

Accepted Manuscript

Title: The rigid steroid 21-hydroxy-6,19-epoxyprogesterone (21OH-6,19OP) is a dissociated glucocorticoid receptor modulator potentially useful as a novel coadjuvant in breast cancer chemotherapy

Author: Andres J. Orqueda M. Virginia Dansey Alejandro Español Adriana S. Veleiro Elisa Bal de Kier Joffe M. Elena Sales Gerardo Burton Adali Pecci

PII: S0006-2952(14)00230-5
DOI: <http://dx.doi.org/doi:10.1016/j.bcp.2014.04.006>
Reference: BCP 11954

To appear in: *BCP*

Received date: 31-1-2014
Revised date: 2-4-2014
Accepted date: 2-4-2014

Please cite this article as: Orqueda AJ, Dansey MV, Español A, Veleiro AS, Joffe EBK, Sales ME, Burton G, Pecci A, The rigid steroid 21-hydroxy-6,19-epoxyprogesterone (21OH-6,19OP) is a dissociated glucocorticoid receptor modulator potentially useful as a novel coadjuvant in breast cancer chemotherapy, *Biochemical Pharmacology* (2014), <http://dx.doi.org/10.1016/j.bcp.2014.04.006>

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



The rigid steroid 21-hydroxy-6,19-epoxyprogesterone (21OH-6,19OP) is a dissociated glucocorticoid receptor modulator potentially useful as a novel adjuvant in breast cancer chemotherapy

Andres J Orqueda^{a,c}, M Virginia Dansey^{b,c}, Alejandro Español^e, Adriana S Veleiro^{b,d}, Elisa Bal de Kier Joffe^f, M Elena Sales^e, Gerardo Burton^{b,d} and Adali Pecci^{a,c}

^a*Departamento de Química Biológica and* ^b*Departamento de Química Orgánica;* ^c*IFIBYNE and* ^d*UMYMFOR (CONICET), Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria (C1428EGA), Buenos Aires, Argentina.* ^e*CEFyBo (CONICET) and* ^f*Área Investigación Instituto de Oncología Ángel H. Roffo, Facultad de Medicina, Paraguay 2155 (C1121AB), , Universidad de Buenos Aires, Buenos Aires, Argentina*

AJO: aorqueda@qb.fcen.uba.ar; MVD: virg_dansey@hotmail.com; AE: aespan_1999@yahoo.com;

ASV: adrianav@qo.fcen.uba.ar; EBdeKJ: balelisa2002@yahoo.com.ar; MES: malegazpio@yahoo.com.ar; GB: burton@qo.fcen.uba.ar; AP: apecchi@qb.fcen.uba.ar

Address correspondence to: Prof. Dr. Adali Pecci, Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires C1428EGA, Argentina. Phone/Fax: 54-11-4576-3342, e-mail: apecchi@qb.fcen.uba.ar; pecciadali@gmail.com

Abstract

Glucocorticoids (GCs) are steroid hormones widely used as adjuvants in the treatment of solid tumors due to their anti-inflammatory effects. However, evidence shows that they also may induce chemotherapy resistance, probably through their capacity to inhibit apoptosis triggered by antineoplastic drugs. GCs exert their action by regulating gene expression through two main mechanisms: transactivation, where the activated glucocorticoid receptor (GR) directly binds to certain genes; and transrepression, an indirect mechanism by which GR regulates other transcription factors activities. Recently, our group has shown that the rigid steroid 21-hydroxy-6,19-epoxyprogesterone (21OH-6,19OP) is a selective GR ligand that behaves as an agonist in transrepression assays and as an antagonist in transactivation ones. Here, we have evaluated the anti-inflammatory activity of 21OH-6,19OP, its capacity to generate chemoresistance, as well as its mechanism of action. We found that 21OH-6,19OP inhibits nitrites formation and the inducible nitric oxide synthase (*Nos-2*) expression in macrophages. It also blocks the expression of both cyclooxygenase-2 (COX-2) and interleukin-8 (IL-8) triggered by tumor necrosis factor-alpha (TNF- α) in epithelial lung cancer cells. However, contrary to dexamethasone (DEX), 21OH-6,19OP neither reverts the paclitaxel-induced caspase-3 activity, nor induces the anti-apoptotic *Bcl-X_L* gene expression in murine tumor mammary epithelial cells; and importantly, it lacks GCs-associated chemoresistance in a mouse mammary tumor model. Together, our findings suggest that 21OH-6,19OP behaves as a dissociated GC that keeps anti-inflammatory action without affecting the apoptotic process triggered by chemotherapeutic drugs. For these reasons, this steroid may become a putative novel adjuvant in the treatment of breast cancer.

Key words: GCs, glucocorticoids; GR, glucocorticoid receptor; **21OH-6,19OP**, 21-hydroxy-6,19-epoxyprogesterone; **MKP-1**, mitogen activated protein kinase phosphatase-1; **COX-2**, cyclooxygenase-2; **BCL-X_L**, B cell lymphoma factor X large.

Chemical compounds studied in this article: Dexamethasone (PubChem CID: 5743); Paclitaxel (PubChem CID: 36314).

1. Introduction

GCs are essential steroid hormones that affect multiple organ systems throughout different pleiotropic functions such as the depression of the immune system, the repression of inflammation, activation of glucose mobilization in the fasting state, cell growth, apoptosis and differentiation. Synthetic glucocorticoid analogs are widely prescribed as immune suppressant/anti-inflammatory agents, being often components of chemotherapy regimes used in the treatment of lymphatic malignancies due to their potent pro-apoptotic properties on these cells [1-2]. They are also administered as anti-emetic, anti-pyretic and anti-hypersensitivity agents before treatment of solid tumors with chemotherapeutic drug [1-4]. However, despite their high anti-inflammatory efficacy, the therapeutic use of GCs is often restrained due to their severe side effects that accompany long-term treatments. In fact, the use of GCs as components of chemotherapy regimes has been alerted by several clinical studies, which indicate that, in spite of alleviating toxic effects on healthy tissue, administration of GCs before chemotherapy induces resistance to treatment, probably due to their ability to trigger anti-apoptotic signals on epithelial cells [1].

GCs exert their multiple effects mainly through their interaction with the GR, a hormone-activated transcription factor essential for life [5-6]. The GR contains three distinct functional domains: the transactivation domain AF-1, the DNA-binding domain and the ligand binding domain [7-8]. In the absence of hormone, GR primarily localizes in the cytoplasm bound to a protein complex that includes heat shock protein (HSP) 70, HSP90 and immunophilins, while upon ligand binding the activated GR is mainly nuclear [9]. Once in the nucleus, GR regulates gene expression directly by binding to specific DNA sequences or by the interaction and modulation of other transcription factors [10]. These two main mechanisms of action were historically named GR transactivation and GR transrepression, respectively [11]. Interestingly, a leading hypothesis proposes that those beneficial effects of GCs would be associated with indirect transrepression mechanisms, whereas rescue from chemotherapy dependent cell death would be associated with direct transactivation [12]. Thus, the main goal in drug research of GRs was to chemically alter the

ligand properties in order to selectively modulate receptor activities. In the case of GCs, recent advances in the study of the molecular mechanisms of GR action, together with simulation of the ligand interaction with the tertiary structure of the GR ligand binding domain by molecular dynamics, have promoted increasing interest in the development of dissociated GR modulators that can alter specifically certain GR activities in order to exclusively favor the beneficial effects of GCs [13].

In previous work, we reported the synthesis of new pregnane-derivatives and the screening of their ability to regulate different glucocorticoid parameters as apoptosis in thymocytes, tyrosine-aminotransferase activity in hepatocytes and MMTV- and κ B-reporter induction. One of these compounds, 21OH-6,19OP, is a synthetic conformationally rigid highly bent pregnane steroid that lacks the bulky substituent at C-11 found in active antagonists of the GR [14]. 21OH-6,19OP behaves as a selective GC since it efficiently displaces [3 H]corticosterone from thymus-GR but neither competes with [3 H]aldosterone binding to kidney-mineralocorticoid receptor nor with [3 H]progesterone binding to uterus-progesterone receptors [14]. Upon GR binding, 21OH-6,19OP activates translocation of the receptor to the nucleus and its dimerization [15]. This rigid steroid has the potential function of a dissociated GC since it inhibits Rel A and activator protein 1 (AP-1) dependent gene expression in BHK and Cos-1 cells [15-16] but it is unable to induce tyrosine aminotransferase or to increase glycogen deposits in rat liver [14] and MMTV-LUC reporter gene expression in L929 fibroblasts [17] and in Cos-1 cells transfected with the GR receptor [16]. Moreover, when 21OH-6,19OP was co-incubated with corticosterone or DEX, it antagonized the transactivation pathway [14,16]. 21OH-6,19OP also behaves as a strong anti-glucocorticoid, blocking DEX-dependent apoptosis in thymocytes [17].

Taking these results into account, the aim of the present work was to evaluate the anti-inflammatory properties of 21OH-6,19OP, as well as its ability to promote cell-survival in mammary tumor cells. According to the results, 21OH-6,19OP arises as a dissociated glucocorticoid with potent anti-inflammatory effects in macrophages and epithelial lung cancer

cells. Moreover, at variance with DEX, the rigid steroid did not generate chemoresistance, since it was unable to prevent apoptosis and the antitumoral effect induced with the chemotherapeutic agent paclitaxel (PTX) in mammary tumor cancer cells.

Accepted Manuscript

2. Materials and Methods

2.1. Reagents and hormones: TNF- α was purchased from Thermo Scientific (Rockford, USA). Lipopolysaccharide (LPS), interferon-gamma (INF- γ), DEX, dimethyl sulfoxide (DMSO), PTX, NaNO₂ and Griess reagents were purchased from Sigma (St. Louis, USA). Minimum Essential Medium (MEM), RPMI 1640 media, penicillin-streptomycin, Trizol reagent, deoxynucleotide triphosphates, random primers, MgCl₂ and Taq polymerase were from Invitrogen (Carlsbad, CA, USA) and fetal bovine serum (FBS) from Internegocios S.A. (Buenos Aires, Argentina). Specific oligonucleotides were from Genbiotech (Buenos Aires, Argentina). Sybr Green from Roche (Indianapolis, IN, USA). Acrylamide/bisacrylamide, Moloney murine leukemia virus reverse transcriptase (MMLV), RNasin, EDTA, EGTA, HEPES, β -mercaptoethanol, dithiothreitol (DTT), Triton X-100, SDS, digitonin, β -glycerophosphate and Tris were from Promega (Madison, WI, USA). Caspases substrate acetyl-Asp-Glu-Val-Asp-7-amino-4 p-nitroanilide (Ac-DEVDpNA) and proteases inhibitors were from Calbiochem (San Diego, CA, USA). Charcoal-stripped FCS was prepared with charcoal-dextran as described elsewhere [18]. All solvents and formaldehyde used in this work were from Cicarelli (San Lorenzo, Argentina); inorganic salts were purchased from Biopack (Pcia. de Buenos Aires, Argentina). 21OH-6,19OP was prepared as previously described [14,19]. For *in vitro* experiments, LPS and DEX were dissolved in sterile water and ethanol, respectively, and 21OH-6,19OP and paclitaxel, in DMSO; compounds were prepared as 1000X stock solution and store at -20°C.

2.2. Cell cultures: Both the human lung cancer cell line A549 (American Type Culture Collection, Virginia, USA) and murine mammary tumor cell line LM3 [20] were cultured in MEM supplemented with 5% FBS containing 100 IU/ml penicillin and 100 μ g/ml streptomycin. Raw 264.7 murine macrophage cell line (American Type Culture Collection, Virginia, USA) was cultured in RPMI 1640 supplemented with 10 % FBS, 100 IU/ml penicillin and 100 μ g/ml streptomycin. Cells were maintained at 37 °C in a humidified atmosphere with 5% CO₂.

2.3. Preparation of peritoneal macrophages: Peritoneal macrophages from female BALB/c mice were suspended in Hank's balanced salt solution (1 g/l glucose; 8 g/l NaCl; 0,4 g/l KCl; 0,06 g/l H₂KPO₄; 0,053 g/l HNa₂PO₄; 0,35 g/l NaHCO₃; pH 7,2); after washing twice with ice cold sterile PBS, cells were plated in 24 wells plates in RPMI 1640 medium containing 10% FBS, 100 IU/ml penicillin/streptomycin and incubated for 2 h at 37°C in 5% CO₂ humidified incubator. Then, non-adherent cells were removed by gently washing with PBS and freshly prepared medium was added.

2.4. Animals and treatments. Female 12 week-old BALB/c mice were purchased from the School of Veterinary Sciences from the University of Buenos Aires. Mice were maintained under specific pathogen-free conditions, and all experiments were performed in accordance with the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee from the University of Buenos Aires. Mammary tumors were developed by subcutaneous implantation (s.c) of LM3 cells (4×10^5) into the right flanks of the mice. At day 7 post-inoculation, when tumors were palpable, mice were randomly divided into 4 groups (8 mice per group) and treated with different regimes on day 0: a) 10 mg/kg PTX (i.p); b) 0.2 mg/kg DEX in combination with 15 mg/kg PTX, i.p; c) 25 mg/kg 21OH-6,19OP in combination with 10 mg/kg PTX, i.p; d) Vehicle (VEH) was also prepared as control treatment. The concentration of each compound was decided according to previous reports [21-22]. Steroids were administered 1 h before PTX injection. PTX was diluted in MEM with 15 % DMSO while steroids were diluted in etanol:propilenglicol:sesame oil (1:1:11.5). These regimes were repeated every 2 days for a total of 5 cycles. Each animal was ear-marked and followed individually throughout the experiments. Two perpendicular lengths (width, w , and length, l) of the tumors were measured every 3 days until the animals were sacrificed. Tumor diameters (D) were calculated using the following formula: $D = \sqrt{w \cdot l}$. The curve of tumor growth was drawn based on tumor diameter (mm) versus time of treatment (days). Tumors were fixed in 10 % formalin and embedded in paraffin. Five μ m tumor sections were stained with hematoxilyn and eosin for histopathological

studies and mitosis counting. The presence of spontaneous metastasis was assessed by histological examination of lung sections.

For the analysis of *Cox-2 in vivo* expression, mice were injected i.p. with 5 mg/kg LPS (diluted in PBS), 2 mg/kg DEX, 25 mg/kg 21OH-6,19OP (both steroids prepared same as before) or vehicles. After 16 h, peritoneal macrophages were harvested as described above and then plated (5×10^5 cells) in 24 well dishes in RPMI 1640 supplemented with 25 mM Hepes and 10 % charcoal-stripped FBS. Two hours later cells were washed 3 times with PBS and incubated for another 2 h; finally total RNA was prepared.

For histological analysis, tumors were fixed in 10% neutral buffered formaldehyde, embedded in paraffin, sectioned at 7 μm and routinely stained with hematoxylin-eosin (H&E). Mitotic figures from each tumor section were counted and the presence of parenchymal metastatic nodules in lung sections was determined using a conventional light microscope.

2.5. Plasmids and transfections. Plasmids encoding p65 activated subunit of nuclear factor- κ B (NF- κ B) [23] and cJun activated subunit of AP-1 (kindly provided by Dr. Coso [24]) were used for transient transfection assays. A549 cells were transiently transfected with Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Briefly, 3×10^5 cells were plated in 6 well plates in growth medium without antibiotics. Twenty-four hours later cells were incubated in the presence of 4 μg plasmids mixed with Lipofectamine for 24 h. Finally, cells were starved overnight and stimulated for 16 h with steroids or vehicle.

2.6. Caspase-3-like activity assay: The assay was performed as previously described [25]. Briefly, 3.5×10^5 LM3 cells were plated in p60 plates; after 48 h cells were treated for 36 h with 10 μM PTX, 1 μM DEX or 10 μM 21OH-6,19OP in MEM supplemented with 1 % charcoal-stripped FBS and antibiotics. Cells were harvested in lysis buffer (50 mM Tris-HCl pH 7.4 containing 1 mM EDTA, 10 mM EGTA, 10 μM digitonin, 0.5 mM PMSF, 1.5 μM aprotinin and 14.6 μM pepstatin) for 30 min at 37 °C. Cellular extracts were clarified by centrifugation at maximum speed and 150 μl of the

resultant supernatant were incubated with 146 μ l of incubation buffer (100 mM HEPES pH 7.5, 20% glycerol, 0.5 mM EDTA and 5 mM dithiothreitol (DTT)) plus 4 μ l of Ac-DEVDpNA substrate (100 μ M) at 37 °C for 5 h. Blanks were also run, containing either the substrate or the cellular extract alone, to deduct in each case. Caspase-catalyzed release of the chromophore pNA from the substrate was measured at 405 nm with a multiplate spectrophotometer (Benchmark, Bio-Rad, Hercules, CA, USA) and the cleavage activity was expressed as pNA absorbance units per mg protein. Protein concentration was determined by the method of Bradford [26] using bovine serum albumin as a standard.

2.7. Cell viability by crystal violet staining. The number of viable cells attached to the culture plate at the end of each treatment was evaluated by crystal violet staining. Briefly, LM3 cells (1×10^4) were plated in 96-multiwell plates and allowed to grow for 48 h. Then, apoptosis was induced by treating cells with 10 μ M PTX for 48 h in MEM supplemented with 1 % charcoal-stripped FBS and antibiotics. After this, cells were fixed with 100 μ l ice-cold glutaraldehyde (1.1% in PBS) for 15 min at 4 °C, washed three times by submersion in de-ionized water, air-dried, and stained for 20 min with 100 μ l of crystal violet 0.1% in 200 mM phosphoric acid, pH=6. After careful aspiration of the crystal violet solution, extensive washing with de-ionized water eliminated the excess of dye. Then, samples were air-dried and treated with 100 μ l of acetic acid 10% for 30 min at room temperature. Optical density was measured at 595 nm with a multiplate spectrophotometer (Benchmark, Bio-Rad, Hercules, CA, USA).

2.8. Protein analysis by western blot. For inflammation experiments, A549 cells seeded in 6 well plates were incubated for 2 h with 10 ng/ml TNF- α with or without 0.1 μ M DEX or 10 μ M 21OH-6,19OP. For chemoresistance experiments, LM3 cells were seeded in 6 well plates and incubated for 5 h with 10 μ M PTX in the presence or absence of 0.1 μ M DEX or 10 μ M 21OH-6,19OP. Then, total proteins were extracted in RIPA protein extraction buffer (50 mM Tris-HCl, 150 mM NaCl, 1% triton, 0.25% sodium deoxycholate, 1 mM EDTA pH 7.4) supplemented with protease (Protease

inhibitor cocktail set I, Calbiochem) and phosphatase inhibitors (1mM NaF, β -glycerophosphate and 1 mM Na₂VO₄). The lysates were centrifuged at 12500 \times g and 4 °C for 15 min, and the pellet discarded. Cleared lysates were combined with SDS sample buffer (50 mM Tris pH 6.8, 1% SDS, 0.1% bromophenol blue, 10% glycerol, 100 mM DTT). For western blot samples were boiled for 5 min and electrophoresed for 3 h at 100 V in 15% SDS-polyacrylamide gel, transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA) by electroblotting in transfer buffer containing 20% methanol, 0.19 M glycine, 0.025 M Tris-base (pH 8.3) at 300 mA for 1.5 h at 0 °C. Blots were blocked 1 h at room temperature in TBS (20 mM Tris-HCl, pH 7.5, 500 mM NaCl) containing low-fat powdered milk (5%) and Tween 20 (0.1%). The incubations with primary antibodies were performed at 4 °C for 12 h in blocking buffer (3% skim milk and 0.1% Tween in Tris-buffered saline). The membranes were then incubated with the corresponding counter-antibody and the proteins evidenced by enhanced chemiluminescence detection (ECL Plus System, GE Health Care, Little Chalfont, Buckinghamshire, UK). As primary antibodies the following were used: anti-BCL-X_L/s (sc-634); anti-BCL-2 (sc-492), anti-Actin (sc-1616-R), anti-p-p38 (sc-7973), anti-MKP-1 (sc-1102) and anti-p38 (sc-535-G), from Santa Cruz Biotechnology (Santa Cruz, CA, USA). As secondary antibody a peroxidase labeled anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used. Densitometric analysis of protein levels was performed with ImageJ 1.34s software (Wayne Rasband, National Institutes of Health, USA. <http://rsb.info.nih.gov/ij/>).

2.9. RNA analysis by RT-qPCR. RAW 264.7, LM3 and A549 cells were incubated for 24 h with steroids and 10 μ g/ml LPS plus 100 U/ml IFN- γ or 10 ng/ml TNF- α , respectively. LM3 cells were treated with different stimuli for 4 h. Total RNA was extracted with Trizol reagent according to the manufacturer's instructions. For reverse transcription, 1 μ g of total RNA was used. The first cDNA strand was synthesized with 200 U MMLV reverse transcriptase; 25 ng/l random primers; 2 g/l RNasin and 1.5 mM deoxynucleotide triphosphates. Retrotranscription was performed at 37 °C for 60 min followed by 15 min at 72 °C. For the quantitative real-time RT-PCR (qRT-PCR), an aliquot

of 0.5 μ l cDNA was used. All reactions were conducted in a volume of 25 μ l containing 2 - 4 mM $MgCl_2$, deoxynucleotide triphosphates 0.2 mM, Taq polymerase 0.75 U, Sybr Green 1:30, and specific oligonucleotides for each gene (as shown below) in a DNA Engine Opticon instrument (MJ Research, Waltham, MA). The oligonucleotides used for each gene were as follows: *Bcl-X_L*, 5'-AACGGCGGCTGGGAC-3' and 5'-TGAAGAGTGAGCCCAGCAGAAC-3'; *Bcl-2*, 5'-AGCAGGAGAGGAGGAACAG-3' and 5'-GTAGATGGCGAACAGGAAGG-3'; *COX-2*, 5'-TTCAAATGAGATTGTGGGAAAATTGCT-3' and 5'-AGATCATCTCTGCCTGAGTATCTT-3'; *IL-8*, 5'-CTGCGCCAACACAGAAATTA-3' and 5'-ATTGCATCTGGCAACCCTAC-3'; *Nos-2*, 5'-TCAGACATGGCTTGCCCTGGA-3' and 5'-TGCCCCAGTTTTTGATCCTCACA-3'; human *GAPDH*, 5'-GAGTCAACGGATTTGGTCGT-3' and 5'-TTGATTTTGGAGGGATCTCG-3'; and mouse *Actin*, 5'-CCACACCCGCCACCAGTTC-3' and 5'-GACCCATTCCCACCATCACACC-3'. Reactions were run for 40 cycles under the following conditions: 25 s at 94 °C, 25 s at 58–65 °C (according to the primers), and 25 s at 72 °C. The amplification of unique products in each reaction was verified by melting curve and ethidium bromide (Sigma Aldrich)-stained agarose gel electrophoresis. The expression level of each gene was normalized to Actin or GAPDH expression level using standard curve method and specific primers. Results are expressed as mean \pm standard deviation (SD) from at least three experiments, and shown as fold changes respect to basal values.

2.10. Nitrite production. Nitric oxide (NO) levels were evaluated through the release of nitrite (NO_2^-) in the supernatants of peritoneal macrophages and RAW 264.7 cells cultured with 10 μ g/ml LPS plus 100 U/ml IFN- γ and 0.1 μ M DEX or 10 μ M 21OH-6,19OP for 24 h by the Griess reaction, as previously described [27]. Briefly, 100 μ l of supernatants were incubated with an equal volume of the Griess reagent (NEED – N-ethyl-N-diamine 0.1% and sulfanilamide 1% in H_3PO_4 5%) at room temperature, and the absorbance was evaluated at 540 nm using a microtiter plate spectrophotometer. The NO_2^- concentration was determined using a standard curve spanning from 1 to 100 μ M NO_2^- .

2.11. Statistical analysis. Results were expressed as means \pm SE, as it is indicated in the figure legends. ANOVA followed by Tukey's multiple comparisons tests were used to detect significant differences among treatments. Statistical analyses were performed with Infostat (J.A. Di Rienzo, F. Casanoves, M.G. Balzarini, L. Gonzalez, M. Tablada, C.W. Robledo. InfoStat versión 2011. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina. URL <http://www.infostat.com.ar>). Differences were regarded as significant if $p < 0.05$. Before, statistical analysis data were tested for normality and homoscedasticity using Shapiro-Wilks and Levene's tests, respectively.

3. Results

3.1. 21OH-6,19OP as a potential anti-inflammatory drug

To analyze the putative role of 21OH-6,19OP as an anti-inflammatory drug, we first evaluated the effects of 21OH-6,19OP on Nitric oxide (NO) production. NO acts as a messenger molecule mediating diverse inflammatory pathways [28]. In our system, NO was assessed by determining NO_2^- levels in supernatants of LPS/IFN- γ -activated peritoneal macrophages. Results from Figure 1 show that 21OH-6,19OP (10^{-5}M) inhibited LPS-induced nitrite production to basal levels, similarly to DEX (10^{-7}M) (FIG. 1A). Although, a similar concentration-response relationship was obtained with the murine macrophage cell line RAW 264.7 treated with LPS/IFN- γ together with increasing amounts of 21OH-6,19OP, this compound seems to be less potent in these cells than in activated macrophages. In fact, 21OH-6,19OP inhibited nitrite formation in a concentration-dependent manner with an estimated IC_{50} of 55 μM in RAW264.7 cells (FIG. 1B). It is well known that NO is synthesized from L-arginine by three Nos isoforms. Nos-2 is one of the enzyme isoform that is mainly expressed in response to pro-inflammatory mediators. Thus, we evaluated whether inhibition of NO production by 21OH-6,19OP is a consequence on the regulation of NOS-2 gene expression. RAW 264.7 cells were incubated with LPS in the presence of 21OH-6,19OP (10^{-5}M) or DEX (10^{-7}M). After 24 h, cells were harvested and total RNA was prepared. The expression of *NOS-2* was assessed by RT-qPCR using specific primers. LPS increased *NOS-2* expression 33 fold above basal levels (FIG 2 A) and these were partially but significantly inhibited by 21OH-6,19OP. Thus, the ability of 21OH-6,19OP to inhibit NO could involve the down regulation of *NOS-2* gene.

Other inflammation markers usually evaluated in different cell lines, are *COX-2*, that catalyses the synthesis of prostaglandins [29-30] and IL-8, a cytokine involved in neutrophil chemo-attraction and activation [31]. Both *COX-2* and *IL-8* gene expression are regulated by NF- κB [32-33] and AP-1 [34-35] transcription factors. To test whether 21OH-6,19OP modulates their expression, A549 lung cancer epithelial cells were treated with TNF- α in the absence or presence of 21OH-6,19OP

(10^{-5} M) or DEX (10^{-7} M) and total RNA was prepared and quantified by RT-qPCR. Results show that 21OH-6,19OP, similarly to DEX, completely blocks both *COX-2* (FIG 2B) and *IL-8* (FIG 2C) gene expression, supporting the anti-inflammatory effect of this compound.

The regulation of *COX-2* expression was also assessed *in vivo*. Inflammation was provoked by injecting LPS (5 mg/kg, i.p.) into female BALB/c in the absence or presence of DEX (2 mg/kg) or 21OH-6,19OP (25 mg/kg). After 16 h, animals were sacrificed and peritoneal macrophages were obtained. Then, total RNA was prepared and quantified by RT-qPCR. Similar to DEX, the steroid completely inhibited *COX-2* expression induced by LPS, confirming the *in vivo* anti-inflammatory effect of this steroid (FIG. 2D).

3.2. 21OH-6,19OP inhibits Cox-2 expression throughout p38 mitogen activated protein kinase inactivation

It is well known that GCs exert their anti-inflammatory effects mainly through the inhibition of NF- κ B and AP-1 signaling pathways. In this sense, pRelA (the active subunit of NF- κ B) and pc-Jun (a component of AP-1 factor) were transiently transfected in A549 cells treated or not with DEX or 21OH-6,19OP. After transfections, *COX-2* expression levels were assayed by RT-qPCR. As expected, DEX inhibited *COX-2* expression induced by both RelA (FIG. 3A) or pcJun (FIG 3B) while the rigid steroid was ineffective, suggesting that in this cellular system 21OH-6,19-OP decreases *COX-2* expression triggered by TNF- α by a different mechanism than that involving NF- κ B or AP-1 activities.

Taking into account that GCs also exert their anti-inflammatory effects through the regulation of p38 MAPK activity, we analyzed the effect of 21OH-6,19OP on p38 activation. Thus, western blots were performed in A549 cells treated with the cytokine TNF- α in the absence or presence of the rigid compound or DEX. As expected, phospho-p38 levels increased, roughly 2.5 fold, upon TNF- α addition (FIG 4A, lane 2). This increment was inhibited by both DEX and 21OH-6,19OP (Figure

4A, lane 5 and 6, respectively). Cells were then co-incubated with TNF- α without or with the steroids in the presence of Na₂VO₄ (NaOV), a dual phosphatase inhibitor. Results show that phospho-p38 levels were recovered when NaOV was added (Figure 4A, lanes 7 and 8), supporting the idea that in these cells, GCs would induce a p38 phosphatase. To further examine the involvement of dual phosphatases on 21OH-6,19OP effects, we quantified *COX-2* expression levels from A549 cells treated with TNF- α and the rigid steroid in the presence of NaOV. Interestingly, inhibition of dual phosphatases completely blocked 21OH-6,19OP repression of TNF- α -dependent *COX-2* expression (FIG 4B, compare lane 4 with lane 3). The inhibition of p38 activity with SB203580 (SB) (FIG 4B, lane 5) or its activation with anisomicin (ANISO) (FIG 4B, lane 6), reduced and increased *Cox-2* expression, respectively. Together these results support the hypothesis that the rigid steroid would decrease *Cox-2* expression mainly by reducing p38 phosphorylation.

Taking into account that the expression of the specific p38 phosphatase MKP-1 is regulated by GCs [35-39], we then evaluated MKP-1 expression by western blot assays. MKP-1 protein levels increased upon the addition of both 21OH-6,19OP or DEX to cells treated with TNF- α (FIG 4C), indicating that the rigid steroid would down-regulate *COX-2* expression by inactivating p38 through the increase of MKP-1 levels. Together these results support the role of 21OH-6,19OP as a new anti-inflammatory compound.

3.3. 21OH-6,19OP does not induce chemoresistance to paclitaxel in mammary tumor cells

As mentioned above, synthetic GCs as DEX are often used as coadjuvants in the chemotherapy of solid tumors, such as mammary tumors. However, their use was also associated to chemoresistance induction, probably due to the anti-apoptotic effects of GCs in epithelial cells. In order to test whether the new steroid provokes resistance effects as DEX, murine mammary tumor cells LM3 were treated with 10 μ M PTX in the presence of 1 μ M DEX or 10 μ M 21OH-6,19OP during 36 h. The concentration of PTX was used according to MTT assays whose results showed that 10 μ M

PTX inhibits at least 50 % cell viability (data not shown). After incubation, Caspase-3 activity was determined as a parameter of apoptosis. Figure 5A shows that while DEX significantly inhibited Caspase-3 activity provoked by the chemotherapeutic drug (Figure 5 A, lane 3 vs lane 2), 21OH-6,19OP was unable to modify this protease activity (Figure 5 A, lane 4 vs lane 2). These results correlate with those obtained from crystal violet cell viability assays where 68% of the cells died in the presence of the antineoplastic drug but diminished to 58% in the presence of DEX, while in cells treated with PTX plus 21OH-6,19OP the percentage of death cells was 77% (Figure 5 B).

In mammary epithelial cells, Caspase-3 activity is mainly controlled by the activity of the anti-apoptotic protein BCL-X_L [40]. In this sense, RT-qPCR and western blot assays were performed in order to analyze the expression levels of BCL-X_L in LM3 cells treated with PTX in the presence of DEX or 21OH-6,19-OP. Results shown in Figures 6 A and B indicate that DEX increases *Bcl-X_L* transcript levels (2.8 fold vs. Control) and BCL-X_L protein (1.7 fold vs Control), independently of the presence of PTX. However, 21OH-6,19OP was unable to induce BCL-X_L expression either alone or in the presence of PTX. Expression of the anti-apoptotic member BCL-2 was neither modified by the chemotherapeutic agent nor by the GCs (Figure 6 C and D). Together these results suggest that the rigid steroid, contrary to DEX, does not inhibit the induction of apoptosis by PTX in mammary tumor epithelial cells.

In order to investigate whether these results had an *in vivo* correlate, mammary tumor growth assays were performed in syngeneic BALB/c female mice. For this, animals were inoculated *s.c* with 4×10^5 LM3 cells to induce tumor development and after 7 days, when tumors were palpable, mice were injected *i.p.* with PTX without or with DEX or 21OH-6,19OP. As shown in Figure 7 PTX significantly reduced LM3 tumor growth as compared with tumors in control animals treated with the vehicle alone. While the combined treatment with DEX prevented PTX-induced tumor growth inhibition, the rigid steroid did not affect PTX *in vivo* antitumoral activity (Fig. 7A). Statistical analyses of tumor size performed at days 7, 14 and 30 after cell inoculation support the idea that 21OH-6,19-OP is not able to induce *in vivo* chemoresistance in mammary cells (Fig. 7B).

Microscopic examination did not reveal any effect of treatments on the histopathological features of LM3 tumors, which maintained their adenocarcinoma poorly differentiated phenotype. However, we found that PTX induced a significant marked reduction in the number of mitotic figures (Fig. 7C) as well as extensive areas of apoptotic and necrotic cells (data not shown). Interestingly, while DEX completely reverted PTX effect, 21OH-6,19OP did not affect it (Fig. 7C).

To evaluate whether these combined treatments could impair the potential antimetastatic activity of PTX, we analyzed the lung sections for the presence of parenchymal metastatic nodules. We found that PTX induced a significant reduction in metastasis incidence, from 75% in control mice to 40 % in the treated ones. In agreement with their effects on *s.c.* tumor growth, 80 % of DEX-PTX treated mice developed metastases while only 20% of those treated with 21OH-6,19OP-PTX did so.

4. Discussion

GCs activities are a consequence of specific and independent glucocorticoid actions at various sites. Although the complex mechanisms by which GR regulates transcription is not completely understood, it is well known that these mechanisms involve the interaction of GR with proteins that make up the basal transcription apparatus.

In the present work we describe several anti-inflammatory actions of the synthetic steroid 21OH-6,19OP. This compound, similar to the full GR agonist DEX and other dissociated GCs [38,41-44] has been demonstrated to inhibit the LPS/IFN- γ mediated NO formation in a concentration-dependent manner and to repress *NOS-2* and *COX-2* gene expression in macrophages. 21OH-6,19OP also inhibits TNF- α -induced *COX-2* and *IL-8* expression in human lung cancer A549 cells. In fact, our results are similar to those reported with Mapracorat, a non-steroidal GR selective ligand that inhibits *COX-2* expression and p38 activation in RAW 264.7 cells [37] and IL-6, IL-8 and G-CSF secretion in different types of ocular cells [41]. 21OH-6,19OP also behaves similar to RU24858, a dissociated steroid analog that inhibits nitrite formation induced by LPS in macrophages [43] and *COX-2* expression as well as activity and IL-8 secretion induced by IL-1 β in A549 cells [42]. Like DEX and RU24958, 21OH-6,19OP also partially reduces *NOS-2* transcript levels [43]. Taking into account that these steroids presented strong inhibitory effects on NO formation, the apparent dissimilar result observed when comparing with glucocorticoid dependent *NOS-2* decrease may be explained by glucocorticoid mediated post-transcriptional events that lead to *Nos-2* protein degradation [45-46].

The anti-inflammatory effects carried out by GCs involve several simultaneous pathways triggered by the activated GR. These include both mechanisms, the GR-mediated tethering of transcription factors and the transactivation of anti-inflammatory proteins. According to our results, the pathways triggered upon DEX or 21OH-6,19OP binding to GR, seem to be slightly dissimilar. While *COX-2* and *IL-8* expression levels are controlled by the DEX-activated GR at different sites,

i.e. by direct tethering of NF- κ B and AP-1 transcription factors [35,47], by down-regulating the p38 activity through the induction of MKP-1 phosphatase [48] and by inducing the expression of factors involved in controlling mRNA stability [49], 21-OH-6,19OP was unable to repress pRelA- and pcJun-dependent *COX-2* induction but it did increase MKP-1 protein levels and consequently led to a decrease in *COX-2* levels by p38 inactivation in A549 cells. This suggests a certain tissue selectivity of this compound towards the up-regulation of the MKP-1 mediated pathway over the direct transrepression mechanism, in accordance with the fact that the rigid steroid was shown to inhibit NF- κ B and AP-1 activities in other cell types [15-16].

Taking into account that most of the anti-inflammatory and immune-suppressive actions of dissociated GCs involve the negative regulation of gene expression throughout the direct GR-mediated tethering of transcription factors, being the most-studied crosstalk mechanisms of those between GR and NF- κ B and AP-1 [29,41,50-53], this selective behavior of the GR/21OH-6,19OP complex observed in lung cancer epithelial cells makes this compound a potential therapeutic drug. Besides, it is interesting to note that 21OH-6,19OP incapacity to trigger the GR-mediated transrepression mechanism might be also inherent to the incapacity of the GR/21OH-6,19OP complex to recruit some transcription co-modulators. In fact, upon binding of 21OH-6,19OP, the GR has been shown to be unable to recruit TIF2, a co-activator involved in GR mediated direct transactivation of certain genes and transrepression of NF- κ B [47].

Whereas MKP-1 is one of the few GC-inducible genes mediating some of the anti-inflammatory properties of GR, many other genes positively regulated by GR would also play a putative role in mediating 21OH-6,19OP actions [54-57]. In this sense, further studies are necessary to evaluate the effects of 21OH-6,19OP on the induction of other anti-inflammatory factors. Nevertheless, identification of those genes and their functionality is still far from being fully understood, indicating the complexity of the anti-inflammatory roles of the GR.

How GCs regulate MKP-1 expression is still a matter of discussion. In fact, Johansson-Haque et al. have suggested that the GR would activate MKP-1 promoter by its indirect recruitment to C/EBP β response elements, since DEX addition to a GR variant unable to directly transactivate gene expression, was found to maintain its ability to induce MKP-1 through the C/EBP β elements present in the promoter [58]. On the other hand, ChIP experiments have also been demonstrated to recruit GR to specific GREs located in the promoter region of MKP-1 [39]. Therefore, at least two mechanisms could be involved simultaneously in the induction of MKP-1 by GCs. Moreover, although studies performed using the knock-in of a GRdim mutant mouse suggested that dimerization of GR is necessary for MKP-1 induction [59], it was also demonstrated that the GRdim/DEX complexes provoked an increase in MKP-1 levels in macrophages [48]. Nevertheless, the ability of the GRdim/DEX mutant to dimerize is actually under revision. Taking into account that the GR/21OH-6,19OP was able to dimerize but unable to induce the expression of genes driven by GREs [15], it seems possible that the increased levels of MKP-1 could be a consequence of the indirect transactivation pathway triggered by the rigid compound.

As we mentioned above, like others dissociated glucocorticoids 21OH-6,19OP is unable to induce the direct GR/GRE action [44]. In this sense, it was demonstrated that VBP1, a Δ -9,11 analog, was more effective than full agonists, to down-regulate anti-inflammatory genes whose promoters contain both GREs and NF- κ B promoter elements, probably due to incapacity of the GR to directly bind DNA [44]. This would not be the case for this rigid compound, since GR/21OH-5,19OP complexes bind DNA [60].

Taken together, these results support the idea that 21OH-6,19OP exerts its anti-inflammatory effects by regulating only some of the pathways involved in the GR cellular response.

In the present work we also demonstrate that 21OH-6,19OP, contrary to DEX, was unable to induce chemoresistance in the LM3 cell line treated with PTX, a murine mammary tumor model, both *in vitro* and *in vivo*. It is worth mentioning that similar results were obtained in LM3 cells

treated with the anthracycline doxorubicin. In fact, DEX (1 μM) inhibited cell death triggered by 10 μM doxorubicin, whereas 21OH-6,19OP (10 μM) did not affect it (AJ Orqueda, personal communication). The lack of effect observed in tumor mammary cells treated with this compound makes it a good candidate to be used in anti-neoplastic regimes, although before leaping to human application, further experiments with other chemotherapy agents and other cancer cell types should be performed. The inability of 21OH-6,19OP to prevent cell death correlates with its failure to prevent PTX-dependent caspase-3 activation and to induce the expression of the anti-apoptotic BCL-X_L isoform. Glucocorticoid-dependent up-regulation of BCL-X_L was also observed in the murine mammary epithelial cells HC11 by a mechanism that involves the recruitment of the activated GR to specific GREs located at the promoter region of the gene [22,61-62].

The anti-apoptotic effect of GCs in the mammary gland has been associated with the inhibition of Caspases-3, -7 and -8 activities and the decrease of AP-1 dependent transcription [63-64]. Moreover, in chemotherapy-treated breast cancer cells, GCs also prevent cell death by a mechanism that involves SGK-1 and MPK-1 kinases [65]. Interestingly, in these cells GCs induce cell-cycle arrest and increase expression of the cell-cycle inhibitors p21CIP1 and p27KIP1 [66-68]. Considering the DNA damaging action of chemotherapeutic drugs, death resistance produced by GCs may be an epiphenomenon of cell-cycle inhibition, since arrested cells are not chemotherapeutic targets. Therefore, glucocorticoid-induced chemotherapy resistance might occur together with tumor-suppressor and anti-apoptotic gene induction.

In summary, the above results suggest that 21OH-6,19OP conserves the so-called beneficial glucocorticoid properties by regulating only some of the pathways triggered by the GR. It is now well established that the ability of ligands to regulate gene expression is influenced by both the identity and relative expression of receptor co-activators and co-repressors [69]. In fact, increasing evidence suggests that the effects of GCs depend significantly on the co-regulators interacting with the receptor, rather than on the state of GR oligomerization or GR ability to bind DNA [60]. The design of new GCs able to provoke the recruitment of specific co-activator subsets by determined

GR conformations acquired upon ligand binding is thus highly desirable. 21OH-6,19OP binding to GR induces subtle conformational changes in the receptor [16]. These compounds would modulate the recruitment of certain co-factors, which consequently would lead to the activation of only a subset of GR functions. In this sense, 21OH-6,19OP arises as a dissociated GC with potential therapeutic effects.

Figure Legends

Figure 1. *21OH-6,19OP exerts GC anti-inflammatory effects.* **A.** Peritoneal macrophages were stimulated with 10 µg/ml LPS and 100 U/ml IFN-γ, and 10 µM 21OH-6,19OP or 0.1 µM DEX as control. After 24 h nitrite production was quantified by the Griess reaction. Values were expressed as µM concentration ± SE from three independent experiments. **P* < 0.05. **B.** RAW 264.7 macrophage tumor cells were incubated with 10 µg/ml LPS and 100 U/ml IFN-γ, and 1 - 100 µM 21OH-6,19OP. After 24 h nitrites production was quantified by the Griess reaction. Values are expressed as fold change relative to the LPS + IFN-γ treatment.

Figure 2. *21OH-6,19OP, similarly to DEX, inhibits TNF-α induced expression of NOS-2 (A), COX-2 (B and D) and IL-8 (C).* **A.** RAW 264.7 cells were incubated with 10 µg/ml LPS and 100 U/ml IFN-γ, and 10 µM 21OH-6,19OP or 0.1 µM DEX for 24 h. Then, total RNA was extracted and the levels of *NOS-2* mRNA were quantified by RT-qPCR. Mean ± SE values of *NOS-2* relative to *actin* levels are shown. **B** and **C.** A549 lung cancer cells were incubated with 10 ng/ml TNF-α and 10 µM 21OH-6,19OP or 0.1 µM DEX. After 24 h *COX-2* (**B**) and *IL-8* (**C**) mRNA levels were measured by RT-qPCR. Mean ± SE values of *COX-2* or *IL-8* relative to *GAPDH* levels from three independent experiments are shown. **D.** Adult female BALB/c mice were injected intraperitoneally with 5 mg/kg LPS without or with 2 mg/kg DEX or 25 mg/kg 21OH-6,19OP. Sixteen hours later, peritoneal macrophages were collected and *COX-2* mRNA levels were quantified by RT-qPCR. Mean ± SE values of *Cox-2* relative to *actin* levels are shown, n = 6. **P* < 0.05.

Figure 3. *21OH-6,19OP inhibitory effects are independent of Rel A and AP-1 pathways.* A549 cells transfected with pRelA (**A**) or pcJun (**B**) expression vectors were incubated with 0.1 µM DEX or 10 µM 21OH-6,19OP. After 16 h total RNA was collected and *COX-2* mRNA levels were determined

by RT-qPCR. Mean \pm SE values of *COX-2* mRNA levels relative to *GAPDH* from three independent experiments are shown. * $P < 0.05$.

Figure 4. *MKP-1-dependent effects of 21OH-6,19OP on COX-2 expression.* 21OH-6,19OP modulates MKP-1 activity. **A.** A549 cells were incubated with 10 ng/ml TNF- α and 10 μ M 21OH-6,19OP or 0.1 μ M DEX for 2 h. Then, activated p38 protein levels were determined by western blot assay. A representative image (upper panel) and the mean values of phospho-p38 relative to p38 protein levels \pm SE from three independent experiments (lower panel) are shown. **B.** A549 cells were incubated with 10 ng/ml TNF- α , with or without 10 μ M 21OH-6,19OP and 100 μ M NaOV. Treatments with the p38 inhibitor SB203580 (SB) or the p38 activator anisomicin (ANISO) were also performed. Twenty four hours later total RNA was collected, and *COX-2* mRNA levels were determined by RT-qPCR. Mean \pm SE values of *COX-2* mRNA levels relative to *GAPDH* from three independent experiments are shown. **C.** A549 cells were incubated with 10 ng/ml TNF- α and 10 μ M 21OH-6,19OP or 0.1 μ M DEX. After 2 h MKP-1 protein levels were determined by western blot. A representative image (upper panel) and the mean values of MKP-1 protein levels relative to actin \pm SE from three independent experiments (lower panel) are shown. * $P < 0.05$.

Figure 5. *21OH-6,19OP does not inhibit apoptosis triggered by PTX in mammary tumor cells.* **A.** LM3 mammary tumor cells were treated with 10 μ M PTX and 10 μ M 21OH-6,19OP or 1 μ M DEX for 36 h. Caspase-3 like enzymatic activity was determined by quantifying the absorbance of the chromophore pNA at 415 nm; the cleavage activity was expressed as pNA absorbance units/mg protein \pm SE. **B.** Cell viability was quantified by crystal violet staining in LM3 cells incubated in the presence of 10 μ M PTX and 10 μ M 21OH-6,19OP or 1 μ M DEX for 48 h. Data are plotted as % cell viability \pm SE. n = 4, * $P < 0.05$.

Figure 6. *21OH-6,19OP does not increase BCL-X_L levels in mammary tumor cells.* LM3 cells were stimulated with 10 μ M PTX and 10 μ M 21OH-6,19OP or 1 μ M DEX. After 4h, total RNA was collected and *Bcl-X_L* and *Bcl-2* mRNA levels were determined by RT-qPCR (**A** and **C**, respectively). Mean \pm SE values of *Bcl-X_L* and *Bcl-2* mRNA levels, both relative to actin, are shown. Proteins were extracted after 5 h treatments, and BCL-X_L and BCL-2 levels were determined by Western blot assays (**B** and **D**, respectively). A representative image (upper panel) and the mean \pm SE values of BCL-X_L and BCL-2 protein levels, both relative to actin, are shown. n = 3, **P* < 0.05.

Figure 7. *21OH-6,19OP does not decrease the effectiveness of PTX on LM3 tumors.* **A.** LM3 cells (4×10^5) were injected *s.c.* into the left flank of 12 week old female BALB/c mice. Seven days later, mice were randomly divided into four treatment groups: Vehicle; PTX (10 mg/kg/day); 21OH-6,19OP (25 mg/kg/day) + PTX; and DEX (0.2 mg/kg/day) + PTX. Tumor dimensions were measured 3 times every week with calipers and two perpendicular diameters (width, *w*, and length, *l*) were determined; D parameters were calculated according to the equation: $D = \sqrt{w \cdot l}$. **B.** Details of the results obtained at days 7, 14 and 30, corresponding to those used in the ANOVA for repeated measures, are shown. Error bars represent SE. n = 3, **P* < 0.05. **C.** Mitotic figures from H&E stained sections of LM3 tumors (three fields from each sample) were counted using a conventional light microscope. Data are plotted as % mitotic cells \pm SE. n = 5, **P* < 0.05.

Acknowledgements:

We thank Dr. G. Dmytrenko, Cefybo-CONICET and Dr. A. Pozzi, IFIByNE- CONICET for their useful help in histological analysis, Dr. C. Paz, School of Medicine-University of Buenos Aires for MKP-1 antibodies, Dr. O. Coso, IFIByNE- CONICET for pREIA and pcJun vectors and Dr. Lautaro Alvarez for his help in the graphical summary.

Footnotes

This work was supported by the grants from the CONICET, ANPCyT and the University of Buenos Aires. The authors are members of the CONICET.

References

- [1] C. Zhang, B. Beckermann, G. Kallifatidis, Z. Liu, W. Rittgen, L. Edler, P. Buchler, K.M. Debatin, M.W. Buchler, H. Friess, I. Herr, Corticosteroids induce chemotherapy resistance in the majority of tumour cells from bone, brain, breast, cervix, melanoma and neuroblastoma, *Int J Oncol* 29 (2006) 1295-1301.
- [2] M. Sui, F. Chen, Z. Chen, W. Fan, Glucocorticoids interfere with therapeutic efficacy of paclitaxel against human breast and ovarian xenograft tumors, *Int J Cancer* 119 (2006) 712-717.
- [3] S. Meyer, T. Eden, H. Kalirai, Dexamethasone protects against Cisplatin-induced activation of the mitochondrial apoptotic pathway in human osteosarcoma cells, *Cancer Biol Ther* 5 (2006) 915-920.
- [4] C. Zhang, T. Wenger, J. Mattern, S. Ilea, C. Frey, P. Gutwein, P. Altevogt, W. Bodenmuller, N. Gassler, P.A. Schnabel, H. Dienemann, A. Marme, M. Hohenfellner, A. Haferkamp, J. Pfitzenmaier, H.J. Grone, A. Kolb, P. Buchler, M. Buchler, H. Friess, W. Rittgen, L. Edler, K.M. Debatin, P.H. Krammer, H.P. Rutz, I. Herr, Clinical and mechanistic aspects of glucocorticoid-induced chemotherapy resistance in the majority of solid tumors, *Cancer Biol Ther* 6 (2007) 278-287.
- [5] T.J. Cole, J.A. Blendy, A.P. Monaghan, K. Krieglstein, W. Schmid, A. Aguzzi, G. Fantuzzi, E. Hummler, K. Unsicker, G. Schutz, Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation, *Genes Dev* 9 (1995) 1608-1621.
- [6] S. Finotto, K. Krieglstein, A. Schober, F. Deimling, K. Lindner, B. Bruhl, K. Beier, J. Metz, J.E. Garcia-Ararras, J.L. Roig-Lopez, P. Monaghan, W. Schmid, T.J. Cole, C. Kellendonk, F. Tronche, G. Schutz, K. Unsicker, Analysis of mice carrying targeted mutations of the

- glucocorticoid receptor gene argues against an essential role of glucocorticoid signalling for generating adrenal chromaffin cells, *Development* 126 (1999) 2935-2944.
- [7] S.M. Hollenberg, C. Weinberger, E.S. Ong, G. Cerelli, A. Oro, R. Lebo, E.B. Thompson, M.G. Rosenfeld, R.M. Evans, Primary structure and expression of a functional human glucocorticoid receptor cDNA, *Nature* 318 (1985) 635-641.
- [8] P. Moutsatsou, A.G. Papavassiliou, The glucocorticoid receptor signalling in breast cancer, *J Cell Mol Med* 12 (2008) 145-163.
- [9] M. Mandal, D.J. Olson, T. Sharma, R.K. Vadlamudi, R. Kumar, Butyric acid induces apoptosis by up-regulating Bax expression via stimulation of the c-Jun N-terminal kinase/activation protein-1 pathway in human colon cancer cells, *Gastroenterology* 120 (2001) 71-78.
- [10] B.M. Necela, J.A. Cidlowski, Mechanisms of glucocorticoid receptor action in noninflammatory and inflammatory cells, *Proc Am Thorac Soc* 1 (2004) 239-246.
- [11] A.R. Clark, M.G. Belvisi, Maps and legends: the quest for dissociated ligands of the glucocorticoid receptor, *Pharmacol Ther* 134 (2012) 54-67.
- [12] B.M. Necela, J.A. Cidlowski, Crystallization of the human glucocorticoid receptor ligand binding domain: a step towards selective glucocorticoids, *Trends Pharmacol Sci* 24 (2003) 58-61.
- [13] K. De Bosscher, G. Haegeman, Minireview: latest perspectives on antiinflammatory actions of glucocorticoids, *Mol Endocrinol* 23 (2009) 281-291.
- [14] G.P. Vicent, M.C. Monteserin, A.S. Veleiro, G. Burton, C.P. Lantos, M.D. Galigniana, 21-Hydroxy-6,19-oxidoprogesterone: a novel synthetic steroid with specific antiglucocorticoid properties in the rat, *Mol Pharmacol* 52 (1997) 749-753.
- [15] D.M. Presman, L.D. Alvarez, V. Levi, S. Eduardo, M.A. Digan, M.A. Marti, A.S. Veleiro, G. Burton, A. Pecci, Insights on glucocorticoid receptor activity modulation through the binding of rigid steroids, *PLoS One* 5 (2010) e13279.

- [16] L.D. Alvarez, M.A. Marti, A.S. Veleiro, D.M. Presman, D.A. Estrin, A. Pecci, G. Burton, Exploring the molecular basis of action of the passive antiglucocorticoid 21-hydroxy-6,19-epoxyprogesterone, *J Med Chem* 51 (2008) 1352-1360.
- [17] L.D. Alvarez LD, M.A. Martí, A.S. Veleiro, R.I. Misico, D.A. Estrin, A. Pecci, G. Burton, Hemisuccinate of 21-hydroxy-6,19-epoxyprogesterone: a tissue-specific modulator of the glucocorticoid receptor, *ChemMedChem* 3 (2008) 1869-77.
- [18] M. Lippman, G. Bolan, K. Huff, The effects of androgens and antiandrogens on hormone-responsive human breast cancer in long-term tissue culture, *Cancer Res* 36 (1976) 4610-4618.
- [19] L.C. Burton G, Veleiro A, Method for the preparation of 21-hydroxy-6,19-oxidoprogesterone (21OH-6,19OP). U.S. Patent ed. 7,071,328, (2006).
- [20] A.J. Urtreger, J.A. Aguirre Ghiso, S.E. Werbajh, L.I. Puricelli, A.F. Muro, E. Bal De Kier Joffe, Involvement of fibronectin in the regulation of urokinase production and binding in murine mammary tumor cells, *Int. J. Cancer* 82 (1999) 748-753.
- [21] D. Pang, M. Kocherginsky, T. Krausz, S.Y. Kim, S.D. Conzen, Dexamethasone decreases xenograft response to Paclitaxel through inhibition of tumor cell apoptosis, *Cancer Biol Ther* 5 (2006) 933-940.
- [22] E. Hoiyman, L. Rocha-Viegas, S.G. Kalko, N. Rubinstein, M. Morales-Ruiz, E.B. Joffe, E.C. Kordon, A. Pecci, Glucocorticoid alternative effects on proliferating and differentiated mammary epithelium are associated to opposite regulation of cell-cycle inhibitor expression, *J Cell Physiol* 227 (2012) 1721-1730.
- [23] L.L. Molinero, M.B. Fuertes, M.V. Girart, L. Fainboim, G.A. Rabinovich, M.A. Costas, N.W. Zwirner, NF-kappa B regulates expression of the MHC class I-related chain A gene in activated T lymphocytes, *J Immunol* 173 (2004) 5583-5590.

- [24] T. Tanos, M.J. Marinissen, F.C. Leskow, D. Hochbaum, H. Martinetto, J.S. Gutkind, O.A. Coso, Phosphorylation of c-Fos by members of the p38 MAPK family. Role in the AP-1 response to UV light, *J Biol Chem* 280 (2005) 18842-18852.
- [25] L. Romorini, O.A. Coso, A. Pecci, Bcl-XL mediates epidermal growth factor dependent cell survival in HC11 mammary epithelial cells, *Biochim Biophys Acta* 1793 (2009) 496-505.
- [26] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal Biochem* 72 (1976) 248-54.
- [27] C.B. Cymeryng, L.A. Dada, C. Colonna, C.F. Mendez, E.J. Podesta, Effects of L-arginine in rat adrenal cells: involvement of nitric oxide synthase, *Endocrinology* 140 (1999) 2962-2967.
- [28] S. Moncada, E.A. Higgs, Endogenous nitric oxide: physiology, pathology and clinical relevance, *Eur J Clin Invest* 21 (1991) 361-374.
- [29] C. Tsatsanis, A. Androulidaki, M. Venihaki, A.N. Margioris, Signalling networks regulating cyclooxygenase-2, *Int J Biochem Cell Biol* 38 (2006) 1654-1661.
- [30] M.I. Luster, P.P. Simeonova, Asbestos induces inflammatory cytokines in the lung through redox sensitive transcription factors, *Toxicol Lett* 102-103 (1998) 271-275.
- [31] A.D. Luster, Chemokines--chemotactic cytokines that mediate inflammation, *N Engl J Med* 338 (1998) 436-445.
- [32] F. D'Acquisto, T. Iuvone, L. Rombola, L. Sautebin, M. Di Rosa, R. Carnuccio, Involvement of NF-kappaB in the regulation of cyclooxygenase-2 protein expression in LPS-stimulated J774 macrophages, *FEBS Lett* 418 (1997) 175-178.
- [33] N. Mukaida, M. Morita, Y. Ishikawa, N. Rice, S. Okamoto, T. Kasahara, K. Matsushima, Novel mechanism of glucocorticoid-mediated gene repression. Nuclear factor-kappa B is target for glucocorticoid-mediated interleukin 8 gene repression, *J Biol Chem* 269 (1994) 13289-13295.

- [34] K. Subbaramaiah, L. Norton, W. Gerald, A.J. Dannenberg, Cyclooxygenase-2 is overexpressed in HER-2/neu-positive breast cancer: evidence for involvement of AP-1 and PEA3, *J Biol Chem* 277 (2002) 18649-18657.
- [35] E.M. King, N.S. Holden, W. Gong, C.F. Rider, R. Newton, Inhibition of NF-kappaB-dependent transcription by MKP-1: transcriptional repression by glucocorticoids occurring via p38 MAPK, *J Biol Chem* 284 (2009) 26803-26815.
- [36] Kassel O, Sancono A, Kratzschmar J, Kreft B, Stassen M, Cato AC. Glucocorticoids inhibit MAP kinase via increased expression and decreased degradation of MKP-1. *EMBO J* 2001; 20:7108-7116.
- [37] T.R. Vollmer, A. Stockhausen, J.Z. Zhang, Anti-inflammatory effects of mapracorat, a novel selective glucocorticoid receptor agonist, is partially mediated by MAP kinase phosphatase-1 (MKP-1), *J Biol Chem* 287 (2012) 35212-35221.
- [38] E. Joanny, Q. Ding, L. Gong, P. Kong, J. Saklatvala, A.R. Clark, Anti-inflammatory effects of selective glucocorticoid receptor modulators are partially dependent on up-regulation of dual specificity phosphatase 1, *Br J Pharmacol* 165 (2012) 1124-1136.
- [39] L.E. Shipp, J.V. Lee, C.Y. Yu, M. Pufall, P. Zhang, D.K. Scott, J.C. Wang, Transcriptional regulation of human dual specificity protein phosphatase 1 (DUSP1) gene by glucocorticoids, *PLoS One* 5 (2010) e13754.
- [40] K.D. Walton, K.U. Wagner, E.B. Rucker, 3rd, J.M. Shillingford, K. Miyoshi, L. Hennighausen, Conditional deletion of the bcl-x gene from mouse mammary epithelium results in accelerated apoptosis during involution but does not compromise cell function during lactation, *Mech Dev* 109 (2001) 281-293.
- [41] J.Z. Zhang, M.E. Cavet, K.R. VanderMeid, M. Salvador-Silva, F.J. Lopez, K.W. Ward, BOL-303242-X, a novel selective glucocorticoid receptor agonist, with full anti-inflammatory properties in human ocular cells, *Mol Vis* 15 (2009) 2606-2616.

- [42] J.E. Chivers, W. Gong, E.M. King, J. Seybold, J.C. Mak, L.E. Donnelly, N.S. Holden, R. Newton, Analysis of the dissociated steroid RU24858 does not exclude a role for inducible genes in the anti-inflammatory actions of glucocorticoids, *Mol Pharmacol* 70 (2006) 2084-2095.
- [43] R. Korhonen, A. Lahti, M. Hamalainen, H. Kankaanranta, E. Moilanen, Dexamethasone inhibits inducible nitric-oxide synthase expression and nitric oxide production by destabilizing mRNA in lipopolysaccharide-treated macrophages, *Mol Pharmacol* 62 (2002) 698-704.
- [44] A.R. Baudy, E.K. Reeves, J.M. Damsker, C. Heier, L.M. Garvin, B.C. Dillingham, J. McCall, S. Rayavarapu, Z. Wang, J.H. Vandermeulen, A. Sali, V. Jahnke, S. Duguez, D. DuBois, M.C. Rose, K. Nagaraju, E.P. Hoffman, Delta-9,11 modification of glucocorticoids dissociates nuclear factor-kappaB inhibitory efficacy from glucocorticoid response element-associated side effects, *J Pharmacol Exp Ther* 343 (2012) 225-232.
- [45] G. Walker, J. Pfeilschifter, U. Otten, D. Kunz, Proteolytic cleavage of inducible nitric oxide synthase (iNOS) by calpain I, *Biochim Biophys Acta* 1568 (2001) 216-224.
- [46] G. Walker, J. Pfeilschifter, D. Kunz, Mechanisms of suppression of inducible nitric-oxide synthase (iNOS) expression in interferon (IFN)-gamma-stimulated RAW 264.7 cells by dexamethasone. Evidence for glucocorticoid-induced degradation of iNOS protein by calpain as a key step in post-transcriptional regulation, *J Biol Chem* 272 (1997) 16679-16687.
- [47] I.J. Cho, S.G. Kim, A novel mitogen-activated protein kinase phosphatase-1 and glucocorticoid receptor (GR) interacting protein-1-dependent combinatorial mechanism of gene transrepression by GR, *Mol Endocrinol* 23 (2009) 86-99.
- [48] S.M. Abraham, T. Lawrence, A. Kleiman, P. Warden, M. Medghalchi, J. Tuckermann, J. Saklatvala, A.R. Clark, Antiinflammatory effects of dexamethasone are partly dependent on induction of dual specificity phosphatase 1, *J Exp Med* 203 (2006) 1883-1889.

- [49] K. Smoak, J.A. Cidlowski, Glucocorticoids regulate tristetraprolin synthesis and posttranscriptionally regulate tumor necrosis factor alpha inflammatory signaling, *Mol Cell Biol* 26 (2006) 9126-9135.
- [50] R. Newton, N.S. Holden, M.C. Catley, W. Oyelusi, R. Leigh, D. Proud, P.J. Barnes, Repression of inflammatory gene expression in human pulmonary epithelial cells by small-molecule IkappaB kinase inhibitors, *J Pharmacol Exp Ther* 321 (2007) 734-742.
- [51] De Bosscher K, Haegeman G, Elewaut D. Targeting inflammation using selective glucocorticoid receptor modulators. *Curr Opin Pharmacol* 2010; 10:497-504.
- [52] Schacke H, Rehwinkel H, Asadullah K. Dissociated glucocorticoid receptor ligands: compounds with an improved therapeutic index. *Curr Opin Investig Drugs* 2005; 6:503-507.
- [53] C.K. Glass, K. Saijo, Nuclear receptor transrepression pathways that regulate inflammation in macrophages and T cells, *Nat Rev Immunol* 10 (2010) 365-376.
- [54] R. Frijters, W. Fleuren, E.J. Toonen, J.P. Tuckermann, H.M. Reichardt, H. van der Maaden, A. van Elsas, M.J. van Lierop, W. Dokter, J. de Vlieg, W. Alkema, Prednisolone-induced differential gene expression in mouse liver carrying wild type or a dimerization-defective glucocorticoid receptor, *BMC Genomics* 11 (2010) 359.
- [55] A. Kleiman, S. Hubner, J.M. Rodriguez Parkitna, A. Neumann, S. Hofer, M.A. Weigand, M. Bauer, W. Schmid, G. Schutz, C. Libert, H.M. Reichardt, J.P. Tuckermann, Glucocorticoid receptor dimerization is required for survival in septic shock via suppression of interleukin-1 in macrophages, *FASEB J* 26 (2012) 722-729.
- [56] J. Ehrchen, L. Steinmuller, K. Barczyk, K. Tenbrock, W. Nacken, M. Eisenacher, U. Nordhues, C. Sorg, C. Sunderkotter, J. Roth, Glucocorticoids induce differentiation of a specifically activated, anti-inflammatory subtype of human monocytes, *Blood* 109 (2007) 1265-1274.
- [57] A.R. Clark, MAP kinase phosphatase 1: a novel mediator of biological effects of glucocorticoids?, *J Endocrinol* 178 (2003) 5-12.

- [58] K. Johansson-Haque, E. Palanichamy, S. Okret, Stimulation of MAPK-phosphatase 1 gene expression by glucocorticoids occurs through a tethering mechanism involving C/EBP, *J Mol Endocrinol* 41 (2008) 239-249.
- [59] S. Vandevyver, L. Dejager, T. Van Bogaert, A. Kleyman, Y. Liu, J. Tuckermann, C. Libert, Glucocorticoid receptor dimerization induces MKP1 to protect against TNF-induced inflammation, *J Clin Invest* 122 (2012) 2130-2140.
- [60] D.M. Presman, M.F. Ogara, M. Stortz, L.D. Alvarez, J.R. Pooley, R.L. Schiltz, L. Grontved, T.A. Johnson, P.R. Mittelstadt, J.D. Ashwell, S. Ganesan, G. Burton, V. Levi, G.L. Hager, A. Pecci, Live Cell Imaging Unveils Multiple Domain Requirements for In Vivo Dimerization of the Glucocorticoid Receptor, *PLoS Biol* 12 (2014) e1001813.
- [61] K. Schorr, P.A. Furth, Induction of bcl-xL expression in mammary epithelial cells is glucocorticoid-dependent but not signal transducer and activator of transcription 5-dependent, *Cancer Res* 60 (2000) 5950-5953.
- [62] L.R. Viegas, G.P. Vicent, J.L. Baranao, M. Beato, A. Pecci, Steroid hormones induce bcl-X gene expression through direct activation of distal promoter P4, *J Biol Chem* 279 (2004) 9831-9839.
- [63] Z. Feng, A. Marti, B. Jehn, H.J. Altermatt, G. Chicaiza, R. Jaggi, Glucocorticoid and progesterone inhibit involution and programmed cell death in the mouse mammary gland, *J Cell Biol* 131 (1995) 1095-1103.
- [64] A. Marti, P.M. Ritter, R. Jager, H. Lazar, A. Baltzer, J. Schenkel, W. Declercq, P. Vandenameele, R. Jaggi, Mouse mammary gland involution is associated with cytochrome c release and caspase activation, *Mech Dev* 104 (2001) 89-98.
- [65] W. Wu, S. Chaudhuri, D.R. Brickley, D. Pang, T. Karrison, S.D. Conzen, Microarray analysis reveals glucocorticoid-regulated survival genes that are associated with inhibition of apoptosis in breast epithelial cells, *Cancer Res* 64 (2004) 1757-1764.

- [66] M.D. Heitzer, D.B. DeFranco, Mechanism of action of Hic-5/androgen receptor activator 55, a LIM domain-containing nuclear receptor coactivator, *Mol Endocrinol* 20 (2006) 56-64.
- [67] Jiang W, Zhu Z, Bhatia N, Agarwal R, Thompson HJ. Mechanisms of energy restriction: effects of corticosterone on cell growth, cell cycle machinery, and apoptosis. *Cancer Res* 2002; 62:5280-5287.
- [68] I. Rogatsky, J.M. Trowbridge, M.J. Garabedian, Glucocorticoid receptor-mediated cell cycle arrest is achieved through distinct cell-specific transcriptional regulatory mechanisms, *Mol Cell Biol* 17 (1997) 3181-3193.
- [69] C.L. Smith, B.W. O'Malley, Coregulator function: a key to understanding tissue specificity of selective receptor modulators, *Endocr Rev* 25 (2004) 45-71.

Figure 1

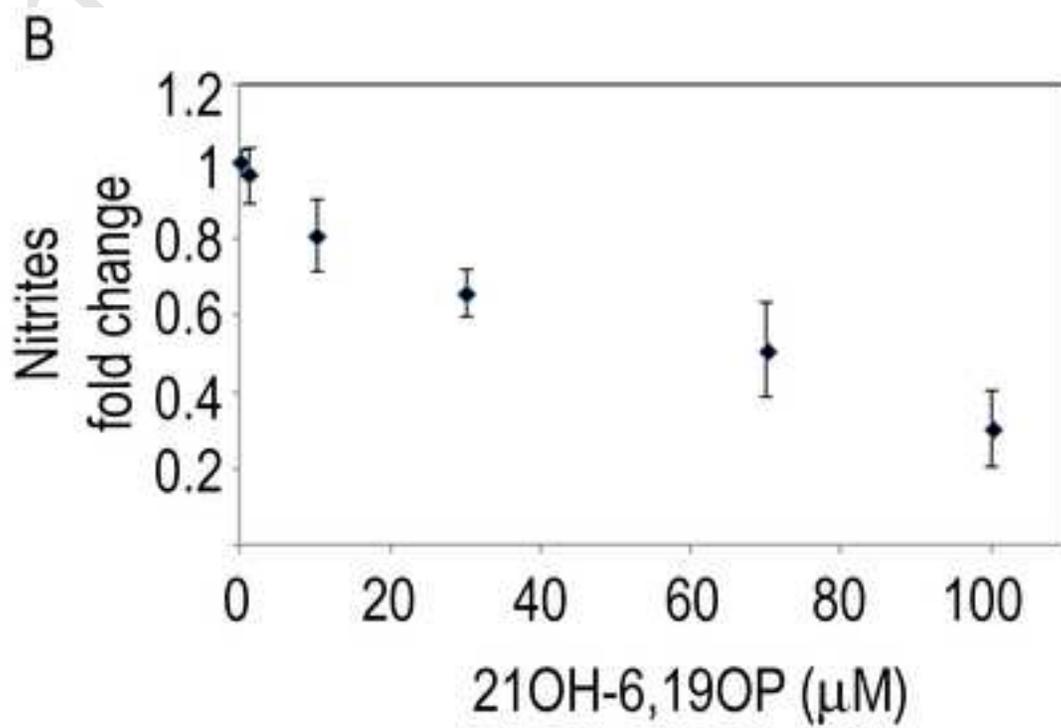
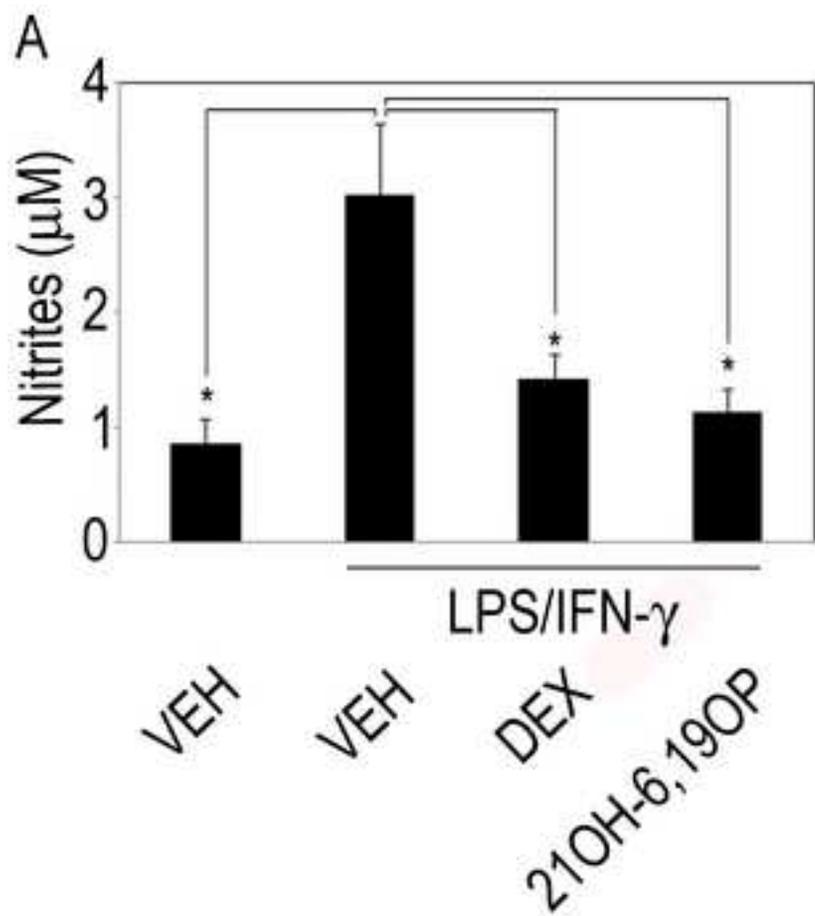
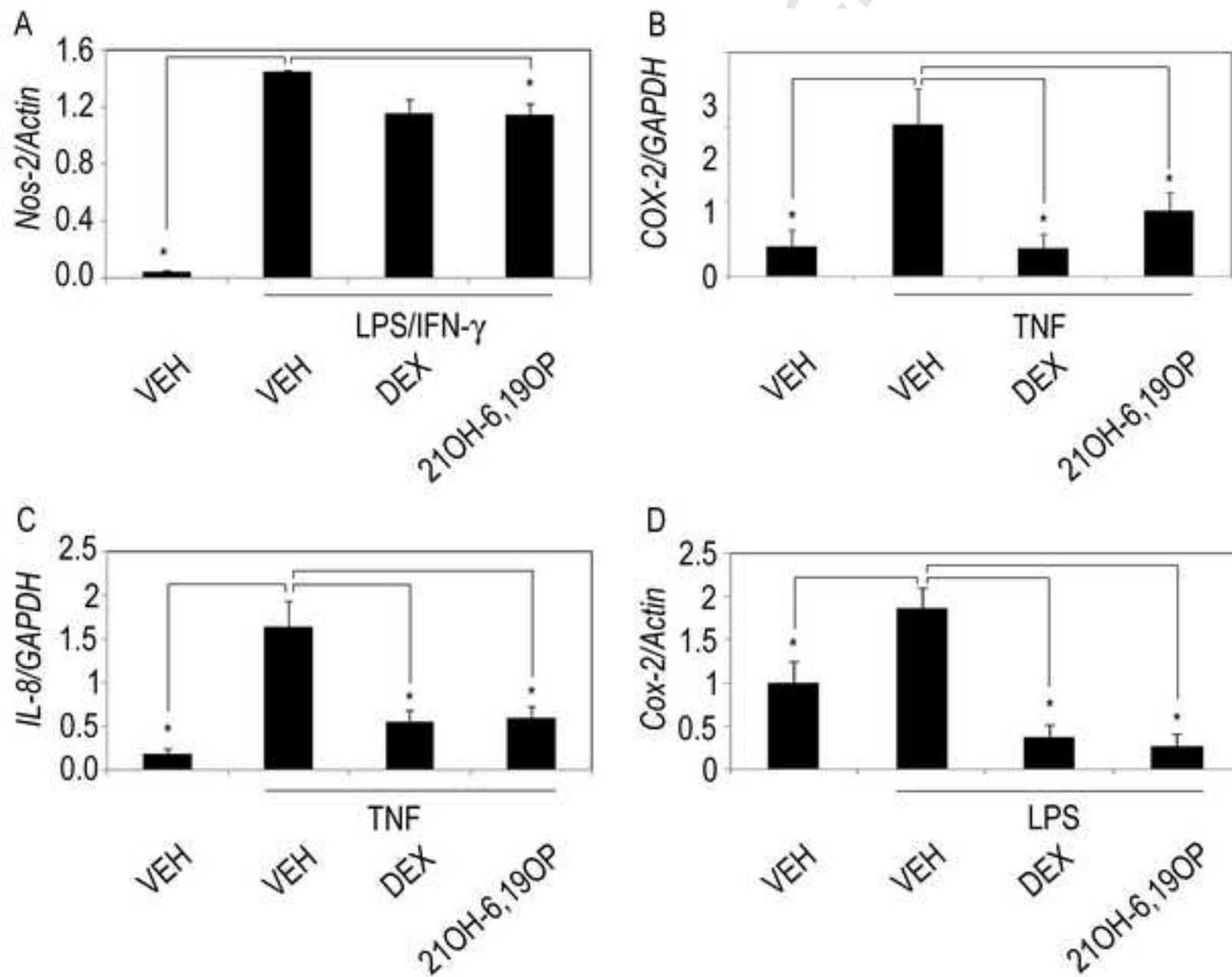
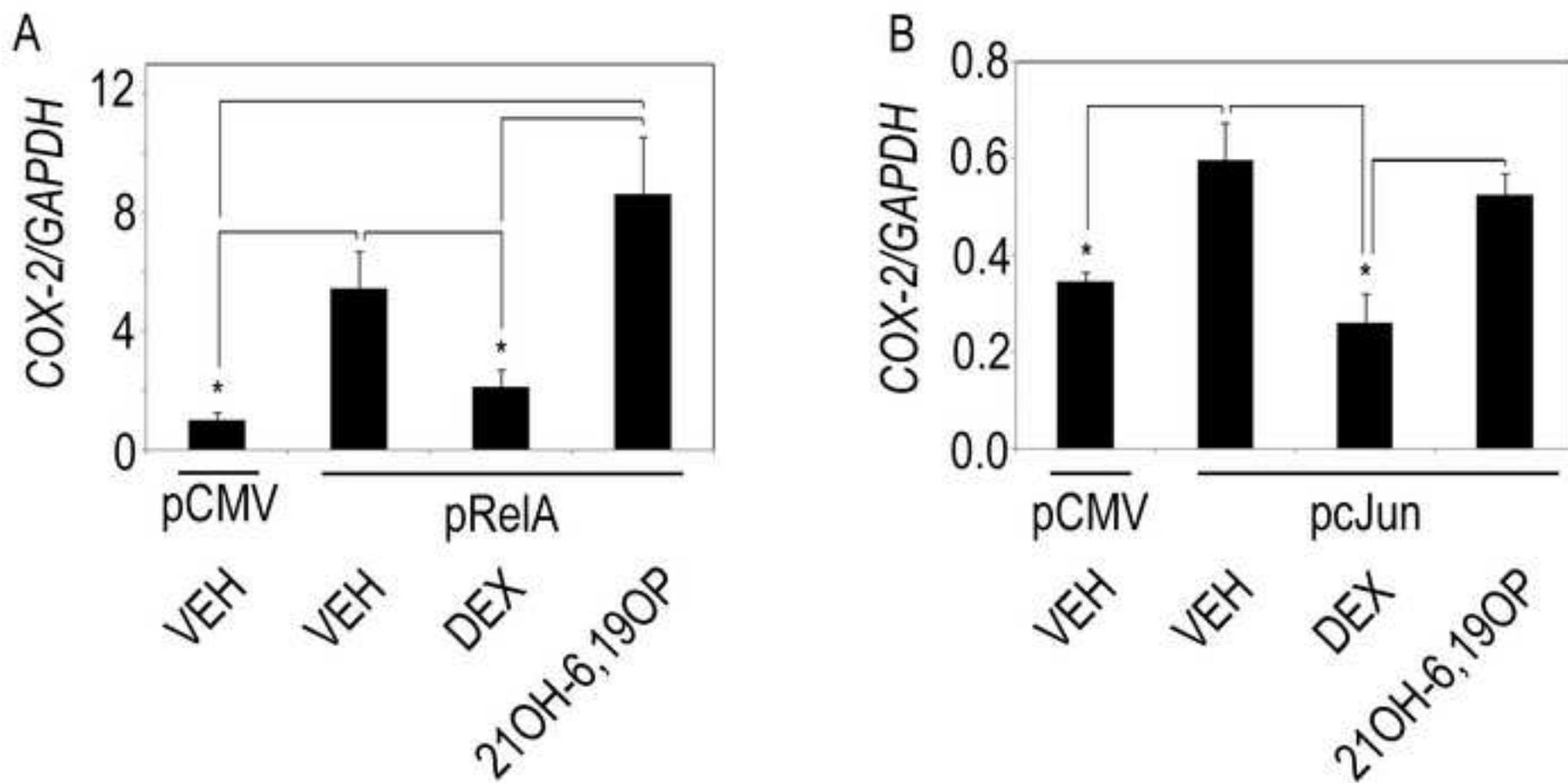


Figure 2





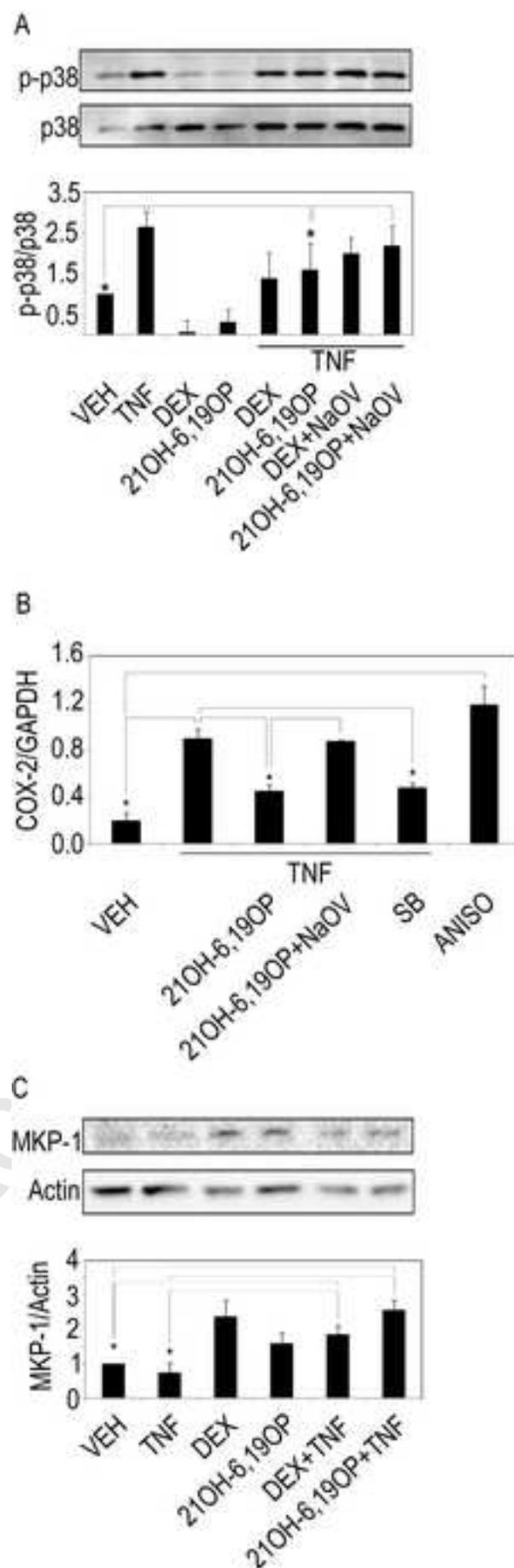
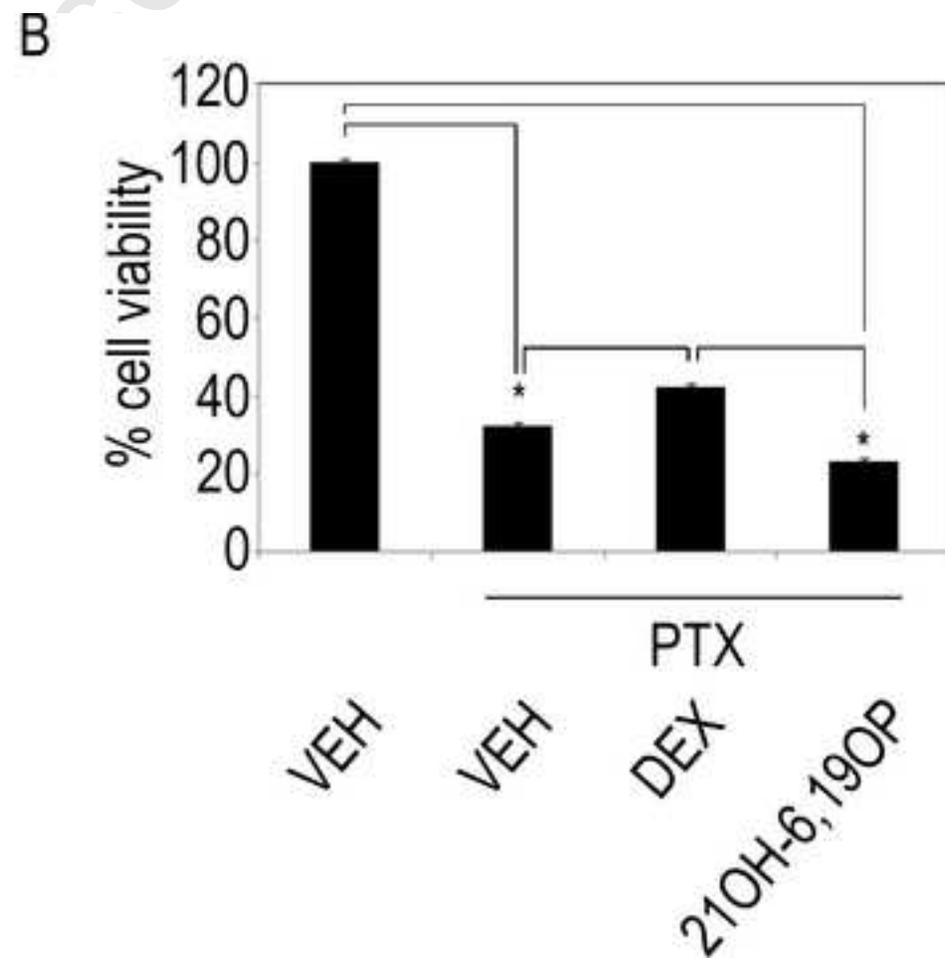
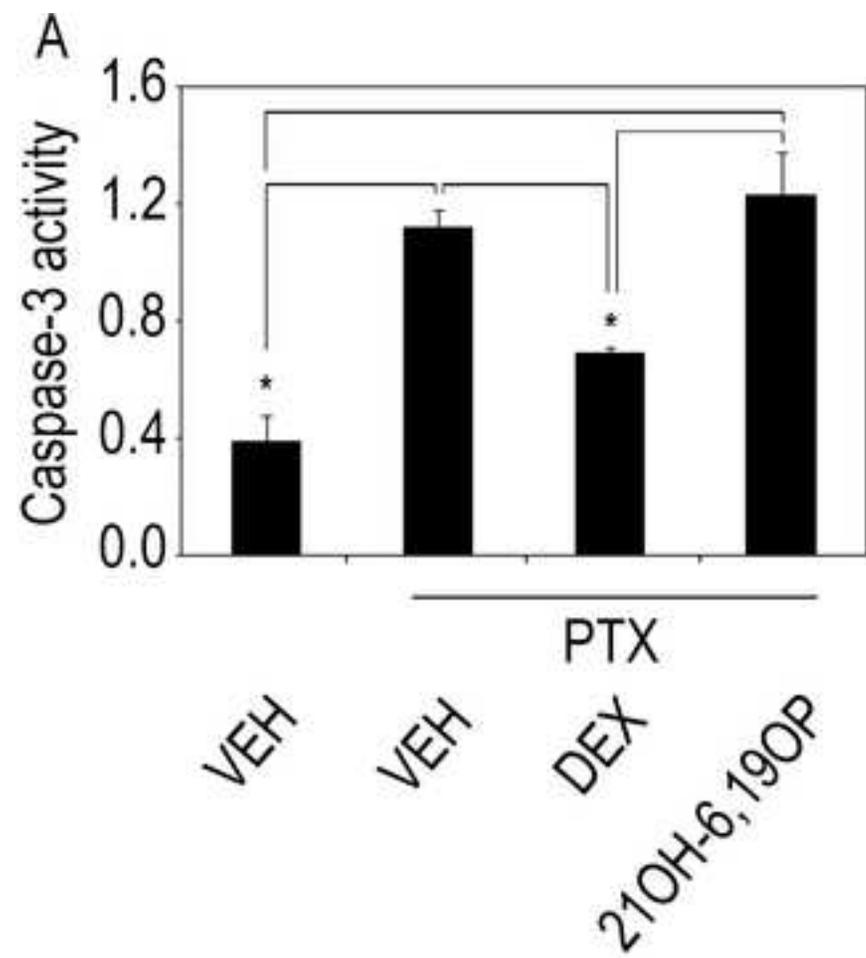
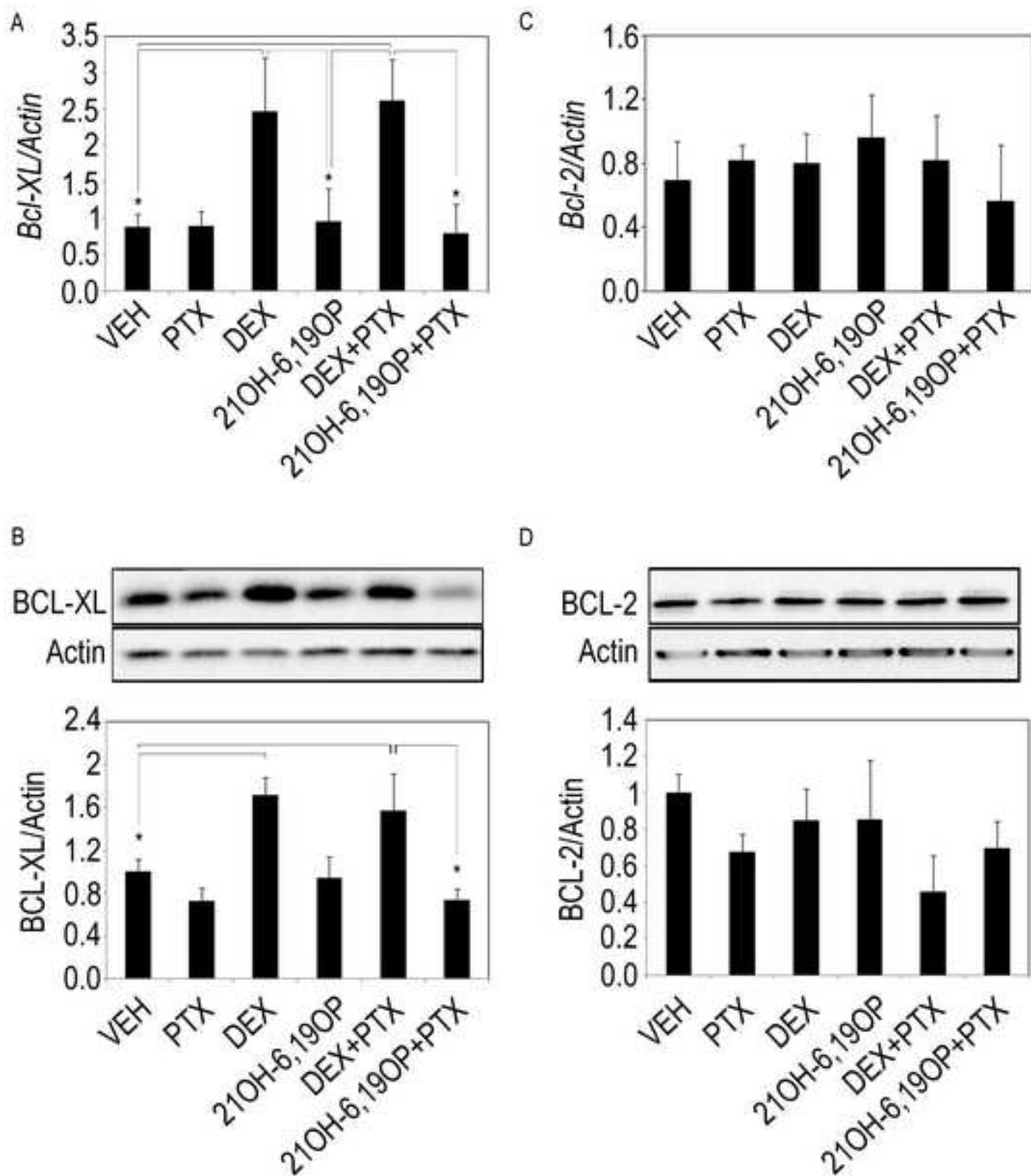
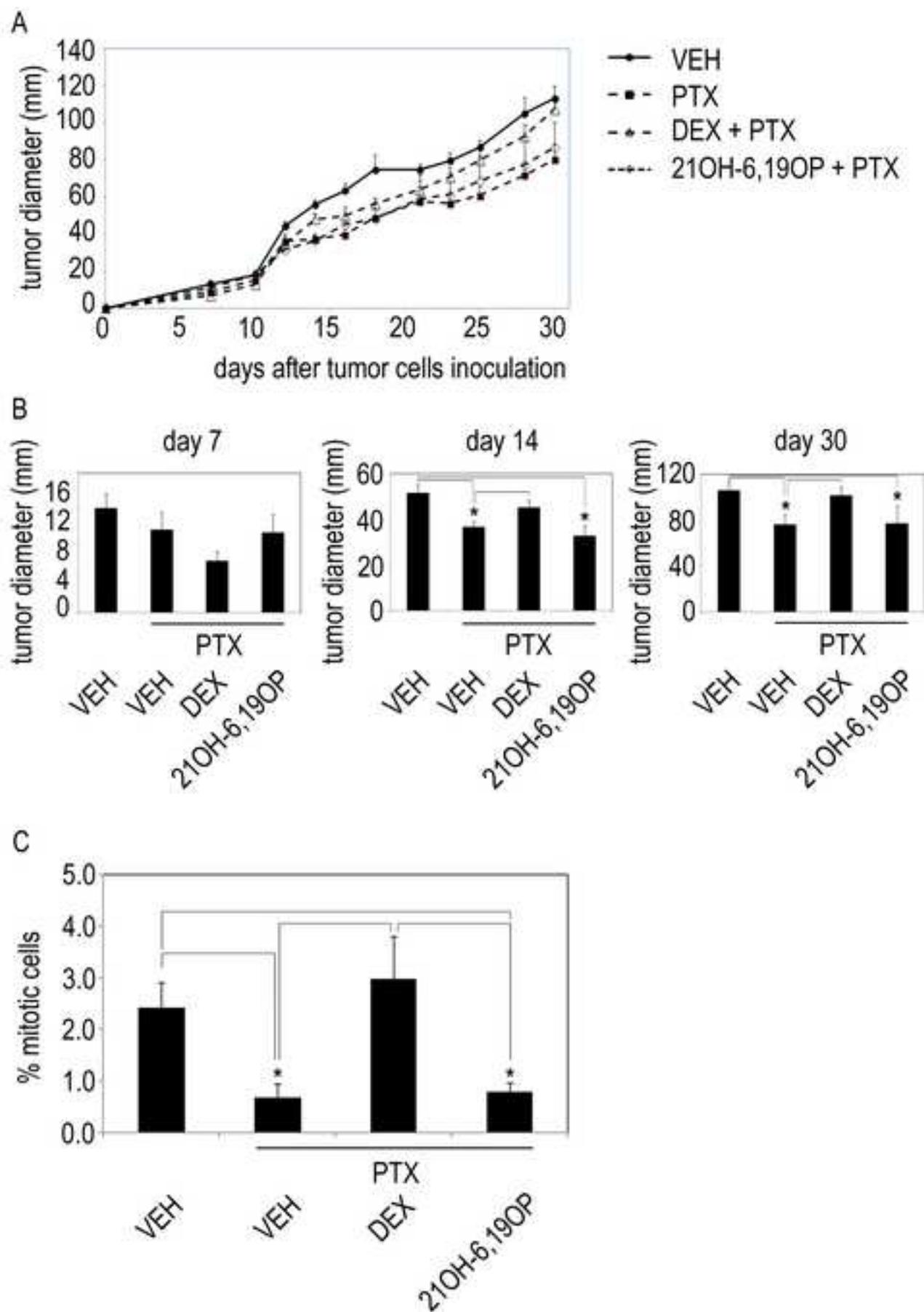


Figure 5







Manuscript

