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Barbora Orlikova, Deniz Tasdemir, Frantisek Golais, Mario Dicato, Marc Diederich. The aromatic ketone 4'-hydroxychalcone inhibits TNF α -induced NF- κ B activation proteasome inhibition. *Biochemical Pharmacology*, 2011, 82 (6), pp.620. <10.1016/j.bcp.2011.06.012>. <hal-00722843>

HAL Id: hal-00722843

<https://hal.science/hal-00722843v1>

Submitted on 5 Aug 2012

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HAL Authorization

Accepted Manuscript

Title: The aromatic ketone 4'-hydroxychalcone inhibits TNF α -induced NF- κ B activation via proteasome inhibition

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PII: S0006-2952(11)00377-7
DOI: doi:10.1016/j.bcp.2011.06.012
Reference: BCP 10936

To appear in: *BCP*

Received date: 24-4-2011
Revised date: 27-5-2011
Accepted date: 7-6-2011

Please cite this article as: Orlikova B, Tasdemir D, Golais F, Dicato M, Diederich M, The aromatic ketone 4'-hydroxychalcone inhibits TNF α -induced NF- κ B activation via proteasome inhibition, *Biochemical Pharmacology* (2010), doi:10.1016/j.bcp.2011.06.012

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The aromatic ketone 4'-hydroxychalcone inhibits TNF α -induced NF- κ B activation *via* proteasome inhibition

Authors: Barbora Orlikova, Deniz Tasdemir¹, Frantisek Golais², Mario Dicato and Marc Diederich

Laboratoire de Biologie Moléculaire et Cellulaire du Cancer, Fondation de Recherche Cancer et Sang - Hôpital Kirchberg, 9 Rue Edward Steichen, 2540 Luxembourg, Luxembourg

¹The School of Pharmacy, University of London, 29-39 Brunswick Square, London, WC1N 1AX, United Kingdom

²Comenius University, Faculty of Sciences, Department of Microbiology and Virology, Bratislava, Slovakia

Address correspondence to: Marc Diederich, Laboratoire de Biologie Moléculaire et Cellulaire du Cancer, Hôpital Kirchberg, 9 rue Edward Steichen, L-2540 Luxembourg, Luxembourg. Tel: +352-2468-4040; Fax: +352-2468-4060; E-mail address: marc.diederich@lbmcc.lu

Abstract

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4 Chalcones are aromatic ketones, known to exhibit anti-microbial, anti-inflammatory
5 and anti-cancer activities. The aim of this study was to investigate the anti-
6 inflammatory and anti-cancer activity of 4'-hydroxychalcone. Here, we report that 4'-
7 hydroxychalcone inhibits TNF α -induced NF- κ B pathway activation in a dose-
8 dependent manner. To investigate the underlying molecular mechanisms we
9 demonstrate that 4'-hydroxychalcone inhibits proteasome activity in a dose-dependent
10 manner but has no effect on IKK activity. Results show that 4'-hydroxychalcone
11 inhibits TNF α -dependent degradation of I κ B α and subsequently prevents p50/p65
12 nuclear translocation leading to 4'-hydroxychalcone-inhibited expression of NF- κ B
13 target genes. Most importantly, inhibition of NF- κ B activation by 4'-hydroxychalcone
14 is not leukemia cell-type specific and has no significant effect on non-transformed
15 cell viability, thus highlighting the compound's potential in both prevention and
16 treatment.
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Key Words:

31 Polyphenol; chalcone; NF- κ B; inflammation; cancer
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1. Introduction

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4 Recently anti-cancer research refocused on the power of Mother Nature to efficiently
5 fight cancer. Natural sources provide a rich repository of save compounds and
6 examination of these natural products has provided evidence for their anticancer
7 potential. One major advantage of naturally occurring drugs relative to synthetic
8 chemicals is their lack of toxicity towards healthy tissues. Additionally, natural
9 products have specific bioactivity and bioavailability. Thus, the trend to treat illnesses
10 with naturally discovered lead compounds has established a basis for the development
11 of novel cancer therapies.
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14 Chalcones belong to the polyphenol family, represent natural antioxidants, are
15 commonly found in fruits and vegetables, and serve as precursors for flavonoid
16 synthesis. Because they are abundant in edible plants, they seem to be promising
17 representatives for dietary cancer prevention strategies [1]. Additionally, their simple
18 and efficient synthesis makes them also attractive for cancer therapy.
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21 Several studies have shown that the anti-proliferative effects on cancer cells are
22 associated with the presence of hydroxyl group/s in chalcone molecules; therefore,
23 hydroxyl-derivates of chalcone have more potent anti-proliferation properties
24 compared to the other chalcone derivatives [2-4]. Additionally, the position of the
25 hydroxyl group within the chalcone molecule seems to play an important role in the
26 activity of the molecule. Chemically, 4'-hydroxychalcone is an alpha, beta-
27 unsaturated ketone with the core structure of chalcone (1,3-diphenyl-2-propenone)
28 and one hydroxyl-substituent on the 4' position of the A ring (Figure 1).
29

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31 Because nuclear factor (NF)- κ B is irreversibly connected with inflammatory
32 processes involved in wide range of severe diseases including cancer and because it
33 has been shown to induce resistance to various chemotherapeutic agents, targeting
34 this pathway by natural compounds is an attractive strategy to attenuate and overcome
35 cancer development [5-10]. The NF- κ B family includes 5 family members: RelA
36 (p65), RelB, c-Rel, p50/p105 (NF- κ B1) and p52/p100 (NF- κ B2), which can form
37 homo- and hetero-dimeric protein complexes. By binding of the dimers to
38 corresponding DNA sequences in promoter or enhancer regions, the expression of
39 pro-inflammatory genes is increased while the expression of other genes is repressed
40 [11]. The most common NF- κ B dimer is the p50/p65 heterodimer, which is involved
41 in canonical NF- κ B pathway activation. In the presence of divergent extrinsic signals
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1 (cytokines, carcinogens, UV light, physical and chemical stress or proliferating
2 agents), inhibitor of NF- κ B (I κ B) is phosphorylated by I κ B kinase (IKK),
3 ubiquitinated and marked for the proteasomal degradation [12]. Subsequently, the
4 NF- κ B dimer is no longer sequestered in the cytoplasm and rapidly translocates to the
5 nucleus where it can transactivate more than 550 genes.
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9 To date, a large spectrum of natural, plant-derived, polyphenols was identified and
10 investigated for its NF- κ B inhibition potential. Additionally, the ability to synthesize
11 original natural compounds or to modify their structures to improve their bioactivity
12 opens a new horizon of potential novel molecules with anticancer potential.
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16 In this study, we provided a detailed biological analysis of the involvement of 4'-
17 hydroxychalcone in NF- κ B regulation in different leukemic cell lines and tried to
18 elucidate the mechanistic background underlying the inhibitory potential of 4'-
19 hydroxychalcone. We showed that 4'-hydroxychalcone significantly repressed the
20 tumor necrosis factor alpha (TNF α)-induced NF- κ B pathway and NF- κ B target genes.
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22 Interestingly, we discovered a strong negative effect of 4'-hydroxychalcone on
23 proteasomal activities, explaining its involvement in the NF- κ B cascade. Moreover,
24 we provided evidence of the selective cytotoxic effect of 4'-hydroxychalcone on
25 cancer cells by a comparative study with a non-cancer cells. Thus, we identified
26 mechanisms, which make 4'-hydroxychalcone an interesting, safe and promising
27 inhibitor of TNF α -induced NF- κ B activation pathway.
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2. Material and Methods

2.1. Compounds and purification

TNF α was purchased from Sigma-Aldrich (Bornem, Belgium) and dissolved to a concentration of 10 mg/mL in 1X 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 % CO₂. The cells were harvested every 3 days. After thawing, the cells were kept in normal culture conditions for 10 days before experiments.

At T₀, the cells were treated with 4'-hydroxychalcone at various concentrations. At T₀ + 2 h the cells were activated by addition of TNF α (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 x g, 20 min) to isolate mononuclear cells. The isolated peripheral blood mononuclear cells (PBMCs) were kept in culture at 37 °C and 5% CO₂ 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 3x10⁵ cells/mL and were pre-treated in the presence

1 and absence of 4'-hydroxychalcone (30 μ M) for 4, 8 or 16 h before being activated by
2 TNF α (20 ng/mL) for 30 min, or for indicated time period. Cells were then harvested
3 and washed twice in 1X PBS. Supernatants were discarded and cell pellets were
4 stored overnight at -80 $^{\circ}$ C. Nuclear protein extractions were performed as described
5 previously, and nuclear extracts were stored at -80 $^{\circ}$ C [13]. The oligonucleotide NF-
6 κ Bc (consensus NF- κ B site) (Eurogentec, Liège, Belgium) (5'-
7 AGTTGAGGGGACTTTCCCAGGC-3') and its complementary sequence were used
8 as probes. After hybridization, the probes were radiolabeled using [g- 32 P]-ATP (MP-
9 Biomedicals, Illkirch, France) and the EMSA assay was performed according to
10 conditions established before [13]. First, 10 μ g of nuclear proteins were incubated
11 with the radiolabeled probe in binding buffer for 20 min on ice. Then the DNA-
12 protein complexes were separated on a 5 % polyacrylamide gel followed by
13 visualization by autoradiography.
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26 **2.4. Transient transfection and luciferase reporter gene assay**

27 K562 cells were transiently transfected as described previously [13]. For each
28 electroporation, we used 5 μ g of a luciferase reporter gene construct containing 5
29 repeats of a consensus NF- κ B site (Stratagene, Genomics Agilent, Diegem, Belgium)
30 and 5 μ g of a *Renilla* luciferase plasmid (Promega, Leiden, Netherlands). The ICAM-
31 1 LUC reporter plasmid was a generous gift from W. Vanden Berghe (University of
32 Antwerp, Belgium). The full-length ICAM-1 promoter construct contains
33 approximately 1.4 kb of ICAM-1 5'-flanking DNA linked to the firefly luciferase
34 (LUC) gene. Promoter sequences between 393 and 176 bp upstream of the gene,
35 contain binding sites for C/EBP and NF- κ B. For co-transfection assays, 5 μ g of
36 expression plasmids coding for proteins of the NF- κ B pathway (receptor of TNF α -1
37 (TNFR-1), TNFR-associated factor-2 (TRAF2), TNFR-1 associated death domain
38 (TRADD), inhibitor of κ B kinase beta (IKK β), tumor growth factor activated kinase 1
39 (TAK1) and TAK1-binding protein 1 (TAB1)) were added to the electroporation
40 mixture. Cells were resuspended in normal culture medium (RPMI, 10 % FCS) after
41 electroporation and cultured at 37 $^{\circ}$ C and 5 % CO $_2$ for 24 h. After the required
42 incubation time, cells were harvested and resuspended in fresh growth medium
43 (RPMI, 0.1 % FCS) to a final concentration of 1×10^6 cells/mL and pre-treated for 2 h
44 with 4'-hydroxychalcone at varying concentrations. Activation with 20 ng/mL of
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TNF α for 6 h followed the pre-treatment. For co-transfection experiments only TNF indicated control was activated by TNF α . After the 8 h incubation, 75 μ L of Dual-GloTM Luciferase Reagent (Promega, Leiden, Netherlands) was added to 75 μ L of the cellular suspension for a 10 min incubation at 22 °C before luciferase activity measurement. Subsequently, 75 mL of Dual-GloTM 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 Mg²⁺, 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.

2.7. Extraction of cellular proteins

After the indicated incubation times with 4'-hydroxychalcone and TNF α , the K562 and Jurkat cells were lysed, and the nuclear and cytoplasmic extracts were prepared according to [13]. Briefly, 10⁷ 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

1 centrifuged at 15000 x g for 15 min at + 4 °C for suspension clearing. Afterwards,
2 supernatants were removed and aliquots were stored at -80 °C until use.
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5 **2.8. Western Blot analysis**

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7 Proteins of nuclear and cytoplasmic extracts were separated by size using
8 sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 10 %),
9 transferred onto nitrocellulose membranes and blocked with 5 % non-fat milk in
10 phosphate buffered saline (PBS)-Tween overnight. Lamin B for cytoplasmic extracts
11 and α -tubulin for nuclear extracts, were used as loading controls. Blots were then
12 incubated with primary antibodies: anti-pIkB α (1/300, Cell Signaling 9246, Bioké,
13 Leiden, Netherlands), anti-IkB α (1/500 Santa Cruz SC-371, Tebu-Bio, Boechout,
14 Belgium), anti-p50 (1/5000, Santa Cruz SC-7178X), anti-p65 (1/5000, Santa Cruz
15 SC-8008), anti- α -tubulin (1/5000, Calbiochem CP06, VWR, Leuven, Belgium) or
16 anti-lamin B (1/1000, Santa Cruz SC-6216). All antibodies were diluted in a PBS-
17 Tween solution containing 5 % bovine serum albumin (BSA) or 5 % milk according
18 to the providers' protocols. After incubation with primary antibodies, membranes
19 were washed 3 x 10 min with PBS-Tween followed by an incubation of 1 h at RT
20 with the corresponding secondary (HRP-conjugated) antibodies. After washing 3 x 10
21 min with PBS-Tween, specific immunoreactive proteins were visualized by
22 autoradiography using the ECL Plus Western Blotting Detection System Kit ® (GE
23 Healthcare, Roosendaal, Netherlands).
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40 **2.9. Human CXCL8/IL-8 Immunoassay**

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42 IL-8 concentrations in culture supernatants of activated K562 cells were
43 measured by sandwich ELISA (R&D Systems, Abingdon, United Kingdom).
44 According to the manufacturer's guide, 50 μ L of cell supernatants were added with
45 100 μ L of Assay Diluent to anti-IL-8 pre-coated wells followed by 2 h incubation at
46 RT. After washing, a polyclonal peroxidase-conjugated anti-IL-8 antibody was added
47 for another 60 min at RT. Colorimetric visualization and protein dosage were
48 developed by addition of the H₂O₂+TMB (tetramethylbenzidine) containing substrate.
49 After a 30 min reaction at room temperature (RT) in the dark, the enzymatic
50 reaction was stopped by addition of H₂SO₄ and optical densities were measured at a
51 wavelength of 450 nm.
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2.10. IKK kinase activity assay

The K-LISA™ IKK β -Inhibitor Screening Kit (Calbiochem, San Diego, CA) was used for rapid in vitro screening of IKK β inhibitors. The ELISA-based activity assay utilizes a 50-amino acid GST-I κ B α fusion polypeptide substrate with Ser32 and Ser36 IKK β phosphorylation sites. The GST-I κ B α substrate and IKK β were incubated in the presence of IKK β inhibitors in a Glutathione-Coated 96-Well plate. The incubation of GST-I κ B α substrate and IKK β allowed for substrate phosphorylation and capture in a single step. The phosphorylated GST-I κ B α substrate was detected using an Anti-Phospho I κ B α (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 IKK β activity. IKK-2 Inhibitor IV ([5-(p-fluorophenyl)-2-ureido]thiophene-3-carboxamide) (Calbiochem, San Diego, CA) was used as a positive control.

2.11. 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 μ M) (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 of 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 hours, 25 μ l (25×10^3) cells per well in 96 well plate were mixed with 25 μ l 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.

2.12. Statistical analysis

1 Data are expressed as mean \pm S.D. Significance was determined by the
2 Student's T-tests. P-values below 0,05 were considered as statistically significant.
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3. Results

3.1. 4'-hydroxychalcone inhibits TNF α -induced NF- κ B pathway activation in a dose-dependent manner

The NF- κ B 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 TNF α -induced NF- κ B transcriptional pathway.

To examine the inhibitory potential of 4'-hydroxychalcone on NF- κ B 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 hours and then treated with TNF α (20 ng/ml) for an additional 6 hours. Results showed that TNF α led to strong activation of the NF- κ B pathway in control cells and that 4'-hydroxychalcone inhibited the TNF α -driven NF- κ B pathway activation in a dose-dependent manner with an IC₅₀ of 30 μ M (Figure 2A). The IC₅₀ value was calculated using XY scatter dependency chart applying the best fitting trend formula model. The average of three IC₅₀ values from three independent experiments was applied.

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

1 indicate involvement of 4'-hydroxychalcone on the receptor and the primary signaling
2 complex.
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5 **3. 2. 4'-hydroxychalcone has no direct effect on IKK activity**

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9 Degradation of I κ B α depends either on IKK activity or on proteasome activity.
10 Chronologically, IKK activity occurs prior to proteasomal activity. To further confirm
11 our previous results showing the inability of 4'-hydroxychalcone to effect directly
12 IKK activity, we monitored IKK β activity by ELISA in the presence of 4'-
13 hydroxychalcone. The ELISA used an I κ B α substrate and IKK β His-Tag[®] protein.
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16 We tested a wide spectrum of concentrations (from 10 μ M to 1000 μ M) of 4'-
17 hydroxychalcone and showed that 4'-hydroxychalcone was not able to significantly
18 inhibit IKK β activity at any tested concentrations (Figure 3).
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21 We concluded that the inhibitory potential of 4'-hydroxychalcone on NF- κ B is
22 independent of direct IKK activity.
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25 **3. 3. 4'-hydroxychalcone inhibits proteasome activity in a dose-dependent** 26 **manner**

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29 We were interested if proteasomal activity was involved in the mechanism by which
30 the chalcone derivative inhibits the NF- κ B pathway. We evaluated the protease
31 inhibition activity of 4'-hydroxychalcone on three different proteolytic activities
32 (chymotrypsin-, trypsin- and caspase-like) of the 26S proteasome at a concentration
33 range between 0.1 μ M to 25 μ M 4'-hydroxychalcone in K562 and U937 leukemia
34 cells. 4'-hydroxychalcone was able to inhibit each of the 3 major proteolytic activities
35 in a dose-dependent manner in both cell lines (Figure 4A and 4B).
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38 Thus, 4'-hydroxychalcone significantly repressed all three proteasomal activities. We
39 showed a positive correlation between dose of 4'-hydroxychalcone and its inhibitory
40 potential.
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43 **3. 4. 4'-hydroxychalcone inhibits TNF α -dependent degradation of I κ B α and** 44 **prevents p50/p65 nuclear translocation**

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1 Degradation of NF- κ B natural inhibitor I κ B α represents the initial step in NF- κ B
2 activation. Thus, we assessed I κ B α stability in the cytoplasm and translocation of the
3 NF- κ B subunits p65 and p50, from the cytoplasm to the nucleus by Western blot
4 analysis. Control cells activated by TNF α showed the classical model of NF- κ B
5 pathway activation: degradation of I κ B α appearing at 10 minutes after stimulation and
6 the re-appearance of I κ B α at 60 minutes after stimulation. Degradation occurs by a
7 negative feedback loop because p50/p65 binding to DNA induces I κ B α gene
8 expression. We also observed nuclear translocation of p50 and p65 15 minutes after
9 TNF α stimulation. However, 4'-hydroxychalcone was able to considerably postpone
10 and attenuate I κ B α degradation and prevented the cytoplasmic to nuclear
11 translocation of both the p50 and p65 subunits of the NF- κ B heterodimer (Figure 5).

12 To confirm previous results showing that 4'-hydroxychalcone can block TNF α -
13 dependent p50/p65 nuclear translocation, we performed an EMSA to determine if 4'-
14 hydroxychalcone can prevent NF- κ B binding to DNA. K562 cells were exposed to 4'-
15 hydroxychalcone for different time intervals and were then treated for 30 minutes
16 with TNF α . Results presented in figure 6A show that 30 μ M 4'-hydroxychalcone
17 suppressed TNF α induced NF- κ B activation for up to 16 hours (Figure 6A).

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

25 **3. 5. Inhibition of NF- κ B activation by 4'-hydroxychalcone is not cell-type** 26 **specific**

27 Because NF- κ B induction can be mediated in different cell types by specific signal
28 transduction pathways, we examined whether 4'-hydroxychalcone is able to suppress
29 the activity of NF- κ B in different cell types. We treated Jurkat cells with 4'-
30 hydroxychalcone at a concentration corresponding to the IC₅₀ of NF- κ B inhibition (30
31 μ M) and then further cultured the cells in the presence or absence of TNF α . EMSA
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1 analysis, shown in figure 6C, confirmed that 4'-hydroxychalcone is able to inhibit
2 TNF α -induced NF- κ B activation in cells other than the K562 leukemic cell line,
3 specifically in the Jurkat T-cell leukemia cell line. This indicates that the effect of 4'-
4 hydroxychalcone on TNF α -induced NF- κ B activation is not cell type specific.
5 Additionally, 4'-hydroxychalcone alone did not activate NF- κ B signaling pathway
6 (Figure 6C).
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11 **3. 6. 4'-hydroxychalcone inhibits the expression of NF- κ B target genes**

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16 TNF α is known to induce the expression of many genes involved in cancer
17 proliferation and metastasis. Additionally, many of these genes have an NF- κ B
18 binding site in their promoters. As described above, 4'-hydroxychalcone is able to
19 down-regulate NF- κ B activity. We were interested if this negative regulation affected
20 NF- κ B target gene expression. To further investigate the effects of 4'-
21 hydroxychalcone on downstream NF- κ B signaling, we focused on two proteins,
22 which are known to be under the NF- κ B control: IL-8 and ICAM-1. We applied two
23 different strategies to study possible changes in gene expression. First, we used an
24 ELISA assay to measure changes in IL-8 production 24 hours after treatment with 4'-
25 hydroxychalcone. IL-8 is a pro-inflammatory CXC chemokine and its expression is
26 primarily regulated by NF- κ B. Two hours after treatment with 4'-hydroxychalcone,
27 K562 cells were incubated in the presence or absence of TNF α . Control cells
28 produced markedly low amounts of IL-8 (165 pg/ml) while TNF α stimulation led to a
29 rapid increase of IL-8 levels (1305 pg/ml). We observed that cells pretreated with 4'-
30 hydroxychalcone at a concentration of 60 μ M produced significantly lower levels of
31 IL-8 (702 pg/ml). Observed decrease in IL-8 release relative to cells stimulated with
32 TNF α but not treated with 4'-hydroxychalcone is up to 40% (Figure 7A).
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48 Secondly, we examined whether 4'-hydroxychalcone affects ICAM-1 gene
49 transcription. ICAM-1 is involved in cell invasion [15]. K562 cells were transiently
50 transfected with ICAM-1 plasmid along with the *Renilla* reporter plasmid and after
51 24 hours incubation the cells were pre-treated with 30 μ M of 4'-hydroxychalcone
52 (concentration corresponding to IC₅₀ for NF- κ B inhibition) for 2 hours. The cells were
53 then treated with TNF α for an additional 6 hours. The induction of ICAM-1 by TNF
54 occurred within 6 h and was rapidly down-regulated by 4'-hydroxychalcone (Figure
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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 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 μ M to 60 μ M. We first studied viability after 8 hours of treatment because this time frame corresponds to the length of the treatment for several of our previous experiments. Cell viability after 8 hours 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. Figure 8A shows that after 8 hours 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 hours of treatment with 4'-hydroxychalcone, cell viability was dramatically affected in all three cancer cell lines (Figure 8B). A significant decrease in cell viability was observed at 10 μ M 4'-hydroxychalcone in K562 and Jurkat cell lines and even at 5 μ M 4'-hydroxychalcone in U937 cells. At 60 μ M, 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 μ M (Figure 8B). At 32 μ M, 4'-hydroxychalcone, we observed a slight attenuation of cell viability in PBMCs. At 60 μ M of 4'-hydroxychalcone, PBMC 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.

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4. Discussion

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4 The goal of the present study was to determine if 4'-hydroxychalcone is able to
5 inhibit the TNF α -induced NF- κ B pathway and if so, how this inhibition is mediated.
6 Several hydroxyl-derivatives of chalcones that do not contain other substituent groups
7 have been shown to possess potent anti-inflammatory properties [3, 4]. Naringenin
8 chalcone, which is abundant in tomato skin and has 3 hydroxyl-groups, inhibited the
9 production of TNF α in lipopolysaccharide (LPS)-stimulated macrophages [16].
10 Isoliquiritigenin (also with three hydroxyl-groups) down-regulated NF- κ B activation
11 by suppression of IKK, resulting in inhibition of the phosphorylation and subsequent
12 degradation of I κ B α [17-19]. Butein (with four hydroxyl-groups) has been shown to
13 attenuate I κ B α phosphorylation by direct inactivation of IKK through cysteine residue
14 179 [20]. Based on structure-activity relationship studies, natural chalcone-based NF-
15 κ B inhibitors contain preferentially hydroxyl or/and methoxy functional groups [21].
16 Yadav *et al* summarized and compared the dose necessary for NF- κ B inhibitory
17 activity within a group of different chalcone derivatives focusing on the functional
18 group/s they possess in their molecules. Interestingly, 2'-hydroxychalcone,
19 structurally closely resembling our chalcone derivative in the present study, was able
20 to inhibit NF- κ B activity already at a three times lower dose compared to the basic
21 chalcone or chalcone derivatives with more hydroxyl groups in their molecules, such
22 as isoliquiritigenin and butein [22]. Based on these findings 4'-hydroxychalcone was
23 a tempting candidate as an anti-inflammatory agent because it bears only one
24 hydroxyl-group.

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41 Here, we provided evidence showing the inhibitory potential of 4'-hydroxychalcone
42 on the TNF α -induced NF- κ B pathway in leukemic cell lines and elucidated the
43 mechanism underlying this activity for the first time.

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

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65 Additionally, we were also interested where 4'-hydroxychalcone is involved in the
66 NF- κ B cascade. We demonstrated that 4'-hydroxychalcone has no direct effect on
67 IKK but nevertheless may attenuate its activity indirectly through inhibition of

1 particular members of the primary signaling complex. IKK activation has been linked
2 to various kinases and signaling molecules [24, 25]. We found that 4'-
3 hydroxychalcone blocked TNFR1-, TRAF2- and TAK1-mediated NF- κ B activation.
4 The importance of TRAF2, which is recruited to TNFR1 through TRADD-interaction
5 and subsequently leads to activation of NF- κ B, has been previously reported [26].
6 Similarly, TNF α activation of the NF- κ B pathway is associated with inducible
7 binding of TAK1 to TRAF2 and thus to both IKK α and IKK β [14]. TRAF2 and
8 cellular inhibitors of apoptosis (cIAPs) are believed to cooperate by recruiting
9 ubiquitin chains to receptor interacting protein (RIP). While TRAF2 seems to play a
10 structural role, allowing recruitment of cIAPs to the vicinity of RIP, cIAPs can
11 directly ubiquitinate RIP [27, 28]. This ubiquitination leads to attachment and
12 activation of TAK1 kinase complex, which consist of TAK1 kinase and its associated
13 proteins TAB1, TAB2 and TAB3, which in turn phosphorylates and activates IKK
14 complex [29-32]. The ability of 4'-hydroxychalcone to inhibit NF- κ B activity induced
15 in cells overexpressing TAK1 supports recent studies showing TAK1 as a critical
16 component required for I κ B kinase-mediated activation of the NF- κ B pathway [33].
17 Contrariwise, when we induced the NF- κ B pathway at the step of overexpressed
18 TRADD, IKK or TAB1, we observed an induction instead of inhibition of NF- κ B-
19 driven reporter gene activity. As we showed, 4'-OH induces the basal level of NF- κ B
20 activity (Figure 2B, vector). We hypothesized, that we can expect/observe the similar
21 effect on the NF- κ B activity driven by overexpression of proteins, which are not
22 directly down-regulated by 4'-OH. Apparently, the effect of 4'-OH on down-
23 regulation of NF- κ B pathway is mediated by abrogation of other step/s of NF- κ B
24 canonical cascade. In the case of an up-regulation of the NF- κ B activity driven by
25 TRADD overexpression, the future analysis has to be done. In several cases, up-
26 regulation of TRADD was linked to activation of apoptotic cell death [34, 35]. As we
27 showed, that 4'-OH had a strong impact on cancer cell viability, up-regulation of
28 TRADD upon 4'-OH treatment could be connected to activation of cell death
29 mechanism. Nevertheless, these findings need to be further elaborated.

30 NF- κ B pathway can be activated in different manners according to a presence of
31 specific dimer of NF- κ B family. We used cells overexpressing NIK to get an insight
32 into the second major NF- κ B activation – the alternative pathway. The alternative
33 pathway is involved during B and T cell organ development and is activated by the
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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 can not abrogate the alternative pathway.

The next step in the NF- κ B activation cascade is through 26S proteasome function. Once the IKK complex is activated, it phosphorylates I κ B 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 I κ B α . Interestingly, by focusing on three main proteolytic sites, 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 our knowledge, our study is the first to indicate that 4'-hydroxychalcone inhibits NF- κ B activation by suppression of proteasomal activities in leukemic cells.

Our proposed model of involvement of 4'-hydroxychalcone in the canonical cascade NF- κ B is displayed in figure 9.

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

Excessive production of TNF α by the tumor itself and by the tumor microenvironment is well documented. Many cancer cells constitutively secrete TNF α , which appears to contribute directly to oncogene activation, DNA damage and tumor growth [37]. TNF α can act as an endogenous tumor promoter and induce other inflammatory mediators such as IL-1, IL-6, IL-8 and TNF α 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]. We provide here evidence, that 4'-hydroxychalcone is able to inhibit the TNF α -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

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contains, among others, binding sites for the transcription factors AP-1, C-EBP/NF-IL6 and NF- κ B [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 TNF α 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- κ B, 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- κ B 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 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 TNF α -induced NF- κ B 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.

5. Acknowledgments

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

Fig. 1. Structure of 4'-hydroxychalcone

Fig. 2. Inhibition of TNF α -induced NF- κ B activation by 4'-hydroxychalcone (A)

K562 cells were transiently transfected with firefly luciferase vector (NF- κ B pGL4), ph-RG-tk Renilla plasmid and with or not I κ B α dominant negative (DN) for 24 hours. After transfection, K562 cells were treated with 4'-hydroxychalcone at the different concentrations for 2 hours followed by a TNF α -treatment (20 ng/ml) during 6 hours. Results are expressed as a ratio of the measured luminescence of the firefly luciferase vector and the luminescence of *Renilla* plasmid. Negative control corresponds to untreated cells, positive control refers to TNF α only treated cells. Results are presented as a mean \pm S.D. of three independent experiments. Negative control (Co-) corresponds to DMSO treated cells, without TNF α activation, positive control (Co+) corresponds to DMSO treated cells activated by TNF α . (B) K562 cells were transiently transfected with TNFR1, TRADD, TRAF2, NIK, IKK β , TAK1 or TAB1 along with NF- κ B pGL4 and ph-RG-tk Renilla plasmids for 24 hours. After transfection, K562 cells were treated or not with 4'-hydroxychalcone at 30 μ M for 2 hours followed by or not a TNF α -treatment (20 ng/ml) during 6 hours. 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 TNF α activation.

Fig. 3. Effect of 4'-hydroxychalcone on IKK activity. Effect of 4'-hydroxychalcone

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

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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 hours and the proteolytic activities of 26S proteasome were assayed. 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 μ M of epoxomicin – known selective proteasome inhibitor refers to positive inhibitory control. Each value is a mean \pm SD of three determinations. The asterisk indicates a significant difference compared to the negative control analyzed by t-test (* $p < 0.05$).

Fig. 5. Effect of 4'-hydroxychalcone on the degradation of I κ B α and translocation of p65 and p50 to the nucleus. Jurkat cells were pre-treated with 4'-hydroxychalcone (60 μ M) for 2 hours followed by activation with TNF α (20 ng/ml) for indicated time periods. Cytoplasmic and nuclear extracts were tested for I κ B α , 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 TNF α -dependent p50/p65 nuclear translocation and prevent NF- κ B binding to DNA. (A) Time-dependent effect of 4'-hydroxychalcone on TNF α -induced NF- κ B activation. K562 cells were pre-incubated with 30 μ M of 4'-hydroxychalcone for 4, 8 or 16 hours, treated with TNF (20ng/ml) for 30 minutes, and then subjected to EMSA. (B) Supershift (SS) with antibodies p50 and p65. NF- κ B induced by TNF α is composed of p50 and p65 subunits. Nuclear extracts from TNF α activated K562 cells were prior to EMSA treated with p50 or p65 antibodies. (C) Inhibition of NF- κ B activation by 4'-hydroxychalcone is not cell-type specific. Jurkat cells were incubated with or without 4'-hydroxychalcone at 30 μ M for 4 hours and then activated or not with 20 ng/ml TNF for 30 minutes. Nuclear extracts were then prepared and assayed for NF- κ B activation by EMSA. (D) Supershift (SS) with antibodies p50 and p65. NF- κ B induced by TNF α is composed of p50 and p65 subunits. Nuclear extracts from TNF α 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 TNF α activation, positive control (Co+) corresponds to nuclear extracts of DMSO treated cells activated by TNF α .

Fig. 7. Effect of 4'-hydroxychalcone on TNF α -induced NF- κ B – dependent gene products. (A) 4'-hydroxychalcone inhibits NF- κ B-dependent IL-8 genes expression. K562 cells were treated or not at indicated concentrations with 4'-hydroxychalcone for 2 hours, before being activated with TNF α (20 ng/ml) during 22 hours. After total 24 hours incubation IL-8 concentration in supernatants were measured. Negative control (Co-) corresponds to untreated cells, positive control (Co+) refers to TNF α only treated cells. (B) 4'-hydroxychalcone inhibits NF- κ B-dependent ICAM-1 genes expression. K562 cells were transiently transfected with ICAM-1 along with ph-RG-tk Renilla plasmid for 24 hours. After transfection, K562 cells were treated with 4'-hydroxychalcone at 30 μ M for 2 hours followed by a TNF α -treatment (20 ng/ml) during 6 hours. The cells were assayed for Luciferase activity. Each value is a mean \pm 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 TNF α activation, positive control (Co+) corresponds to transfected and DMSO treated cells activated by TNF α .

Fig. 8. Effect of 4'-hydroxychalcone on cancer cells versus non-transformed cell viability. (A) Short-term effect (8 hours) 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 hours. Negative control (Co-) corresponds to untreated cells. Each value is a mean \pm SD of three determinations. The asterisk indicates a significant difference compared to the negative control analyzed by t-test (*p < 0.05).

Fig. 9. Schematic representation of involvement of 4'-hydroxychalcone in canonical NF- κ B pathway. (A) 4'-OH abolished NF- κ B 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- κ B target genes (ICAM-1, IL-8) was abrogated.

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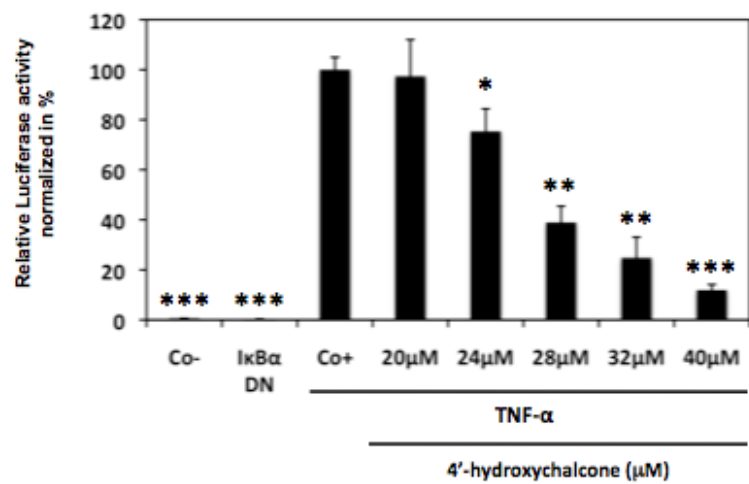
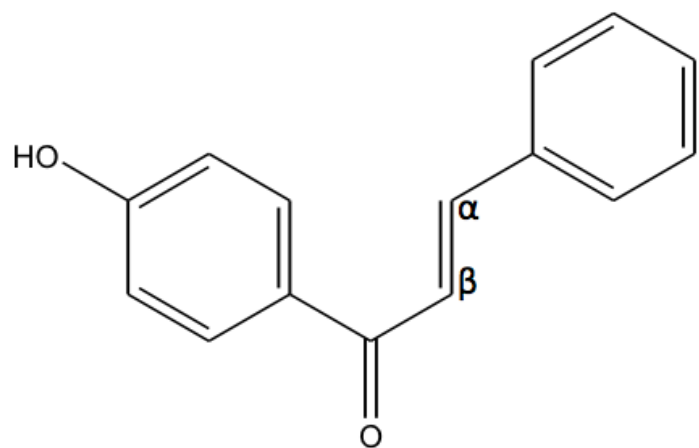
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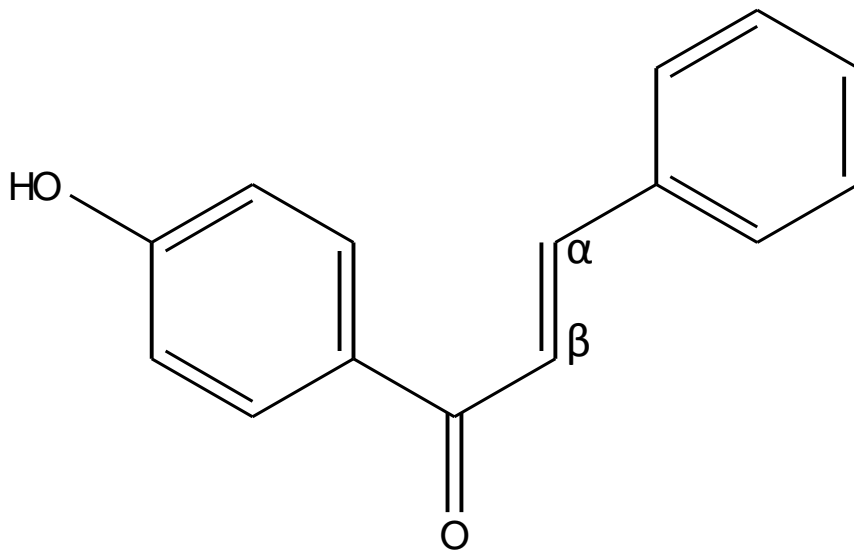
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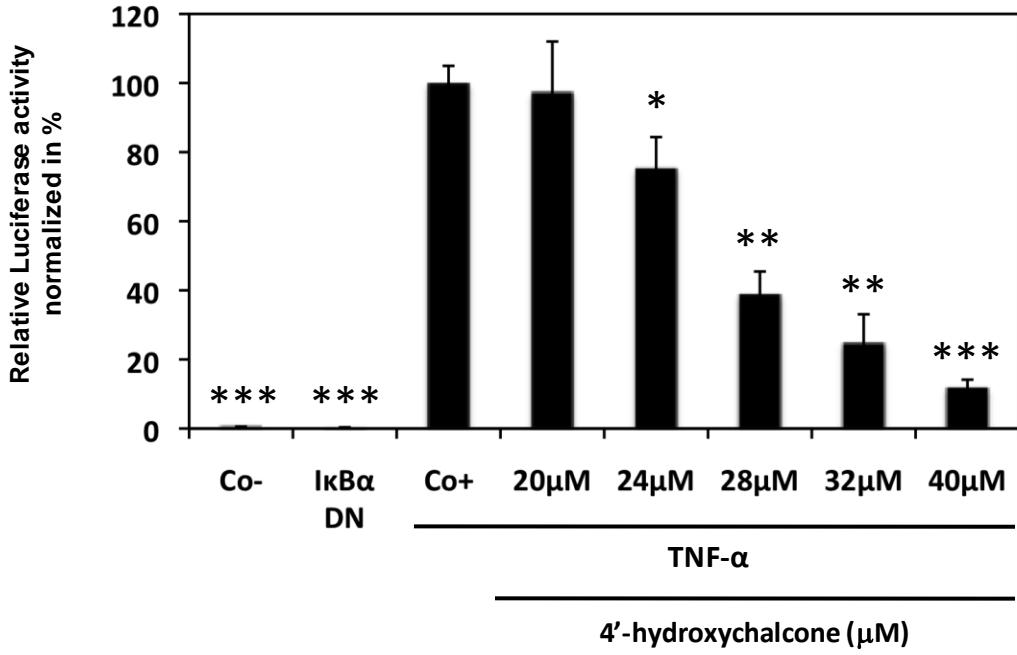
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A.



B.

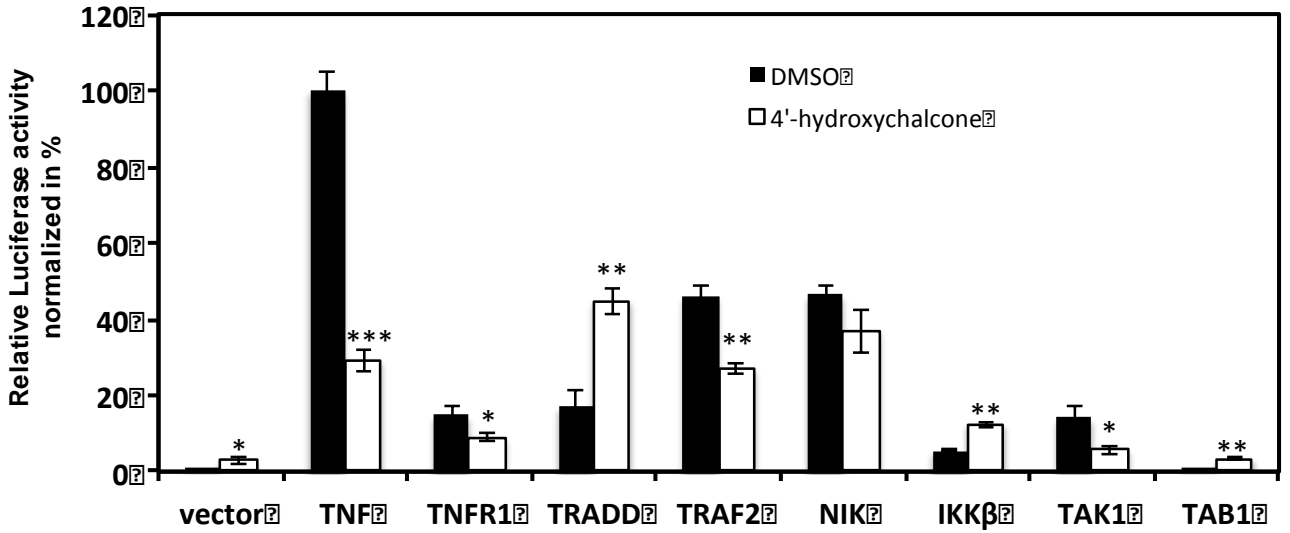


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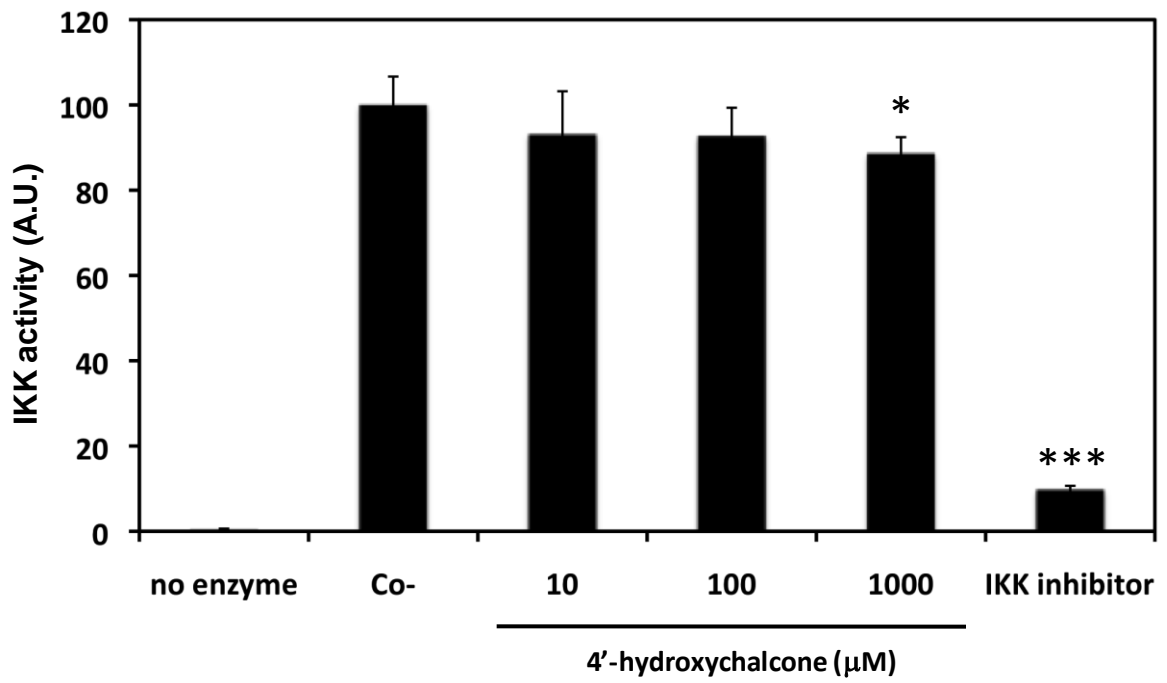
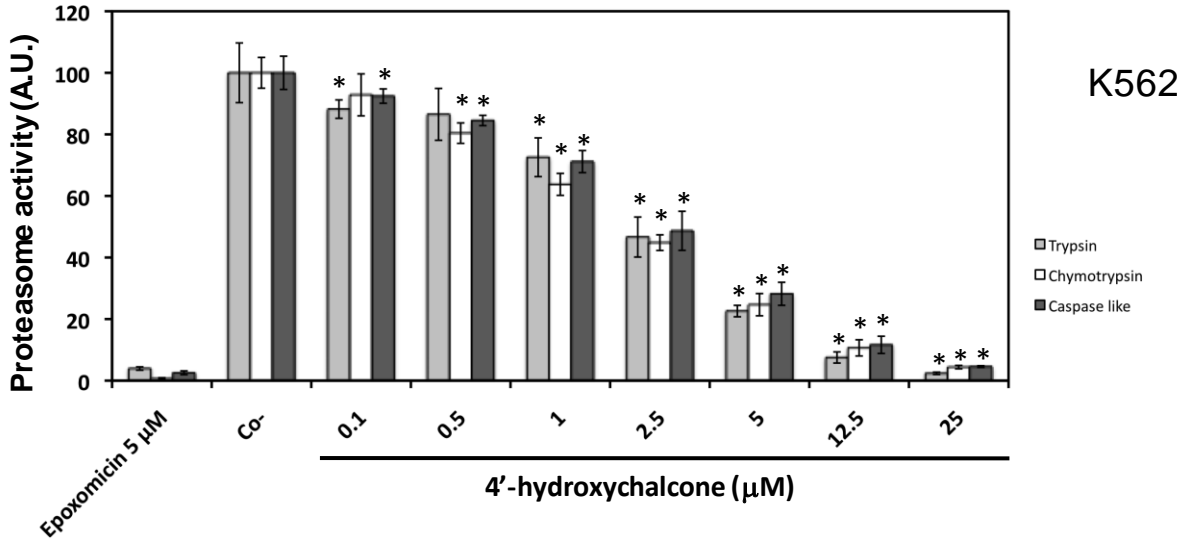


Figure 3

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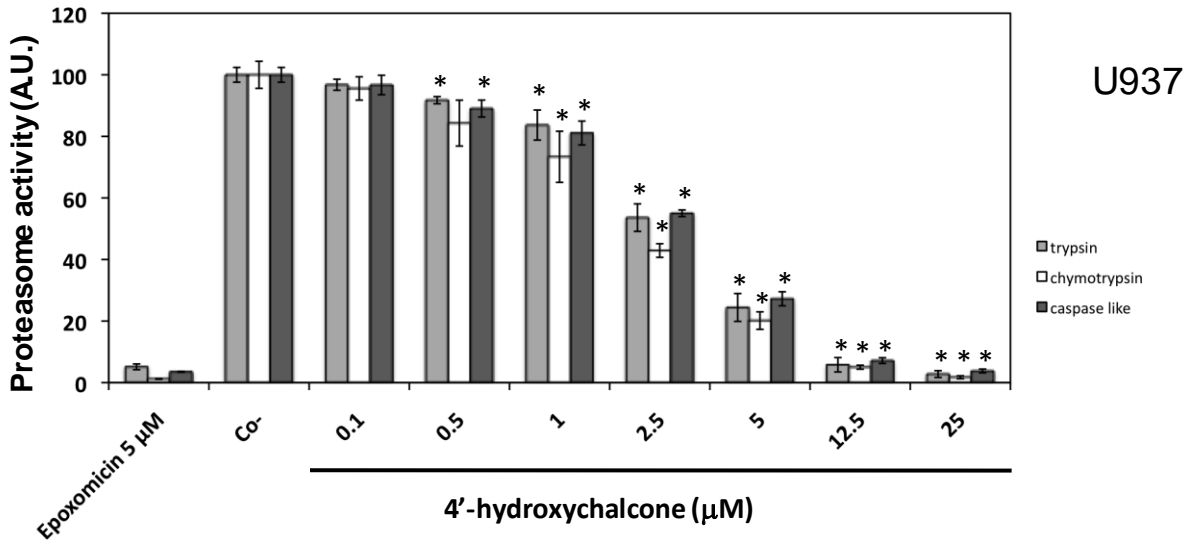


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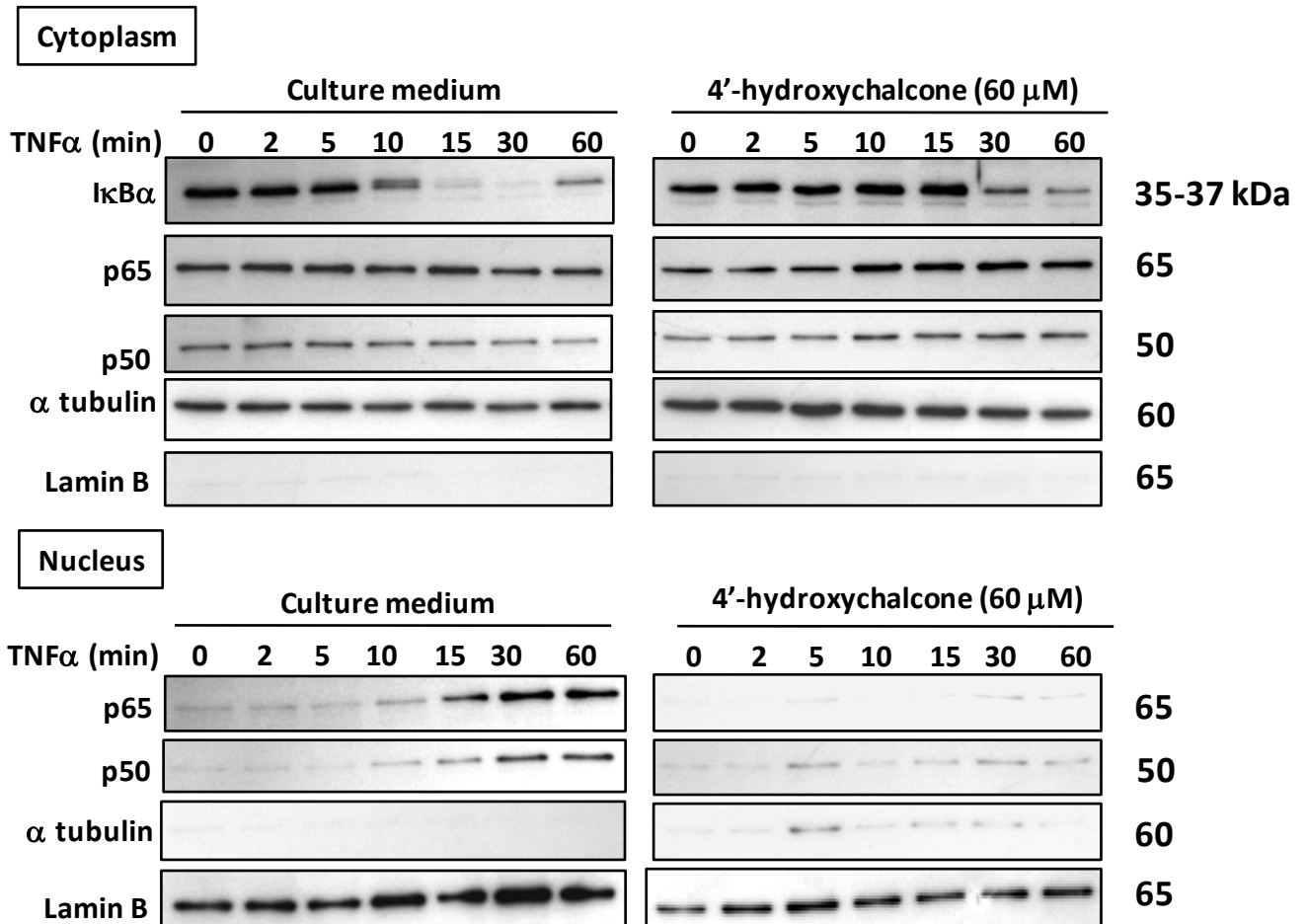


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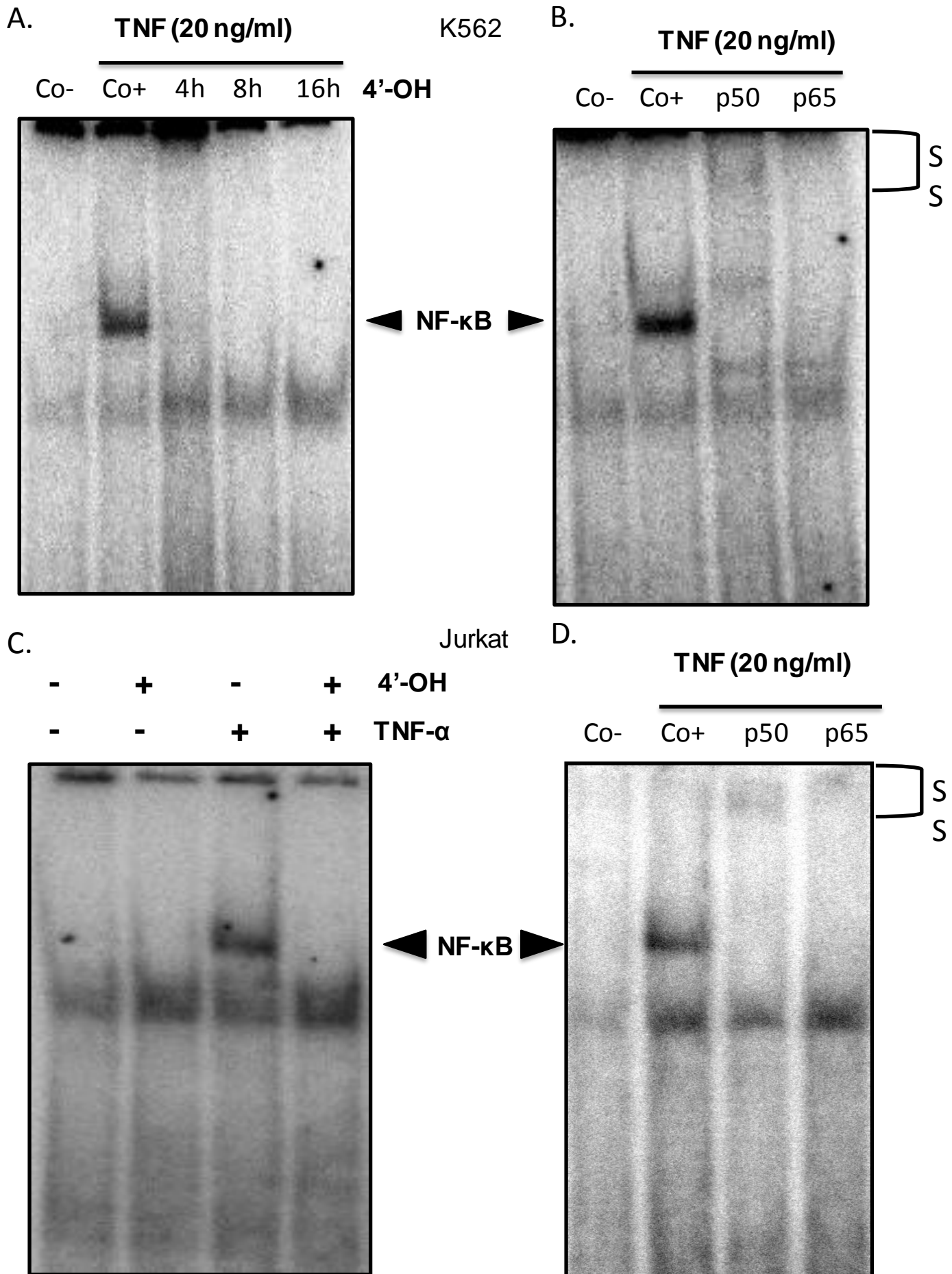


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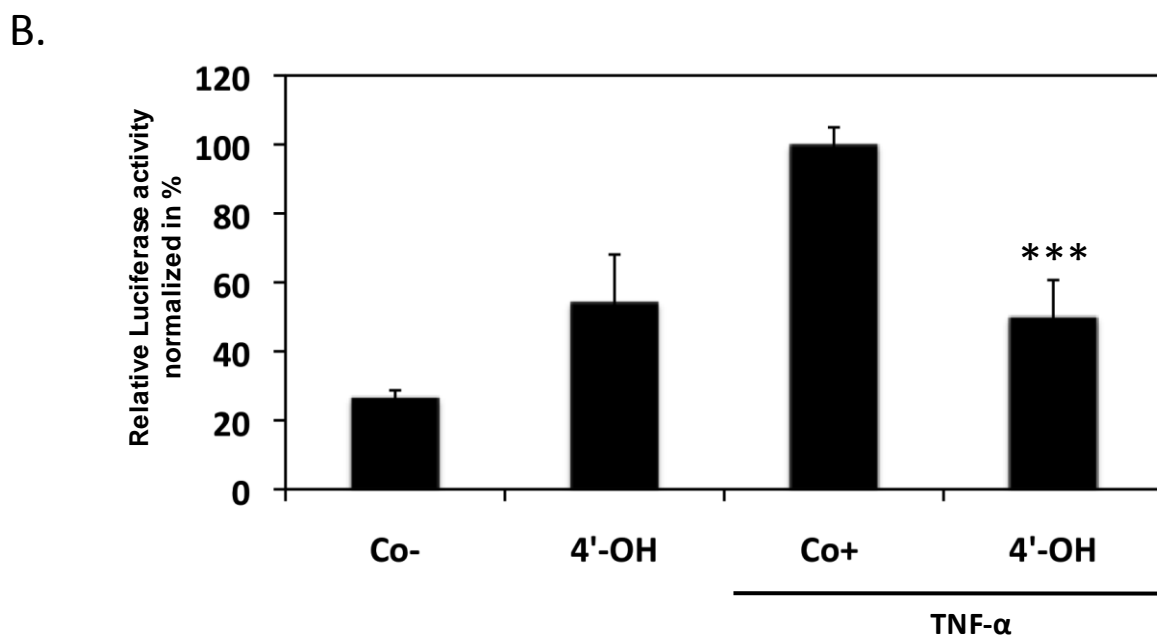
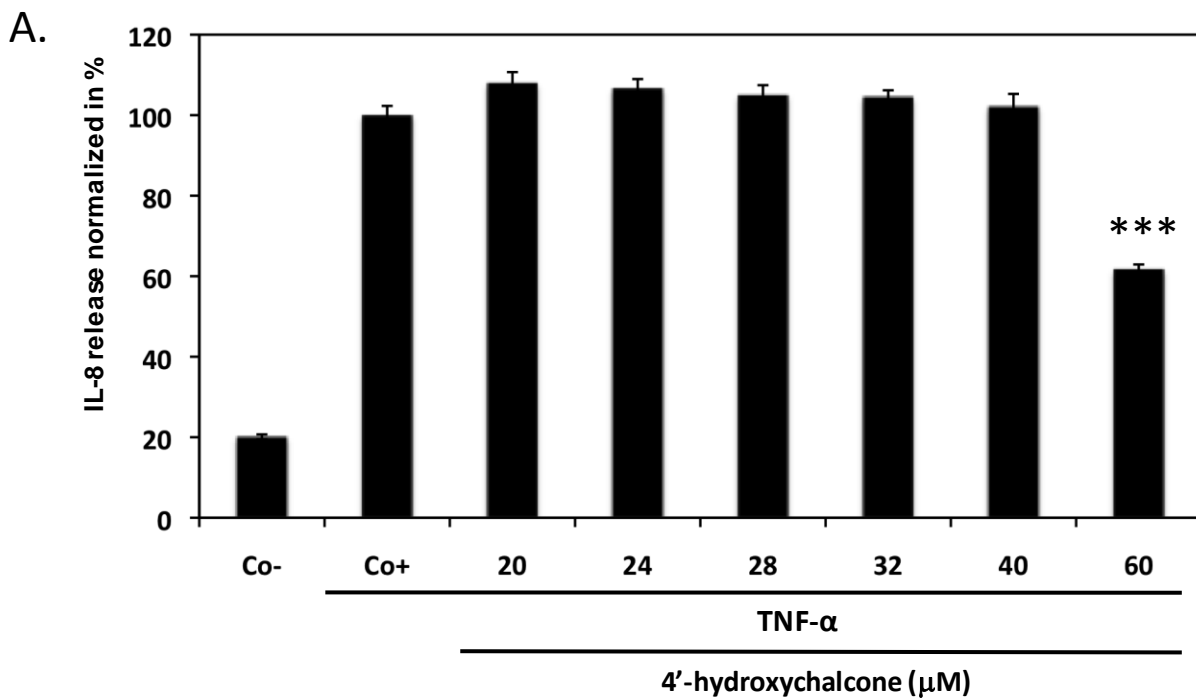
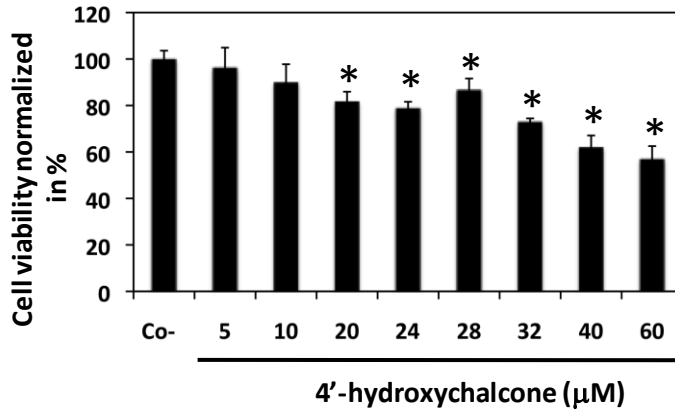


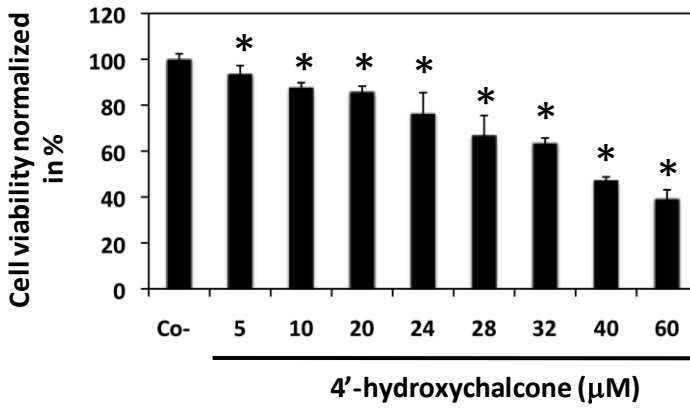
Figure 7

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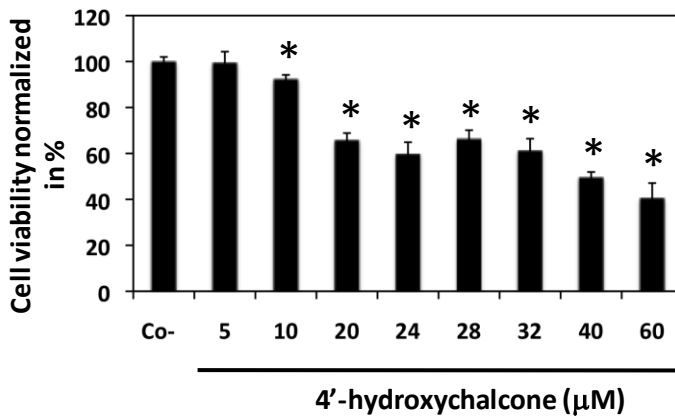
K562



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PBMC's

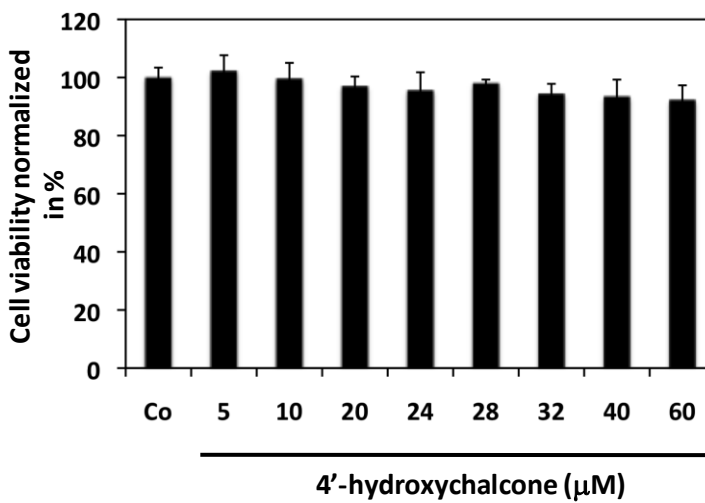
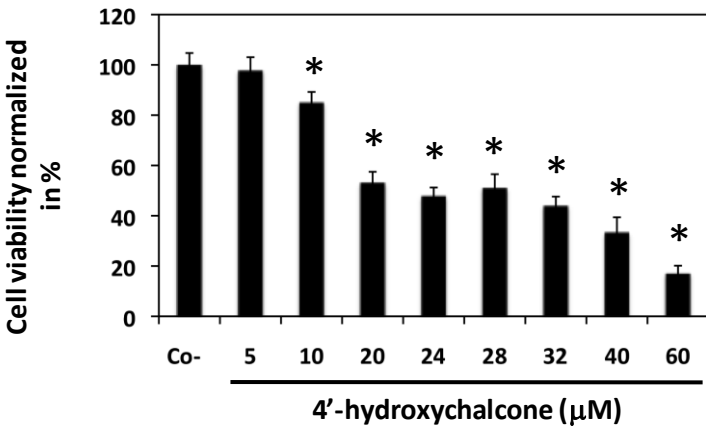


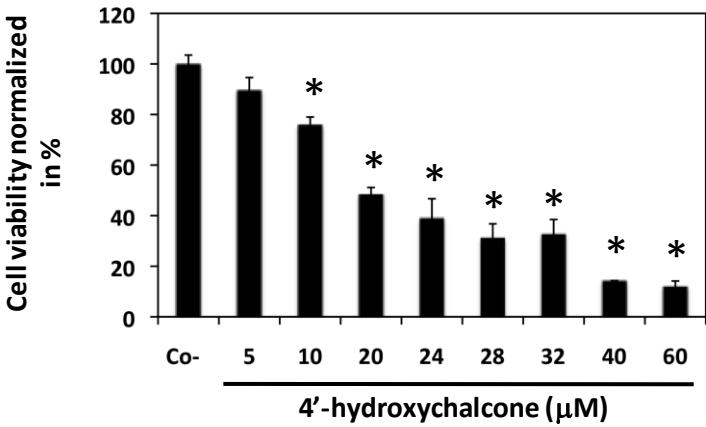
Figure 8

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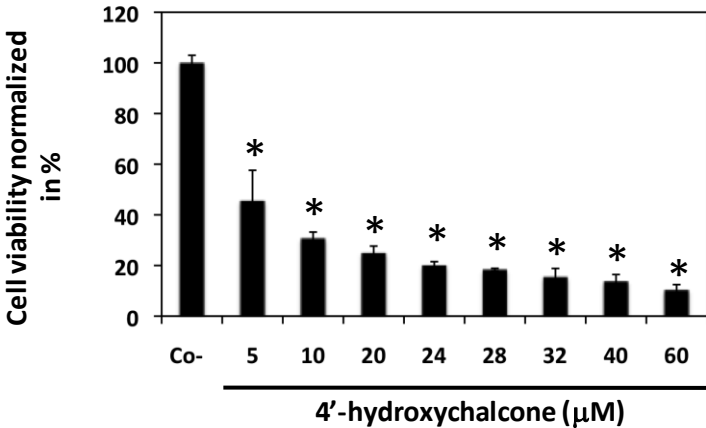
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Jurkat



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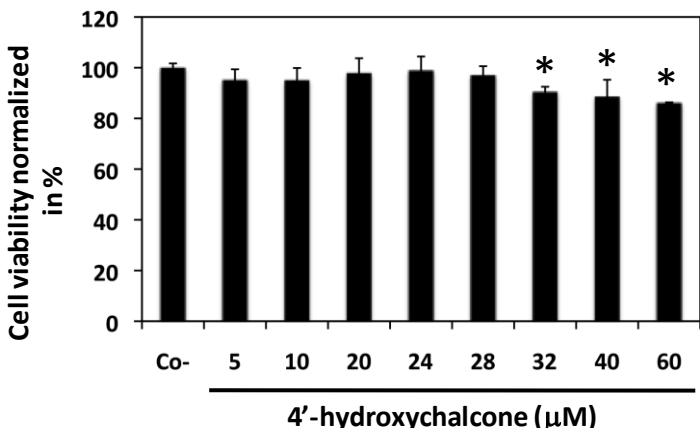


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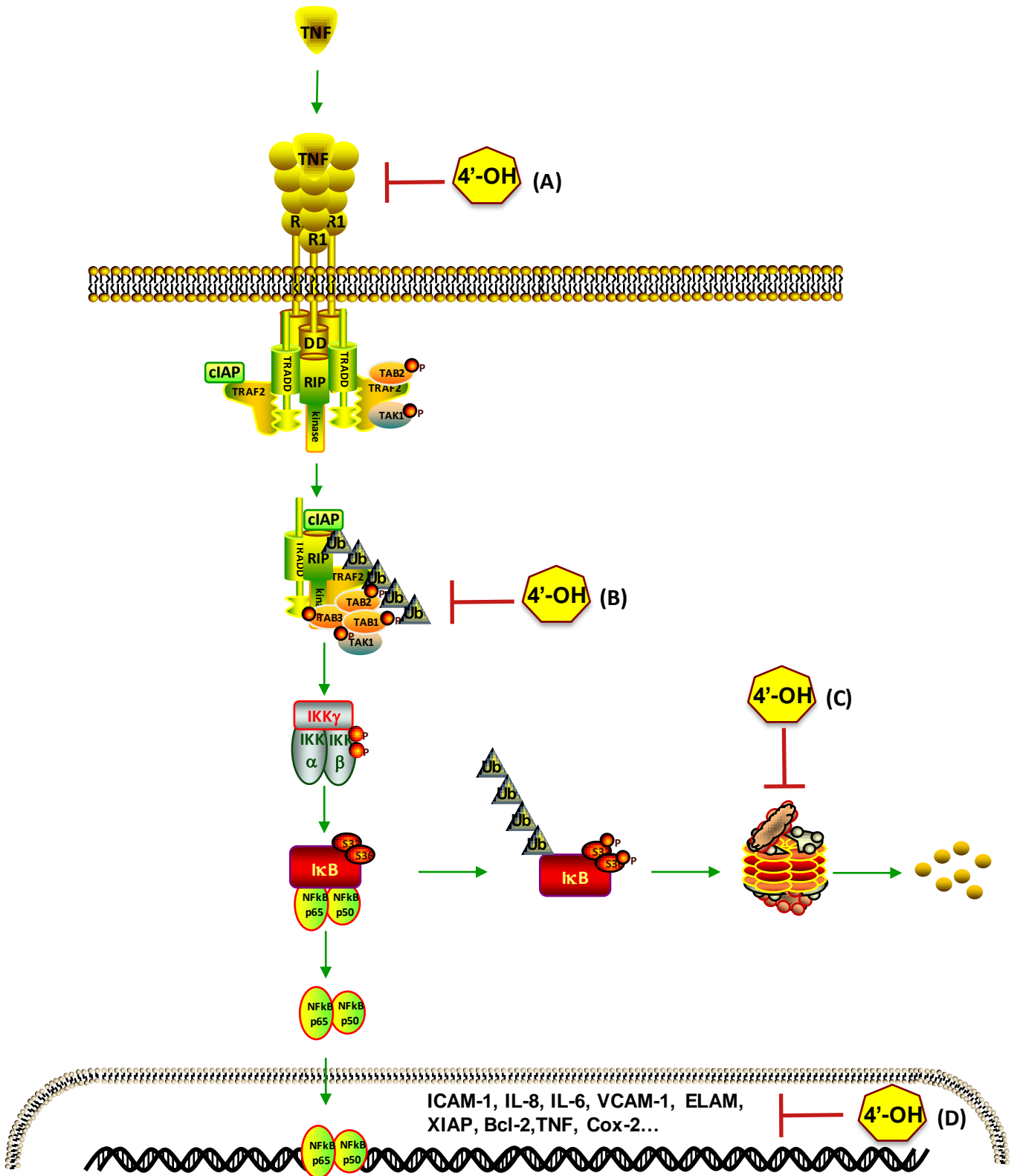


Figure 9