Biochemical Pharmacology 82 (2011) 620–631

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

The aromatic ketone 4′-hydroxychalcone inhibits TNFα-induced NF-κB activation *via* proteasome inhibition

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ARTICLE INFO

Article history:
Received 24 April 2011
Accepted 7 June 2011
Available online 14 June 2011

Keywords:
Polyphenol
Chalcone
NF-κB
Inflammation
Cancer

ABSTRACT

Chalcones are aromatic ketones, known to exhibit anti-microbial, anti-inflammatory and anti-cancer activities. The aim of this study was to investigate the anti-inflammatory and anti-cancer activity of 4′-hydroxychalcone. Here, we report that 4′-hydroxychalcone inhibits TNFα-induced NF-κB pathway activation in a dose-dependent manner. To investigate the underlying molecular mechanisms we demonstrate that 4′-hydroxychalcone inhibits proteasome activity in a dose-dependent manner but has no effect on IKK activity. Results show that 4′-hydroxychalcone inhibits TNFα-dependent degradation of IκBα and subsequently prevents p50/p65 nuclear translocation leading to 4′-hydroxychalcone-inhibited expression of NF-κB target genes. Most importantly, inhibition of NF-κB activation by 4′-hydroxychalcone is not leukemia cell-type specific and has no significant effect on non-transformed cell viability, thus highlighting the compound's potential in both prevention and treatment.

1. Introduction

Recently anti-cancer research refocused on the power of Mother Nature to efficiently fight cancer. Natural sources provide a rich repository of save compounds and examination of these natural products has provided evidence for their anticancer potential. One major advantage of naturally occurring drugs relative to synthetic chemicals is their lack of toxicity towards healthy tissues. Additionally, natural products have specific bioactivity and bioavailability. Thus, the trend to treat illnesses with naturally discovered lead compounds has established a basis for the development of novel cancer therapies.

Chalcones belong to the polyphenol family, represent natural antioxidants, are commonly found in fruits and vegetables, and serve as precursors for flavonoid synthesis. Because they are abundant in edible plants, they seem to be promising representatives for dietary cancer prevention strategies [1]. Additionally, their simple and efficient synthesis makes them also attractive for cancer therapy.

Several studies have shown that the anti-proliferative effects on cancer cells are associated with the presence of hydroxyl group/s in chalcone molecules; therefore, hydroxyl-derivates of chalcone have more potent anti-proliferation properties compared to the other chalcone derivatives [2–4]. Additionally, the position of the hydroxyl group within the chalcone molecule seems to play an important role in the activity of the molecule. Chemically, 4′-hydroxychalcone is an alpha, beta-unsaturated ketone with the core structure of chalcone (1,3-diphenyl-2-propenone) and one hydroxyl-substituent on the 4′ position of the A ring (Fig. 1).

Because nuclear factor (NF)-κB is irreversibly connected with inflammatory processes involved in wide range of severe diseases including cancer and because it has been shown to induce resistance to various chemotherapeutic agents, targeting this pathway by natural compounds is an attractive strategy to attenuate and overcome cancer development [5–10]. The NF-κB family includes 5 family members: RelA (p65), RelB, c-Rel, p50/p105 (NF-κB1) and p52/p100 (NF-κB2), which can form homo- and hetero-dimeric protein complexes. By binding of the dimers to corresponding DNA sequences in promoter or enhancer regions, the expression of pro-inflammatory genes is increased while the expression of other genes is repressed [11]. The most common NF-κB dimer is the p50/p65 heterodimer, which is involved in canonical NF-κB pathway activation. In the presence of divergent extrinsic signals (cytokines, carcinogens, UV light, physical and chemical stress or proliferating agents), inhibitor of NF-κB (IκB) is phosphorylated by IκB kinase (IKK), ubiquitinated and marked for the proteasomal degradation [12]. Subsequently, the NF-κB dimer is no longer sequestered in the cytoplasm and rapidly translocates to the nucleus where it can transactivate more than 550 genes.

To date, a large spectrum of natural, plant-derived, polyphenols was identified and investigated for its NF-κB inhibition potential. Additionally, the ability to synthesize original natural compounds

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doi:10.1016/j.bcp.2011.06.012

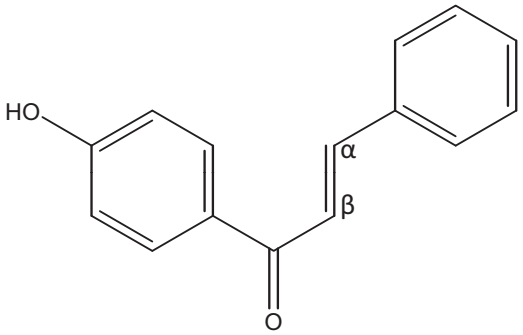

Fig. 1. Structure of 4′-hydroxychalcone.

or to modify their structures to improve their bioactivity opens a new horizon of potential novel molecules with anticancer potential.

In this study, we provided a detailed biological analysis of the involvement of 4′-hydroxychalcone in NF-κB regulation in different leukemic cell lines and tried to elucidate the mechanistic background underlying the inhibitory potential of 4′-hydroxychalcone. We showed that 4′-hydroxychalcone significantly repressed the tumor necrosis factor alpha (TNFα)-induced NF-κB pathway and NF-κB target genes. Interestingly, we discovered a strong negative effect of 4′-hydroxychalcone on proteasomal activities, explaining its involvement in the NF-κB cascade. Moreover, we provided evidence of the selective cytotoxic effect of 4′-hydroxychalcone on cancer cells by a comparative study with a non-cancer cells. Thus, we identified mechanisms, which make 4′-hydroxychalcone an interesting, safe and promising inhibitor of TNFα-induced NF-κB activation pathway.

2. Materials 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 1× PBS supplemented with 0.5% (w/v) BSA, according to the manufacturer's instructions.

4′-Hydroxychalcone was a gift from laboratory of Dr. Deniz Tasdemir (Department of Pharmaceutical and Biological Chemistry, School of Pharmacy of University of London, England). The compound, with a molecular weight of 224.27 g/mol, was received as a powder and solubilized in 100% DMSO (Sigma–Aldrich, Bornem, Belgium) to obtain a stock concentration of 100 mM. This stock solution was then further diluted in DMSO to obtain working aliquots. Both stock and working solutions 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) and Jurkat (T-cell 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 (Bio-Whittaker, Verviers, Belgium) at 37 °C and 5% CO_2. The cells were harvested every 3 days. After thawing, the cells were kept in normal culture conditions for 10 days before experiments.

At T_0, the cells were treated with 4′-hydroxychalcone at various concentrations. At T_0 + 2 h the cells were activated by addition of TNFα (20 ng/mL). Controls and test samples were then incubated for the appropriate time at 37 °C.

Healthy blood samples were kindly donated as buffy coats by the Red Cross (Luxembourg, Luxembourg). Diluted (1/3) blood in RPMI 1640 was layered onto Ficoll and centrifuged (400 × *g*, 20 min) to isolate mononuclear cells. The isolated peripheral blood mononuclear cells (PBMCs) 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 that were cultured in growth medium (RPMI, 0.1% FCS) at a concentration of 3×10^5 cells/mL and were pre-treated in the presence and absence of 4′-hydroxychalcone (30 μM) for 4, 8 or 16 h before being activated by TNFα (20 ng/mL) for 30 min, or for indicated time period. Cells were then harvested and washed twice in 1× PBS. Supernatants were discarded and cell pellets were stored overnight at −80 °C. Nuclear protein extractions were performed as described previously, and nuclear extracts were stored at −80 °C [13]. The oligonucleotide NF-κBc (consensus NF-κB site) (Eurogentec, Liège, Belgium) (5′-AGTTGAGGGGACTTTCCCAGGC-3′) and its complementary sequence were used as probes. After hybridization, the probes were radiolabeled using [g-^{32}P]-ATP (MP-Biomedicals, Illkirch, France) and the EMSA assay was performed according to conditions established before [13]. First, 10 μg of nuclear proteins were incubated with the radiolabeled probe in binding buffer for 20 min on ice. Then the DNA–protein complexes were separated on a 5% polyacrylamide gel followed by visualization by autoradiography.

2.4. Transient transfection and luciferase reporter gene assay

K562 cells were transiently transfected as described previously [13]. For each electroporation, we used 5 μg of a luciferase reporter gene construct containing 5 repeats of a consensus NF-κB site (Stratagene, Genomics Agilent, Diegem, Belgium) and 5 μg of a *Renilla* luciferase plasmid (Promega, Leiden, Netherlands). The ICAM-1 LUC reporter plasmid was a generous gift from W. Vanden Berghe (University of Antwerp, Belgium). The full-length ICAM-1 promoter construct contains approximately 1.4 kb of ICAM-1 5′-flanking DNA linked to the firefly luciferase (LUC) gene. Promoter sequences between 393 bp and 176 bp upstream of the gene, contain binding sites for C/EBP and NF-κB. For co-transfection assays, 5 μg of expression plasmids coding for proteins of the NF-κB pathway (receptor of TNFα-1 (TNFR-1), TNFR-associated factor-2 (TRAF2), TNFR-1 associated death domain (TRADD), inhibitor of κB kinase beta (IKKβ), tumor growth factor activated kinase 1 (TAK1) and TAK1-binding protein 1 (TAB1)) were added to the electroporation mixture. 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, 0.1% FCS) to a final concentration of 1×10^6 cells/mL and pre-treated for 2 h with 4′-hydroxychalcone at varying concentrations. Activation with 20 ng/mL of TNFα for 6 h followed the pre-treatment. For co-transfection experiments only TNF indicated control was activated by TNFα. After the 8 h incubation, 75 μL of Dual-Glo™ Luciferase Reagent (Promega, Leiden, Netherlands) was added to 75 μL of the cellular suspension for 10 min incubation at 22 °C before luciferase activity measurement. Subsequently, 75 μL of Dual-Glo™ Stop&Glo® Reagent (Promega, Leiden, Netherlands) was added for 10 min at 22 °C to the cell suspension to measure *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 to *Renilla* luciferase activity.

2.5. Cell viability assessment

Cell survival percentages were evaluated using Promega's CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega, Leiden, Netherlands) according to the manufacturer's instructions. Briefly, the assay determines the number of viable cells based on the quantification of ATP as an indicator of metabolic activity. ATP is measured using the luciferase catalysis of luciferin to oxyluciferin and light in the presence of Mg^{2+}, ATP and oxygen. The lyophilized enzyme/substrate mixture was reconstituted with the provided buffer. Then, an equal volume of the reaction buffer was added to medium containing K562, U937, Jurkat or PBMC cells (untreated or treated with 4′-hydroxychalcone at the indicated concentrations and times). The mixture was shaken on a rocking platform for 2 min and then incubated for 10 min in the dark at RT. The luminescence signal, which is proportional to the amount of ATP present, was quantified using a Berthold Orion microplate luminometer and converted into the number of viable cells according to the manufacturer's instructions. Data were normalized to the control and reported as percentage of viable cells.

2.6. Extraction of cellular proteins

After the indicated incubation times with 4′-hydroxychalcone and TNFα, the K562 and Jurkat cells were lysed, and the nuclear and cytoplasmic extracts were prepared according to [13]. Briefly, 10^7 cells per sample were lysed in a hypertonic detergent medium containing protease inhibitor cocktail (Complete®, Roche, Luxembourg). The extraction was performed on ice to avoid protein degradation. The suspension was put on a vertical agitation shaker for 15 min at + 4 °C and was then centrifuged at 15,000 × *g* for 15 min at +4 °C for suspension clearing. Afterwards, supernatants were removed and aliquots were stored at −80 °C until use.

2.7. Western Blot analysis

Proteins of nuclear and cytoplasmic 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 for 1 h. Lamin B for cytoplasmic extracts and α-tubulin for nuclear extracts, were used as loading controls. Blots were then incubated with primary antibodies: anti-pIκBα (1/300, Cell Signaling 9246, Biokê, 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) or anti-lamin B (1/1000, Santa Cruz SC-6216). All antibodies were diluted in a PBS-Tween solution containing 5% bovine serum albumin (BSA) or 5% 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).

2.8. Human CXCL8/IL-8 immunoassay

IL-8 concentrations in culture supernatants of activated K562 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 with 100 μL of Assay Diluent to anti-IL-8 pre-coated wells followed by 2 h incubation at RT. After washing, a polyclonal peroxidase-conjugated anti-IL-8 antibody was added for another 60 min at RT. Colorimetric visualization and protein dosage were developed by addition of the H_2O_2 + TMB (tetramethylbenzidine) containing substrate. After a 30 min reaction at room temperature (RT) in the dark, the enzymatic reaction was stopped by addition of H_2SO_4 and optical densities were measured at a wavelength of 450 nm.

2.9. IKK kinase activity assay

The K-LISA™ IKKβ-Inhibitor Screening Kit (Calbiochem, San Diego, CA) was used for rapid in vitro screening of IKKβ inhibitors. The ELISA-based activity assay utilizes a 50-amino acid GST-IκBα fusion polypeptide substrate with Ser32 and Ser36 IKKβ phosphorylation sites. The GST-IκBα substrate and IKKβ were incubated in the presence of IKKβ inhibitors in a Glutathione-Coated 96-Well plate. The incubation of GST-IκBα substrate and IKKβ allowed for substrate phosphorylation and capture in a single step. The phosphorylated GST-IκBα substrate was detected using an Anti-Phospho IκBα (Ser32/Ser36) antibody, followed addition of the HRP-Conjugate and colorimetric development with TMB Substrate. ELISA Stop Solution was used to stop the colorimetric development and the absorbance was read at 450 nm, preferably with a reference wavelength of 540–600 nm, using a SpectraCount UV-spectrometer (Packard, Groningen, Netherlands). The absorbance is directly related to the level of IKKβ activity. IKK-2 Inhibitor IV ([5-(p-fluorophenyl)-2-ureido]thiophene-3-carboxamide) (Calbiochem, San Diego, CA) was used as a positive control.

2.10. Proteasome inhibition activity

The Proteasome-Glo™ Chymotrypsin-like Cell-Based Assay (Promega, Leiden, Netherlands) was used in addition to the Trypsin-like and Caspase-like Assays to evaluate the three major types of proteolytic activities. Epoxomicin (5 μM) (Sigma, Bornem, Belgium) was used as positive inhibitory control. The assays were performed as indicated in the manufacturers' protocols. Briefly, K562 or U937 cells at a concentration 10^6 cells/mL in RPMI 1640 medium containing 0.1% FCS were treated with indicated concentrations of 4′-hydroxychalcone. After an incubation period of 8 h, 25 μl (25×10^3) cells per well in 96 well plate were mixed with 25 μl of the recombined cell-based reagent. After vigorous shaking for 2 min and an additional incubation period of 10 min at room temperature, the luminescence was measured on a luminometer (Berthold). A viability assay using CellTiter-Glo® Luminescent Cell Viability Assay Kit (Promega, Leiden, Netherlands) was performed in parallel to normalize the proteasome activity to the number of viable cells.

2.11. Statistical analysis

Data are expressed as mean ± SD. Significance was determined by the Student's *t*-tests. *P*-values below 0.05 were considered as statistically significant.

3. Results

3.1. 4′-Hydroxychalcone inhibits TNFα-induced NF-κB pathway activation in a dose-dependent manner

The NF-κB pathway is engaged in many inflammatory processes. Its involvement in various malignancies suggests that its activation is linked to cancer development and progression. Therefore, we were interested to see how 4′-hydroxychalcone interferes with the classical model of pro-inflammatory signaling through the TNFα-induced NF-κB transcriptional pathway.

To examine the inhibitory potential of 4′-hydroxychalcone on NF-κB activity we used a reporter gene assay. K562 cells transfected with a luciferase reporter gene were pre-treated with different concentrations of 4′-hydroxychalcone for 2 h and then treated with TNFα (20 ng/mL) for an additional 6 h. Results showed that TNFα led to strong activation of the NF-κB pathway in control cells and that 4′-hydroxychalcone inhibited the TNFα-driven NF-κB pathway activation in a dose-dependent manner with an IC_{50} of 30 μM (Fig. 2A). The IC_{50} value was calculated using XY scatter dependency chart applying the best fitting trend formula model. The average of three IC_{50} values from three independent experiments was applied.

Additionally, because TNFα triggers the NF-κB pathway by sequentially activating TNFR1, TRADD, TRAF2 and IKK, thus leading to IκBα phosphorylation, we wanted to elucidate where exactly 4′-hydroxychalcone inhibits this cascade. Recently, the importance of TAK1 in the TNF-induced NF-κB activation pathway has been reported [14]. These studies indicated that TNFα stimulation leads to formation of the TAK1, TRAF2, IKKα and IKKβ complex and results in IKK activation. Thus, we transiently co-transfected K562 cells with TNFR1-, TRADD-, TRAF2-, NIK-, TAK1-, TAB1-, and IKKβ-expressing plasmids along with a NF-κB-regulated luciferase reporter construct. The luciferase assay revealed that cells co-transfected with each of these plasmids expressed the NF-κB-regulated reporter gene and that 4′-hydroxychalcone significantly suppressed the NF-κB reporter activity in cells co-transfected with TNFR1, TRAF2 or TAK1, but had no inhibitory effect on the activity in cells overexpressing TRADD, IKKβ, NIK or TAB1 (Fig. 2B). These results indicate involvement of 4′-hydroxychalcone on the receptor and the primary signaling complex.

3.2. 4′-Hydroxychalcone has no direct effect on IKK activity

Degradation of IκBα depends either on IKK activity or on proteasome activity. Chronologically, IKK activity occurs prior to proteasomal activity. To further confirm our previous results showing the inability of 4′-hydroxychalcone to effect directly IKK activity, we monitored IKKβ activity by ELISA in the presence of 4′-hydroxychalcone. The ELISA used an IκBα substrate and IKKβ His-Tag® protein.

We tested a wide spectrum of concentrations (from 10 μM to 1000 μM) of 4′-hydroxychalcone and showed that 4′-hydroxychalcone was not able to significantly inhibit IKKβ activity at any tested concentrations (Fig. 3).

We concluded that the inhibitory potential of 4′-hydroxychalcone on NF-κB is independent of direct IKK activity.

3.3. 4′-Hydroxychalcone inhibits proteasome activity in a dose-dependent manner

We were interested if proteasomal activity was involved in the mechanism by which the chalcone derivative inhibits the NF-κB

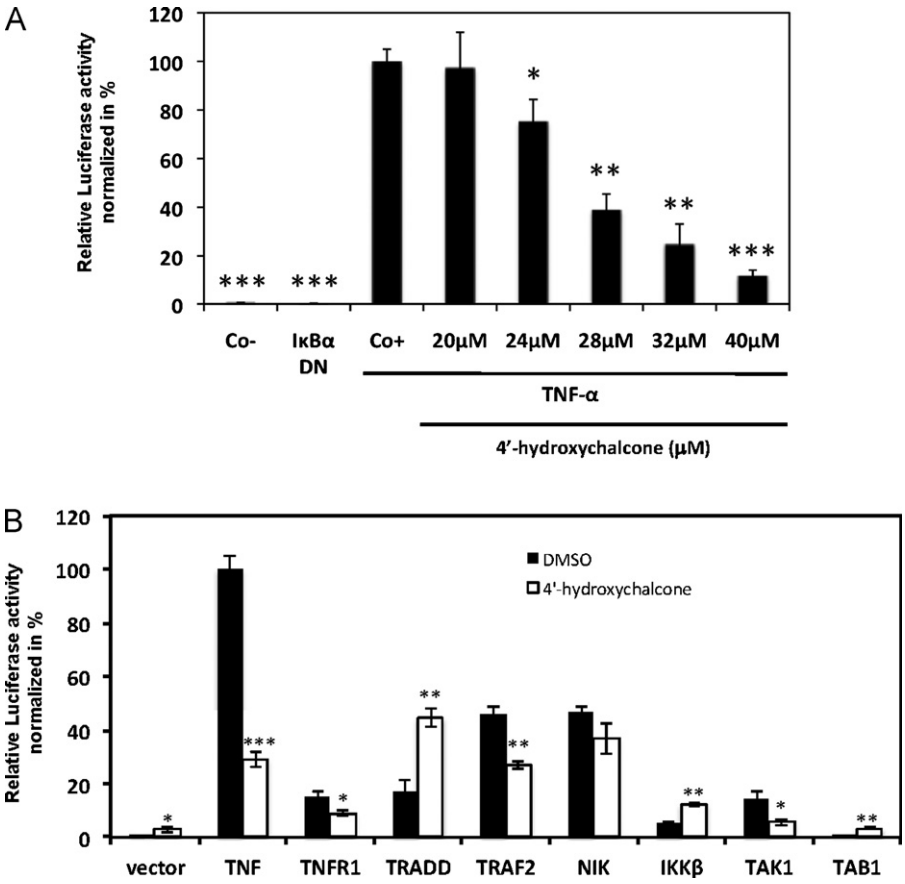

Fig. 2. Inhibition of TNFα-induced NF-κB activation by 4′-hydroxychalcone. (A) K562 cells were transiently transfected with firefly luciferase vector (NF-κB pGL4), ph-RG-tk *Renilla* plasmid and with or not IκBα dominant negative (DN) for 24 h. After transfection, K562 cells were treated with 4′-hydroxychalcone at the different concentrations for 2 h followed by a TNFα-treatment (20 ng/mL) during 6 h. Results are expressed as a ratio of the measured luminescence of the firefly luciferase vector and the luminescence of *Renilla* plasmid. Results are presented as a mean ± SD of three independent experiments. Negative control (Co−) corresponds to DMSO treated cells, without TNFα activation, positive control (Co+) corresponds to DMSO treated cells activated by TNFα. (B) K562 cells were transiently transfected with TNFR1, TRADD, TRAF2, NIK, IKKβ, TAK1 or TAB1 along with NF-κB pGL4 and ph-RG-tk *Renilla* plasmids for 24 h. After transfection, K562 cells were treated or not with 4′-hydroxychalcone at 30 μM for 2 h followed by or not a TNFα-treatment (20 ng/mL) during 6 h. The cells were assayed for Luciferase activity. Asterisks indicate a significant difference between untreated and 4′-hydroxychalcone treated cells as analyzed by *t*-test ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). Vector refers to untreated or 4′-OH treated cells without TNFα activation.

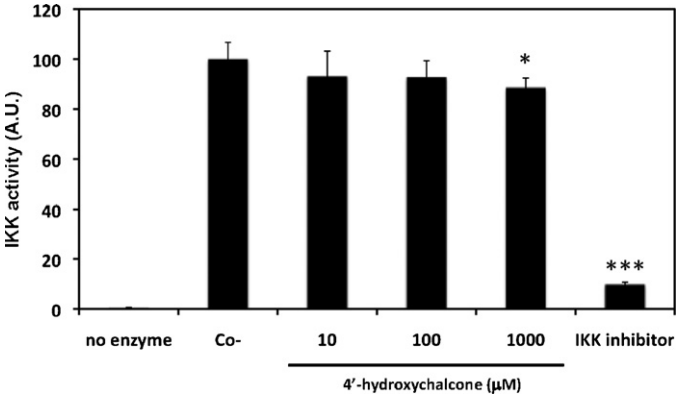

Fig. 3. Effect of 4′-hydroxychalcone on IKK activity. Effect of 4′-hydroxychalcone at the indicated concentrations on the kinase activity IKKβ with an incubation period of 30 min at 30 °C. 'no enzyme' was performed without IKKβ and negative control (Co−) was determined without any test compound but in the presence of IKKβ. IKK-2 Inhibitor IV (Calbiochem) was used at the recommended concentration of 1 μM. Each value is a mean ± SD of three determinations. Asterisks indicate a significant difference compared to the negative control as analyzed by *t*-test ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

pathway. We evaluated the protease inhibition activity of 4′-hydroxychalcone on three different proteolytic activities (chymotrypsin-, trypsin- and caspase-like) of the 26S proteasome at a concentration range between 0.1 μM and 25 μM 4′-hydroxychalcone in K562 and U937 leukemia cells. 4′-hydroxychalcone was able to inhibit each of the 3 major proteolytic activities in a dose-dependent manner in both cell lines (Fig. 4A and B).

Thus, 4′-hydroxychalcone significantly repressed all three proteasomal activities. We showed a positive correlation between dose of 4′-hydroxychalcone and its inhibitory potential.

3.4. 4′-Hydroxychalcone inhibits TNFα-dependent degradation of IκBα and prevents p50/p65 nuclear translocation

Degradation of NF-κB natural inhibitor IκBα represents the initial step in NF-κB activation. Thus, we assessed IκBα stability in the cytoplasm and translocation of the NF-κB subunits p65 and p50, from the cytoplasm to the nucleus by Western blot analysis. Control cells activated by TNFα showed the classical model of NF-κB pathway activation:degradation of IκBα appearing at 10 min after stimulation and the re-appearance of IκBα at 60 min after stimulation. Degradation occurs by a negative feedback loop because p50/p65 binding to DNA induces IκBα gene expression. We also observed nuclear translocation of p50 and p65 15 min

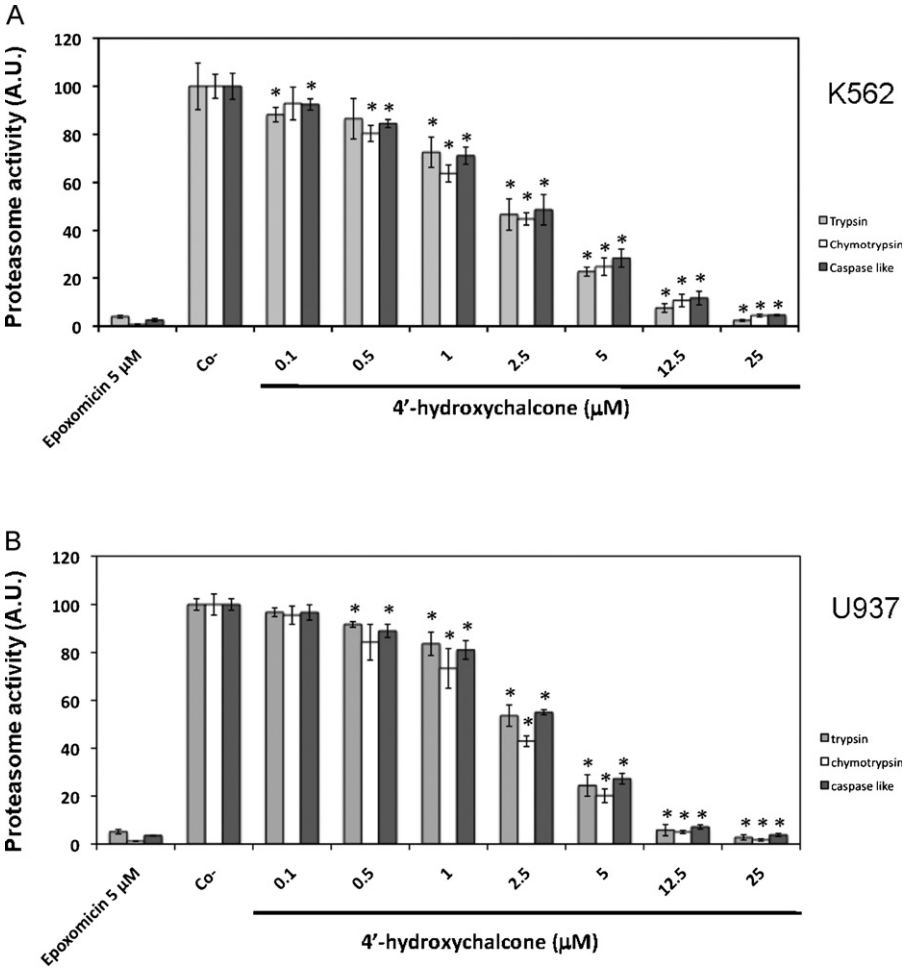

Fig. 4. Effect of 4′-hydroxychalcone on 3 major proteasome activities. (A) K562 and (B) U937 cells were treated with 4′-hydroxychalcone at indicated concentrations for 8 h and the proteolytic activities of 26S proteasome were assessed. Results are expressed as a ratio of the firefly luciferase activity luminescence of the proteasome kit over the firefly luciferase activity of the viability kit. Negative control (Co−) corresponds to untreated cells, 5 μM of epoxomicin-known selective proteasome inhibitor refers to positive inhibitory control. Each value is a mean ± SD of three determinations. The asterisk indicates a significant difference compared to the negative control analyzed by *t*-test ($^{*}p < 0.05$).

after TNFα stimulation. However, 4′-hydroxychalcone was able to considerably postpone and attenuate IκBα degradation and prevented the cytoplasmic to nuclear translocation of both the p50 and p65 subunits of the NF-κB heterodimer (Fig. 5).

To confirm previous results showing that 4′-hydroxychalcone can block TNFα-dependent p50/p65 nuclear translocation, we performed an EMSA to determine if 4′-hydroxychalcone can prevent NF-κB binding to DNA. K562 cells were exposed to 4′-hydroxychalcone for different time intervals and were then treated for 30 min TNFα. Results presented in Fig. 6A shows that 30 μM 4′-hydroxychalcone suppressed TNFα induced NF-κB activation for up to 16 h (Fig. 6A).

As the active NF-κB dimer may consist of various combinations of Rel/NF-κB proteins, we wanted to show that the band visualized by EMSA represented the p50/p65 NF-κB heterodimer. We incubated the nuclear extract from TNFα-stimulated cells with anti-p50 or anti-p65 antibodies. The results shown in Fig. 6B and D indicate that in K562 and Jurkat cells, respectively, TNFα-activated complex consisted of p50 and p65 subunits because the major bands were shifted to a higher molecular mass (Fig. 6B and D).

3.5. Inhibition of NF-κB activation by 4′-hydroxychalcone is not cell-type specific

Because NF-κB induction can be mediated in different cell types by specific signal transduction pathways, we examined whether 4′-hydroxychalcone is able to suppress the activity of NF-κB in different cell types. We treated Jurkat cells with 4′-hydroxychalcone at a concentration corresponding to the IC_{50} of NF-κB inhibition (30 μM) and then further cultured the cells in the presence or absence of TNFα. EMSA analysis, shown in Fig. 6C, confirmed that 4′-hydroxychalcone is able to inhibit TNFα-induced NF-κB activation in cells other than the K562 leukemic cell line, specifically in the Jurkat T-cell leukemia cell line. This indicates that the effect of 4′-hydroxychalcone on TNFα-induced NF-κB activation is not cell type specific. Additionally, 4′-hydroxychalcone alone did not activate NF-κB signaling pathway (Fig. 6C).

3.6. 4′-Hydroxychalcone inhibits the expression of NF-κB target genes

TNFα is known to induce the expression of many genes involved in cancer proliferation and metastasis. Additionally, many of these genes have an NF-κB binding site in their promoters. As described above, 4′-hydroxychalcone is able to down-regulate NF-κB activity. We were interested if this negative regulation affected NF-κB target gene expression. To further investigate the effects of 4′-hydroxychalcone on downstream NF-κB signaling, we focused on two proteins, which are known to be under the NF-κB control: IL-8 and ICAM-1. We applied two different strategies to study possible changes in gene expression. First, we used an ELISA assay to measure changes in IL-8 production 24 h after treatment with 4′-hydroxychalcone. IL-8 is a pro-inflammatory CXC chemokine and its expression is primarily regulated by NF-κB. Two hours after treatment with 4′-hydroxychalcone, K562 cells were incubated in the presence or absence of TNFα. Control cells produced markedly low amounts of IL-8 (165 pg/ml) while TNFα stimulation led to a rapid increase of IL-8 levels (1305 pg/ml). We observed that cells pretreated with 4′-hydroxychalcone at a concentration of 60 μM produced significantly lower levels of IL-8 (702 pg/ml). Observed decrease in IL-8 release relative to cells stimulated with TNFα but not treated with 4′-hydroxychalcone is up to 40% (Fig. 7A).

Secondly, we examined whether 4′-hydroxychalcone affects ICAM-1 gene transcription. ICAM-1 is involved in cell invasion [15]. K562 cells were transiently transfected with ICAM-1 plasmid along with the *Renilla* reporter plasmid and after 24 h incubation the cells were pre-treated with 30 μM of 4′-hydroxychalcone(concentration corresponding to IC_{50} for NF-κB inhibition) for 2 h. The cells were then treated with TNFα for an additional 6 h. The induction of ICAM-1 by TNF occurred within 6 h and was rapidly down-regulated by 4′-hydroxychalcone (Fig. 7B).

3.7. 4′-Hydroxychalcone affects cancer cell viability but has no significant effect on non-transformed cell viability

A current limitation in cancer treatment is the toxicity of active compounds to non-cancerous tissues. Thus, we were interested to

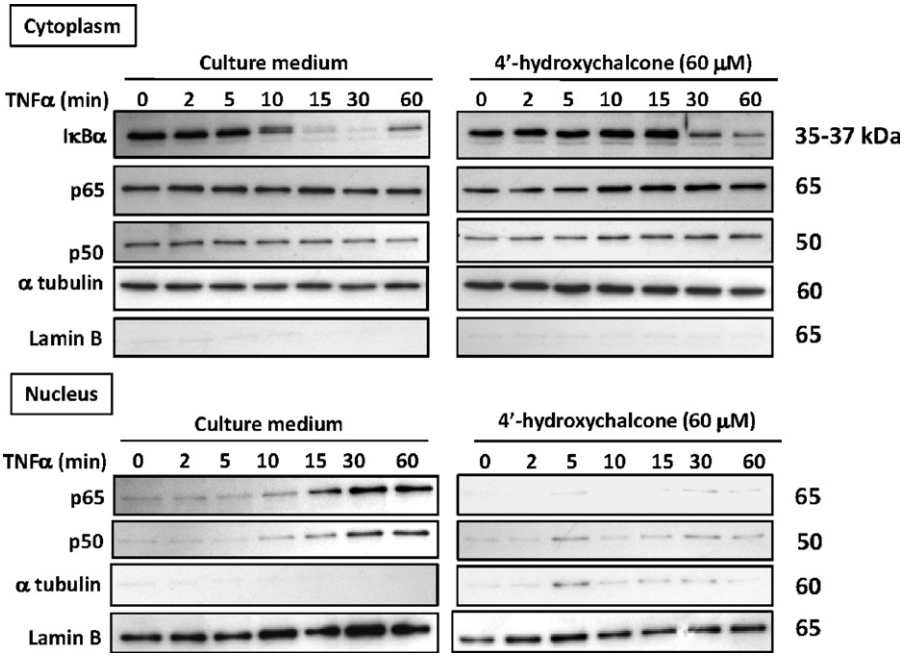

Fig. 5. Effect of 4′-hydroxychalone on the degradation of IκBα and translocation of p65 and p50 to the nucleus. Jurkat cells were pre-treated with 4′-hydroxychalcone (60 μM) for 2 h followed by activation with TNFα (20 ng/mL) for indicated time periods. Cytoplasmic and nuclear extracts were tested for IκBα, p50 and p65. Protein loading and purity of extracts were verified by lamin B and α-tubulin Western blots. Shown data are representative out of three independent experiments with similar results.

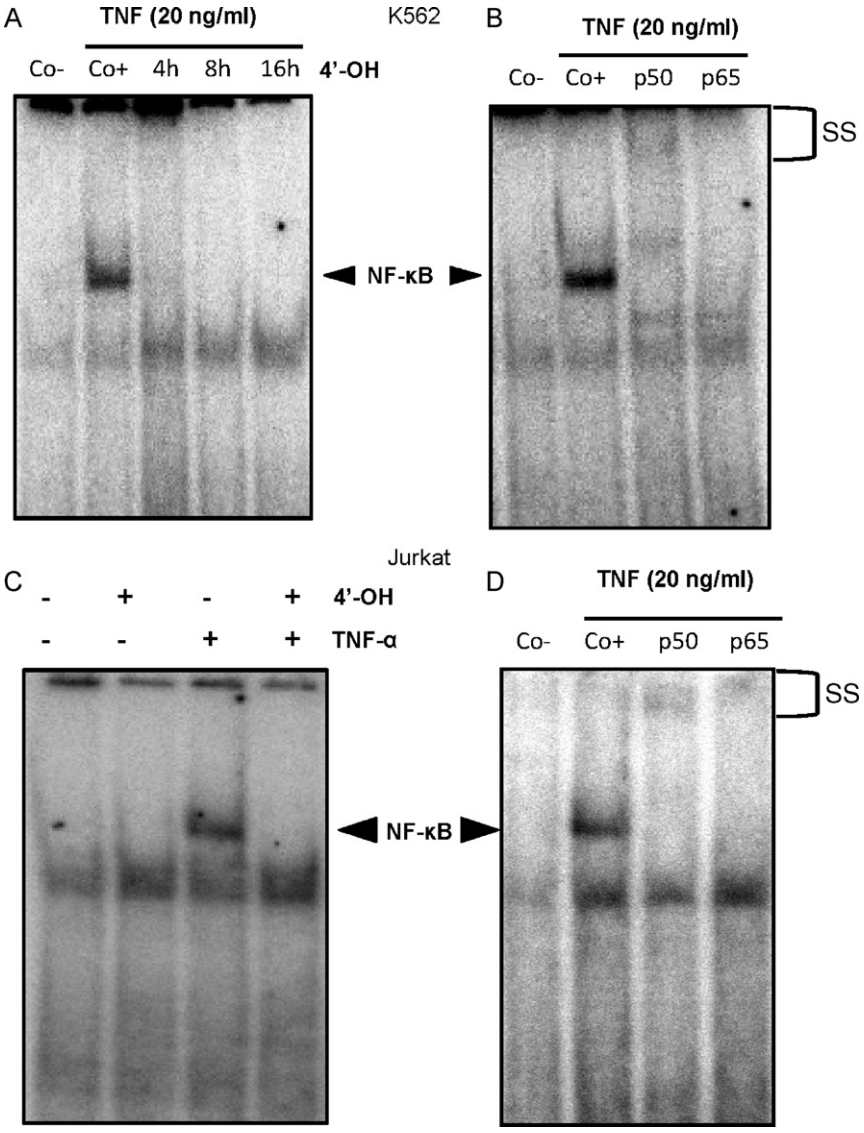

Fig. 6. 4′-Hydroxychalcone blocks TNFα-dependent p50/p65 nuclear translocation and prevent NF-κB binding to DNA. (A) Time-dependent effect of 4′-hydroxychalcone on TNFα-induced NF-κB activation. K562 cells were pre-incubated with 30 μM of 4′-hydroxychalcone for 4, 8 or 16 h, treated with TNF (20 ng/mL) for 30 min, and then subjected to EMSA. (B) Supershift (SS) with antibodies p50 and p65. NF-κB induced by TNFα is composed of p50 and p65 subunits. Nuclear extracts from TNFα activated K562 cells were prior to EMSA treated with p50 or p65 antibodies. (C) Inhibition of NF-κB activation by 4′-hydroxychalcone is not cell-type specific. Jurkat cells were incubated with or without 4′-hydroxychalcone at 30 μM for 4 h and then activated or not with 20 ng/mL TNF for 30 min. Nuclear extracts were then prepared and assayed for NF-κB activation by EMSA. (D) Supershift (SS) with antibodies p50 and p65. NF-κB induced by TNFα is composed of p50 and p65 subunits. Nuclear extracts from TNFα activated Jurkat cells were prior to EMSA treated with p50 or p65 antibodies. Negative control (Co−) corresponds to nuclear extracts of DMSO treated cells, without TNFα activation, positive control (Co+) corresponds to nuclear extracts of DMSO treated cells activated by TNFα.

compare the cytotoxic effect of 4′-hydroxychalcone on 3 different cancer cell lines (K562, Jurkat and U937) with non-transformed peripheral blood mononuclear cells (PBMCs).

To assess whether 4′-hydroxychalcone could inhibit cell growth, K562, U937, Jurkat cells and PBMCs were treated with 4′-hydroxychalcone at concentrations ranging from 5 μM to 60 μM. We first studied viability after 8 h of treatment because this time frame corresponds to the length of the treatment for several of our previous experiments. Cell viability after 8 h was determined using the CellTiter-Glo® Reagent, which quantitatively measures ATP levels to signify the presence of metabolically active cells. The inhibitory potential of 4′-hydroxychalcone was calculated relative to cells without any treatment. Fig. 8A shows that after 8 h 4′-hydroxychalcone significantly inhibited cell viability in all three cancer cell lines in a dose-dependent manner. However, the cell viability of PBMCs was not affected by 4′-hydroxychalcone even at the highest concentrations.

We also wanted to know how cell viability might change after prolonged 4′-hydroxychalcone treatment. After 24 h of treatment with 4′-hydroxychalcone, cell viability was dramatically affected in all three cancer cell lines (Fig. 8B). A significant decrease in cell viability was observed at 10 μM 4′-hydroxychalcone in K562 and Jurkat cell lines and even at 5 μM 4′-hydroxychalcone in U937 cells. At 60 μM, 4′-hydroxychalcone cell viability dropped to 17% for K562 cells, 12% for Jurkat cells and 10.3% for U937 cells. Interestingly, the cell viability of PBMCs was not inhibited by 4′-hydroxychalcone doses up to 28 μM (Fig. 8B). At 32 μM, 4′-hydroxychalcone, we observed a slight attenuation of cell viability in PBMCs. At 60 μM of 4′-hydroxychalcone, PBMCs viability dropped to 86.1%. However, relative to the cancer cell lines, the effect of 4′-hydroxychalcone on non-cancer cells is dramatically weaker. These results indicate that 4′-hydroxychalcone specifically targets cell viability in cancer cell lines. The mechanism of cell death induced by 4′-hydroxychalcone remains to be elucidated.

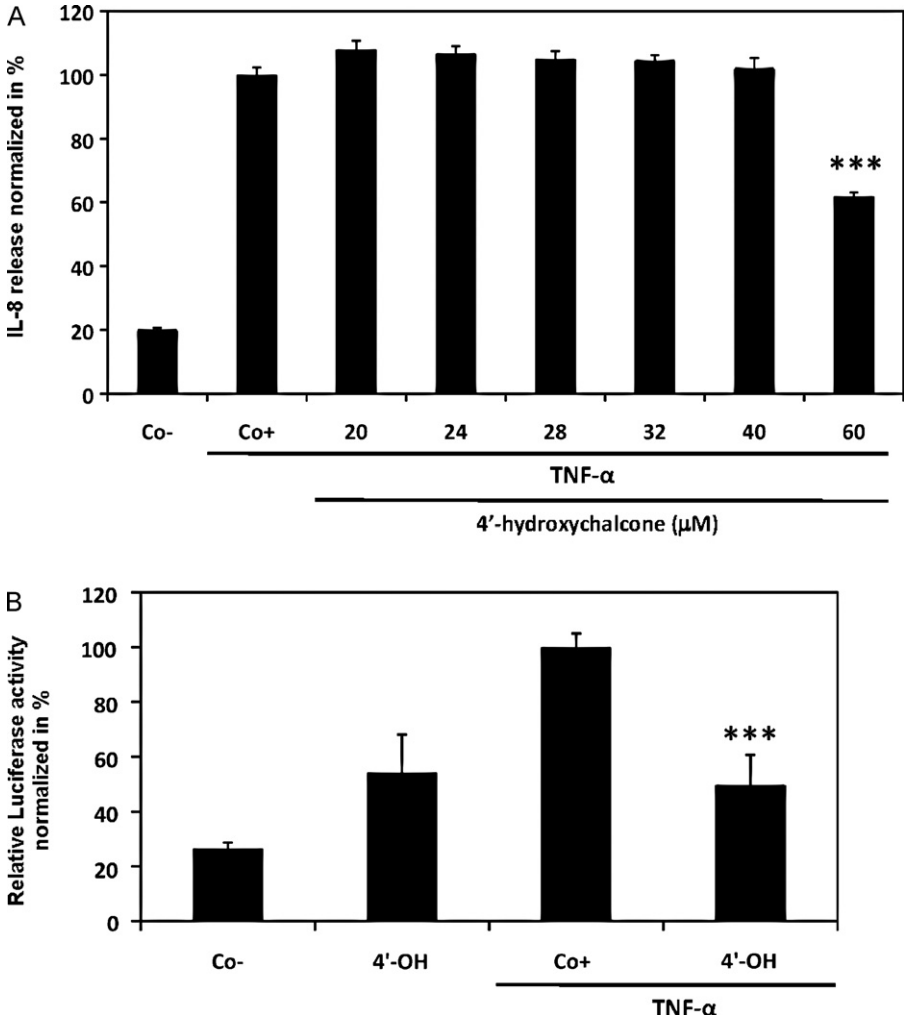

Fig. 7. Effect of 4′-hydroxychalcone on TNFα-induced NF-κB-dependent gene products. (A) 4′-Hydroxychalcone inhibits NF-κB-dependent IL-8 genes expression. K562 cells were treated or not at indicated concentrations with 4′-hydroxychalcone for 2 h, before being activated with TNFα (20 ng/mL) during 22 h. After total 24 h incubation IL-8 concentration in supernatants were measured. Negative control (Co−) corresponds to untreated cells, positive control (Co+) refers to TNFα only treated cells. (B) 4′-Hydroxychalcone inhibits NF-κB-dependent ICAM-1 genes expression. K562 cells were transiently transfected with ICAM-1 along with ph-RG-tk *Renilla* plasmid for 24 h. After transfection, K562 cells were treated with 4′-hydroxychalcone at 30 μM for 2 h followed by a TNFα-treatment (20 ng/mL) during 6 h. The cells were assayed for Luciferase activity. Each value is a mean ± SD of three determinations. Asterisks indicate a significant difference compared to control positive as analyzed by *t*-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Negative control (Co−) corresponds to transfected and DMSO only treated cells, without TNFα activation, positive control (Co+) corresponds to transfected and DMSO treated cells activated by TNFα.

4. Discussion

The goal of the present study was to determine if 4′-hydroxychalcone is able to inhibit the TNFα-induced NF-κB pathway and if so, how this inhibition is mediated. Several hydroxyl-derivatives of chalcones that do not contain other substituent groups have been shown to possess potent anti-inflammatory properties [3,4]. Naringenin chalcone, which is abundant in tomato skin and has 3 hydroxyl-groups, inhibited the production of TNFα in lipopolysaccharide (LPS)-stimulated macrophages [16]. Isoliquiritigenin (also with three hydroxyl-groups) down-regulated NF-κB activation by suppression of IKK, resulting in inhibition of the phosphorylation and subsequent degradation of IκBα [17–19]. Butein (with four hydroxyl-groups) has been shown to attenuate IκBα phosphorylation by direct inactivation of IKK through cysteine residue 179 [20]. Based on structure–activity relationship studies, natural chalcone-based NF-κB inhibitors contain preferentially hydroxyl or/and methoxy functional groups [21]. Yadav et al. summarized and compared the dose necessary for NF-κB inhibitory activity within a group of different chalcone derivatives focusing on the functional group/s they possess in their molecules. Interestingly, 2′-hydroxychalcone, structurally closely resembling our chalcone derivative in the present study, was able to inhibit NF-κB activity already at a three times lower dose compared to the basic chalcone or chalcone derivatives with more hydroxyl groups in their molecules, such as isoliquiritigenin and butein [22]. Based on these findings 4′-hydroxychalcone was a tempting candidate as an anti-inflammatory agent because it bears only one hydroxyl-group.

Here, we provided evidence showing the inhibitory potential of 4′-hydroxychalcone on the TNFα-induced NF-κB pathway in leukemic cell lines and elucidated the mechanism underlying this activity for the first time.

Specifically, we found that 4′-hydroxychalcone suppressed NF-κB activation induced by TNFα in a dose-dependent manner. As aberrant NF-κB activity is typically linked to high TNFα concentrations in patients with myelodysplastic syndromes and because this high level of TNFα is believed to be responsible for leukemic properties, we used this model to study potential effects of 4′-hydroxychalcone on NF-κB [23].

Additionally, we were also interested where 4′-hydroxychalcone is involved in the NF-κB cascade. We demonstrated that 4′-

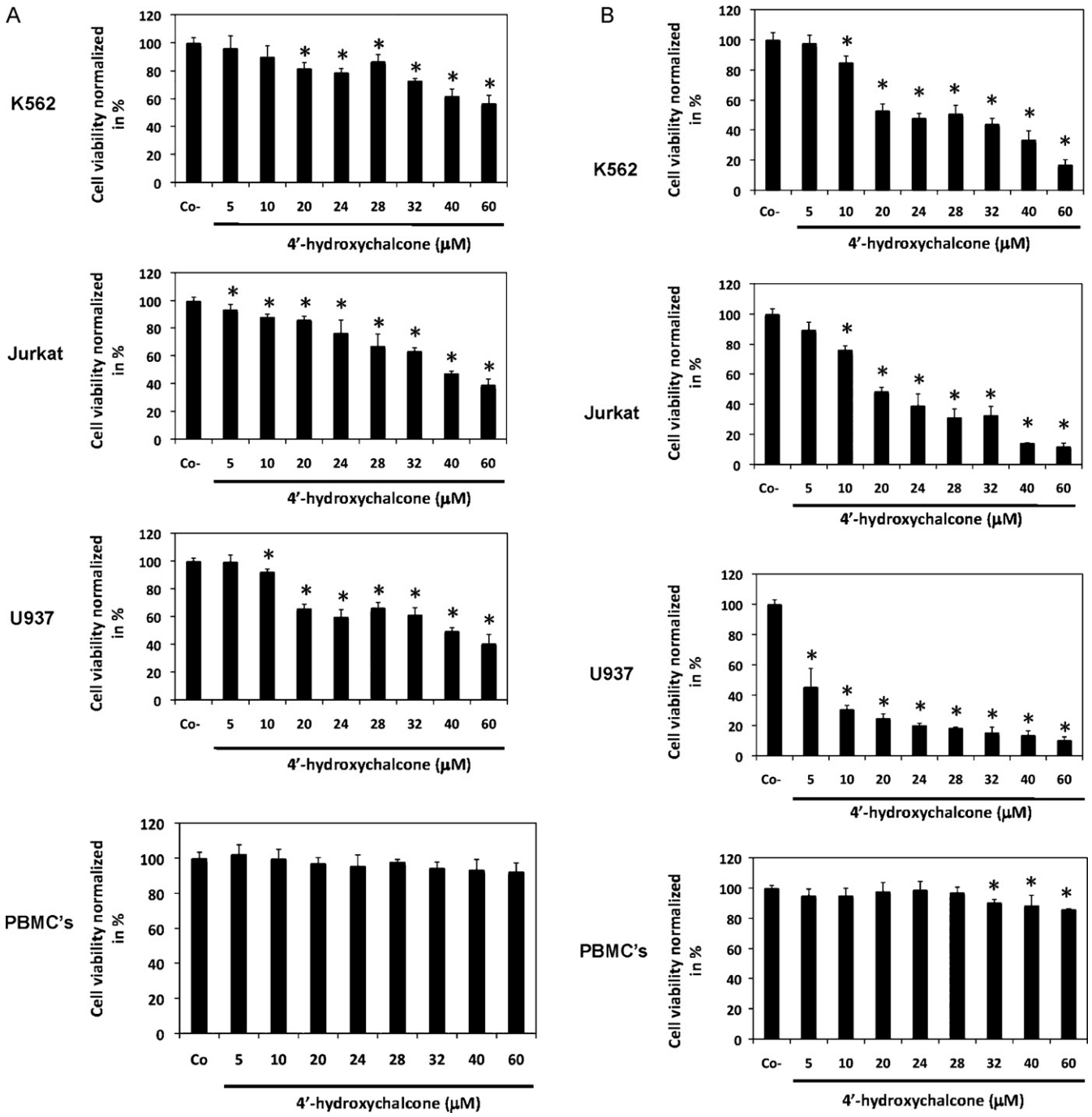

Fig. 8. Effect of 4′-hydroxychalcone on cancer cells versus non-transformed cell viability. (A) Short-term effect (8 h) of 4′-hydroxychalcone at the indicated concentrations on the different cancer cells (K562, Jurkat, U937) and PBMC's cell viability. (B) Effect of 4′-hydroxychalcone at the indicated concentrations on different cancer cells (K562, Jurkat, U937) and PBMC's cell viability analyzed after 24 h. Negative control (Co−) corresponds to untreated cells. Each value is a mean ± SD of three determinations. The asterisk indicates a significant difference compared to the negative control analyzed by *t*-test (*$p < 0.05$).

hydroxychalcone has no direct effect on IKK but nevertheless may attenuate its activity indirectly through inhibition of particular members of the primary signaling complex. IKK activation has been linked to various kinases and signaling molecules [24,25]. We found that 4′-hydroxychalcone blocked TNFR1-, TRAF2- and TAK1-mediated NF-κB activation. The importance of TRAF2, which is recruited to TNFR1 through TRADD-interaction and subsequently leads to activation of NF-κB, has been previously reported [26]. Similarly, TNFα activation of the NF-κB pathway is associated with inducible binding of TAK1 to TRAF2 and thus to both IKKα and IKKβ [14]. TRAF2 and cellular inhibitors of apoptosis (cIAPs) are believed to cooperate by recruiting ubiquitin chains to receptor interacting protein (RIP). While TRAF2 seems to play a structural role, allowing recruitment of cIAPs to the vicinity of RIP, cIAPs can directly ubiquitinate RIP [27,28]. This ubiquitination leads to attachment and activation of TAK1 kinase complex, which consist of TAK1 kinase and its associated proteins TAB1, TAB2 and TAB3, which in turn phosphorylates and activates IKK complex [29–32]. The ability of 4′-hydroxychalcone to inhibit NF-κB activity induced in cells overexpressing TAK1 supports recent studies showing TAK1 as a critical component required for IκB kinase-mediated activation of the NF-κB pathway [33]. Contrariwise, when we

induced the NF-κB pathway at the step of overexpressed TRADD, IKK or TAB1, we observed an induction instead of inhibition of NF-κB-driven reporter gene activity. As we showed, 4′-OH induces the basal level of NF-κB activity (Fig. 2B, vector). We hypothesized, that we can expect/observe the similar effect on the NF-κB activity driven by overexpression of proteins, which are not directly down-regulated by 4′-OH. Apparently, the effect of 4′-OH on down-regulation of NF-κB pathway is mediated by abrogation of other step/s of NF-κB canonical cascade. In the case of an up-regulation of the NF-κB activity driven by TRADD overexpression, the future analysis has to be done. In several cases, up-regulation of TRADD was linked to activation of apoptotic cell death [34,35]. As we showed, that 4′-OH had a strong impact on cancer cell viability, up-regulation of TRADD upon 4′-OH treatment could be connected to activation of cell death mechanism. Nevertheless, these findings need to be further elaborated.

NF-κB pathway can be activated in different manners according to a presence of specific dimer of NF-κB family. We used cells overexpressing NIK to get an insight into the second major NF-κB activation – the alternative pathway. The alternative pathway is involved during B and T cell organ development and is activated by the receptor controlled NIK kinase that promotes the processing of p52 precursor (p100) and translocation of the hetero-dimers p52-RelB to the nucleus [36]. Nevertheless, we did not observe any significant difference in NIK-transfected cells treated with 4′-OH in comparison to untreated cells. These results indicate that 4′-OH is not involved and cannot abrogate the alternative pathway.

The next step in the NF-κB activation cascade is through 26S proteasome function. Once the IKK complex is activated, it phosphorylates IκB proteins, which leads to their degradation by the 26S proteasome [24]. We hypothesize that even if 4′-hydroxychalcone does not directly affect IKK, it might prevent the proteasomal degradation of IκBα. Interestingly, by focusing on three main proteolytic sides, we showed that 4′-hydroxychalcone significantly down-regulated all three proteolytic activities in a dose-dependent manner in both K562 and U937 cell lines. To the

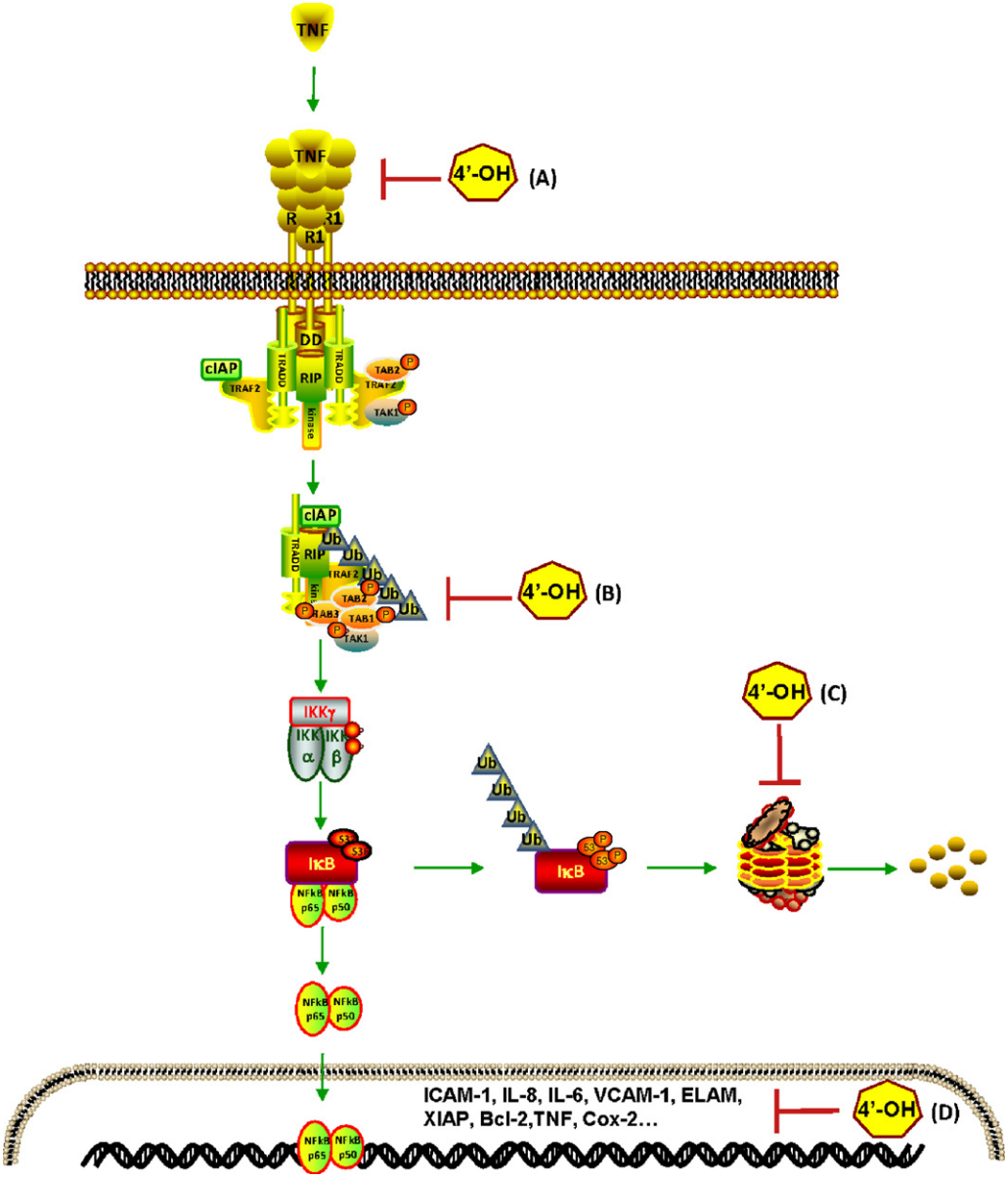

Fig. 9. Schematic representation of involvement of 4′-hydroxychalcone in canonical NF-κB pathway. 4′-OH abolished NF-κB pathway at (A) receptor TNFR1, (B) primary signaling complex side (TRAF2, TAK1) and (C) by inhibition of 3 major proteasomal activities. (D) As a secondary effect, expression of NF-κB target genes (ICAM-1, IL-8) was abrogated.

best of our knowledge, our study is the first to indicate that 4′-hydroxychalcone inhibits NF-κB activation by suppression of proteasomal activities in leukemic cells.

Our proposed model of involvement of 4′-hydroxychalcone in the canonical cascade NF-κB is displayed in Fig. 9.

Furthermore, we confirmed that 4′-hydroxychalcone reduced induction of NF-κB transcriptional activity by blocking TNFα-induced p50/p65 heterodimer translocation to the nucleus and thus reduced and prevented NF-κB binding to target DNA.

Excessive production of TNFα by the tumor itself and by the tumor microenvironment is well documented. Many cancer cells constitutively secrete TNFα, which appears to contribute directly to oncogene activation, DNA damage and tumor growth [37]. TNFα can act as an endogenous tumor promoter and induce other inflammatory mediators such as IL-1, IL-6, IL-8 and TNFα itself [38]. Interestingly, IL-8 has been characterized as a significant regulatory factor within the tumor microenvironment and elevated concentrations of IL-8 were found in patients serum with different cancer malignancies such as breast [39,40], ovarian [41,42], prostate [43,44], lung [45], pancreatic [46] and melanoma cancer [47]. Moreover, IL-8 has been linked to angiogenesis, tumorigenicity and tumor metastasis in several xenograft and orthotopic *in vivo* models [48–50]. Here, we provided evidence, that 4′-hydroxychalcone is able to inhibit the TNFα-induced IL-8 production in chronic myelogenous leukemia cells. The higher concentration of 4′-OH needed to reduce IL-8 production could be explained by activity of other transcription factors known to be involved in positive or negative regulation of IL-8 expression. The IL-8 promoter contains, among others, binding sites for the transcription factors AP-1, C-EBP/NF-IL6 and NF-κB [51]. Moreover, new findings indicate that besides other cytokines, oncogenic Ras up-regulates also the expression of IL-8 [52] and EGFR signaling positively regulates its synthesis and secretion in tumor cells [53]. Thus, the higher concentration of 4′-OH necessary for IL-8 inhibition could be explained due to the activity of other transcription factors, which boosted IL-8 expression.

Because TNFα is known to be one of the major inducers of divergent adhesion molecules in human endothelial cells and because the expression of TNF requires the activation of NF-κB, we examined the effect of 4′-hydroxychalcone on the TNF-mediated induction of intracellular adhesion molecule ICAM-1. We found that the expression of ICAM-1 was abrogated by 4′-hydroxychalcone.

We also analyzed the effect of 4′-hydroxychalcone on cell viability. Loa et al. recently reported that 4′-hydroxychalcone had a cytotoxic effect on HepG2 cells [2]. Chemotherapeutic agents and radiation therapy unfortunately can activate NF-κB and can promote a cytotoxic effect on healthy tissues as well as cancer cells, causing side effects such as nephrotoxicity, hepatotoxicity, ototoxicity or neurotoxicity [54]. One strategy to overcome these adverse effects is to identify novel drugs lacking these side effects and develop the utility of these compounds in combinatory treatment with commercially available chemotherapy agents. *Via* comparative analysis, we determined the cytotoxic effect of 4′-hydroxychalcone on different cancer cell lines (K562, Jurkat, U937) and on healthy peripheral blood mononuclear cells (PBMCs). We showed that 4′-hydroxychalcone has selective cytotoxicity to the leukemic cells, which makes it an attractive candidate for cancer prevention and treatment.

In conclusion, we determined that 4′-hydroxychalcone is a potent inhibitor of TNFα-induced NF-κB activation and described the mechanism by which 4′-hydroxychalcone executes this inhibition. Moreover, we identified the 26S proteasome as a major molecular target of 4′-hydroxychalcone. The absence of toxicity on healthy cells makes 4′-hydroxychalcone an inviting candidate as a potential anti-cancer drug, whether used alone or in combinatory treatment with other chemotherapeutics.

Acknowledgments

BO is a recipient of a PhD Télévie grant. Research at the Laboratoire de Biologie Moléculaire et Cellulaire du Cancer is supported by the "Recherche Cancer et Sang" foundation, by the "Recherches Scientifiques Luxembourg" association, by "Een Haerz fir kriibskrank Kanner" association, by the Action Lions "Vaincre le Cancer" association and by Télévie Luxembourg. Editing and print costs are covered by the Fonds National de la Recherche (FNR), Luxembourg.

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