



Helenalin bypasses Bcl-2-mediated cell death resistance by inhibiting NF- κ B and promoting reactive oxygen species generation

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ABSTRACT

Evasion of cell death by overexpression of anti-apoptotic proteins, such as Bcl-2, is commonly observed in cancer cells leading to a lack of response to chemotherapy. Hence, there is a need to find new chemotherapeutic agents that are able to overcome chemoresistance mediated by Bcl-2 and to understand their mechanisms of action. Helenalin, a sesquiterpene lactone (STL), induces cell death and abrogates clonal survival in a highly apoptosis-resistant Bcl-2 overexpressing Jurkat cell line as well as in two other Bcl-2 overexpressing solid tumor cell lines (mammary MCF-7; pancreatic L6.3pl). This effect is not achieved by directly affecting the mitochondria-protective function of Bcl-2 in the intrinsic pathway of apoptosis since Bcl-2 overexpressing Jurkat cells do not show cytochrome *c* release and dissipation of mitochondrial membrane potential upon helenalin treatment. Moreover, helenalin induces an atypical form of cell death with necrotic features in Bcl-2 overexpressing cells, neither activating classical mediators of apoptosis (caspases, AIF, Omi/HtrA2, Apaf/apoptosome) nor ER-stress mediators (BiP/GRP78 and CHOP/GADD153), nor autophagy pathways (LC3 conversion). In contrast, helenalin was found to inhibit NF- κ B activation that was considerably increased in Bcl-2 overexpressing Jurkat cells and promotes cell survival. Moreover, we identified reactive oxygen species (ROS) and free intracellular iron as mediators of helenalin-induced cell death whereas activation of JNK and abrogation of Akt activity did not contribute to helenalin-elicited cell death. Our results highlight the NF- κ B inhibitor helenalin as a promising chemotherapeutic agent to overcome Bcl-2-induced cell death resistance.

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1. Introduction

Chemotherapy remains a fundamental part in the treatment of cancer. Chemoresistance of cancer cells, however, continues to be a major problem [1]. Current anti-cancer treatments kill tumor cells by inducing apoptosis or other forms of programmed cell death [2]. Apoptosis can be triggered either at the cell surface (extrinsic pathway) or at the mitochondria (intrinsic pathway) [2]. Many standard chemotherapeutics act *via* mitochondria, inducing cytochrome *c* release into the cytosol, which results in the assembly of the apoptosome and the activation of caspase-9, initiating a caspase cascade leading to apoptotic cell death. In contrast, when AIF (apoptosis-inducing factor), Omi/HtrA2 (high-

temperature-requirement protein A2) or endonuclease G are released into the cytosol, a caspase-independent cell death can occur [3].

Members of the Bcl-2 (B-cell lymphoma) family are important regulators of the intrinsic pathway of apoptosis [4,5]. They control mitochondrial outer membrane permeability promoting or inhibiting the release of mitochondrial proteins. Bcl-2, the first member of this family discovered, protects cells from apoptosis by inhibiting oligomerisation and pore formation induced by the pro-apoptotic Bcl-2 family members Bax and Bak or by sequestering pro-apoptotic BH3-only proteins. Since overexpression of Bcl-2 often confers chemoresistance, Bcl-2 is a promising therapeutic target [1,6,7]. Bcl-2 is also localized at membranes of the endoplasmic reticulum (ER) where it regulates Ca²⁺ homeostasis by desensitizing cells to stimuli that cause the release of ER-sequestered Ca²⁺ [8,9]. Autophagy is another strongly regulated cellular program, in which regulation Bcl-2 is involved [10]. Autophagy has been interpreted as a form of non-apoptotic death as well as a survival strategy during stress [11]. Recently, a link between Bcl-2 and constitutive activation of nuclear factor κ B

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(NF- κ B) [12–15], another crucial player for cell survival and apoptosis resistance in cancer [16], has been described.

We could previously show that the sesquiterpene lactone helenalin, a secondary plant metabolite, induces apoptosis in Jurkat leukemia cells, which was caspase dependent and moreover cell death in Jurkat cells overexpressing Bcl-2 [17]. Obviously, helenalin is able to overcome Bcl-2-mediated chemoresistance, however the mechanism is still unknown. Aim of this study, therefore, was to characterize the mechanism of cell death induced by helenalin in Bcl-2 overexpressing cancer cells.

2. Materials and methods

2.1. Compounds

Helenalin, purchased from Axxora (Lörrach, Germany), was dissolved in DMSO (final concentration <0.1%). Other chemicals were purchased from Merck (Darmstadt, Germany) (i.e. 3-MA, Etoposide, Necrostatin-1 Q-VD-OPh); Sigma-Aldrich (Taufkirchen, Germany) (BMS-345541, deferoxamine mesylate, DMSO, N-acetyl-L-cysteine, paclitaxel, propidium iodide, β -phenylethyl isothiocyanate); Axxora (Lörrach, Germany) (DCDHF diacetate, JC-1-iodide); Molecular Probes/Invitrogen (Karlsruhe, Germany) (goat anti-mouse IgGs), Bachem (Bubendorf, Germany) (Ac-DEVD-AFC, Ac-LETD-AFC), BIOTREND GmbH (Cologne, Germany) (Fura-2-AM), Bender MedSystems (Vienna, Austria) (Human Annexin V-FITC kit) or Repro Tech GmbH (Hamburg, Germany) (TNF- α).

2.2. Cell culture

Human leukemia Jurkat T cells (J16), and Jurkat T cells transfected with vector control (Neo Jurkat) or Bcl-2 (Bcl-2 Jurkat) (both kindly provided by Drs. P.H. Krammer and H. Walczak, Heidelberg, Germany) were cultured as previously described [17]. MCF-7 cells were purchased from DSMZ (ACC 115). The highly metastatic human pancreatic cancer cell line L3.6pl was cultivated as originally described [18]. Bcl-2 overexpressing MCF-7 or L3.6pl cells (Bcl-2 MCF-7, Bcl-2 L3.6pl) and the corresponding empty-vector control cell lines (Neo MCF-7, Neo L3.6pl) were created by transfection with the pcDNA3 Bcl-2 vector (Addgene plasmid 8768) and empty vector pcDNA3 (Invitrogen/Molecular Probes, Karlsruhe, Germany), respectively, using the Amaxa[®] Cell Line Nucleofector Kit[®] V (LONZA Cologne AG, Cologne, Germany).

2.3. Quantification of cell death

The following protocols were used: (A) subdiploid-DNA content was quantified as previously described [17,19]; (B) propidium iodide (PI, 5 μ g/ml, 30 min at room temperature in the dark) staining was monitored by flow cytometry (FACSCalibur; Becton Dickinson, Heidelberg, Germany). (C) Annexin-V/PI double staining was performed using a human Annexin V-FITC Kit (Bender MedSystems, Vienna, Austria).

2.4. Clonogenic assay

The clonogenic assay was carried out as previously described [20] and colonies were scored after 7 days of culture using the analysis system from S.CO LifeScience (Garching, Germany). The clonogenic assay with Neo L3.6pl and Bcl-2 L3.6pl cells was performed as previously described [18]. Cells were stained with crystal violet (0.5% crystal violet in 20% methanol) and quantified by absorption measurement (550 nm; SpectraFluor Plus[™] (Tecan; Männedorf, Austria)).

2.5. Apaf-1 and AIF siRNA

Sense and antisense siRNA oligonucleotides corresponding to nucleotides 978–998 of Apaf-1 [21] (AATTGGTGCACCTTTTACGTGA), to the AIF nucleotides [22] (GGAAATATGGGAAAGA-TCCdTdT) and oligonucleotides corresponding to a scramble sequence were purchased from Biomers.net GmbH (Ulm, Germany). Bcl-2 Jurkat cells were transfected with 3 μ g of scramble, Apaf-1 or AIF siRNAs using the Amaxa[®] Cell Line Nucleofector Kit[®] V (LONZA Cologne AG, Cologne, Germany). Cells were treated 48 h after transfection.

2.6. Measurement of cytochrome c release

Release of cytochrome c from mitochondria was analyzed as previously described [17]. Purity of cytosolic fractions was assessed by incubating membranes with VDAC antibody (Cell Signalling, Frankfurt, Germany).

2.7. Western blot analysis

Samples were treated as described before [20] and membranes were incubated overnight at 4 °C with the following specific antibodies: Apaf-1 (BD Transduction Laboratories, Heidelberg, Germany), Bcl-2, eIF2 α (D-3), caspase-3 (Santa Cruz Biotechnology, Heidelberg, Germany), cytochrome c, JNK, pJNK (Thr183/Tyr185), VDAC, AKT, mTOR, Beclin-1, LC3B (G40), p-c-Jun (Ser63) II, p-eIF2 α (Ser 51), caspase-9, pAKT (Ser473), p-mTOR (Ser2448) (Cell Signalling, Frankfurt, Germany), ANTI-GADD153/CHOP10 (Sigma, Saint Louis, MO, USA), BiP/GRP78 (BD Transduction Laboratories, Heidelberg, Germany). Equal protein loading was controlled by β -actin (Millipore, Billerica, MA, USA) staining of membranes.

2.8. Measurement of ROS generation

Bcl-2 Jurkat cells were stained with 2',7'-dichlorodihydrofluorescein diacetate (DCDHF diacetate, 10 μ M) for 30 min at 37 °C in the dark and analyzed by flow cytometry (FACSCalibur, Becton Dickinson, Heidelberg, Germany).

2.9. Reporter gene assay

Bcl-2 and Neo Jurkat cells were co-transfected with a plasmid containing 5.7 kB of the human NF- κ B promoter driving a firefly luciferase gene (pNF- κ B-Luc, Stratagene, La Jolla, CA, USA; 4 μ g) and a β -galactosidase plasmid (p β -Gal, 6.83 kB, Promega, Mannheim, Germany; 506 ng) using the Nucleofector[®] II device (LONZA Cologne AG, Cologne, Germany). Luciferase activity was measured with Orion II Microplate Luminometer (Berthold Detection Systems, Pforzheim, Germany) and normalized to β -galactosidase activity.

2.10. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared and EMSA was carried out using p³²-labeled NF- κ B consensus sequence oligonucleotides (Promega, Heidelberg, Germany) as previously described [23]. Gels were exposed to Cyclone Storage Phosphor Screens (Canberra-Packard, Schwadorf, Austria) followed by analysis with a phosphor imager station (Cyclone Storage Phosphor System, Canberra-Packard). The composition of p65/p50 of NF- κ B was confirmed by supershift analysis using antibodies against p65 and p50, respectively [24].

2.11. Measurement of mitochondrial potential

The measurement was performed as previously described [17]. Cells were stained with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-

benzimidazol-carbocyanine iodide (JC-1) (Axxora, Lörrach, Germany) at a final concentration of 1.25 μ M for 15 min at 37 °C and analyzed by flow cytometry.

2.12. Caspase activity assay

Caspase activity was determined as described before [17]. Caspase substrates: Ac-LETD-AFC for caspase-8 or Ac-DEVD-AFC (Bachem, Bubendorf, Germany) for caspase-3, respectively. Caspase activity was monitored by a plate-reading multifunction photometer (SpectraFluor Plus™, Tecan, Männedorf, Austria) after 2 h at 37 °C.

2.13. Transmission electron microscopy (TEM)

Jurkat and Bcl-2 Jurkat cells were left untreated (Co) or treated with helenalin for 8 h and TEM was carried out as described before [25].

2.14. Statistical analysis

All experiments were performed at least three times. Results are expressed as mean value \pm SEM. Statistical analysis was performed with GraphPad Prism™ version 3.0 for Windows (GraphPad Software, San Diego, CA, USA). One-way ANOVA with Bonferroni multiple comparison post-test or unpaired two-tailed Student's *t*-test were performed. *P* values <0.05 were considered significant.

3. Results

3.1. Helenalin overcomes Bcl-2-mediated resistance

Overexpression of the antiapoptotic protein Bcl-2 in Jurkat T-cells confers death resistance against etoposide but not helenalin, confirming published data [17]. Moreover, helenalin almost completely inhibited colony growth in Jurkat cells overexpressing Bcl-2. In contrast, the classical chemotherapeutic agent etoposide is not able to suppress the clonogenic survival of Bcl-2 overexpressing Jurkat cells (Fig. 1a). To verify that helenalin not only

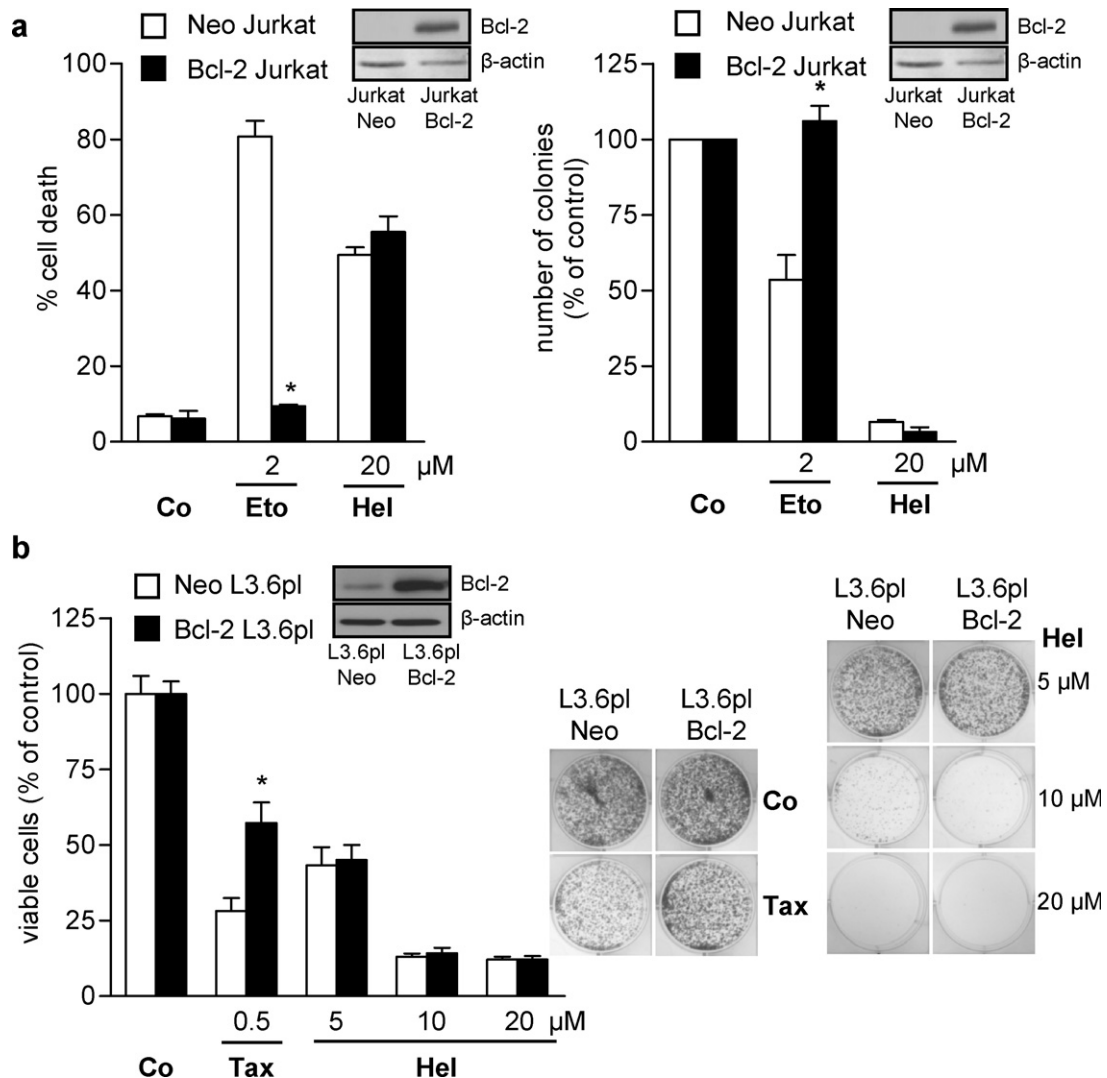


Fig. 1. Helenalin overcomes Bcl-2-mediated chemoresistance. (A) Neo Jurkat or Bcl-2 Jurkat were either left untreated (Co) or treated with helenalin (Hel, 20 μ M) or etoposide (Eto, 2 μ M) for 24 h (cell death, left panel) or 2 h (colony growth, right panel). Cell death was quantified by measuring nuclei with a subdiploid DNA content and colony growth was quantified by a clonogenic assay; both assays are described under Section 2. Results of colony growth are represented as the number of colonies referred to untreated cells (Co) (right panel). **p* < 0.001 (ANOVA, Bonferroni). (B) Neo L3.6pl and Bcl-2 L3.6pl cells were either left untreated (Co) or stimulated with paclitaxel (Tax) or helenalin (Hel) for 2 h and a clonogenic assay was performed. **p* < 0.001 (ANOVA, Bonferroni). Data are expressed as mean \pm SEM (*n* = 3). Bcl-2 protein level of Neo and Bcl-2 Jurkat cells, and Neo and Bcl-2 L3.6pl cells were analyzed by Western blot.

overcomes Bcl-2 resistance in leukemia cells, we stably overexpressed Bcl-2 in the breast cancer cell line MCF-7 and the highly metastatic human pancreatic carcinoma cell line L3.6pl. Helenalin induced cell death in both cell lines (Supplement Fig. 1). Moreover, helenalin inhibited colony growth in Bcl-2 overexpressing L3.6pl cells that were clearly insensitive towards paclitaxel (Fig. 1b).

3.2. Helenalin does not abrogate mitochondrial function of Bcl-2 and acts independently of mitochondria and the apoptosome

Next, we focused on the mechanism leading to cell death in Bcl-2 overexpressing Jurkat cells. Helenalin did not alter Bcl-2 protein level, nor did it induce Bcl-2 phosphorylation as seen after paclitaxel treatment (Fig. 2a). Moreover, helenalin does not abrogate the function of Bcl-2 on mitochondria since the mitochondrial membrane potential was kept nearly intact and only minute amounts of cytochrome *c* were released into the

cytosol in Bcl-2 overexpressing compared to vector control (Neo Jurkat) cells treated with helenalin (Fig. 2b and c).

To underline these findings, transmission electron microscopy (TEM) was performed. In *wild-type* Jurkat cells exposed to 20 μ M helenalin mitochondria showed severe ultrastructural changes: all stages of disintegration were observed starting from swelling of the cristae and compaction of the matrix, up to mitochondrial “ghosts” with only residual inner membrane and cristae visible (Fig. 3, upper part). These structural findings concur with biochemical data: in *wild-type* Jurkat cells, loss of mitochondrial membrane potential, cytochrome *c* release and caspase activation was observed in response to helenalin [17]. In contrast, in Bcl-2 overexpressing Jurkat cells exposed to 20 μ M helenalin mitochondria remain structurally more or less uninfluenced (Fig. 3, lower part) which is in accordance with a lack of cytochrome *c* release and an intact mitochondrial membrane potential (Fig. 2b and c). Notably, the intermembrane space of the nuclear envelope drastically swells and numerous vesicles form in the cytoplasm in helenalin treated Bcl-2 overexpressing cells.

Silencing of Apaf-1 *via* siRNA did not influence the induction of cell death by helenalin (Fig. 4a). This fact further suggests that helenalin-induced cell death occurs independently of the apoptosome and mitochondria. Accordingly, caspase activity measurements (Fig. 4b and Supplement Fig. 2 a and b) and employment of the pan-caspase inhibitor Q-VD-OPh (Fig. 4c) negated a role for caspases in helenalin-induced cell death in Bcl-2 overexpressing cells. The chemical β -phenylethyl isothiocyanate (PEITC) was used as positive control since it is known to induce a caspase-dependent cell death in Bcl-2 Jurkat cells [26].

Examining caspase-independent apoptotic factors such as the serine protease Omi/HtrA2 and the endonuclease AIF by use of siRNA against AIF and the Omi inhibitor UCF showed that these factors are also not involved in helenalin-mediated cell death (Supplement Fig. 2c and d). Moreover, phosphatidylserine translocation to the outer cell membrane (analyzed by Annexin V staining), a classical sign of early apoptosis, was not visible in Bcl-2 overexpressing cells after helenalin treatment for 8 h, whereas the vector control cells (Neo Jurkat cells) were clearly Annexin V positive (Fig. 4d).

3.3. Potential mechanisms used by helenalin to bypass Bcl-2-mediated cytoprotection

We analyzed potential non-apoptotic events leading to helenalin-mediated cell death, such as autophagy and ER-stress. ER-stress induction has recently been linked to induction of autophagy [11,27]. Helenalin did not affect the expression of BiP/GRP78 and CHOP/GADD153, two critical UPR-upregulated proteins during ER-stress (Fig. 5a). Furthermore, increased Ca^{2+} -levels, as seen after treatment with thapsigargin, an inhibitor of the endoplasmic reticulum Ca^{2+} ATPase (SERCA), was not observed after stimulation with helenalin (Supplement Fig. 3a and b). However, a strong transient activation of JNK, a rapid phosphorylation of eIF2 α and a slightly reduced phosphorylation of mTOR in Bcl-2 overexpressing cells was detected upon helenalin treatment (Fig. 5b and Supplement Fig. 3b and c). All three events have been linked to induction of autophagy [11,27]. Nevertheless, no accumulation of LC3 II, commonly observed during autophagy, could be detected by Western blot analysis (Fig. 5c), and 3-MA, a well established inhibitor of autophagy [28], partly diminished helenalin-induced cell death after 16 h stimulation but not after 48 h (Fig. 5d). Furthermore TEM analysis of helenalin treated cells did not reveal significant occurrence of autophagolysosomes (Fig. 3).

We then examined mediators involved in signaling events of programmed necrosis. Necrostatin-1 (Nec-1) inhibits RIP1 and acts as a specific inhibitor of necroptotic cell death [29]. Exposure of

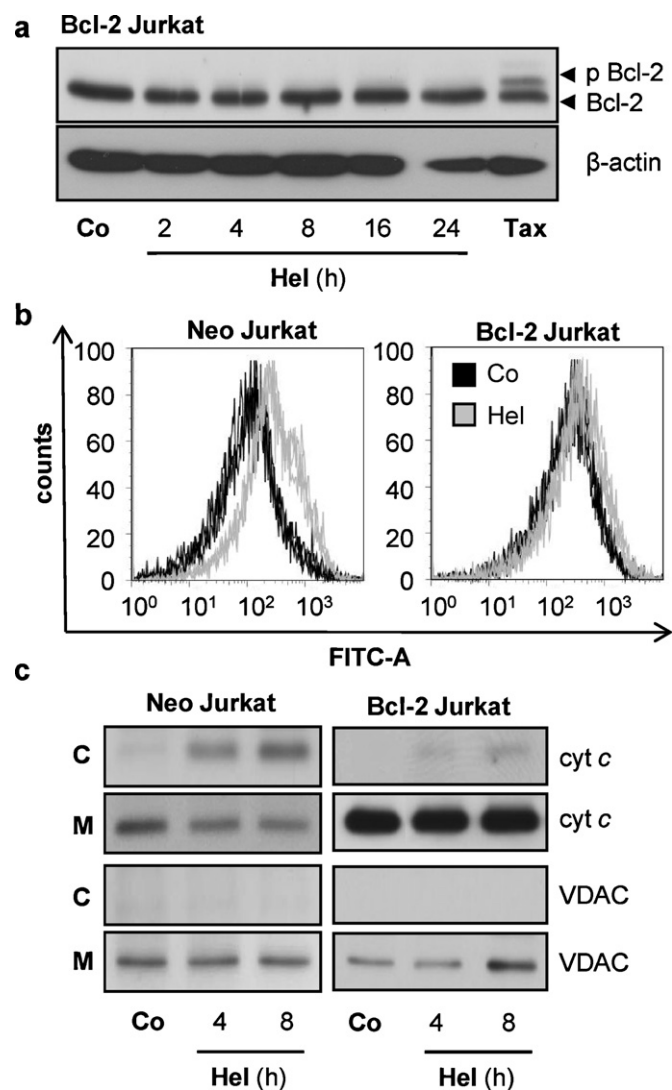


Fig. 2. Helenalin does not abrogate mitochondrial function of Bcl-2. (A) Bcl-2 Jurkat cells were treated with helenalin (Hel; 20 μ M) for the indicated times. Paclitaxel (Tax; 1 μ M, 16 h) was used as control. Bcl-2 expression pattern was analyzed by Western blot. (B) Neo Jurkat and Bcl-2 Jurkat cells were treated with helenalin (Hel; 20 μ M, 6 h) and mitochondrial membrane potential (MMP) was analyzed by staining with JC-1. Histograms of one representative experiment out of three are shown. (C) For Western blot analysis of cytochrome *c*, Neo Jurkat and Bcl-2 Jurkat cells were treated with helenalin (Hel; 20 μ M) for the indicated times. Cytosolic and membrane fractions were prepared. Purity of cytosolic fractions was verified by use of a VDAC antibody.

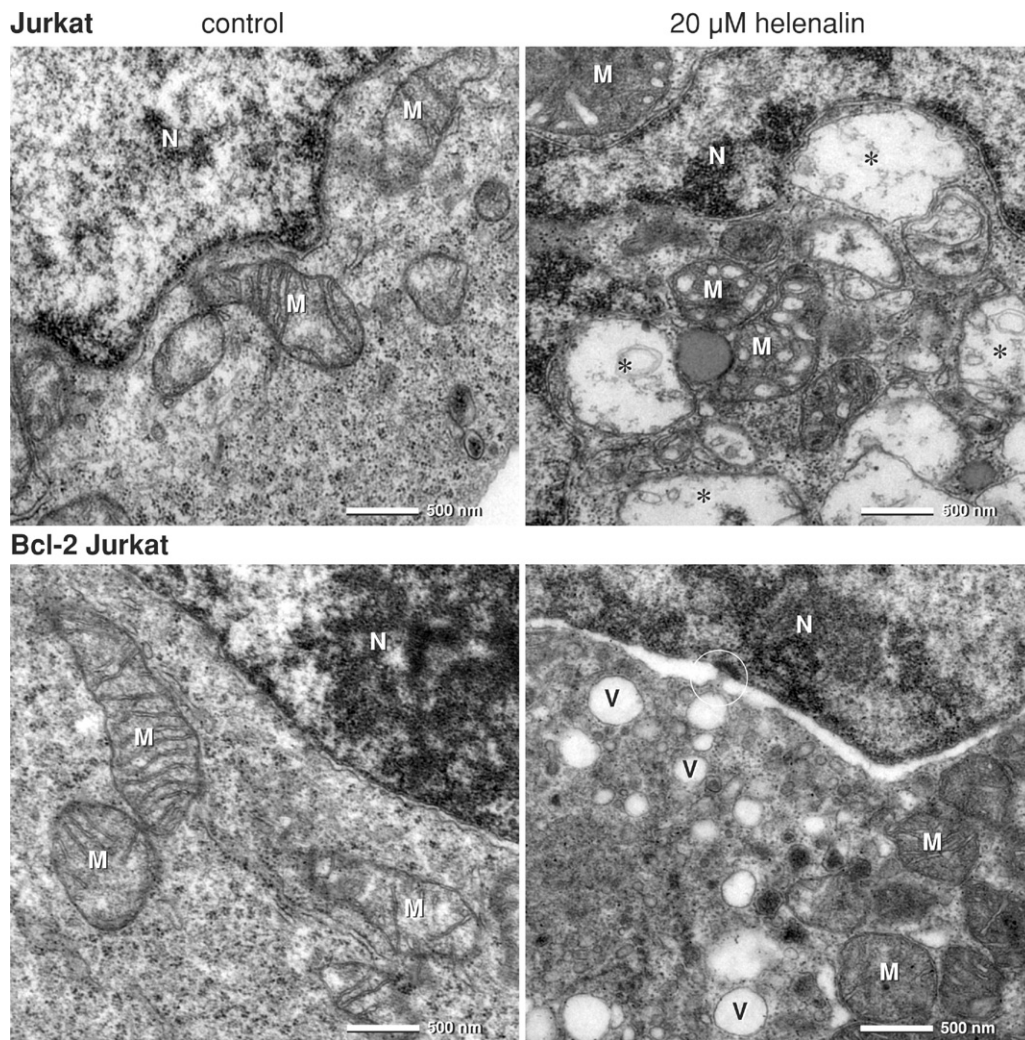


Fig. 3. Mitochondria of Bcl-2 overexpressing cells are not altered ultrastructurally by helenalin. Transmission electron micrographs of ultrathin sections of *wild-type* Jurkats and Bcl-2 Jurkats untreated (control) and treated with 20 μ M helenalin for 8 h. *Wild-type* Jurkat cells show all stages of mitochondrial disintegration starting from swelling of the cristae and compaction of the matrix, up to electron translucent “ghosts” (asterisks) with only residual inner membranes and cristae visible. Mitochondria of Bcl-2 overexpressing Jurkat cells remain structurally intact but the intermembrane space of the nuclear envelope swells while maintaining the nuclear pores (circle); numerous vesicles are observed in the cytoplasm. N = nucleus; M = mitochondrion; V = vesicle.

Bcl-2 Jurkat cells to Nec-1 prior to helenalin treatment did not prevent cell death (Fig. 5e), arguing against necroptosis as a mode of cell death induced by helenalin. In contrast, Nec-1 abrogated necroptosis induced by TNF- α in the presence of cycloheximide and the pan-caspase inhibitor Q-VD-OPH in Jurkat cells (positive control) [30] (Fig. 5e insert). An early JNK activation as observed in response to helenalin has been associated with programmed necrosis [29]. Yet, JNK activation by helenalin does not contribute to cell death as indicated by the use of SP600125, a commonly used JNK inhibitor (activity was confirmed by analyzing the phosphorylation of the JNK downstream target c-Jun) (Fig. 5f and insert).

In addition, helenalin reduced Akt activation (Supplement Fig. 3d), which is an important survival factor in cancer cells [31]. However, in Bcl-2 overexpressing cells reduction of Akt activity does not seem to be involved in cell death as wortmannin, a potent inhibitor of Akt phosphorylation, does neither induce cell death itself nor adds to the toxicity of helenalin in Bcl-2 overexpressing Jurkat cells (Supplement Fig. 3e).

3.4. Role of NF- κ B

Since a link of Bcl-2 and the NF- κ B signaling pathway has been described for other cell lines [12–15,32], we examined (1) whether

Bcl-2 overexpressing Jurkat cells show increased NF- κ B activity and if so, (2) whether helenalin is able to interfere with it. As shown in Fig. 6a, NF- κ B-dependent gene transactivation as well as DNA binding activity were increased in Bcl-2 overexpressing Jurkat cells. NF- κ B is known to be an important mediator of survival and death resistance in leukemia cells [33]. Helenalin significantly reduced NF- κ B-dependent gene transactivation and DNA binding activity in Bcl-2 overexpressing cells (Fig. 6b). In addition, also the NF- κ B inhibitor BMS-345541, acting on the I κ B kinase complex, induced cell death in Bcl-2-protected cells (Supplement Fig. 4a) pointing to NF- κ B as an attractive target against Bcl-2-conferred cell death resistance. In search of a potential mechanism downstream of NF- κ B we measured intracellular ROS level. Helenalin (20 μ M) increased intracellular ROS level comparable to H₂O₂ (17, 6 μ M), which was used as a positive control (Fig. 7a). An antioxidant such as NAC completely prevented and the radical scavenger Tiron attenuated cell death induced by helenalin in Bcl-2 overexpressing Jurkat cells indicating that the Bcl-2 resistance overcoming mechanism of helenalin requires ROS production (Fig. 7b). Moreover, helenalin-induced cell death involves iron ions since the use of the iron chelator desferrioxamine (DFO) inhibits cell death induction by helenalin (Fig. 7c). Thus, ROS production by helenalin might partly depend on the Fenton reaction as reported

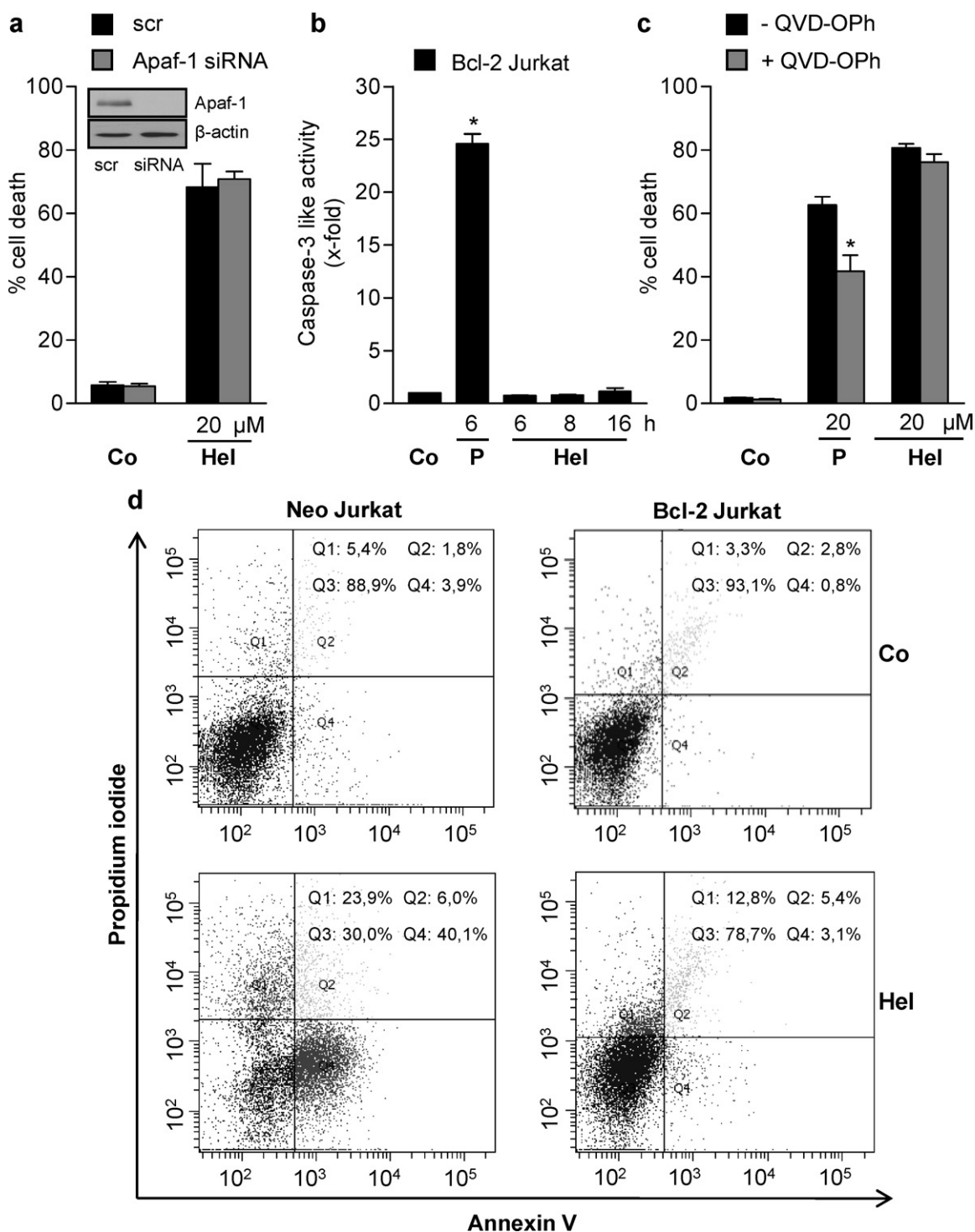


Fig. 4. The classical mitochondrial pathway and caspases are not involved in helenalin-induced cell death overcoming Bcl-2 chemoresistance. (A) Bcl-2 Jurkat cells were transiently transfected with scrambled (scr) or Apaf-1 siRNA oligonucleotides as described under Section 2 and stimulated with helenalin (Hel; 20 μ M) for 24 h. Cell death was quantified by measuring nuclei with a subdiploid DNA content. Apaf-1 protein levels in transfected cells were analyzed by Western blot. (B) Cells were treated with helenalin (Hel; 20 μ M) or β -phenylethyl isothiocyanate (PEITC) (P, 20 μ M) as a positive control for caspase activation in Bcl-2 Jurkat cells for the indicated time points. Caspase-3-like and caspase-8 activity in Bcl-2 Jurkat cells was determined fluorometrically. * $p < 0.001$ (ANOVA, Bonferroni). (C) Bcl-2 Jurkat cells were preincubated with the pan-caspase inhibitor Q-VD-OPh (10 μ M, 1 h, were indicated) before stimulation with PEITC (P, 20 μ M) or helenalin (Hel; 20 μ M) for 16 h and cell death was measured by the propidium iodide exclusion. * $p < 0.001$ (ANOVA, Bonferroni). Data are expressed as mean \pm SEM ($n = 3$). (D) Neo Jurkat and Bcl-2 Jurkat cells were left untreated (upper panels) or treated with helenalin (Hel; 20 μ M, lower panels) for 8 h. PI/Annexin V-FITC staining was performed. Dot plots of one representative experiment out of three are shown.

by others [33]. Since BMS induced inhibition of NF- κ B only partly abrogated ROS response by helenalin (Supplement Fig. 4b) ROS induction by helenalin seems to occur both upstream as well as downstream of NF- κ B.

4. Discussion

The present paper highlights helenalin as a small molecule that is able to overcome Bcl-2-mediated chemoresistance in tumor

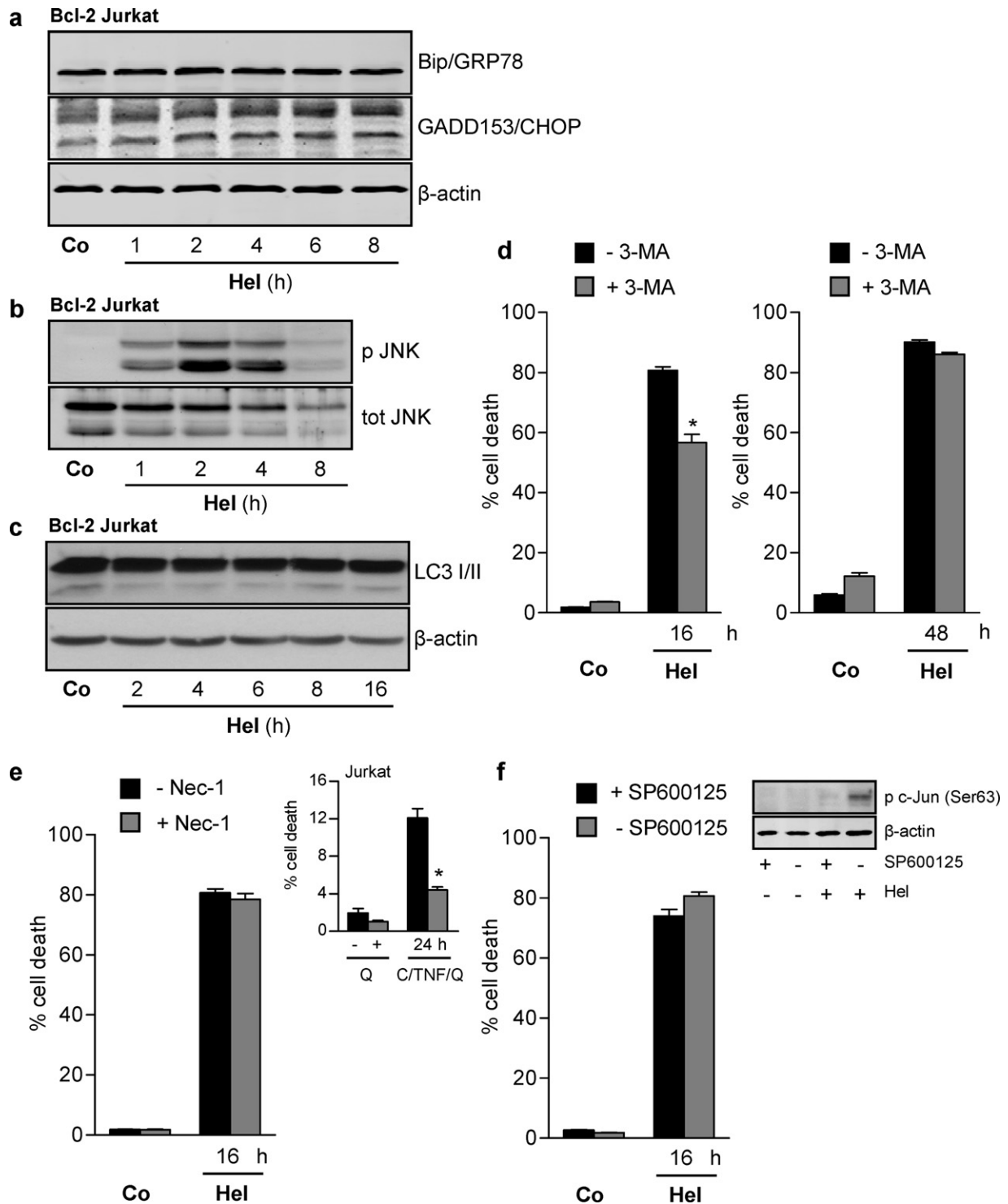


Fig. 5. Potential mechanisms used by helenalin to bypass Bcl-2-mediated cytoprotection. (A) Bcl-2 Jurkat cells were treated with helenalin (Hel; 20 μ M) for the indicated time points and proteins involved in ER stress were detected Western blot analysis. (B) Bcl-2 Jurkat cells were treated with helenalin (Hel; 20 μ M) for the indicated time points and the phosphorylation status of JNK was determined by Western blot analysis. (C) For the Western blot analysis of the autophagy-induced conversion of LC3 I to LC3 II, Bcl-2 Jurkat cells were treated with helenalin (Hel; 20 μ M) for the indicated times. (D) Bcl-2 Jurkat cells were treated with helenalin (Hel; 20 μ M) with or without a 1 h pretreatment with the autophagy inhibitor 3-MA (10 mM). Cell death was quantified by PI exclusion. * $p < 0.001$ (ANOVA, Bonferroni). (E) Where indicated, Bcl-2 Jurkat cells were treated with necrostatin-1 (Nec-1; 30 μ M), a selective inhibitor of necroptosis, 1 h before treatment with helenalin (Hel; 20 μ M, 16 h). *Insert:* induction of necroptotic cell death in Jurkat cells by a combination of TNF α (10 ng/ml), cycloheximide (C; 1 μ g/ml) and the pan-caspase inhibitor Q-VD-OPh (Q, 10 μ M), which is significantly reduced by a 1 h-pretreatment with Nec-1 (30 μ M). Cell death was quantified by PI exclusion. * $p < 0.001$ (ANOVA, Bonferroni). (F) Bcl-2 Jurkat cells were treated with helenalin (Hel; 20 μ M, 16 h) with or without a 1 h pretreatment with the specific JNK-inhibitor SP600125 (10 μ M). Cell death was quantified by PI exclusion. *Insert:* Western blot analysis of the JNK downstream target p-c-Jun (Ser63) after helenalin and SP600125 treatment of Bcl-2 Jurkat cells. Data are expressed as mean \pm SEM ($n = 3$).

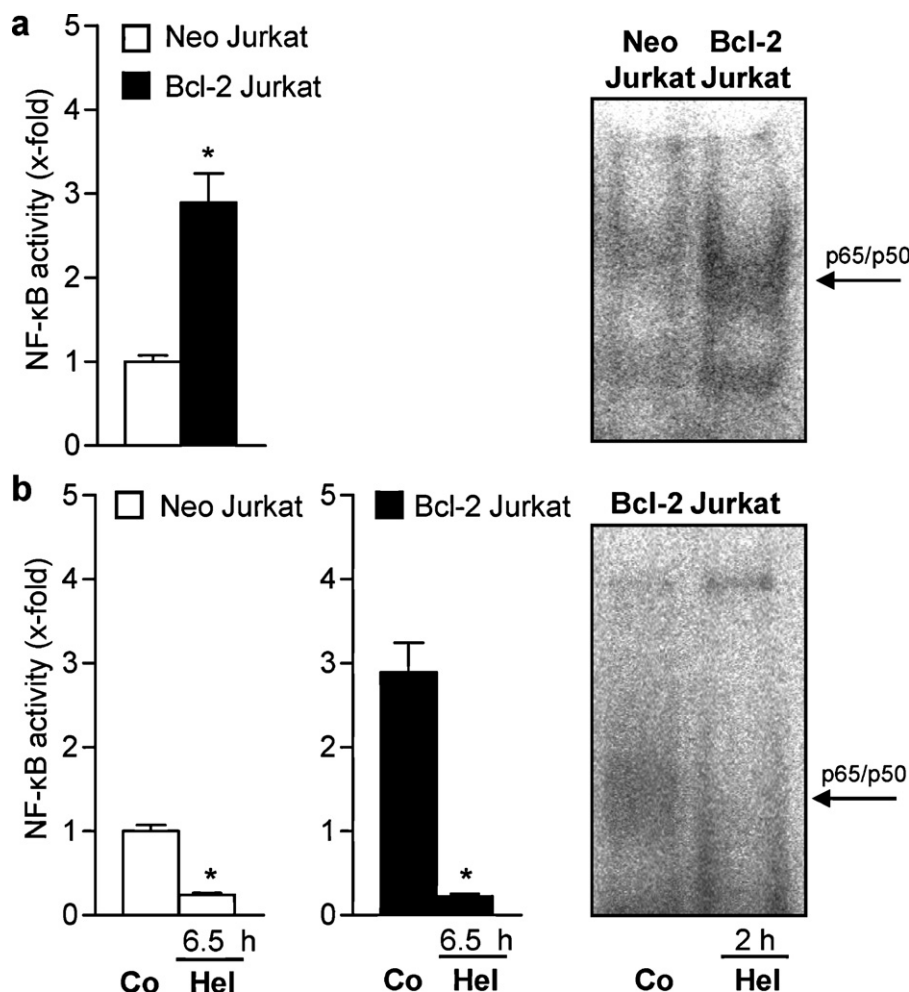


Fig. 6. Bcl-2 Jurkat cells show increased NF-κB activity, which is inhibited by helenalin. (A) NF-κB-dependent transactivation and DNA-binding activity was analyzed in untreated Neo Jurkat and Bcl-2 Jurkat cells, using a reporter-gene assay as well as an EMSA performed as described in Section 2. NF-κB activity is diagrammed as x-fold activity referring to untreated Neo Jurkat cells. * $p < 0.001$ (Student's *t*-test). (B) Neo or Bcl-2 Jurkat cells were treated with helenalin (Hel, 20 μ M) for 6.5 h and NF-κB-dependent transactivation and DNA binding activity was determined by a reported-gene assay (left panel) and EMSA (right panel), respectively. * $p < 0.0001$ (Student's *t*-test) versus controls.

cells. The major mechanisms that mediate this important feature are (1) the inhibition of Bcl-2-induced upregulation of NF-κB activity and (2) the generation of reactive oxygen species.

Sesquiterpene lactones (STL) such as helenalin comprise a large group of secondary plant metabolites with various biological activities that are linked to the high reactivity of their α,β -unsaturated carbonyl structures with thiol groups by a Michael-type reaction [34]. Interestingly, helenalin has recently gained considerable attention as a lead structure for the treatment of inflammation [35,36], whereas the related STL parthenolide has been preferentially appreciated as experimental tumor drug [37–39]. However, our work and several other groups attribute an *in vitro* as well as *in vivo* antitumor activity also to helenalin [17,40–42]. Both compounds have been shown to interfere with the signal transduction of NF-κB [35,43] playing a pivotal role both in inflammation and cancer [44,45]. One of the most documented functions of NF-κB in cancer is its ability to promote cell survival by the induction of target gene products that inhibit the apoptotic machinery in both normal and malignant cells [46]. However, NF-κB can also prevent programmed necrosis by inducing genes that encode antioxidant proteins [47]. Thus, helenalin-induced ROS production in conjugation with an impaired antioxidative defense of helenalin-treated tumor might account for its success.

Interestingly, in most cases NF-κB is activated in malignant cells in response to inflammatory stimuli originating from the microenvironment [16]. In addition, intrinsic mutations of the upstream regulators of NF-κB such as receptor activator of NF-κB (RANK), Notch1 or CD40 activity are discussed. Importantly, especially with respect to our work, a link between Bcl-2 and constitutive activation of NF-κB has been described in embryonic kidney cells as well as in myocytes [12,13]. Moreover, down-regulation of Bcl-2 was shown to attenuate NF-κB activation [13]. However, others report that Bcl-2 inhibits NF-κB activity and subsequently apoptosis in serum-deprived embryonic kidney cells [14].

In this study we show that Bcl-2 overexpressing Jurkat T cells have increased NF-κB activity compared to empty vector control cells. These data are supported by several other observations: (1) lymphoma cells with the t(14;18) translocation show high levels of NF-κB [15] and (2) in breast cancer and leukemic (CLL) cells high NF-κB expression was associated with high Bcl-2 expression [48]. In fact, it has been shown for MCF-7 breast cancer cells that Bcl-2 overexpression enhances NF-κB activity and induces MMP-9 transcription [32]. Thus Bcl-2 might not only prevent cell death by heterodimerizing with pro-apoptotic BAX or BAK proteins, subsequently abrogating the intrinsic mitochondrial pathway of

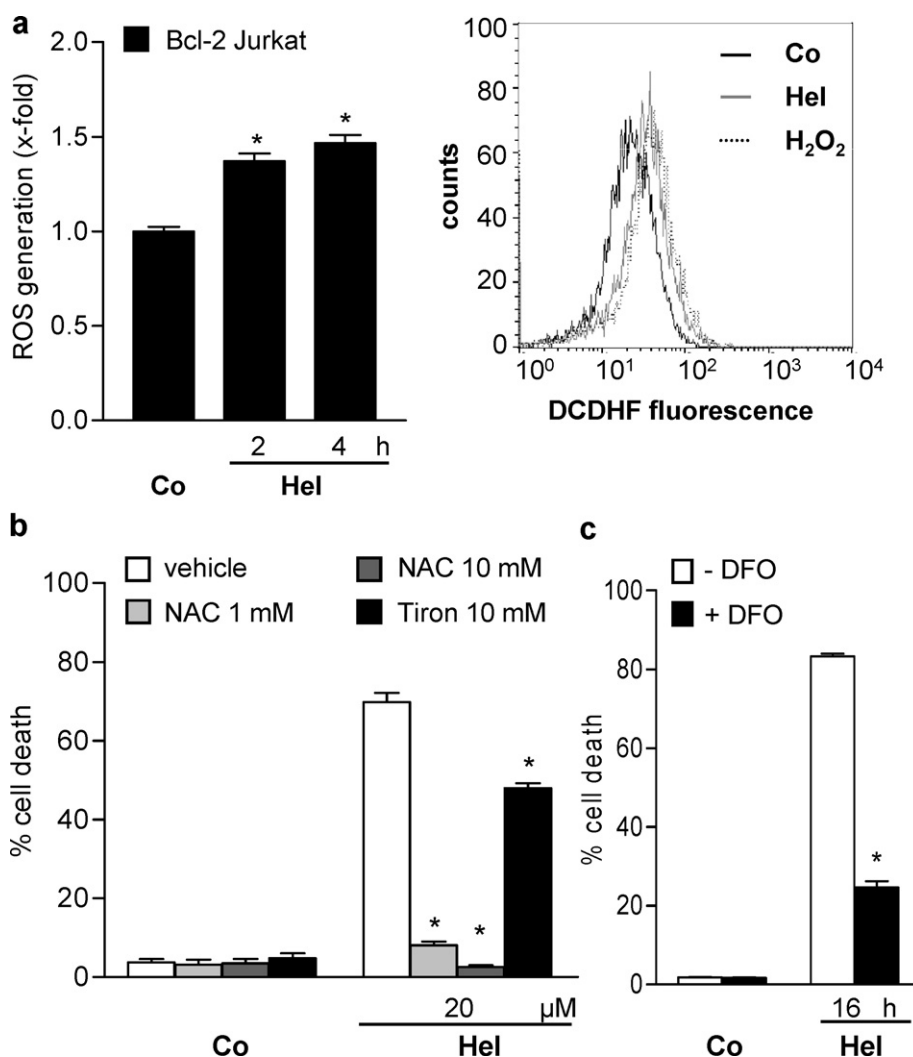


Fig. 7. Helenalin induces cell death by generating ROS. (A) After stimulation with helenalin (Hel; 20 μ M) for the indicated times, Bcl-2 Jurkat cells were stained with DCDHF diacetate and analyzed by flow cytometry. The x-fold increase of ROS generation compared to untreated (Co) cells is shown. * $p < 0.001$ (ANOVA, Bonferroni) (left panel). A representative histogram plot is shown (Hel, 20 μ M and H_2O_2 , 17.6 μ M, 30 min) on the right panel. (B) Bcl-2 Jurkat cells were either treated with vehicle control or helenalin (Hel; 20 μ M) for 24 h. When indicated, cells were preincubated with NAC or Tiron for 1 h before treatment with helenalin. * $p < 0.001$ (ANOVA, Bonferroni). (C) Bcl-2 Jurkat cells were treated with helenalin alone (Hel; 20 μ M), or in combination with DFO (20 μ M) for 16 h. * $p < 0.001$ (ANOVA, Bonferroni). Data are expressed as mean \pm SEM ($n = 3$).

apoptosis, but also by inducing a NF- κ B-driven survival machinery. Using helenalin as tool we could confirm this notion. Helenalin is a known inhibitor of NF- κ B [35] and although the inhibitory action of STLs on NF- κ B is undisputed, their molecular mechanism of action remains a matter of debate. Surface plasmon resonance analysis supported earlier reports that helenalin exerts a direct inhibitory effect on the activated p65 moiety rather than targeting I κ B kinase β (IKK β) [35,49]. Importantly, next to helenalin the NF- κ B inhibitor BMS is able to block Bcl-2-mediated death resistance. This might open up a novel strategy to overcome Bcl-2-induced chemoresistance at least in leukemic cells.

Interestingly, helenalin does not act as specific Bcl-2 inhibitor since neither typical mitochondrial activation features such as cytochrome *c* release, disruption of mitochondrial membrane potential and caspase activation nor classical apoptosis (phosphatidylserine translocation) could be seen in response to helenalin but are reported for specific Bcl-2 inhibitors such as the compound ABT-737 [7]. In contrast, helenalin is obviously able to bypass the survival mechanism of Bcl-2 at mitochondria. This is certainly a very important and promising feature with respect to chemoresistance that is frequently a consequence of deregulated apoptotic signaling.

Next to mitochondria, Bcl-2 may act also at the endoplasmic reticulum (ER) influencing ER-stress-mediated cell death. The ER represents the most important storage site for Ca^{2+} in the cell. Upon ER stress, high amounts of Ca^{2+} can be released into the cytosol, mediating further downstream effects such as apoptosis [50] or autophagy [51]. We could not observe increased calcium level in response to helenalin treatment, although helenalin has been reported to decrease cellular glutathione levels [52], which is frequently associated with an increase of resting intracellular calcium, leading to cytotoxicity [53]. Furthermore, helenalin does not alter the expression of BiP/GRP78 and CHOP/GADD153, two markers for ER-stress [54,55]. Also distinct hallmarks of autophagy such as conversion of LC3 I to membrane-bound LC3 II were missing [27,28]. Finally, the lack of phosphatidylserine translocation in helenalin-treated Bcl-2 overexpressing cells and the early onset of membrane permeability (PI positive staining) lead us to the assumption that helenalin induces cell death by necrosis. Necrosis often occurs when cell death programs such as apoptosis or autophagy are blocked [56]. Thus, necrosis is a mechanism that overcomes resistance to apoptosis as has been observed in a variety of human tumors [2] and in apoptosis-defective breast carcinomas treated with an anthracycline-based therapy [57]. There

is now evidence that necrosis, traditionally considered an accidental form of cell death, can be initiated or modulated by programmed control mechanisms. ROS, calcium-ions, poly-ADP-ribose polymerase (PARP), calcium activated non-lysosomal proteases (calpains) and cathepsins are some of the mediators of necrosis [58].

Recently two forms of programmed necrosis have been described: necroptosis and PARP1-mediated necrotic death [29]. Necroptosis is a form of programmed necrotic cell death under conditions where apoptosis is impeded [30]. Although induction of autophagy has been observed in a number of cell lines by necroptotic signaling, autophagy seems to be a downstream consequence of necroptosis rather than a contributing factor. RIP1 translocates to mitochondria causing disruption of the bonding of ANT with cyclophilin D leading to rapid mitochondrial dysfunction that is associated with necroptosis [29]. Necrostatins are well characterized inhibitors of RIP1 and therefore commonly used inhibitors of necroptosis. Since the RIP-1 inhibitor necrostatin-1 did not influence helenalin-induced cell death and we did not observe induction of autophagy, helenalin induced cell death is not due to necroptosis. Another form of programmed necrosis, PARP1-mediated necrosis, is also unlikely to be induced by helenalin in Bcl-2 overexpressing Jurkat cells since the two major players of this pathway, AIF and JNK [29], are not involved in helenalin-induced cell death, shown by use of AIF siRNA and the JNK inhibitor SP600125.

ROS represent important signaling molecules that help to transduce necrotic signals, e.g. upon stimulation with TNF α [55]. We could show that helenalin induces early ROS generation, which is responsible for cell death induction in Bcl-2 Jurkat cells since helenalin-mediated cell death was completely blocked by treatment with the antioxidant NAC and attenuated by the radical scavenger Tiron. Massive ROS generation can lead to both apoptosis and necrosis. Apoptosis induction by H₂O₂ for instance is mediated by the release of cytochrome *c* and the activation of transcription factors like NF- κ B, which may upregulate death proteins or produce inhibitors of survival proteins [55]. As Bcl-2 overexpression protects cells from cytochrome *c* release and helenalin inhibits NF- κ B, cells are potentially forced to switch to necrosis. Inhibition of constitutively active NF- κ B causes the downregulation of ferritin heavy chain that leads to an increase of free intracellular iron, which can in turn induce massive production of ROS as reported in cutaneous T-cell lymphoma [33]. Our own data support this notion for Bcl-2 overexpressing leukemia cells as well: helenalin-induced cell death is significantly decreased following treatment with the iron-chelator DFO. Thus, the inhibition of NF- κ B, free intracellular iron and ROS are mediators of helenalin-induced cell death in Bcl-2 overexpressing cells. However, helenalin might also stimulate ROS upstream of NF- κ B as a combination of BMS and helenalin partly abrogates ROS production. Neither inhibition of Akt nor JNK activation contributed to helenalin-mediated cell death in Bcl-2 Jurkat cells.

In summary, helenalin abrogates Bcl-2-mediated chemoresistance not by directly targeting Bcl-2-induced mitochondrial resistance; helenalin rather inhibits augmented NF- κ B activity in Bcl-2 overexpressing tumor cells and promotes production of ROS leading to necrosis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2011.05.029.

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