

# **Induction of Apoptosis in Estrogen-Receptor Negative Breast Cancer Cells by Natural and Synthetic Cyclopentenones: Role of the IKK/NF- $\kappa$ B Pathway**

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## Running title page

### **Running title:**

*Cyclopentenone-induced apoptosis in ER-negative breast cancer*

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**Number** of text pages: 32

**Number** of Figures: 7

**Number** of References: 40

**Number** of words in the *Abstract*: 205

**Number** of words in the *Introduction*: 741

**Number** of words in the *Discussion*: 1484

**Abbreviations:** AA, arachidonic acid; cyPG, cyclopentenone prostaglandins; 2-Cy, 2-cyclopenten-1-one; 15d-PGJ<sub>2</sub>, 15-deoxy- $\Delta^{12,14}$ prostaglandin J<sub>2</sub>; EMSA, Electrophoretic Mobility Shift Assay; ER, Estrogen Receptor; IKK, I $\kappa$ B kinase; NF- $\kappa$ B, Nuclear Factor- $\kappa$ B.

## ABSTRACT

Nuclear factor- $\kappa$ B (NF- $\kappa$ B), a transcription factor with a critical role in promoting inflammation and cell survival, is constitutively activated in estrogen-receptor (ER)-negative breast cancer, and is considered a potential therapeutic target for this type of neoplasia. We have previously demonstrated that cyclopentenone prostaglandins are potent inhibitors of NF- $\kappa$ B activation by inflammatory cytokines, mitogens and viral infection, via direct binding and modification of the  $\beta$  subunit of the I $\kappa$ B kinase (IKK). Herein we describe the NF- $\kappa$ B-dependent anticancer activity of natural and synthetic cyclopentenone IKK inhibitors. We demonstrate that the natural cyclopentenone 15-deoxy- $\Delta^{12,14}$ prostaglandin J<sub>2</sub> (15d-PGJ<sub>2</sub>) is a potent inhibitor of constitutive I $\kappa$ B-kinase and NF- $\kappa$ B activities in chemotherapy-resistant ER-negative breast cancer cells. 15d-PGJ<sub>2</sub>-induced inhibition of NF- $\kappa$ B function is rapidly followed by down-regulation of NF- $\kappa$ B-dependent anti-apoptotic proteins cIAP1/2, Bcl-X<sub>L</sub> and cFLIP, leading to caspase activation and induction of apoptosis in breast cancer cells resistant to treatment with paclitaxel and doxorubicin. We then demonstrate that the cyclopentenone ring structure is responsible for these activities, and identify a new synthetic cyclopentenone derivative, 3-tert-butyldimethylsilyloxy-5-(E)-iso-propylmethylenecyclopent-2-enone (CTC-35), as a potent NF- $\kappa$ B inhibitor with pro-apoptotic activity in ER-negative breast cancer cells. The results open new perspectives in the search for novel pro-apoptotic molecules effective in the treatment of cancers presenting aberrant NF- $\kappa$ B regulation.

## INTRODUCTION

Apoptosis is an essential physiologic process required for embryonic development, immune system function and the maintenance of tissue homeostasis in multicellular organisms (Hengartner, 2000). When the decision to undergo apoptosis is made in response to physiologic signals, a proteolytic cascade involving different caspases is triggered in the suicidal cell, which ultimately results in activation of nucleases that degrade chromosomal DNA (Hengartner, 2000). One of the major regulators of life or death decisions is the nuclear factor- $\kappa$ B (NF- $\kappa$ B) (Karin et al., 2002; Karin and Lin, 2002). NF- $\kappa$ B normally exists as an inactive cytoplasmic complex, whose predominant form is a heterodimer composed of p50 and p65 (Rel A) subunits, bound to inhibitory proteins of the I $\kappa$ B family (I $\kappa$ Bs), and is induced in response to a variety of pathogenic stimuli, including UV radiation, exposure to proinflammatory cytokines or mitogens, and to bacterial or viral infection (Karin and Lin, 2002; Li and Verma, 2002; Santoro et al., 2003). In most instances, induction requires the activation of the I $\kappa$ B kinase IKK, a multisubunit complex, containing two catalytic subunits (IKK- $\alpha$  and IKK- $\beta$ ) and the IKK- $\gamma$  regulatory subunit (Rothwarf et al., 1998; Israel, 2000). The IKK complex phosphorylates I $\kappa$ Bs triggering their ubiquitination and proteasome-mediated degradation (Karin and Ben-Neriah, 2000). Release of I $\kappa$ Bs results in nuclear translocation of NF- $\kappa$ B and its binding to DNA at specific  $\kappa$ B-sites, rapidly inducing a variety of genes encoding, among others, cell adhesion molecules, matrix metalloproteinases, inflammatory and chemotactic cytokines, cytokine receptors and enzymes that produce inflammatory mediators, such as cyclooxygenase-2 and the inducible form of nitric oxide synthase (Karin and Lin, 2002; Li and Verma, 2002).

As a consequence, NF- $\kappa$ B is considered a critical regulator of the inflammatory and immune responses (Li and Verma, 2002).

More recently, NF- $\kappa$ B activation has been connected with multiple aspects of oncogenesis, including the control of apoptosis (Baldwin, 2001). NF- $\kappa$ B acts as an anti-apoptotic factor and its activation can suppress cell death pathways by switching on genes that dampen pro-apoptotic signals, including members of the Bcl-2 family (Bcl-X<sub>L</sub> and A1/Bfl-1), cellular inhibitors of apoptosis (cIAP-1, cIAP-2 and XIAP), TNF receptor (TNFR)-associated factors 1 and 2 (TRAF1 and TRAF2), and the caspase 8/FADD-like IL-1 $\beta$ -converting enzyme (FLICE)-inhibitory protein cFLIP (Karin et al., 2002; Karin and Lin, 2002). Interestingly, NF- $\kappa$ B has been recently shown to be constitutively activated in several types of cancer cells, including lymphoid malignancies and some types of breast cancer (Rayet and Gelinas, 1999; Nakshatri et al., 1997). In particular, both the I $\kappa$ B kinase and NF- $\kappa$ B have been shown to be constitutively activated in estrogen-receptor (ER) negative breast cancer cell lines and primary tumors (Nakshatri et al., 1997; Biswas et al., 2000; Romieu-Mourez et al., 2001; Sovak et al., 1997), and this event has been associated with resistance to apoptosis induced by chemotherapeutic drugs (Patel et al., 2000; Weldon et al., 2001).

Cyclopentenone prostaglandins (cyPG) are potent bioactive molecules that possess anti-viral (Santoro, 1997) and anticancer activity (Straus and Glass, 2001; Santoro et al., 1989). The unique characteristic of cyPG is the presence of an  $\alpha,\beta$ -unsaturated carbonyl group in the cyclopentane ring, which renders this portion of the molecule able to form Michael adducts with cellular nucleophiles and to covalently bind to thiol groups of target cysteine residues of specific proteins (Chen et al., 1999). The cyclopentenone structure has been shown to be essential for the biological activity (Rossi et al., 1996; Santoro, 1997; Straus and Glass, 2001).

We have shown that natural cyPG are potent inhibitors of NF- $\kappa$ B activation by inflammatory cytokines, mitogens and viral infection, via direct binding and modification of the  $\beta$  subunit of the I $\kappa$ B kinase (Rossi et al., 1997,2000; Amici et al., 2001, 2004). Starting from these observations, in the present study we investigated the effect of cyPG on NF- $\kappa$ B activity and survival of ER-positive (ER+) and ER-negative (ER-) breast cancer cells. We report that the natural prostanoid 15-deoxy- $\Delta^{12,14}$ -PGJ<sub>2</sub> (15d-PGJ<sub>2</sub>) is able to inhibit constitutive NF- $\kappa$ B activity in different chemoresistant ER- breast cancer cell lines, including MDA-MB-231, SKBR-3 and MDA-MB-468 cells, at concentrations that have no effect in ER+ cells. NF- $\kappa$ B inhibition is rapidly followed by down-regulation of NF- $\kappa$ B-dependent anti-apoptotic proteins cIAP1/2, Bcl-X<sub>L</sub> and cFLIP, leading to caspase activation and induction of apoptosis in chemoresistant ER- breast cancer cells. We also show that these effects are mimicked by the 2-cyclopenten-1-one (2-Cy) ring by itself, and two 2-Cy synthetic derivatives, 4(S)-tert-butyldimethylsilyloxy cyclopent-2-enone (CTC-8) and 3-tert-butyldimethylsilyloxy-5-(E)-iso-propylmethylenecyclopent-2-enone (CTC-35). Finally, we have identified CTC-35 as a potent inducer of apoptosis in chemoresistant ER-breast cancer cells.

## MATERIALS AND METHODS

**Reagents.** Arachidonic acid (AA), prostaglandins A<sub>1</sub> (PGA<sub>1</sub>), E<sub>2</sub> (PGE<sub>2</sub>), F<sub>2α</sub> (PGF<sub>2α</sub>) and 15-deoxy-Δ<sup>12,14</sup>-prostaglandin J<sub>2</sub> were obtained from Cayman Chemical (Ann Arbor, MI). 2-Cyclopenten-1-one, doxorubicin-hydrochloride and paclitaxel were obtained from Sigma Chemical Co. (St. Louis, MO). 4(S)-tert-butyldimethylsilyloxy cyclopent-2-enone (CTC-8) and 3-tert-butyldimethylsilyloxy-5-(E)-iso-propylmethylenecyclopent-2-enone (CTC-35) were synthesized as described (Bickley et al., 2004). TNFα and antibodies to human cIAP-1, cIAP-2 were purchased from R&D Systems (Minneapolis, MN). Monoclonal antibodies to α-tubulin were obtained from Sigma, to Hsp70 from Stressgen (Victoria, BC), and to Bcl-2 from DAKO (Glostrup, DK). Rabbit polyclonal antibodies to caspase-8, caspase-9, caspase-3 and Bcl-X<sub>L</sub> were from Santa-Cruz Biotechnology (Santa Cruz, CA), to cFLIP were from Upstate Biotechnology, and to anti-IKKα from PharMingen (San Diego, CA).

**Cell culture and Treatments.** Human breast cancer cell lines MCF-7 (ER+) and MDA-MB-231 (ER-) were obtained from ATCC (Manassas, VA). SKBR-3 and MDA-MB-468 cell lines were kindly provided by Dr. O. Segatto (Regina Elena Institute, Rome, Italy). T-47D cells were kindly provided by Dr. G. Forni (University of Turin, Turin, Italy). All cell lines were grown at 37°C in 5% CO<sub>2</sub> and 95% humidified air in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 2mM glutamine and antibiotics (Life Technologies, Inc.), and supplemented with 1mM sodium pyruvate (MCF-7) or 1x non-essential amino acids (MDA-MB-231). AA, prostaglandins and cyclopentenones were dissolved in absolute ethanol and diluted in the culture medium immediately before use. Control cells received the same amount of ethanol diluent. Cell viability was determined by

vital-dye exclusion assay (Trypan blue, 0.1%).

**Flow Cytometry.** For DNA content determination, cells fixed in 70% ethanol were treated with RNase (0.25 mg/ml) and stained with propidium iodide (5  $\mu$ g/ml). The subG<sub>1</sub>/G<sub>0</sub>-phase fraction was calculated using CellQuest program (Becton-Dickinson). For Annexin-V staining, cells suspended in staining buffer (10mM HEPES pH 7.4, 140mM NaCl, 2.5mM CaCl) with Annexin-V-PI (Annexin-V-FITC, Becton-Dickinson) were analyzed by FACScan, using CellQuest Program.

**DNA Fragmentation.** Cytoplasmic histone-bound DNA fragments (mono- and oligonucleosomes) generated during apoptosis were measured by Cell-Death-Detection ELISA kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. Histone-associated DNA fragments were quantified spectrophotometrically using antibodies against DNA and histones in a colorimetric assay. Enrichment in cytoplasmic nucleosomes was expressed as fold-induction of levels in untreated controls.

**DAPI Staining.** Cells grown on coverslips and treated with 20  $\mu$ M 15d-PGJ<sub>2</sub> for 36 h were fixed with 3.7% paraformaldehyde, permeabilized with 0.5% Triton X-100 and stained with 4',6-diamidino-2-phenylindole (DAPI) (SIGMA) (5  $\mu$ g/ml). Fluorescence microscopy was performed on a Leica DM-IL microscope equipped with UV excitation filters, and images were captured on a Leica DC-300 camera using Leica Image-Manager500 software.

**Electrophoretic Mobility Shift Assay (EMSA).** For nuclear extract preparation, cells (2 x 10<sup>6</sup> cells/sample) were lysed in hypotonic lysis buffer (10mM NaCl, 3mM MgCl<sub>2</sub>, 10mM Tris-HCl pH 7.8, 0.5% NP40, 1mM DTT) and then in high-salt extraction buffer (50mM Tris-HCl, 400mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton, 0.5% NP-40, 10% Glycerol,



1mM DTT, 1mM PMSF, 0.5  $\mu$ g/ml leupeptin, 0.7  $\mu$ g/ml pepstatin, 0.2 % aprotinin). Equal amounts of protein (6  $\mu$ g/sample) were incubated with  $^{32}$ P-labeled  $\kappa$ B-DNA probe (5'-AGCTTCAGAGGGGACTTTCCGAGAGG-3' and 3'-AGTCTCCCCTGAAAGGCTCTCCAGCT-5) (Rossi et al., 1997), followed by analysis of DNA-binding activity by EMSA. Binding reactions were performed as described (Rossi et al., 1997). Complexes were analyzed by nondenaturing 4% polyacrylamide gel electrophoresis. Specificity of protein-DNA complexes was verified by immunoreactivity with polyclonal antibodies specific for p65/RelA. Quantitative evaluation of NF- $\kappa$ B/ $\kappa$ B complex formation was determined by Typhoon-8600 Imager (Molecular Dynamics), using ImageQuant software (MDP analysis). For control of equal loading, NF- $\kappa$ B values were normalized to the level of the nonspecific protein-DNA complex (ns) in the same lane.

**Western Blot Analysis.** Whole-cell extracts were prepared after lysis in buffer containing 50mM Tris-Cl pH 7.4, 0.15M NaCl, 5mM EDTA, 0.1% Triton X-100, 1mM Na<sub>3</sub>VO<sub>4</sub>, 1mM PMSF and protease inhibitors. Equal amounts of protein (20  $\mu$ g/sample) were separated by SDS-PAGE, transferred onto nitrocellulose membranes and incubated with specific antibodies, followed by labeling with peroxidase-labeled anti-mouse or anti-rabbit IgG (ECL, Amersham Pharmacia Biotech, UK).  $\alpha$ -Tubulin (Sigma) Western blot analysis was performed as a control of equal sample loading. Membranes were stripped by washing in glycine stripping buffer (0.2M glycine, 0.1% SDS, 1% polyoxyethylenesorbitan monolaurate [Tween-20, Sigma], pH 2.4) for 60 min at room temperature, before exposure to different antibodies. Quantitative evaluation of proteins was determined by Versadoc 1000 analysis using the Quantity One software program (BIO-RAD Laboratories, Hercules, CA). Levels of the different proteins analyzed were normalized to the level of  $\alpha$ -tubulin in the same blot.

**Kinase assay.** For IKK activity determination, cell lysates were immunoprecipitated with anti-IKK $\alpha$  antibody in the presence of 15  $\mu$ l protein-A-Sepharose (Sigma) at 4 °C for 16 h. After extensive washing, endogenous IKK activity was determined using GST-IkB $\alpha$ (1-54) as substrate (Rossi et al., 2000). The reaction mixtures were resolved by SDS-PAGE and transferred to nitrocellulose membranes, followed by autoradiography. Western blot analysis of IKK $\alpha$  was performed as kinase loading control.

**RT-PCR.** Total RNA was extracted using the TRIzol reagent (Life Technologies, Inc.) RT-PCR was performed (1  $\mu$ g RNA) according to the manufacturer's protocols (Invitrogen). The oligonucleotide primer pairs used for RT-PCR were the following: human cIAP-1 (984bp), 5'-ATGAACATAGTAGAAAACAGC-3' and 5'- CCTGTCCTTTAATTCTTATCA-3'; human cIAP-2 (900bp), 5'-TGACTTTTCCTGTGAACTCT-3' and 5'-GCCTTTCATTCGTATCAAGA-3'; human c-FLIP (851bp), 5'-GCTGAAGTCATCCATCAGGT-3 and 5'- CATACTGAGATGCAAGAATT-3'; human Bcl-X<sub>L</sub> (601bp), 5'-TGTGGAAGAGAACAGGACTGAGGC-3' and 5'-GAAAGAGTGAGCCCAGCAGAACCA-3'; human  $\beta$ -actin (210bp) 5'-GCGCTCAGGAGGAGCAAT-3' and 5'-GCACTCTTCCAGCCTTCC-3'. PCR was performed using the following thermocycler parameters: denaturing temperatures of 95 °C for 1 min, annealing temperatures of 58 °C for 1 min and elongation temperatures of 72 °C for 2 min for 30 cycles. The cycles for human  $\beta$ -actin were 28. PCR products were electrophoresed alongside DNA Molecular Weight Marker-IX (Roche, Basel, CH) in 2% agarose gels and then stained with ethidium bromide.

**Statistical analysis.** Statistical analysis was performed using the Student's *t* test for unpaired data. Data are expressed as the mean  $\pm$  S.E. of duplicate or triplicate samples. *P* values of  $< 0,05$  were considered significant.

## RESULTS

### **Differential sensitivity of ER-positive and ER-negative breast cancer cells to conventional chemotherapeutic drugs and to NF- $\kappa$ B inhibitors.**

Antiestrogen-resistant breast cancers are generally invasive and respond poorly to chemotherapy as compared to ER-positive tumors (Clarke et al., 1993). In order to compare the effect of conventional chemotherapeutic drugs on apoptosis induction in ER+ and ER- breast cancer cells, the human cell lines MCF-7, ER-positive and hormone-dependent, and MDA-MB-231, ER-negative and hormone-independent, were utilized. Cells were plated at the density of  $2 \times 10^5$  cells/ml and treated with 0.1  $\mu$ M paclitaxel, 1  $\mu$ M doxorubicin or control diluent for 48 hours. Apoptosis was determined by analysis of DNA fragmentation. As shown in Fig. 1A (top panel), treatment with paclitaxel or doxorubicin was very effective in inducing apoptosis in ER+ MCF-7 cells, whereas MDA-MB-231 cells were found to be resistant to doxorubicin and to respond poorly to paclitaxel. Similar results were obtained when cells were treated with higher concentrations of both drugs (data not shown).

Since constitutive NF- $\kappa$ B DNA-binding activity has been associated with resistance to chemotherapy in breast cancer, in a parallel experiment the effect of two NF- $\kappa$ B inhibitors, sodium arsenite (NaAsO<sub>2</sub>) (Mathas et al., 2003) and the proteasome inhibitor MG132 (Dai et al., 2003), on the induction of apoptosis was examined in MCF-7 and MDA-MB-231 cells. MG132 (1  $\mu$ M) and NaAsO<sub>2</sub> (50  $\mu$ M) were found to be ineffective in inducing DNA

fragmentation in MCF-7 cells up to 48 hours after treatment (Fig. 1A, lower panel). Interestingly, both molecules had a potent pro-apoptotic effect in MDA-MB-231 cells.

**15d-PGJ<sub>2</sub> induces apoptosis in ER- breast cancer cells which are resistant to conventional chemotherapeutic drugs.**

We have previously shown that cyclopentenone prostanoids, that are known to induce cell death in many tumor cell lines (Straus and Glass, 2001), are potent inhibitors of NF- $\kappa$ B activation (Rossi et al., 1997,2000; Amici et al., 2001, 2004). We then investigated the effect of the natural cyPG 15d-PGJ<sub>2</sub> on cell death of ER<sup>+</sup> and ER<sup>-</sup> breast cancer cells. MCF-7 and MDA-MB-231 were plated at the density of  $2 \times 10^5$  cells/ml and treated with different concentrations of 15d-PGJ<sub>2</sub> or with control diluent. Apoptosis was evaluated at 48 hours after treatment by measuring DNA fragmentation. In a parallel experiment, the percentage of Annexin-V<sup>+</sup> cells was determined by flow-cytometry after annexin-V-FITC/PI staining at 24 and 48 hours after treatment with 15d-PGJ<sub>2</sub>. As shown in Fig. 1C, 15d-PGJ<sub>2</sub> caused a dose-dependent increase in cytoplasmic mono- and oligonucleosomes in MDA-MB-231 cells, while it had no effect on ER<sup>+</sup> MCF-7 cells at the same concentrations. The pro-apoptotic activity of 15d-PGJ<sub>2</sub> on ER<sup>-</sup> breast cancer cells was confirmed by FACS analysis of Annexin-V<sup>+</sup> cells. After 24 hours of exposure, 15d-PGJ<sub>2</sub> induced apoptosis in MDA-MB-231 cells in a dose-dependent manner, reaching 40% of positive cells at the concentration of 30  $\mu$ M (Fig 1D, lower panel). At 48 hours, the percentage of Annexin-V<sup>+</sup> MDA-MB-231 cells was increased to 70% at 20  $\mu$ M and 80% at 30  $\mu$ M 15d-PGJ<sub>2</sub>. In contrast, no significant change in Annexin-V binding was observed in MCF-7 cells up to 48 hours of treatment (Fig. 1D, top panel).

The differential sensitivity of ER<sup>-</sup> and ER<sup>+</sup> breast cancer cells to 15d-PGJ<sub>2</sub>-induced apoptosis was also shown by determination of DNA content through FACS analysis of hypodiploid cells (sub G<sub>0</sub>/G<sub>1</sub> phase). As shown in Fig. 2A, untreated MCF-7 and MDA-MB-

231 cells showed the expected pattern of cell cycle distribution. After 48 hours of treatment, a sub G<sub>0</sub>/G<sub>1</sub> peak, indicative of a 48% increase of apoptotic cells, was evident in 15d-PGJ<sub>2</sub>-treated MDA-MB-231 cells (Fig. 2A, left panels). In contrast, no change in the percent of the sub G<sub>0</sub>/G<sub>1</sub> population was observed in MCF-7 cells (Fig. 2A, right panels). This observation was further confirmed by analysis of nuclear morphology. In MDA-MB-231 cells treatment with 15d-PGJ<sub>2</sub> (20  $\mu$ M) for 36 h caused a marked increase in nuclear fragmentation and condensation, as indicated by the pattern of DAPI staining, while no nuclear morphology changes were observed in MCF-7 cells (Fig. 2B).

To investigate whether 15d-PGJ<sub>2</sub> treatment induced caspase activation, MCF-7 and MDA-MB-231 cells were treated with 20  $\mu$ M 15d-PGJ<sub>2</sub> or control diluent and, after 24 hours, whole-cell lysates were processed for pro-caspase 8, 9 and 3 detection by immunoblotting. As shown in Fig. 2C, treatment with 15d-PGJ<sub>2</sub> caused a reduction in both pro-caspase-8 and -9 levels in MDA-MB-231 cells. Also the level pro-caspase-3 was decreased in the ER- cell line (data not shown). No changes in the level of caspases 8 and 9 (MCF-7 cells lack caspase 3) were instead detected in ER+ cells treated with 15d-PGJ<sub>2</sub> (Fig. 2C).

### **15d-PGJ<sub>2</sub> inhibits constitutive IKK and NF- $\kappa$ B activities in ER- breast cancer cells.**

NF- $\kappa$ B has been shown to be constitutively activated in ER- breast cancer cells, and this event has been associated with resistance to apoptosis induced by chemotherapeutic drugs (Sovak et al., 1997; Patel et al., 2000; Weldon et al., 2001). CyPG inhibit TNF $\alpha$ - and TPA-induced NF- $\kappa$ B activation by blocking IKK activity (Rossi et al., 2000). To determine the levels of constitutive IKK and NF- $\kappa$ B activities in MCF-7 and MDA-MB-231 cells and to investigate whether these would be affected by 15d-PGJ<sub>2</sub>, ER+ and ER- breast cancer cells were treated with different concentrations of 15d-PGJ<sub>2</sub> and, after 3 hours, whole-cell and nuclear extracts were analyzed for IKK activity by kinase assay and NF- $\kappa$ B DNA-

binding activity by EMSA, respectively. The levels of IKK and NF- $\kappa$ B activities were then quantified by MDP analysis. As shown in Fig. 3A, IKK activity was found to be three times higher in MDA-MB-231 cells than in MCF-7 cells. Furthermore, the levels of IKK activity correlated strictly with the levels of NF- $\kappa$ B DNA-binding activity (Fig. 3B), confirming an increased constitutive DNA-binding activity of the nuclear factor in more malignant tumors. A three-hour treatment with 15d-PGJ<sub>2</sub> was found to inhibit constitutive IKK activity in both cell lines; however, the prostanoid appeared to be more effective in MDA-MB-231 cells, where IKK activity was decreased by more than 35% and 80% after treatment with 10 or 20  $\mu$ M 15d-PGJ<sub>2</sub>, respectively. Inhibition of IKK was associated with a dramatic inhibition of NF- $\kappa$ B DNA-binding activity in ER- breast cancer cells. Quantitative evaluation by MDP analysis revealed that 15d-PGJ<sub>2</sub>, at the concentration of 10 and 20  $\mu$ M, inhibited NF- $\kappa$ B DNA-binding activity by over 65% and 95% respectively in MDA-MB-231 cells, whereas it was inactive in the ER+ cells (Fig. 3B and C).

In order to investigate whether the proapoptotic effect of 15d-PGJ<sub>2</sub> was specific for MDA-MB-231 cells or could be a general property of the prostanoid in ER- breast cancer cells, two human ER- (SKBR-3 and MDA-MB-468) and one ER+ (T-47D) breast cancer cell lines were examined for their sensitivity to 15d-PGJ<sub>2</sub> treatment. Breast cancer cells were treated with different concentrations of 15d-PGJ<sub>2</sub> and, after 3 hours, nuclear extracts were analyzed for NF- $\kappa$ B DNA-binding activity by EMSA. Levels of NF- $\kappa$ B activity were quantified by MDP analysis. The results shown in Fig. 4A indicate that ER- SKBR-3 and MDA-MB-468 cells expressed constitutively high levels of NF- $\kappa$ B DNA-binding activity, while NF- $\kappa$ B activity was barely detected in ER+ T-47D cells. Quantitative evaluation by MDP analysis revealed that 15d-PGJ<sub>2</sub>, at the concentration of 20  $\mu$ M, inhibited NF- $\kappa$ B DNA-binding activity by 64% and >90% in SKBR-3 and MDA-MB-468 cells respectively, whereas it was inactive in ER+ T-47D cells. In a parallel experiment, the effect of 15d-PGJ<sub>2</sub>

on induction of apoptosis was determined in the same cell lines after 48 hours of treatment. As shown in Fig. 4B, inhibition of NF- $\kappa$ B was associated with a dose-dependent increase in the number of Annexin V+ cells in both ER- cell lines. In contrast, no significant change in the number of apoptotic cells was observed in the ER+ T-47D cell line, which presented low levels of NF- $\kappa$ B DNA-binding activity not altered by 15d-PGJ<sub>2</sub> treatment.

### **15d-PGJ<sub>2</sub> down-regulates the expression of anti-apoptotic proteins in ER- breast cancer cells.**

Since induction of apoptosis by 15d-PGJ<sub>2</sub> was found to be associated with NF- $\kappa$ B inhibition in ER- breast cancer cells, we evaluated the effect of this prostanoid on the level of several survival-regulating proteins, whose expression is modulated by the transcriptional activity of NF- $\kappa$ B, using as a model MDA-MB-231 cells. At different times after treatment with 20  $\mu$ M 15d-PGJ<sub>2</sub>, MDA-MB-231 nuclear extracts were analyzed for NF- $\kappa$ B DNA-binding activity by EMSA, whereas the expression of NF- $\kappa$ B-dependent gene products was determined by Western blot analysis in whole-cell extracts. As a control of 15d-PGJ<sub>2</sub> activity we determined the level of the heat shock protein Hsp70, whose expression is known to be induced by cyclopentenone prostanoids via the activation of the heat shock transcription factor HSF-1 (Rossi et al., 1996). The high level of NF- $\kappa$ B DNA-binding activity in MDA-MB-231 cells was found to be dramatically decreased by 15d-PGJ<sub>2</sub> also at 24 and 48 hours after treatment (Fig. 5A, top panel). At the same times, 15d-PGJ<sub>2</sub> was found to decrease the expression of anti-apoptotic proteins, including cIAP-1 and cIAP-2, cFLIP and Bcl-X<sub>L</sub> (Fig. 5A, lower panel, and Fig. 5B). In contrast, Bcl-2 protein levels were not reduced, while the level of Hsp70 was greatly increased as expected. In the same experiment the number of Annexin V+ cells in 15d-PGJ<sub>2</sub>-treated samples was increased to 70%, as compared to 9% of control, at 48 hours after treatment.

To determine whether the decrease in the anti-apoptotic proteins was a consequence of reduced mRNA levels, the accumulation of the survival protein mRNAs was evaluated in MDA-MB-231 cells treated with 15d-PGJ<sub>2</sub> at different concentrations. In the same experiment, NF- $\kappa$ B activity was analyzed by EMSA 3 hours after treatment, whereas mRNA levels were determined by RT-PCR 24 hours after 15d-PGJ<sub>2</sub> administration. As expected, MDA-MB-231 NF- $\kappa$ B constitutive activity was dramatically decreased already 3 hours after treatment (Fig. 5C, top panel). The analysis of NF- $\kappa$ B-modulated genes showed that treatment with 15d-PGJ<sub>2</sub> caused a dose-dependent reduction in the levels of cIAP-1, cIAP-2, cFLIP and Bcl-X<sub>L</sub> mRNAs (Fig. 5C, lower panel), indicating that the decrease in the anti-apoptotic proteins described above is due to inhibition of NF- $\kappa$ B-dependent transcriptional activity.

**The cyclopentenone ring structure of natural prostaglandins, 2-cyclopenten-1-one, is responsible for NF- $\kappa$ B inhibition and cell death induction in ER- breast cancer cells.**

We have previously shown that, among the different arachidonic acid (AA) metabolites, only the cyclopentenone prostaglandins are able to inhibit TNF $\alpha$ - or mitogen-induced NF- $\kappa$ B activation and that, in fact, the presence of a cyclopentenone structure is essential for NF- $\kappa$ B inhibition (Rossi et al., 1997, 2000; Amici et al., 2001). We then evaluated the effect of AA and different AA metabolites on NF- $\kappa$ B inhibition and induction of apoptosis in ER-breast cancer cells. MDA-MB-231 cells were treated with 30  $\mu$ M AA, the non-cyclopentenone AA-derivatives PGE<sub>2</sub> and PGF<sub>2 $\alpha$</sub> , the reactive cyclopentenone prostanoids PGA<sub>1</sub> and 15d-PGJ<sub>2</sub>, or control diluent. After 3 hours, nuclear extracts were assayed for NF- $\kappa$ B activity by EMSA, whereas apoptosis was evaluated by FACS analysis of Annexin-V<sup>+</sup> cells 48 hours after treatment. As shown in Fig. 6, among the different AA derivatives,



only the cyclopentenone prostanoids were found to reduce NF- $\kappa$ B DNA-binding activity and to induce apoptosis, with 15d-PGJ<sub>2</sub> being the most active compound. On the other hand, the non-cyclopentenone prostaglandins PGE<sub>2</sub> and PGF<sub>2 $\alpha$</sub>  and AA itself had no effect on either NF- $\kappa$ B activity or apoptosis.

As indicated in the Introduction, the unique characteristic of cyPG is the presence of an  $\alpha,\beta$ -unsaturated carbonyl group in the cyclopentane ring, 2-cyclopenten-1-one, which renders this portion of the molecule able to bind to specific proteins (Chen et al., 1999). To investigate whether the cyclopentenone ring structure itself, in the absence of the lateral aliphatic side chains, could be effective in inhibiting NF- $\kappa$ B and inducing apoptosis in ER-breast cancer cells, MDA-MB-231 cells were treated with different concentrations of 2-cyclopenten-1-one, and NF- $\kappa$ B activity and the number of Annexin-V<sup>+</sup> cells were determined at 3 or 48 hours after treatment, respectively. In a parallel experiment, the activity of 2-Cy was compared with the activity of the 2-Cy derivatives 4(S)-tert-butyltrimethylsilyloxy cyclopent-2-enone (CTC-8) and 3-tert-butyltrimethylsilyloxy-5-(E)-isopropylmethylenecyclopent-2-enone (CTC-35), described in Fig. 7A, which were found to inhibit NF- $\kappa$ B much more potently than the parent compound in a luciferase reporter assay (Bickley et al., 2004). As shown in Fig. 7B and C (right panels), 2-cyclopenten-1-one was found to inhibit NF- $\kappa$ B and induce apoptosis, though at concentrations much higher than the natural prostaglandins (>500  $\mu$ M). Concentrations as high as 1 mM were necessary to induce apoptosis in 60% of MDA-MB-231. On the other hand, the 2-Cy derivatives CTC-8 and CTC-35 were found to be much more active than 2-Cy, inhibiting NF- $\kappa$ B and inducing apoptosis in ER- breast cancer cells at concentration similar to the natural prostanoids. Data shown in Fig. 7 indicate that the chemical modification of the cyclopentenone ring (Fig. 7A) caused an increase in the pro-apoptotic activity of CTC-8 and CTC-35 between 30 and 150-fold as compared to 2-Cy. In particular, CTC-35 was found to be more active

than 15d-PGJ<sub>2</sub>, being able to induce apoptosis in approximately 70% of the cells at the concentration of 5  $\mu$ M (Fig. 7C).

## DISCUSSION

Breast cancer represents one of the leading causes of death among women between the ages of 40-55 (Sovak et al., 1997). Estrogen is known to play a major role in the progression of the disease (McGuire et al., 1991). The estrogen-receptor, which is required for estrogen-dependent growth, is expressed in more than 60% of breast cancers. Interaction of  $17\beta$ -estradiol (E2) with ER initiates a sequence of events leading to ER activation and binding to its response element (ERE), which results in the modulation of the expression of hormone-responsive genes responsible for enhanced proliferation of mammary epithelial cells (Biswas et al., 2000). Antihormones such as tamoxifen bind to ER without conferring it an active configuration, thus blocking consequent downstream events (Jordan, 1992). As the breast cancer progresses, tumor cells can acquire growth autonomy and no longer require estrogen, becoming resistant to antihormones (Clarke et al., 1993). These antiestrogen-resistant tumors are usually highly invasive and metastatic, and respond poorly to chemotherapy and radiotherapy. Mutation of ER, downregulation of ER expression, dysregulation of ER-responsive genes and clonal selection of ER-negative cells, are among the factors believed to be responsible for antiestrogen-resistant growth of breast cancer. ER- breast cancers, that lack the E2-ER-ERE-mediated hormone-dependent cell proliferation pathway, have been shown to contain constitutively elevated levels of NF- $\kappa$ B (Nakshatri et al., 1997; Biswas et al., 2000), and this event has been associated with resistance to apoptosis induced by chemotherapeutic drugs (Patel et al., 2000; Weldon et al., 2001).

In ER- breast cancer, constitutive NF- $\kappa$ B activation is considered to be a downstream consequence of the abnormal epidermal growth factor-receptor (EGFR) signaling (Biswas et al., 2000), and to be linked to elevated expression and activity of IKK (Romieu-Mourez et al., 2001). Inhibition of NF- $\kappa$ B by stable expression of IKK $\beta$  dominant-negative mutants

or treatment with Go6976, that blocks NF- $\kappa$ B activation via PKC inhibition, was found to inhibit the growth and cause regression of ER- mammary tumors *in vitro* and *in vivo* (Biswas et al., 2003). The IKK inhibitor curcumin was also shown to possess antiinvasive activity in ER- breast cancer cells by downregulating the expression of NF- $\kappa$ B-dependent genes that promote tumor invasion, including MMP-2 and VEGF (Bharti et al., 2003). Collectively, these findings identify IKK and NF- $\kappa$ B as potential therapeutic targets for estrogen-receptor negative breast cancer.

On the basis of our previous observation that cyclopentenone prostanoids prevent virus- and inflammatory cytokines-induced NF- $\kappa$ B activation by direct modification of the IKK complex via binding to the IKK  $\beta$ -subunit (Rossi et al., 1997, 2000; Amici et al., 2001, 2004), in the present study we investigated the effect of natural and synthetic cyclopentenones on constitutive NF- $\kappa$ B activity and survival of ER+ and ER- breast cancer cells. In particular we focused our attention on the natural prostanoid 15d-PGJ<sub>2</sub>, that has been shown to possess potent anti-neoplastic activity in several *in vitro* and *in vivo* tumor models, including breast cancer (Straus and Glass, 2001; Qin et al., 2003; Clay et al., 2001).

Since antiestrogen-resistant breast cancers are generally invasive and respond poorly to chemotherapy as compared to ER+ tumors (Clarke et al., 1993), we first compared the effect of the conventional chemotherapeutic drugs paclitaxel and doxorubicin on apoptosis induction in ER+ and hormone-dependent MCF-7 cells, and ER- and hormone-independent MDA-MB-231 cells. The results confirmed that the anticancer treatment was very effective in inducing apoptosis in ER+ cells, whereas MDA-MB-231 cells were found to be resistant to doxorubicin and to respond poorly to paclitaxel. Conversely, two well known NF- $\kappa$ B inhibitors, sodium arsenite (Mathas et al., 2003) and the proteasome inhibitor MG132 (Dai et al., 2003), were found to have a potent pro-apoptotic activity in antiestrogen-resistant

MDA-MB-231 cells, whereas they had no effect in ER<sup>+</sup> MCF7 cells. 15d-PGJ<sub>2</sub> behaved similarly to the second group of compounds, being extremely effective in inducing apoptosis selectively in ER<sup>-</sup> breast cancer cells. At 48 hours after treatment, the percentage of apoptotic MDA-MB-231 cells was increased to 70% at 20  $\mu$ M and 80% at 30  $\mu$ M 15d-PGJ<sub>2</sub>, while no significant change was observed in MCF-7 cells. The differential sensitivity of ER<sup>-</sup> and ER<sup>+</sup> breast cancer cells to 15d-PGJ<sub>2</sub>-induced apoptosis was also confirmed by analysis of nuclear condensation and DNA fragmentation. In agreement with previous observations (Clay et al., 2001), apoptosis of MDA-MB-231 cells was mediated by simultaneous activation of multiple caspases demonstrated by the cleavage of initiator caspases 8 and 9, and executioner caspase 3.

When levels of constitutive IKK and NF- $\kappa$ B activities were analyzed in MCF-7 and MDA-MB-231 cells, both activities were found to be three times higher in ER<sup>-</sup> MDA-MB-231 cells than in MCF-7 cells, confirming an increased constitutive DNA-binding activity of the nuclear factor in more malignant tumors. A short treatment with 15d-PGJ<sub>2</sub> was found to be effective in inhibiting constitutive IKK activity in MDA-MB-231 cells, where the kinase activity was decreased by more than 35% and 80% already 3 hours after treatment with 10 or 20  $\mu$ M 15d-PGJ<sub>2</sub>, respectively. At the higher concentration, 15d-PGJ<sub>2</sub> inhibited the low level of IKK activity also in MCF-7 cells. In ER<sup>-</sup> breast cancer cells, IKK inhibition was associated with a dramatic inhibition of NF- $\kappa$ B DNA-binding activity. Quantitative determination by MDP analysis revealed that 15d-PGJ<sub>2</sub>, at the concentration of 10  $\mu$ M, markedly (>65%) inhibited constitutive NF- $\kappa$ B DNA-binding activity already 3 hours after treatment in MDA-MB-231 cells (whereas it was inactive in ER<sup>+</sup> cells), and almost completely suppressed it at higher concentrations up to 48 hours after treatment. Similar results were obtained in two other human ER<sup>-</sup> breast cancer cell lines (SKBR-3 and MDA-MB-468), indicating a general effect of 15d-PGJ<sub>2</sub> in this type of cancer.

Inhibition of NF- $\kappa$ B by 15d-PGJ<sub>2</sub> in ER- breast cancer cells was found to lead to down-regulation of the expression of several NF- $\kappa$ B-dependent anti-apoptotic gene products, including Bcl-X<sub>L</sub>, cIAP-1/2 and cFLIP, followed by activation of multiple caspases. All together these findings indicate that 15d-PGJ<sub>2</sub> is able to control IKK and NF- $\kappa$ B aberrant regulation in malignant antihormone-resistant breast cancer cells.

As indicated above, cyclopentenone prostaglandins have been shown to possess potent antitumor activity in different *in vitro* and *in vivo* cancer models (Straus and Glass, 2001; Piva et al., 2005). Multiple mechanisms of pro-apoptotic activity have been suggested, including binding to peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ), activation of oxidative stress-mediated mitochondrial apoptosis pathway, and stimulation of MAPK kinases (Straus and Glass, 2001, Padilla et al., 2000; Nencioni et al., 2003; Liu et al., 2003). We now suggest that inhibition of NF- $\kappa$ B activity and NF- $\kappa$ B-dependent expression of cell survival proteins may play a major role in the pro-apoptotic activity of 15d-PGJ<sub>2</sub> in ER- breast cancer. However, we cannot exclude that different mechanisms, other than inhibition of NF- $\kappa$ B activity, may participate in the pro-apoptotic activity of cyclopentenone prostanoids.

Among several prostaglandins tested, only the cyclopentenone prostanoids PGA<sub>1</sub> and 15d-PGJ<sub>2</sub> were found to reduce NF- $\kappa$ B DNA-binding activity and to induce apoptosis in ER- breast cancer cells, with 15d-PGJ<sub>2</sub> being the most active compound, whereas the non-cyclopentenone prostaglandins PGE<sub>2</sub> and PGF<sub>2 $\alpha$</sub>  and the prostaglandin precursor arachidonic acid had no effect on either NF- $\kappa$ B activity or apoptosis. As indicated above, the unique characteristic of cyPG is the presence of an  $\alpha,\beta$ -unsaturated carbonyl group in the cyclopentane ring, 2-cyclopenten-1-one, which renders this portion of the molecule able to bind to cysteine-179 in the IKK $\beta$  activation loop (Rossi et al., 2000). We then investigated whether the cyclopentenone ring structure itself, in the absence of the lateral

aliphatic side chains, could be effective in inhibiting NF- $\kappa$ B and inducing apoptosis in ER-MDA-MB-231 cells. In addition, the activity of 2-cyclopenten-1-one was compared with the activity of two 2-Cy derivatives, 4(S)-tert-butyldimethylsilyloxy cyclopent-2-enone (CTC-8) and 3-tert-butyldimethylsilyloxy-5-(E)-iso-propylmethylenecyclopent-2-enone (CTC-35), whose synthesis has been recently described (Bickley et al., 2004). 2-Cyclopenten-1-one was found to be able to inhibit NF- $\kappa$ B and induce apoptosis in MDA-MB-231 cells, but at concentrations much higher than the natural prostaglandins ( $>500\ \mu\text{M}$ ). Interestingly, 2-Cy derivatives CTC8 and CTC35 were much more effective than the parent compound, with an activity comparable or greater than the natural prostanoids, both in inhibiting NF- $\kappa$ B and inducing apoptosis in ER- breast cancer cells. These results indicate that a simple substitution of a hydrophobic group into a cyclopentenone molecule enhances the biological activity of the 5-membered ring system, and identify CTC35 as a new potent anticancer agent.

Prostaglandins are used clinically in the treatment of gastroduodenal ulcers, congenital heart disease, erectile dysfunction and to facilitate labor, and are generally effective and well tolerated (Vane and O'Grady, 1993). The results described in the present report indicate that cyclopentenone prostanoids are potent inducers of apoptosis in aggressive ER- breast cancer that is often resistant to chemotherapy. We also show that the cyclopentenone ring structure itself is essential for the anticancer activity. The fact that 2-cyclopenten-1-one and the 2-Cy derivatives CTC-8 and CTC-35 were able to inhibit constitutive NF- $\kappa$ B activity and induce apoptosis in ER- breast cancer cells, suggests that a new class of novel NF- $\kappa$ B inhibitors could be designed, which could be of therapeutic value in the treatment of breast cancers presenting aberrant NF- $\kappa$ B regulation.

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## FOOTNOTES

### Footnotes to the title page

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This work was supported by grants from the Italian Ministry of University and Scientific Research (MIUR) and Charterhouse Therapeutics, Ltd., Oxford, UK.

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## FIGURE LEGENDS

**Figure 1. Differential response of ER+ and ER- breast cancer cells to conventional chemotherapeutic drugs and to NF- $\kappa$ B inhibitors. (A)** ER+ MCF-7 (filled bars) and ER- MDA-MB-231 (empty bars) cells were treated with paclitaxel (0.1  $\mu$ M) and doxorubicin (5  $\mu$ M) (top panel) or with MG132 (1  $\mu$ M) and NaAsO<sub>2</sub> (50  $\mu$ M) (bottom panel). After 48 h, apoptosis was evaluated by measuring DNA fragmentation, as described in the Methods section. The enrichment of nucleosomes in the cytoplasm of treated cells is expressed as fold induction of levels in untreated controls. **(B)** Structure of 15-deoxy- $\Delta^{12,14}$ -PGJ<sub>2</sub> (15d-PGJ<sub>2</sub>). **(C, D)** MCF-7 and MDA-MB-231 cells were treated with different concentrations of 15d-PGJ<sub>2</sub> or with control diluent. Apoptosis was evaluated at 48 hours after treatment by measuring DNA fragmentation **(C)**. In a parallel experiment, the percentage of Annexin-V positive (annexin V<sup>+</sup>) cells was determined by flow cytometry after Annexin-V-FITC/PI staining at 24 (empty bars) and 48 (filled bars) hours after treatment with 15d-PGJ<sub>2</sub> **(D)**. Data represent the mean  $\pm$  SEM of three independent experiments, each in duplicate. \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ .

**Figure 2. 15d-PGJ<sub>2</sub> induces apoptosis through multiple caspase activation in ER- MDA-MB-231 cells, but not in ER+ MCF-7 cells.** Unsynchronised MCF-7 and MDA-MB-231 cells were treated with 20  $\mu$ M 15d-PGJ<sub>2</sub> or control diluent (Control). Cell cycle profile was evaluated at 48 hours after treatment by PI staining using flow cytometry **(A)**. The percentage of sub-G<sub>0</sub>/G<sub>1</sub> cells is indicated on each panel. Alternatively, cells were fixed, permeabilized and stained with DAPI at 36 h after treatment **(B)**. Nuclear morphology was analysed by fluorescent microscopy (magnification 400X). Apoptotic cells containing condensed and fragmented fluorescent nuclei are visible in 15d-PGJ<sub>2</sub>-treated MDA-MB-

231 cells, but not in MCF-7 cells. **(C)** MCF-7 and MDA-MB-231 cells were treated with 20  $\mu$ M 15d-PGJ<sub>2</sub> or control diluent (Control) for 24 h. Whole-cell lysates were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with the indicated antibodies. The levels of  $\alpha$ -tubulin are shown as control. Data are representative of three separate experiments with similar results.

**Figure 3. 15d-PGJ<sub>2</sub> inhibits constitutive IKK and NF- $\kappa$ B activities in ER- breast cancer cells.** MCF-7 and MDA-MB-231 cells were treated with the indicated concentrations of 15d-PGJ<sub>2</sub> or with control diluent. After 3 hours, protein extracts were analyzed for IKK activity by kinase assay (KA: IKK) **(A)** and NF- $\kappa$ B activation by EMSA **(B)**. Endogenous IKK recovery was determined in the same samples by immunoblot analysis for IKK $\alpha$  (IB: IKK $\alpha$ ). A section of the fluorograms is shown (right panels). Positions of NF- $\kappa$ B-DNA (NF- $\kappa$ B) and non-specific protein-DNA (ns) complexes are indicated. The levels of IKK **(A)** and NF- $\kappa$ B **(B)** activities in MCF-7 (●) and MDA-MB-231 (○) cells were quantified by MDP analysis and expressed as arbitrary units (left panels). Data are representative of three separate experiments with similar results. **C.** NF- $\kappa$ B-DNA binding activity in MCF-7 and MDA-MB-231 cells treated with 15d-PGJ<sub>2</sub> (10  $\mu$ M) or control diluent is expressed as the mean  $\pm$  SEM of three independent experiments. \*\*\* =  $p < 0.001$ . **D.** Specificity of NF- $\kappa$ B-DNA complexes in MCF-7 and MDA-MB-231 cells, as determined by supershift analysis using anti-p65 antibodies (1:100 dilution). The shifted NF- $\kappa$ B-DNA complexes are indicated by the arrow.

**Figure 4. 15d-PGJ<sub>2</sub> inhibits constitutive NF- $\kappa$ B binding activity and induces apoptosis in ER- breast cancer cells.** ER-positive T-47D cells, and ER-negative SKBR-3 and MDA-MB-468 cells were treated with the indicated concentrations of 15d-PGJ<sub>2</sub> or with

control diluent. **(A)** After 3 hours nuclear extracts were analyzed for NF- $\kappa$ B activation by EMSA (right panels). Levels of NF- $\kappa$ B DNA-binding activity quantified by MDP analysis are expressed as arbitrary units (left panels). **(B)** In a parallel experiment, the percentage of Annexin-V positive (Annexin V<sup>+</sup>) cells was determined by flow cytometry after Annexin-V-FITC/PI staining at 48 hours after treatment with 15d-PGJ<sub>2</sub> at the indicated concentrations. Data represent the mean  $\pm$  SEM of two independent experiments, each in duplicate. \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$ .

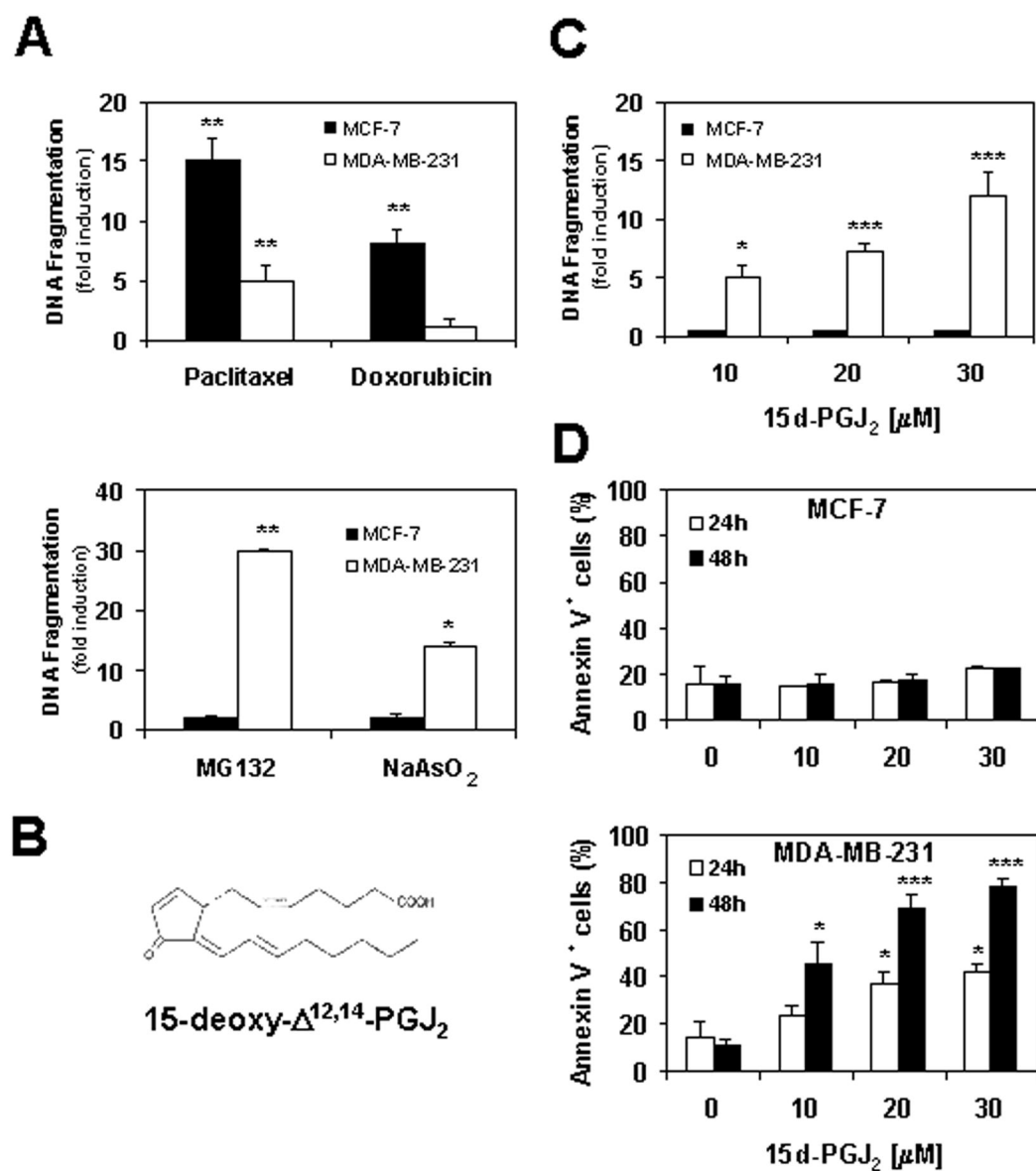
**Figure 5. 15d-PGJ<sub>2</sub> down-regulates the expression of cellular inhibitor-of-apoptosis proteins (cIAPs), cFLIP and Bcl-X<sub>L</sub> in ER- breast cancer cells.** **(A)** MDA-MB-231 cells were treated with 20  $\mu$ M 15d-PGJ<sub>2</sub> or control diluent. At the indicated times nuclear extracts were analyzed for NF- $\kappa$ B DNA-binding activity by EMSA (top panel). A section of the fluorograms is shown. Positions of NF- $\kappa$ B-DNA (NF- $\kappa$ B) and non-specific protein-DNA (ns) complexes are indicated. In the same experiment, whole-cell lysates were immunoblotted with the indicated antibodies. The different proteins were detected on the same blot after stripping (bottom panel). The levels of Hsp70 and  $\alpha$ -tubulin are shown as positive control and loading control respectively. Inhibition of NF- $\kappa$ B by 15d-PGJ<sub>2</sub> is associated with a decrease in the expression of cIAP-1, cIAP-2, cFLIP and Bcl-X<sub>L</sub> anti-apoptotic proteins. **(B)** Levels of proteins shown in (A), quantified by densitometric analysis, are expressed as arbitrary units after normalization to  $\alpha$ -tubulin levels in the same sample. Empty, grey and filled bars represent protein levels at 0, 24 and 48 hours after 15d-PGJ<sub>2</sub> treatment respectively. **(C)** MDA-MB-231 cells were treated with different concentrations of 15d-PGJ<sub>2</sub>. At 3 hours after treatment, nuclear extracts were analyzed for NF- $\kappa$ B activity by EMSA (top panel). Levels of cIAP-1, cIAP-2, XIAP, cFLIP and Bcl-X<sub>L</sub>



mRNA in whole-cell extracts were analyzed by RT-PCR 24 hours after treatment (bottom panel).  $\beta$ -Actin mRNA levels were determined and used for normalization.

**Figure 6. Effects of arachidonic acid metabolites on cell death and NF- $\kappa$ B activity in MDA-MB-231 cells.** MDA-MB-231 cells were treated with 30  $\mu$ M arachidonic acid (AA),  $\text{PGA}_1$ ,  $\text{PGE}_2$ ,  $\text{PGF}_{2\alpha}$  and  $15\text{-d-PGJ}_2$  or control diluent. **(A)** After 3 hours, nuclear extracts were assayed for NF- $\kappa$ B DNA-binding activity by EMSA (top panel). The levels of NF- $\kappa$ B DNA-binding activity were quantified by MDP analysis and expressed as percent of control (bottom panel). **(B)** At 48 hours after treatment, apoptosis was evaluated by FACS analysis of Annexin- $\text{V}^+$  cells. Data represent the mean  $\pm$  SEM of duplicate samples. \* =  $p < 0.05$ ; \*\*\* =  $p < 0.001$ .

**Figure 7. 2-Cyclopenten-1-one (2-Cy) and 2-Cy derivatives CTC-8 and CTC-35 inhibit NF- $\kappa$ B activity and induce apoptosis in ER- breast cancer cells.** **(A)** Structure of 2-Cyclopenten-1-one, CTC-8 and CTC-35. **(B, C)** MDA-MB-231 cells were treated with different concentrations of 2-Cy (right panels), CTC-8 (middle panels) and CTC-35 (left panels). After 3 hours nuclear extracts were assayed for NF- $\kappa$ B DNA-binding activity by EMSA. The levels of NF- $\kappa$ B DNA-binding activity were quantified by MDP analysis and expressed as percent of control **(B)**. In the same experiment apoptosis was measured 48 h after treatment by FACS analysis of Annexin- $\text{V}^+$  cells **(C)**. Data represent the mean  $\pm$  SEM of duplicate samples. \* =  $p < 0.05$ ; \*\*\* =  $p < 0.001$ .



**Figure 1**

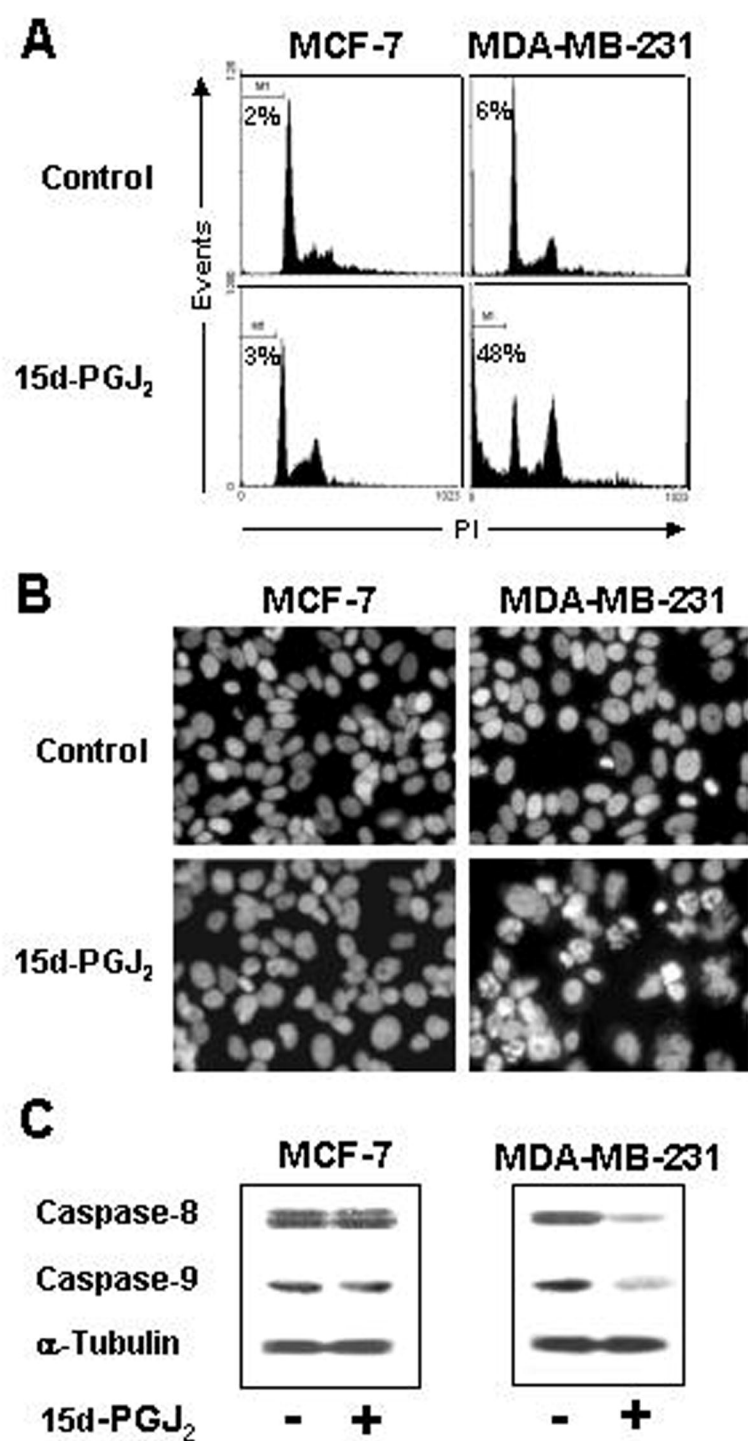
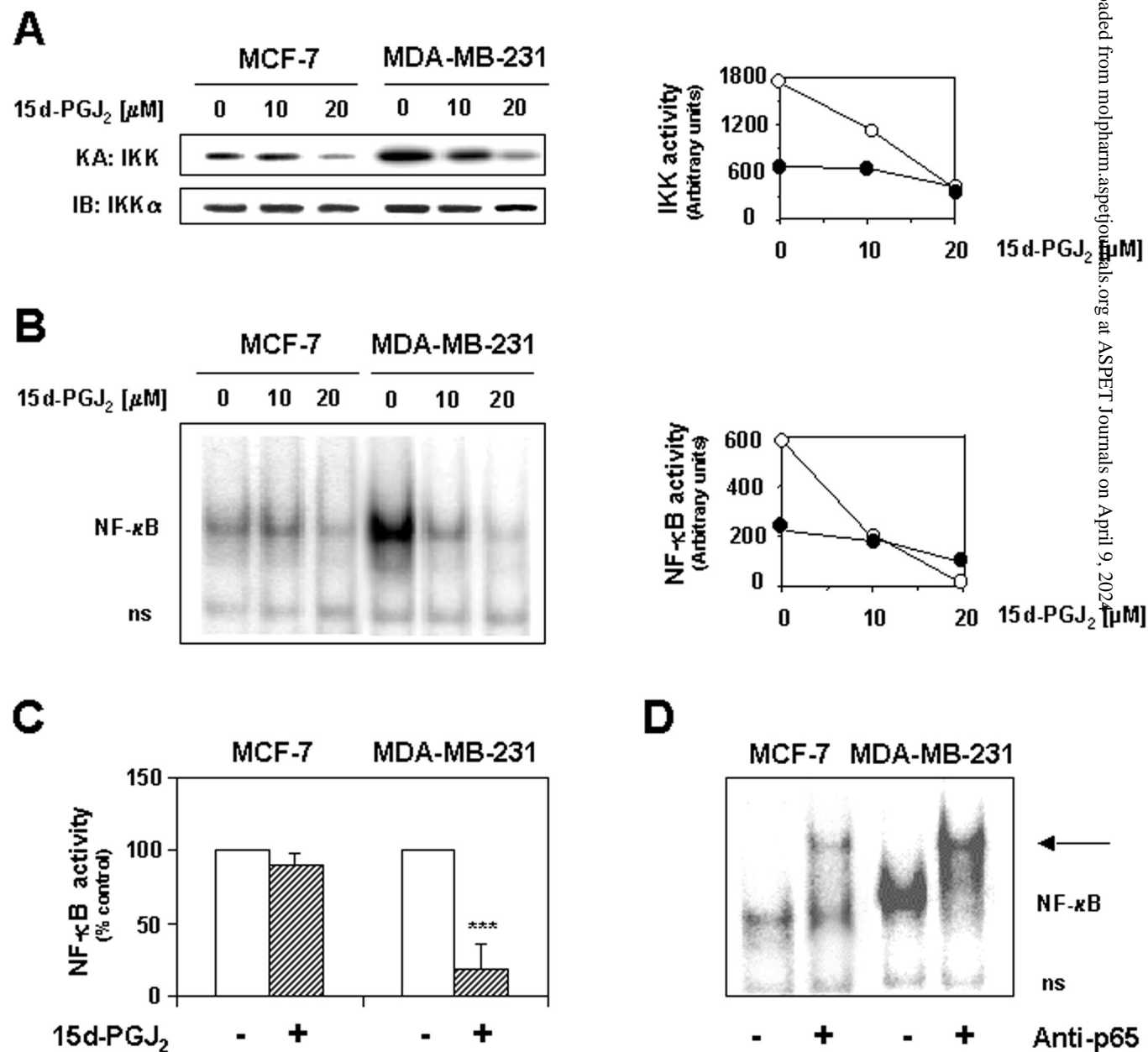
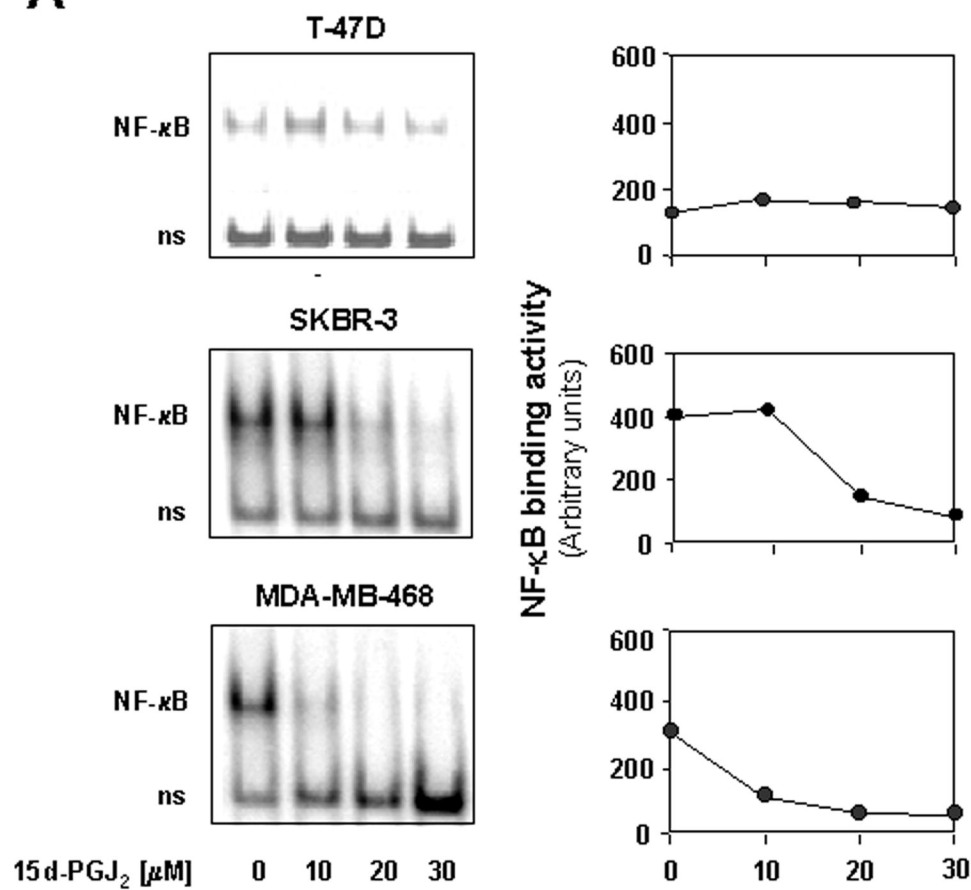
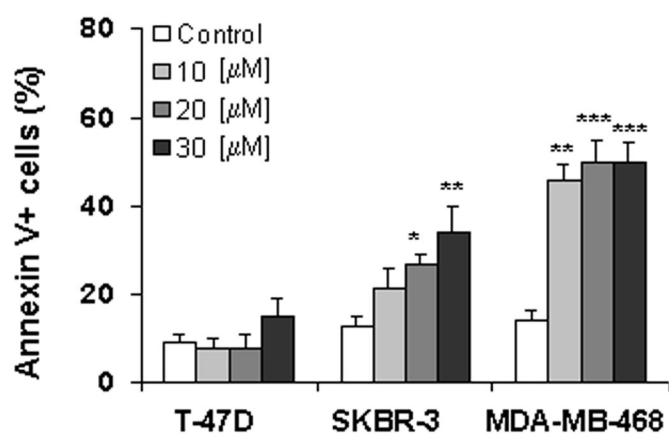
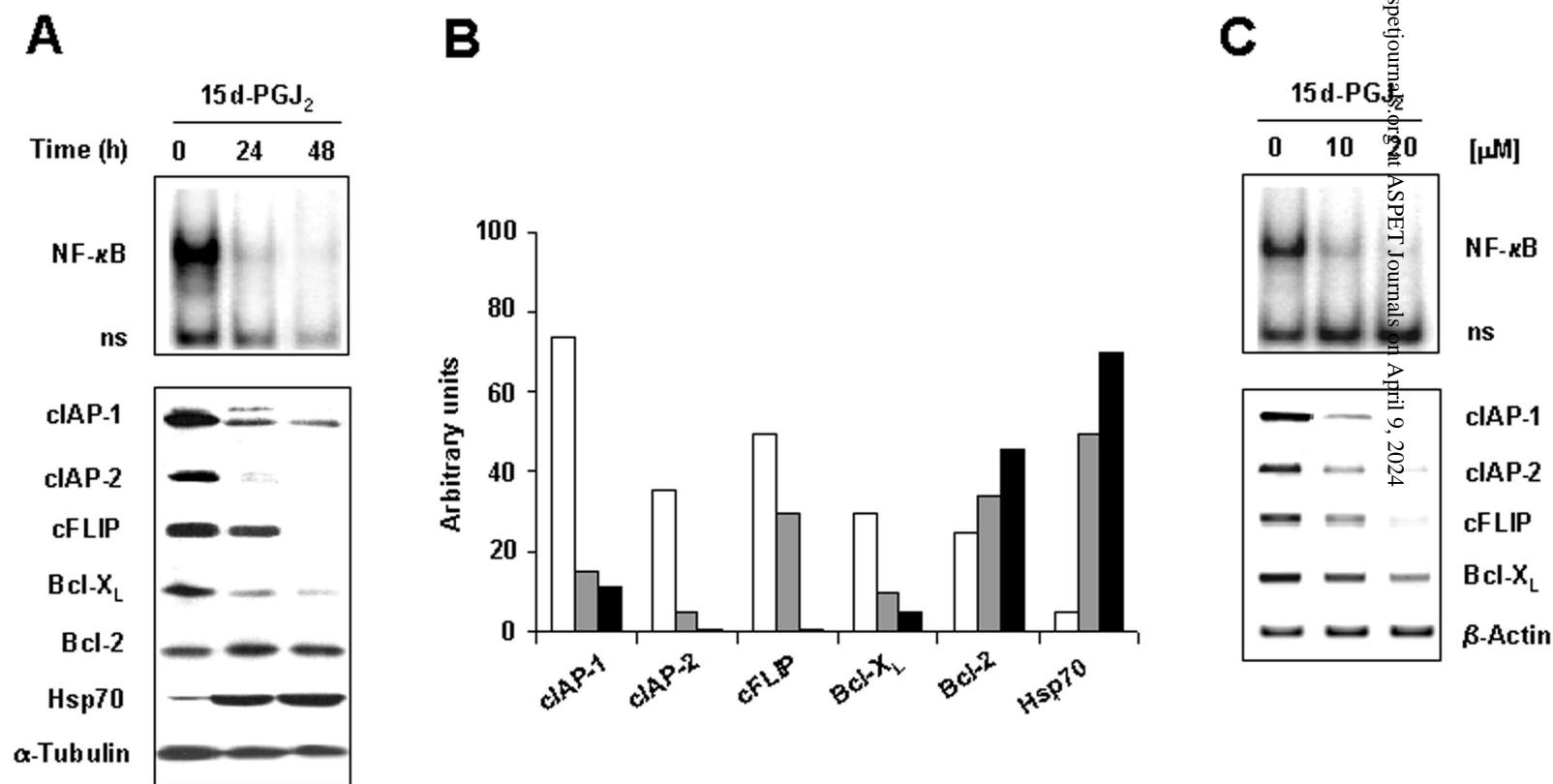


Figure 2

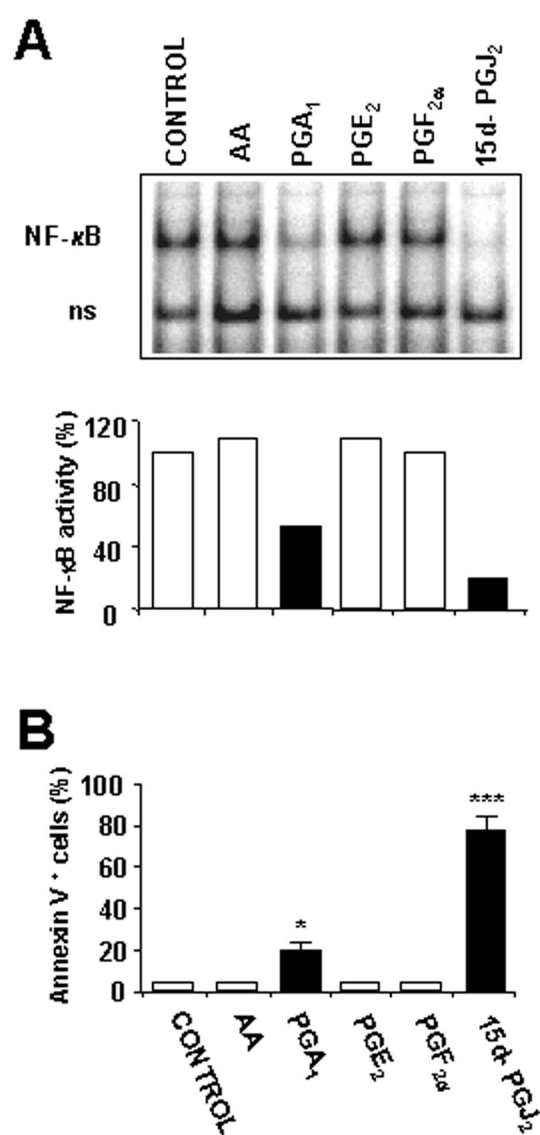


**Figure 3**

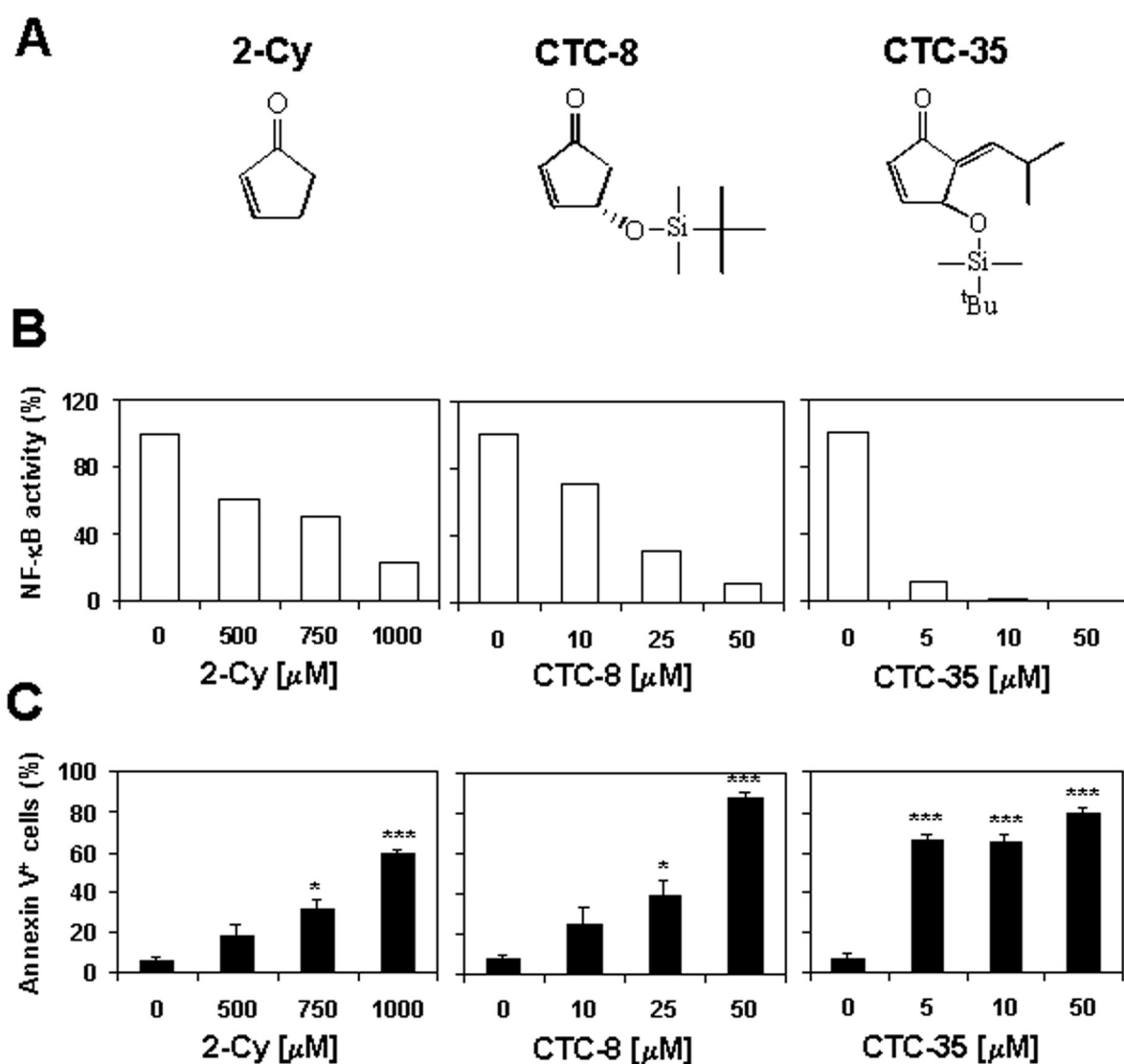
**A****B****Figure 4**



**Figure 5**



**Figure 6**



**Figure 7**