (**b**, **c**, **f**). In **c**, a section of the vacuoles containing cytoplasm of the prazosin-treated HEL cell in **b** (bounded by the *rectangle*) is pictured at higher magnification. The figure of the vacuoles from a prazosin-treated K562 cell (**f**) originates from a cell not shown as a whole in this compilation. The *arrow* in **e** indicates a nuclear region, which connects separate lobes of the complex nucleus of a prazosin-treated K562 cell. **e** Extracellular space, *fN* fragmented nucleus, *m* mitochondria, *N* nucleus, *n* nucleoli, *t* telolysosomes, *v* vacuoles

Kikuchi et al. [17] that in the megakaryocytic differentiation process, the polyploidization of the cells and the functional maturation are distinct processes, which are regulated independently.

In contrast to prazosin, benoxathian effectively suppressed the expression of GPA but induced just a low level of CD41a expression and did not induce endomitosis. In light of these data, it can be concluded that benoxathian inhibits the development of the erythroid phenotype but insufficiently or just partially induces the megakaryocyte differentiation program in erythroleukemia cells. A possible role of α1-adrenergic receptor signaling in the regulation of erythroid and megakaryocytic differentiation can be assumed.

The fact that prazosin and benoxathian inhibit erythroid differentiation is in line with the results of an *in vivo* study in mice, which showed that prazosin administration leads to a significant depression of erythropoiesis [18]. Our results suggest that this depression is caused by increased apoptosis and a shift toward megakaryocytic differentiation of immature progenitor cells. A similar effect of K562 and HEL has been well described after treatment with the proteinkinase C modulators, phorbol 12-myristate 13-acetate (PMA) and staurosporine, which leads to a shift toward megakaryocytic differentiation of erythroleukemia cells [19–24]. In comparison to PMA, which causes endomitosis only in the HEL cells but not in K562 cells [23], prazosin is able to induce endomitosis in both cell lines.

As to our knowledge, benoxathian and prazosin are the first described extracellular-acting ligands, which induce megakaryopoiesis in K562 and HEL cells. Comparing the effects of PMA, benoxathian and prazosin on the molecular level could shed new light on the signaling mechanisms of the megakaryocytic differentiation process. Potential targets of adrenergic antagonist treatment could be the Akt-mTOR (mammalian target of rapamycin) pathway and the mitogen-activated protein kinase pathway, including the extracellular signal-regulated kinases ERK1/2 and p38. Both pathways have been shown to be involved in α1-adrenergic signaling [25–28] and in regulation of megakaryocytic differentiation [29–31]. Guerriero et al. [29] have shown that inhibition of the Akt-pathway inhibits endomitosis, whereas inhibition of MEK1/2, upstream activators of ERK1/2, enhances endomitosis in megakaryocyte progenitors. Jacquel et al. [30] have demonstrated that inhibition of p38 MAP kinase by a synthetic kinase inhibitor supports the PMA-induced megakaryocytic differentiation of K562 cells. Supplementary results

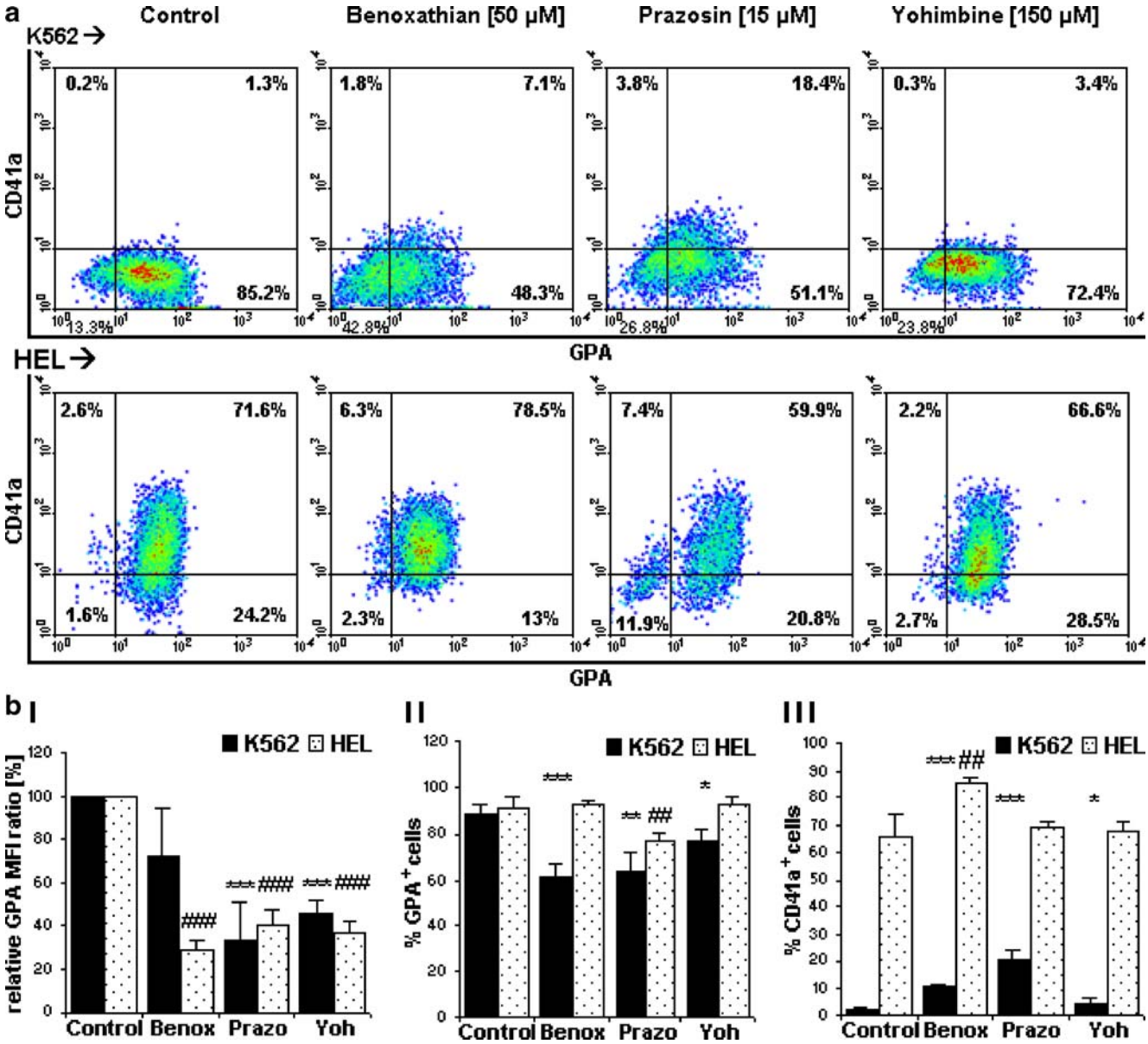

Fig. 6 α1-Adrenergic antagonist treatment reduces the expression of the erythroid marker GPA and induces the expression of the megakaryocytic marker CD41a. **a** Density blots, generated after dual color flow cytometric analyses, of human erythroleukemia cells are shown for the expression of GPA and CD41a. K562 and HEL cells were incubated 48 h with benoxathian, prazosin, or yohimbine at different concentrations. The criterion for gating the cells expressing the respective antigen was set on the basis of light scatter intensity of unstained cells. Treatment of K562 cells (*upper line*) with 50 µM benoxathian, 15 µM prazosin, or 150 µM yohimbine results in a reduction of the expression of GPA. CD41a expression was induced by benoxathian and prazosin but only marginally by yohimbine. In HEL cells (*lower line*), 50 µM benoxathian reduced the percentage of $CD235a^{+}/CD41a^{-}$ cells. Administration of 15 µM prazosin enriched a mixed population of $CD235a^{-}/CD41a^{-}$ and $CD235a^{-}/CD41a^{+}$ HEL cells. Yohimbine-treatment up to 150 µM had just a low impact on the expression of both markers in comparison to benoxathian and prazosin. The figure shows one representative experiment series of a total of four replications. **b** Statistical analysis of GPA/CD41a flow cytometry data after 48 h cultivation of K562 and HEL cells with benoxathian (*Benox*), prazosin (*Prazo*) and yohimbine (*Yoh*). *I* Fluorescent-activated cell sorting analysis revealed a significant decrease of GPA expression after α-antagonist treatment. Data are presented as mean fluorescence intensity ratio (MFI ratio, mean fluorescence of CD235a antibody stained probe/mean fluorescence of unstained cells) in comparison to untreated control (=100%). II/III α-Antagonist treatment resulted in a decrease of GPA^{+} cells parallel to a significant increase of $CD41a^{+}$ cells in the K562 cell line, whereas in HEL cells, only prazosin significantly decreased the level of GPA^{+} cells and benoxathian significantly increased the percentage of $CD41a^{+}$ cells. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ versus control of K562; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0,001$ versus control of HEL according to unpaired Student's *t* test

of our experiments suggest a possible involvement of p38 in the effect of adrenergic antagonists on erythroleukemia cells, since the p38 inhibitor SB203580 inhibited the expression of GPA and induced a slight expression of CD41a without induction of endomitosis in K562 cells (data not shown). This is similar to what has been observed with benoxathian.

The results of our study provide evidence for a receptor-specific effect of the drugs, since the α1-adrenergic antagonists, prazosin and benoxathian, are structurally different, and the α2-adrenergic antagonist, yohimbine, does not exert the same effects concerning apoptosis and differentiation. α1-Adrenergic antagonist treatment may either inhibit a possible autocrine control circuit of the cells or mediate their effect solely through inverse agonism via α1-adrenergic receptors [32, 33]. Nevertheless, receptor dependence of the effects has to be further investigated, since prazosin has been shown to induce also receptor-independent apoptosis in prostate cancer cells [34].

Based on our data, benoxathian and prazosin may serve as new tools to study megakaryocytic differentiation, especially through the selectivity of prazosin to induce endomitosis. Furthermore, keeping in mind the effects of benoxathian and prazosin on differentiation and apoptosis, this study could be the basis for future research elucidating the use of adrenergic antagonists as alternatives and/or additives to traditional chemotherapeutics.

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