Cyclooxygenase-2 inhibitors prevent the development of chemoresistance phenotype in a breast cancer cell line by inhibiting glycoprotein p-170 expression

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Abstract

Breast cancer cells are usually sensitive to several chemotherapeutic regimens, but they can develop chemoresistance after prolonged exposure to cytotoxic drugs, acquiring a more aggressive phenotype. Drug resistance might involve the multi-drug resistance (MDR) 1 gene, encoding a transmembrane glycoprotein p-170 (P-gp), which antagonizes intracellular accumulation of cytotoxic agents, such as doxorubicin. We previously demonstrated that type 2 cyclooxygenase (COX-2) inhibitors can reverse the chemoresistance phenotype of a medullary thyroid carcinoma cell line by inhibiting P-gp expression and function. The aim of our study was to investigate the role of COX-2 inhibitors in modulating chemoresistance in a human breast cancer cell line, MCF7. MCF7 cells, expressing COX-2 but not MDR1, were treated with increasing doses of doxorubicin, and they became chemoresistant after 10 days of treatment, in association with MDR1 expression induction. This effect was reversed by doxorubicin withdrawal and prevented by co-incubation with N-[2-(cyclohexyloxy)4-nitrophenyl]-methanesulfonamide (NS-398), a selective COX-2 inhibitor. Treatment with NS-398 alone did not influence cell viability of a resistant MCF7 cell clone (rMCF7), but sensitized rMCF7 cells to the cytotoxic effects of doxorubicin. Moreover, treatment with NS-398 significantly reduced MDR1 expression in rMCF7 cells. Doxorubicin-induced membrane P-gp expression and function was also greatly impaired. Our data therefore support the hypothesis that COX-2 inhibitors can prevent or reduce the development of the chemoresistance phenotype in breast cancer cells by inhibiting P-gp expression and function.

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Introduction

The chemotherapeutic approach might become unsuccessful in several malignant tumors due to the development of chemoresistance. In breast cancer, chemoresistance often follows exposure to cytotoxic drugs and accompanies the progression from a hormone-dependent, nonmetastatic, antiestrogen-sensitive phenotype to a hormone-independent, invasive, metastatic, antiestrogen-resistant phenotype (Campbell et al. 2001). Understanding the mechanisms of drug resistance is the key to developing new therapeutic options for cancer.

Drug resistance might involve the multi-drug resistance (MDR) 1 gene, encoding a transmembrane glycoprotein p-170 (P-gp), which antagonizes intracellular accumulation of cytotoxic agents, such as doxorubicin (Doxo; Ambudkar et al. 1999, Yasui et al. 2004). We previously demonstrated that type 2 cyclooxygenase (COX-2) inhibitors can reverse the chemoresistance phenotype of a medullary thyroid carcinoma cell line by inhibiting P-gp expression and function (Zatelli et al. 2005a). It has been previously demonstrated that selective COX-2 inhibitors significantly reduce carcinogen-induced rat mammary tumors (Dannenberg & Howe 2003) and may have a role in chemoprevention (Arun & Goss 2004). Indeed, nonsteroidal anti-inflammatory drugs can reduce the risk of breast cancer by ~20% (Khuder & Mutgi 2001). The aim of our study was therefore to investigate the role of COX-2 inhibitors in modulating the development of drug resistance in breast cancer cells.
of the chemoresistance phenotype in a human breast cancer cell line, MCF7.

Materials and methods
MCF7 cell line
The MCF7 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in culture with Dulbecco’s Minimum Essential Medium (DMEM, Eagle) with 2 mM L-glutamine, supplemented with 10% fetal bovine serum at 37 °C in 95% air and 5% CO₂. The MCF7 cell line was derived from a pleural effusion of a patient with metastatic breast cancer (Soule et al. 1973), and it retains several characteristics of differentiated mammary epithelium, including the capability of forming domes and the ability to respond to estradiol via cytoplasmic estrogen receptors (Levenson & Jordan 1997). 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 2 months in the presence of Doxo 50 nM. The obtained cell strain was then cultured in DMEM supplemented with Doxo 5 nM.

Compounds
R(+)–verapamil hydrochloride and rhodamine 123 (R123) were purchased from Sigma. Doxorubicin hydrochloride (Adriblastin) was obtained from Pharmacia. 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.

Isolation of RNA and RT-PCR
In order to evaluate MDR1 and COX-2 expression in MCF7 cells, RT-PCR analysis was performed. Total RNA was isolated from subconfluent MCF7 cells using TRIzol reagent (Invitrogen), according to the manufacturer’s protocol, and was subjected to reverse transcription (RT) with random hexamers, as previously described (Zatelli et al. 2003, 2005b). To prevent DNA contamination, RNA was treated with ribonuclease-free DNase (Promega). The cDNA was then amplified by PCR using the PCR Master Mix (Promega), in the conditions recommended by suppliers. PCRs were carried on using the oligonucleotide primers and conditions previously described (Zatelli et al. 2005a). PCR products were analyzed on a 2% agarose gel and visualized by ethidium bromide staining. Each PCR product was subjected to restriction enzyme digestion and analyzed on 2% agarose gel to further confirm correct identification of the amplicons (data not shown).

Viable cell number assessment
Variations in cell number were assessed by the Cell Proliferation Kit (Roche), as previously described (Zatelli et al. 2002). MCF7 and rMCF7 cells were incubated without or with the test substances for the indicated time intervals. After incubation, the revealing solution was added and the absorbance at 560 nm was recorded using the Wallac Victor 1420 Multilabel Counter (Perkin–Elmer, Monza, Italy) in at least three experiments in six replicates.

Cell death assessment
The number of dead cells was evaluated by trypan blue dye exclusion assay, as previously described (Pon et al. 2005). Briefly, MCF7 cells treated as indicated were harvested by trypsinization. Cells were stained with 0.2% trypan blue solution (Sigma). Cell death was expressed as the percentage of trypan blue stained cells in treated versus control cells. At least 250 cells per treatment were counted, and assays were performed in six replicates at least three times.

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 (Tagliati et al. 2006). For P-gp protein evaluation, cell proteins were fractionated on 7.5% SDS-PAGE, as previously described (Sarkadi et al. 1992), and transferred by electrophoresis to Nitrocellulose Transfer Membrane (PROTRAN, Dassel, Germany). Membranes were incubated with an anti-P-gp monoclonal antibody (Sigma) at 1:500. Horseradish peroxidase-conjugated antibody IgG (Sigma) was used at 1:2000 and binding was revealed using enhanced chemiluminescence (Amersham Biosciences). For COX-2 protein evaluation, cell proteins were fractionated on 12% SDS-PAGE and transferred by electrophoresis to Nitrocellulose Transfer Membrane (PROTRAN). The membranes were incubated with an anti-COX-2 monoclonal antibody (Santa Cruz Biotechnology, M C Zatelli et al.: COX-2 inhibitors prevent chemoresistance

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Heidelberg, Germany) at 1:200. Horseradish peroxidase-conjugated antibody IgG (Sigma) was used at 1:2000 and binding was revealed using enhanced chemiluminescence (Amersham Biosciences). The blots were then stripped and used for further blotting with anti-actin antibody (Sigma).

**Rhodamine 123 assay**

R123 assay was performed as previously described (Zatelli et al. 2005a). Briefly, MCF7 cells were seeded in 24-well plates at 2×10^5 cells/well 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 thrice with 1 ml of ice-cold serum-free culture medium to remove any extracellular R123. Cells were then incubated with serum-free culture medium with or without 100 μM verapamil at 37 °C. After 1 h, cells were washed, solubilized with NaOH 0.2 M overnight, and assayed for R123 and protein contents as described above. The concentration of R123 in each sample was determined quantitatively by fluorescence spectroscopy using the Wallac Victor 1420 Multilabel Counter (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.

**Fluorescence microscopy**

Native and resistant MCF7 (MCF7 and rMCF7) cells were cultured for 10 days in culture medium supplemented without or with 5 nM Doxo, 1 μM NS-398 or both. Cells (2×10^4/well) were then seeded in 8-well chamber slides (Lab-Tek Chamber Slide System, Nalgene Nunc International, Naperville, IL, USA) and incubated 30 min in a humified atmosphere at 37 °C with a monoclonal anti-human P-gp antibody (1:500; Sigma). The cells were then fixed in methanol–acetone (1:1) for 10 min 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) for 45 min at room temperature. Slides were then mounted with the ProLong Gold antifade reagent (Invitrogen Molecular Probes) containing DAPI under glass coverslips (Menzel-Glaser). The slides were examined for Doxo fluorescence using fluorescence microscopy, as described above, with the Nikon TRITC filter. Nuclear staining with DAPI was detected with the Nikon u.v. filter. Images of control and treated cultures were acquired using the DS-5M Nikon color CCD digital camera and cell fluorescence was analyzed and quantified with the Multi-Analyst software (Bio-Rad). Values were expressed as mean fluorescence (arbitrary units) per cell ± S.E.M., corrected for background, as previously described (van Wijngaarden et al. 2007). All experiments were carried out at least three times independently, analyzing 50±10 individual cells.

**Statistical analysis**

Results of cell viability experiments are expressed as the mean ± S.E.M. 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 ± S.E.M. 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.

**Results**

**MDR1 and COX-2 expression in MCF7 cells**

In order to assess MDR1 and COX-2 expression in MCF7 cells, the expression of these two genes was evaluated in MCF7 cells by RT-PCR. As shown in Fig. 1A, the native
representative of at least three different experiments. Bands are indicated with an arrow. Displayed data are SDS-PAGE, transferred on nitrocellulose membrane, and (panel C). Cell extracts (100 μg) from native MCF7 cells were fractionated on 7.5% SDS-PAGE, transferred on nitrocellulose membrane, and incubated with an anti-P-gp antibody positive control; ct (panel A) and COX-2 (panel B; lane M, 100-bp PCR marker; expected PCR products are indicated with arrows for MDR1 and COX-2. PCR products were resolved on a 2% agarose gel. The were subjected to subsequent PCR amplification of MDR1 and COX-2 expression in MCF7 cells. Isolated RNA (1 μg/reaction) from native MCF7 cells was treated with DNase and subjected to RT. Aliquots from the generated cDNA were subjected to subsequent PCR amplification of MDR1 and COX-2. PCR products were resolved on a 2% agarose gel. The expected PCR products are indicated with arrows for MDR1 (panel A) and COX-2 (panel B; lane M, 100-bp PCR marker; MCF7, native MCF7 cell line; TT, TT cell line, considered as positive control; ct-, negative control). Whole cell extracts (100 μg) from native MCF7 cells (MCF7) and from TT cells (TT) were fractionated on 7.5% SDS-PAGE, transferred on nitrocellulose membrane, and incubated with an anti-P-gp antibody (panel C). Cell extracts (100 μg) were also fractionated on 12% SDS-PAGE, transferred on nitrocellulose membrane, and incubated with an anti-COX-2 antibody (panel D). The expected bands are indicated with an arrow. Displayed data are representative of at least three different experiments.

MCF7 cell line lacks MDR1 expression, while it expresses COX-2 at mRNA level (Fig. 1B). These data are further strengthened by the results of western blot analysis for P-gp (Fig. 1C), showing that P-gp protein is expressed in TT cells but not in MCF7 cells. On the contrary, COX-2 protein was demonstrated in the protein extracts of both cell lines (Fig. 1D).

**MCF7 chemoresistance**

In order to evaluate whether native MCF7 cells are resistant to treatment with chemotherapeutic agents, cells were treated with Doxo at concentrations ranging from 5 nM to 50 μM up to 10 days. As shown in Fig. 2A, Doxo significantly reduced native MCF7 cell number at concentrations ranging from 50 nM (P < 0.05) to 50 μM (P < 0.01) after 3 and 7 days. However, after 10 days MCF7 cell viability significantly increased under treatment with Doxo at 5 and 50 nM (P < 0.01), while it decreased for higher Doxo concentrations. In parallel, cell death was evaluated by trypan blue dye exclusion assay. Figure 2B shows that Doxo significantly induced native MCF7 cell death at concentrations ranging from 50 nM (P < 0.05) to 50 μM (P < 0.01) after 3 and 7 days. However, after 10 days cell death was not significantly modified under treatment with Doxo at 50 nM, while it significantly increased for higher Doxo concentrations.

These data indicate that, after prolonged exposure to the cytotoxic drug, MCF7 cells became resistant to low doses of Doxo.

In order to verify whether the chemoresistance phenotype was associated with MDR1 gene expression induction, total RNA was isolated from MCF7 cells cultured for 10 days in the presence of 50 nM Doxo. RT-PCR analysis then showed evidence for MDR1 expression in these cells (Fig. 2C). These data are further strengthened by the results of western blot analysis for P-gp (Fig. 2D), showing P-gp protein expression in MCF7 cells cultured for 10 days in the presence of 50 nM Doxo.

To verify whether P-gp was functional in MCF7 cells expressing the efflux pump after treatment with 50 nM Doxo up to 10 days, R123 assay was performed on these cells treated without or with 100 μM verapamil (V) for 1 h. As shown in Fig. 2E, R123 was retained in untreated native MCF7 cells throughout the experiment, and incubation with verapamil did not modify R123 intracellular accumulation, in keeping with the absence of the efflux pump in these cells. R123 retention in native MCF7 cells was also not modified after 3 and 7 days of treatment with 50 nM Doxo. On the contrary, in MCF7 cells treated with 50 nM Doxo for 10 days R123 accumulation significantly decreased (−68%; P < 0.01), an effect completely abrogated by incubation with V, in keeping with P-gp expression and function in these cells.

**Effects of NS-398 on MDR1 gene expression and P-gp protein levels**

To verify whether MDR1 gene expression could be influenced by incubation with the COX-2 inhibitor NS-398, PCR analysis was performed on cDNA from MCF7 cells previously treated for 10 days without or with 1 μM NS-398, 50 nM Doxo, or both. As shown in Fig. 3A, MDR1 expression was not detected in untreated MCF7 cells and in cells treated with NS-398, while it was apparent in cells treated with 50 nM Doxo.

In order to verify whether the observed effects at mRNA level are mirrored by parallel changes in P-gp protein levels, western blot analysis for P-gp was performed with protein extracts from MCF7 cells treated for 10 days without or with 1 μM NS-398, 50 nM Doxo, or both. As indicated in Fig. 3B, P-gp immunoreactivity was evident only in protein extracts from cells treated with 50 nM Doxo. Again, treatment with NS-398 did not influence basal P-gp protein...
levels, but was capable of completely blocking Dox-induced P-gp protein expression.

To evaluate whether the observed effects of the selective COX-2 inhibitor on P-gp involve protein expression at the membrane level, the presence of P-gp was investigated by immunofluorescence on native MCF7 cells treated for 10 days without or with 50 nM Doxo, or both. As shown in Fig. 4 (Panel A), in native MCF7 cells no specific membrane immunofluorescence for P-gp was detected in untreated control cells, as well as in cells treated with NS-398 alone. On the contrary, treatment with Doxo clearly induced P-gp expression at the membrane level, and immunofluorescence intensity was strongly reduced by co-treatment with NS-398.

To verify whether the inhibitory effects of NS-398 on P-gp expression could be observed also in chemoresistant MCF7, cells belonging to the rMCF7 clone were treated for 10 days without or with 1 μM NS-398, 50 nM Doxo, or both. Cells were then incubated with the specific P-gp antibody, fixed, incubated with the secondary FITC-conjugated antibody, and observed for membrane immunofluorescence. As shown in Fig. 4 (Panel D), untreated control rMCF7 cells showed slight membrane immunofluorescence, which was significantly reduced in cells treated with NS-398. On the contrary, rMCF7 cells treated with Doxo displayed a strong membrane P-gp immunofluorescence, which was significantly reduced by co-treatment with NS-398.

Influence of NS-398 on cytotoxic effects of doxorubicin

Preliminary dose–response and time-course studies demonstrated that NS-398 does not affect native MCF7 cell proliferation at doses ranging from 0.1 to 100 μM (data not shown).
In order to evaluate whether COX-2 inhibitors might sensitize MCF7 cells to the cytotoxic effects of Doxo, native MCF7 cells and rMCF7 cells were treated for 7 days without or with 1 \( \mu \)M NS-398, 50 nM Doxo, or both. As shown in Fig. 5A, Doxo was capable of significantly reducing (−21%; \( P < 0.05 \) versus control native MCF7 cells) native MCF7 cell viability (white columns). On the contrary, treatment with NS-398 did not influence native MCF7 cell viability, either alone or in combination with Doxo. On the other hand, in rMCF cells (Fig. 5A, grey columns) treatment for 7 days with Doxo or NS-398 did not influence cell viability. However, co-treatment with Doxo and NS-398 significantly reduced cell viability (−32%; \( P < 0.01 \) versus control rMCF7 cells and \( P < 0.01 \) versus rMCF7 cells treated with Doxo alone).

In order to evaluate P-gp expression under these conditions, western blot analysis for P-gp was performed with protein extracts from native MCF7 cells and from rMCF7 cells treated for 7 days without or with 1 \( \mu \)M NS-398, 50 nM Doxo, or both. As indicated in Fig. 5B, a slight P-gp immunoreactivity was evident only in protein extracts from native MCF7 cells treated with 50 nM Doxo. On the other hand, the expected 170 kDa band was detected in all samples derived from rMCF7 cells.

In order to evaluate P-gp activity, R123 assay was performed in the same settings. As shown in Fig. 5C, in native MCF7 cells (white columns) treatment with NS-398 did not significantly modify R123 retention, either alone or in combination with Doxo. Treatment with Doxo slightly but not significantly reduced R123 retention in native MCF7 cells. On the contrary, R123 retention was significantly inhibited (−40%; \( P < 0.01 \)) in rMCF7 cells treated with Doxo, in keeping with Doxo-induced P-gp expression and function. This effect was completely counteracted by co-treatment with NS-398.

**Effects on doxorubicin accumulation**

Doxorubicin is an auto-fluorescent compound that enables the visualization of its intracellular presence by fluorescence microscopy. Figure 6 shows the fluorescence of intracellularly accumulated Doxo, which is mainly visible in the nuclei, in native MCF7 cells and in rMCF7 cells treated for 24 h without or with 10 \( \mu \)M NS-398, 5 \( \mu \)M Doxo, or both. As shown in the upper panels (Fig. 6, Panel A), intracellular fluorescence in native MCF7 cells treated with Doxo was not influenced by treatment with NS-398. On the contrary, intracellular fluorescence was reduced in rMCF7 cells treated with Doxo alone, but was significantly induced (+60%; \( P < 0.05 \) versus cell treated with Doxo alone; Fig. 6, Panel C) by co-treatment with NS-398.

**Discussion**

In this study, we show that the selective COX-2 inhibitor NS-398 sensitizes chemoresistant breast cancer cells to the cytotoxic effects of Doxo and significantly increases intracellular Doxo accumulation and retention in vitro. Our data indicate that these effects depend on the inhibition of P-gp expression and function in both native and chemoresistant MCF7 cells.

MCF7 cells represent a useful tool for the study of breast cancer resistance to chemotherapy, because they appear to mirror the heterogeneity of tumor cells in vivo. Our data show that these cells become drug resistant after long-term exposure to chemotherapeutic drugs, in agreement with previous evidence (Simstein et al. 2003). It has been demonstrated, in fact, that though cancer cells initially respond to therapy, they can subsequently gain resistance to these treatments and survive, resulting in a more phenotypically aggressive cell variant with an inclination...
Figure 4 Effects of NS-398 and Doxo on P-gp membrane expression. MCF7 cells were incubated for 10 days in culture medium supplemented without (0) or with 1 μM NS-398 (NS-398), 50 nM doxorubicin (Doxo), or both (NS-398 + Doxo). 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 × 60 objective magnification. Panel A: native MCF7 cells observed with the FITC filter, revealing membrane immunofluorescence. Panel B: native MCF7 cells observed with the u.v. filter, revealing nuclear immunofluorescence. Panel C: merged FITC (Panel A) and u.v. (Panel B) images. Panel D: rMCF7 cells observed with the FITC filter, revealing membrane immunofluorescence. Panel E: rMCF7 cells observed with the u.v. filter, revealing nuclear immunofluorescence. Panel F: merged FITC (Panel D) and u.v. (Panel E) images. Membrane-associated fluorescence was measured and values were expressed in the bar graph as mean fluorescence per cell (arbitrary units) ± S.E.M., corrected for background, in native MCF7 cells and in MCF7 cells treated without (white bars) or with NS-398 (grey bars), Doxo (black bars) or both (dashed bars). All experiments were carried out at least three times independently and 50 ± 10 individual cells have been analyzed. *P < 0.05 versus untreated control cells. §P < 0.05 versus cells treated with Doxo alone.

to metastasize (Simstein et al. 2003). Responsiveness of breast cancer to chemotherapy, especially in metastatic settings, might strongly decrease over time during treatment with cytotoxic drugs, significantly impacting on survival (Gasparini et al. 2005). We demonstrated that the chemoresistance phenotype associates with the induction of MDR1 gene expression and P-gp appearance on the plasma membrane. The overexpression of ATP-binding cassette (ABC) transporters, such as P-gp, has been reported to induce an increased efflux of chemotherapeutic drugs such as anthracyclines, epipodophyllotoxins, and vinca-alkaloids. P-gp overexpression has been associated with resistance to a wide range of unrelated anticancer drugs, differing widely with respect to molecular structure and target specificity, including many natural product agents (e.g., paclitaxel, vincristine, and Doxo) as well as new targeted anticancer agents (e.g., Gleevec; Gottesman 2002). In agreement with previous evidence, we demonstrate that MDR1 expressing cells are resistant to the cytotoxic effects of Doxo, which appears to be actively extruded from the cytosolic compartment. Therefore, our data support the hypothesis that treatment of breast cancer cells with a chemotherapeutic drug, such as Doxo, induces MDR1 gene expression and consequent appearance of a functionally active P-gp on the plasma membrane, which, in turn, protects the cell from the cytotoxic effects of Doxo by pumping the drug out of the cytoplasm and making chemotherapy ineffective. This effect seems to be reversible, since Doxo withdrawal for 10 days results in strongly reduced P-gp levels on the cell membrane. This finding indicates a possible explanation for the increased efficacy of chemotherapeutic treatments delivered in cycles as compared with continuous administrations (Saltz 2006).

We previously demonstrated that the chemoresistance phenotype associated with MDR1 gene expression and function in medullary thyroid cancer can be reversed by COX-2 inhibitors (Zatelli et al. 2005a). COX-2 expression in breast cancer associates with tumor size, axillary node metastasis, hormone receptor-negative disease, invasiveness, and poor prognosis (Arun & Goss 2004). Indeed, there is evidence indicating that COX-2 promotes angiogenesis, invasiveness, inflammation, and aromatase induction (Gasparini et al. 2003). These data support the hypothesis that COX-2 inhibition might represent a new tool for treating breast cancer. A selective COX-2 inhibitor, celecoxib, has been already
replicates are expressed as the mean
three individual experiments evaluated independently with three
measured and standardized by the protein content. Data from
Cells were then washed and lysed. Sample fluorescence was
retention was assessed by incubating the cells with 4
least three different experiments. Panel C. R123 assay. R123
indicated with an arrow. Displayed data are representative of at
7 days without (0) or with 1
extracts from native MCF7 and from rMCF7 cells incubated for
levels. Western blot analysis for P-gp and actin of protein
rMCF7 cells treated with Doxo alone. Panel B: P-gp protein
expression and function. Native MCF7 cells (white
bars) and rMCF7 cells (grey bars) were incubated in 96-well
plates for 7 days in culture medium supplemented without or
with 1 µM NS-398 (NS-398), 50 nM Doxo (Doxo), or both
(Doxo + NS-398). Panel A: cell viability. Cell viability was
evaluated by a colorimetric method, as described. Data from
three individual experiments evaluated independently with six
replicates are expressed as the mean ± S.E.M. percent cell
proliferation inhibition versus untreated control cells. *P < 0.05
and **P < 0.01 versus untreated control cells; §P < 0.01 versus
rMCF7 cells treated with Doxo alone. Panel B: P-gp protein
levels. Western blot analysis for P-gp and actin of protein
extracts from native MCF7 and from rMCF7 cells incubated for
7 days without (0) or with 1 µM NS-398 (NS-398), 50 nM Doxo
(Doxo), or both (NS-398 + Doxo). The expected bands are
indicated with an arrow. Displayed data are representative of at
least three different experiments. Panel C. R123 assay. R123
retention was assessed by incubating the cells with 4 µM R123.
Cells were then washed and lysed. Sample fluorescence was
measured and standardized by the protein content. Data from
three individual experiments evaluated independently with three
replicates are expressed as the mean ± S.E.M. percentage of
R123 retention versus control cells. **P < 0.01 versus control.

Figure 5 Effects of Doxo and NS-398 on MCF7 cell viability and
on P-gp expression and function. Native MCF7 cells (white
bars) and rMCF7 cells (grey bars) were incubated in 96-well
plates for 7 days in culture medium supplemented without or
with 1 µM NS-398 (NS-398), 50 nM Doxo (Doxo), or both
(Doxo + NS-398). Panel A: cell viability. Cell viability was
evaluated by a colorimetric method, as described. Data from
three individual experiments evaluated independently with six
replicates are expressed as the mean ± S.E.M. percent cell
proliferation inhibition versus untreated control cells. *P < 0.05
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three individual experiments evaluated independently with three
replicates are expressed as the mean ± S.E.M. percentage of
R123 retention versus control cells. **P < 0.01 versus control.

employed as neoadjuvant treatment in combination with
5-fluorouracil/epirubicin/cyclophosphamide, demonstrat-
ing a greater effect as compared with chemotherapy
alone (Chow et al. 2003). Moreover, celecoxib has been
demonstrated to increase the intracellular Doxo accumu-
lation and enhance Doxo-induced cytotoxicity in MDA-
MB231 breast cancer cells, most likely via the
modulation of NF-kB activity (van Wijngaarden et al.
2007). In keeping with this evidence, our data show that a
selective COX-2 inhibitor is capable of preventing the
chemoresistance phenotype and of sensitizing chemoresis-
tant MCF7 cells to the cytotoxic effects of Doxo. As
previously underlined, in our experimental model,
 punished of native MCF7 cells to Doxo promotes
resistance to chemotherapeutic drugs in association
with MDR1 expression. Our data clearly show that
treatment of native MCF7 cells with the COX-2
inhibitor NS-398 prevents MDR1 gene expression,
despite prolonged exposure to Doxo. In addition, we
show that the rMCF7 clone, which expresses both COX-
2 and MDR1, displays resistance to the cytotoxic effects
of Doxo. However, in these resistant cells, treatment with
NS-398 restrains Doxo-induced P-gp expression and
promotes intracellular Doxo retention, indicating that the
COX-2 inhibitor is indeed capable of reducing P-gp
function in rMCF7 cells. The reduced P-gp protein levels
might account for the decreased P-gp function in cells
treated with NS-398, which therefore become sensitive to
Doxo. Indeed, due to the decreased transmembrane
efflux, the chemotherapeutic agent accumulates in the
cell and exerts its antiproliferative effects.

Previous evidence showed that COX-2 inhibitors
might per se induce a dose-dependent increase in
apoptosis and growth inhibition in breast cancer cells
(McFadden et al. 2006). However, in our model, no
cytotoxic effect of the employed selective COX-2
inhibitor was observed at any of the tested concen-
trations. On the other hand, NS-398 reduces P-gp
protein levels, in keeping with previous evidence
showing a causal link between COX-2 and P-gp
activities (Patel et al. 2002). Several mechanisms
have been demonstrated to influence MDR1 gene
expression, such as transactivation and genetic
amplification. In addition, an inverse correlation
between functional gene expression and MDR1
promoter methylation has been found (Roberti et al.
2006). Further studies are needed to elucidate the
precise mechanism by which Doxo and NS-398 are
capable of regulating P-gp expression.

Our results suggest 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. These encoura-
ging results should, however, undergo strict clinical
verification, given the relevant cardiovascular and
thrombo-embolic toxicity correlated to the prolonged
use of COX-2 inhibitors (Bresalier et al. 2005,
Nussmeier et al. 2005, Solomon et al. 2005). It should
be noted, however, that our data support the use of
these compounds only in association with che-
motherapy, i.e., for short time periods.
In conclusion, our findings provide evidence that a selective COX-2 inhibitor reduces P-gp level and function in the MCF7 breast cancer cell line. These data may indicate a possible application of selective COX-2 inhibitors in combination with chemotherapy and/or as neo-adjuvant therapy in the medical treatment of metastatic breast cancer.

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