

Antiproliferative effects of COX-2 inhibitor celecoxib on human breast cancer cell lines

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Abstract The inducible COX-2 enzyme is over-expressed in human breast cancer and its over-expression generally correlates with angiogenesis, deregulation of apoptosis and worse prognosis. This observation may explain the beneficial effect of nonsteroidal anti-inflammatory drugs and COX-2 inhibitors on breast cancer treatment. Here, we evaluated the antiproliferative activity of celecoxib, a selective COX-2 inhibitor, and its nitro-oxy derivative on human breast cancer cells characterized by low and high COX-2 expression, respectively. In ER α (+) MCF-7 cells celecoxib and its derivative induce a strong inhibition of cell growth, inhibition that is associated with the reduction of ER α expression and activation. These effects may be directly associated with ERK and Akt suppression and with PP2A and PTEN induction. In this cell line the drugs exert only weak effect on COX-2 level while they are able to reduce aromatase expression. On the contrary, in ER α (-) MDA-MB-231 cells, both drugs induce a marked inhibition of COX-2, inhibition that is associated with the reduction of aromatase expression and of cell proliferation. In both cell lines the effects of the drugs are associated with the suppression of cell invasion.

Keywords Breast cancer · COX-2 · Celecoxib · PI3K/Akt · ER

Introduction

Breast cancer is the second leading cause of cancer-related death in women [1]. The mainstay of treatment has generally been surgical intervention with roles for chemotherapy, radiotherapy and drugs acting against the effect of oestrogen directly at the receptor level in the case of tamoxifen or by inhibiting the peripheral synthesis of oestrogen with aromatase inhibitors [2].

Numerous studies have demonstrated the presence of elevated levels of prostaglandin in breast cancer compared with normal tissue [3, 4]. Cyclooxygenase-2 (COX-2), the inducible form of COX, has been found to be up-regulated in human breast cancer and its overexpression has been linked to a number of critical components of breast carcinogenesis including inhibition of apoptosis and aromatase-catalyzed oestrogen biosynthesis [5].

As a consequence of this, over the last two decades the possible link between the use of COX-2 selective inhibitors (coxibs) and the risk of breast cancer has developed increasing interest [6]. Coxibs, through the inhibition of COX-2 activity, are able to reduce the synthesis of prostaglandins involved in the induction of aromatase gene expression and thereby in the stimulation of oestrogen biosynthesis [7, 8].

Of note is the accumulating evidence that COX-2 inhibitors decreased both aromatase expression and activity in breast cancer cells [9], and the use of combinations of COX-2 and aromatase inhibitors was more effective than single agents in decreasing estradiol production [10].

Aromatase, which catalyzes the formation of aromatic C18 oestrogen from C19 androgens, is regarded as responsible for the local production of oestrogen in cancer. Studies from aromatase transfected breast cancer cells and from transgenic mice overexpressing aromatase

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demonstrated that in situ produced oestrogen plays more important roles than circulating oestrogens in breast tumour promotion and progression [11, 12].

Oestrogens play a crucial role in the development and progression of breast cancer. The classic effects of oestrogens are mediated through binding to oestrogen receptors ($ER\alpha$ and $ER\beta$) and stimulation of transcription at nuclear levels. Recently, the non-genomic actions of oestrogens have been reported through binding to membrane-associated ER [13], which resides in or near the cell membrane and cross-talks with the signal transduction pathways, including the c-Src/Ras/MAPK and PI3K/Akt pathways [14–16].

Celecoxib is a COX-2-selective inhibitor that was developed to reduce the incidence of gastrointestinal side effects associated with non-selective long-term inhibition of COX-1 and COX-2 by traditional NSAIDs. There is increasing epidemiological and experimental evidence that celecoxib and its derivatives have a protective effect in the development of certain malignancies especially colorectal, prostate and possibly breast cancer [17, 18]. In vitro, celecoxib reduces cell proliferation and initiates apoptosis in different types of cancer cells [19].

In any case, enthusiasm for the use of selective COX-2 celecoxib in chemoprevention of breast cancer and other malignancies has been tempered by reports of adverse effects on the cardiovascular system. Based on the observation that nitric oxide (NO) possesses some of the same useful pharmacological properties as prostaglandins within the gastric mucosa, such as vascular relaxation and inhibition of platelet activation, a new class of coxibs able to release NO has been synthesized [20]. NO-coxibs consist of a known coxib molecule and a NO-releasing group (typically $-NO_2$) linked to it via a chemical spacer. This coupling might deliver NO to the site of coxib-induced damage and thus decrease gastric and cardiovascular toxicity.

Ongoing work in our laboratory evaluates the biological effects of celecoxib and a series of its nitro-oxy analogues on human colon cancer cells. We recently reported that celecoxib and the nitro-oxy derivative with two nitro-oxy functions on the phenyl rings showed significant biological features such as: (a) antiproliferative activity, (b) inhibition of COX-2, (c) inhibition of ERK/MAPK and PI3K/Akt pathways, (d) reorganization of E-cadherin/ β -catenin system [21].

On the basis of these observations, in this study we evaluated the potential inhibitory action exerted by celecoxib and its nitro-oxy derivative on proliferation of human tumour breast cancer cells, on signalling pathways involved in $ER\alpha$ activation and on aromatase expression. We used, as model systems, human $ER\alpha(+)$ (MCF-7) and $ER\alpha(-)$ (MDA-MB-231) breast cancer cells, which were, respectively, characterized by low and high COX-2 expression.

Materials and methods

Reagents

Celecoxib was obtained from LC Laboratories (Woburn, MA, USA), whereas the nitro-oxy derivative of celecoxib (Fig. 1) was designed and synthesized at the Department of Scienza e Tecnologia del Farmaco, University of Torino, Italy [22]. The drugs were solubilized in dimethylsulphoxide (DMSO) (Sigma Chemical) and freshly diluted in culture medium before each experiment. The final DMSO concentration never exceeded 0.1% and this condition was used as a control in each experiment.

Mouse monoclonal antibody specific for β -actin and rabbit polyclonal antibody specific for aromatase were purchased from Sigma Chemical Co. (MO, USA). Rabbit polyclonal antibody specific for ERK1, $ER\alpha$, pAkt1/2/3 or pGSK3 β , mouse monoclonal antibody specific for PTEN, COX-2, pERK1/2 or PI3K-p85, goat polyclonal antibodies specific for PP2A, pERK or Akt1/2/3, goat anti-rabbit, goat anti-mouse and rabbit anti-goat secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. (CA, USA). Anti-rabbit Cy3-conjugated secondary antibody was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden), while anti-goat Cy3-conjugated secondary antibody was from Sigma Chemical Co. (MO, USA). The Transam ER kit was obtained from Vinci-Biochem, (Firenze, Italy).

Cell culture

MDA-MB-231 and MCF-7 breast cancer cell lines were obtained from American Type Culture Collection (ATCC, USA). The cells were cultured at 37°C in a humidified incubator with 5% CO₂ and 95% air in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% foetal bovine serum (FBS), 100 U/ml penicillin, 100 μ g/ml streptomycin and 25 μ g/ml amphotericin B.

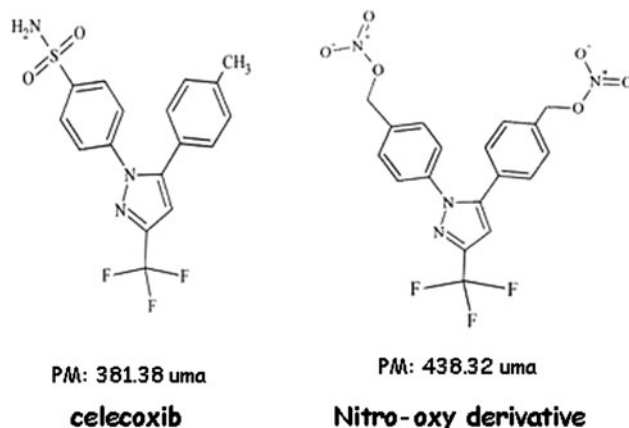


Fig. 1 Molecular structure of celecoxib and its nitro-oxy derivative

For treatments, cells were seeded at a density of 3×10^4 cells/cm² and cultured for 24 h to allow them to adhere to the substratum. The medium was then replaced with DMEM supplemented with the drugs or DMSO.

Proliferation assay

Cells were seeded in 12-well culture plates and properly treated. Cell viability was assessed by the trypan blue exclusion assay. Aliquots of cell suspension were incubated with trypan blue solution (0.5% in NaCl) for 5 min. Finally, cells were transferred to the Bürker chamber and counted by light microscope. Dead cells were defined as those stained with the dye.

Western blot analysis

Cells were seeded in 75 cm² plates and properly treated. Collected cells were suspended in lysis buffer containing 20 mM Tris/HCl (pH 7.4), 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 0.1 mM phenylmethyl-sulphonyl fluoride, 0.05% aprotinin, 0.1% Igepal and then incubated for 30 min at 4°C. The suspension was centrifuged for 25 min at 12,000 rpm; the supernatant from this centrifugation was saved as the total extracts.

Total extracts were measured using a commercially available assay (Protein Assay Kit 2, Biorad) with bovine serum albumin as a standard. Extracts were then subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis on 12, 10 or 7.5% acrylamide gels. Proteins were transferred onto nitrocellulose for 2 h in a Biorad electroblotting device. Nitrocellulose matrices were blocked with 5% milk in TBST (1 M Tris buffer saline, pH 7.4, 5 M NaCl, 0.1% Tween-20) buffer for 1 h at room temperature. For immunodetection, blocked matrices were incubated overnight at 4°C with primary antibody, incubated with peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulins in Tris-buffered saline–Tween containing 2% (wt/vol) non-fat dry milk for 1 h at room temperature and developed with the enhanced chemiluminescence reagents. Band intensities were quantified by densitometry and the expression of proteins was reported as a proportion of β -actin, Akt1/2/3 or ERK1 protein expression to monitor any discrepancies in gel loading (VersaDoc Imaging System 3000, Biorad).

Fluorescence microscopy

Cells were seeded on 6-well culture plates, allowed to adhere for 24 h and then treated. After treatment, the cells were fixed and permeabilized for 20 min at -20°C with methanol/acetone (1:1). Cells were then incubated with

primary antibody specific for ER α , pER α or aromatase followed by anti-rabbit or anti-goat Cy3-conjugated secondary antibody. Then the cells were stained with 4',6-diamidino-2-phenylindole (DAPI, 1 mg/ml in methanol) for 30 min at 37°C to detect nuclei. After washings with PBS, slides were mounted with H₂O/glycerol (1:1) and viewed under a fluorescence microscope equipped with a UV light filter (Dialux 20, Leitz).

ER transcription factor assay

Cells were seeded in 75 cm² plates and properly treated. The Nuclear Extract Kit (Active Motif) was developed for the preparation of nuclear extract from cells.

ER α transactivation was checked by Transam ER Kit (Active Motif), a highly sensitive ELISA based assay, according the manufacturer's directions. 5 μg of nuclear extracts of treated or untreated MCF-7 cells were added into a 96-well plate to which oligonucleotide containing a consensus-binding site was immobilized. ER α binds specifically to the oligonucleotide (5'-GGTCACAGTGACC-3'). The revelation was done by incubation with a primary antibody that recognized an accessible epitope on ER α protein upon DNA binding, followed by incubation with an HRP-conjugated secondary antibody. After incubation with standard developing solution, the samples were quantified by an ELISA plate reader (450 nm). Positive and negative controls were run in parallel.

Migration assay

Cells were seeded on 6-well culture plates, and properly treated. After treatment, cell migration was evaluated with Boyden chambers equipped with 8 μm porosity polyvinylpyrrolidone-free polycarbonate filters that were coated with 50 $\mu\text{g}/\text{ml}$ of Matrigel solution. With a Zeiss microscope (Oberkochen, Germany) equipped with bright field optics (40 \times) invasiveness was quantified by counting crystal violet-stained cells that invaded Matrigel. For each filter/Matrigel, the number of cells in 10 randomly chosen fields was counted, and the counts were averaged (means \pm SD). Results are expressed as the number of migrated cells per high-power field.

Statistical analysis

Differences between the means were analyzed for significance using the one-way ANOVA test with Bonferroni post hoc multiple comparisons used to assess the differences between independent groups. All values are expressed as means \pm SD, and differences were considered significant at $P < 0.05$.

Results

Effect on cell viability and on ERK–MAPK pathway

To assess the effect exerted by celecoxib and its nitro-oxy derivative on human breast cancer cell viability, MCF-7 (Fig. 2A) and MDA-MB-231 (Fig. 2B) breast cancer cells were treated with increasing concentrations (1–50 μM) for 48 h. In both cell lines the inhibition of viability was dose-dependent and characterized by a significant decrease of total and living cells as compared to control conditions. In all untreated and drug-treated cells the percentage of trypan blue positive cells was <10% respect to total cell number (not shown), indicating that no cytotoxic effect was induced by the drugs. The most effective condition in inhibiting cell survival was detected after 48 h treatment with celecoxib and derivative at 25 and 50 μM concentrations, an experimental condition that was used for all further experiments. In both cell lines neither celecoxib nor its derivative induced apoptosis as they did not affect bak,

procaspase and bax expression. These results were confirmed by TUNEL assay and cytofluorimetric analysis (data not shown).

To explore the nature of the antiproliferative activity exhibited by celecoxib and its nitro-oxy derivative, we analyzed their effects on the ERK/MAPK pathway, a large network of signalling molecules regulating cell growth and differentiation. As regards MCF-7 cells, in accordance with the results obtained from the cell viability assay, both drugs determined a down-regulation of the phosphorylation state of ERK, with a most pronounced reduction at 50 μM concentration. The reduction of the active form of ERK most likely resulted in the reduction of c-myc expression, a downstream target of the ERK cascade involved in the regulation of cell proliferation (Fig. 2C).

On the contrary, at the same experimental conditions, celecoxib and its derivative were not effective on the phosphorylation state of ERK and on c-myc expression level in MDA-MB-231 cells (Fig. 2D).

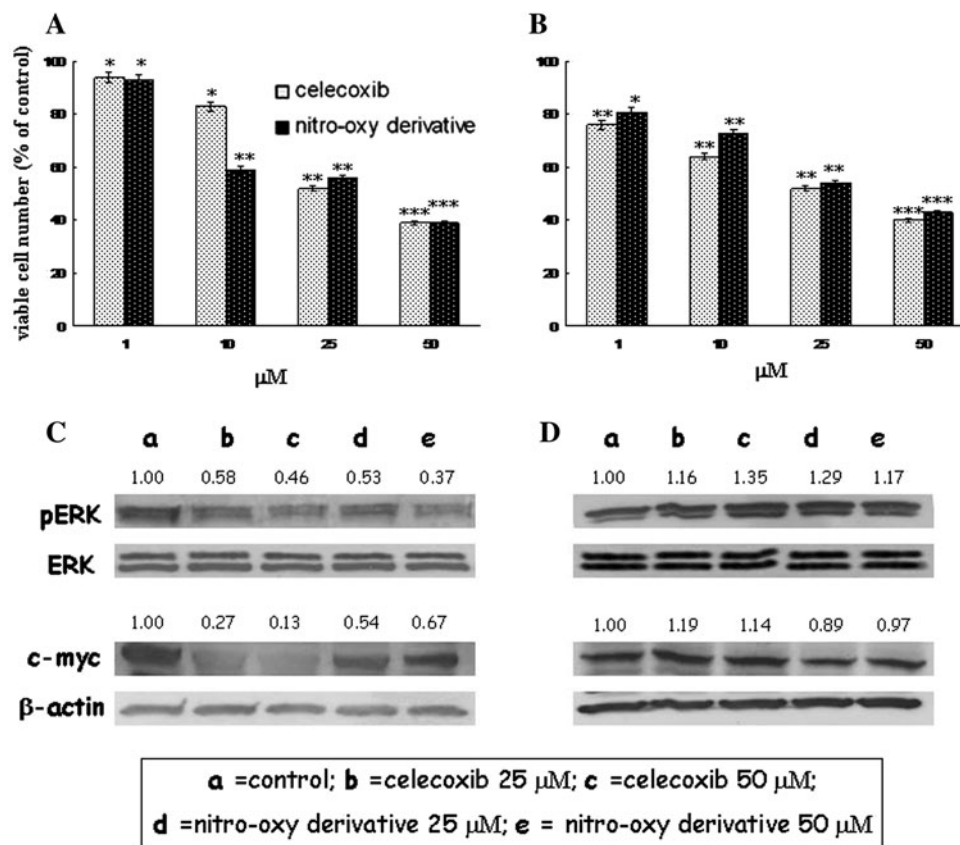


Fig. 2 Effect on cell growth and on ERK pathway. MCF-7 (A) and MDA-MB-231 (B) cells were incubated for 48 h with different concentrations of the drugs (1–50 μM). Cell viability was determined by the trypan blue exclusion test and calculated by standardizing viable untreated cells to 100%. The values represent the mean of three independent experiments, each performed in triplicate (bars, SD). Statistical significance compared with untreated control: * $P < 0.05$,

** $P < 0.01$, *** $P < 0.001$, by one-way ANOVA test with the Bonferroni. MCF-7 (C) and MDA-MB-231 (D) cells were exposed for 48 h to the drugs (25 and 50 μM) and processed for western blot analysis with anti-pERK1/2 or anti-c-myc antibody. Protein contents were normalized with anti-ERK1 or anti- β -actin antibody and quantified by densitometry (SD < 10%). The blots shown are from a representative experiment repeated three times with similar results

Effect on PI3K/Akt signalling pathway

The PI3-kinase pathway is involved in the regulation of cell survival and apoptosis, and its activation is associated with tumour growth and progression [23]. In line with the data regarding ERK phosphorylation, in MCF-7 cells celecoxib and its derivative decreased the expression of p85 α , the regulatory subunit of PI3-kinase pathways, and phospho-Akt (active form), the downstream effector of the cascade (Fig. 3A), while in MDA-MB-231 cells no alteration was revealed after incubation with both drugs (Fig. 3B).

The activation of PI3-kinase pathways also induces a phosphorylation and inactivation of GSK3 β , followed by nuclear translocation of β -catenin and SNAIL and down-regulation of E-cadherin that can increase cellular proliferation and invasiveness [24]. In accordance with the results on the Akt cascade, in MCF-7 cells only the exposure to celecoxib resulted in a decrease of phosphorylation of GSK3 β , while its derivatives was not able to activate GSK3 β (Fig. 3A). These results demonstrated that there was a correlation between Akt inactivation and GSK3 β activation.

Thus, to better clarify the effect of celecoxib and its derivative on the PI3K/Akt and ERK/MAPK cascades, we analyzed the expression level of PTEN and PP2A, both involved in the regulation of these pathways. As expected,

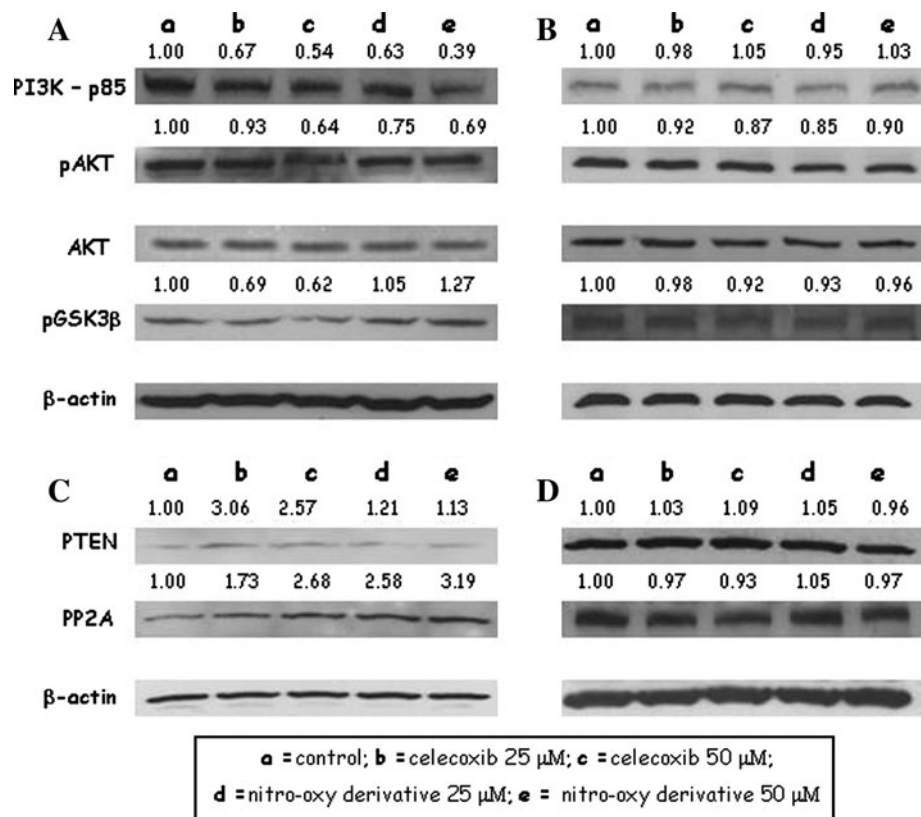
in MCF-7 cells celecoxib and its derivative trigger a strong increase in PP2A expression level, while only celecoxib is able to induce PTEN expression (Fig. 3C). On the contrary, the expression of both phosphatases is not modified in MDA-MB-231 cells (Fig. 3D).

Involvement of the ER α pathway

Since celecoxib and its derivative inhibited ERK/MAPK and PI3K/Akt cascades and induced PP2A and PTEN expressions only in the ER α (+) MCF-7 cells we evaluated the possibility of cross-talk between these drugs and the ER α signalling pathway. It is well known [25, 26] that the transcriptional activity of ER α depends on its phosphorylation status. In this regard, the best understood phosphorylation-related event involves MAPK and Akt, and the dephosphorylation involves phosphatases such as PP2A.

Initially we sought to determine if celecoxib and its derivative may affect the activation state of ER α through the evaluation of the serine phosphorylation state of the receptor as well as of the total ER α expression level. As shown in Fig. 4A, 48 h treatment with celecoxib and its derivative at all the concentrations tested strongly reduces both the phosphorylation level and the total expression of ER α . The phosphorylation status of ER α is closely linked with oestrogen response element (ERE) binding and with

Fig. 3 Effect on PI3K/Akt pathway. MCF-7 (A, C) and MDA-MB-231 (B, D) cells were treated for 48 h with the drugs (25 and 50 μ M). Total cell lysates were probed with anti-PI3K-p85, anti-pAkt, anti-pGSK-3 β , anti-PTEN or anti-PP2A antibody. Protein contents were normalized with anti-Akt or anti- β -actin antibody and quantified by densitometry (SD < 10%). The blots shown are from a representative experiment repeated three times with similar results



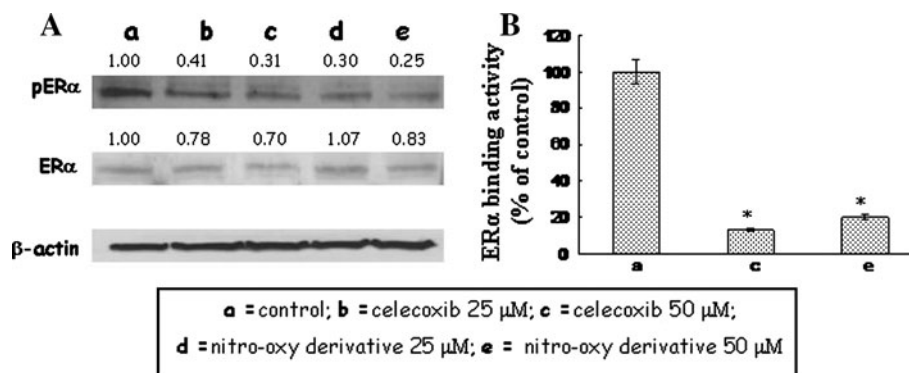


Fig. 4 Effect on ER α expression and transactivation. In **A**, MCF-7 cells were treated for 48 h with the drugs (25 and 50 μ M). Total cell lysates were probed with anti-pER α or anti-ER α antibody, normalized with anti- β -actin antibody and quantified by densitometry (SD < 10%). The blots shown are from a representative experiment repeated three times with similar results. In **B**, nuclear lysates of MCF-7 cells,

treated as described in **A**, were probed with primary antibody specific for the active form of bound ER α , and then with HRP-conjugated secondary antibody. The values represent the mean of three independent experiments each performed in triplicate (bars, SD). Statistical significance compared with untreated control: * P < 0.001, by one-way ANOVA test with the Bonferroni

the subsequent transcriptional activation of target genes involved in cell growth [27]. The results from western blotting analysis were confirmed by transactivation assays as nuclear extracts from MCF-7 cells treated with 50 μ M celecoxib and its derivative showed decreased binding activity to the canonical ERE (Fig. 4B).

The activation state of ER α was also evaluated through the analysis of ER α cellular immunolocalization. Immunofluorescence staining showed that treatment of MCF-7 cells with 25 and 50 μ M celecoxib and its derivative (Fig. 5) induces ER α translocation from the nucleus to the cytoplasm and cell membrane. ER α translocation was accompanied with a reduction of the nuclear localization of the active form of the receptor (phosphorylated form: pER α) (Fig. 6) and, consequently, of ER α transcriptional activity. In fact, the phosphorylation of ER α and the consequent translocation to the nucleus represents a mechanism whereby the activity of ER α can be enhanced by hormone independent activation.

COX-2 and aromatase regulation

Over-expression of COX-2 has been shown to contribute to tumourigenesis [28], and has the potential to induce aromatase expression in different cancer cells, including breast cancer cells [9]. To investigate the possible link between COX-2 and aromatase in breast cancer cells, we analyzed the expression status of these proteins in MCF-7 and MDA-MB-231 cells, which exhibit low and high COX-2 expression levels, respectively.

Western blot analysis showed that in MCF-7 cells celecoxib and derivative exerted a weak downregulation of COX-2 protein (Fig. 7A), while in MDA-MB-231 cells the inhibitory effect on COX-2 expression was significant, above all after cell treatment with nitro-oxy derivative at

50 μ M concentration (Fig. 7B). In MDA-MB-231 cells, the ability of celecoxib to inhibit PGE₂ release is comparable to that of nitro-oxy derivative as confirmed by Amersham PGE₂ EIA System (data not shown).

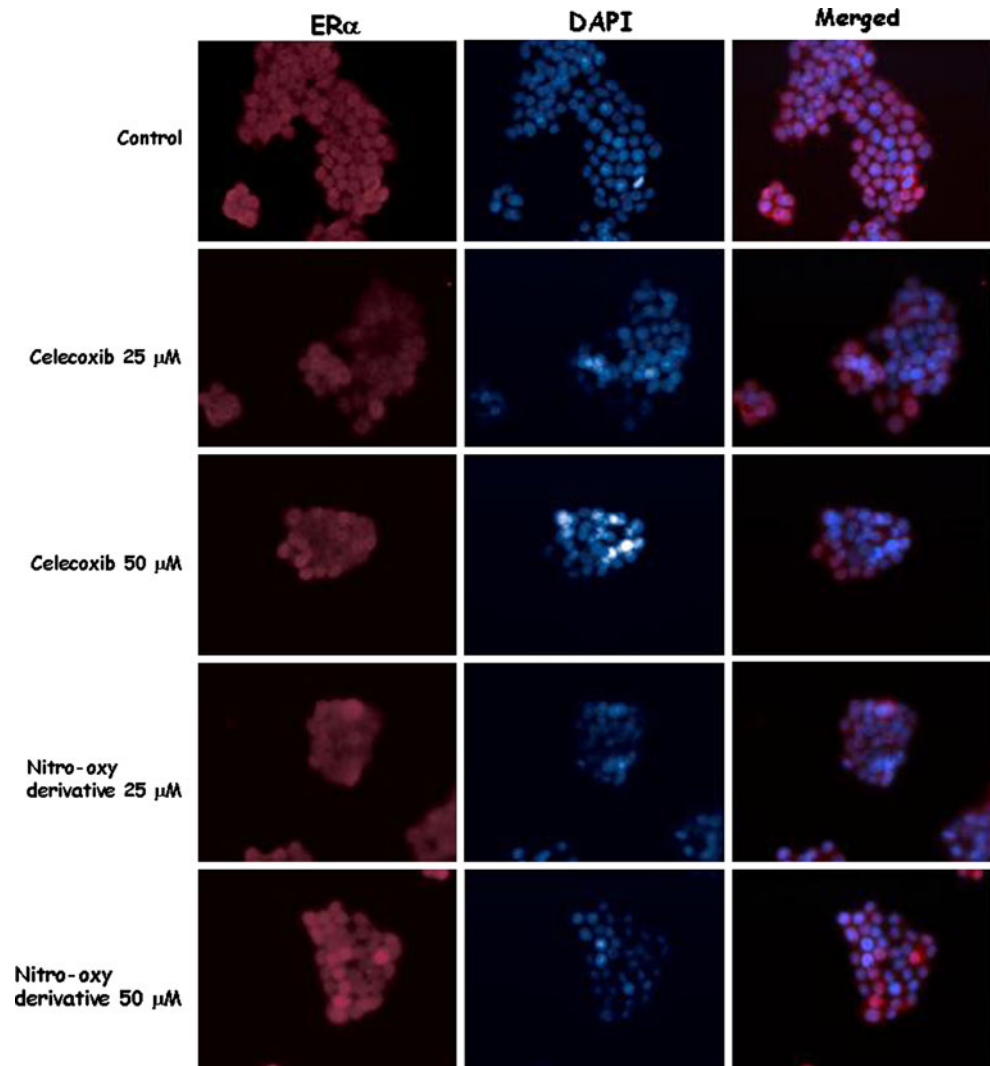
In accordance with the results on COX-2 expression, celecoxib and its derivative strongly inhibited aromatase expression in MDA-MB-231 cells and, in this case as well, the main reduction was detected after cell treatment with nitro-oxy derivative at 50 μ M concentration (Fig. 7B), while in MCF-7 cells the reduction was lower (Fig. 7A). In addition, in MCF-7 cells the up-regulation of COX-2 induced by the administration of 10 nM 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) for 24 h was not associated with aromatase over-expression (data not shown).

The effect of celecoxib and its derivative on aromatase expression was also confirmed by the analysis of aromatase intracellular distribution. In MCF-7 cells, only 50 μ M celecoxib was able to reduce aromatase expression at cytoplasmic level distributions (Fig. 8) while in MDA-MB-231 cells both drugs induce a strong decrease of aromatase expression in the cytoplasmic compartment as compared to control cells (Fig. 9).

Effect on invasiveness

Over-expression of aromatase is highly related to the aggressiveness of breast cancer cells including invasion and metastasis [29]. Since celecoxib and its derivative reduced aromatase expression levels, we determined the ability of these compounds to reduce breast cancer cell migration using the classic Boyden chamber assay. Figure 10A, B showed that in both cell lines celecoxib and its derivative at 10 μ M concentration significantly decreased cell invasiveness, quantified by counting the number of cells that invaded Matrigel.

Fig. 5 Effect on intracellular localization of ER α . MCF-7 cells were incubated with the drugs (25 and 50 μ M) for 48 h, and then exposed to anti-ER α antibody followed by anti-rabbit or anti-goat Cy3-conjugated secondary antibody. To detect nuclei the cells were stained with DAPI. The *right panels* show the overlaid pictures ($\times 400$ final magnification). The images shown are from a representative experiment repeated three times with similar results



Discussion

Breast cancer is the most common malignancy in women worldwide with over one million cases diagnosed each year [30].

The importance of COX-2 in breast tumourigenesis is suggested by the fact that breast tumours, but not normal mammary epithelium, highly express the enzyme; COX-2 overexpression is associated with multifunctional activity in cancer biology, such as tumour aggressiveness and growth [4, 31]. Additionally, high COX-2 levels were sufficient to induce mammary tumourigenesis in transgenic mice [32], while the inhibition of COX-2 activity exerted protective effects following the tumourigenesis process in animal models of breast cancer [33]. COX-2 expression is regulated through the activation of ERK1/2 and PI3K/Akt in HER-2/neu transformed cells, whereas the amount of COX-2 was significantly decreased by inhibitors of these pathways [34].

As regards PI3K/Akt signalling pathways, activation of the Akt in breast tumour samples is associated with resistance to endocrine therapy and worse outcomes in breast cancer patients [35, 36]. Akt signalling plays a crucial role in the initiation and progression of breast cancer and also regulates several downstream targets that are responsible for cell proliferation and survival.

In our work we observed that celecoxib, a selective COX-2 inhibitor, and its derivative exert a strong inhibitory effect on the phosphorylation state of ERK in ER α (+) MCF-7 cells, while they are ineffective in ER α (-) MDA-MB-231 cells. Our results correspond well with reports showing that inhibition of the MAPK pathway significantly reduces c-myc protein levels and cell growth [37]. In fact, in MCF-7 cells we found reduced levels of the transcription factor c-myc and a lack of apoptosis (data not shown).

At the same time, in MCF-7 cells celecoxib and its derivative induce the reduction of p85 levels, a reduction that leads to the inhibition of Akt, the downstream target of

Fig. 6 Effect on intracellular localization of pER α . MCF-7 cells were incubated with the drugs (25 and 50 μ M) for 48 h, and then exposed to anti-pER α antibody followed by anti-rabbit or anti-goat Cy3-conjugated secondary antibody. To detect nuclei the cells were stained with DAPI. The right panels show the overlaid pictures ($\times 400$ final magnification). The images shown are from a representative experiment repeated three times with similar results

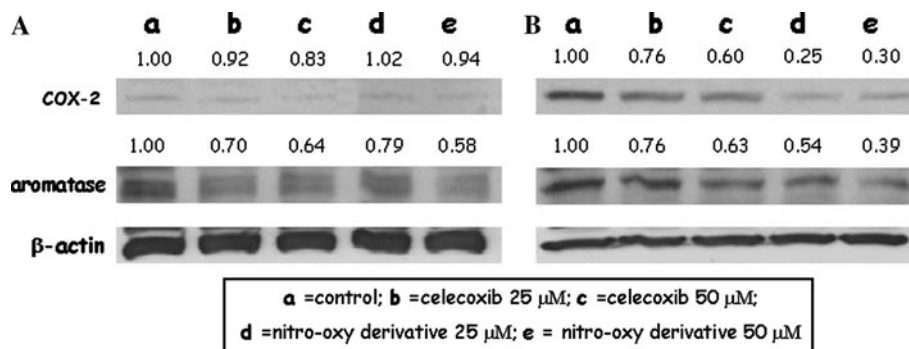
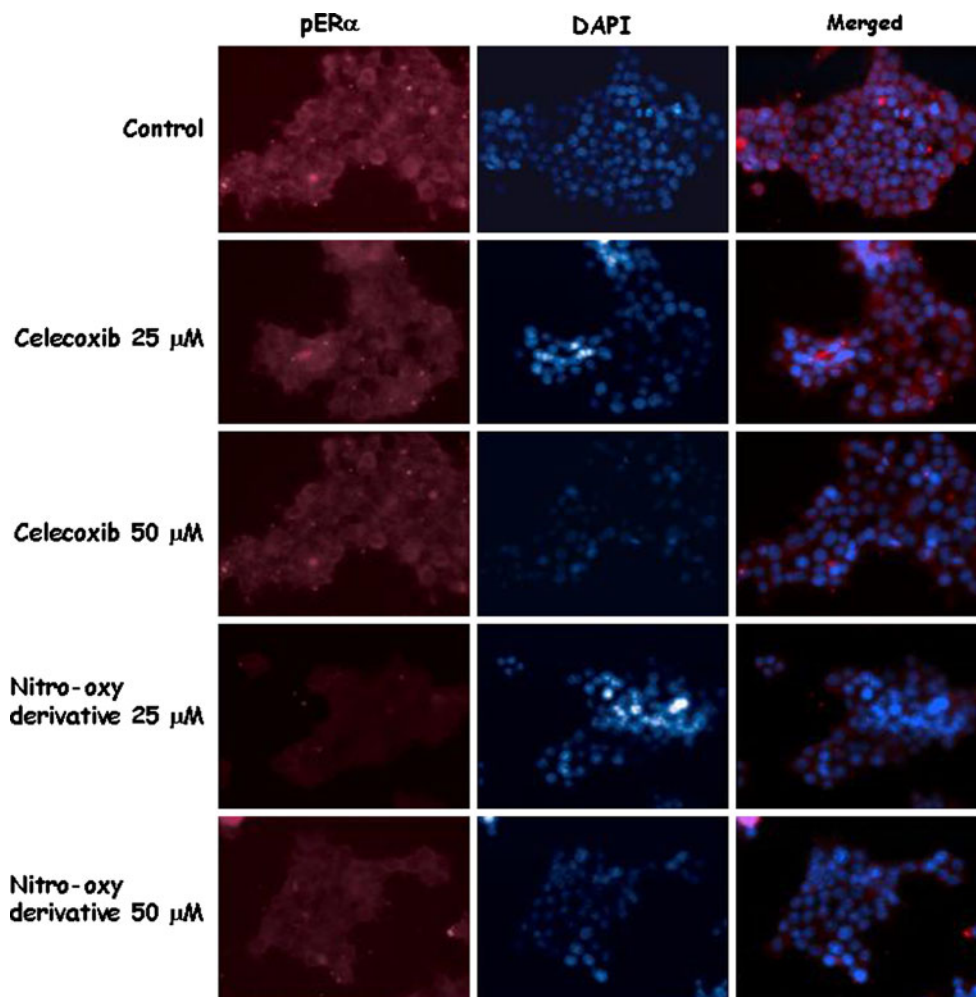


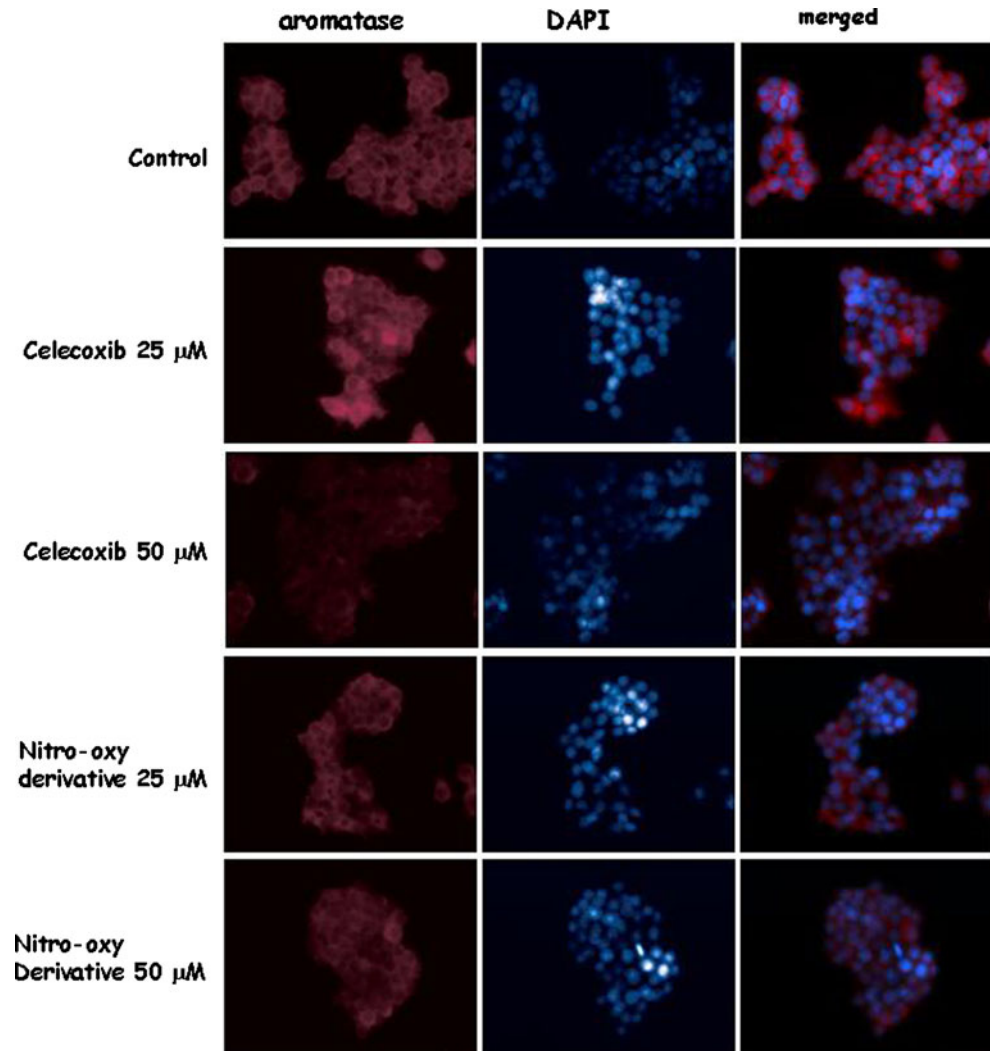
Fig. 7 Effect on COX-2 and aromatase expression. MCF-7 (A) and MDA-MB-231 (B) cells were incubated for 48 h with the drugs (25 and 50 μ M). Total cell lysates were probed with anti-COX-2 or anti-

aromatase antibody, normalized with anti- β -actin antibody and quantified by densitometry (SD < 10%). The blots shown are from a representative experiment repeated three times with similar results

PI3K, while in MDA-MB-231 cells they are ineffective. These findings, together with the observation that MCF-7 cells are characterized by low COX-2 levels, suggest that celecoxib mediates antiproliferative effects through the inhibition of Akt and ERK signalling independently of COX-2.

ERK and PI3K/Akt, as well as PP2A and PTEN, exert a crucial role in the regulation of ER α activation. ERK and Akt up-regulate ER α , transcriptional activity [38, 39] by phosphorylating discrete residues of the receptors, while PP2A and PTEN down-regulate ER α by dephosphorylating them.

Fig. 8 Effect on aromatase intracellular localization in MCF-7 cells. MCF-7 cells were treated for 48 h with the drugs (25 and 50 μM) and exposed to anti-aromatase antibody followed by anti-rabbit Cy3-conjugated secondary antibody. To detect nuclei cells were stained with DAPI. The *right panels* show the overlaid pictures ($\times 400$ final magnification). The images shown are from a representative experiment repeated three times with similar results



ERs are localized to many sites within the cell, potentially contributing to overall oestrogen action. In the nucleus, ERs mainly modulate gene transcription and the resulting protein products determine the cell biological actions of the sex steroid. In addition, a small pool of ERs localizes to the plasma membrane and its signal is mainly coupled to G proteins. Cross-talk from membrane-localized ERs to nuclear ERs can be mediated in a steroid ligand-independent fashion [16] through growth factor receptor tyrosine kinases, such as epidermal growth factor receptor and IGF-I receptor. Kinase-induced phosphorylation of nuclear ER α enhances cyclin D1 transcription and, consequently, promotes breast cancer cell proliferation [13].

Our data indicate that in ER α (+) MCF-7 cells celecoxib and its nitro-oxy derivative reduce ER α phosphorylated form and consequently, ER α transcriptional activity by promoting phosphatase expression and by inactivating signal transduction pathways (ERK and Akt) that up-regulate ER α activation. The consequence of these events is the decrease of MCF-7 cell growth.

In addition, PI3K/Akt inhibition induced by celecoxib and its derivative reduce the phosphorylation level of GSK-3 β and, consequently, promoted the repressive effect of GSK-3 β on ER α phosphorylation and activation.

It has been reported that aromatase gene expression is correlated with COX-1 and COX-2 levels in human breast cancer and that COX-2 inhibitors can suppress aromatase activity in breast cancer cells by suppressing aromatase transcription [9]. This biochemical mechanism may explain epidemiological observations of the beneficial effect of nonsteroidal anti-inflammatory drugs on breast cancer. Aromatase catalyzes the formation of aromatic C18 oestrogens from C19 androgens and is considered responsible local production of oestrogen in breast cancer. Several studies from transfected breast cancer cells and from transgenic mouse which overexpressed aromatase demonstrated that in situ produced oestrogen plays more important roles than circulating oestrogens in breast tumour promotion and progression [32]. From our results, celecoxib and its derivative reduce aromatase expression in

Fig. 9 Effect on aromatase intracellular localization in MDA-MB-231 cells. MDA-MB-231 cells were incubated for 48 h with the drugs (50 μ M) and exposed to anti-aromatase antibody followed by anti-rabbit Cy3-conjugated secondary antibody. To detect nuclei cells were stained with DAPI. The right panels show the overlaid pictures ($\times 400$ final magnification). The images shown are from a representative experiment repeated three times with similar results

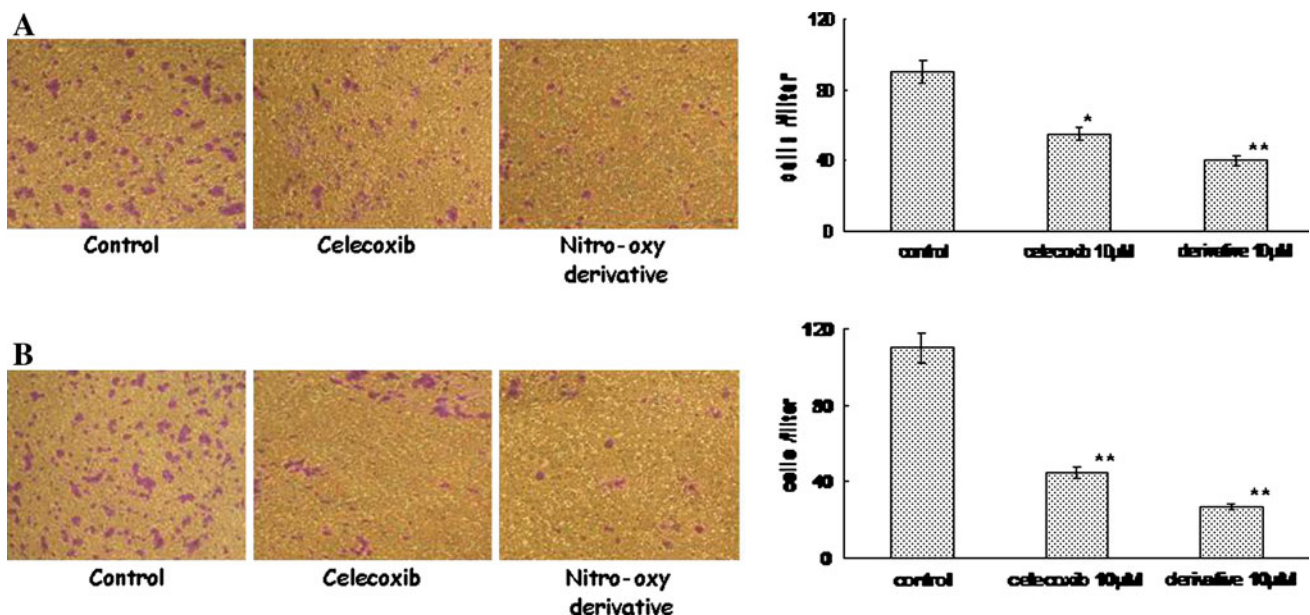
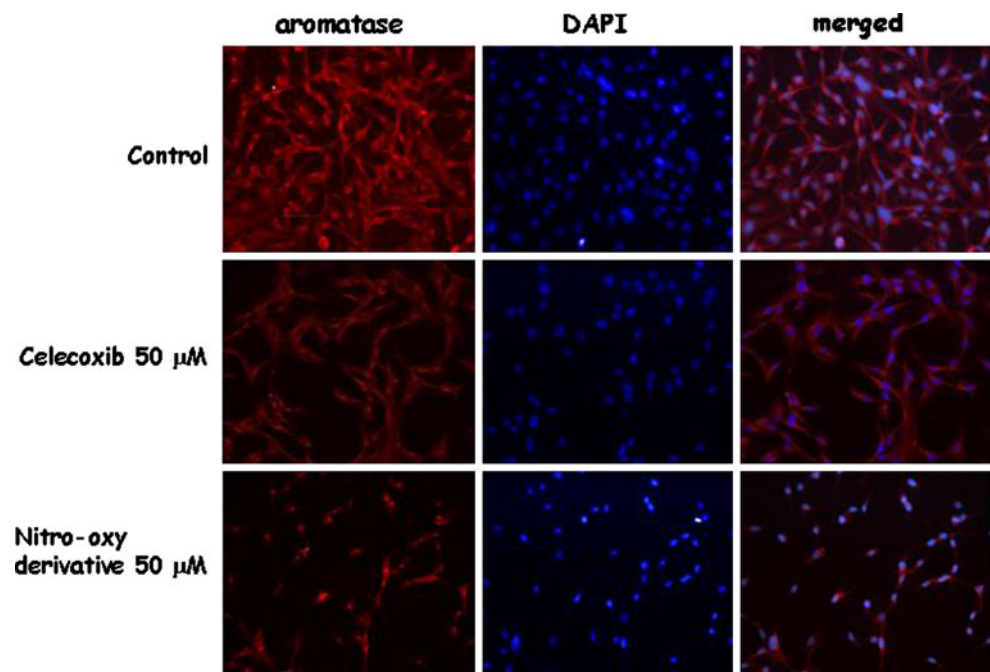


Fig. 10 Effect on cell migration. MCF-7 (A) and MDA-MB-231 (B) were incubated with the drugs (10 μ M) for 48 h. Matrigel invasion was evaluated with a Boyden chamber assay; each filter was examined with a Zeiss microscope and the number of cells was counted. Results are expressed as number of migrated cells (means)

per high-power field. The values represent the mean of three independent experiments, each performed in triplicate (bars, SD). Statistical significance compared with untreated control: * $P < 0.01$, ** $P < 0.001$, by one-way ANOVA test with the Bonferroni

both cell lines with a more marked effect in ER α (-) MDA-MB-231 cells that express high COX-2 level. These data are in agreement with literature data and suggest a correlation between COX-2 and aromatase expression in ER α (-) MDA-MB-231 cells.

In conclusion, we provided evidence that celecoxib and its nitro-oxy derivative significantly suppresses the in vitro growth of human breast cancer cells. In ER α (+) MCF-7 cells we also evidenced a pharmacological regulation of aromatase by celecoxib and its derivative, that can act to

decrease the ER α transcriptional activity. The suppression of ER α activation may be associated with ERK and Akt inhibition and with PP2A and PTEN induction. These effects are not detected in ER α (–) MDA-MB-231 cells in which, on the contrary, we showed a correlation between COX-2 and aromatase expression. Finally, the nitro-oxy derivative of celecoxib seems to exert antiproliferative effects similar to that detect with celecoxib, but it may implement gastric tolerance and reduce cardiotoxicity of the parent drug [40, 41].

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Conflicts of interest The authors declare that there are no conflicts of interest.

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