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Use of a novel histone deacetylase inhibitor to induce apoptosis in cell lines of acute lymphoblastic leukemia

ANNETTE ROMANSKI
BISERKA BACIC
GESINE BUG
HEIKE PFEIFER
HILAL GUL
STACY REMISZEWSKI
DIETER HOELZER
PETER ATADJA
MARTIN RUTHARDT
OLIVER G. OTTMANN

A B S T R A C T

Background and Objectives. Chromatin structure and thereby transcription is controlled by the level of acetylation of histones, which is determined by the balance between histone acetyl transferase (HAT) activity and histone deacetylase (HDAC) activity. HDAC inhibitors are a class of compounds able to regulate gene expression by modulating chromatin structure. There are two major classes of HDAC inhibitors: the hydroxamic acid derivatives such as trichostatin A (TSA) or SAHA, and the butyrates such as phenylbutyrate. HDAC inhibitors interfere with differentiation, proliferation and apoptosis in tumor cells. Here, we investigated the activity of a new hydroxamic acid derivative, LAQ824, on lymphoblastic cells.

Design and Methods. Four different pre-B lymphoblastic cell lines: Sup-B15 and TMD-5, both t(9;22) positive, SEM, t(4;11) positive, and NALM-6 cells were exposed to the hydroxamic acid derivatives, LAQ824 and TSA. Histone hyperacetylation, apoptosis, cell cycle and related pathways were assessed by flow cytometry and Western blotting.

Results. LAQ824 significantly inhibited the proliferation of leukemic lymphoblastic cell lines. The effect of LAQ824 was due to increased apoptosis accompanied by activation of caspase-3 and caspase-9, cleavage of poly(ADP-ribose)-polymerase (PARP) as well as by down-regulation of Bcl-2 and disruption of the mitochondrial membrane potential. Surprisingly, LAQ824-induced apoptosis was at least partially independent of caspase activation as indicated by the fact that LAQ824-induced apoptosis was inhibited only partially in both t(9;22) positive Sup-B15 and TMD-5 cells, whereas no inhibition was observed in t(4;11) positive SEM cells upon exposure to the polycaspase inhibitor zVAD-fmk.

Interpretations and Conclusions. Our study establishes that LAQ824 is a promising agent for the therapy of acute lymphoblastic leukemia.

Key words: HDAC inhibitor, apoptosis, LAQ824.

From the Med. Klinik III/Abtl. Hämatologie, Johann Wolfgang Goethe-Universität, 60590 Frankfurt, Germany (AR, BB, GB, HP, HL, DH, MR, OGO); Oncology Research, Novartis Institute for Biomedical Research, One Health Plaza, East Hanover, NJ, USA (SR, PA).

Correspondence: Oliver Gerhard Ottmann, Med. Klinik III/Abtl. Hämatologie, Klinikum der J.W. Goethe Universität Frankfurt Theodor Stern Kai 7, 60590 Frankfurt, Germany. E-mail: ottmann@em.uni-frankfurt.de

The induction of a leukemic phenotype is considered to be mainly related to impaired transcription, leading to uncontrolled proliferation, defects in apoptosis and an impaired differentiation potential of leukemic blasts. It has recently been shown that the induction of the leukemic phenotype in acute myeloid leukemia is strictly related to aberrant chromatin modeling, the major mechanism of transcriptional control.[1-3] Chromatin modeling is controlled by the balance between histone acetylation and deacetylation. Deacetylation of histones leads to compact nucleosomes and condensed chromatin which is inaccessible for the transcriptional machinery of transcription factors and the RNA-polymerase. Thus condensed chromatin is transcriptionally silent. The deacetylation of histones is maintained by the continuous action of histone deacetylases (HDAC).

Transcription is initiated by local inactivation of HDAC and concomitant activation of histone acetylation in a chromatin segment. Histone acetylation is due to the inhibition of HDAC activity and the activation of histone acetyl transferases (HAT). When the balance is shifted towards histone acetylation, the nucleosome structure opens followed by unfolding of the associated DNA. Uncondensed chromatin then allows transcription activation by access to transcription factors.[4-7]

HDAC inhibitors are members of a new class of agents able to regulate gene expression by modulating chromatin structure. Several structurally diverse HDAC inhibitors, such as trichostatin A (TSA), a microbial metabolite, or butyrates, have been identified and their *in vitro* activity in transformed cells makes them promising agents for cancer therapy.[8,9] There is

increasing evidence that HDAC inhibitors are effective therapeutic agents in the treatment of a variety of cancers refractory to conventional anticancer agents. Different classes of agents with HDAC inhibitor activity exert different biological effects, possibly because of distinct effects on additional mechanisms of cell regulation, e.g. phosphorylation.

Acute lymphoblastic leukemia (ALL) is an aggressive disease with a poor prognosis which is further exacerbated by the presence of the Philadelphia chromosome (Ph) — the cytogenetic substrate of the t(9;22) — or by the t(4;11).[10-12] With the currently applied chemotherapy regimens survival ranges between 0-10%, even though initial complete remission rates of 80% are comparable to those achieved in Ph negative patients.[13] Allogeneic stem cell transplantation is the only treatment with curative potential, but treatment-related mortality and the relapse rate both remain high.[11,14-17]

Considering that HDAC inhibitors are able to induce apoptosis in different cell types,[18-21] we investigated their potential to induce apoptosis in poor prognosis ALL cells. We compared the activity of TSA with that of LAQ824, a novel cinnamic acid hydroxamate which has been shown to induce hyperacetylation in several tumor cell lines through blockage of the deacetylation of core histones H3 and H4. Furthermore it has recently been shown that LAQ824 is effective in cell line models of chronic myeloid leukemia as well as in multiple myeloma models.[22,23]

Here we show that LAQ824 is able to induce apoptosis in cell lines derived from high risk ALL, establishing that LAQ824 is a promising agent for the treatment of high risk ALL patients.

Design and Methods

Cell culture, HDAC inhibitor treatment

Cells were cultured at 37°C in 5% CO_2 and a humidified atmosphere. Human leukemic cell lines, NALM-6, SEM (acute Ph negative B precursor ALL) and Sup-B15 (acute Ph positive B precursor ALL), were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and TMD-5 (acute Ph positive B precursor ALL) was kindly provided by Dr. Nobuo Nara (Tokyo Medical and Dental University).[24] NALM-6 and SEM were maintained in RPMI-1640 medium (GibcoBRL, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS) and Sup-B15 with 15% FCS (Hyclone, UT, USA). TMD-5 cells were cultured in MEMα medium (GibcoBRL, Karlsruhe, Germany) supplemented with 15% FCS (Hyclone, UT, USA). Cells in exponential growth phase were plated at 2.5×10^5 cells/mL and incubated with 100nM TSA, 10 nM LAQ824 or 50nM for 1-4 days.

Cell growth, cell cycle, and apoptosis assays

Viability of cells was detected by the trypan blue dye exclusion assay. Cell cycle analysis was determined by propidium iodide (PI) staining. Briefly, 2×10^6 cells were incubated in Cellgro (CellGenix, Freiburg, Germany) in a fully humidified atmosphere of 5% CO_2 in air with 10 μM BrdU (Sigma-Aldrich) at 37°C for 25 min. Cells were fixed by ice-cold 70% ethanol and denatured by 4N HCl. Then fluorescein isiothiocyanate (FITC)-conjugated monoclonal antibody (MoAb) against BrdU (Becton-Dickinson, Heidelberg, Germany) was bound to the BrdU incorporated in DNA and the cells were stained with PI (5μg/mL, Sigma-Aldrich). Stained cells were analyzed by FACScan (Becton-Dickinson, Heidelberg, Germany). At least 2×10^4 cells were subjected to replicate analyses.

Apoptosis was determined by staining cells with annexin V-FITC and PI labeling, because annexin V can identify the externalization of phosphatidylserine during the apoptotic progression and, therefore, detect early apoptotic cells.[25] To quantify the apoptosis of cells, 10^6 cells/mL were washed with cold phosphate-buffered saline (PBS) and then resuspended in annexin-V-FLUOS labeling solution (Roche Diagnostics GmbH, Mannheim, Germany) and incubated for 10 min. Apoptosis was quantified after 6, 24, 48, and 72h by FACScan flow cytometry (LysisII, Becton Dickinson, Heidelberg, Germany). To inhibit activation of caspases, cells were pretreated for 1h with 20μM polycaspase inhibitor N-tert-butoxy-carbonyl-Val-Ala-Asp-fluoromethylketon (zVAD-fmk).

Western blotting

Polyclonal anti-$p27^{Kip1}$ (C19), polyclonal anti-caspase-3 (N-19) and polyclonal anti-caspase-9 (H-83) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); polyclonal anti-poly(ADP-ribose)-polymerase (PARP) came from Roche Diagnostics GmbH (Mannheim, Germany); monoclonal anti-Bcl-2 (124) from DAKO (Hamburg, Germany); monoclonal anti-β tubulin (DM1A/DM1B) from Dianova (Hamburg, Germany) and polyclonal anti-acetyl-H3, polyclonal anti-acetyl-H4 and mixed monoclonal anti-$p21^{WAF1/Cip1}$ (CP36,CP74) from Upstate Biotechnology, Inc. (Lake Placid, NY, USA); and the secondary horseradish-peroxidase conjugated anti-mouse as well as anti-rabbit antibodies and anti-goat antibodies from Dianova (Hamburg, Germany). Briefly, cells (2×10^6) were pelleted and fractionated by SDS-PAGE (8–15% gradient gels), and proteins were transferred to a nitrocellulose membrane by semi-dry electroblotting (Bio-Rad, München, Germany). The membrane was blocked with 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%

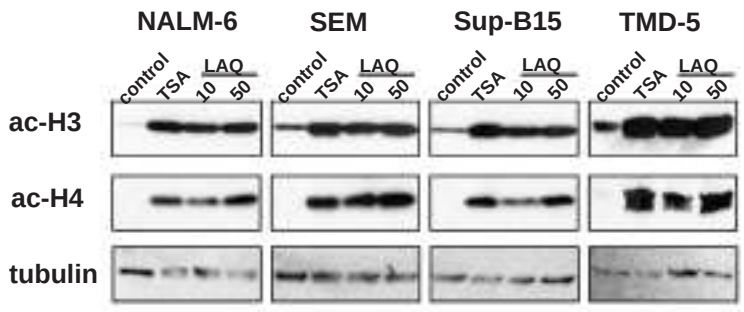

Figure 1. Increase in histone H3 and H4 acetylation in NALM-6, SEM, Sup-B15 and TMD-5 cells. Cells were treated with 10nM as well as 50nM LAQ824 and 100nM TSA for 4h. After cell harvesting histones were analyzed by SDS-PAGE and Western blotting with specific antibodies against either acetylated H3 or acetylated H4.

Tween 20 (TBS-T). The membrane was then incubated with the secondary horseradish-peroxidase conjugated antibody. After extensive washing with TBS-T, blots were developed by chemoluminescence. For *stripping* the blots were treated with *Restore Western Blot Stripping Buffer* according to the manufacturer's instructions (PIERCE, Rockford, USA).

Analysis of the mitochondrial membrane potential ($\Delta\psi_m$)

Changes in the mitochondrial membrane potential ($\Delta\psi_m$) were measured by flow cytometry using the intramitochondrial dye JC-1 (Alexis Biochemicals, Gruenberg, Germany) after 16 and 24 h, as described in the manufacturer's instructions. Data were converted to dot plots using CellQuest software (Becton Dickinson, Heidelberg, Germany).

Results

LAQ824 causes hyperacetylation of histones H3 and H4 in ALL blasts

LAQ824 is a derivative of hydroxamic acid similar to TSA.[26,27] To investigate the effect of LAQ824 on the acetylation of histones 3 and 4 (H3, H4) in cell lines derived from patients suffering from B-lineage ALL we exposed these cells to 10nM or 50nM LAQ824 for 4h. As a control the cells were treated with 100nM TSA. Hyperacetylation of H3 and H4 histones was determined by Western blot analysis using anti-acetyl-H3 and anti-acetyl-H4 antibodies. All employed cell lines (NALM-6 , SEM, Sup-B15 and TMD-5) have an almost identical differentiation status at the pre B-level. SEM cells have the t(4;11), whereas Sup-B15 and TMD-5 have the t(9;22), both conferring a poor prognosis when present in ALL patients. In all cell lines treatment with 10nM of LAQ824 induced strong H3 hyperacetylation (Figure 1). In contrast, the effect of LAQ824 on H4-hyperacetylation was dose-dependent, but a concentration of at least 50nM was necessary to achieve the effects induced by TSA. The dosages of LAQ824 which led to hyperacetylation of histones also had a strong anti-proliferative effect on all tested cell lines (*data not shown*).

These data confirm the potent HDAC inhibitor activity of LAQ824 in malignant lymphoblasts.

LAQ824 causes cell cycle arrest and up-regulates $p21^{WAF1/Cip1}$ without influencing $p27^{Kip}$ expression

Since chemotherapeutic drugs may act not only by inducing cell death but also by inhibiting proliferation, we studied the effect of LAQ824 on cell cycle progression. Thus we exposed the above described cell lines to LAQ824 (10nM and 50nM) and TSA (100nM) for 16h. Cell cycle progression was analyzed by FACS analysis of cells stained with PI and an anti-BrdU-antibody. In NALM-6 cells treatment with 10nM LAQ824 or TSA induced a G0/G1 block and 50nM of LAQ824 led to a further increase of cells in G0/G1 and a higher percentage of cells in G2/M-phase (Figure 2A, Table 1).

A small percentage of SEM cells treated with TSA showed a G0/G1 block, whereas 10nM and 50nM of LAQ824 induced G0/G1 block in nearly all cells. A similar picture was seen in the t(9;22) positive Sup-B15 cells. In contrast, neither LAQ824 nor TSA significantly influenced cell cycle progression of TMD-5 cells (Figure 2A, Table 1).

Taken together these data indicate that the hydroxamic acid derived HDAC inhibitors, LAQ824 and TSA, are able to induce a G0/G1 block in lymphoblastic cells, although this effect seems to be cell type specific.

The induction of $p21^{WAF1/Cip1}$ and the resulting blockage of cell cycle progression are critical for the anti-tumor activities of all HDAC inhibitors.[28-31] Therefore we treated the different lymphoblastic cell lines with LAQ824 (10 and 50nM) and TSA (100nM) for 6h. The expression of $p21^{WAF1/Cip1}$ was studied by Western blot analysis. As shown in Figure 2B expression of $p21^{WAF1/Cip1}$ was induced after 6h of treatment with LAQ824 or TSA. The induction of $p21^{WAF1/Cip1}$ by LAQ824 was dose-dependent.

Another key player in regulation of cell cycle pro-

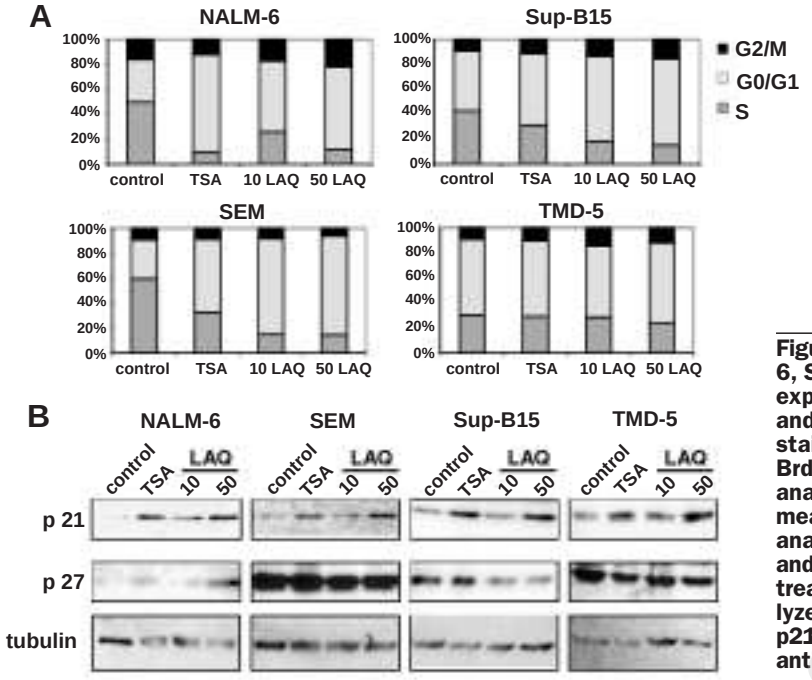

Figure 2. A Cell cycle analysis of NALM-6, SEM, Sup-B15 and TMD-5 following exposure to LAQ824 (10 and 50 nM) and TSA (100 nM). The cells were stained after 16h of treatment with BrdU and PI, and analyzed by FACS analysis. The bar graphs show the means for 3 experiments of cell cycle analysis. B. Expression of $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ after 6h of HDAC inhibitor treatment. Total cell lysates were analyzed by Western blots using anti-$p21^{WAF1/Cip1}$, anti-$p27^{Kip1}$ and anti-β-tubulin antibody.

gression is $p27^{Kip1}$.[32] We, therefore, analyzed concomitant changes in $p27^{Kip1}$ expression in NALM-6, SEM, Sup-B15 and TMD-5. As depicted in Figure 2B, $p27^{Kip1}$ was constitutively expressed in all tested cell lines. Neither LAQ824 nor TSA induced alterations in the $p27^{Kip1}$ expression levels, even after 24 h (Figure 2B and *data not shown*). These data suggest that the LAQ824-mediated cell cycle arrest involves induction of $p21^{WAF1/Cip1}$ but not of $p27^{Kip1}$.

LAQ824 induces apoptosis in pre B lymphoblasts

We next investigated whether the LAQ824-related cell cycle block leads to apoptosis. The rate of apoptosis induced by 10 and 50nM of LAQ824 and 100nM of TSA was assessed by annexin V staining and subsequent FACS analysis. With the exception of in NALM-6 cells, 10nM LAQ824 induced a high rate of apoptosis after 24h in all cell lines. Treatment of NALM-6 cells with 50nM LAQ824 achieved a level of apoptosis corresponding to the effect of 100nM of TSA. About 51% of the SEM cells were annexin V positive after 24h exposure to 10nM LAQ824. With 50nM of LAQ824 the rate of apoptosis in SEM cells could be increased to 89% (Figure 3). In summary, these results suggest that LAQ824 is a potent elicitor of apoptosis in lymphoblastic cell lines and it exhibits its activity at a lower molarity than TSA.

LAQ824 induced apoptosis is associated with caspase-9 and -3 activation

To address whether LAQ824-induced apoptosis is mediated by caspase activitation we investigated the cleavage of PARP, a major substrate of executioner caspases, including caspase-3. Thus we treated the above described cell lines with LAQ824 (10 and 50nM) and TSA (100nM) for 24h. Cleavage of endogenous PARP was observed after 24h of treatment with 10nM and 50nM of LAQ824 and 100nM TSA in NALM-6, SEM and TMD-5 cells (Figure 4). PARP cleavage was observed

Table 1. HDAC inhibitors induce G0/G1 and/or G2/M arrest in lymphoblastic cells.

	% cells											
	NALM-6			*SEM*			*Sup-B15*			*TMD-5*		
	G1	*S*	*G2/M*	*G1*	*S*	*G2/M*	*G1*	*S*	*G2/M*	*G1*	*S*	*G2/M*
control	33.0±5.4	48.3±7.1	15.9±0.5	30.8± 3.4	58.4± 8.0	9.4±3.8	47.1±8.8	42.1±14.2	9.4±0.5	63.4±5.2	29.3±2.6	7.0±2.4
TSA	71.6±7.5	9.0±0.2	11.3±0.1	56.0± 7.3	31.0±10.5	8.6±2.2	56.7±3.4	30.7±5.4	11.6±2.8	57.4±3.5	27.4±3.6	10.0±1.4
10 LAQ	54.7±4.8	24.8±6.5	17.9±2.1	74.1±15.7	14.3± 2.0	5.1±4.8	67.4±3.7	17.6±4.4	13.5±3.9	55.3±5.5	28.0±1.2	14.3±4.2
50 LAQ	62.1±0.8	11.3±4.7	21.2±0.1	76.3±10.9	14.0± 8.1	6.2±4.0	73.4±12.5	14.9±7.5	9.8±7.6	61.2±7.2	23.3±3.1	12.1±4.0

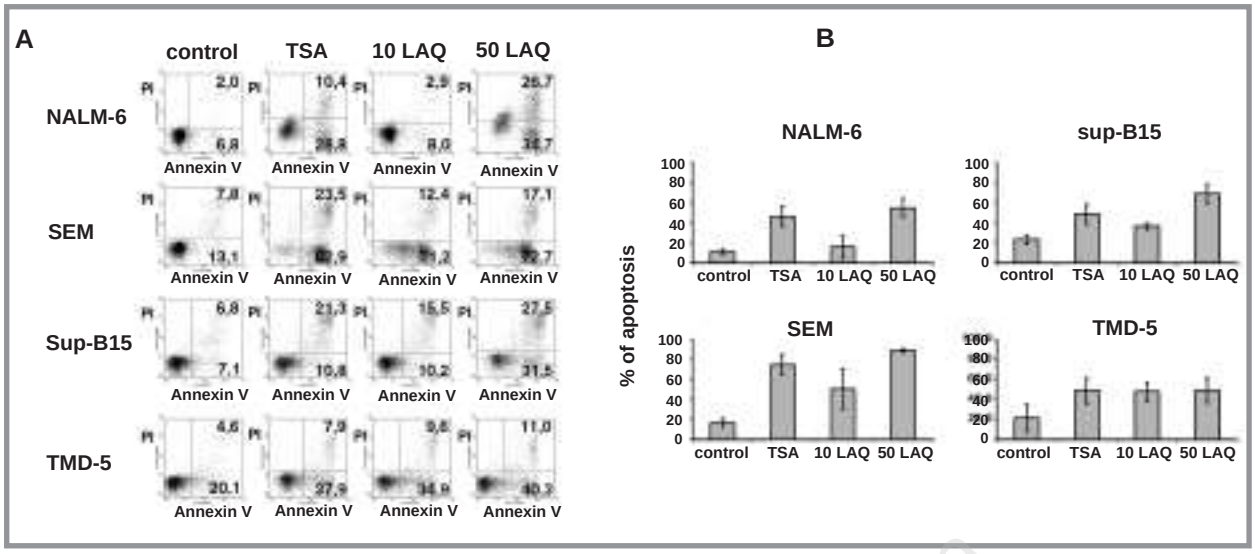

Figure 3. Induction of apoptosis in ALL cells treated with LAQ824 (10 and 50 nM) or TSA (100 nM). Apoptosis in these cells was detected by annexin V/PI assay after treatment with or without these HDAC inhibitors for 24h. A. Cells that were annexin V positive and PI negative were in early stage of apoptosis (lower right quadrant), whereas cells that were both annexin V positive and PI positive were in the late state of apoptosis or necrosis (upper right quadrant). Panels show representative FACS blots after 24h of exposure to HDAC inhibitor. The figure shows one representative experiment of three which gave nearly identical results. B. The bar graphs show the means of annexin V positive cells for 3 independent experiments with standard deviation (+/-).

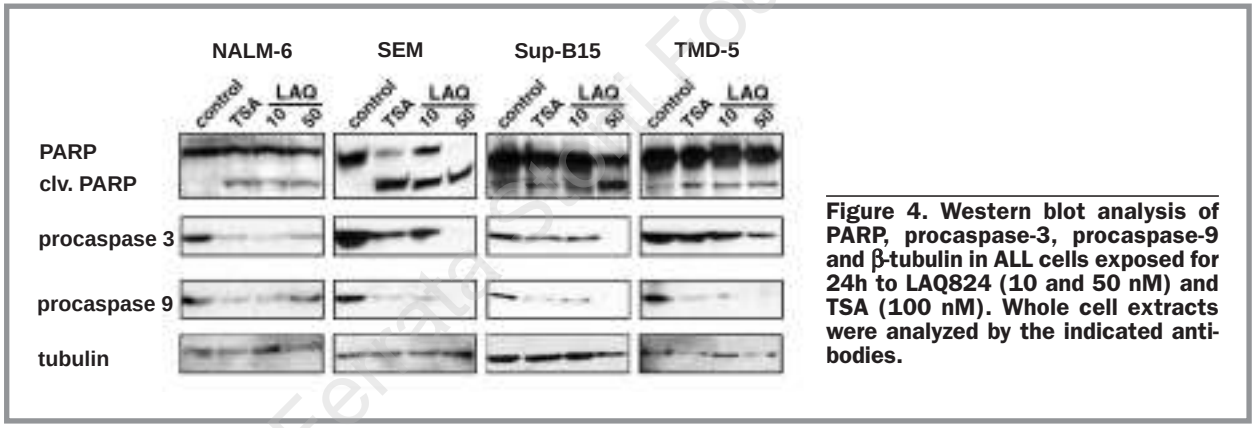

Figure 4. Western blot analysis of PARP, procaspase-3, procaspase-9 and β-tubulin in ALL cells exposed for 24h to LAQ824 (10 and 50 nM) and TSA (100 nM). Whole cell extracts were analyzed by the indicated antibodies.

in Sup-B15 cells only when these were exposed to 50nM LAQ824 or to TSA. These data show that activation of effector caspases is involved in LAQ824-induced apoptosis. Activation of caspases is organized as a cascade with upstream (initiator) caspases responsible for activating the downstream (effector) caspases. The downstream caspases are activated by proteolytic cleavage by upstream caspases.[33] To answer the question which caspases are activated by LAQ824 we studied the levels of procaspase-3 and procaspase-9 in all four ALL cell lines. Exposure to LAQ824 (10 and 50 nM) and TSA (100 nM) led to a considerable reduction of the procaspase-3 signal in all cell lines. The procaspase-9 signal was completely abolished by treatment with LAQ824 already at a concentration of 10 nM (Figure 4).

In addition we investigated the activation of the *extrinsic* apoptotic pathway through the determination of caspase 8-cleavage. In our experiments we did not detect an activation of caspase 8 by TSA or LAQ824 (*data not shown*). To determine the significance of LAQ824-induced caspase activation we analyzed apoptosis by annexin V staining under exposure to the broad range caspase inhibitor *z*VAD-fmk, which inhibits caspases-3, -7, -8 and -9. Interestingly, the effect of *z*VAD-fmk on LAQ824-induced apoptosis was cell type-specific. In NALM-6 cells LAQ824-induced apoptosis was completely inhibited by *z*VAD-fmk, whereas in the t(9;22) positive TMD-5 and Sup-B15 cells only partial inhibition was seen (Figure 5). *z*VAD-fmk was unable to inhibit LAQ824- or TSA-induced apoptosis in SEM cells. Taken together these data indicate that activation of caspases-3 and -9 is at least partially dispensable for LAQ824- and TSA-induced apoptosis.

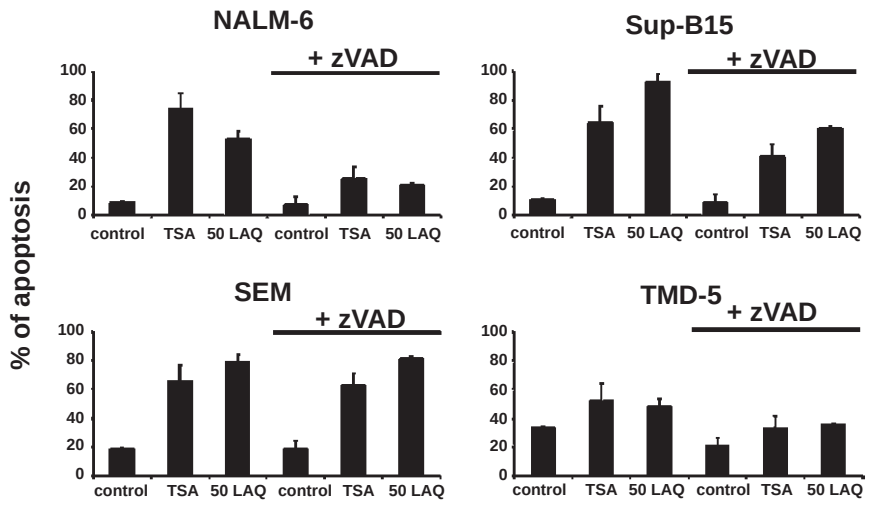

Figure 5 [left]. Inhibition of LAQ824- and TSA-induced apoptosis by the pan-caspase inhibitor zVAD-fmk in ALL cells. Cells were treated with LAQ824 (50 nM) and TSA (100 nM) for 24h. In some wells cells were pretreated with zVAD-fmk for 1h. Cells were stained with annexin V and analyzed by flow cytometry. The percentage of annexin V positive cells is presented and data are means of 3 independent experiments with standard deviation (+/-).

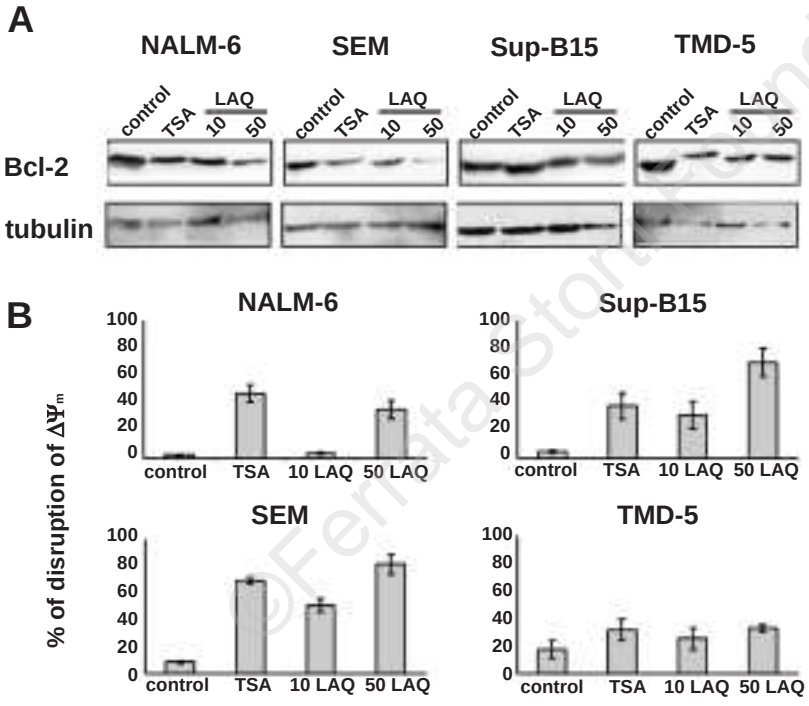

Figure 6. A Western blot analysis of Bcl-2 in ALL cells exposed for 24h to LAQ824 (10 and 50nM) or TSA (100nM). Total cell lysates were analyzed by Western blots using anti-Bcl-2 and anti-β-tubulin antibody. B. Treatment with HDAC inhibitor (10 and 50 nM LAQ824 and 100 nM TSA) affect disruption of $\Delta\psi_m$. The percentage of cells exhibiting disruption of $\Delta\psi_m$ over time was assessed by FACS. The values at 24h are summarized in the diagram. The values are means of 3 independent experiments with standard deviation (+/-).

Treatment with LAQ824 leads to down-regulation of Bcl-2 and disruption of the mitochondrial membrane potential ($\Delta\psi m$)

Activation of caspase-9 upon exposure to LAQ824 or to TSA, and the fact that caspase-9 is cleaved as part of the apoptosome suggests that the apoptosis induced by HDAC inhibitors requires disruption of the mitochondrial membrane potential ($\Delta\psi m$). The down-regulation of Bcl-2 is critical for the disruption of the $\Delta\psi m$ in many cell types. Investigating the Bcl-2 expression after 24h of treatment with LAQ824 (10 and 50nM) or TSA (100nM) we found that the level of Bcl-2 was decreased in all cell lines (Figure 6A) even in the presence of zVAD-fmk (*data not shown*). To evaluate whether the down-regulation of Bcl-2 by LAQ824 and TSA leads to premature and/or enhanced loss of $\Delta\psi m$ the shift in fluorescence of JC-1, which indicates a disruption of $\Delta\psi m$, was assessed by FACS analysis after incubation for 16h and 24h with 10nM or 50nM LAQ824 or 100nM TSA. After 16h of exposure all cell lines except for TMD-5 cells exhibited a considerable change of $\Delta\psi m$ upon treatment with 50 nM of LAQ824 or TSA; 10 nM LAQ824 was unable to induce a significant change of $\Delta\psi m$ in NALM-6 and TMD-5 cells. In contrast, SEM and Sup-B15

cells exhibited significant alterations in $\Delta\psi m$ after treatment with 10nM LAQ824 and this effect was further amplified after 24h (Figure 6B). Taken together these data clearly show that apoptosis induced by LAQ824 involves mitochondria-associated signaling pathways.

Discussion

Several structurally diverse HDAC inhibitors have been identified. Their activity in transformed cells *in vitro* makes them promising agents for anti-tumor therapy.[9,20,34] However, most HDAC inhibitors (e.g. TSA, SAHA, trapoxin, butyrates) are of limited therapeutic value due to their poor bioavailability *in vivo*[35] as well as their presumed toxic side effects.[36-38]

In the present study, we examined the *in vitro* effects of the novel HDAC inhibitor LAQ824 on ALL-derived cell lines. LAQ824 inhibits intracellular HDAC activity, inducing an accumulation of acetylated histone species within 4h. When compared to TSA, a ten-fold lower molarity of LAQ824 was sufficient to induce an identical extent of histone acetylation.

At the dosage necessary for histone hyperacetylation LAQ824 inhibited proliferation in three of the four tested cell lines, and only NALM-6 cells needed 50nM of LAQ824 for the same effect. It is noteworthy that the three cell lines which responded already to 10nM of LAQ824 harbor t(4;11) or t(9;22), both translocations associated with a poor prognosis. The growth inhibition induced by LAQ824 was associated in particular with a G0/G1 phase arrest exception in the t(9;22) positive TMD-5 cell line. In fact, 10nM of LAQ824 induced a G0/G1 arrest in NALM-6 cells to the same extent as in SEM and in Sup-B15 cells. Furthermore we demonstrated that treatment with LAQ824 leads to increased levels of the cell cycle inhibitor $p21^{WAF1/Cip1}$ in all tested cells which is considered to be associated with a G1 block in cell cycle progression,[30,39-42] whereas expression of $p27^{Kip1}$ was not modified by either LAQ824 or TSA. The lack of cell cycle arrest in TMD-5 cells, despite there being an up-regulation of $p21^{WAF1/Cip1}$ in the presence of the HDAC inhibitor, indicates that cell cycle regulation in these cells may be uncoupled from $p21^{WAF1/Cip1}$ expression and that the induction of apoptosis is only due to a down-regulation of Bcl-2.

We demonstrated that LAQ824 induces a considerable rate of apoptosis at 24h in all tested cell lines. The majority of cells blocked in G0/G1 most likely subsequently undergo apoptosis. We thus outline the major steps within programmed cell death induced by LAQ824 as being down-regulation of Bcl-2, disruption of $\Delta\psi m$, PARP-cleavage and concomitant activation of caspase-9 and -3. Moreover we show that LAQ824 induces apoptosis in cell lines harboring translocations, which define high risk ALL patients in the clinical setting. The t(4;11) positive SEM cells exhibit a yet greater responsiveness to LAQ824 than do NALM-6 cells or the t(9;22) positive Sup-B15 and TMD-5 cells. It is noteworthy that in the SEM cells, activation of caspases-3 and -9 is dispensable for LAQ824-induced apoptosis. The t(9;22) positive Sup-B15 and TMD-5 also undergo LAQ824-induced apoptosis in the presence of the caspase inhibitor zVAD-fmk. These findings are in accordance with data recently published by Peart[43] on T-cell leukemia cells treated with the HDAC inhibitors SAHA, depsipeptide and oxamflatin. Exposure of these cells to zVAD-fmk did not significantly reduce the rate of apoptosis upon treatment with HDAC inhibitors.[43] This was attributed to the fact that the HDAC inhibitors may induce a novel transcription dependent apoptosis signaling pathway, which leads to disruption of $\Delta\psi m$ related to the cleavage of Bid. This novel apoptosis signaling pathway was mitochondria-mediated as indicated by the fact that it was susceptible to inhibition by up-regulation of Bcl-2.[43] Cell type specificity of the effect of zVAD-fmk indicates that the presence of the t(4;11) or t(9;22) translocation may interfere with apoptosis induction. A loss of $\Delta\psi m$ and caspase activation are not necessarily linked.[43] In fact we found a relationship between loss of $\Delta\psi m$ and caspase activation only for NALM-6, SEM and Sup-B15 but not for TMD-5. This could explain our finding that LAQ824 induces cell cycle arrest in NALM-6, SEM and Sup-B15 but not in TMD-5 cells.

In summary, we demonstrated that LAQ824 is a novel HDAC inhibitor with significant anti-leukemia activity *in vitro*. The anti-tumor activity correlates well with its ability to arrest the cell cycle and to induce apoptosis even in cells derived from high risk ALL. Collectively, these findings generate the rationale to investigate the clinical efficacy of LAQ824 in the treatment of ALL.

AR: implemented the study, coordinated the experiments, performed the analyses of cell cycle and apoptosis and drafted the manuscript; BB: performed the experiments for caspase activation and determination of apopotosis; GB: analyzed $p21^{waf1/Cip1}$ and $p27^{Kip1}$ expression; HP: performed the analysis for acetylate histone 3 and histone 4; HG: performed the Bcl-2 analysis; SR: developed and synthesized the novel HDAC inhibitor LAQ824; DH: overall planning of the experiments and critical reviewing of the paper; PA: validated the novel substance LAQ824; MR: designed the experimental approach, supervised the experiments on mitochondrial membrane potential, critically reviewed the dta analysis and participated in preparation of the manuscript; OGO: conceived and designed the study, criticall reviewed the report and had primarily responsibility for the implementation of the study.

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