

# Cyclo-oxygenase 2 modulates chemoresistance in breast cancer cells involving NF- $\kappa$ B

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**Abstract.** *Background:* Breast cancer cells can develop chemoresistance after prolonged exposure to cytotoxic drugs due to expression of the multi drug resistance (MDR) 1 gene. Type 2 cyclo-oxygenase (COX-2) inhibitors reverse the chemoresistance phenotype of a medullary thyroid carcinoma cell line, TT, and of a breast cancer cell line, MCF7, by inhibiting *MDR1* expression and P-gp function.

*Aim:* investigate the role of prostaglandin (PG) in modulating chemoresistance in MCF7 cells and to explore the involved intracellular mechanisms.

*Methods:* native and chemoresistant MCF7 cells were treated with PGH<sub>2</sub> and resistance to Doxorubicin was tested in the presence or absence of COX-2 inhibitors.

*Results:* PGH<sub>2</sub> restores resistance to the cytotoxic effects of Doxo, with concomitant nuclear translocation of the transcription factor NF- $\kappa$ B.

*Conclusions:* COX-2 inhibitors prevent chemoresistance development in breast cancer cells by inhibiting P-gp expression and function by a mechanism that involves PGH<sub>2</sub> generation and NF- $\kappa$ B activation.

Keywords: PGH<sub>2</sub>, P-gp, COX-2 inhibitors, NF- $\kappa$ B, chemoresistance, breast cancer

## 1. Introduction

The mechanism by which several human cancers develop resistance to chemotherapeutic drugs has been extensively investigated over the last years. Finding a way to overcome drug resistance is becoming increasingly important, since chemotherapy approaches have been extended in the attempt to treat cancers not responding to targeted therapy [25] and new markers are under intense investigation [11].

We previously demonstrated that the multi drug resistance (MDR) 1 gene, encoding a transmembrane glycoprotein p-170 (P-gp), is responsible for chemoresistance phenotype development in a breast cancer cell line, the MCF7 cells, exposed to a cytotoxic drug for

long time [31]. Indeed, P-gp is capable of antagonizing intracellular accumulation of cytotoxic agents, such as doxorubicin (Doxo), by actively extruding the drug from the cytoplasm to the extra-cellular environment [1,22]. Evidence has been provided that type 2 cyclo-oxygenase (COX-2) inhibitors can reverse the chemoresistance phenotype of a medullary thyroid carcinoma cell line [30] and can prevent the development of chemoresistance in MCF7 cells by inhibiting P-gp expression and function [31].

Prostaglandin H synthase, also termed COX, converts arachidonic acid to hydroxy-endoperoxide prostaglandin H<sub>2</sub> (PGH<sub>2</sub>), the common precursor for prostanoids biosynthesis [10]. The ultimate produced prostanoid may be different due to differential regulation of COX-1 and COX-2, tissue distribution, and availability. Therefore, PGH<sub>2</sub> represents the first COX-2 product and may mediate many of the effects blocked by COX-2 inhibitors. This evidence suggests that COX-2 inhibitors might exert their effect by repressing prostaglandin production and/or by influenc-

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ing *MDR1* transcription. Previous studies showed that *MDR1* expression can be controlled by *c-fos* [21] and by Nuclear Factor- $\kappa$ B (NF- $\kappa$ B) [8]. Moreover, COX-2 inhibitors have been demonstrated to inhibit NF- $\kappa$ B signalling [9,26,29] and COX-2 expression has been associated to NF- $\kappa$ B transcription factor activation in stromal myofibroblasts surrounding colon adenocarcinomas [13].

The aim of our study was therefore to investigate the mechanism by which COX-2 inhibitors may modulate the chemoresistance phenotype in the human breast cancer cell line, MCF7. In particular, we evaluated the involvement of PGH<sub>2</sub>, *c-fos* and NF- $\kappa$ B in COX-2 inhibitors-dependent *MDR1* expression modulation.

## 2. Materials and methods

### 2.1. MCF7 cell line

The MCF7 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in culture as previously described [31]. MCF7 cells not previously treated with Doxo will be referred to as "native" MCF7 for clarity. A clone of resistant MCF7 cells was also selected (rMCF7) by incubating native MCF7 cells for at least 2 months in the presence of 50 nM Doxo, as described previously [31]. The obtained cell strain was then cultured in DMEM supplemented with 5 nM Doxo.

### 2.2. Compounds

Prostaglandin H<sub>2</sub> (PGH<sub>2</sub>) and Rhodamine 123 (R123) were purchased from Sigma (Milano, Italy). Doxorubicin hydrochloride (Adriablastin) was obtained from Pharmacia (Milano, Italy). N-[2-(cyclohexyloxy) 4-nitrophenyl]-methanesulfonamide (NS-398), a specific COX-2 inhibitor, was purchased from Alexis Biochemicals (Lausen, Switzerland). All other reagents, if not otherwise specified, were purchased from Sigma (Milano, Italy).

### 2.3. DNA synthesis

DNA synthesis was evaluated by measuring [<sup>3</sup>H]thymidine ([<sup>3</sup>H]thy) incorporation in native MCF7 and rMCF7 cells, as described [23]. Native MCF7 and rMCF7 cells (5 × 10<sup>4</sup>/well) were incubated in the absence of serum for 24 h, and then cultured in medium supplemented with 10% FBS, without or with

the test compounds for up to 10 days in the presence of [<sup>3</sup>H]thy (1.5 μCi/ml; 87 Ci/mmol, Amersham-Pharmacia Biotech Italia, Cologno Monzese, Italy) for the last 24 h before harvesting the cells. After incubation, cell-associated radioactivity was determined after harvesting cells on glass fibers, and liquid scintillation counting of quadruplicate wells in at least three separate experiments. Results are expressed as mean cpm ± SE.

### 2.4. Rhodamine 123 assay

R123 assay was performed as previously described [30,31]. Briefly, native MCF7 and rMCF7 cells were cultured without or with 50 nM Doxo, 10 μM NS-398, 10 μM PGH<sub>2</sub> alone or in combination. Cells (2 × 10<sup>5</sup>/well) were then seeded in 24-well plates in culture medium the day before the experiment. Cells were washed and incubated with or without 4 μM R123 for 1 h at 37°C. After incubation, cells were washed three times with 1 ml of ice-cold serum-free culture medium to remove any extracellular R123, and then solubilized with NaOH 0.2 M over night and assayed for R123 and protein content. The concentration of R123 in each sample was determined quantitatively by fluorescence spectrophotometry using the Wallac Victor™ 1420 Multilabel Counter (Perkin Elmer, Milano, Italy) (λ<sub>ex</sub> = 485 nm, λ<sub>em</sub> = 535 nm) and standardized by the protein content of each sample. All experiments were carried out in triplicate.

### 2.5. Western blot analysis

For immunoblotting, MCF7 cells were resuspended in Sample Buffer (60 mM Tris, pH 6.8, containing 5% SDS, 10% glycerol, 2.5% β-mercaptoethanol and 0.02% bromphenol blue). Samples were lysed at 100°C for 10 min, and protein concentration was measured by BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA), as previously described [31]. Proteins were fractionated on SDS-PAGE and transferred by electrophoresis to Nitrocellulose Transfer Membrane (PROTRAN®, Dassel, Germany). Membranes were incubated with appropriate primary (human *c-fos* antibody from Sigma or NF- $\kappa$ B p65 (C-20) from Santa Cruz Biotechnology, Santa Cruz, CA, USA) and secondary antibodies. Horseradish peroxidase-conjugated antibody IgG (Sigma) was used and binding was revealed using enhanced chemiluminescence (Amersham Biosciences, Uppsala, Sweden). The blots were then stripped and used for further blotting with anti-actin antibody (Sigma).

## 2.6. Fluorescence microscopy

Native MCF7 and rMCF7 cells were cultured in culture medium supplemented without or with 50 nM Doxo, 10  $\mu$ M NS-398, 10  $\mu$ M PGH<sub>2</sub> alone or in combination. Cells ( $2 \times 10^4$ /well) were then seeded in 8-well chamber slides (Lab-Tek Chamber Slide System, Nalgene Nunc International, Naperville, IL, USA). For P-gp immunofluorescence, cells were incubated for 30 min in a humidified atmosphere at 37°C with a mouse monoclonal anti human P-gp antibody (1:500) (Sigma), then fixed in methanol-acetone (1:1) for 10 minutes at -20°C, blocked for 1 h with a blocking buffer (5% goat serum in PBS), and incubated with a secondary fluorescein isothiocyanate (FITC)-conjugated goat anti mouse antibody (1:200; Invitrogen Molecular Probes, Eugene, OR, USA) for 45 min at room temperature. Slides were then mounted with the ProLong Gold antifade reagent (Invitrogen Molecular Probes) containing the nuclear stain 4',6'-diamidino-2-phenylindole (DAPI) under glass coverslips (Menzel-Glaser, Braunschweig, Germany). The slides were visualized with the Nikon Eclipse TE2000-U fluorescent microscope, photographed with a  $\times 60$  objective magnification with the DS-5M Nikon colour CCD digital camera and analyzed with the Multi-Analyst software (Bio-Rad, Milano, Italy). Preimmune serum and antigen-absorbed antibody were used as controls. All experiments were carried out at least three times independently, analysing  $50 \pm 10$  individual cells.

## 2.7. Determination of NF- $\kappa$ B nuclear translocation

Native MCF7 and rMCF7 cells were seeded in 75 cm<sup>2</sup> culture flasks, starved over night, and then treated with 5  $\mu$ M Doxo. Cells were harvested after 24, 48 and 72 h and subsequently submitted to protein extraction. NE-PER<sup>®</sup> nuclear and Cytoplasmic Extraction Reagents kit (Pierce Biotechnology, Rockford, IL, USA) was employed to isolate nuclear from cytoplasmic proteins. Protein concentration of both fractions was determined by using the BCA Protein Assay Reagent Kit (Pierce). In order to determine NF- $\kappa$ B nuclear translocation, the NF- $\kappa$ B/p65 ActivELISA<sup>™</sup> kit was performed according to the manufacturer's instructions (Imgenex Corporation, San Diego, CA, USA).

## 2.8. Transfection and luciferase assay

Lipofectamine<sup>™</sup> LTX Reagent-mediated transfection was performed according to the manufacturer's in-

structions (Invitrogen). All transfections included extra empty vector to ensure that total amount of transfected DNA was kept constant in each culture plate. For luciferase assay, native MCF7 and rMCF7 cells ( $6 \times 10^3$ /well) were seeded in 96-well plates in culture medium and then staved over night. Transfection was then performed with 100 ng/well c-fos luciferase reporter plasmid, kindly provided by J. Schwartz, Department of Molecular and Integrative Physiology, University of Michigan, Ann Arbor, MI, USA, using the pmaxGFP plasmid (100 ng/well) (Amaxa, Cologne, Germany) as control for transfection efficiency. In order to evaluate transfection efficiency, cells were inspected 24 and 48 h after transfection using a light microscope (Zeiss Axiovert 25, Jena, Germany). The proportion of dead to living cells was determined by adding 20  $\mu$ l culture medium to 80  $\mu$ l trypan-blue and counting dead cells in a Burkler chamber. The proportion of transfected to untransfected cells was evaluated by counting fluorescent vs. nonfluorescent cells under the Nikon Eclipse TE2000-U fluorescent microscope. Transfection efficiency was expressed as the ratio between fluorescent and total living cells. Forty-eight hours after transfection, cells were treated with 50 nM Doxo, 10  $\mu$ M NS-398, 10  $\mu$ M PGH<sub>2</sub> alone or in combination for 8 h. Reporter gene activity was then determined by using the luciferase assay system (Dual-Glo<sup>™</sup> Luciferase Assay System; Promega, Milano, Italy), following the manufacturer's instructions. The basal c-fos promoter activity (set to 1) was measured in the absence of treatment and/or in the presence of vector DNA, using Phorbol Myristate Acetate 100 nM as positive control. Luciferase activity is presented as mean  $\pm$  SE for at least three independent experiments, each performed in triplicate, as previously indicated [7].

## 2.9. Statistical analysis

Results of DNA synthesis experiments are expressed as the mean  $\pm$  standard error (SE). A preliminary analysis was carried out to determine whether the datasets conformed to a normal distribution, and a computation of homogeneity of variance was performed using Bartlett's test. The results were compared within each group and between groups using ANOVA. If the *F*-values were significant ( $p < 0.05$ ), Student's paired or unpaired *t*-test was used to evaluate individual differences between means. *p*-values  $< 0.05$  were considered significant. For all the other experiments, results are expressed as the mean  $\pm$  standard error (SE)

among at least three replicates. Student's paired or unpaired *t*-test was used to evaluate individual differences between means and *p*-values < 0.05 were considered significant.

### 3. Results

#### 3.1. Effects of PGH<sub>2</sub> on cell proliferation

Preliminary dose-response and time-course studies demonstrated that PGH<sub>2</sub> does not affect native MCF7 and rMCF7 cell proliferation at doses ranging from 0.1 to 100 μM (data not shown). Cells were then treated with 10 μM PGH<sub>2</sub>, a concentration 10-fold the effective dose used for functional studies [6], in order to exclude PGH<sub>2</sub> degradation.

To investigate whether PGH<sub>2</sub> could influence the reported chemosensitizing effects of COX-2 inhibitors in breast cancer cells, DNA synthesis was evaluated

by measuring [<sup>3</sup>H]thy incorporation in native MCF7 and rMCF7 cells, as described [23]. Native MCF7 and rMCF7 cells were cultured without or with 50 nM Doxo, 10 μM NS-398, 10 μM PGH<sub>2</sub> alone or in combination for up to 10 days in the presence of [<sup>3</sup>H]thy, for further DNA synthesis evaluation.

NS-398 10 μM dose has been selected since it has been reported to inhibit COX-2 activity by almost 80% [15], without significantly affect COX-1 activity.

As shown in Fig. 1 (upper panel), Doxo was capable of significantly reducing DNA synthesis in native MCF7 cells after 3 and 7 days (−24% and −39%, respectively; *p* < 0.05 vs. control native MCF7 cells). However, after a 10 days treatment, cells were no longer sensitive to the cytotoxic effects of Doxo, in keeping with previous evidence [31]. Treatment with NS-398 alone did not significantly influence DNA synthesis in native MCF7 cells. On the other hand, cotreatment with Doxo determined a significant reduction in DNA synthesis, also after 10 days (−26%;

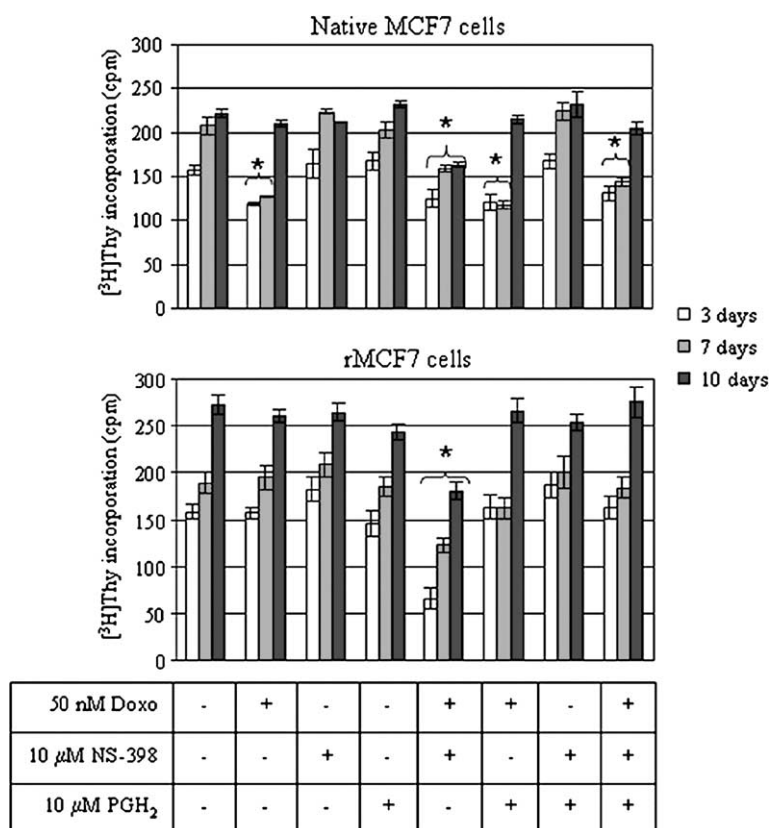


Fig. 1. Effects of PGH<sub>2</sub> on MCF7 cell proliferation. Native MCF7 and rMCF7 cells were incubated in 24-well plates up to 10 days in the presence of [<sup>3</sup>H]thy in culture medium supplemented without or with 50 nM Doxo, 10 μM NS-398, 10 μM PGH<sub>2</sub> alone or in combination and then DNA synthesis was evaluated. Data from three individual experiments evaluated independently with four replicates are expressed as the mean ± SE [<sup>3</sup>H]thy incorporation (cpm). \**p* < 0.05 vs. control untreated cells.

$p < 0.05$  vs. control). Treatment with  $\text{PGH}_2$  did not modify DNA synthesis in native MCF7 cells, either alone or in combination with NS-398. Moreover,  $\text{PGH}_2$  did not influence the cytotoxic activity of Doxo after 3 and 7 days, which was lost after 10 days. The same growth pattern was observed in the presence of NS-398, indicating that in native MCF7 cells  $\text{PGH}_2$  blocks the chemosensitizing effects of NS-398.

As shown in Fig. 1 (lower panel), treatment with Doxo did not influence DNA synthesis in rMCF7 cells in keeping with previous evidence [31]. Treatment with NS-398 alone did not significantly influence DNA synthesis in rMCF7 cells. On the other hand, co-treatment with Doxo determined a significant reduction in DNA synthesis ( $-59\%$ ,  $-35\%$ ,  $-34\%$ , respectively after 3, 7 and 10 days;  $p < 0.05$  vs. control). Treatment with  $\text{PGH}_2$  (alone or in combination with either Doxo or NS-398) did not modify DNA synthesis in rMCF7 cells, but was capable of blocking the sensitizing effects of NS-398 treatment.

### 3.2. Effects of $\text{PGH}_2$ on P-gp expression and function

To evaluate whether  $\text{PGH}_2$  can block the sensitizing effects of NS-398 by modulating P-gp protein expression, the presence of P-gp at the membrane level was investigated by immunofluorescence in native MCF7 cells and rMCF7 cells treated for 2 days without or with 50 nM Doxo, 10  $\mu\text{M}$  NS-398, 10  $\mu\text{M}$   $\text{PGH}_2$  alone or in combination. P-gp protein was not detected on plasma membrane of native MCF7 cells, as previously reported [31] (data not shown). On the other hand, in keeping with previous evidence, specific membrane immunofluorescence for P-gp was detected in untreated control rMCF7 cells, as shown in Fig. 2 (Panels B and D). Treatment with Doxo further enhanced P-gp expression at the membrane level, while NS-398 strongly reduced basal and Doxo-induced immunofluorescence intensity. On the contrary,  $\text{PGH}_2$  did not modify basal and Doxo-induced P-gp protein expression, while it restored membrane immunofluorescence in cells treated with NS-398, in the presence or absence of Doxo.

In order to evaluate P-gp activity, R123 assay was performed. In native MCF7 cells, R123 intracellular accumulation was not significantly influenced by treatment with Doxo, NS-398 or  $\text{PGH}_2$  for 2 days, in keeping with the absence of P-gp protein (not shown). On the contrary, in rMCF7 cells treatment for 2 days with 50 nM Doxo, 10  $\mu\text{M}$  NS-398, 10  $\mu\text{M}$   $\text{PGH}_2$  alone or in combination significantly modified R123 accumu-

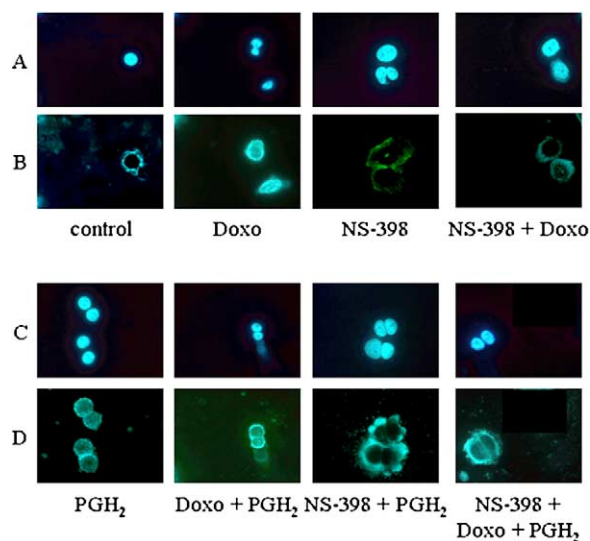


Fig. 2. Effects of  $\text{PGH}_2$  on P-gp expression in rMCF7 cells. rMCF7 cells were treated for 2 days without (control) or with 50 nM Doxo, 10  $\mu\text{M}$  NS-398, 10  $\mu\text{M}$   $\text{PGH}_2$  alone or in combination. Cells were then incubated with the specific P-gp antibody, fixed, incubated with the secondary FITC-conjugated antibody, mounted with the antifade reagent containing DAPI, and photographed with a  $\times 60$  objective magnification. (A and C) rMCF7 cells observed with the UV filter, revealing nuclear immunofluorescence. (B and D) rMCF7 cells observed with the FITC filter, revealing P-gp specific membrane immunofluorescence.

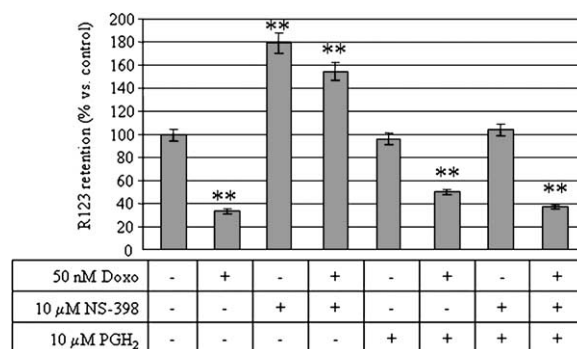


Fig. 3. R123 assay. rMCF7 cells were treated for 2 days without (control) or with 50 nM Doxo, 10  $\mu\text{M}$  NS-398, 10  $\mu\text{M}$   $\text{PGH}_2$  alone or in combination. Cells were then incubated with 4  $\mu\text{M}$  R123, washed, and lysed. Sample fluorescence was measured and normalized by the protein content. Data from three individual experiments evaluated independently with three replicates are expressed as the mean  $\pm$  SE percent R123 retention as compared to untreated control cells. \*\*\* $p < 0.01$  vs. untreated control rMCF7 cells.

lation (Fig. 3). R123 accumulation in control rMCF7 cells was weak, in keeping with the presence of an active P-gp protein, and was significantly reduced after treatment with Doxo ( $-67\%$  vs. control;  $p < 0.01$ ). On the contrary, treatment with NS-398 significantly

enhanced intracellular R123 accumulation in rMCF7 cells (+80% vs. control;  $p < 0.01$ ), and completely blocked Doxo-induced R123 efflux. Treatment with PGH<sub>2</sub> did not significantly influence basal intracellular R123 fluorescence, but completely blocked R123 accumulation induced by NS-398. This effect is further confirmed by the evidence that in the presence of both PGH<sub>2</sub> and NS-398, treatment with Doxo is still capable of reducing R123 intracellular fluorescence (−62.5% vs. control;  $p < 0.01$ ), in keeping with the presence of an active P-gp protein in these cells.

### 3.3. *c-fos* transcriptional activity

In order to explore whether the observed effects on P-gp expression are due to transcriptional modifications, we evaluated *c-fos* transcriptional activity in native MCF7 cells and rMCF7 cells. Cells were transfected with a *c-fos* luciferase reporter plasmid as described in Section 2, with a transfection efficiency of  $43 \pm 8\%$ . After 48 h, treatment without or with 50 nM Doxo, 10  $\mu$ M NS-398, 10  $\mu$ M PGH<sub>2</sub> alone or in combination for 8 h was performed, with subsequent luciferase activity evaluation. We found that treatment with the test substances failed to modify basal luciferase activity in both native MCF7 and rMCF7 cells transfected with the luciferase reporter plasmid under the control of *c-fos* promoter (not shown). The absence of *c-fos* involvement in Doxo-induced chemoresistance in our model is further confirmed by the absence of *c-fos* expression modifications in native MCF7 cells and rMCF7 cells, as assessed by Western blot (not shown).

### 3.4. Doxorubicin, NS-398 and PGH<sub>2</sub> influence NF- $\kappa$ B activity

The pleiotropic transcription factor NF- $\kappa$ B, when inactive, is located in the cytoplasm, where it is bound to NF- $\kappa$ B inhibitory proteins, termed I $\kappa$ Bs [27]. Upon extracellular signals, I $\kappa$ Bs are phosphorylated and then degraded [4,19] and, consequently, NF- $\kappa$ B can enter the nucleus and activate target gene transcription [27]. To verify whether NF- $\kappa$ B was involved in the observed modifications concerning P-gp expression, we evaluated NF- $\kappa$ B nuclear translocation in native MCF7 cells starved overnight and treated for up to 72 h with 50 nM Doxo in the presence of 10% FBS. As shown in Fig. 4A, in native MCF7 cells NF- $\kappa$ B serum-induced nuclear translocation peaked after 24 h, gradually declining thereafter. On the other hand, treat-

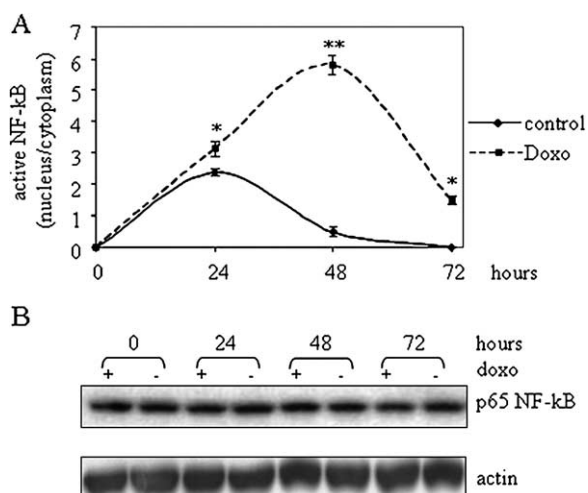


Fig. 4. Doxorubicin induces NF- $\kappa$ B nuclear translocation in MCF7 cells. (A) Native MCF7 cells were starved overnight and treated for up to 72 h with 50 nM Doxo in the presence of 10% FBS. NF- $\kappa$ B/p65 content was then evaluated in cytoplasmic and nuclear proteins by ELISA. Results are expressed as the ratio between nuclear and cytoplasmic NF- $\kappa$ B immunoreactivity in control untreated native MCF7 cells (control) and in native MCF7 cells treated with 50 nM Doxo (Doxo). \* $p < 0.05$  and \*\* $p < 0.01$  vs. control untreated native MCF7 cells. (B) Native MCF7 cells were starved overnight and treated for up to 72 h with 50 nM Doxo in the presence of 10% FBS. NF- $\kappa$ B/p65 total cellular content was then evaluated by Western blot. Upper panel: total cellular NF- $\kappa$ B protein levels in native MCF7 cells treated without (−) and with 50 nM Doxo (+). Lower panel: total actin protein in native MCF7 cells treated without (−) and with 50 nM Doxo (+).

ment with Doxo induced a significantly greater nuclear translocation both after 24 and 48 h ( $p < 0.05$  and  $p < 0.01$  vs. control cells, respectively). Western blot analysis showed that total cellular NF- $\kappa$ B expression level in these cells did not significantly vary in this time frame, both in the presence and in the absence of Doxo (Fig. 4B). Further experiments evaluating NF- $\kappa$ B nuclear translocation were therefore performed after 48 h treatments.

To evaluate whether Doxo-induced NF- $\kappa$ B nuclear translocation could be influenced by COX-2 inhibitors, native MCF7 cells and rMCF7 cells were treated for 48 h without or with 50 nM Doxo, 10  $\mu$ M NS-398, 10  $\mu$ M PGH<sub>2</sub> alone or in combination and then NF- $\kappa$ B nuclear translocation was evaluated.

In native MCF7 cells, NF- $\kappa$ B nuclear translocation significantly increased after treatment with Doxo (+62% vs. control;  $p < 0.01$ ) (white bars, Fig. 5). On the other hand, treatment with NS-398 did not significantly influence basal intracellular NF- $\kappa$ B localization, while it completely blocked Doxo-induced NF- $\kappa$ B nuclear translocation. Moreover, treatment with PGH<sub>2</sub>

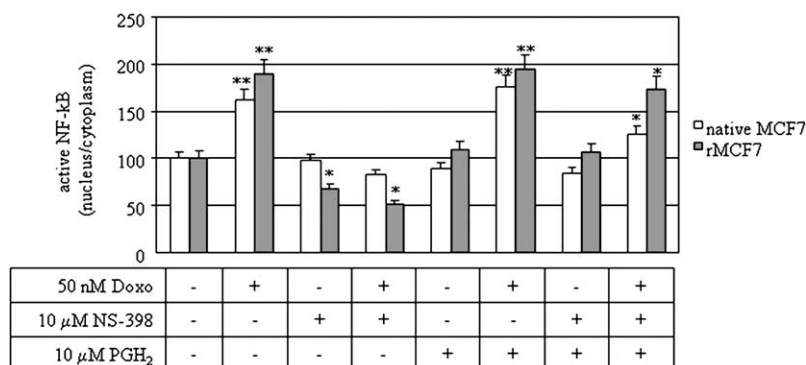


Fig. 5. Effects of PGH<sub>2</sub> on NF-κB nuclear translocation in native MCF7 cells. Native MCF7 (white bars) and rMCF7 cells (grey bars) were treated for 48 h without or with 50 nM Doxo, 10 μM NS-398, 10 μM PGH<sub>2</sub> alone or in combination. NF-κB/p65 content was then evaluated in cytoplasmic and nuclear proteins by ELISA. Results are expressed as the mean ± SE percent active NF-κB (nuclear to cytoplasmic NF-κB immunoreactivity ratio) in treated samples as compared to untreated control cells. All experiments were carried out at least three times independently. \* $p < 0.05$  and \*\* $p < 0.01$  vs. untreated control cells.

did not significantly influence basal and Doxo-induced intracellular NF-κB localization, while it promoted a significant NF-κB nuclear translocation in cells treated with both Doxo and NS-398 (+26% vs. control;  $p < 0.05$ ) (white bars, Fig. 5).

In rMCF7 cells, nuclear NF-κB translocation significantly increased after treatment with Doxo (+90% vs. control;  $p < 0.01$ ) (grey bars, Fig. 5). In these settings, P-gp function is stimulated by Doxo, but is unable to completely extrude the drug. As a consequence, Doxo can stimulate NF-κB nuclear translocation even in rMCF7 cells. On the other hand, treatment with NS-398 significantly reduced both basal (-32% vs. control;  $p < 0.05$ ) and Doxo-induced NF-κB nuclear translocation (-50% vs. control;  $p < 0.01$ ). On the other hand, treatment with PGH<sub>2</sub> did not significantly influence basal and Doxo-induced intracellular NF-κB localization, while it completely blocked the effects of NS-398 on NF-κB nuclear translocation (grey bars, Fig. 5).

#### 4. Discussion

Reduced cellular drug uptake resulting from P-gp overexpression represents one of the several described mechanisms of cancer cell resistance to chemotherapeutic agents. In this study, we confirm that the sensitizing effect of selective COX-2 inhibitors towards the cytotoxic action of Doxo in chemoresistant breast cancer cells depends on P-gp expression modulation. We also demonstrate that this effect is due to prostaglandin synthesis blockade. The importance of COX-2 blockade in breast cancer has been highlighted by previous

studies, showing that COX-2 expression and function is associated with greater aggressivity and poor prognosis [2]. Prostaglandin synthesis therefore is implicated in cancer promotion and progression and we here demonstrate that it plays a key role also in chemoresistance phenotype development. COX implication in carcinogenesis has been thoroughly studied in the settings of colon cancer [5]. However, whether and how prostaglandins are involved in this process is less certain. Treatment with carcinogens of knockout mice deficient in prostaglandin receptor show significant suppression of aberrant colonic crypt foci [28], suggesting that indeed prostaglandins play an important role in inducing the initial steps of carcinogenesis, preventing apoptosis of transformed cells and stimulating tumor-associated angiogenesis [16].

Our data show that PGH<sub>2</sub> generation by COX-2 might represent an important step in drug-induced chemoresistance, even if in these settings it does not seem to influence basal cell proliferation. Indeed, incubation with PGH<sub>2</sub> of both native MCF7 and rMCF7 cells does not influence DNA synthesis, but completely blocks the sensitizing effects of COX-2 inhibitors towards the cytotoxic action of Doxo. This effect is due to restoration of membrane P-gp expression, which is down-regulated in COX-2 treated rMCF7 cells. Restored P-gp expression is associated with the capacity of actively extruding cytotoxic drugs from the cell, as demonstrated by Doxo accumulation assays. Taken together, our data indicate that COX-2 enzymatic activity is responsible for drug-induced chemoresistance in a breast cancer cell line and that inhibition of COX-2 results in phenotype reversal. Which prostaglandin down-stream from PGH<sub>2</sub> is involved in this process has still to be elucidated.

Our data confirm that in MCF7 cells the chemoresistance phenotype depends, at least in part, on P-gp expression at membrane level. However, the involvement of other multidrug resistance associated proteins cannot be completely ruled out. We previously demonstrated that Doxo-induced P-gp protein parallels with *MDR1* gene expression [31], which is controlled by several transcription factors [3]. Previous reports demonstrated that a protein complex consisting of NF- $\kappa$ B/p65 and c-fos transcription factors interacts with the promoter region in MCF7 cells, negatively regulating the human *MDR1* promoter activity [17]. However, we failed to demonstrate any modification in basal c-fos promoter activity both in native MCF7 and in rMCF7 cells treated either with Doxo or with a specific COX-2 inhibitor. Our data therefore suggest that this transcription factor is not directly involved in the modulation of *MDR1* expression in our experimental conditions. NF- $\kappa$ B was reported to induce *MDR1* expression in rat hepatoma [32] and kidney cells [24], where COX-2 is highly expressed [20]. Accordingly, our results indicate that induction of P-gp expression and function in both native and chemoresistant MCF7 cells is associated with NF- $\kappa$ B nuclear translocation, which accounts for its activation. NF- $\kappa$ B is a pleiotropic transcription factor playing a key role in determining the death threshold of human cells, especially in response to chemotherapeutic drugs [14]. NF- $\kappa$ B is kept silent in the cytoplasm via interaction with the inhibitory protein I $\kappa$ B $\alpha$  and translocates into the nucleus upon activation and I $\kappa$ B $\alpha$  degradation, inducing transcription of target genes. Our data are consistent with NF- $\kappa$ B activation by treatment with Doxo, which is inhibited by COX-2 inhibitors. The evidence that PGH<sub>2</sub> restores NF- $\kappa$ B nuclear translocation supports the hypothesis that prostaglandin generation is important for Doxo-induced NF- $\kappa$ B activation and suggests that blockade of PGH<sub>2</sub> synthesis by COX-2 inhibitors might hamper NF- $\kappa$ B induced *MDR1* expression. The role of NF- $\kappa$ B in solid tumors has been well documented in several studies performed on primary tumors and neoplastic cell lines derived from different human tissues [18]. These studies show that the inhibition of constitutive NF- $\kappa$ B activity blocks the oncogenic potential of neoplastic cells by sensitizing tumor cells to chemotherapeutic drug-induced apoptosis, decreasing the highly proliferative rate which characterizes transformed cells, and inhibiting tissue invasiveness and metastatic potential of highly malignant cells [12].

In conclusion, our data further confirm that chemoresistance phenotype can be induced in MCF7

cells by long-lasting treatment with chemotherapeutic drugs and that COX-2 inhibitors might prevent the development of this phenotype by blocking prostaglandin generation. Moreover, our results demonstrate that treatment with Doxo induces NF- $\kappa$ B nuclear translocation, which is in turn blocked by COX-2 inhibitors and restored by PGH<sub>2</sub>, indicating that NF- $\kappa$ B is involved in the development of the chemoresistance phenotype. These data further support the hypothesis that selective COX-2 inhibitors might be useful in combination with chemotherapy and/or as neo-adjuvant therapy in the medical treatment of metastatic breast cancer in order to prevent chemoresistance.

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