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In bcr-abl-positive myeloid cells resistant to conventional chemotherapeutic agents, expression of Par-4 increases sensitivity to imatinib (STI571) and histone deacetylase-inhibitors

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Abstract

In a variety of malignant cells the prostate-apoptosis-response-gene-4 (Par-4) induces increased sensitivity towards chemotherapeutic agents by down-regulating anti-apoptotic B-cell lymphoma-gene 2 (Bcl-2). Hypothesizing that Par-4 also influences apoptosis in myeloid cell lines, we tested this hypothesis by stably transfecting bcr-abl transformed-K562 cells with a Par-4-expressing vector. Here we demonstrate that over-expression of Par-4 in K562 cells up-regulates expression levels of Bcl-2 and death-associated protein (Daxx). Upon treatment with different chemotherapeutic agents, Fas- or TRAIL agonistic antibodies, Par-4-positive cells did not exhibit an increased rate of apoptosis as compared to Par-4-negative control cells. However, incubation with histone deacetylase (HDAC)-inhibitors Trichostatin A (TSA) and LAQ824 or the tyrosinkinase inhibitor Imatinib (STI571) increased the rate of apoptosis in Par-4-positive K562 cells. Assessing the underlying molecular mechanisms for the Par-4-induced response to HDAC-inhibitors and STI571 we provide evidence, that these effects are associated with a down-regulation of Daxx, enforced activation of caspases and enhanced cleavage of cellular inhibitor of apoptosis (cIAP)-1 and -2.

Keywords: Par-4; Daxx; Bcl-2; Bax; Imatinib; Histone deacetylase-inhibitors

1. Introduction

The prostate-apoptosis-response-gene-4 (Par-4) gene is known to be a pro-apoptotic transcriptional repressor gene originally identified in prostate cancer cells [1]. Functional studies demonstrated that activation of Par-4 is not sufficient to cause apoptosis but rather sensitizes cells to the action of apoptotic agents [2,3].

Par-4 transcriptionally regulates B-cell lymphoma-gene 2 (Bcl-2) through a Wilms-tumor-1-gene (WT-1) binding site in the Bcl-2 promoter [4]. Studies on various malignancies such as solid tumors as well as hematological neoplasias of lymphatic origin provided evidence that the pro-apoptotic role of Par-4 relies on a down-regulation of the anti-apoptotic protein Bcl-2 [5–7]. In blast cells of acute myeloid leukemia Bcl-2 posseses anti-apoptotic properties through its influence on mitochondrial apoptosis pathways and plays an important role in drug-resistance [8]. The expression levels of Bcl-2 in AML and especially the ratio of Bcl-2 with other pro-apoptotic Bcl-2-family members such as Bcl-associated x-protein (Bax) are of prognostic relevance [9].

Abbreviations: Par-4, prostate-apoptosis-response-gene-4; Daxx, death-associated protein; WT-1, Wilms-tumor-1-gene; PML, pro-myelocytic leukemia; XIAP, x-linked inhibitor of apoptosis; cIAP, cellular inhibitor of apoptosis; caspases, cysteine proteases that cleave after aspartatic acids; Bcl-2, B-cell lymphoma-gene 2; Bax, Bcl-associated x-protein; STI, signal-transduction inhibitor; HDAC, histone deacetylase; LAQ, histone deacetylase-inhibitor; TSA, Trichostatin A; bcr-abl, break cluster region-protooncogen abl (t9;22)

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Death-associated protein (Daxx) is an ubiquitously expressed and highly conserved protein [10] with multiple putative functions and subcellular localizations in the context of apoptosis. Early reports depict Daxx as a Fas-receptor interacting and Jun N-terminal kinase (JNK) activating protein [11,12]. More recent studies underline a function of Daxx in the nucleus as part of pro-myelocytic leukemia (PML) oncogenic domains (PODs/nuclear bodies) where it plays a role in regulating transcription [10,13,14] and interacts with histone deacetylases [15]. Whether Daxx should be regarded as a pro- or anti-apoptotic protein remains controversial [16,17]. Newest findings establish a connection between Daxx and Par-4 in PODs via ZIP kinase (ZIPK), a pro-apototic protein implicated in apoptosis suggesting a novel nuclear pathway for apoptosis involving the interaction of these three proteins [18]. In this regard we evaluated the hypothesis that the pro-apoptotic function of Par-4 is also present in myeloid cells by means of assessing apoptotic mechanisms in stably Par-4 over-expressing K562 cells as compared to mock transfected control cells. As apoptosis inducing agents, CD95 and TRAIL agonistic antibodies and conventional cytotoxic anti-cancer drugs such as Ara-C, cladribine, doxorubicin, mitoxantrone and the new alkylating agent bendamustine were used. Bendamustine is a bifunctional alkylating agent characterized by a nitrogen mustard group and an additional purine-like benzimidazol nucleus [19]. Several phase II studies have proven its efficacy in the treatment of low- and high-grade non-Hodgkin's lymphomas [20,21]. Furthermore, the effects of new therapeutics with specific targets such as the tyrosine kinase inhibitor STI571 (Imatinib mesylate) (for review see [22]) or the histone deacetylase (HDAC)-inhibitors Trichostatin A (TSA) or LAQ824 (NVP-LAQ824) (for review see [23]) were investigated in this cell-line model.

The latter substances were chosen or favored over other comparable experimental agents because all of them are already in clinical use or in promising clinical trials.

2. Material and methods

2.1. Cell culture

K562 cells were cultured at 37 °C under 5% CO_2 in RPMI 1640 (Life Technologies Inc.) supplemented with 10% FCS (Life Technologies Inc.), 2 mM L-glutamine (Life Technologies Inc.), and a 1% penicillin–streptomycin mixture (Life Technologies Inc.). For all experiments cells were seeded in a concentration of 25×10^4 cells/ml.

2.2. Antibodies

Anti-Bcl-2 (clone 124) was purchased from DAKO; anti-caspase-10 (4C1) from Medical and Biological Laboratories Co.; anti-caspase-6 (B93-4), anti-caspase-7 (B94-1), the polyclonal anti-caspase-8, anti-Daxx (M-112), anti-Par-4 (R-334) were purchased from PharMingen; anti-tubulin from Dunn; the polyclonal anti-XIAP, anti-cIAP-1 and anti-cIAP-2 from R&D Systems; anti-Bax (P-19), anti-caspase-3 (N19), anti caspase-9 (H-83) and the secondary horseradish-peroxidase conjugated antibodies from Santa Cruz Biotechnology; anti-CD95 (CH11) from Beckman Coulter Company.

2.3. Construction of plasmids and stable transfection

The pCB6+ expression vector containing the entire open reading frame of Par-4 was a gift of Dr. Vivek M. Rangnekar (University of Kentucky, Lexington, USA). Par-4 cDNA was excised by digestion at *Eco*RI and cloned into the pcDNA3.1+ expression vector (Invitrogen).

K562-cells, obtained from the American Type Culture Collection were transfected using TransFast™ Transfection Reagent essentially according to the manufacturers instructions (Promega). Briefly, 2×10^6 K562 cells were transfected using 9 μl TransFast™ Reagent and 3 μg pcDNA3.1+/Par-4 or pcDNA3.1+, respectively.

Fourty-eight hour after transfection cells were selected by treatment with G418 (Serva) and subcloned under limiting dilution conditions. Clones were tested for successful pcDNA3.1+/Par-4 or pcDNA3.1+ control vector transfection by PCR using 5′-ctc act ata ggg aga ccc aag c-3′ (sense) and 5′-ttc ctc cag gaa agt ccg tgg t-3′ (antisense) or 5′-ctc act ata ggg aga ccc aag c-3′ (sense) and 5′-ggc tgg caa cta gaa ggc aca a-3′ (antisense), respectively. In addition, Par-4 expression was tested by Western blotting.

2.4. Induction and detection of apoptosis

Apoptosis of Par-4 transfected K562 cells and mock control cells was induced by incubating $2–5 \times 10^6$ cells/ml with the commonly used chemotherapeutics: 3 μg/ml (12.3 μM) Ara-C, 0.1 μg/ml (0.285 μM) and 0.5 μg/ml (1.425 μM) cladribine (Janssen-Cilag), 0.2 μg/ml (0.35 μM) doxorubicin (Pharmacia), 0.2 μg/ml (0.39 μM) mitoxantrone (Lederle), 75 μg/ml (19 μM) and 100 μg/ml (25.33 μM) bendamustine (Ribosepharm). These dosages represent the drug concentrations causing 50% apoptosis (IC50) in other comparable cell-lines such as HEL, Jurkat, WSU-NHL or DOHH-2 previously established in or laboratory by dose response experiments with cytometrical assessment of the induced rates of apoptosis by Annexin V/Propidium iodide staining and analysis of the mitochondrial membrane potential (MMP) with JC-1. Furthermore the impact on apoptosis was determined by incubating $2–5 \times 10^6$ cells/ml with: 250 ng/ml Fas/CD95 agonistic antibody (Immunotec), 100 ng/ml TRAIL agonistic antibody (CH11) (Immunotec), 1 and 5 μM Imatinib (STI571) (Novartis), 0.1 and 0.5 μM Trichostatin A (Sigma) or 0.1 and 1 μM LAQ824 (Novartis). Apoptosis was quantified by a FACScan flow cytometer (Lysis II; Becton Dickinson)

after 4 and 24 h using an Annexin V/Propidium iodide kit (Roche). Error bars in the graphs represent standard error of the mean (S.E.M.) of triplicates calculated using the GraphPad Prism Software.

2.5. Western blot analysis

The proteins were separated on denaturing 10 or 15% SDS-polyacrylamide gels, followed by Western blotting on nitrocellulose-membranes. The membranes were blocked with a 5% non-fat, dry milk and incubated with the primary antibody. Unbound antibody was removed by washing with Tris-buffered saline (pH 7.2) containing 0.5% Tween 20. The membranes were then incubated with the secondary antibody and detected with the ECL-Kit (Amersham Pharmacia Biotech). Blots were stripped using Restore™ Western blot stripping buffer according to the manufacturers instructions (Pierce). All Western blot experiments were carried out three times and blots presented are representative for data obtained thereby. Intensity of protein expression was compared to tubulin and measured quantitatively by densitometric software (Scion Image for Windows, Scion Corporation).

3. Results

3.1. In malignant myeloid cells Par-4 fails to promote apoptosis upon stimulation with conventional chemotherapeutic agents, Fas- or TRAIL-agonistic antibodies

To test the hypothesis that Par-4 sensitizes malignant myeloid cells towards apoptosis induced by conventional chemotherapeutic agents K562 cells were transfected with a Par-4-containing vector or with the corresponding Par-4-negative "mock" vector. After clones were selected via limiting dilution, three Par-4-positive and two Par-4-negative clones were chosen and subsequently incubated with Ara-C, cladribine, doxorubicin, mitoxantrone or bendamustine. Since K562 cells responded only to induction of apoptosis upon incubation with bendamustine (data not shown), but were insensitive to treatment with the other cytotoxic drugs, all experiments were carried out using bendamustine. As depicted in Fig. 1A, Par-4 transfected K562 cells exhibited no significant enhancement of the rate of apoptosis in comparison to mock-transfected control cells under treatment with 100 μg/ml (25.33 μM) bendamustine.

Additionally, apoptosis was induced by application of Fas and TRAIL-agonistic antibodies in order to assess the standard apoptosis pathways activated by the corresponding receptors in this cell-line model. As demonstrated in Fig. 1B, treatment with 250 ng/ml of Fas-L or 100 ng/ml TRAIL agonistic antibody only led to a negligible increase of the rate of apoptosis after 4 h as compared to untreated control cells. Furthermore, no significant differences between Par-4-positive or -negative cells emerged. In fact, mock-transfected control cells exhibited slightly higher rates of apoptosis than Par-4-expressing cells.

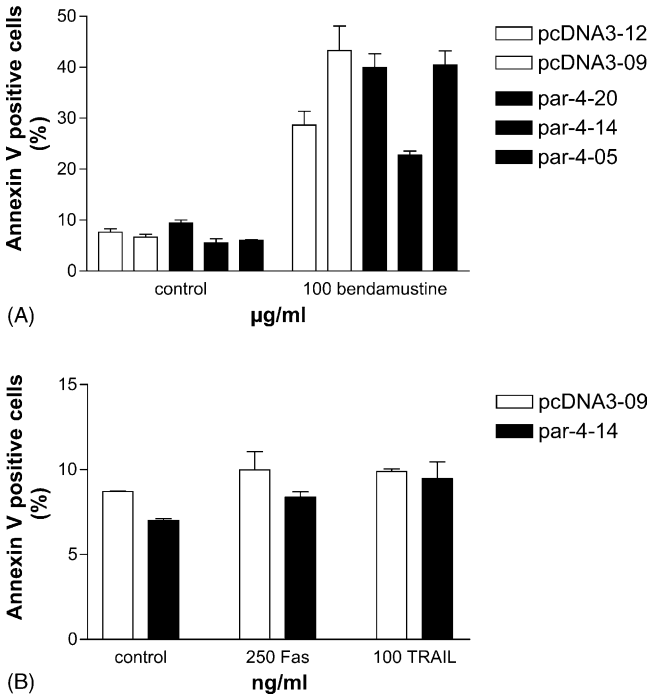

Fig. 1. (A) Expression of Par-4 in K562 cells does not enhance the rate of apoptosis induced by conventional drugs (here: bendamustine) as compared to mock transfected control cells. K562 clones stably transfected with pcDNA3.1+/Par-4 or pcDNA3.1+, respectively, were treated with IC50 concentrations of conventional cytotoxic drugs (Ara-C, cladribine, doxorubicin, mitoxantron and bendamustin) for 4 and 24 h and subsequently the rate of apoptosis was determined cytometrically with Annexin V/Propidium iodide. The tested cells only responded to treatment with bendamustine and were resitant against the other drugs mentioned above (data not shown). (B) Expression of Par-4 in K562 cells does not enhance the rate of apoptosis induced by Fas-L or TRAIL. The cell clones described above were treated with apoptosis inducing Fas antibody and TRAIL agonistic antibody for 4 and 24 h and consecutively stained with Annexin V/Propidium iodide and analyzed cytometrically to determine the rate of apoptosis. The diagram above shows the rate of apoptosis measured after 4 h, values after 24 h were comparable (data not shown). Treatment with 250 ng/ml of Fas or 100 ng/ml TRAIL antibodies had only marginal effects on the rate of apoptosis as compared to untreated control cells and no difference between Par-4-positive and -negative cells could be detected.

We thus demonstrate that in this model using malignant cells of myeloid origin, Par-4 fails to exhibit an apoptosis augmenting effect upon induction of apoptosis by conventional chemotherapeutic agents, Fas or TRAIL agonistic antibodies.

3.2. Upon incubation with STI571 or HDAC-inhibitors Trichostatin A (TSA) or LAQ824, expression of Par-4 sensitizes bcr-abl-positive myeloid cells to apoptosis

After demonstrating that expression of Par-4 does not influence the rate of apoptosis of K562 cells incubated with conventional chemotherapeutic agents, we tested whether more specific molecular-targeting agents may involve

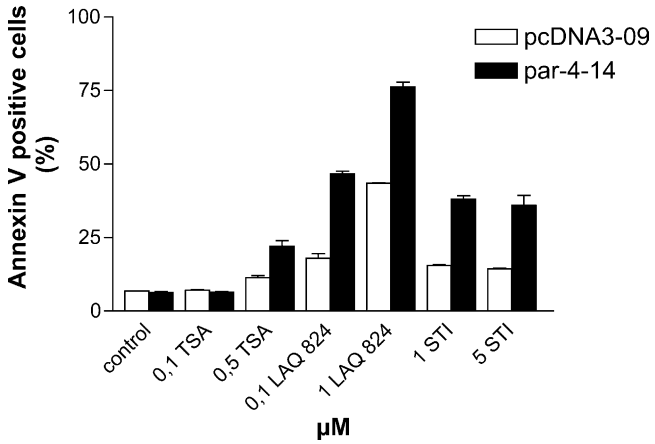

Fig. 2. Expression of Par-4 in K562 cells augments apoptosis induced by tyrosine-kinase-inhibitor STI571 or histone deacetylase (HDAC)-inhibitors Trichostatin A or LAQ824. As depicted in this diagram, treatment of Par-4-positive and -negative K562 clones with escalating dosages of the HDAC-inhibitors TSA or LAQ824 led to approximately two-fold increases in apoptosis in Par-4 transfected cells as compared to their mock transfected control cells. Apoptosis was determined cytometrically with Annexin V/Propidium iodide staining after 24 h.

different mechanisms regarding induction of apoptosis in this model. Since K562 cells contain the bcr-abl translocation [24], the influence of STI571, a drug able to repress deregulated tyrosine kinases, was assessed on the Par-4 over-expressing cell clones. As depicted in Fig. 2, Par-4 over-expressing K562 cell clones exhibited an increased rate of apoptosis upon incubation with STI571 as compared to the Par-4-negative clones. The percentage of apoptotic cells in Par-4-negative K562 incubated with 1 μM STI571 for 24 h reached a median of approximately 15%, whereas the rate of apoptosis was more than doubled upon Par-4 expression. The rate of apoptosis was not further increased by higher dosages of signal-transduction inhibitor (STI) (Fig. 2). HDAC-inhibitors function by promoting acetylation of histones, leading to uncoiling of chromatin and activation of a variety of genes implicated in the regulation of cell survival [25] and are thus considered—together with STI571—to belong to a new group of agents specifically targeting molecular mechanisms, whose deregulations are causative in the leukemogenesis. Comparable to the results obtained with STI571, the histone deacetylase-inhibitors TSA and LAQ824 led to an increase in apoptosis in Par-4-positive cells as compared to Par-4-negative cells. In Par-4 transfected cells approximately two-fold increases of apoptosis rates was observed (Fig. 2). Both HDAC-inhibitors induced higher rates of apoptosis upon augmentation of dosage, an effect that was maintained in Par-4-expressing cells clones.

3.3. In neoplastic cells of myeloid origin expression of Par-4 up-regulates Bcl-2

After providing evidence that in K562 cells expression of Par-4 remains without influence upon induction of apoptosis with conventional chemotherapeutic agents, but increases sensitivity towards the newer and more specific therapeutics such as STI571 and HDAC-inhibitors, we next evaluated the molecular mechanisms underlying the different responses. Since previous studies demonstrated that the pro-apoptotic function of Par-4 relies on down-regulation of Bcl-2 [5,6] we first addressed the question whether the failure of Par-4 to promote apoptosis in an AML cell line might be due to a lack of Bcl-2 down-regulation. Interestingly, as shown in Fig. 3A, expression of Bcl-2 in K562 cells was stronger in Par-4-positive than in Par-4-negative cell clones. Since it is established, that the anti-apoptotic function of Bcl-2 can be antagonized by Bax [9], we consequently assessed the expression of this pro-apoptotic protein in Par-4-positive and -negative cell clones. However, expression of Par-4 remained without influence on expression of Bax (Fig. 3A).

3.4. Upon expression of Par-4, bcr-abl-positive myeloid leukemia cells augment protein expression of Daxx

As a consequence of newest findings implicating a collaboration of Par-4 and Daxx in a new nuclear pathway for apoptosis [18,26], the basal expression levels of Daxx in the Par-4-positive and -negative cell clones were assessed to resolve the question whether the described effects of Par-4 in myeloid cells may be associated with a differential expression of Daxx. As depicted in Fig. 3A, expression of Par-4 led to an up-regulation of Daxx-levels as compared to Par-4-negative cell clones.

3.5. Par-4-positive myeloid cells exhibit a higher expression of the pro-caspases-8, -9 and -10 than Par-4-negative cells, whereas the levels of pro-caspase-3 and of the inhibitors of apoptosis proteins (IAP's) remain unaltered

In order to evaluate whether the differential expression of the anti-apototic protein Bcl-2 in Par-4-positive and -negative clones entails altered expression levels of the initiator and executioner caspases, protein levels of the respective pro-caspases were assessed. Fig. 3B demonstrates, that Par-4-negative cell clones—which contain less Bcl-2—exhibit lower levels of the initiator pro-caspases-8, -9 and -10. The levels of caspase-3, -6 and -7 remained unaltered by the expression of Par-4 (Fig. 3B and data not shown). As expression and activation of caspases are regulated in part by inhibitors of apoptosis proteins (IAPs) and as in Par-4 expressing lymphatic cells cellular inhibitor of apoptosis (cIAP)-1/-2 and x-linked inhibitor of apoptosis (XIAP) were down-regulated [6], we studied if the altered caspase-activation in myeloid cells is also associated with a decrease of IAPs. Of note, levels of the major IAPs, among them cIAP-1, XIAP (Fig. 3C), cIAP-2 and survivin (data not shown) were retained independently of the Par-4-expression level.

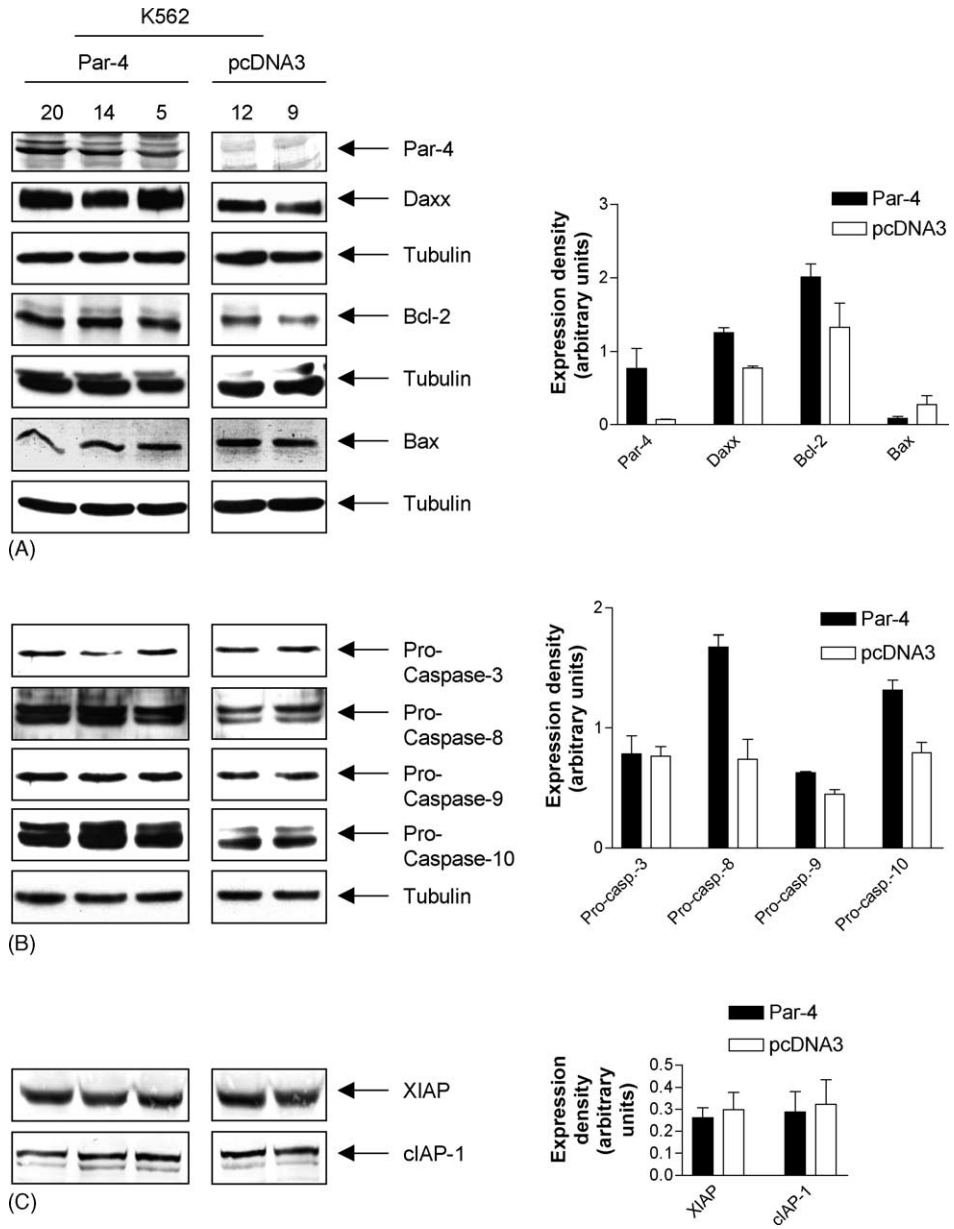

Fig. 3. Western blot analysis and densitometrical analysis of the protein expression levels of K562 cells (whole cell lysates) stably transfectected with pcDNA3.1+/Par-4 (left lanes) and mock transfected with pcDNA3.1+ (right lanes) without treatment. Error bars represent the standard deviation of protein levels of the analyzed and depicted clones. (A) In Par-4 over-expressing clones basal expression levels of death-associated protein and anti-apoptotic Bcl-2 were elevated as compared to Par-4-negative control cells. Expression levels of pro-apoptotic Bax were unaffected by over-expression of Par-4. (B) Basal expression levels of the pro-forms of caspases-8, -9 and -10 were up-regulated in Par-4 over-expressing K562 cell clones as compared to Par-4-negative control clones. Pro-caspase -3 expession levels remained uninfluenced by Par-4 over-expression. The same applied for the inhibitor of apoptosis proteins (IAP's) XIAP and cIAP-1 (C).

3.6. Upon incubation with the apoptosis-inducing agents STI571 or LAQ824 expression of Par-4 reverses the up-regulation of Daxx

After outlining the molecular changes elicited by Par-4-expression alone, i.e. in absence of a pro-apoptotic stimulus, we further examined the consequences of Par-4 in myeloid cells in presence of pro-apoptotic stimuli. While none of the conventional chemotherapeutic agents (Ara-C, bendamustine, cladribine, doxorubicin, mitoxantrone) induced a further change in the protein expression of Bcl-2, Bax, or Daxx after 24 or 48 h incubation time (data not shown), the newer agents STI571 and LAQ824 which were the only agents with the ability to increase the rate of apoptosis, led to an altered protein expression of the previously described targets. As demonstrated in Fig. 4B—upon induction of apoptosis with STI571 or LAQ824—Par-4 expression led to a down-regulation of Daxx as compared to Par-4-negative cell clones, an effect not detectable upon incubation of cells with conventional chemotherapeutic agents, Fas or TRAIL antibodies. An interaction with the expression of CD95 was ruled out: Fig. 4B shows that the expression levels of CD95

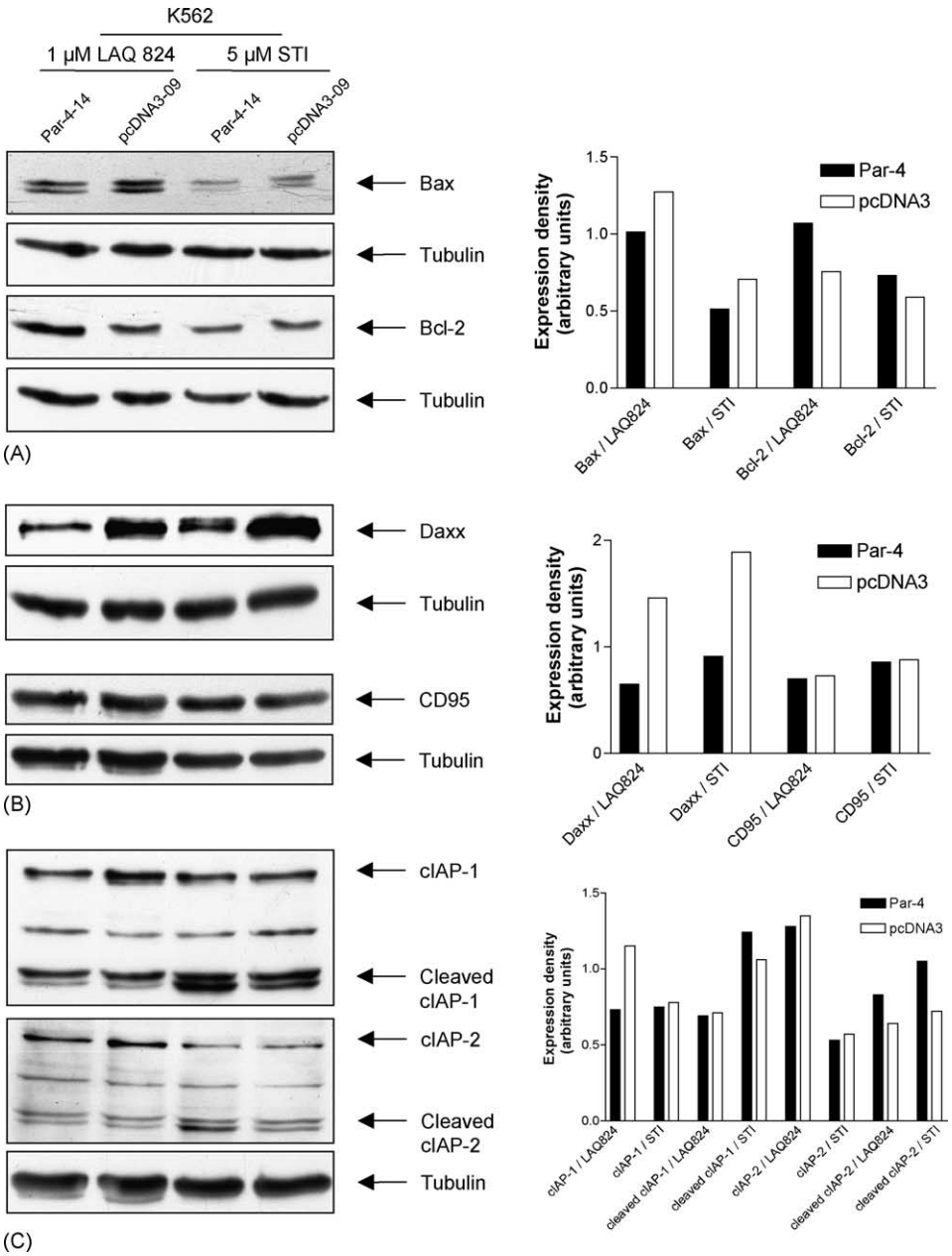

Fig. 4. Western blot analysis (including densitometrical analysis of the protein expression levels) of K562 cells (whole cell lysates) stably transfected with pcDNA3.1+/Par-4 or pcDNA3.1+, treated with 1 μM histone deacetylase inhibitor LAQ824 (left lanes) or 5 μM tyrosin kinase inhibitor STI571 (right lanes) for 24 h. (A) Bcl-2 expression levels remained elevated in Par-4 expressing K562 cells as compared to the Par-4-negative controls when treated with LAQ824. Under treatment with STI571 Bcl-2 levels were down-regulated when compared to treatment with LAQ824 but no difference between the Par-4-positive and the Par-4-negative clone could be ascertained. Bax was down-regulated under treatment with STI571 as compared to treatment with LAQ824. However, in both cases protein levels were slightly higher in control cells as compared to Par-4 expressing cells. (B) Par-4 over-expression in K562 cells led to an enhanced Down-regulation of Daxx under treatment with STI571 or LAQ824 as compared to Par-4-negative cells whereas CD95 levels remained unaltered under all conditions. (C) Cleavage of cIAP-1 and -2 was more accentuated in Par-4 expressing clones as compared to the mock transfected controls.

remained unchanged under all conditions. Of note, treatment with STI571 led to a down-regulation of Bcl-2 as compared to treatment with LAQ824 and in both cases Bcl-2 levels were slightly higher in Par-4-positive clones than in control cells. Concerning the expression of Bax, STI571 treatment caused a down-regulation of protein levels as compared to treatment with LAQ824 but in both cases protein levels were slightly higher in control cells as compared to Par-4-positive cells (Fig. 4A).

3.7. Upon incubation with the apoptosis-inducing agents STI571 and LAQ824 Par-4 expression in myeloid cells leads to decreased levels of pro-caspase-8, -9 and -3 and concomittant down-regulation and cleavage of cIAP-1 and -2

Fig. 5 demonstrates that induction of apoptosis in Par-4 expressing K562 cells by STI571 and LAQ824 caused a stronger decrease of the protein levels of the proforms of

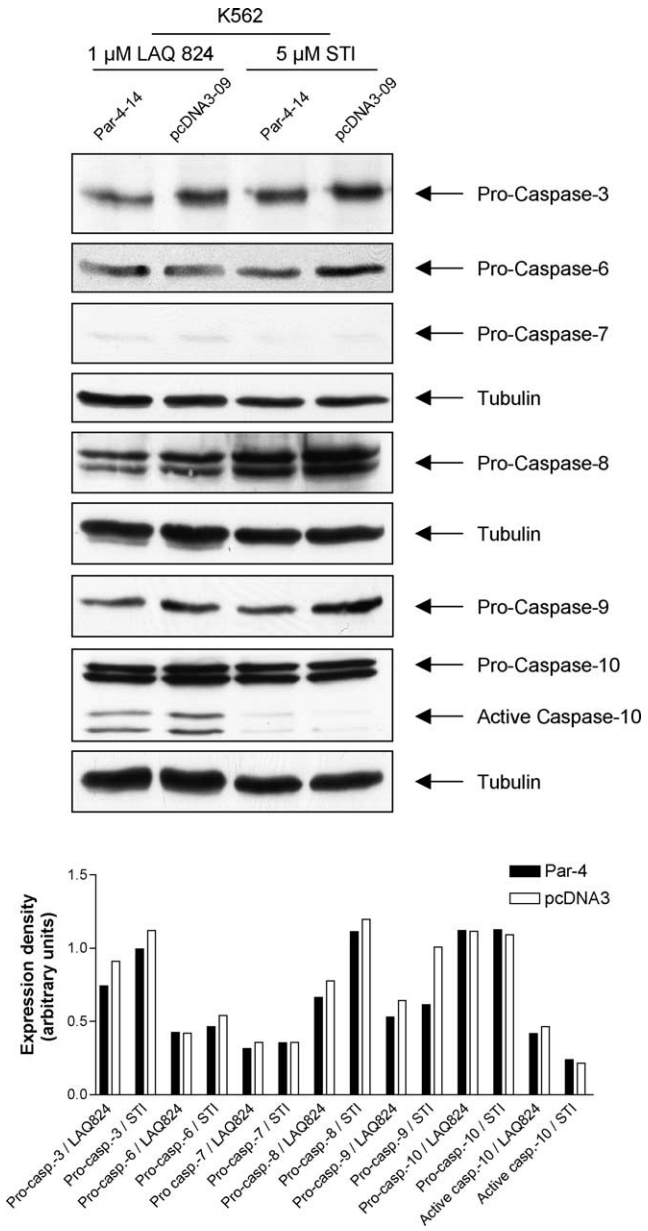

Fig. 5. Western blot analysis (including densitometrical analysis of the protein expression levels) of K562 cells (whole cell lysates) stably transfected with pcDNA3.1+/Par-4 or pcDNA3.1+, treated with 1 μM Histone deacetylase inhibitor LAQ824 (left lanes) or 5 μM tyrosin kinase inhibitor STI571 (right lanes) for 24 h. Upon treatment with LAQ824 or STI571 the initial pattern of stronger expression levels of pro-caspases-8, -9 and -10 in Par-4 over-expressing cells was largely reversed into an enhanced decrease of the pro-form of caspases-8 and -9 implying their stronger activation in Par-4 expressing cells under treatment with these agents. A stronger down-regulation of pro-forms, i.e. activation could also be ascertained for the executioner caspases-3 and -6 in Par-4-positive cells treated with these substances as compared to the Par-4-negative control cells.

caspases-8, -9 and -3 implicating an enhanced cleavage into their active subunits under the influence of Par-4 as compared to mock transfected control cells, especially when considering that these pro-caspases were initially constitutively up-regulated in Par-4-positive cells. Again this effect could not be observed under treatment with the conventional cytotoxic drugs described above (data not shown). It is noteworthy that the increased activation of these caspases was associated with a slightly stronger down-regulation of cIAP-1 and -2 in Par-4 expressing clones under treatment with LAQ824 and an enforced cleavage of cIAP-1 and -2 in STI571 treated Par-4 containing cells as compared to control clones (Fig. 4C).

Of note, the levels of pro-caspase-8 were reduced under treatment with LAQ824 as compared to treatment with STI571, but the difference in protein levels between Par-4-positive versus Par-4-negative cells as described above could still be demonstrated. In contrast, treatment with LAQ824 led to an activation of caspase-10 as compared to treatment with STI571 but no real difference between the Par-4-positive and -negative clone could be ascertained. Expression levels of pro-caspase-6 were nearly equal in both clones treated with STI571 or LAQ824. The same applied for pro-caspase-7 (Fig. 5).

4. Discussion

The development of therapies aiming at specific molecular targets enables the clinician to successfully treat patients suffering from hematopoetic malignancies resistant to conventional chemotherapeutic agents [27]. Nevertheless, similarly to established cytotoxic drugs, the majority of malignant cells eventually also becomes resistant to these new drugs and recent research focuses on the possible mechanisms of such resistance. Thus, resistance against STI571 for example is so far (largely) associated with mutations of the abl-kinase or progressive bcr-abl gene amplification in advanced stages of disease [28,29]. After we previously demonstrated that Par-4 sensitizes malignant lymphocytes to conventional chemotherapeutic agents [6] we hypothesized that Par-4 exhibits comparable attributes in malignant myeloid cells, a cell type, which has not been studied regarding the function of Par-4. Surprisingly, Par-4 failed to promote apoptosis in a myeloid cell line model with K562 cells upon incubation with conventional chemotherapeutic agents, Fas or TRAIL agonistic antibodies but increases sensitivity of these cells to the treatment with STI571 and HDAC-inhibitors. Evaluating the underlying molecular mechanisms governing sensitivity and resistance in malignant myeloid cells we show that—in contrast to results obtained on malignant lymphocytes and solid tumor cells [4,6,7]—Par-4 expression unexpectedly does not lead to a down-regulation of Bcl-2. In contrast, the basal expression levels of this anti-apoptotic protein are increased in myeloid cells over-expressing Par-4. The transcriptional down-regulation of Bcl-2 by Par-4 through a WT-1-binding site on the Bcl-2 promotor described by Cheema et al. [4] requires high levels of WT-1 to cause a decrease in Bcl-2 expression. In Western blot analyses of WT-1 levels in these experiments with various antibodies, WT-1 was almost not detectable (data not shown) giving a possible explanation

for the failure of Par-4 to down-regulate Bcl-2 in this system. As described by various authors, the expression level of Bcl-2 is considered crucial in myeloid cells, since it plays a major role in drug resistance and is of prognostic significance [8,9,30,31]. However, the fact that Bcl-2 remains elevated in Par-4-positive cells as compared to Par-4-negative cells, despite incubation with the apoptosis-inducing agents STI571 and LAQ824, demonstrates that in this system, induction of apoptosis by these agents does not depend on a down-regulation of Bcl-2.

Of note are the elevated basal expression levels of Daxx in Par-4 over-expressing clones and then the reversal of this condition into an enhanced down-regulation of Daxx in Par-4-positive cells incubated with STI571 or the HDAC-inhibitors. This down-regulation of Daxx was not elicited by conventional chemotherapeutic agents and, may therefore, potentially represent the molecular mechanism responsible for the increased susceptibility of Par-4-positive myeloid cells to these new therapeutics. Daxx is an ubiquitously expressed and highly conserved protein [10] implicated in a number of cellular contexts of apoptosis but also in virus replication. Initially Daxx was identified as a Fas-receptor interacting protein promoting Fas induced apoptosis [11], a function not evident in our experiments (Figs. 1B, 4B). Under certain circumstances Daxx was shown to activate the Jun N-terminal kinase pathway through interaction with the apoptosis signaling-regulating kinase-1 (ASK1) [12]. Subsequent reports concentrated more on a function of Daxx in the nucleus depicting Daxx as a potent regulator of transcription [13,14,32,33] and established a connection of Daxx to PML oncogenic domains (PODs/nuclear bodies/ND10) [34,35]. It is becoming apparent that Daxx is involved in transcriptional repression through interaction with histone deacetylases (HDAC's) [15] which is also corroborated by the observation that its expression levels can be influenced by treatment with HDAC-inhibitors [36], also without affecting expression of Bcl-2 and Bax. Whether Daxx should be regarded as pro- or anti-apoptotic remains controversial up to the present [16,17]. A possible interdependency or interaction between Par-4 and Daxx was recently demonstrated by Kawai et al. [18] who is suggesting a novel nuclear pathway for apoptosis by interaction of Daxx and Par-4 via ZIP-kinase in nuclear bodies. Furthermore a possible cohesion between Daxx and Par-4 has also been implicated by results of our group while assessing the molecular mechanisms involved in apoptosis induction by synergistic anti-cancer drug combinations [26]. Exactly in which way the expression levels of Daxx are of relevance for the sensitization of myeloid cells to apoptosis induced by the employed new molecular anti-cancer agents remains a matter of speculation. However, it has become obvious in our experiments that an enforced induction of apoptosis, either by synergistic drug combinations [26] or, as in this case, by STI571 or LAQ824 in myeloid cells sensitized by Par-4, is always associated with an enhanced down-regulation of Daxx. As demonstrated by Kawai et al. [18] ZIP kinase recruits Daxx to nuclear bodies in collaboration with Par-4. In turn, sequestration of Daxx into nuclear bodies has been associated with an inhibition of Daxx mediated repression of transcription [32]. These interrelations encourage to hypothesize that in Par-4 over-expressing myeloid cells the higher levels of Par-4 lead to an increased sequestration of Daxx to nuclear bodies and possibly its subsequent degradation, thereby abrogating its repressive activity on transcription and enhancing apoptosis pathways activated by the molecular agents STI571 and HDAC-inhibitors.

Similarly to effects on Daxx expression, Par-4 over-expression also leads to increased basal expression levels of the pro-forms of initiator caspases-8, -9 and -10 as compared to control cells indicating a weaker activation of these proteases in untreated Par-4-positive cells. However, when treated with the apoptosis inducing agents STI571 or LAQ824 this pattern is reversed into and enhanced activation of caspases-8, -9 and -10 and also the executioner caspase-3 in Par-4 over-expressing clones demonstrated by the stronger decrease of the pro-caspase levels in these cells. In accordance with enhanced apoptosis and stronger caspase activation under these conditions a slightly stronger degradation or cleavage of the IAP's cIAP-1 and -2 can be observed. How Par-4 leads to a basal up-regulation of pro-caspase levels of initiator caspases in comparison to control cells remains unclear. Assuming that with more pro-caspases at disposal more caspases can in turn be activated to mediate apoptosis, the subsequent enforced apoptosis in Par-4 expressing cells treated with STI571 or LAQ824 may be explained.

Taken together a pro-apoptotic function of Par-4 under treatment with conventional cytotoxic anti-cancer drugs described in various malignancies seems impaired in K562 cells. However, when Par-4 over-expression is combined with new specific therapeutics such as thyrosine kinase inhibitor STI571 or HDAC-inhibitor LAQ824 the apoptosis enhancing properties of Par-4 can largely be reestablished and significantly higher rates of apoptosis in Par-4 over-expessing cells as compared to mock transfected control cells can be observed. This phenomenon is accompanied by an initial increase of basal Daxx expression in untreated Par-4 transfected myeloid cells and subsequently enhanced down-regulation of Daxx upon treatment with new specific therapies in these cells. This implicates a possible functional interdependency between Daxx and Par-4 in a novel apoptosis pathway with the ability to enhance apoptosis in cells otherwise largely resistant against therapy.

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