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UNBS1450, a steroid cardiac glycoside inducing apoptotic cell death in human leukemia cells

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ABSTRACT

Cardiac steroids are used to treat various diseases including congestive heart failure and cancer. The aim of this study was to investigate the anti-leukemic activity of UNBS1450, a hemi-synthetic cardenolide belonging to the cardiac steroid glycoside family. Here, we report that, at low nanomolar concentrations, UNBS1450 induces apoptotic cell death. Subsequently, we have investigated the molecular mechanisms leading to apoptosis activation. Our results show that UNBS1450 inhibits NF-κB transactivation and triggers apoptosis by cleavage of pro-caspases 8, 9 and 3/7, by decreasing expression of anti-apoptotic Mcl-1 and by recruitment of pro-apoptotic Bak and Bax protein eventually resulting in cell death.

1. Introduction

Most anti-cancer drugs are isolated from natural sources or are at least bearing close structural relationships to natural compounds. Both, marine and terrestrial organisms are considered today as important sources for novel lead compounds.

Non-cardiotonic steroid glycosides obtained from the tropical plant *Calotropis procera* possess anti-cancer potential via binding to the α3β1 Na^+/K^+-ATPase isozymes. The cardenolide UNBS1450 (Fig. 1A) has been analyzed *in vitro* as well as *in vivo* on non small cell lung cancer (NSCLC) and glioblastoma xenograft mice models [1,2]. UNBS1450 is a chemically modified form of 2-oxovoruscharin, a derivative of voruscharin; despite its already promising anti-cancer potential, the high toxicity of 2-oxovoruscharin required chemical modifications to improve tolerance *in vivo* [3]. The improved hemi-synthetic compound UNBS1450 revealed itself to be significantly less toxic when compared to its mother compound and in the same time was proven to be even more potent in cancer therapy: with an average MTD of up to 120 mg/kg, chronic administration increased mouse survival and reduced glioblastoma cell migration. Mijatovic et al. showed that UNBS1450-mediated antitumor activity is due to activation of non-apoptotic cell death mechanisms in Na^+/K^+-ATPase α1 over-expressing glioma cells [4]. UNBS1450 has the capacity to disorganize nucleolar structure and functions via impairment of cyclin-dependent kinase and c-Myc expressions accompanied by disorganization of cancer cell-specific perinucleolar bodies. This non-apoptotic cancer cell death was a new cardenolide-induced mechanism of antitumor action [5] and this drug was described to be able to circumvent glioblastoma resistance to apoptosis [6].

Nuclear factor (NF)-κB has been shown previously to be implicated in (1) cell death (apoptosis), (2) cell adhesion, (3) cell proliferation, (4) innate- and adaptive-immune response and (5) cancer development. This dimeric protein complex can both induce and repress gene expression, by binding to DNA sequences found in promoter and enhancer regions. NF-κB hetero- and homodimers can be constituted of five different family members: RelA (p65), RelB, c-Rel, p50/p105 (NF-κB1) and p52/p100 (NF-κB2) [7]. In the absence of an extrinsic cell signal, NF-κB dimers remain sequestered in the cytoplasm, inactivated by the NF-κB inhibitor IκB. Upon cell stimulation by cytokines, proliferating agents, carcinogens or physical stress, IκB phosphorylation by the Iκ kinase (IKK) frees the NF-κB dimer, then rapidly translocating into the nucleus and transactivating more than 550 genes.

Deregulation of NF-κB leads to various pathologies including chronic inflammations and cancer [8]. NF-κB has become a major target in drug engineering. To date, a number of natural NF-κB inhibitors are being investigated, many of them being plant-derived isoprenoids and polyphenols.

As NF-κB is responsible for increased cell resistance towards possible cytocidal treatments currently applied in clinics, apoptosis

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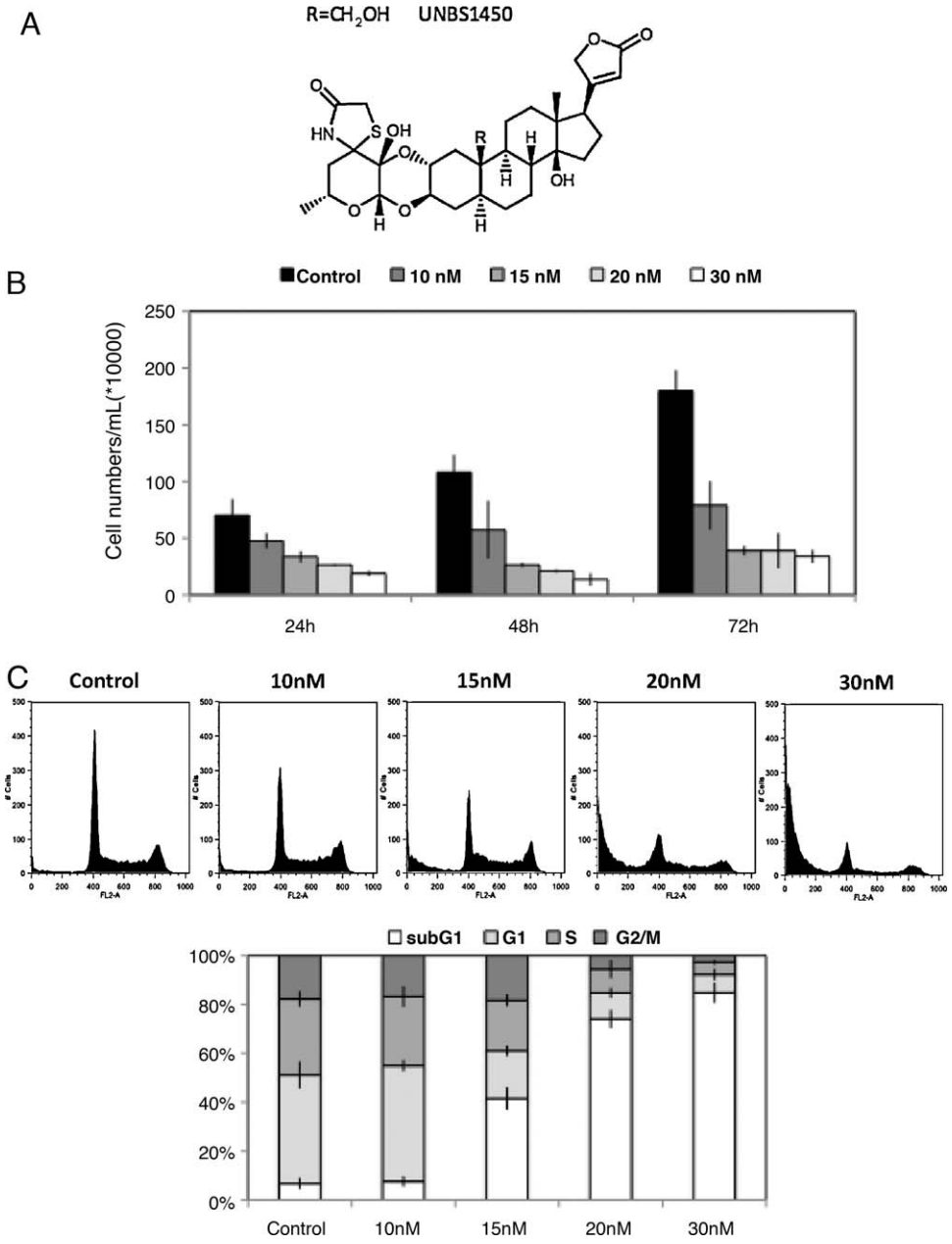

Fig. 1. UNBS1450 induces apoptotic cell death in U937 cells. (A) Molecular structure. (B) Analysis of UNBS1450-induced cell death was performed by Trypan Blue staining after 24, 48 and 72 h of treatment with UNBS1450 at 10, 15, 20 and 30 nM. (C) Cell cycle analysis after 24 h of incubation time with indicated concentrations. (D) Hoechst staining (upper panel) and quantification (lower panel) of the fraction of cells presenting fragmented nuclei. (E) Flow cytometry analysis after 24 h of incubation time at indicated concentrations. (F) Analysis of PBMCs viability after UNBS1450 treatment. PBMCs were seeded at 2×10^6, then after 24 h treated for 24 h with various concentrations (0–100 nM) of UNBS1450. Cells were then stained either by Trypan Blue (upper panel) or by Hoechst (panel below) to analyze either cell integrity or apoptosis induction. The data shown here were representative for three independent experiments.

induction through NF-κB inhibition and/or extrinsic and intrinsic pathway activation is considered as a main aim in cancer research. Apoptosis induction through NF-κB inhibition and subsequent extrinsic and intrinsic cell death pathway activation is considered a main target in cancer research. During apoptosis induction, many different activation cascades eventually lead to caspase-3 or -7 cleavage and subsequent cell death [9].

In the absence of concluding investigations of anti-cancer effects of UNBS1450 in human leukemia cells, we hereby analyzed the effect of UNBS1450 on activation of cell death mechanisms and NF-κB pathway inhibition in human leukemia cells and demonstrate for the first time that nanomolar concentrations of UNBS1450 induce canonical apoptotic cell death mechanisms.

2. Material and methods

2.1. Compounds and purification

TNFα was purchased from Sigma–Aldrich (Bornem, Belgium) and dissolved to a concentration of 10 mg/mL in PBS 1× supplemented with 0.5% (w/v) BSA according to the manufacturer's instructions.

UNBS1450 was a gift of Unibioscreen (Pr Robert Kiss, Brussels, Belgium): the compound, with a molecular weight of 605.8 g and received as dry powder, was solubilized in 52.95% DMSO (Sigma–Aldrich, Bornem, Belgium) and then further diluted to 5% DMSO/95% H_2O to get working aliquots with a concentration of 5 mM.

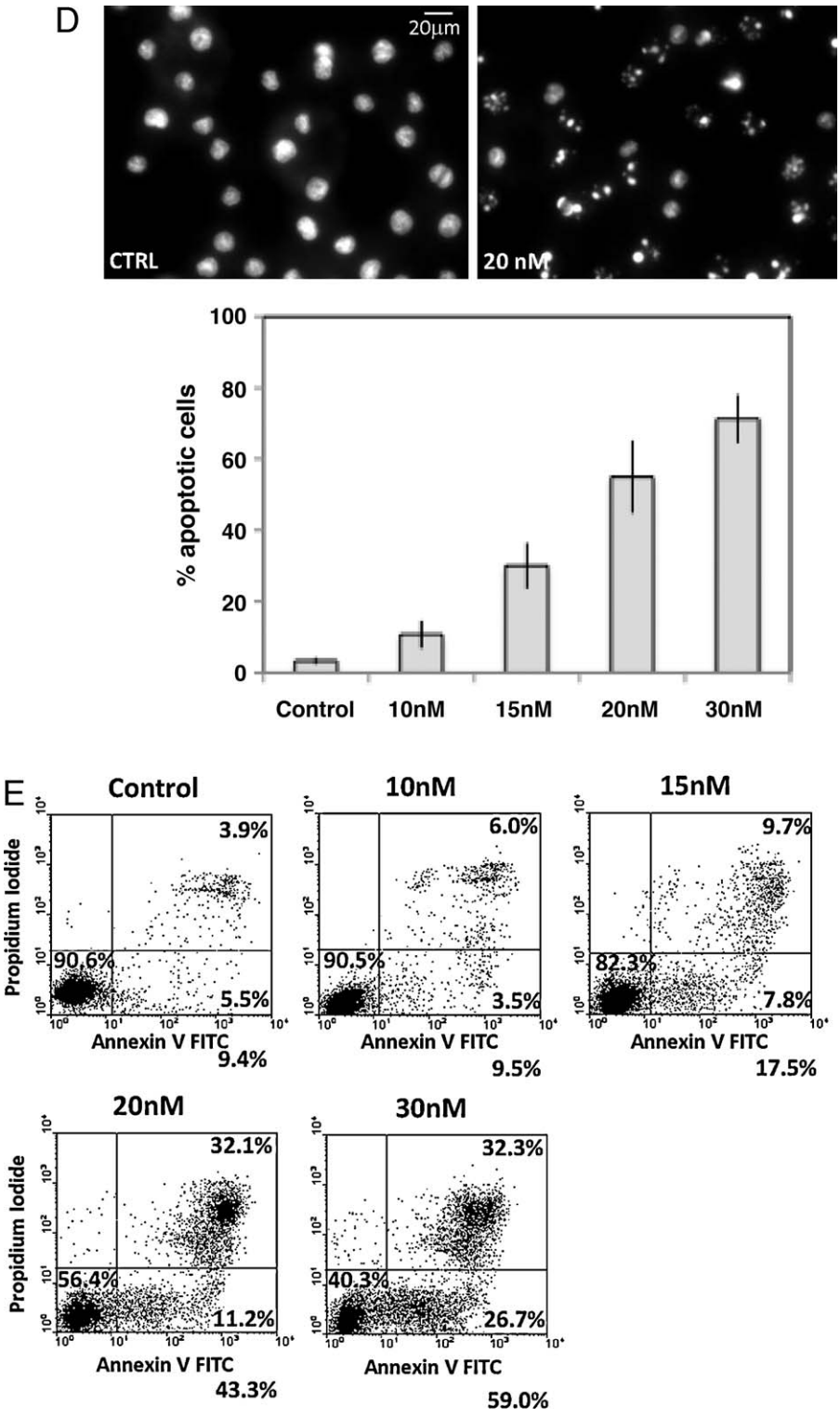

Fig. 1. (*Continued*).

Both were frozen at −20 °C. Control cells were treated with equivalent amounts of DMSO.

2.2. Cell culture

K562 (human chronic myelogenous leukemia), U937 (histiocytic lymphoma), Jurkat (T-cell leukemia), Raji (Burkitt's Lymphoma), Hel (erythroleukemia), Molt (human acute lymphoblastic leukemia), Meg01 (human megacaryoblastic cells), HL60 (human promyelocytic leukemia), TF1 (erythroleukemia) and KBM5 (chronic myelogenous leukemia) cells (DSMZ) were cultured in RPMI medium (Lonza, Verviers, Belgium) supplemented with 10% (v/v) fetal calf serum (Lonza, Verviers, Belgium) and 1% (v/v) antibiotic–antimycotic (BioWhittaker, Verviers, Belgium) at 37 °C and 5% of CO_2. At T_0 cells had been pre-treated with UNBS1450 at various concentrations. At T_0 + 2 h cells were activated by TNFα (20 ng/mL).

Healthy blood samples were kindly donated as buffy coats by the Red Cross (Luxembourg, Luxembourg). By applying diluted (1/3)

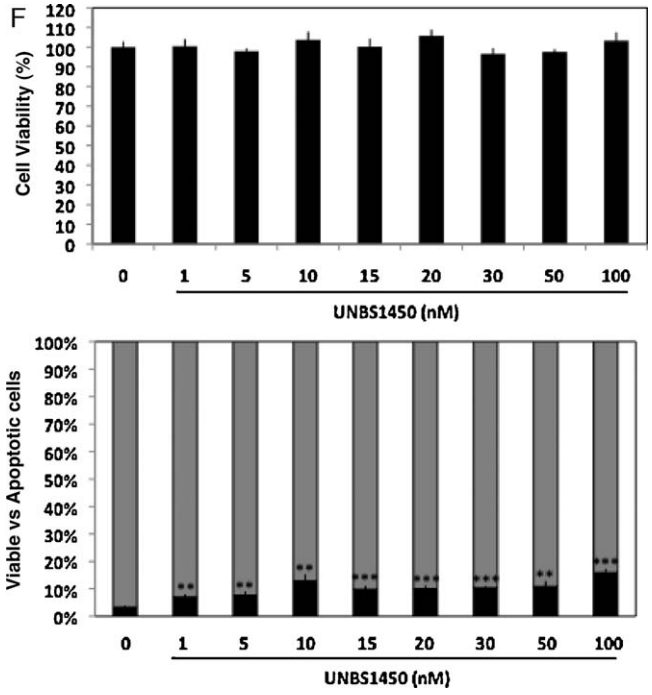

Fig. 1. (*Continued*).

blood onto a Ficoll layer followed by centrifugation (400 × *g*, 20 min), mononuclear cells were isolated and collected. The isolated peripheral blood mononuclear cells (PBMC) were kept in culture at 37 °C and 5% CO_2 for 24 h before they were subjected to treatments.

2.3. Electrophoretic mobility shift assay (EMSA)

Both, K562 and Jurkat cells, cultured in growth medium (RPMI, 10% FCS) at a concentration of 3×10^5 cells/mL, were pre-treated or not for 2 h with UNBS1450, respectively at 40 nM and 20 nM before being activated by TNFα (20 ng/mL) for 6 h. Cells were then harvested and washed twice in PBS 1×; supernatants were discarded and cell pellets were stored overnight at −80 °C. Nuclear protein extractions were performed as described previously before being stored at −80 °C [10]. The oligonucleotide NF-κBc (consensus NF-κB site) (Eurogentec, Liège, Belgium) (5′-AGTTGAGGGGACTTTCCCAGGC-3′) and its complementary sequence were used as probe. After being hybridized, the probes were radiolabelled using [g-^{32}P]ATP (MP Biomedicals, Illkirch, France) and the EMSA assay was realized according to conditions established before [10].

2.4. Transient transfection and luciferase reporter gene assay

K562 and Jurkat cells were transiently transfected as described previously [10]. For each electroporation we used 5 μg of a luciferase reporter gene construct containing five repeats of a consensus NF-κB site (Stratagene, Genomics Agilent, Diegem, Belgium) and 5 μg of a *Renilla* luciferase plasmid (Promega, Leiden, Netherlands). Cells were resuspended in normal culture medium (RPMI, 10% FCS) after electroporation and cultured at 37 °C and 5% CO_2 for 24 h. After the required incubation time, cells were harvested and resuspended in fresh growth medium (RPMI, 10% FCS) to a final concentration of 3×10^5 cells/mL and pre-treated for 2 h with UNBS1450 40 nM for K562 and 20 nM for Jurkat. Activation with 20 ng/mL of TNFα for 6 h followed the pre-treatment. After the 8 h of total challenge, 75 μL of Dual-Glo™ luciferase reagent (Promega, Leiden, Netherlands) were added to 75 μL of the cellular suspension for 10 min incubation at 22 °C before luciferase activity measurement. Further on, 75 μL of Dual-Glo™ Stop&Glo® Reagent (Promega, Leiden, Netherlands) were added for 10 min at 22 °C to the cell suspension to assay *Renilla* activity. An Orion microplate luminometer (Berthold) was used to measure luciferase and *Renilla* activity. The results are expressed as a ratio of arbitrary units of firefly luciferase activity normalized to *Renilla* luciferase activity.

2.5. Cell viability assessment

Percentages of cell survival were evaluated using Promega's CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Leiden, Netherlands) kit, according to the manufacturer's instructions. Alternatively, Trypan Blue staining was used to determine cell integrity. Data were normalized to the control and reported as percentage of viable cells.

2.6. Analysis of apoptosis

2.6.1. Analysis of nuclear fragmentation

Percentages of apoptotic cells, quantified as the fraction of apoptotic nuclei, were assessed by fluorescence microscopy (Leica-DM IRB microscope, Lecuit, Luxembourg) upon staining with the DNA-specific dye Hoechst 33342 (Sigma–Aldrich, Bornem, Belgium) as previously described [11]. The fraction of cells with nuclear apoptotic morphology was counted (at least 300 cells in at least three independent fields). The images were analyzed using the Image J software (http://rsb.info.nih.gov/ij/docs/index.html).

2.6.2. Flow cytometric analysis (Annexin V-FITC/propidium iodide staining) of phosphatidylserine exposure

At the indicated times and doses of treatment, K562, U937 and Jurkat cells were assayed for phosphatidylserine exposure, by using the Annexin V-FITC Apoptosis Detection Kit I (Becton Dickinson Biosciences, Erembodegem, Belgium) according to the manufacturer's instructions. Stained samples were analyzed by FACS (FACSCalibur, Becton Dickinson, San Jose, CA, USA). Data were recorded using the CellQuest software (http://www.bdbiosciences.com/features/products) for further analysis.

2.7. Extraction of cellular proteins

After indicated incubation times with UNBS1450 and TNFα, K562, U937 and Jurkat cells were lysed; nuclear and cytoplasmic extracts were prepared according to Duvoix et al. [10].

2.8. RT-PCR analysis

Isolation of total RNA was performed using a NucleoSpin® (Macherey–Nagel, Hoerdt, France) kit. One microgram of total RNA were submitted to reverse transcription (RT) using Oligo(dT) primers (SuperScript First-Strand Synthesis System, Invitrogen, Fisher Scientific, Tournai, Belgium). The resulting RT products were used as templates for PCR amplification using SYBR® Green (Power SYBR Green PCR Master mix 1×, Applied Biosystems, Halle, Belgium) and Na^+/K^+-ATPase α-subunit mRNA specific primers (F: 5′-CTA-CCT-GGC-TTG-CTC-TGT-CC-3′; R: 5′-GCT-GAC-TCA-GAG-GCA-TCT-CC-3′) (Eurogentec, Liège, Belgium). The amount of cDNA synthesized was evaluated by amplification of the GAPDH gene (F: 5′-ACA-GTC-AGC-CGC-ATC-TTC-TT-3′; R: 5′-ACG-ACC-AAA-TCC-GTT-GAC-TC-3′) (Eurogentec, Liège, Belgium) as a standard. After amplification, the PCR products were separated on a 2% agarose gel.

2.9. Immunofluorescence staining

U937 cells were fixed and permeabilised according to the manufacturer's instructions using the BD Cytofix/Cytoperm Kit

(Becton Dickinson, Erembodegem, Belgium). Incubations with primary antibodies were performed in BD Perm/Wash solution for 1 h at room temperature with 10 mg/ml of one of the following antibodies: anti-Bax (6A7) [mouse, monoclonal (Santa Cruz Biotechnology, Boechout, Belgium)], anti-Bak (AB-1) [mouse, monoclonal, (Calbiochem, Leuven, Belgium)], both recognizing active/activated forms of the proteins. After washing twice with PBS, cells were incubated with the corresponding secondary antibody at concentrations of 8 mg/ml at room temperature for 30 min on a shaking Platform (mouse, Alexafluor 488, green fluorescence; rabbit or mouse, Alexafluor 568, red fluorescence; Invitrogen/Molecular Probes, Merelbeke, Belgium). After two additional washing steps with PBS, cells were counterstained with Hoechst 33342 (DNA specific, blue fluorescence) and monitored by fluorescence microscopy (Olympus, Hamburg, Germany). The images were analyzed and elaborated using the Cell^M software (Olympus Soft Images Solutions GMBH, Germany).

2.10. Western blot analysis

Proteins of total extracts were separated by size using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 10%), transferred onto nitrocellulose membranes and blocked with 5% non-fat milk in phosphate buffered saline (PBS)–Tween overnight. Equal loading of samples was controlled using β-actin or lamin B and α-tubulin for cytosolic and nuclear extracts.

Blots were incubated with primary antibodies: anti-pIκBα (1/3000, Cell Signaling 9246, Bioké, Leiden, Netherlands), anti-IκBα (1/500, Santa Cruz SC-371, Tebu-Bio, Boechout, Belgium), anti-p50 (1/5000, Santa Cruz SC-7178X), anti-p65 (1/5000, Santa Cruz SC-8008), anti-α-tubulin (1/5000, Calbiochem CP06, VWR, Leuven, Belgium), anti-lamin-β (1/1000, Santa Cruz SC-6216), anti-β-actin (1/10000, Sigma–Aldrich A5441, Bornem, Belgium), anti-caspase-3 (1/5000, Cell Signaling 9668), anti-caspase-7 (1/1000, Cell Signaling 9494), anti-caspase-8 (1/1000, Cell Signaling 9746), anti-caspase-9 (1/1000, Cell Signaling 9502), anti-XIAP (1/1000, BD Pharmingen 610763, Erembodegem, Belgium), anti-Bcl-2 (1/2000, Calbiochem OP60) and anti-Mcl-1 (1/1000, Cell Signaling 4572). All antibodies were diluted in a PBS–Tween solution containing 5% of bovine serum albumin (BSA) or 5% of milk according to the providers' protocols. After incubation with primary antibodies, membranes were washed 3 × 10 min with PBS–Tween, followed by an incubation of 1 h at RT with the corresponding secondary (HRP-conjugated) antibodies. After washing 3× 10 min with PBS–Tween, specific immunoreactive proteins were visualized by autoradiography using the ECL Plus Western Blotting Detection System Kit® (GE Healthcare, Roosendaal, Netherlands).

2.11. Elisa

IL-8 concentrations in culture supernatants of activated K562, U937 and Jurkat cells were measured by sandwich ELISA (R&D Systems, Abingdon, United Kingdom). According to the manufacturer's guide, 50 μL of cell supernatants were added together with 100 μL of assay diluent to anti-IL-8 pre-coated wells, followed by 2 h of incubation at RT. After washing, a polyclonal peroxydase-conjugated anti-IL-8 antibody was added for another 60 min at RT. Colorimetric visualization and protein dosage were rendered possible by addition of the H_2O_2 and TMB (tetramethylbenzidine) containing substrate. After 30 min of reaction at RT, in the dark, the enzymatic reaction was stopped by H_2SO_4 and optical densities were measured at a wavelength of 450 nm.

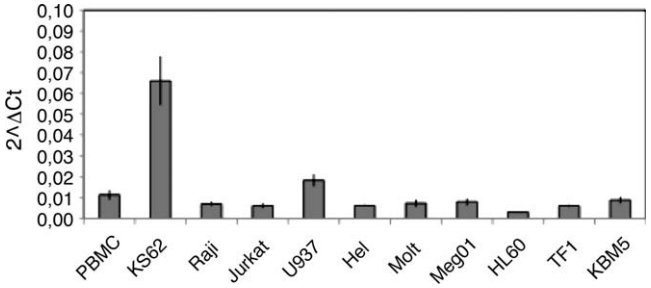

Fig. 2. Na^+/K^+-ATPase subunit α1 mRNA quantification. Na^+/K^+-ATPase subunit α1 mRNA content of untreated PBMCs and a wide panel of hematological cancer cell lines including K562, Jurkat and U937 cells was transcribed and then quantified by RT-PCR. The quantification of three independent experiments is expressed in brute 2^ΔCt values ± S.D.

2.12. Statistical analysis

Data is expressed as mean ± S.D. and its signification degree was analyzed by Student's *t*-tests. *P*-values below 0.05 were considered as statistically significant.

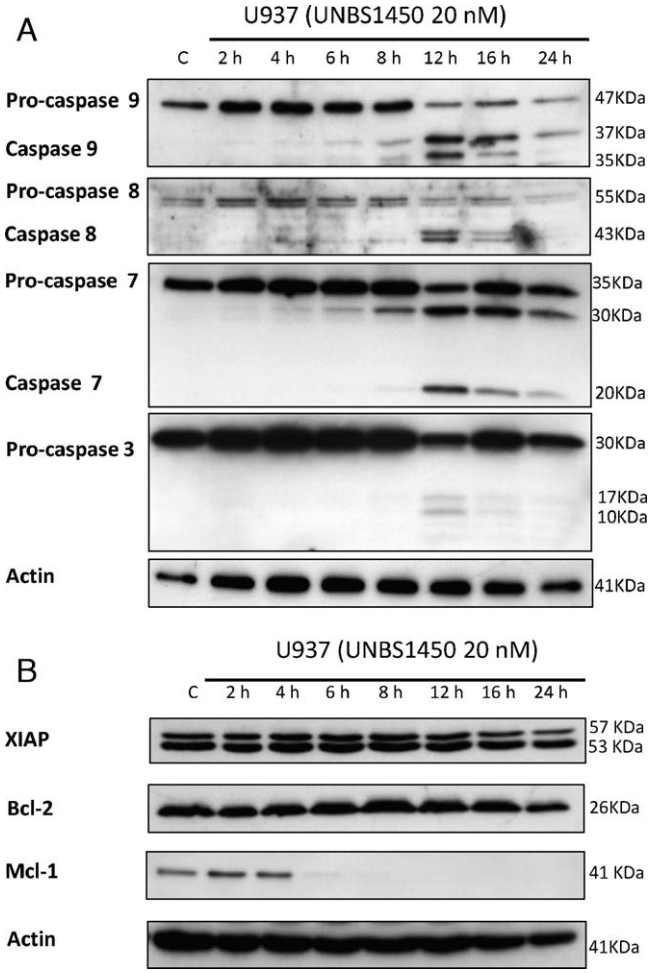

Fig. 3. (A) Caspase activation. U937 cells were incubated in RPMI + 10% FCS ± UNBS1450 20 nM up to 24 h. Western blot analysis of UNBS1450-induced cleavage of pro-caspases-9, -8, -7 and -3. (B) Analysis of expression levels of anti-apoptotic proteins. UNBS1450-induced expression level alterations of XIAP, Bcl-2 and Mcl-1. The data shown here were representative for three independent experiments.

3. Results

3.1. UNBS1450 induces apoptosis in human leukemia cells

We first evaluated the effect of UNBS1450 on the cell growth of chronic myeloid leukemia K562, histiocytic lymphoma U937 and acute T-cell leukemia Jurkat. Cells were treated for 24, 48 and 72 h with different concentrations of UNBS1450 and their culture concentrations estimated by Trypan Blue exclusion assay. As reported in Fig. 1B, UNBS1450 deeply affected cell growth in a dose-dependent manner with an IC_{50} value of 13.37 ± 2.1 nM in U937.

To disentangle whether the effects of UNBS1450 were due to an impact on cell viability rather than an effect on cell metabolism and, therefore growth, we performed at first a cell cycle analysis to detect and quantify the presence of any sub-G1 phase. The analysis reported in Fig. 1C showed that UNBS1450 lead to accumulation of cells in a sub-G1 phase in a dose-dependent manner, significantly starting from 15 nM, thus confirming that UNBS1450 induced cell death. From 20 nM to 30 nM, UNBS1450 increasingly drove U937 cells into a sub-G1 phase (Fig. 1C).

Next, we investigated the nature of cell death induced by UNBS1450, first by analyzing alterations in cell morphology by Hoechst staining and fluorescence microscopy (Fig. 1D, top). As shown, the nuclei of U937 cells treated with UNBS1450 underwent morphological alterations typically occurring during apoptosis [11]. Fig. 1D (bottom) shows the quantification of apoptosis after UNBS1450 treatment, as estimated by counting the fraction of cells stained with Hoechst and presenting fragmented nuclei. Annexin V/PI double staining confirmed apoptotic cell death (Fig. 1E) starting from 15 nM, thus giving similar results to Hoechst analysis.

In order to generalize our effects, we used K562 cells and observed similar findings. Again, UNBS1450 reduced cell proliferation in a dose- and time-dependent manner with an IC_{50} value of 32.65 ± 3.10 nM at 48 h (Supplemental Fig. 1A). Cell cycle analysis (Supplemental Fig. 1B) showed that an incubation time of 48 h in the presence of UNBS1450 50 nM drove a fraction of cells into a sub-G1 phase while overall cell status remained rather unchanged; G1 and G2/M phases still showed control-comparable patterns. Hoechst staining indicated fragmented cell nuclei of cells dying via apoptosis (Supplemental Fig. 1C). FACS analysis for the exposure of phosphatidylserine/plasma membrane integrity by Annexin V/PI analysis confirmed the activation of an apoptotic program (Supplemental Fig. 1D). Similar effects of UNBS1450 on cell death induction were observed in Jurkat T cells (Supplemental Fig. 2A and B).

Concerning the specificity of UNBS1450 for cancer cells, we further analyzed the effects of UNBS1450 on normal, healthy PBMCs (Fig. 1F). After 24 h of incubation time, PBMCs had been treated by

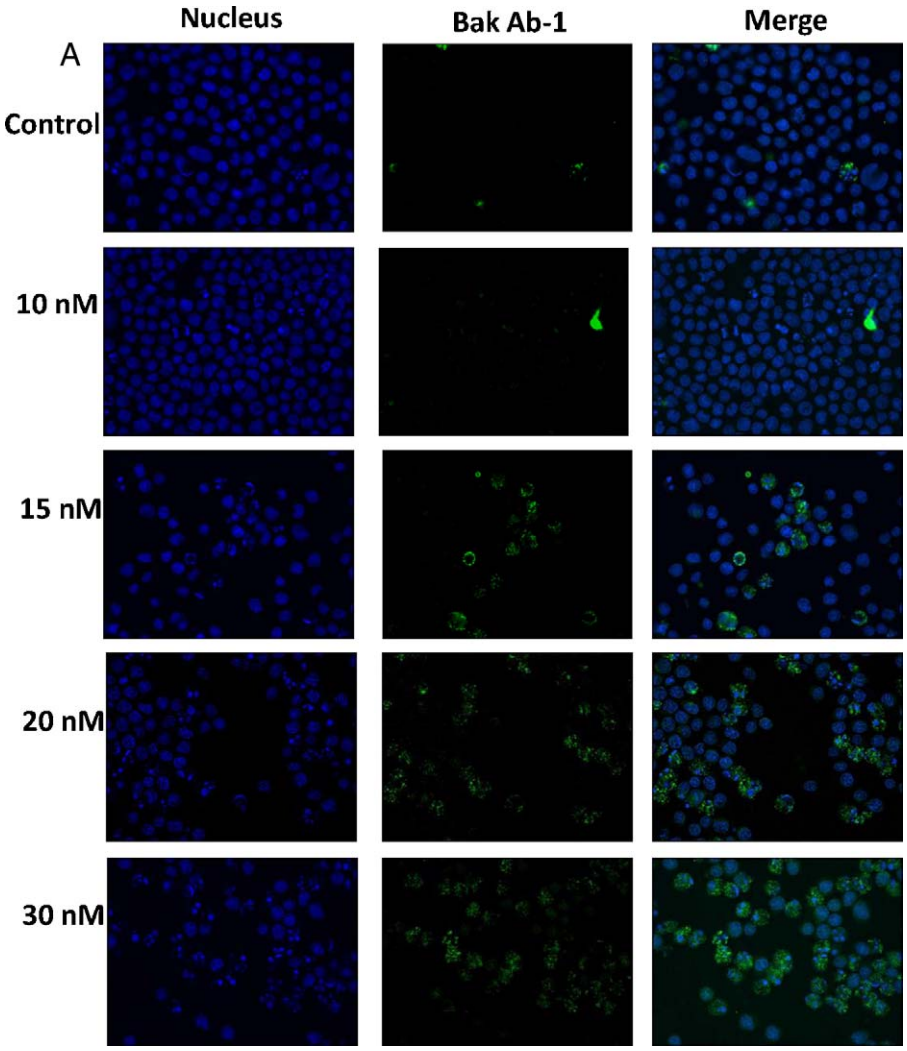

Fig. 4. UNBS1450 enables Bak/Bax activation. U937 cells were incubated for 24 h in RPMI + 10% FCS in presence or in absence of UNBS1450. Bak (A.) and Bax (B.) activation status were assessed by using primary antibodies specifically targeting activated forms of Bak (Ab-1; Calbiochem) and Bax (6A7; Santa Cruz). Counterstaining was done by Hoechst staining to assess apoptotic nuclei. The data shown here were representative for three independent experiments with similar results.

various concentrations of UNBS1450 (0–100 nM) for 24 h. Sample volumes were then divided: Trypan Blue staining was performed to assess cell integrity (upper panel) whereas Hoechst staining allowed determination of apoptotic percentages (lower panel). Altogether, UNBS1450 did not change cell permeability to Trypan Blue thus allowing us to conclude that cell viability of PBMCs is not compromised by UNBS1450. These results were confirmed by quantification of apoptotic cells: only a weak effect was observed at 100 nM ($\leq$16%) which was not comparable to what we obtained in leukemia cell lines (>60% for UNBS1450 at 30 nM in U937; Fig. 1D).

As it has been shown that the α1 subunit of Na^+/K^+-ATPases is a target for UNBS1450 [2] and that it is frequently overexpressed in solid tumor cells where UNBS1450 is severely inhibiting proliferation [2], we compared α1 subunit mRNA expression levels of a panel of hematological cancer cell lines to those of normal healthy PBMCs (Fig. 2). Our results indicate all cell lines express the corresponding mRNA but that only K562 produces significantly higher amounts compared to PBMCs (2^ΔCt values of 0.06 versus 0.011, respectively, * S.D.). For all other cell lines, subunit α1 specific mRNA levels were not significantly different compared to PBMCs.

3.2. Effect on anti-apoptotic cell signaling pathways

We subsequently investigated whether UNBS1450 was able to trigger a caspase-dependent apoptotic cell death (Fig. 3A). To this purpose, a kinetic analysis (0–24 h) was performed on U937 cells treated with UNBS1450 at 20 nM. We demonstrated that UNBS1450 at 20 nM induced the cleavage of the pro-caspases-9 and -8 starting after 12 h of incubation time. The executor pro-caspases-3 and-7, were cleaved into active caspase-3 and 7 (Fig. 3A).

Induction of apoptosis can be triggered by both inhibition of anti-apoptotic- and/or by activation of pro-apoptotic mechanisms. Here, overall expression of XIAP and Bcl-2 were not influenced by UNBS1450 (Fig. 3B). The expression level of Mcl-1 however, another anti-apoptotic Bcl-2 family member, was sensibly affected by the treatment with UNBS1450 as a strong reduction in the protein level was noticeable after 4 h of treatment while after 12 h of treatment, no expression level was detectable anymore.

3.3. Activation of pro-apoptotic mechanisms

We then analyzed *in situ* the activation status of pro-apoptotic Bak and Bax in U937 cells, untreated and treated with 10–30 nM of

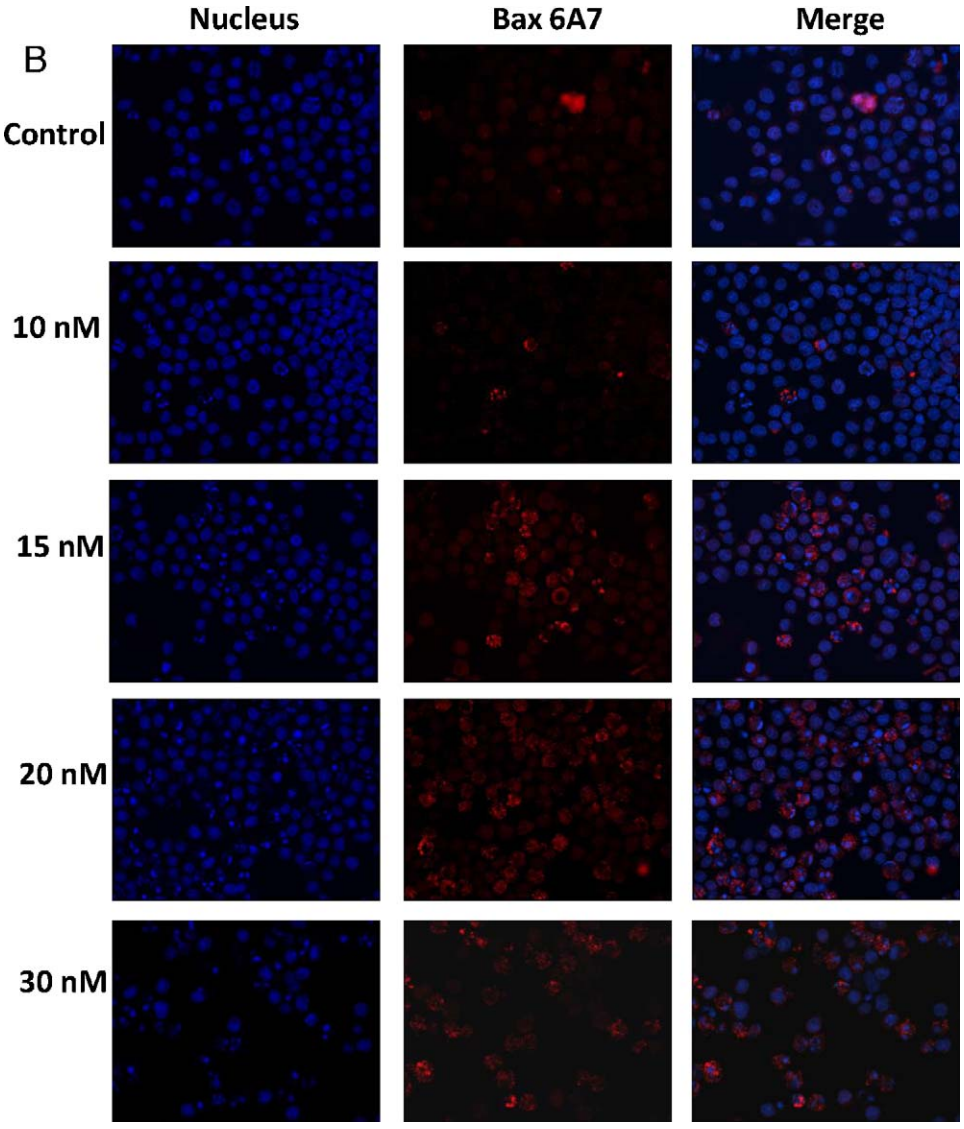

Fig. 4. (*Continued*).

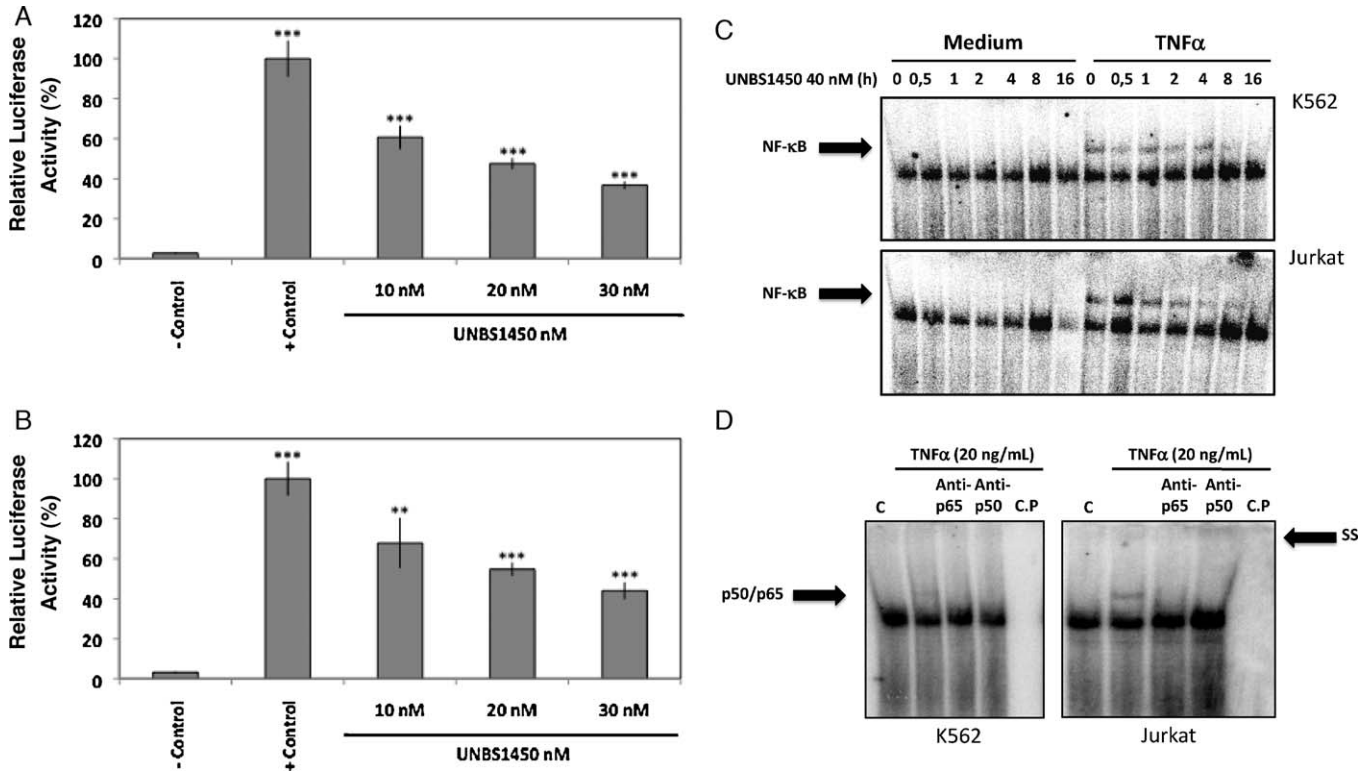

Fig. 5. Inhibition by UNBS1450 of TNFα-induced NF-κB activation. (A) K562 and (B) Jurkat cells were pretreated with UNBS1450 at various concentrations from 10 to 50 nM and incubated for 2 h, followed by TNFα addition (20 ng/ml) and an additional incubation period of 6 h. Results are represented as the ratio of the measured luminescence of the firefly luciferase vector divided by the measured luminescence of the *Renilla* plasmid. Untreated cells were used as a negative control, cells treated with TNFα only as a positive control. Results are presented as mean ± S.D. of 3 individual measurements performed in triplicates. (C) Effect of UNBS1450 on the binding affinity of NF-κB was assessed by an EMSA on the K562 and Jurkat cell lines. The data shown here were representative for three independent experiments with similar results. (D) For supershift/immunodepletion experiments, the nuclear extracts and labelled probes were incubated in the reaction mixture for 30 min on ice prior to a 30 min incubation with 2 μg of anti-p50 or anti-p65 antibodies. SS designates supershifted bands. (E) Jurkat cells were incubated with UNBS1450 (40 nM) for 2 h, followed by a TNFα (20 ng/ml) activation for the indicated time periods. Cytoplasmic and nuclear extracts were prepared, fractionated on a 10% SDS-page gel, transferred to a membrane and then tested for IκBα and p65. Protein loading and purity of nuclear/cytosolic extracts were verified by lamin B and α-tubulin Western blots. Data shown are representative for three independent experiments with similar results. K562 (F), and U937 (G) cells were incubated for 2 h in RPMI + 10% FCS in presence or in absence of various concentrations (10–50 nM) of UNBS1450 before being activated by TNFα during 22 h. After 24 h of incubation IL-8 concentrations in supernatants were measured. Untreated cells served as negative control whereas cells activated by TNFα only were used as a positive control. The data shown here were representative for three independent experiments with similar results.

UNBS1450, by using antibodies specifically recognizing activated forms of both pro-apoptotic Bcl-2 family members. Immunostained cells were counterstained with Hoechst to detect apoptotic nuclei. Bak resulted activated by treatments with 15 nM UNBS1450 and higher (Fig. 4A). Similar results were observed for activated Bax (Bax 6A7) (Fig. 4B). As expected, both activated Bak and Bax co-localized with cells presenting fragmented, apoptotic nuclei, witnessing apoptotic cell death triggered by UNBS1450 a hemi-synthetic cardenolide originally from the plant *C. procera*.

3.4. Inhibition of NF-κB pathway activation

NF-κB transcription factor is well known to act as an inhibitor of apoptotic cell signaling specifically via transactivation. Moreover Mijatovic et al. [12] already provided first insights into UNBS1450-induced inhibition of NF-κB activation. Here we observed that UNBS1450 reduced TNFα-driven NF-κB activation in a dose-dependent manner in K562 and Jurkat cells (Fig. 5A and B). As illustrated in Table 1, UNBS1450 has however no repressing effect on constitutive, basal NF-κB activity in K562 cells.

In order to further confirm the inhibitory effect of UNBS1450 on the TNFα-induced NF-κB signaling pathway, we realized EMSA. Fig. 5C clearly shows that UNBS1450, at a concentration of 40 nM inhibits TNFα-induced NF-κB–DNA binding. By applying various pre-treatment times from 0 to 16 h, we obtained a complete inhibition after 8 h of pre-incubation. Incubation with p50 and p65 antibodies enabled us to identify the NF-κB dimer composition as p50 and p65. Similar results were obtained for Jurkat cells (Fig. 5D).

NF-κB activation is generally initiated by the degradation of its natural inhibitor IκBα; we thus assessed IκBα integrity as well as the translocation of p65 from the cytoplasm to the nucleus by Western blot analysis (Fig. 5E). Both degradation of IκBα as well as translocation of p65 into the nucleus, were analyzed by Western blot. In control cells without UNBS1450 pre-treatment, IκBα degradation was observed after 10 min of TNFα stimulation; consequently, p65 translocation to the nucleus was observed in parallel. In contrast, the presence of UNBS1450 inhibited TNFα-induced degradation of IκBα and considerably prevented the translocations of p65 into the nucleus (Fig. 5E, right panel).

To further investigate the influence of UNBS1450 on downstream NF-κB signaling in K562, U937 and Jurkat cells, we chose IL-8 as it was described that this gene product is under the control of NF-κB. We observed that UNBS1450-pre-treated cells produced significantly lower quantities of IL-8 (less than 600 pg/mL) compared to TNFα only activated K562 cells (1300 pg/mL) (Fig. 5F). Similar results were obtained for U937 (Fig. 5G). Unstimulated U937 cells produced low amounts of IL-8 (92 pg/mL) and TNFα activation resulted in increased IL-8 release (1002 pg/mL). A significant

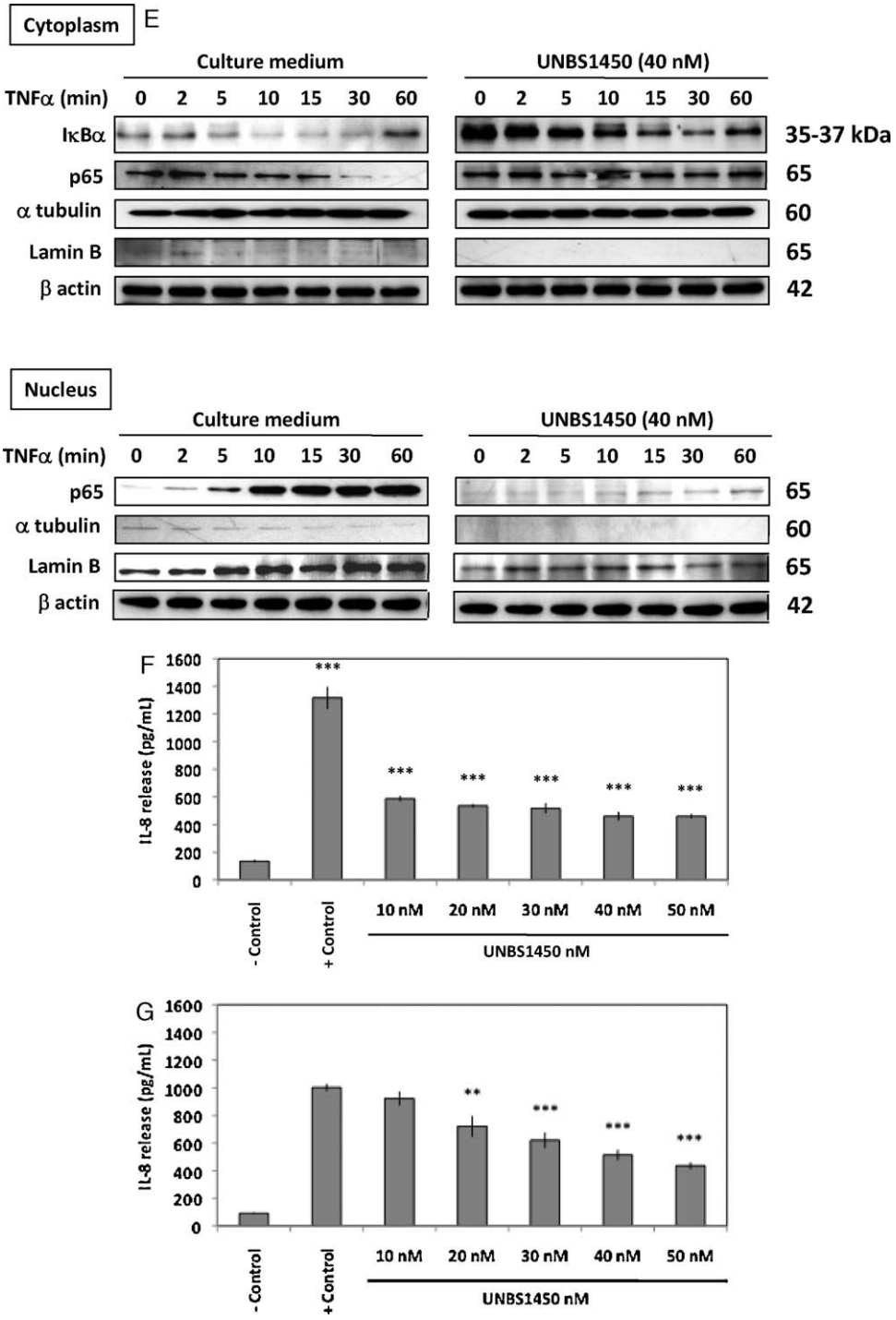

Fig. 5. (*Continued*).

inhibition was obtained after UNBS1450 treatment at 20 nM (IL-8 release: 721 pg/mL). In Jurkat cells, TNFα stimulation could not trigger IL-8 release; neither did UNBS1450 show any significant effect on IL-8 release (results not shown).

Table 1
Effect of UNBS1450 (40 nM) on basal and TNFα-induced NF-κB activity.

K562	R.L.A. (%)	S.D. (%)
Untreated	0.574	0.147
UNBS1450 (40 nM)	0.587	0.178
TNFα	100	2.850
UNBS1450 (40 nM) + TNFα	42.578	7.761

4. Discussion

We show here that UNBS1450, belonging to the cardenolide family of the steroid cardiac glycosides, efficiently reduces viability of leukemia cancer cell lines by inducing apoptotic cell death. We focused our efforts on possible modifications of inflammation and cell death induction by UNBS1450. To this purpose, we used chronic myeloid leukemia K562, histiocytic lymphoma U937 as well as acute T-cell leukemia Jurkat cells stimulated by TNFα as a model of investigation to mimic pro-inflammatory surroundings stimulating the NF-κB transcription factor.

While UNBS1450 has so far mainly been applied on solid tumors [1,12], we hereby confirm that deactivation of NF-κB

activity through UNBS1450 is also true in human leukemia cells as indicated by reduced induction of NF-κB transcriptional activity, reduced binding to target DNA as well as repressed synthesis of NF-κB-dependent genes. TNFα-induced p50/p65 dimer translocation to the nucleus is strongly reduced in the presence of UNBS1450 due to repressed IκBα degradation.

We also analyzed the impact of UNBS1450 on leukemia cell survival capacity. As finally indicated by increased Annexin V/PI staining, fragmented nuclei after Hoechst staining and thus reduced cell numbers, UNBS1450 is beneficial to an apoptosis induction. We observed that nanomolar concentrations of UNBS1450 were able to induce the cleavages of the initiator caspases 8 and 9 as shown by Western blot. Furthermore, we obtained a cleavage induction of the pro-caspase-7, leading to hypothetic thoughts that UNBS1450 might induce apoptosis by processing and concomitant activation of executors caspases such as caspase-7 and caspase-3. Consequently, we assessed expression levels of various Bcl-2 family proteins in order to conclude on a possible amplification of the death signal through activation of the intrinsic mitochondrial cell death pathways [13,14]. The analysis of cell proliferation in the presence of UNBS1450 in both cell lines investigated reveals that the compound is able to affect cell proliferation at concentrations lower than those requested to induce apoptosis (see Fig. 1B and Supplemental Fig. 1A). This is in line with previous findings [15] and prompts to elucidate in the future whether this cytostatic potential may be also implicated in determining the cytotoxic effect.

Several studies underline their pivotal role [16] in apoptosis induction [17–19]. Loss of expression as well as over-expression of Bcl-2 family genes, depending on the pro- or anti-apoptotic potential, can lead to oncogenesis by decreased malignant cell death. It is for instance known that over-expression of anti-apoptotic Bcl-2 or Mcl-1 disables apoptosis induced by TNF-related ligands [20–22]. Consequently, we assessed by Western blot the expression levels of XIAP, a member of the inhibitor of apoptosis family, and Bcl-2 as well as Mcl-1, each a member of the anti-apoptotic Bcl-2 subfamily. Whereas expression levels remained unaltered for XIAP and Bcl-2 in the presence of UNBS1450, Mcl-1 was found to be strongly down-regulated by UNBS1450. Combined to the fact that noticeably low concentration levels in a nanomolar range were largely sufficient to influence the expression of Mcl-1 in the observed manner suggests this anti-apoptotic protein may play a major role in apoptosis induction due to UNBS1450.

Mcl-1 acts by directly binding to Bak and Bax proteins that are two pro-apoptotic Bcl-2 family members. From the perspective of the Bcl-2 family proteins, the intrinsic apoptosis pathway is initiated by various signals. BH3-only proteins such as Bim, Bid, Bad, Noxa or Puma engage with anti-apoptotic members such as Mcl-1 to relieve inhibition of Bak and Bax as reviewed by Kang and Reynolds [23]. Direct or indirect repression of Mcl-1 expression results in Bak and Bax activation and oligomerization eventually leading to mitochondrial outer membrane permeabilization and cell death through apoptosis [24]. Our results strongly suggest this pathway to be stimulated by UNBS1450 in human leukemia cell lines. Further point-by-point investigations are necessary to elucidate the exact molecular cascades closing the gap between Na^+/K^+-ATPase binding of UNBS1450 and Mcl-1 expression inhibition and thus apoptosis induction.

Today, NF-κB is considered by most as a key anti-apoptotic factor [25,26]. Besides frequent constitutive expression of NF-κB in various human cancer cell lines, contributing to the onset of drug resistance of tumor cells or to increased aggressiveness [27–29], it is well documented nowadays that TNFα release by the tumor microenvironment is a characteristic of many malignant tumors and acts as a sort of a master switch between activation of inflammatory processes and oncogenesis [8,30]. Through its receptors both on epithelial and stromal cell membranes, TNFα either directly influences cell proliferation and malignant cell survival or indirectly drives surrounding stromal cells, macrophages, dendritic cells and fibroblasts into releasing general inflammatory cytokines such as TNFα itself, IL-1, IL-6 and IL-8 [31]. Altogether, this creates a beneficial situation for the onset of genetic alterations and enhanced survival and proliferation of the tumor cells.

As a conclusion, we hereby further described the mechanism by which UNBS1450 inhibits tumor expansion by cell death induction. As an addition to existing knowledge of anti-cancer activities of UNBS1450 due to autophagy [4,5] mainly on human glioblastoma and NSCLC, we demonstrate here that this hemi-synthetic cardenolide rapidly and effectively induces cell death through apoptosis in human leukemia cell lines. While down-regulating induced NF-κB pathway, UNBS1450 activates the extrinsic caspase-dependent apoptotic pathway, represses the expression of Mcl-1, activating pro-apoptotic Bak and Bax and eventually leading to cell death via the mitochondrial apoptotic pathway. However, we do not have obvious evidence that a downregulation of the NF-kB pathway is linked to the observed induction of apoptosis as UNBS1450 does not show any inhibitory activities on constitutive NF-κB activity in K562 cells where induced apoptosis is significant.

While additional analysis thus remain requested to further analyze signaling cascades involved, these findings show for the first time that UNBS1450 acts as an apoptogenic inducer, specifically affecting cancer cell lines. This makes of UNBS1450 a promising candidate as a new potential anti-cancer drug for leukemia and lymphoma treatments.

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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.2010.08.025.

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