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ORIGINAL ARTICLE

Apoptosis resistance, mitotic catastrophe, and loss of ploidy control in Burkitt lymphoma

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Abstract
Resistance to cell death is the major cause of chemotherapy failure in most kinds of cancers, including Burkitt lymphoma (BL). When analyzing therapy resistance in Burkitt lymphoma (BL), we discovered a link between apoptosis resistance and ploidy control. We therefore studied systematically a panel of 15 BL lines for apoptosis induction upon treatment with microtubule inhibitors and compared three types of microtubule toxins, i.e., paclitaxel, nocodazole and vincristine. We found an inverse relationship between apoptosis sensitivity and ploidy control. Thus, cells resistant to paclitaxel- or nocodazole-induced apoptosis underwent mitotic catastrophe and developed polyploidy (>4N). Mechanistically, apoptosis resistance was linked to failure of caspase activation, which was most pronounced in cells lacking the pro-apoptotic multidomain Bcl-2 homologs Bax and Bak. Pharmacological caspase inhibition promoted polyploidy upon exposure to paclitaxel and nocodazole supporting the relationship between resistance to apoptosis and polyploidization. Of note, vincristine induced persistent mitotic arrest but no loss of ploidy control. Considering targets to facilitate Bax/Bak-independent cell death and to avoid drug-induced mitotic catastrophe and consecutive mitotic catastrophe should be of great importance to overcome therapy resistance and therapy-related events that result in ploidy changes and tumor progression.

Key message
- Inverse relation of apoptosis and polyploidy induction by paclitaxel or nocodazole in BL.
- Resistant cells undergo mitotic catastrophe and develop polyploidy.
- Lack of Bax/Bak confers resistance and leads to induction of polyploidy in BL.
- Intact apoptosis response protects from polyploidy as a result of mitotic catastrophe.

Keywords Mitotic catastrophe · Aneuploidy · Apoptosis · Bax · Bak · Caspase

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Introduction

Burkitt lymphoma (BL) is the most aggressive non-Hodgkin's lymphoma that originates from mature B cells [1]. It is generally associated with Epstein–Barr virus infection and/or c-myc chromosomal translocations leading to activation of c-myc [1, 2]. Some patients rapidly develop clinical resistance to therapy due

to the high myc-related genetic instability and selection of resistant tumor cell subclones [3]. Therefore, understanding resistance mechanisms is important for combating tumor progression in BL.

Beside deregulation of tumor suppressor and oncogenes in BL like p53 and c-myc, there is mounting evidence that acquired resistance towards cell death induction is a hallmark of most if not all types of cancers [4]. Thus, a significant number of genes involved in the regulation of apoptosis have been reported to be modified in various types of cancers. This includes reduced function of pro-apoptosis molecules (e.g., Bax [5], Bak [6–8], BH3-only proteins [9, 10], CD95/Fas, TRAIL-R1/2 [11], etc.) and increased expression of anti-apoptosis proteins (e.g., Bcl-2, Mcl-1, IAPs) [12, 13]. Acquired resistance to apoptosis is also involved in BL. For instance, p53 or Bax mutations may inhibit the apoptosis response of BL to anticancer drugs [14–16].

Another type of cell death involved in cancer, mitotic (or microtubule) catastrophe (MC) [17], refers to cell death that occurs during mitosis and has gained considerable interest [18]. There are diverse definitions of MC describing it as a special case of apoptosis [19], associated with senescence [20], while others interpret MC to be a cell survival mechanism of tumors [21]. MC is often associated with the formation of giant cells with an altered nuclear morphology, i.e., nuclear swelling, multiple nuclei, or micronuclei [22, 23]. These cells may continue to divide and become polyploid and/or aneuploid. Eventually, they may die by delayed apoptosis or necrosis. MC is particularly prevalent in cells with compromised p53 function, as p53 is a major regulator of both G1 and G2 cell cycle checkpoints [22–26]. The implications of MC and ploidy control in development of resistance in cancer, especially during therapy-related events, remain nevertheless enigmatic.

Combination chemotherapy with vincristine-containing regimens is the treatment of choice in BL [27]. Here, we analyzed the molecular mechanisms linking apoptosis and mitotic catastrophe in BL upon treatment with the different types of mitotic spindle toxins, i.e., vincristine and paclitaxel as irreversible inhibitors of microtubule degradation, and a reversible inhibitor of tubulin polymerization, nocodazole. A comparison of BL cell lines showed an inverse relationship between apoptosis and polyploidy induction upon treatment with either paclitaxel or nocodazole. Cells resistant to apoptosis, e.g., in consequence of loss of Bax and Bak, showed a prolonged mitotic arrest with a >4N DNA content following exposure to paclitaxel or nocodazole. In analogy, inhibition of apoptosis by caspase inhibitors promoted polyploidy confirming the inverse relationship between apoptosis and polyploidization. This suggests that an intact apoptosis response protects from ploidy changes in consequence of mitotic catastrophe. Surprisingly, vincristine treatment did not trigger polyploidy indicating that mechanisms involved in vincristine-induced cell death differ from those triggered by paclitaxel or nocodazole.

Materials and methods

Cell culture

Human Burkitt lymphoma cell lines (BL) and mouse factor-dependent myeloid cells (FDM) were cultured in RPMI 1640 supplemented with 10 % FBS (Life Technologies Inc) as described [28]. For FDM cells, IL-3 was added to the culture medium [29]. Paclitaxel (Bristrol-Myers Squibb), vincristine (GRY-Pharma), and nocodazole (Sigma-Aldrich) were used at 100 nM unless otherwise stated.

Cell viability assays

Viability and cell membrane integrity were determined by propidium iodide (PI) uptake. PI uptake was detected by flow cytometric analysis of cells in the FL3 channel of a FACScan (Becton Dickinson) using CellQuest software. Viability was also determined by an XTT assay using the Cell Proliferation Kit II (Roche Molecular Biochemicals).

DNA fragmentation and cell cycle analysis

Cells were processed as described [7], and genomic DNA fragmentation and polyploidy were determined by flow cytometric relative quantification of cellular DNA. To this end, fixed and permeabilized cells were RNAse treated, resuspended in PI containing PBS, and analyzed in the FL3 channel of a FACScan cytometer (Becton Dickinson) using CellQuest software. Data are given in percentage hypodiploid cells (sub G1) and percentage of hyperploid cells (>4N DNA content), respectively. Alternatively, apoptotic cell death was determined by Annexin-V-FITC and PI staining followed by flow cytometric analysis [6].

Determination of mitotic index

Cells were labeled by use of a MPM2 antibody (Upstate Biotechnology) followed by FITC-labeled goat-anti-mouse antibody (Jackson ImmunoResearch) as described [26]. Mitotic cells distinct from cells in G2 phase were identified by flow cytometry as MPM2-positive events with 4N DNA content.

Caspase activation and inhibition

Cells were labeled with the cell permeable caspase-3/7-like inhibitor FAM-DEVD-fmk to detect active caspase-3/7-like caspases (DEVDases). Caspase-9-like activities (LEHDases) were detected by using FAM-labeled LEHD-fmk

(Immunochemistry Technologies) followed by flow cytometric detection of cells with green fluorescence resulting from irreversible binding of FAM-DEVD-fmk or FAM-LEHD-fmk to active caspases. For inhibition of pan-caspase activity, cells were incubated with 10 μM cell-permeable, irreversible, broad-spectrum caspase inhibitor Q-VD-Oph (Calbiochem) 2 h prior to drug treatment with microtubule inhibitors.

Antibodies and Western blot analysis

Rabbit anti-Mcl-1 (H-260) was from Santa Cruz Biotechnology. Polyclonal mouse anti-p53 was from BD Biosciences. Polyclonal goat anti-human caspase-3 and goat anti-caspase-9 were from R&D Systems. The polyclonal rabbit anti-Bak antibody was from Sigma-Aldrich, monoclonal mouse anti-Bax from Upstate. Mouse anti-human caspase-8 was from Alexis and polyclonal anti-human actin was from Sigma-Aldrich. Secondary anti-rabbit, anti-goat, and anti-mouse HRP-conjugated antibodies were from Promega or Southern Biotechnology. Western blot analysis was performed as described [6]. Equal amounts of protein (20 μg per lane) were separated by SDS-PAGE. Following immunoblotting, antibody labeling, and washing, visualization of the proteins was performed by ECL.

Measurement of mitochondrial permeability transition

Cells incubated 30 min in the presence of JC-1 (Molecular Probes, Leaden, Netherlands) and mitochondrial permeability transition was then assessed by flow cytometric detection of cells with increased green fluorescence, i.e., cells with mitochondria displaying a reduced membrane potential ($\Delta\Psi_m$) as described [30].

Results

Inverse relationship between apoptosis and polyploidy induction in Burkitt lymphoma

To understand the mechanism of cell death induced by anticancer drugs in Burkitt lymphoma (BL), 15 BL cell lines were investigated for their sensitivity to apoptosis upon treatment with microtubule inhibitors (Fig. 1). To determine percentages of apoptotic cells, genomic DNA fragmentation was analyzed by flow cytometry following exposure to paclitaxel (Fig. 1a, upper panel). There, we observed an inverse correlation between induction of apoptosis and development of hypertetraploid (>4N) cells upon exposure to paclitaxel (Fig. 1a, lower panel). Thus, resistance to apoptosis coincided with increasing numbers of cells with a >4N DNA content. Hypertetraploid cells showed characteristics of mitotic catastrophe, i.e., nuclear swelling and increase in cell size as described [23]. Similarly to paclitaxel, nocodazole induced

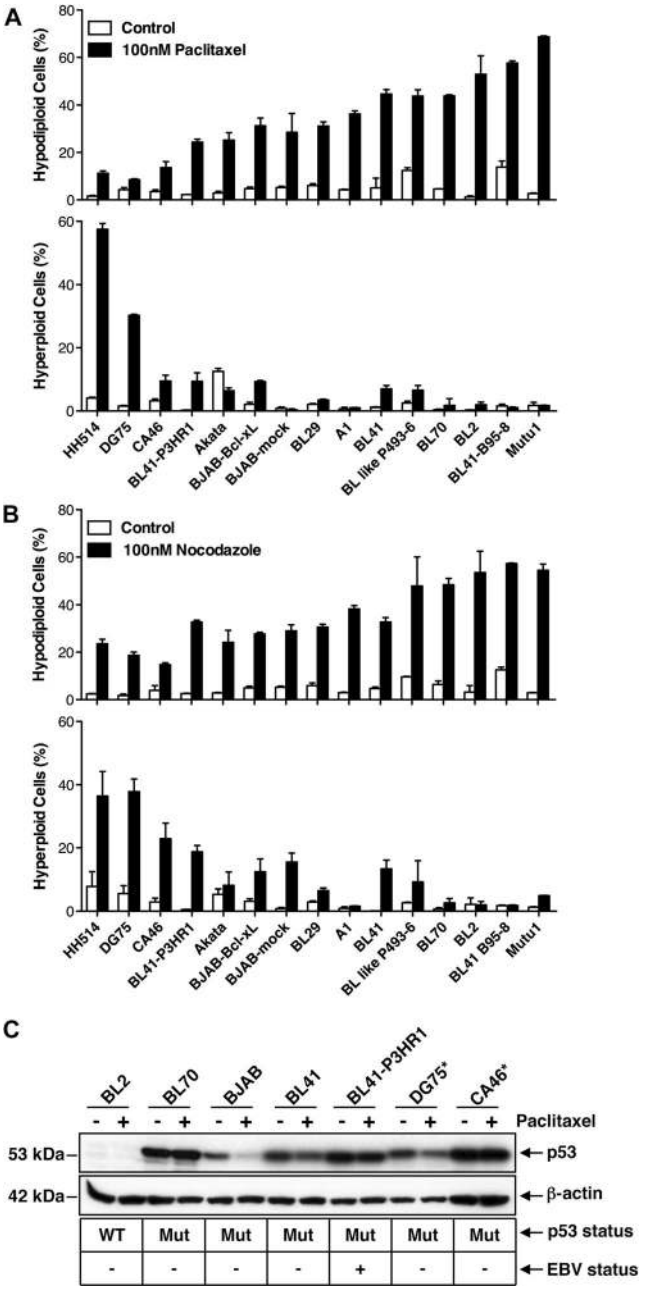

Fig. 1 Paclitaxel- and nocodazole-treated BL cells show an inverse relationship between induction of apoptosis or hyperploidy. Fifteen different BL cell lines were cultured for 72 h in presence or absence of 100 nM paclitaxel (**a**) or nocodazole (**b**) and subjected to flow cytometric analysis of DNA content. Cells with sub-G1, hypodiploid (<2N) DNA content were considered apoptotic (**a**, **b**, upper panels). Cells showing hypertetraploid (>4N) DNA content were considered polyploid (**a**, **b**, lower panels). The data show the means±s.d. of triplicates from a single experiment, representative of two independent experiments giving similar results. **c** Western blot analysis in the absence or presence of paclitaxel. The table below indicates the p53 genotype (*WT*, wild type; *Mut*, mutant) and the EBV status (+, EBV-positive BL41 cells were infected with EBV strain P3HR1). *Asterisk* indicates resistance to paclitaxel

varied percentages of apoptosis and polyploidy in different cell lines, again with an inverse relationship (Fig. 1b). This shows that cells resistant to paclitaxel- or nocodazole-induced apoptosis are prone to develop polyploidy. Paclitaxel- and nocodazole-induced polyploidy increased over time. Measurement of DNA content 3, 6, and 12 days post treatment revealed formation of cells with 8N, 16N, and 32N (and up to 64N for nocodazole), respectively (Fig. S1). To study the mechanism underlying the inverse relationship between induction of apoptosis and polyploidy, apoptosis-sensitive cell lines, BL2, BL70, BJAB, and BL41 encompassing cell lines with high and moderate apoptosis sensitivity were selected.

To corroborate that cell death of these sensitive cell lines occurs by apoptosis and to confirm resistance of DG75* and CA46* cells, cells stained with PI/annexin-V-FITC 24 and 48 h after paclitaxel treatment were analyzed flow cytometrically. As in the case of DNA fragmentation analysis, paclitaxel induced apoptosis in BL2, BL70, BJAB, and BL41 cells indicated by the detection of cells positive for annexin V-FITC (phosphatidylserine exposure) and negative for PI staining (intact plasma membrane), i.e., early apoptotic, and detection of annexin V/PI positive (late apoptotic) cells (Fig. S2). In contrast, annexin-V-FITC-positive cells were hardly detectable in samples of paclitaxel-treated DG75* and CA46* cells, confirming resistance of these two cell lines to paclitaxel-induced apoptosis.

To address the impact of the EBV status, BL41 infected with EBV strain P3HR1 were included in view of the impact of EBV on G2/M restriction point control in p53-deficient cells. DG75* and CA46* were included as cell lines with prototypic resistance to apoptosis induced by microtubule inhibitors. There was, however, no correlation between susceptibility to microtubule inhibitors, induction of polyploidy, and EBV or p53 mutation status (Fig. 1c). For better distinction, apoptosis-resistant lines are depicted with an asterisk throughout the manuscript. Neither the apoptosis-sensitive cell line BL2 carrying a p53 wild type nor the p53-mutant cells showed induction of p53 expression upon paclitaxel treatment (Fig. 1c). Moreover, cell lines with mutated p53 displayed either sensitivity or resistance to paclitaxel-induced apoptosis. These data are supported by earlier findings indicating that p53 is not involved in paclitaxel-induced apoptosis in BL [28, 30].

Vincristine treatment does not induce hyperploidy in Burkitt lymphoma cells

To further address this phenomenon, we included the vinca alkaloid vincristine, a "microtubule destabilizer" commonly used as chemotherapeutic drug for treatment of BL [31]. Cells sensitive to paclitaxel also displayed sensitivity to vincristine-induced apoptosis (Fig. 2a). In the same vein, the paclitaxel-resistant cell lines DG75* and CA46* were also resistant to vincristine-induced cell death. Analysis of cell cycle distribution upon paclitaxel, nocodazole, or vincristine treatment showed considerable accumulation of cells in the G2/M phase (4N) of the cell cycle for all cell lines (data not shown).

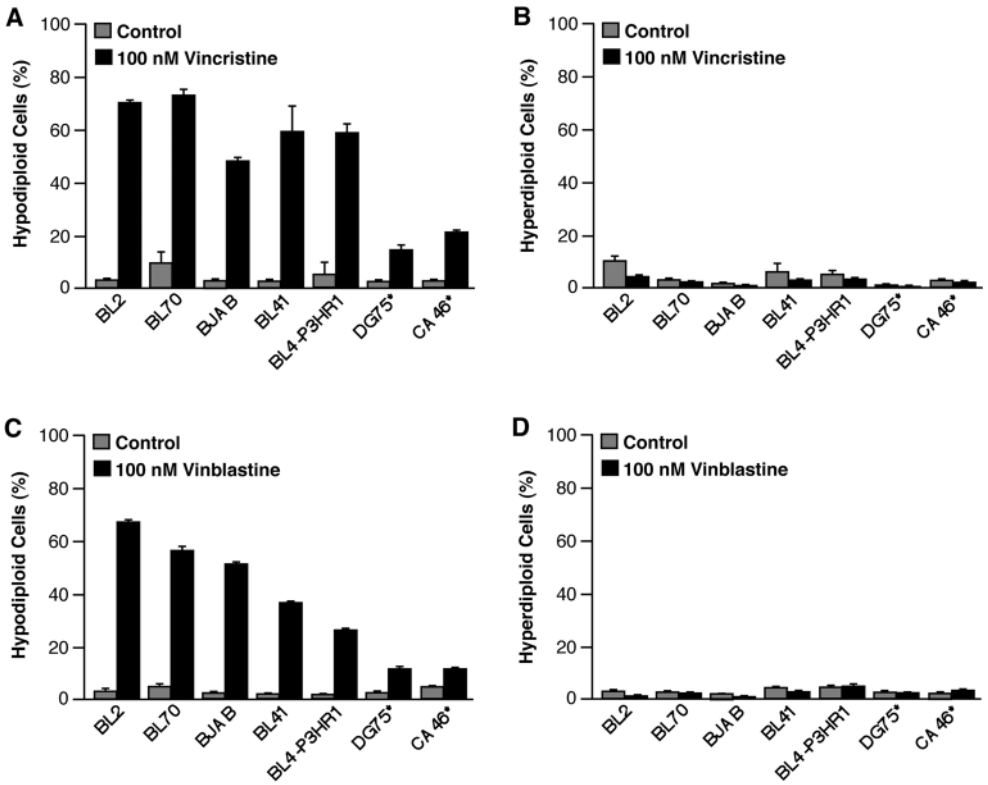

Fig. 2 Vincristine and vinblastine do not induce hyperploidy in apoptosis-resistant BL cells. BL2, BL70, BJAB-WT, BL41, and BL41-P3HR1 BL41 cells and two cell lines resistant to paclitaxel-induced apoptosis, DG75* and CA46*, were treated with 100 nM vincristine (**a**, **b**) or 100 nM vinblastine (**c**, **d**) for 72 h and subjected to flow cytometric analysis of cellular DNA content. **a**, **c** Cells with sub-G1, hypodiploid (<2N) DNA content were considered apoptotic. **b**, **d** Cells showing hypertetraploid (>4N) DNA content were considered polyploid. The data show the means±s.d. of triplicates from a single experiment, representative of three independent experiments giving similar results. *Asterisk* indicates resistance to paclitaxel

Surprisingly, and in contrast to paclitaxel or nocodazole treatment, accumulation of 4N cells upon treatment with 100 nM vincristine did not coincide with hyperploidy induction (Fig. 2b). To confirm that vinca alkaloids do not induce hyperploidy in apoptosis-resistant BL cells, we treated BL cells also with 100 nM vinblastine for 72 h. Vinblastine induced cell death in the sensitive BL cell lines but not in the resistant cell lines DG75* and CA46* (Fig. 2c). Similar to vincristine and in contrast to paclitaxel and nocodazole 100 nM vinblastine did not induce hyperploidy (Fig. 2d). This indicates a different mechanism of cell death induction by vinca alkaloids that may protect cells from developing hyperploidy upon targeting mitotic checkpoint control.

Microtubule inhibitors facilitate endomitosis in apoptosis-resistant cells

Next, we asked if the enhanced ploidy is a result of endomitosis, a form of DNA endoreplication in which cells undergo certain aspects of mitosis but aberrant cytokinesis leads to an increase in the ploidy level [22, 32]. To examine this possibility, the mitotic cell population, positive for MPM2 (Mitosis-specific Phospho-epitope and Mitotic marker antibody 2 [33] that recognizes mitotic phospho-Ser/Thr-Pro epitopes), was quantified by flow cytometry. Treatment with microtubule inhibitors induced enhanced numbers of MPM2-positive cells in all BL cell lines (Fig. 3a) indicating that accumulation of cells in the mitotic phase is independent of apoptosis sensitivity.

At 24 h, microtubule inhibitor treatment had no effect on hyperploidy in the MPM2-positive population (Fig. 3b). Intriguingly, apoptosis-sensitive cell lines did not show mitotic accumulation at prolonged time points (Fig. 3c). At 72 h, their mitotic index was less than 10 %, which was comparable to untreated control cells (Fig. 3c) indicating that mitotic arrest is followed by apoptosis, presumably out of M-phase. In contrast, the apoptosis-resistant DG75* and CA46* cells prevailed in mitotic arrest (Fig. 3c) and this coincided with MPM2-positive (mitotic) cells displaying polyploidy (Fig. 3d). The EBV status of BL41-P3HR1 facilitated both mitotic arrest and, to a lesser extent, hyperploidy of MPM-2-positive cells (Fig. 3c, d). Induction of polyploidy did not coincide with multinucleation (as observed, e.g., in Reed-Sternberg cells of Hodgkin's lymphoma) as evidenced by assessing nuclear morphologies as described [22, 23] supporting the notion of mitotic catastrophe. Cells resistant to microtubule inhibitors nocodazole and paclitaxel prevail in mitosis but undergo endomitosis leading to an increased number of polyploid cells. Of note, vincristine-treated DG75* and CA46* cells showed prolonged mitotic arrest for up to 72 h (Fig. 3c) but no polyploidy in the MPM2-positive compartment (Fig. 3d) even while these cells are resistant to vincristine-induced apoptosis (Fig. 2a).

We next investigated if cells that are resistant to microtubule inhibitors would undergo other means of cell death or growth inhibition. Upon treatment with microtubule inhibitors, apoptosis-sensitive cells showed enhanced PI uptake in contrast to a lack of PI uptake by resistant cell lines (Fig. 4a). This excludes death by non-apoptotic mechanisms in the apoptosis resistant lines up to 72 h post-treatment. However, inhibition of metabolic activity and proliferation, as analyzed by XTT assay, was observed in all the cell lines irrespective of their sensitivity to apoptosis induction (Fig. 4b). Further, to investigate if microtubule inhibitor-induced loss of XTT activity can be rescued, cells were washed after 72 h of incubation with microtubule inhibitor, re-cultured in drug-free medium for an additional 72 h and cell viability was again analyzed by XTT cell proliferation assay. Interestingly, cells that were rescued from microtubule inhibitor treatment did not show restoration of XTT activity (Fig. 4c) but resistant cells remained viable implying that polyploid cells may persist following exposure to paclitaxel or nocodazole.

Bcl-2 family regulation of apoptosis resistance and ploidy control

The pro-apoptotic Bcl-2 family members Bax and Bak constitute an essential apoptosis gateway to regulate the intrinsic apoptosis pathway [34]. Cells showing induction of apoptosis upon paclitaxel or nocodazole treatment were characterized by strong Bak and Bax protein expression. There was, however, no upregulation of Bax and Bak protein expression after paclitaxel treatment. In fact, Bax levels were slightly reduced upon treatment with paclitaxel (Fig. 5a). This may occur in consequence of cleavage of Bax by caspases. In contrast, DG75* and CA46* are highly resistant to treatment, lack Bax, and show very low Bak protein expression (Fig. 5a). This indicates that Bax and Bak are both critically involved in paclitaxel-induced apoptosis. Here, disturbed Bax and Bak expression is not only associated with apoptosis resistance but also facilitates polyploidy in BL upon microtubule inhibitor treatment. Further Western blot analyses revealed that all cell lines express the pro-survival protein Mcl-1 and responded with a decrease of Mcl-1 expression as a unifying feature to exposure to paclitaxel. This decrease of Mcl-1 was also observed in the apoptosis-resistant DG75* and CA46* cells. Mcl-1 down-regulation therefore may be important for apoptosis induction that occurs "upstream" of Bax/Bak and caspase activation as observed recently in a different setting of oncogene/$p14^{ARF}$-induced cell death [12]. However, and in contrast to Bax/Bak deregulation, the down-regulation of Mcl-1 is not sufficient to overcome apoptosis resistance and induction of polyploidy. In contrast to Mcl-1 expression, paclitaxel treatment does not impact on the expression level of Bcl-x_L and expression of Bcl-2 is only marginally reduced in BL41 and BL41-P3HR1 (Fig. 5a). Intriguingly, there is no correlation of sensitivity to paclitaxel and the

expression level of Bcl-x_L or Bcl-2. Bcl-x_L, e.g., is strongly expressed in the sensitive cell lines BL2, BL70, and BJAB. In contrast, expression levels were low in the resistant cell lines DG75* and CA46*. Expression of Bcl-2 was highest in BL2 and moderate in DG75* and CA46* cells. Besides anti-apoptotic Bcl-2 family proteins, we also analyzed the expression of the anti-apoptotic XIAP, which has been shown to inhibit caspase-9 and -3 activation through its function as a substrate-specific E3-ubiquitin ligase [35]. Expression of XIAP is reduced upon paclitaxel treatment, indicating a crucial role of XIAP in paclitaxel-induced apoptosis (Fig. 5a). However, as XIAP is downregulated even in DG75* and CA46* cells, deregulation of XIAP seems not to be the cause of resistance.

The critical role of Bax and Bak as gatekeepers in microtubule inhibitor induced apoptosis and development of hyperploidy was confirmed in murine hematopoietic factor-

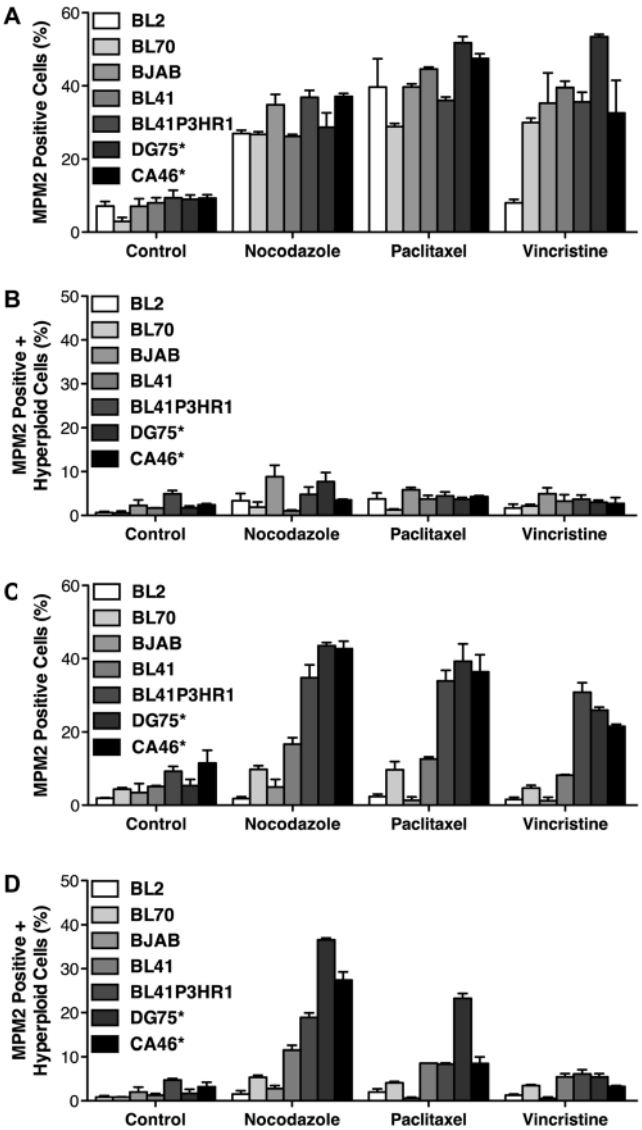

Fig. 3 Microtubule inhibitors induce prolonged mitotic arrest and trigger loss of polidy control in apoptosis-resistant BL cell lines. Cells were cultured for either 24 h (**a**, **b**) or 72 h (**c**, **d**) in presence or absence (control) of 100 nM nocodazole, paclitaxel, or vincristine. Cells were stained with a fluorescein isothiocyanate (FITC)-labeled MPM2 antibody to identify percentages of mitotic cells (**a**, **c**). Bivariate analysis of MPM2 and cellular DNA content was performed in **b** and **d** to identify percentages of mitotic, hypertetraploid (>4N) cells. The data show the means± s.d. of triplicates from a single experiment, representative of three independent experiments giving similar results in relation to the total cell population

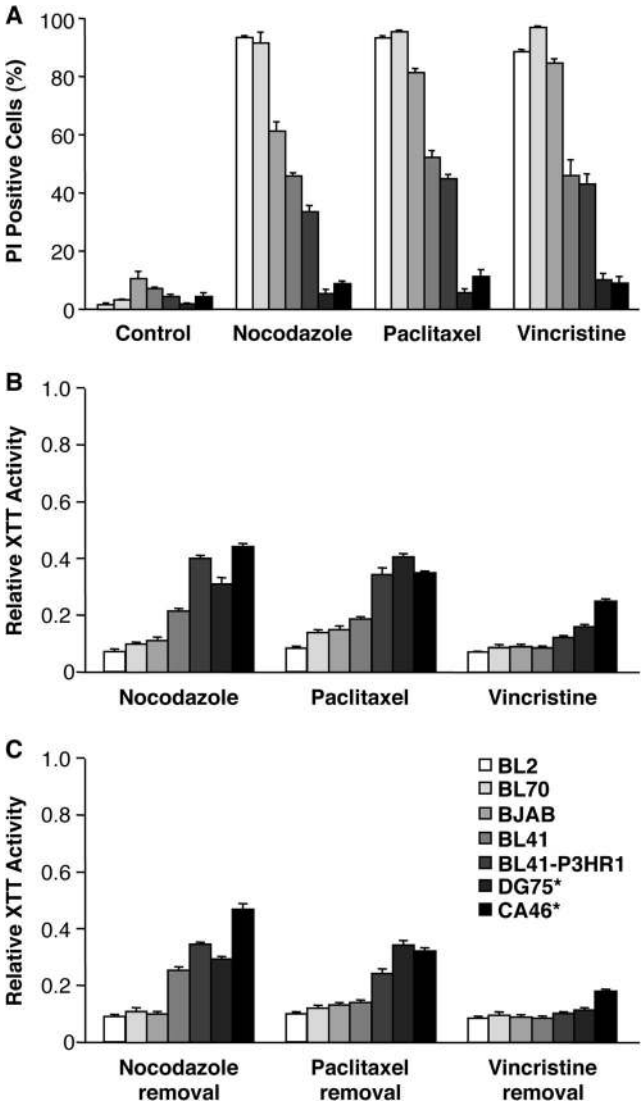

Fig. 4 Microtubule inhibitors inhibit proliferation but do not trigger non-apoptotic cell death in BL cell lines resistant to apoptosis. Cells were cultured for 72 h in presence or absence of 100 nM nocodazole, paclitaxel, or vincristine. **a** Cells were stained with propidium iodide (PI) and percentages of PI-positive (dead) cells were measured using flow cytometric analysis. **b** Cell proliferation/viability measurement by colorimetric XTT assay. **c** Washout experiments. Cells were cultured for 72 h in presence or absence of 100 nM microtubule inhibitors. Thereafter, cells were washed and re-cultured in medium without microtubule inhibitors for an additional 72 h. Cell viability was analyzed by the XTT assay. The XTT activity of treated cells is expressed relative to that of untreated cells, which were set to 1. The data show the means±s.d. of triplicates from a single experiment, representative of three independent experiments giving similar results

dependent myeloid (FDM) cells from bax, bak, or bax/bak knock-out mice. FDM-wild type (WT), FDM-*bax*$^{-/-}$, FDM-*bak*$^{-/-}$, and FDM-*bax*$^{-/-}$/*bak*$^{-/-}$ double deficient cells were treated with paclitaxel and analyzed for the correlation of apoptosis induction and polyploidy. Upon paclitaxel treatment, FDM-WT, FDM-*bax*$^{-/-}$, or FDM-*bak*$^{-/-}$ cells underwent apoptotic cell death, with more than 54 % of the cells showing a hypodiploid DNA content and no hyperploidy (Fig. 5b–d). In contrast, FDM *bax*$^{-/-}$/*bak*$^{-/-}$ cells were highly resistant to paclitaxel-induced apoptosis, with less than 5 % of cells undergoing apoptosis. In accordance with the hypothesis that loss of Bax and Bak predisposes for cell death resistance and polyploidy induction in BL, treatment of Bax/Bak-deficient FDM cells with paclitaxel was followed by the

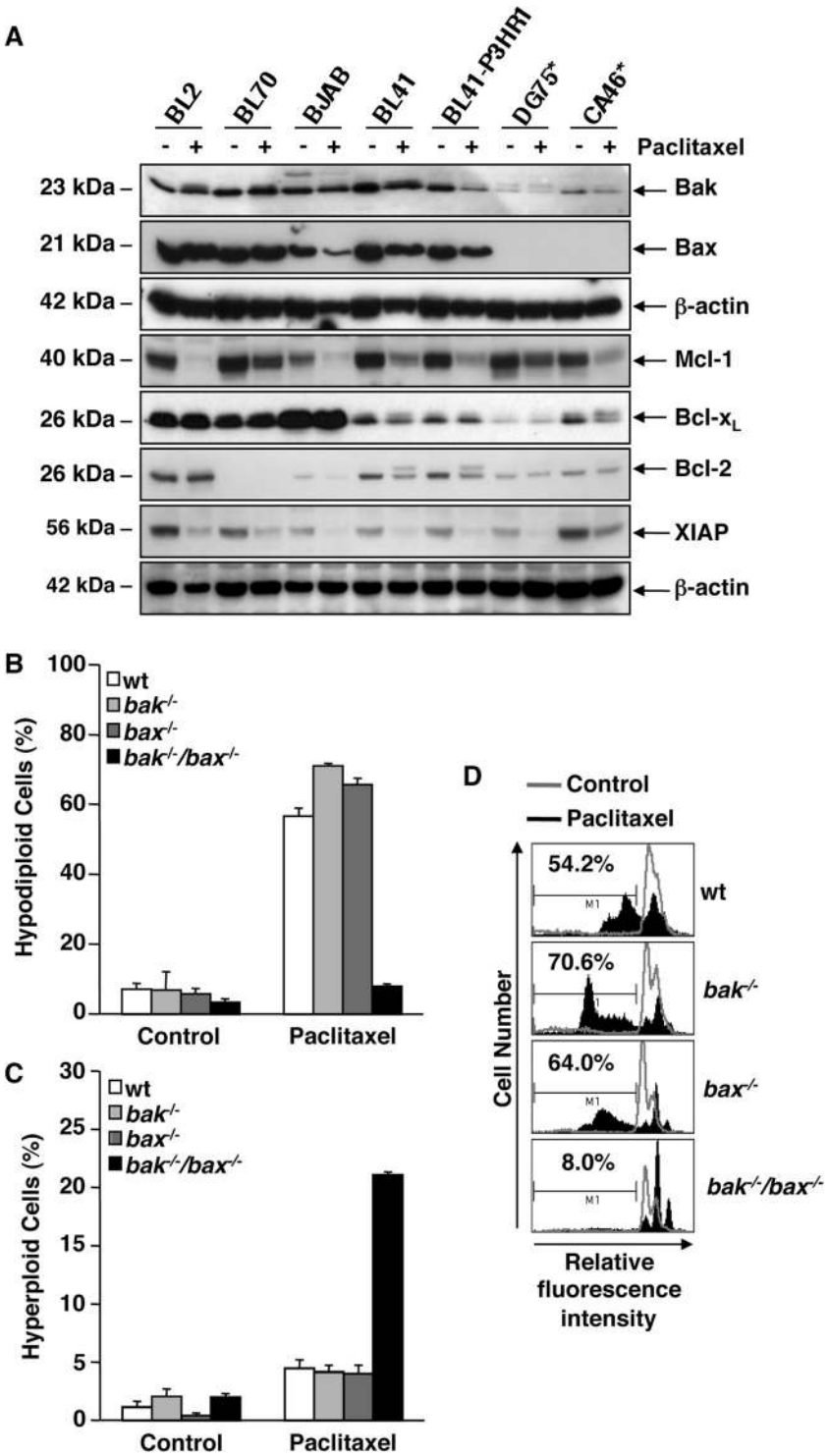

Fig. 5 Combined loss of Bax and Bak coincides with apoptosis resistance to paclitaxel and induction of polyploidy. **a** BL cell lines were cultured in the presence or absence of 100 nM paclitaxel for 48 h and lysates were subjected to immunoblot analysis for expression of pro-apoptotic Bcl-2 family members Bak and Bax (*upper panel*) and the anti-apoptotic Bcl-2 family members Mcl-1, Bcl-2, and Bcl-x_L and the anti-apoptotic XIAP (*lower panel*). β-Actin served as a loading control. Molecular masses are indicated at the left. **b**–**d** Murine FDM-WT, FDM-*bax*$^{-/-}$, FDM-*bak*$^{-/-}$, and FDM-*bax*/*bak*$^{-/-}$ cells were cultured for 72 h in the presence or absence of 100 nM paclitaxel. Measurement of DNA content revealed that double knockout (but not single knockout) of *bax* and *bak* confers resistance to paclitaxel (**b**), which is accompanied by induction of polyploidy (**c**). Representative DNA histograms are shown in **d** and depict induction of DNA fragmentation (percentages of sub-G1, apoptotic cells are shown between markers) and polyploidy in controls (*hallow gray*) or paclitaxel-treated cultures (*solid black*). *FDM*, factor-dependent myeloid cells

induction of polyploidy. After paclitaxel treatment, 22 % of the cells displayed a polyploid DNA content compared to less than 5 % of Bax- and/or Bak-proficient FDM cells (Fig. 5c, d). These results confirm the inverse correlation between apoptosis and polyploidy and the decisive impact of central apoptosis defects.

In addition to Bax, we analyzed the expression level of the BH3-only proteins Bid, Puma Bim, and Noxa. Bid, Puma, and Bim function as direct activators and therefore are crucial for Bax and Bak activation. The sensitizer BH3-only protein Noxa was chosen because Noxa specifically inactivates Mcl-1, thereby regulating Bak activity. However, as in the case of Bcl-2, Bcl-x_L, and Bcl-w, there is no correlation between paclitaxel-induced cell death and expression levels of the BH3-only proteins (Fig. S3 A).

Furthermore, knockout of the respective genes does not increase paclitaxel-induced induction of apoptosis or polyploidy in FDM cells (Fig. S3 B, C, D). These data indicate that none of the tested BH3-only proteins individually has a crucial role in paclitaxel-induced apoptosis. However, BH3-only proteins are known to have redundant and overlapping functions. Thus, we cannot exclude that a combined knockout of certain "*bh3-only* genes" would have a more sustained effect.

To further investigate involvement of Bax and Bak in paclitaxel-induced apoptosis of BL cells, we analyzed Bax and Bak activation. During apoptosis, Bax and Bak undergo a conformational change, leading to the exposure of the N-terminus that is inaccessible in vital, non-apoptotic cells. To study Bax and Bak activation, the most sensitive cell lines (BL2, BL70, and BJAB) were stained by use of conformation-specific antibodies directed against the N-terminus of Bax or Bak, respectively, and analyzed by fluorescence microscopy (Fig. 6a). Paclitaxel treatment resulted in a strong punctuated staining pattern indicating a conformational change, i.e., activation, and clustering of Bax and Bak. Furthermore, Bax and Bak activation is accompanied by breakdown of the mitochondrial membrane potential ($\Delta\Psi$m) in sensitive cell lines, as shown by staining of the cells with JC-1. JC-1 is a cationic dye that accumulates in the mitochondria and forms red fluorescent J-aggregates. Upon dissipation of $\Delta\Psi$m; however, JC-1 locates to the cytosol and fluorescence shifts from red to green. In the most sensitive cell lines, BL2, BL70, and Bjab and to a lesser extend in BL41 cells paclitaxel treatment resulted in loss of $\Delta\Psi_m$ 24 h post treatment as indicated by the increased number of cells with green fluorescence (Fig. 6b). In BL41P3HR1 cells, the onset of apoptosis is delayed and loss of $\Delta\Psi_m$ is detectable 48 h after paclitaxel treatment. In contrast, in DG75* and CA46* cells, paclitaxel does not induce breakdown of the mitochondrial membrane potential.

Caspase regulation of apoptosis resistance and ploidy control

To further address the impact of apoptosis defects on polyploidy triggered by microtubule inhibitors, caspase activation was investigated. Paclitaxel induced weak processing of caspase-8 in all apoptosis-sensitive cell lines, except for BL41-P3HR1, indicated by a faint signal of p41/p43 caspase-8 cleavage product (Fig. 7a) in accordance with our previous report on caspase-8 processing occurring secondary to caspase-3 activation [28]. Furthermore, paclitaxel induced processing of pro-caspase-9 in all sensitive cell lines (Fig. 7a). However, despite cleavage of pro-caspase-9, fragments representing active caspase-9 were only detectable in BL2, BL70, and BJAB but not in BL41 and BL41-P3HR1 cells. This pro-caspase-9 processing and activation is accompanied by processing of the effector caspase-3 to active subunits (p17). In contrast, active caspase-3 subunits were not detectable in BL41 and BL41-P3HR1.

Apoptosis-resistant cell lines did not show processing of pro-caspases-3, -8, and -9 to active subunits (Fig. 7a). For functional studies, we employed fluorescent peptide substrates binding irreversibly to the enzyme through a fluoromethylketone (fmk) moiety. Caspase-9-like activities were detected by use of a LEHD peptide (Fig. 7b) whereas caspase-3/7 activities were visualized using a DEVD peptide (Fig. 7c). Nocodazole, paclitaxel, as well as vincristine induced active caspase-9 (LEHDase) and caspase-3/7 (DEVDase) activities in all sensitive cell lines, most pronounced in BL2 and BL70 cells, and, to a diminished extent, in BL41 and BL41-P3HR1 cells. In contrast, such caspase-9-like or caspase-3/7-like activities were not detectable in DG75* and CA46*.

Polyploidy is induced by paclitaxel and nocodazole but not vincristine upon caspase inhibition

To investigate the role of caspases on a functional level, caspase activity was blocked by pre-incubation of the cells with the pan-caspase inhibitor Q-VD-OPh prior to drug exposure. Q-VD-OPh inhibited induction of apoptosis in the sensitive BL lines (Fig. 8a). As expected, there was no effect on apoptosis-resistant cell lines. Interestingly, analysis of cellular DNA DNA content revealed increased polyploidy in most of the sensitive cell lines upon the combined treatment with paclitaxel and caspase inhibitor (Fig. 8b). No further increase of ploidy was observed in BL41, BL41-P3HR1, DG75*, and CA46*, presumably in consequence of the reduced caspase activation or the loss of Bax/Bak (Figs. 5a, 7a, 8b). Consistently, nocodazole-induced apoptosis was inhibited by Q-VD-OPh (Fig. 8c) and nocodazole treatment also induced polyploidy in presence of the pan-caspase inhibitor, as observed in paclitaxel-treated cells (Fig. 8d). This indicates that paclitaxel and nocodazole induce apoptosis through an equivalent

pathway in a caspase-dependent manner and that inhibition of caspase activity facilitates polyploidization. This also confirms our observation of an inverse relationship between induction of apoptosis and polyploidy.

As vincristine did not promote polyploidy in cells resistant to apoptosis (Fig. 2), we also investigated the fate of vincristine-treated cells in the presence of Q-VD-OPh. Similar to our observation with paclitaxel or nocodazole, inhibition of caspases led to a decrease in vincristine-induced apoptosis (Fig. 8e). For example, in BJAB cells, apoptosis was reduced from 42 to 18 %, which accounts to a 57 % reduction. However, less than 3 % of cells showed polyploidization upon caspase inhibition in vincristine-treated cells (Fig. 8f), which is in sharp contrast to paclitaxel and nocodazole treatment. This further confirms our observation that vincristine may induce cell death, depending on apoptosis proficiency, but does not trigger polyploidy (and MC) in apoptosis-resistant cells.

Discussion

BL usually responds well to initial chemotherapy but is prone to rapidly develop resistance that is difficult to overcome by salvage therapies. Reasons for this impressive therapy resistance of clinically advanced disease often remains enigmatic albeit numerous resistance mechanisms have been described.

A defining feature of BL is activation of the c-myc gene at 8q24 through translocation with one of three immunoglobulin

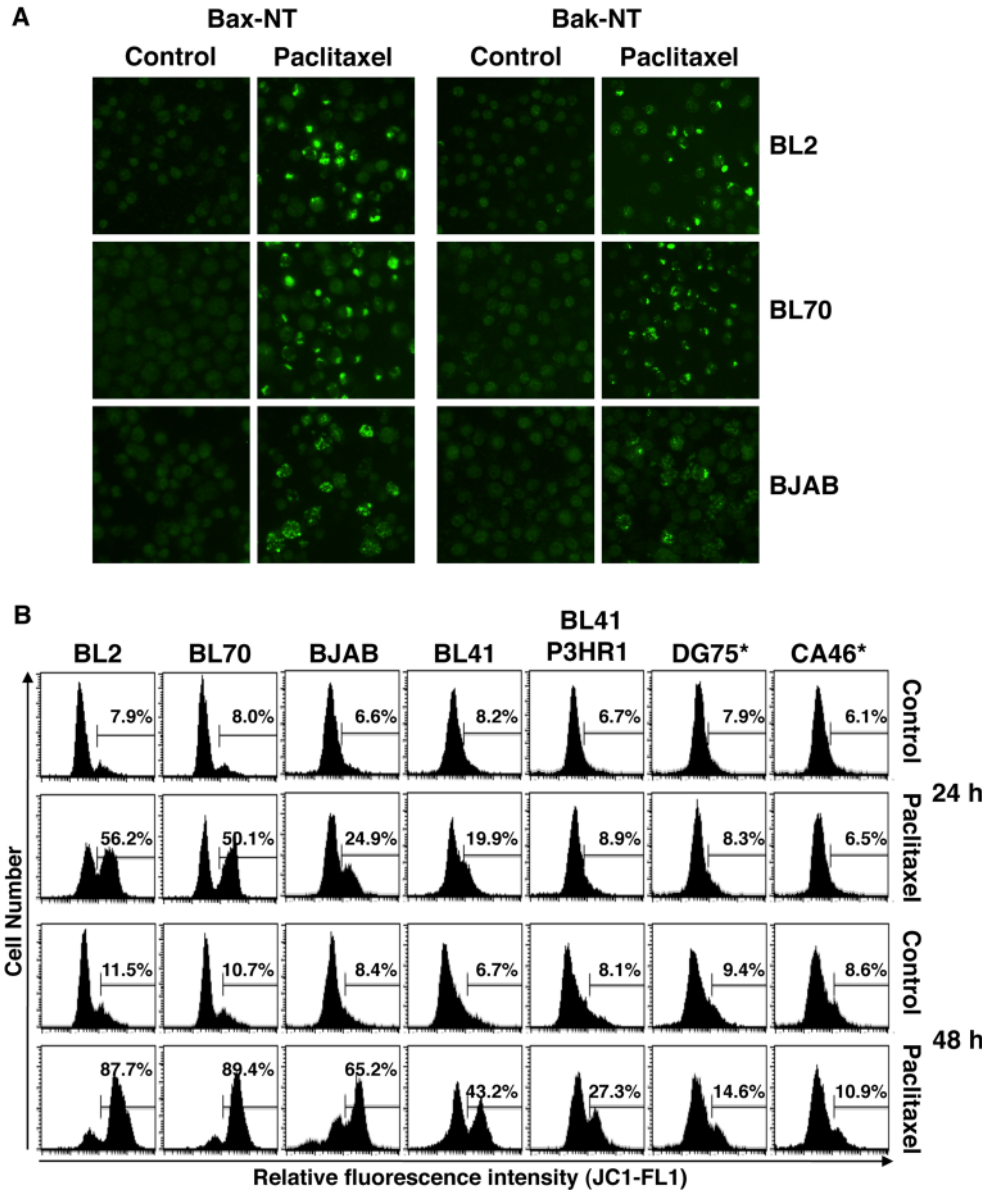

Fig. 6 Bax and Bak activation upon paclitaxel treatment is accompanied by loss of mitochondrial membrane potential. **a** The sensitive BL cell lines BL2, BL70, and BJAB were treated with paclitaxel for 48 h, stained with conformation-specific antibodies against the Bax or Bak N-terminus (NT) and analyzed by fluorescence microscopy. Upon Taxol treatment, cells showed a strong punctuated staining pattern, indicating Bax and Bak clustering and activation, respectively. **b** BL cell lines were treated with paclitaxel as in **a** and stained with JC-1 to analyze loss of mitochondrial membrane potential ($\Delta\Psi_m$). At high membrane potentials, JC-1 accumulates in mitochondria and forms red fluorescent J-aggregates whereas at low membrane potentials JC-1 produces a green cytosolic fluorescence. Increased green fluorescence intensity, indicative of cell with loss of mitochondrial membrane potential, was measured by flow cytometry (representative experiment). The percentage of cells with $\Delta\psi m$ loss is indicated between markers

loci, which introduces a transcriptional enhancer element [36]. Nevertheless, a key feature of BL is the relative simplicity of its karyotype. In a good proportion of cases, the myc translocation is the sole gross chromosomal abnormality indicating that ploidy control is largely intact in BL, at least at the time of diagnosis. This chromosomal stability distinguishes BL from other NHL entities [37]. In contrast to cytogenetic alterations, genomic instability by mutation is frequent in myc-driven lymphomas and facilitates mutations in tumor suppressor genes including p53 and Bax [14, 16, 38]. Pre-treated, relapsed, and hence resistant Burkitt lymphomas may, however, acquire additional cytogenetic and numeric aberrations that coincide with development of aneuploidy.

To gain mechanistic insight, we systematically addressed apoptosis induction by chemotherapeutic compounds in a larger panel of BL cell lines. The microtubule inhibitor vincristine has been used as a key drug in the combination chemotherapy for lymphoid neoplasias [reviewed in [39]]. While studying vinca alkaloids in comparison to other microtubule targeting agents, paclitaxel, and nocodazole, we observed that microtubule targeting agents may trigger an impressive loss of ploidy control with induction of hyperploidy and signs of MC. This was observed only in cells displaying central apoptosis defects.

Evasion of apoptosis by tumor cells has gained much attention since it allows tumor cells to escape from elimination by endogenous default apoptosis mechanisms, immune surveillance, and cancer therapeutics [13]. Here, we observed that paclitaxel, nocodazole, and vincristine induce transient mitotic arrest followed by apoptosis in most of the BL cell lines. Moreover, we found that BL resistant to paclitaxel- or nocodazole-induced apoptosis develop polyploidy, a characteristic feature of mitotic catastrophe, indicating an inverse correlation between apoptosis and polyploidization. Interestingly, there was no polyploidy induction despite prolonged mitotic arrest in apoptosis-resistant cell lines in response to vincristine,

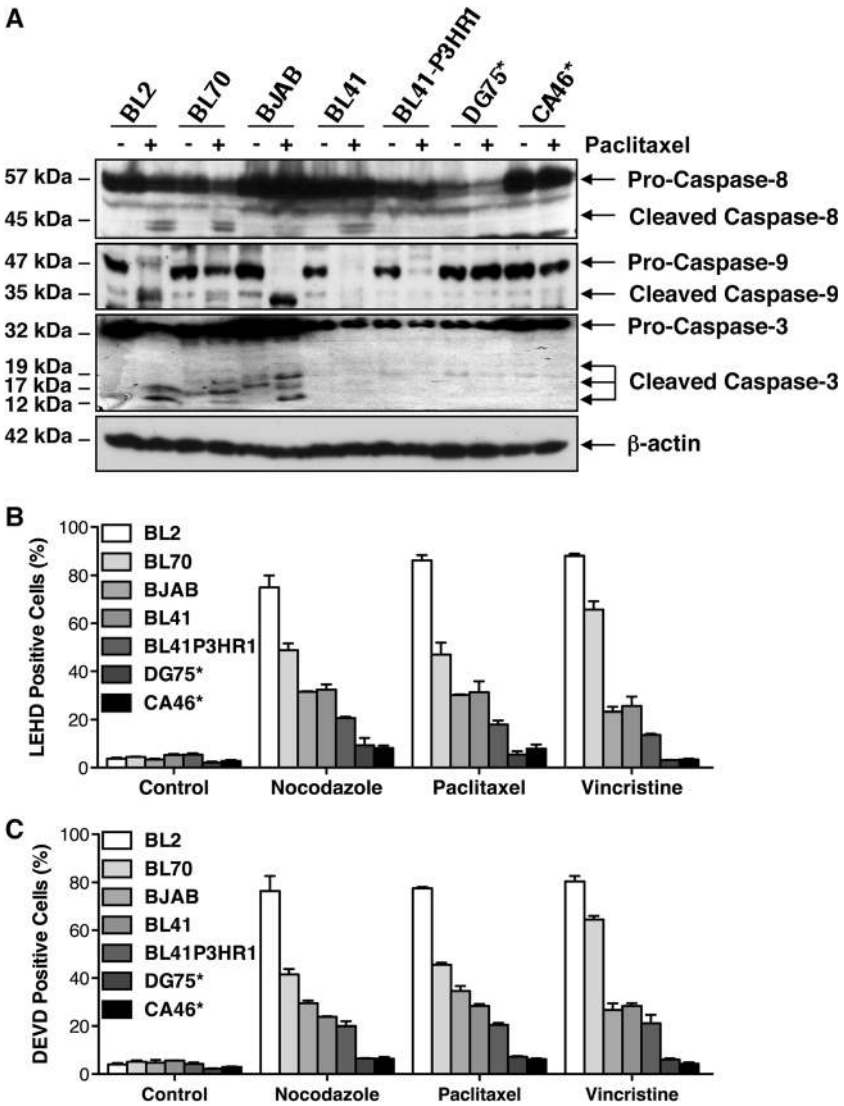

Fig. 7 Caspase-processing, caspase-activation, and apoptosis resistance. **a** Analysis of caspase processing. Cell lysates were subjected to immunoblot analysis for processing of pro-caspase-9, -3, and -8. β-Actin was used as a loading control. **b**, **c** Caspase activities were determined by labeling cells with substrate peptides FAM-LEHD-fmk (for determining caspase-9-like activity (**b**)) or FAM-DEVD-fmk (for determining caspase-3-like activity (**c**)). Percentage of cells with activated caspases was calculated by flow cytometric analysis of green fluorescent cells indicating irreversible binding of the labeled substrate to active caspases. Data are given as percentages of green fluorescent cells indicative of caspase-3-like activity or caspase-9-like activity. The data show the means±s.d. of triplicates from a single experiment, representative of three independent experiments giving similar results

indicating a different mode of action as compared to paclitaxel or nocodazole. Thus, paclitaxel and the reversible microtubule inhibitor nocodazole induce mitotic arrest, endoreplication, and hyperploidy in apoptosis-resistant Burkitt lymphoma while vincristine induces mitotic arrest and does not trigger loss of ploidy control. This may explain the clinical activity of vincristine in therapy of Burkitt and other lymphoma types whereas taxanes and other microtubule polymerization inducing agents failed to take root in lymphoma therapy.

Following exposure to microtubule inhibitors, apoptosis-resistant BL cells were arrested in proliferation but remained viable as determined by cell cycle state and XTT activity. This prolonged mitotic arrest and polyploidization are characteristics of mitotic catastrophe. In support to this, there are reports defining MC as an aberrant form of mitosis associated with the formation of multinucleate giant cells that are temporarily viable but reproductively dead [40, 41]. These cells might later on endure delayed apoptosis or undergo mitotic restitution and re-enter the regular cell division cycle [42, 43]. Thus, exposure to microtubule targeting agents other than vincristine may facilitate development of subclones displaying gross aberrations in ploidy and prone to tumor progression.

Apoptosis defects facilitating polyploidization in BL were not restricted to the p53 or the EBV status. EBV-infected BL41 showed a slightly higher propensity for mitotic arrest and polyploidization upon exposure to paclitaxel indicating that the known impact of EBV on G2/M restriction point control [44] may support polyploidization upon mitotic stress.

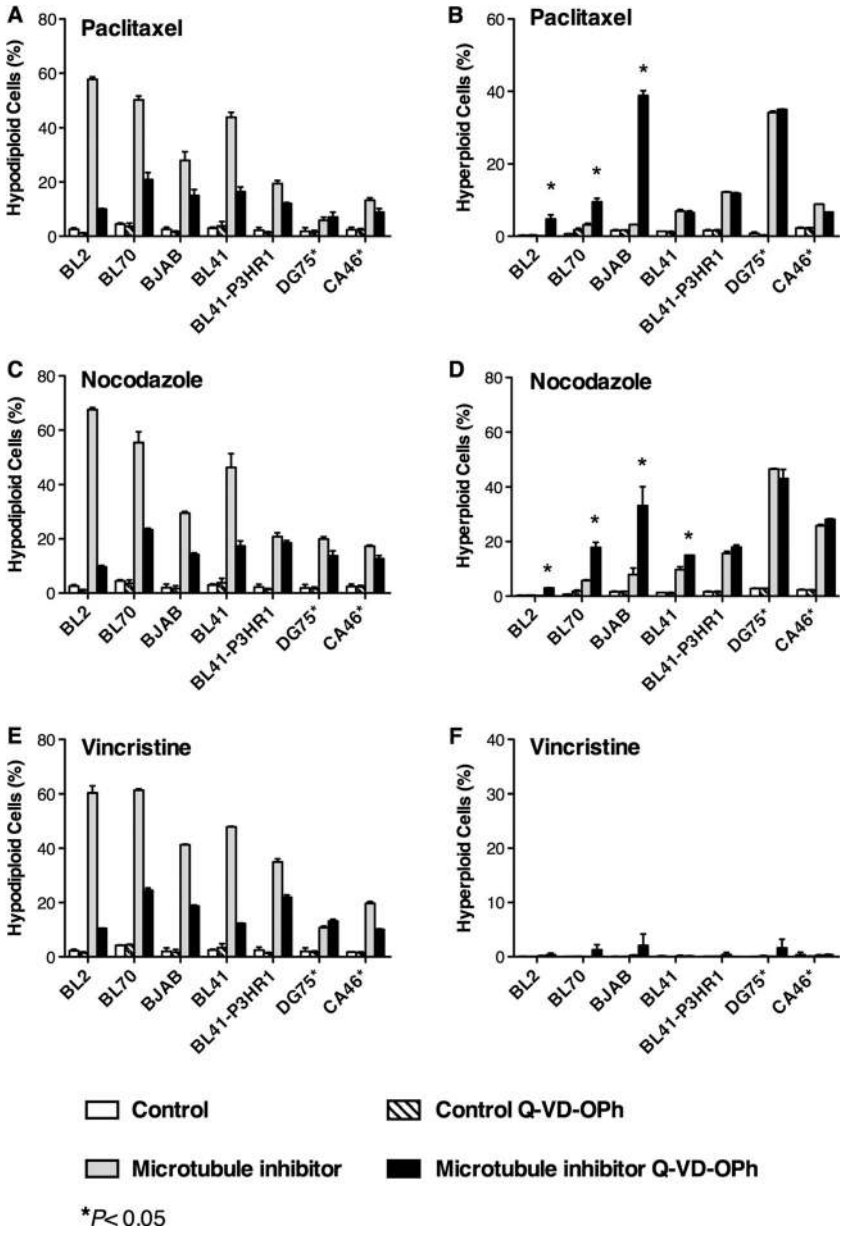

Fig. 8 Inhibition of caspases facilitates development of polyploidy in BL cell lines exposed to paclitaxel and nocodazole but not vincristine. BL cell lines were cultured in the presence or absence of the pan-caspase inhibitor Q-VD-OPh in addition to treatment with or without 100 nM paclitaxel (**a**, **b**), nocodazole (**c**, **d**), or vincristine (**e**, **f**) for 72 h. Thereafter, cells were subjected to flow cytometric analysis of DNA content. Cells with a sub-G1, hypodiploid DNA content were considered apoptotic (**a**, **c**, **e**). Cells showing a hypertetraploid (>4N) DNA content were considered polyploid (**b**, **d**, **f**). The data show the means± s.d. of triplicates from a single experiment, representative of three independent experiments giving similar results

Nevertheless, the consequences of loss of Bax/Bak were far more pronounced. This implies that lack of functional Bax/Bak not only confers resistance against paclitaxel-induced apoptosis but, in turn, leads to induction of polyploidy in BL. Similar data were obtained in murine FDM cells where *bax* and *bak* double knockout cells were resistant to apoptosis but underwent polyploidization upon paclitaxel treatment. This was not the case with single knockouts of *bax* or *bak* indicating their functional redundancy in paclitaxel-induced apoptosis.

Anti-apoptotic Bcl-2 family members like Bcl-x_L and Mcl-1 were shown to be down-regulated in response to paclitaxel treatment in NHL [45] and Bcl-x_L is known to inhibit paclitaxel-mediated apoptosis [30, 46]. Especially Mcl-1 phosphorylation/degradation seems to be specific response to microtubule inhibition and phosphorylation of Mcl-1 by CDK1/cyclin B1 initiates its degradation in cells arrested in mitosis by microtubule poisons [47, 48]. In our study, only Mcl-1 but not Bcl-x_L levels were found to be systematically decreased by paclitaxel treatment in all the cell lines studied. Mcl-1 down-regulation did not occur in consequence of caspase-mediated proteolysis of Mcl-1 but appears to be a common signaling event involved in different BL. Down-regulation of Mcl-1 was, however, not sufficient to overcome apoptosis resistance in those BL lines prone to polyploidization.

Execution of paclitaxel-induced apoptosis in BL involves canonical activation of mitochondria, caspase-9, -3, and -8 cleavage as shown earlier in BJAB cells by our group [28]. In apoptosis-resistant cell lines, exposure to microtubule inhibitors was not accompanied by caspase activation. Caspase inhibition in drug-sensitive BL led to reduced apoptosis and enhanced polyploidy. Interestingly, the cell lines BL41 and BL41-P3HR1 showed decreased caspase activities that might account for the moderate increase of polyploidy in BL41, although cells are not resistant to paclitaxel.

In summary, this study provides evidence of an inverse correlation between apoptosis and mitotic catastrophe in various BLs in response to microtubule inhibitor treatment. We show that microtubule inhibitor-induced apoptosis requires Bax and Bak to proceed via the mitochondrial death signaling pathway followed by activation of caspases-9 and -3. Further, we demonstrate that apoptosis resistance due to Bax/Bak deficiency or caspase inhibition leads to polyploidy in BL under mitotic stress (Fig. 9). Interestingly, vincristine did not induce polyploidy upon conditions of Bax/Bak loss or pan-caspase inhibition. These results provide novel insights into resistance in BL, loss of ploidy control, and genomic instability under specific chemotherapies. Finally, these findings may help to understand the superior activity of vincristine as compared to taxanes in lymphoma therapy.

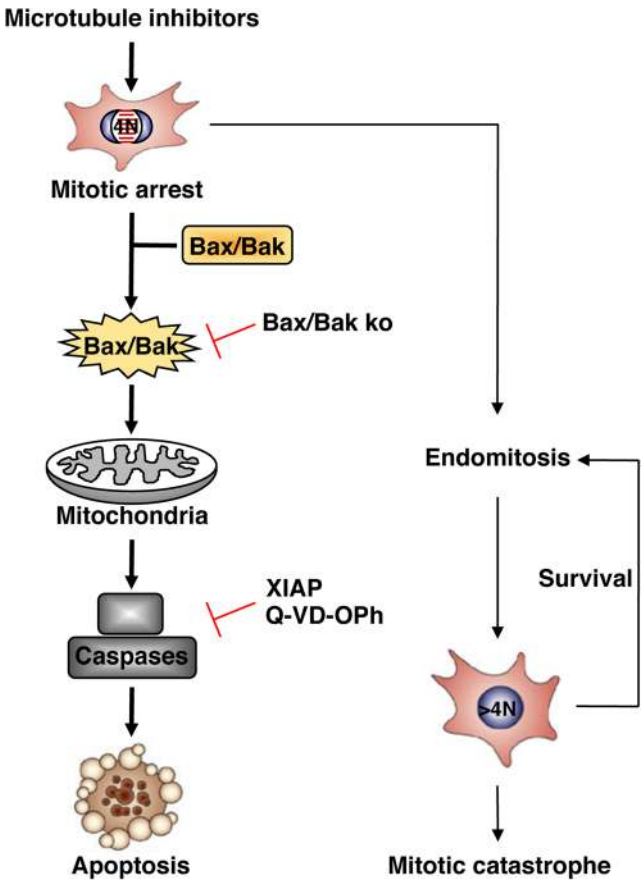

Fig. 9 Model illustrating cell death by apoptosis versus mitotic catastrophe induced by microtubule inhibitors in BL cell lines. Microtubule-active drugs cause cell cycle arrest at a tetraploid (4N) stage and subsequent apoptosis. Apoptosis is induced through the intrinsic mitochondrial pathway that connects microtubule damage to the apoptotic machinery. Loss of Bax and Bak as well as reduced caspase activation or pharmacological pan-caspase inhibitors, here Q-VD-Oph, suppresses the apoptotic response induced by distinct microtubule inhibitors without disruption of their activity on microtubules or on cell cycle arrest at M-phase or the G2/M boundary. However, inhibition of apoptosis results in a prolonged mitotic arrest that is followed by endomitosis and, consequently, induction of polyploidy indicative of mitotic catastrophe. Cells undergoing mitotic arrest may slowly undergo non-apoptotic death (e.g., by necrosis) or survive for prolonged time periods

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Conflict of interest All authors have read and approved the final version of the manuscript. None of the authors has any type of financial interest to disclose.

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