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2 **UNBS1450, a steroid cardiac glycoside inducing apoptotic cell death in human leukemia**  
3 **cells**  
4

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**ABSTRACT**

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

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22 UNBS1450; NF- $\kappa$ B; apoptosis; cardenolide; cancer  
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## 1. Introduction

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4 Most anti-cancer drugs are isolated from natural sources or are at least bearing close  
5 structural relationships to natural compounds. Both, marine and terrestrial organisms are  
6 considered today as important sources for novel lead compounds.  
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9 Non-cardiotonic steroid glycosides obtained from the tropical plant *Calotropis*  
10 *procera* possess anti-cancer potential *via* binding to the  $\alpha 3\beta 1$   $\text{Na}^+/\text{K}^+$ -ATPase isozymes. The  
11 cardenolide UNBS1450 (**Figure 1A**) has been analyzed *in vitro* as well as *in vivo* on non  
12 small cell lung cancer (NSCLC) and glioblastoma xenograft mice models [1, 2]. UNBS1450  
13 is a chemically modified form of 2-oxovoruscharin, a derivative of voruscharin ; despite its  
14 already promising anti-cancer potential, the high toxicity of 2-oxovoruscharin required  
15 chemical modifications to improve tolerance *in vivo* [3]. The improved semi-synthetic  
16 compound UNBS1450 revealed itself to be significantly less toxic when compared to its  
17 mother compound and in the same time was proven to be even more potent in cancer therapy:  
18 with an average MTD of up to 120 mg/kg, chronic administration increased mouse survival  
19 and reduced glioblastoma cell migration. Mijatovic *et al.* showed that UNBS1450-mediated  
20 antitumor activity is due to activation of non-apoptotic cell death mechanisms in  $\text{Na}^+/\text{K}^+$ -  
21 ATPase  $\alpha 1$  over-expressing glioma cells [4]. UNBS1450 has the capacity to disorganize  
22 nucleolar structure and functions *via* impairment of cyclin-dependent kinase and c-Myc  
23 expressions accompanied by disorganization of cancer cell-specific perinucleolar bodies. This  
24 non-apoptotic cancer cell death was a new cardenolide-induced mechanism of antitumor  
25 action [5] and this drug was described to be able to circumvent glioblastoma resistance to  
26 apoptosis [6].  
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42 Nuclear factor (NF)- $\kappa$ B has been shown previously to be implicated in (1) cell death  
43 (apoptosis), (2) cell adhesion, (3) cell proliferation, (4) innate- and adaptive-immune response  
44 and (5) cancer development. This dimeric protein complex can both induce and repress gene  
45 expression, by binding to DNA sequences found in promoter and enhancer regions. NF- $\kappa$ B  
46 hetero- and homodimers can be constituted of five different family members: RelA (p65),  
47 RelB, c-Rel, p50/p105 (NF- $\kappa$ B1) and p52/p100 (NF- $\kappa$ B2) [7]. In the absence of an extrinsic  
48 cell signal, NF- $\kappa$ B dimers remain sequestered in the cytoplasm, inactivated by the NF- $\kappa$ B  
49 inhibitor I $\kappa$ B. Upon cell stimulation by cytokines, proliferating agents, carcinogens or  
50 physical stress, I $\kappa$ B phosphorylation by the I $\kappa$  kinase (IKK) frees the NF- $\kappa$ B dimer, then  
51 rapidly translocating into the nucleus and transactivating more than 550 genes.  
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Deregulation of NF- $\kappa$ B leads to various pathologies including chronic inflammations and cancer [8]. NF- $\kappa$ B has become a major target in drug engineering. To date, a number of natural NF- $\kappa$ B inhibitors are being investigated, many of them being plant-derived isoprenoids and polyphenols.

As NF- $\kappa$ B is responsible for increased cell resistance towards possible cytotoxic treatments currently applied in clinics, apoptosis induction through NF- $\kappa$ B inhibition and/or extrinsic and intrinsic pathway activation is considered as a main aim in cancer research. Apoptosis induction through NF- $\kappa$ B 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- $\kappa$ B 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

TNF $\alpha$  was purchased from Sigma-Aldrich (Bornem, Belgium) and dissolved to a concentration of 10 mg/mL in PBS 1X 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 605.8g and received as dry powder, was solubilized in 52.95 % DMSO (Sigma-Aldrich, Bornem, Belgium) and then further diluted to 5 % DMSO/95 % H<sub>2</sub>O to get working aliquots with a concentration of 5 mM. 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<sub>2</sub>. At T<sub>0</sub> cells had been pre-treated with UNBS1450 at various concentrations. At T<sub>0</sub> + 2 h cells were activated by TNF $\alpha$  (20 ng/mL).

Healthy blood samples were kindly donated as buffy coats by the Red Cross (Luxembourg, Luxembourg). By applying diluted (1/3) 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<sub>2</sub> 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<sup>5</sup> cells/mL, were pre-treated or not for 2 h with UNBS1450 respectively at 40 nM and 20 nM before being activated by TNF $\alpha$  (20 ng/mL) for 6 h. Cells were then harvested and washed twice in PBS 1X; supernatants were discarded and cell pellets were stored overnight at - 80 °C. Nuclear protein extractions were performed as described

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previously before being stored at  $-80\text{ }^{\circ}\text{C}$  [10]. The oligonucleotide NF- $\kappa$ Bc (consensus NF- $\kappa$ B site) (Eurogentec, Liège, Belgium) (5'-AGTTGAGGGGACTTTCCAGGC-3') and its complementary sequence were used as probe. After being hybridized, the probes were radiolabelled using [ $g\text{-}^{32}\text{P}$ ]ATP (MP Biomedicals, Illkirch, France) and the EMSA assay was realized according to conditions established before [10].

#### 11 **2.4. Transient transfection and luciferase reporter gene assay**

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K562 and Jurkat cells were transiently transfected as described previously [10]. For each electroporation we used 5  $\mu\text{g}$  of a luciferase reporter gene construct containing five repeats of a consensus NF- $\kappa$ B site (Stratagene, Genomics Agilent, Diegem, Belgium) and 5  $\mu\text{g}$  of a *Renilla* luciferase plasmid (Promega, Leiden, Netherlands). Cells were resuspended in normal culture medium (RPMI, 10 % FCS) after electroporation and cultured at  $37\text{ }^{\circ}\text{C}$  and 5 %  $\text{CO}_2$  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  $3 \times 10^5$  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 TNF $\alpha$  for 6 h followed the pre-treatment. After the 8 h of total challenge, 75  $\mu\text{L}$  of Dual-Glo<sup>TM</sup> Luciferase Reagent (Promega, Leiden, Netherlands) were added to 75  $\mu\text{L}$  of the cellular suspension for a 10 min incubation at  $22\text{ }^{\circ}\text{C}$  before luciferase activity measurement. Further on, 75  $\mu\text{L}$  of Dual-Glo<sup>TM</sup> Stop&Glo<sup>®</sup> Reagent (Promega, Leiden, Netherlands) were added for 10 min at  $22\text{ }^{\circ}\text{C}$  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.

#### 45 **2.5. Cell viability assessment**

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Percentages of cell survival were evaluated using Promega's CellTiter-Glo<sup>®</sup> Luminescent Cell Viability Assay (Promega, Leiden, Netherlands) kit, according to 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.

#### 55 **2.6. Analysis of apoptosis**

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Analysis of nuclear fragmentation. Percentages of apoptotic cells, quantified as the fraction of apoptotic nuclei, were assessed by fluorescence microscopy (Leica-DM IRB

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microscope, Lecuit, Luxemburg) 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>).

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 TNF $\alpha$ , K562, U937 and Jurkat cells were lysed; nuclear and cytoplasmic extracts were prepared according to [10].

## 2.8. RT-PCR Analysis

Isolation of total RNA was performed using a NucleoSpin® (Macherey-Nagel, Hoerd, France) kit. 1  $\mu$ g 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 1X, Applied Biosystems, Halle, Belgium) and Na<sup>+</sup>/K<sup>+</sup>-ATPase  $\alpha$ -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. Western Blot analysis

1 Proteins of total extracts were separated by size using sodium dodecyl sulfate  
2 polyacrylamide gel electrophoresis (SDS-PAGE, 10 %), transferred onto nitrocellulose  
3 membranes and blocked with 5 % non-fat milk in phosphate buffered saline (PBS)-Tween  
4 overnight. Equal loading of samples was controlled using  $\beta$ -actin or lamin B and  $\alpha$ -tubulin for  
5 cytosolic and nuclear extracts.  
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9 Blots were incubated with primary antibodies: anti-pI $\kappa$ B $\alpha$  (1/3000, Cell Signaling  
10 9246, Bioké, Leiden, Netherlands), anti-I $\kappa$ B $\alpha$  (1/500, Santa Cruz SC-371, Tebu-Bio,  
11 Boechout, Belgium), anti-p50 (1/5000, Santa Cruz SC-7178X), anti-p65 (1/5000, Santa Cruz  
12 SC-8008), anti- $\alpha$ -tubulin (1/5000, Calbiochem CP06, VWR, Leuven, Belgium), anti-lamin- $\beta$   
13 (1/1000, Santa Cruz SC-6216), anti- $\beta$ -actin (1/10000, Sigma-Aldrich A5441, Bornem,  
14 Belgium), anti-caspase-3 (1/5000, Cell Signaling 9668), anti-caspase-7 (1/1000, Cell  
15 Signaling 9494), anti-caspase-8 (1/1000, Cell Signaling 9746), anti-caspase-9 (1/1000, Cell  
16 Signaling 9502), anti-XIAP (1/1000, BD Pharmingen 610763, Erembodegem, Belgium), anti-  
17 Bcl-2 (1/2000, Calbiochem OP60) and anti-Mcl-1 (1/1000, Cell Signaling 4572). All  
18 antibodies were diluted in a PBS-Tween solution containing 5 % of bovine serum albumin  
19 (BSA) or 5 % of milk according to the providers' protocols. After incubation with primary  
20 antibodies, membranes were washed 3 x 10 min with PBS-Tween, followed by an incubation  
21 of 1 h at RT with the corresponding secondary (HRP-conjugated) antibodies. After washing 3  
22 x 10 min with PBS-Tween, specific immunoreactive proteins were visualized by  
23 autoradiography using the ECL Plus Western Blotting Detection System Kit <sup>®</sup> (GE  
24 Healthcare, Roosendaal, Netherlands).  
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## 40 2.10. Elisa

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42 IL-8 concentrations in culture supernatants of activated K562, U937 and Jurkat cells  
43 were measured by sandwich ELISA (R&D Systems, Abingdon, United Kingdom). According  
44 to the manufacturer's guide, 50  $\mu$ L of cell supernatants were added together with 100  $\mu$ L of  
45 Assay Diluent to anti-IL-8 pre-coated wells, followed by 2 h of incubation at RT. After  
46 washing, a polyclonal peroxidase-conjugated anti-IL-8 antibody was added for another 60  
47 min at RT. Colorimetric visualization and protein dosage were rendered possible by addition  
48 of the H<sub>2</sub>O<sub>2</sub> and TMB (tetramethylbenzidine) containing substrate. After 30 min of reaction at  
49 RT, in the dark, the enzymatic reaction was stopped by H<sub>2</sub>SO<sub>4</sub> and optical densities were  
50 measured at a wavelength of 450 nm.  
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### 2.11. Statistical analysis

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2 Data is expressed as mean + - S.D. and its signification degree was analyzed by  
3 Student's T-tests. P-values below 0,05 were considered as statistically significant.  
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### 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 human leukemia. Cells were treated for 24, 48 and 72h with different concentrations of UNBS1450 and their culture concentrations estimated by Trypan Blue exclusion assay. As reported in **Figure 1B**, UNBS1450 deeply affected cell growth in a dose-dependent manner with an  $IC_{50}$  value of  $13.37 \pm 2.1$  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 **Figure 1C** showed that UNBS1450 lead to accumulation of cells in a sub-G1 phase in a dose-dependent manner, significantly starting from 15nM, thus confirming that UNBS1450 induced cell death. From 20nM to 30nM, UNBS1450 increasingly drove U937 cells into a sub-G1 phase (**Figure 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 (**Figure 1D, top**). As shown, the nuclei of U937 cells treated with UNBS1450 underwent morphological alterations typically occurring during apoptosis [11]. **Figure 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 (**Figure 1E**) starting from 15nM, 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  $32.65 \pm 3.10$  nM at 48 h (**Supplemental Figure 1A**). Cell cycle analysis (**Supplemental Figure 1B**) showed that an incubation time of 48 h in the presence of UNBS1450 50nM 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 Figure 1C**). FACS analysis for the exposure of phosphatidylserine/plasma membrane integrity by annexin V/PI analysis confirmed the activation of an apoptotic program

(**Supplemental Figure 1D**). Similar effects of UNBS1450 on cell death induction were observed in Jurkat T cells (**Supplemental Figures 2A and B**).

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

As it has been shown that the  $\alpha 1$  subunit of  $\text{Na}^+/\text{K}^+$ -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  $\alpha 1$  subunit mRNA expression levels of a panel of hematological cancer cell lines to those of normal healthy PBMCs (**Figure 2**). Our results indicate all cell lines express the corresponding mRNA but that only K562 produces significantly higher amounts compared to PBMCs ( $2^{\Delta\Delta\text{Ct}}$  values of 0.06 versus 0.011 respectively,  $\pm$  S.D.). For all other cell lines, subunit  $\alpha 1$  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 (**Figure 3A**). To this purpose, a kinetic analysis (0h to 24h) was performed on U937 cells treated with UNBS1450 at 20nM. We demonstrated that UNBS1450 at 20nM induced the cleavage of the pro-caspases-9 and -8 starting after 12h of incubation time. The executor pro-caspases-3 and -7, substrates of caspase-9, were cleaved into active caspase-3 and 7 (**Figure 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 (**Figure 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

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2 strong reduction in the protein level was noticeable after 4 h of treatment while after 12 h of  
3 treatment, no expression level was detectable anymore.  
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### 5 **3.3. Activation of pro-apoptotic mechanisms**

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7 We then analyzed *in situ* the activation status of pro-apoptotic Bak and Bax in U937  
8 cells, untreated and treated with 10 to 30 nM of UNBS1450, by using antibodies specifically  
9 recognizing activated forms of both pro-apoptotic Bcl-2 family members. Immunostained  
10 cells were counterstained with Hoechst to detect apoptotic nuclei. Bak resulted activated by  
11 treatments with 15 nM UNBS1450 and higher (**Figure 4A**). Similar results were observed for  
12 activated Bax (Bax6A7) (**Figure 4B**). As expected, both activated Bak and Bax co-localized  
13 with cells presenting fragmented, apoptotic nuclei, witnessing apoptotic cell death triggered  
14 by UNBS1450 a semi-synthetic cardenolide originally from the plant *Calotropis Procera*.  
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### 23 **3.4. Inhibition of NF- $\kappa$ B pathway activation**

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25 NF- $\kappa$ B transcription factor is well known to act as an inhibitor of apoptotic cell  
26 signaling specifically *via* transactivation. Moreover Mijatovic *et al.* [12] already provided first  
27 insights into UNBS1450-induced inhibition of NF- $\kappa$ B activation. Here we observed that  
28 UNBS1450 reduced TNF $\alpha$ -driven NF- $\kappa$ B activation in a dose-dependent manner in K562 and  
29 Jurkat cells (**Figure 5A and B**). As illustrated in **Table I**, UNBS1450 has however no  
30 repressing effect on constitutive, basal NF- $\kappa$ B activity in K562 cells.  
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36 In order to further confirm the inhibitory effect of UNBS1450 on the TNF $\alpha$ -induced  
37 NF- $\kappa$ B signaling pathway, we realized EMSA. **Figure 5C** clearly shows that at a  
38 concentration of UNBS1450 40 nM inhibits TNF $\alpha$ -induced NF- $\kappa$ B-DNA binding. By  
39 applying various pre-treatment times from 0 to 16 h, we obtained a complete inhibition after 8  
40 h of pre-incubation. Incubation with p50 and p65 antibodies enabled us to identify the NF- $\kappa$ B  
41 dimer composition as p50 and p65. Similar results were obtained for Jurkat cells (**Figure 5D**).  
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47 NF- $\kappa$ B activation is generally initiated by the degradation of its natural inhibitor I $\kappa$ B $\alpha$ ;  
48 we thus assessed I $\kappa$ B $\alpha$  integrity as well as the translocation of p65 from the cytoplasm to the  
49 nucleus by Western Blot analysis (**Figure 5E**). Both degradation of I $\kappa$ B $\alpha$  as well as  
50 translocation of p65 into the nucleus, were analyzed by Western Blot. In control cells without  
51 UNBS1450 pre-treatment, I $\kappa$ B $\alpha$  degradation was observed after 10 min of TNF $\alpha$  stimulation;  
52 consequently, p65 translocation to the nucleus was observed in parallel. In contrast, the  
53 presence of UNBS1450 inhibited TNF $\alpha$ -induced degradation of I $\kappa$ B $\alpha$  and considerably  
54 prevented the translocations of p65 into the nucleus (**Figure 5E**, right panel).  
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To further investigate the influence of UNBS1450 on downstream NF- $\kappa$ B 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- $\kappa$ B. We observed that UNBS1450-pre-treated cells produced significantly lower quantities of IL-8 (less than 600 pg/mL) compared to TNF $\alpha$  only activated K562 cells (1300 pg/mL) (**Figure 5F**). Similar results were obtained for U937 (**Figure 5G**). Unstimulated U937 cells produced low amounts of IL-8 (92 pg/mL) and TNF $\alpha$  activation resulted in increased IL-8 release (1002 pg/mL). A significant inhibition was obtained after UNBS1450 treatment at 20 nM (IL-8 release: 721 pg/mL). In Jurkat cells, TNF $\alpha$  stimulation could not trigger IL-8 release; neither did UNBS1450 show any significant effect on IL-8 release (results not shown).

#### 4. Discussion

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4 We show here that UNBS1450, belonging to the cardenolide family of the steroid  
5 cardiac glycosides, efficiently reduces viability of leukemia cancer cell lines by inducing  
6 apoptotic cell death. We focused our efforts on possible modifications of inflammation and  
7 cell death induction by UNBS1450. To this purpose, we used chronic myeloid leukemia  
8 K562, histiocytic lymphoma U937 as well as acute T-cell leukemia Jurkat cells stimulated by  
9 TNF $\alpha$  as a model of investigation to mimic pro-inflammatory surroundings stimulating the  
10 NF- $\kappa$ B transcription factor.  
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16 While UNBS1450 has so far mainly been applied on solid tumors [1] [12], we hereby  
17 confirm that deactivation of NF- $\kappa$ B activity through UNBS1450 is also true in human  
18 leukemia cells as indicated by reduced induction of NF- $\kappa$ B transcriptional activity, reduced  
19 binding to target DNA as well as repressed synthesis of NF- $\kappa$ B-dependent genes. TNF $\alpha$ -  
20 induced p50/p65 dimer translocation to the nucleus is strongly reduced in the presence of  
21 UNBS1450 due to repressed I $\kappa$ B $\alpha$  degradation.  
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27 We also analyzed the impact of UNBS1450 on leukemia cell survival capacity. As  
28 finally indicated by increased Annexin V/PI staining, fragmented nuclei after Hoechst  
29 staining and thus reduced cell numbers, UNBS1450 is beneficial to an apoptosis induction.  
30 We observed that nanomolar concentrations of UNBS1450 were able to induce the cleavages  
31 of the initiator caspases 8 and 9 as shown by Western Blot. Furthermore, we obtained a  
32 cleavage induction of the pro-caspase-7, leading to hypothetical thoughts that UNBS1450 might  
33 induce apoptosis by processing and concomitant activation of effector caspases such as  
34 caspase-7 and caspase-3. Consequently, we assessed expression levels of various Bcl-2 family  
35 proteins in order to conclude on a possible amplification of the death signal through activation  
36 of the intrinsic mitochondrial cell death pathways [13] [14]. The analysis of cell proliferation  
37 in the presence of UNBS1450 in both cell lines investigated reveals that the compound is able  
38 to affect cell proliferation at concentrations lower than those requested to induce apoptosis  
39 (see **Figure 1B** and supplemental **Figure 1A**). This is in line with previous findings [15] and  
40 prompts to elucidate in the future whether this cytostatic potential may be also implicated in  
41 determining the cytotoxic effect.  
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54 Several studies underline their pivotal role [16] in apoptosis induction [17, 18] [19].  
55 Loss of expression as well as over-expression of Bcl-2 family genes, depending on the pro- or  
56 anti-apoptotic potential, can lead to oncogenesis by decreased malignant cell death. It is for  
57 instance known that over-expression of anti-apoptotic Bcl-2 or Mcl-1 disables apoptosis  
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1 induced by TNF-related ligands [20] [21] [22]. Consequently, we assessed by Western Blot  
2 the expression levels of XIAP, a member of the inhibitor of apoptosis family, and Bcl-2 as  
3 well as Mcl-1, each a member of the anti-apoptotic Bcl-2 subfamily. Whereas expression  
4 levels remained unaltered for XIAP and Bcl-2 in the presence of UNBS1450, Mcl-1 was  
5 found to be strongly down-regulated by UNBS1450. Combined to the fact that noticeably low  
6 concentration levels in a nanomolar range were largely sufficient to influence the expression  
7 of Mcl-1 in the observed manner suggests this anti-apoptotic protein may play a major role in  
8 apoptosis induction due to UNBS1450.  
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14 Mcl-1 acts by directly binding to Bak and Bax proteins that are two pro-apoptotic Bcl-  
15 2 family members. From the perspective of the Bcl-2 family proteins, the intrinsic apoptosis  
16 pathway is initiated by various signals. BH3-only proteins such as Bim, Bid, Bad, Noxa or  
17 Puma engage with anti-apoptotic members such as Mcl-1 to relieve inhibition of Bak and Bax  
18 as reviewed by Kang and Reynolds [23]. Direct or indirect repression of Mcl-1 expression  
19 results in Bak and Bax activation and oligomerisation eventually leading to mitochondrial  
20 outer membrane permeabilisation and cell death through apoptosis [24]. Our results strongly  
21 suggest this pathway to be stimulated by UNBS1450 in human leukemia cell lines. Further  
22 point-by-point investigations are necessary to elucidate the exact molecular cascades closing  
23 the gap between  $\text{Na}^+/\text{K}^+$ -ATPase binding of UNBS1450 and Mcl-1 expression inhibition and  
24 thus apoptosis induction.  
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34 Today, NF- $\kappa$ B is considered by most as a key anti-apoptotic factor [25, 26]. Besides  
35 frequent constitutive expression of NF- $\kappa$ B in various human cancer cell lines, contributing to  
36 the onset of drug resistance of tumor cells or to increased aggressiveness [27] [28] [29], it is  
37 well documented nowadays that TNF $\alpha$  release by the tumor microenvironment is a  
38 characteristic of many malignant tumors and acts as a sort of a master switch between  
39 activation of inflammatory processes and oncogenesis [8] [30]. Through its receptors both on  
40 epithelial and stromal cell membranes, TNF $\alpha$  either directly influences cell proliferation and  
41 malignant cell survival or indirectly drives surrounding stromal cells, macrophages, dendritic  
42 cells and fibroblasts into releasing general inflammatory cytokines such as TNF $\alpha$  itself, IL-1,  
43 IL-6 and IL-8 [31]. Altogether, this creates a beneficial situation for the onset of genetic  
44 alterations and enhanced survival and proliferation of the tumor cells.  
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54 As a conclusion, we hereby further described the mechanism by which UNBS1450  
55 inhibits tumor expansion by cell death induction. As an addition to existing knowledge of  
56 anti-cancer activities of UNBS1450 due to autophagy [4] [5] mainly on human glioblastoma  
57 and NSCLC, we demonstrate here that this semi-synthetic cardenolide rapidly and effectively  
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1 induces cell death through apoptosis in human leukemia cell lines. While down-regulating  
2 induced NF- $\kappa$ B pathway, UNBS1450 activates the extrinsic caspase-dependent apoptotic  
3 pathway, represses the expression of Mcl-1, activating pro-apoptotic Bak and Bax and  
4 eventually leading to cell death via the mitochondrial apoptotic pathway. However, we do not  
5 have obvious evidence that a downregulation of the NF- $\kappa$ B pathway is linked to the observed  
6 induction of apoptosis as UNBS1450 does not show any inhibitory activities on constitutive  
7 NF- $\kappa$ B activity in K562 cells where induced apoptosis is significant.  
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10 While additional analysis thus remain requested to further analyze signaling cascades  
11 involved, these findings show for the first time that UNBS1450 acts as an apoptogenic  
12 inducer, specifically affecting cancer cell lines. This makes of UNBS1450 a promising  
13 candidate as a new potential anti-cancer drug for leukemia and lymphoma treatments.  
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## Legends to Figures

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4 **Figure 1: UNBS1450 induces apoptotic cell death in U937 cells. A.** Molecular structure. **B.**  
5 Analysis of UNBS1450-induced cell death was performed by trypan blue staining after 24, 48  
6 and 72 h of treatment with UNBS1450 at 10, 15, 20 and 30nM. **C.** Cell cycle analysis after  
7 24h of incubation time with indicated concentrations, **D.** Hoechst staining (upper panel) and  
8 quantification (lower panel) of the fraction of cells presenting fragmented nuclei. **E.** Flow  
9 cytometry analysis after 24h of incubation time at indicated concentrations. **F.** Analysis of  
10 PBMCs viability after UNBS1450 treatment. PBMCs were seeded at  $2 \times 10^6$ , then after 24h  
11 treated for 24 h with various concentrations (0-100 nM) of UNBS1450. Cells were then either  
12 stained by Trypan Blue (upper panel) or by Hoechst (panel below) to either analyze cell  
13 integrity or apoptosis induction. The data shown here were representative for three  
14 independent experiments.  
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25 **Figure 2. Na<sup>+</sup>/K<sup>+</sup>-ATPase subunit  $\alpha 1$  mRNA quantification.** Na<sup>+</sup>/K<sup>+</sup>-ATPase subunit  $\alpha 1$   
26 mRNA content of untreated PBMCs and a wide panel of hematological cancer cell lines  
27 including K562, Jurkat and U937 cells was transcribed and then quantified by RT-PCR. The  
28 quantification of three independent experiments is expressed in brute  $2^{\Delta\Delta Ct}$  values +- S.D.  
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34 **Figure 3. A. Caspase activation.** U937 cells were incubated in RPMI+10% FCS±  
35 UNBS1450 20nM up to 24 h. Western Blot analysis of UNBS1450-induced cleavage of pro-  
36 caspases-9, -8, -7 and -3. **B. Analysis of expression levels of anti-apoptotic proteins.**  
37 UNBS1450-induced expression level alterations of XIAP, Bcl-2 and Mcl-1. The data shown  
38 here were representative for three independent experiments.  
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45 **Figure 4. UNBS1450 enables Bak/Bax activation.** U937 cells were incubated for 24h in  
46 RPMI+10% FCS in presence or in absence of UNBS1450. Bak (**A.**) and Bax (**B.**) activation  
47 status were assessed by using primary antibodies specifically targeting activated forms of Bak  
48 (Ab-1; Calbiochem) and Bax (6A7; Santa Cruz). Counterstaining was done by Hoechst  
49 staining to assess apoptotic nuclei. The data shown here were representative for three  
50 independent experiments with similar results.  
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58 **Figure 5. Inhibition by UNBS1450 of TNF $\alpha$ -induced NF- $\kappa$ B activation. A.** K562 and **B.**  
59 Jurkat cells were pretreated with UNBS1450 at various concentrations from 10-50 nM and  
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1 incubated for 2 hours, followed by TNF $\alpha$  addition (20 ng/ml) and an additional incubation  
2 period of 6 hours. Results are represented as the ratio of the measured luminescence of the  
3 firefly luciferase vector divided by the measured luminescence of the *Renilla* plasmid.  
4 Untreated cells were used as a negative control, cells treated with TNF $\alpha$  only as a positive  
5 control. Results are presented as mean $\pm$ SD of 3 individual measurements performed in  
6 triplicates. **C.** Effect of UNBS1450 on the binding affinity of NF- $\kappa$ B was assessed by an  
7 EMSA on the K562 and Jurkat cell line. The data shown here were representative for three  
8 independent experiments with similar results. **D.** For supershift/immunodepletion  
9 experiments, the nuclear extracts and labeled probes were incubated in the reaction mixture  
10 for 30 min on ice prior to a 30 min incubation with 2  $\mu$ g of anti-p50 or anti-p65, antibodies.  
11 SS designates supershifted bands. **E.** Jurkat cells were incubated with UNBS1450 (40 nM) for  
12 2 hours, followed by a TNF $\alpha$  (20 ng/ml) activation for the indicated time periods.  
13 Cytoplasmic and nuclear extracts were prepared, fractionated on a 10 % SDS-page gel,  
14 transferred to a membrane and then tested for I $\kappa$ B $\alpha$  and p65. Protein loading and purity of  
15 nuclear/cytosolic extracts were verified by lamin B and  $\alpha$ -tubulin Western Blots. Data shown  
16 are representative for three independent experiments with similar results. K562 (**F.**), and  
17 U937 (**G.**) cells were incubated for 2 h in RPMI + 10 % FCS in presence or in absence of  
18 various concentrations (10-50 nM) of UNBS1450 before being activated by TNF $\alpha$  during 22  
19 h. After 24 h of incubation IL-8 concentrations in supernatants were measured. Untreated  
20 cells served as negative control whereas cells activated by TNF $\alpha$  only were used as a positive  
21 control. The data shown here were representative for three independent experiments with  
22 similar results.  
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## Legends to Supplemental Figures

### **Supplemental Figure 1: UNBS1450 induces apoptotic cell death in K562 cells. A.**

Analysis of UNBS1450-induced cell death was performed by trypan blue staining after 24, 48 and 72 h of treatment with UNBS1450 at 10, 30 and 50nM. **B.** Cell cycle analysis after 48h of incubation time at indicated concentrations, **C.** Hoechst staining and **D.** quantification of the fraction of cells presenting fragmented nuclei. **E.** Flow cytometry analysis after 48h of incubation time at indicated concentrations.

### **Supplemental Figure 2: UNBS1450 induces apoptotic cell death in Jurkat cells. A.**

Analysis of UNBS1450-induced cell death was performed by trypan blue staining after 24, 48 and 72 h of treatment with UNBS1450 at 10, 30 and 50nM. **B.** Cell cycle analysis after 24h of incubation time with indicated concentrations.

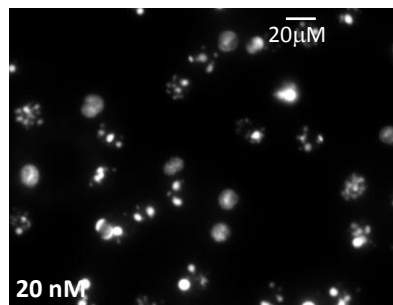
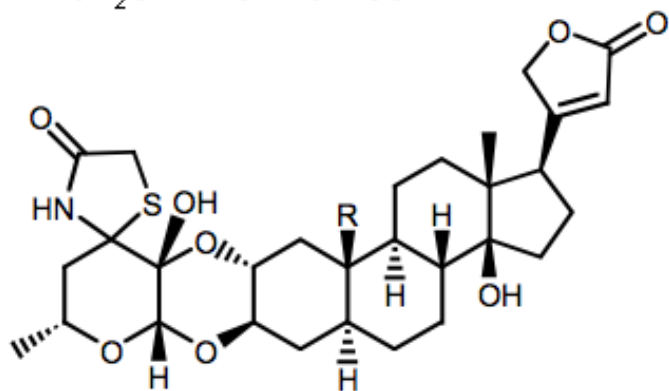
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R=CH<sub>2</sub>OH UNBS1450



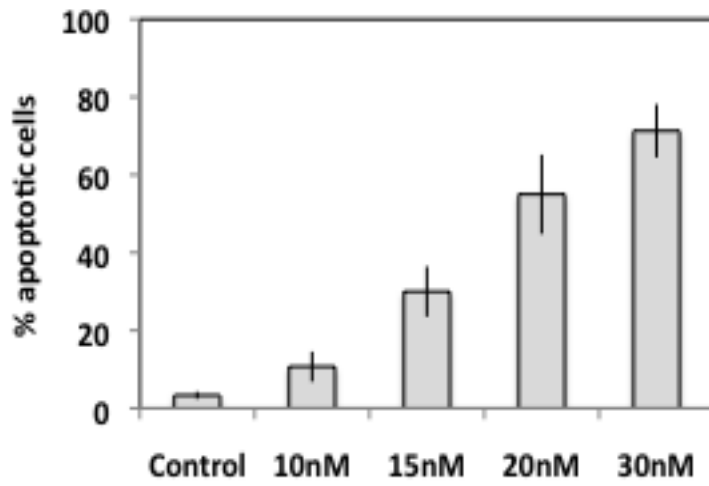
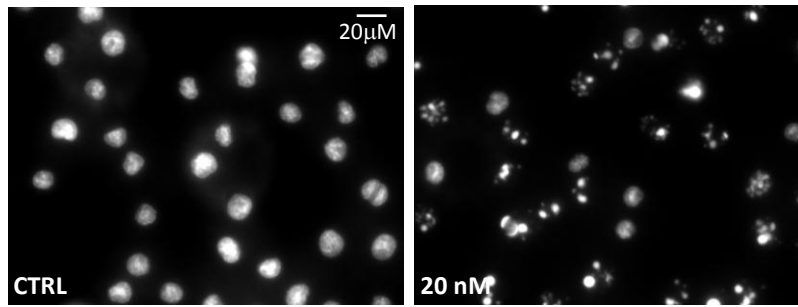
**Table I. Effect of UNBS1450 (40 nM) on basal and TNF $\alpha$ -induced NF- $\kappa$ B activity.**

<b>K562</b>	R.L.A. (%)	S.D. (%)
untreated	0.574	0.147
UNBS1450 (40 nM)	0.587	0.178
TNF $\alpha$	100	2.850
UNBS1450 (40 nM) + TNF $\alpha$	42.578	7.761

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D.



E.

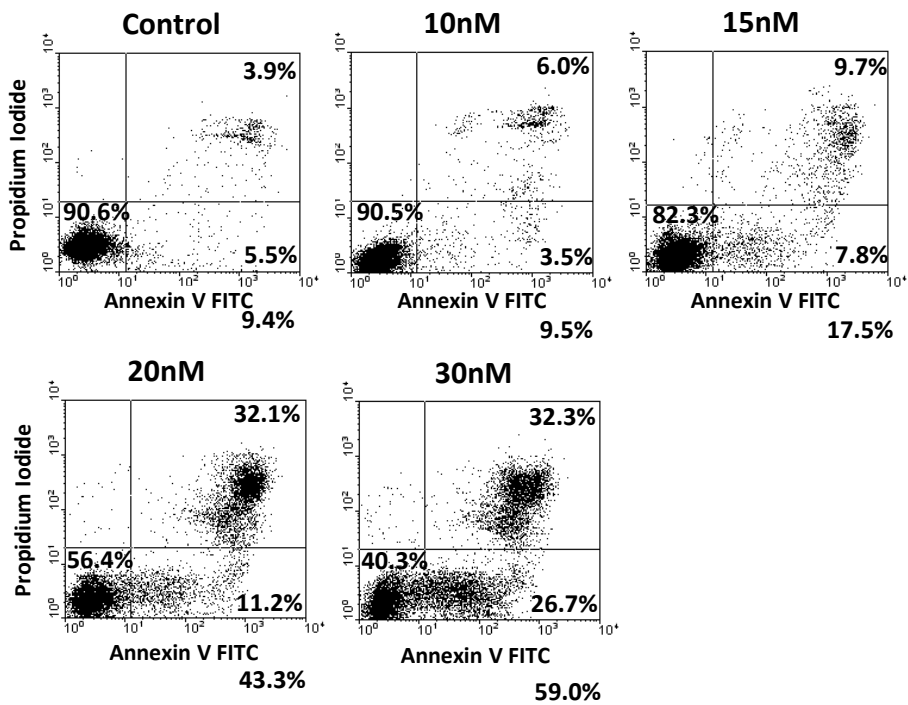
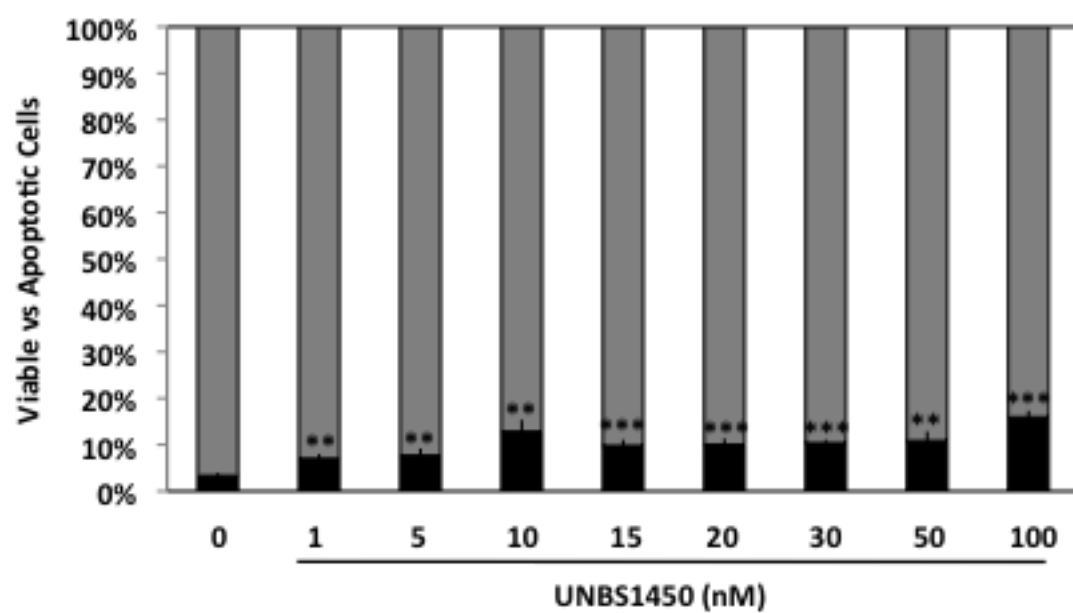
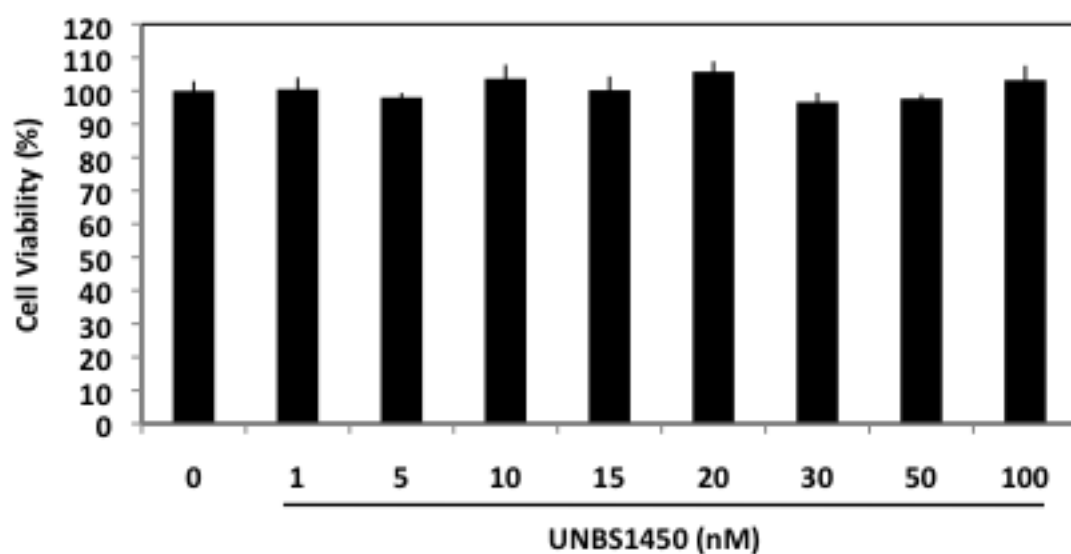


Figure 1

F.



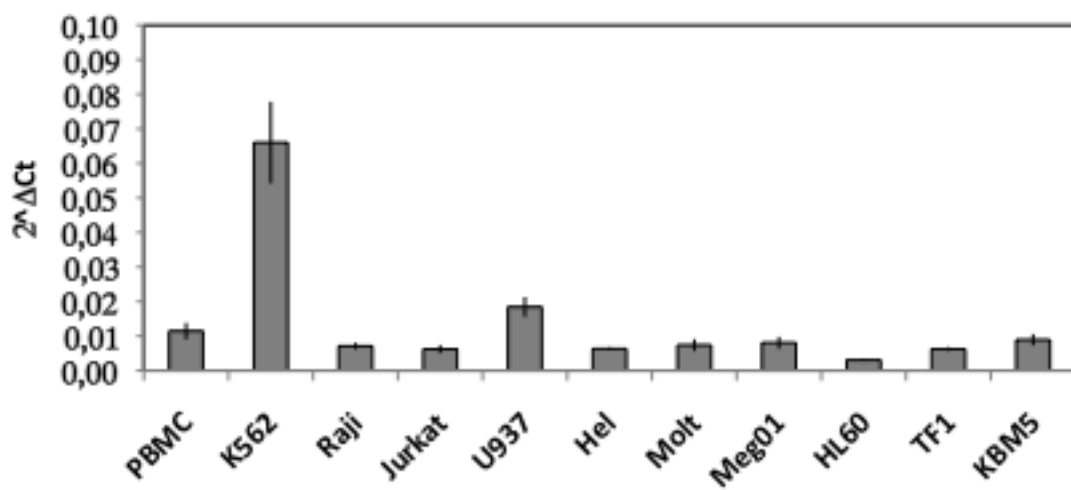
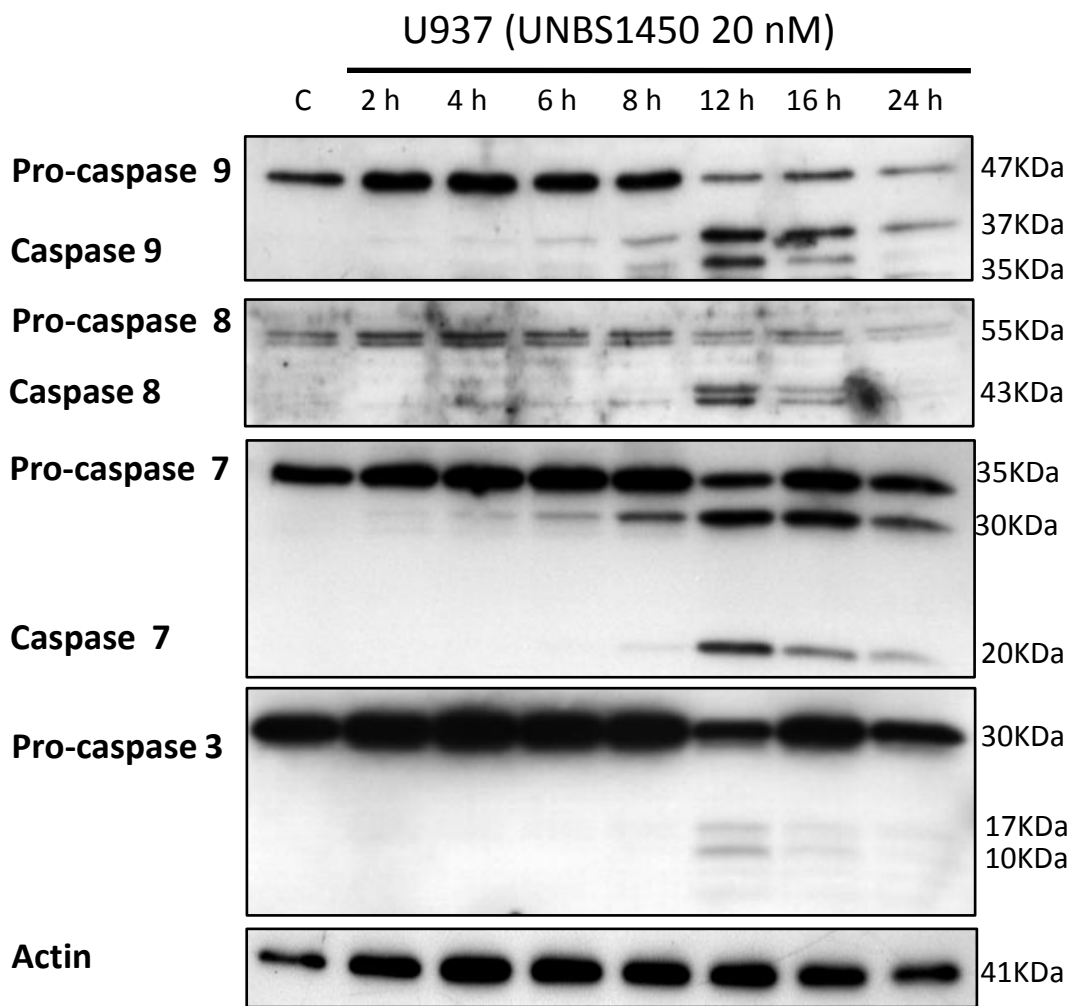


Figure 2

A.



B.

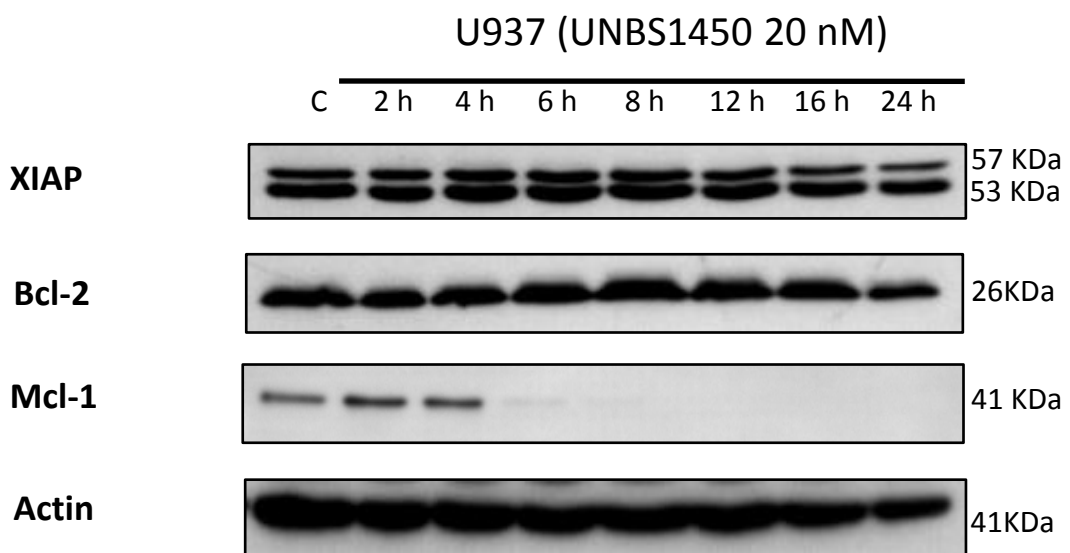


Figure 3

A.

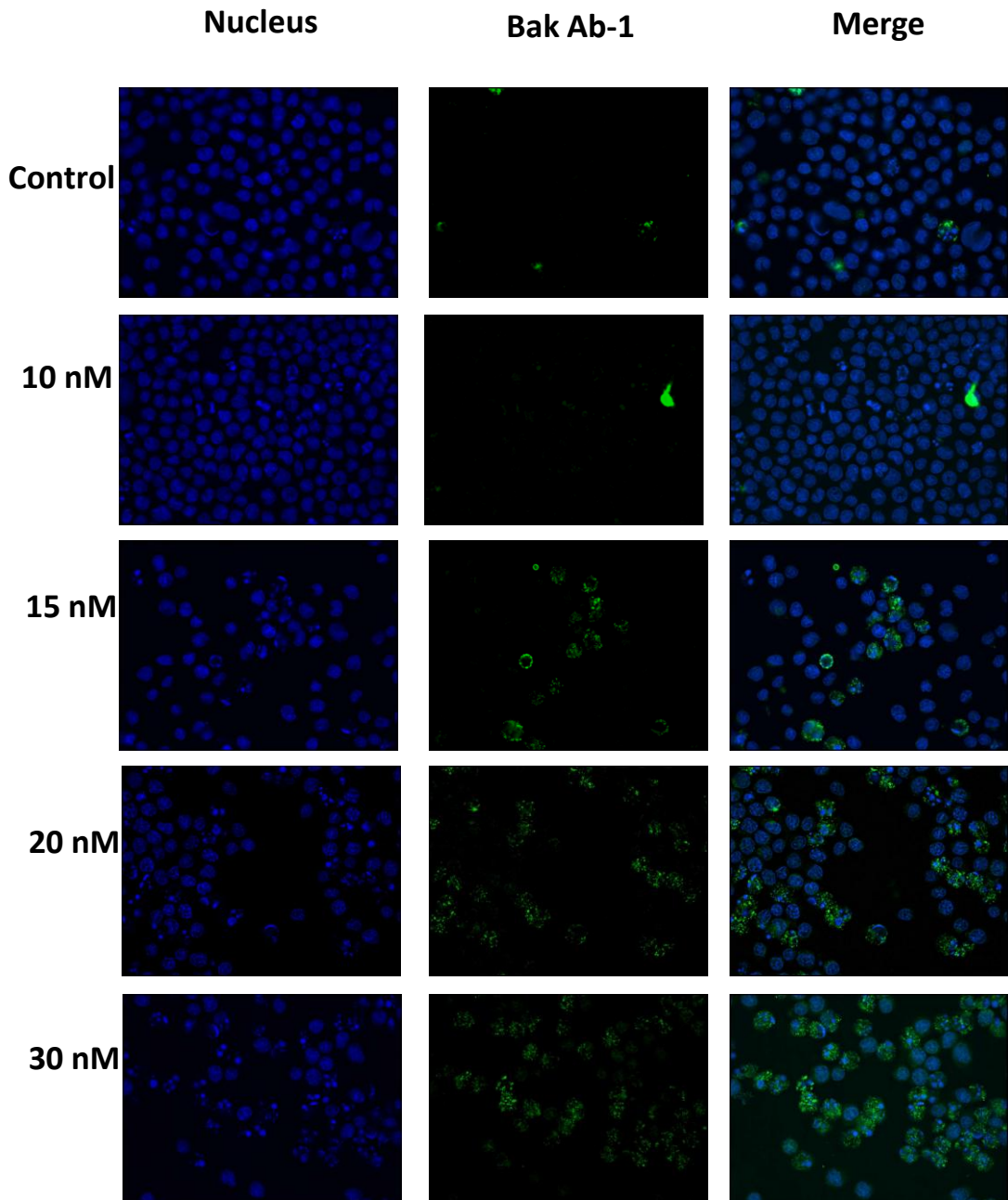


Figure 4

B.

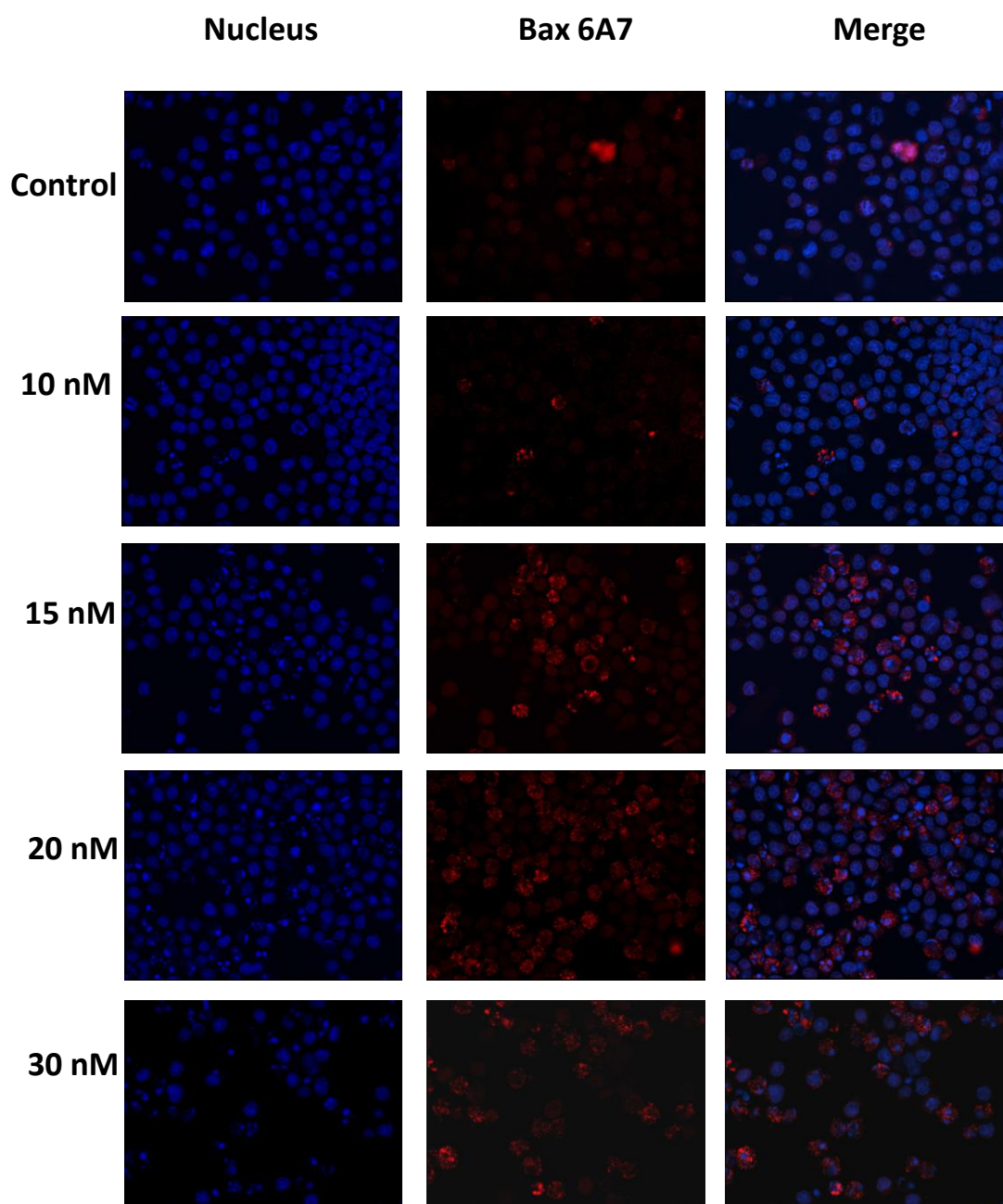
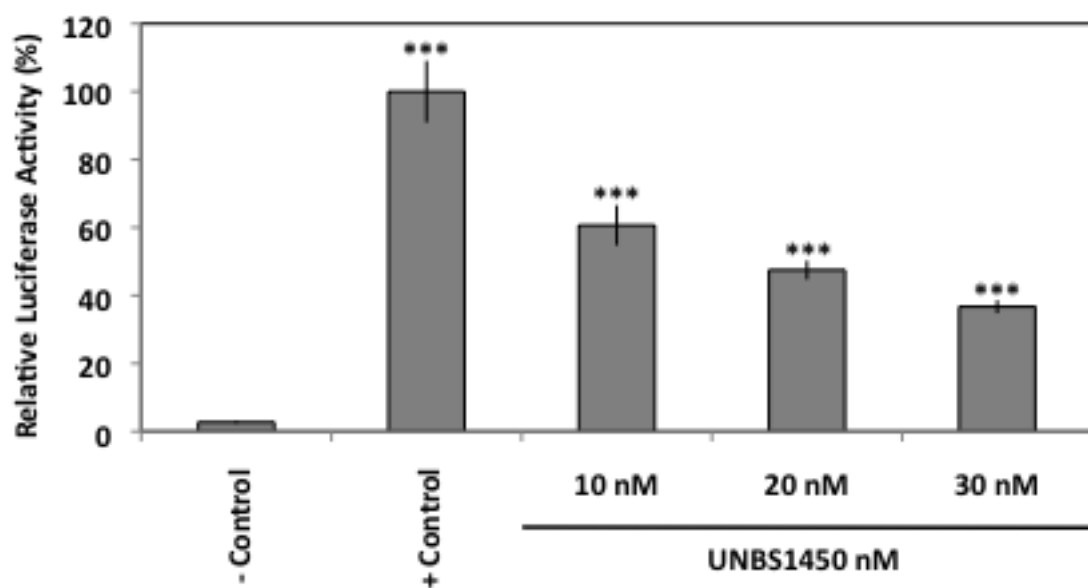


Figure 4

A.



B.

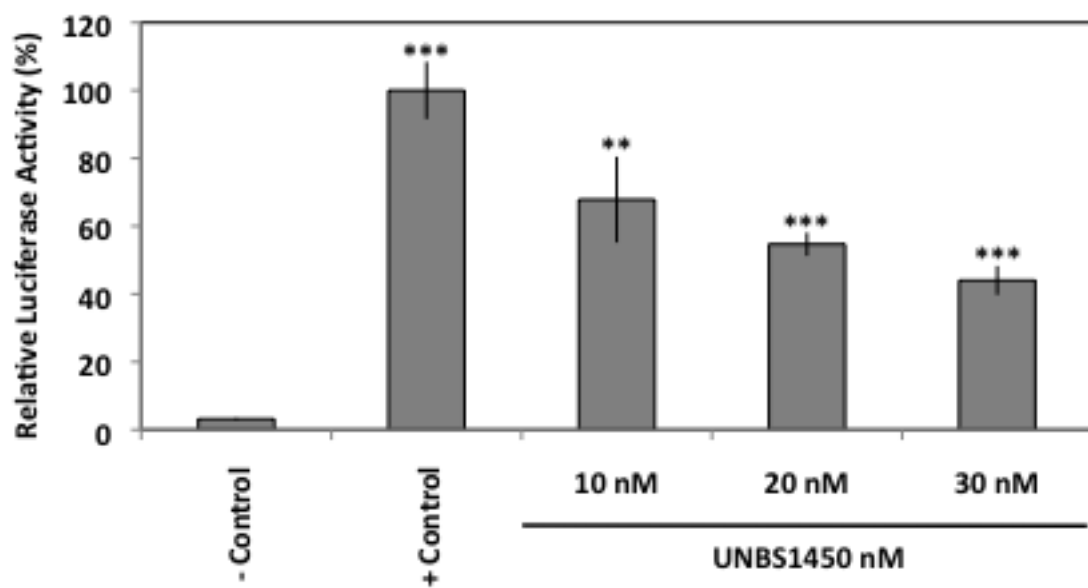


Figure. 5

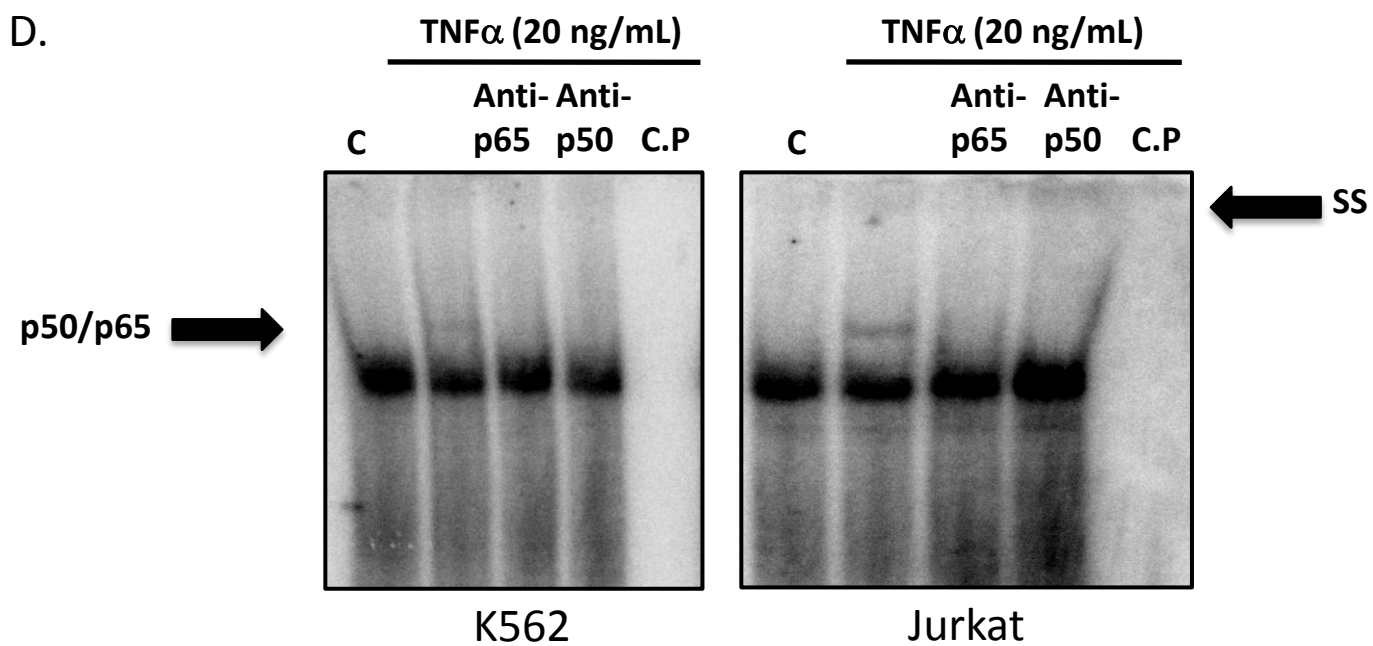
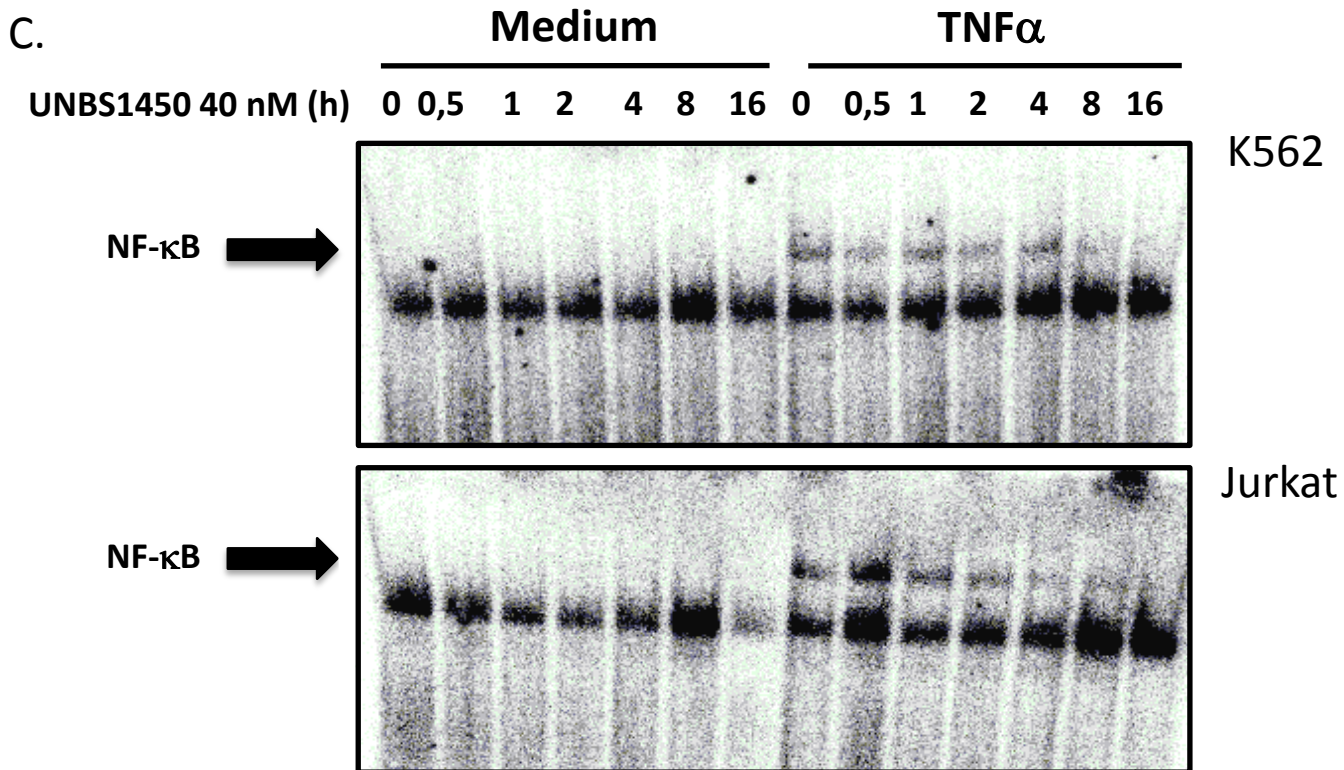


Figure 5

E.

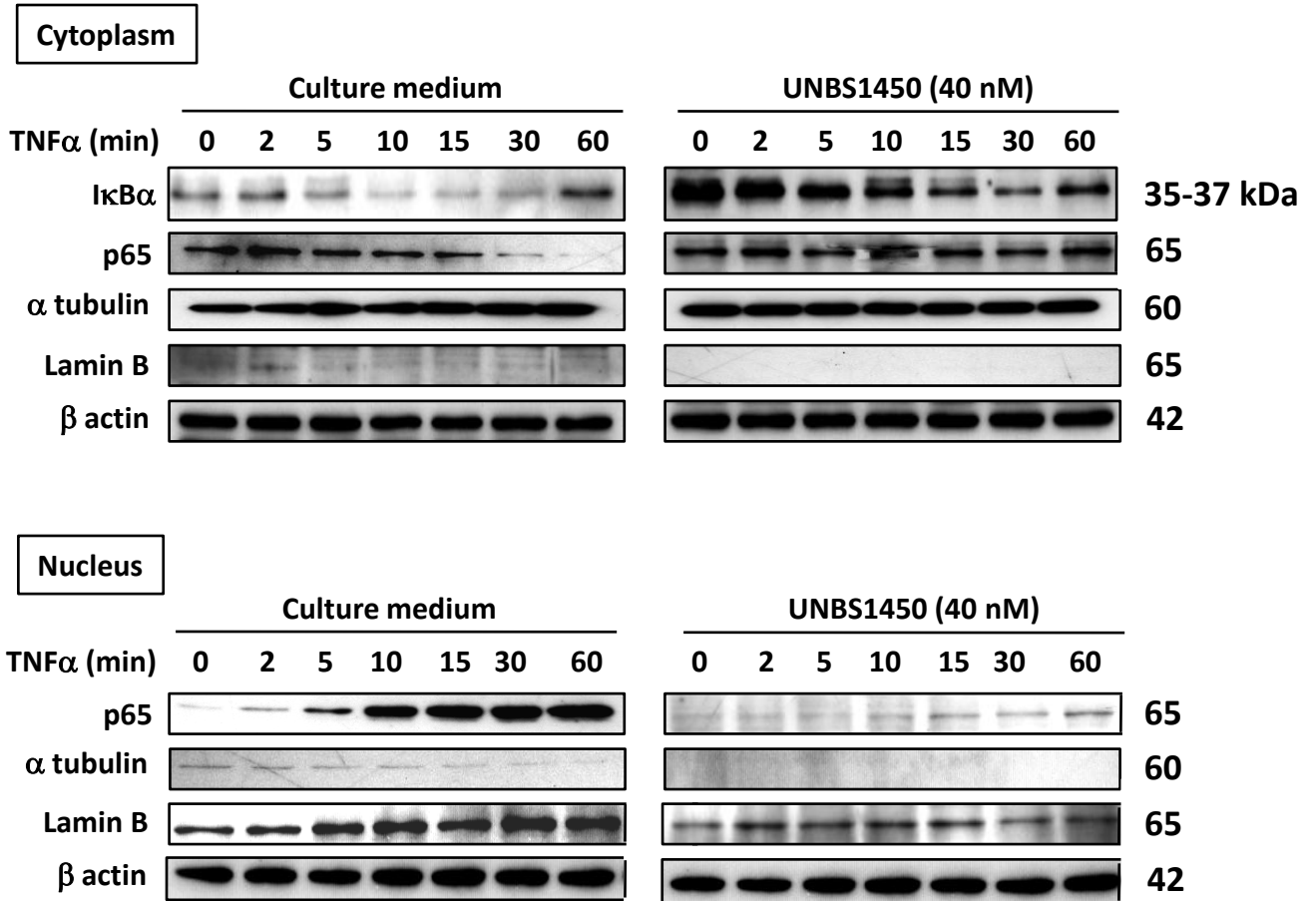
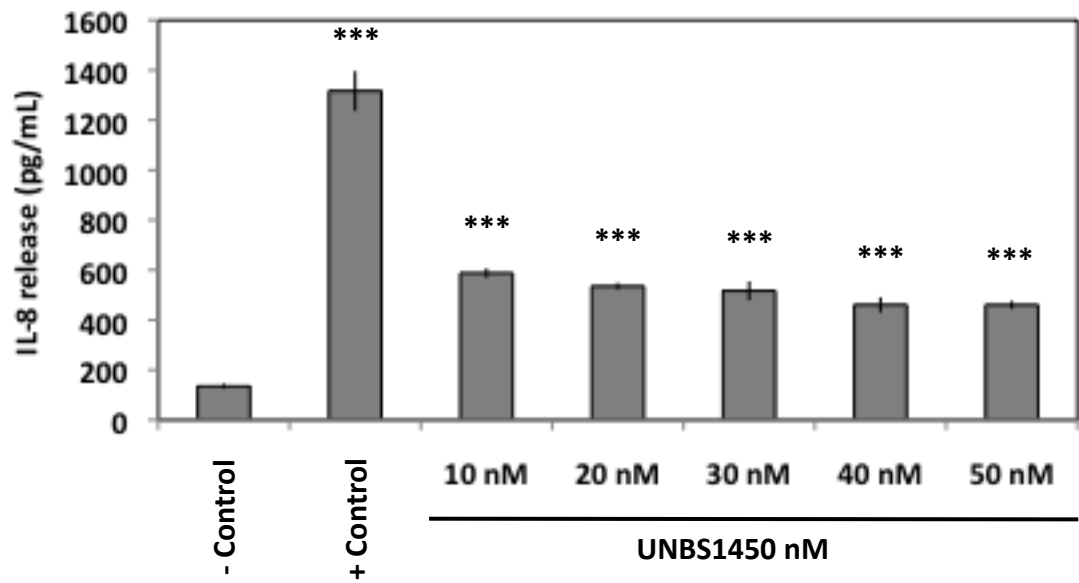


Figure 5

F.



G.

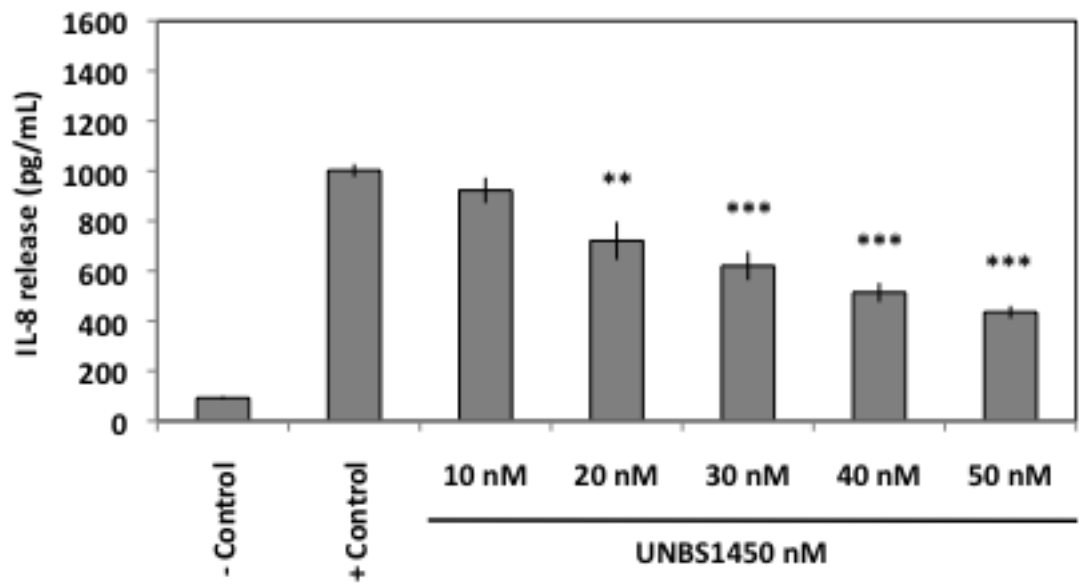


Figure 5