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

Maria Chiara Zatelli, Andrea Luchin, Federico Tagliati, Stefania Leoni, Daniela Piccin, Marta Bondanelli, Roberta Rossi and Ettore C degli Uberti

Section of Endocrinology, Department of Biomedical Sciences and Advanced Therapies, University of Ferrara, Via Savonarola 9, 44100 Ferrara, Italy

(Correspondence should be addressed to E C degli Uberti; Email: dut@unife.it)

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.

Endocrine-Related Cancer (2007) **14** 1029–1038

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*. 2005*a*). 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 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% CO_2. 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, 2005*b*). 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.* 2005*a*). 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,

Heidelberg, Germany) at 1:200. Horseradish per-oxidase-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.* 2005*a*). 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 spectro-photometry using the Wallac Victor 1420 Multilabel Counter ($\lambda_{ex} = 485$ nm, $\lambda_{em} = 535$ nm) and standar-dized 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 supple-mented 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 mouse 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 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 fluor-escent microscope, photographed with a ×60 objective magnification with the DS-5M Nikon color CCD (charge coupled device) digital camera, and analyzed with the Multi-Analyst software (Bio-Rad). Preimmune

serum and antigen-absorbed antibody were used as controls. All experiments were carried out at least thrice independently, analyzing 50 ± 10 individual cells.

Doxorubicin accumulation assay

Native MCF7 and rMCF7 cells (3×10^4) were seeded in eight-well chamber slides (Lab-Tek Chamber Slide System), incubated overnight in culture medium, and then treated for 24 h without or with 5 µM Doxo, 10 µM NS-398, or both. Subsequently, the culture medium was removed and cells were washed thrice with PBS. Cells were fixed in methanol and acetone (1:1) for 10 min at −20 °C, washed thrice with PBS and mounted with the ProLong Gold antifade reagent (Invitrogen Molecular Probes) containing DAPI under glass coverslips (Menzel-Glaser). The cells 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 quanti-fied with the Multi-Analyst software (Bio-Rad). Values were expressed as mean fluorescence (arbitrary units) per cell $\pm$ 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 $\pm$ 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 $\pm$ 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
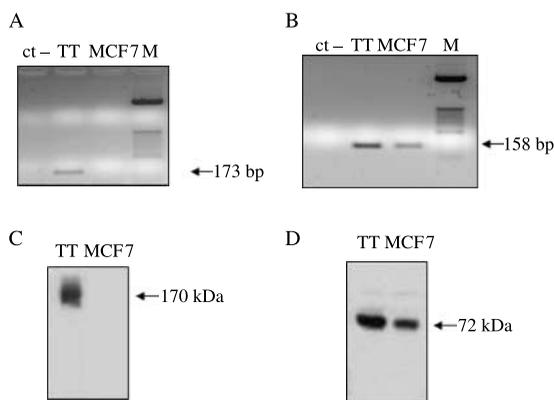
A

←173 bp

B

←158 bp

C

←170 kDa

D

←72 kDa

Figure 1 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 nitro-cellulose 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 signi-ficantly 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 2 B 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 through-out 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 signi-ficantly 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
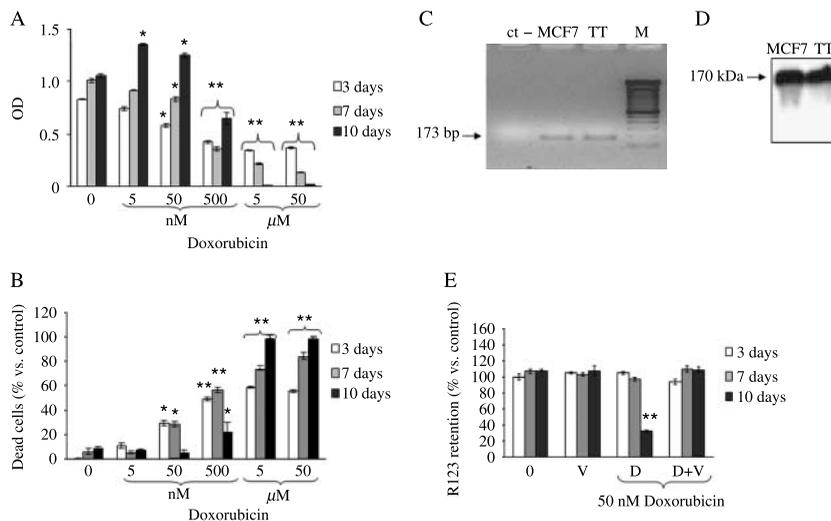
Figure 2 Effects of doxorubicin on MCF7 cell proliferation and on MDR1 expression and function. Panel A: cell viability. Native MCF7 cells were incubated in 96-well plates up to 10 days in culture medium supplemented without or with Doxo at concentrations ranging from 5 nM to 50 μM. Data from three individual experiments evaluated independently with six replicates are expressed as the mean±s.e.m. sample absorbance (OD) at 560 nm. *$P<0.05$ and **$P<0.01$ versus control untreated cells. Panel B: cell death. Native MCF7 cells were incubated in 24-well plates up to 10 days in culture medium supplemented without or with Doxo at concentrations ranging from 5 nM to 50 μM. Trypan blue dye exclusion assay was performed. Data from three individual experiments evaluated independently with four replicates are expressed as the mean±s.e.m. number of dead cells in treated MCF7 versus untreated control cells. *$P<0.05$ and **$P<0.01$ versus control untreated cells. Panel C: MDR-1 expression. Isolated RNA (1 μg/reaction) from native MCF7 cells cultured for 10 days in the presence of 50 nM Doxo was treated with DNase and subjected to RT. Aliquots from the generated cDNA were subjected to PCR amplification of MDR1. PCR products were resolved on a 2% agarose gel. The expected PCR products are indicated with arrows (lane M, 100-bp PCR marker; MCF7, native MCF7 cell line after treatment with Doxo for 10 days; TT, TT cell line, considered as positive control; ct−, negative control). Displayed data are representative of at least three different experiments. Panel D: P-gp expression. Whole cell extracts (100 μg) from native MCF7 cells cultured for 10 days in the presence of 50 nM Doxo (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. The expected bands are indicated with an arrow. Displayed data are representative of at least three different experiments. Panel E: R123 assay. Native untreated MCF7 cells and native MCF7 cells treated with 50 nM Doxo for up to 10 days were incubated with 4 μM R123, washed, incubated for 1 h without or with 100 μM verapamil, and then 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. (0, native untreated control cells; V, native cells incubated with 100 μM verapamil for 1 h; D, cells treated with 50 nM Doxo; D+V, cells treated with 50 nM Doxo incubated with 100 μM verapamil for 1 h). **$P<0.01$ versus native untreated control cells.

levels, but was capable of completely blocking Doxo-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 1 μM NS-398, 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).
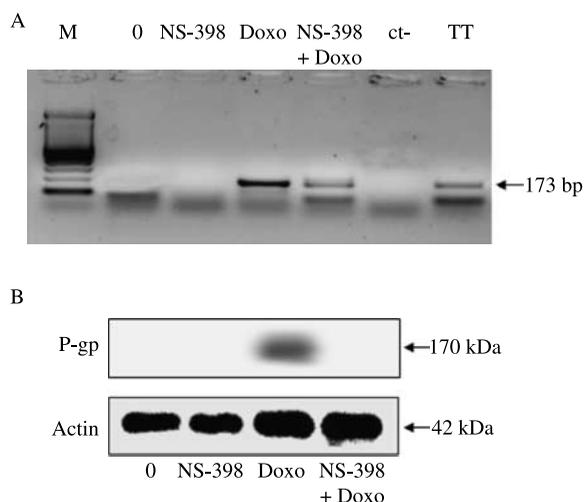
A

B

Figure 3 Effects of NS-398 on MDR1 expression and P-gp protein levels in native MCF7 cells. Panel A: MDR1 expression. Isolated RNA (1 µg/reaction) from native MCF7 cells cultured for 10 days without (0) or with 1 µM NS-398 (NS-398), 50 nM Doxo (Doxo), or both (NS-398 + Doxo) was treated with DNase and subjected to RT. Aliquots from the generated cDNA were subjected to PCR amplification of MDR1. PCR products were resolved on a 2% agarose gel. The expected PCR products are indicated with arrows (lane M, 100-bp PCR marker; TT, TT cell line, considered as positive control; ct−, negative control). Displayed data are representative of at least three different experiments performed in duplicate. Panel B: P-gp protein levels. Western blot analysis for P-gp and actin of protein extracts from native MCF7 cells incubated for 10 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.

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 µM 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 µM 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 µM NS-398, 5 µM 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
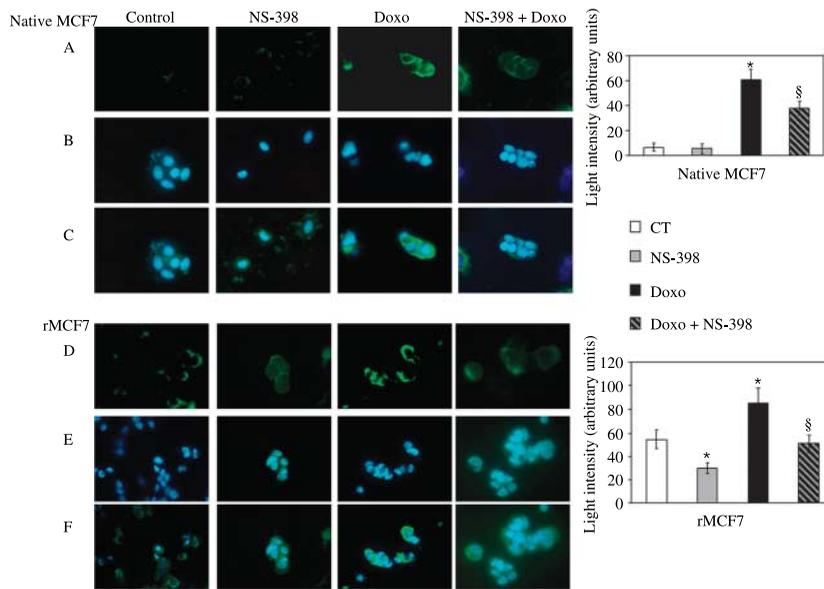
Figure 4 Effects of NS-398 and Doxo on P-gp membrane expression. MCF7 cells were incubated for 10 days in culture medium supplemented without (0) or with 1 μM NS-398 (NS-398), 50 nM doxorubicin (Doxo), or both (NS-398+ Doxo). Cells were then incubated with the specific P-gp antibody, fixed, incubated 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 rMCF7 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.* 2005*a*). 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
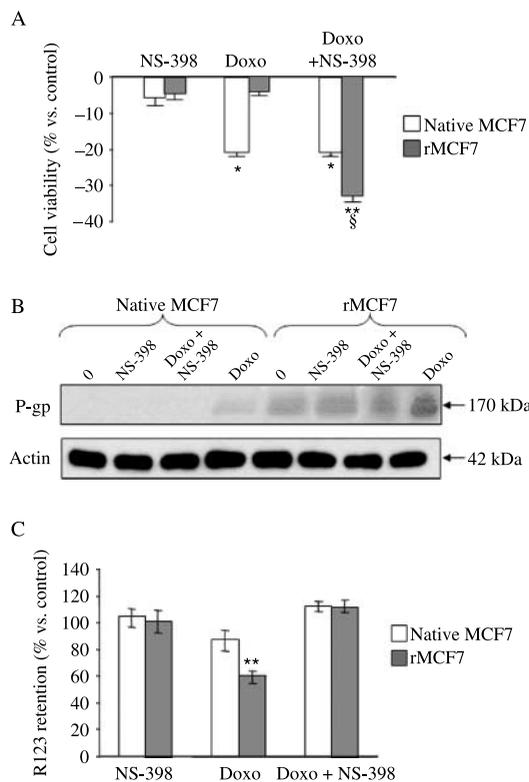
A

B

C

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. *$P < 0.05$ 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.

employed as neoadjuvant treatment in combination with 5-fluorouracil/epirubicin/cyclophosphamide, demonstrating a greater effect as compared with chemotherapy alone (Chow *et al.* 2003). Moreover, celecoxib has been demonstrated to increase the intracellular Doxo accumulation 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 chemoresistant MCF7 cells to the cytotoxic effects of Doxo. As previously underlined, in our experimental model, exposure of native MCF7 cells to Doxo promotes resistance to chemotherapeutic drugs in association with MDR1 expression. Our data clearly show that co-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 concentrations. 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 encouraging 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 chemotherapy, i.e., for short time periods.
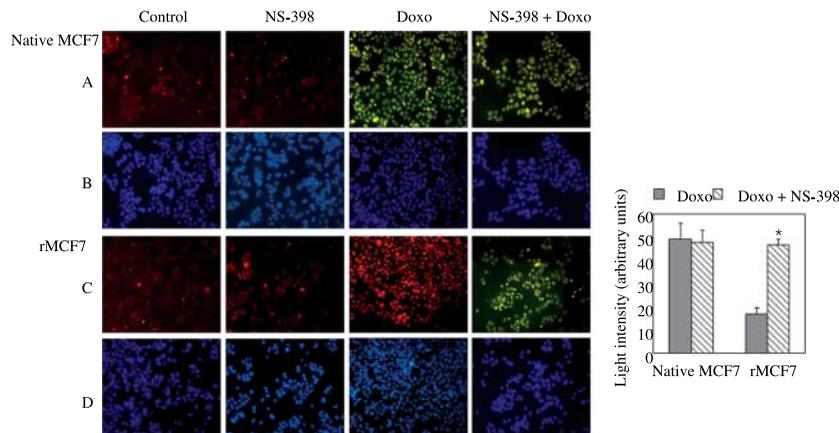
Figure 6 Doxorubicin accumulation in MCF7 cells. Fluorescent microscopic pictures of native MCF7 cells (Panels A and B) and rMCF7 cells (Panels C and D) incubated for 24 h in a medium containing 10 μM NS-398 (NS-398), 5 μM Doxo (Doxo), or both (Doxo + NS-398). Panels A and C: Doxo fluorescence was observed in native MCF7 cells and in rMCF7 cells with the Nikon TRITC filter. Panels B and D: nuclear staining with DAPI was detected with the Nikon u.v. filter. Intracellular Doxo was quantified by using computerized image analysis (Multi-Analyst), after image acquisition by the DS-5M Nikon color CCD digital camera. Fluorescence was measured and values were expressed in the bar graph as mean fluorescence per cell (arbitrary units) $\pm$ s.e.m., corrected for background, in native MCF7 cells and in rMCF7 cells treated with Doxo alone (grey bars) or in combination with NS-398 (dashed bars). All experiments were carried out at least three times independently and 50 $\pm$ 10 individual cells have been analyzed. $*P<0.05$ versus rMCF7 cells treated with Doxo alone.

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.

Acknowledgements

References

Ambudkar SV, Dey S, Hrycyna CA, Ramachandra M, Pastan I & Gottesman MM 1999 Biochemical, cellular and pharmacological aspects of the multidrug transporter. *Annual Review of Pharmacology and Toxicology* **39** 361–398.

Arun B & Goss P 2004 The role of COX-2 inhibition in breast cancer treatment and prevention. *Seminars in Oncology* **31** 22–29.

Bresalier RS, Sandler RS, Quan H, Bolognese JA, Oxenius B, Horgan K, Lines C, Riddell R, Morton D, Lanas A *et al.*

2005 Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial. *New England Journal of Medicine* **352** 1092–1102.

Campbell RA, Bhat-Nakshatri P, Patel NM, Constantinidou D, Ali S & Nakshatri H 2001 Phosphatidylinositol/3-kinase/Akt-mediated activation of estrogen receptor α: a new model for anti-estrogen receptor resistance. *Journal of Biological Chemistry* **276** 9817–9824.

Chow LW, Toi M & Takebayashi Y 2003 Neoadjuvant celecoxib and 5-fluorouracil/epirubicin/cyclophosphamide (FEC) for the treatment of locally advanced breast cancer (LABC). *Proceedings of the American Society of Clinical Oncology* **22** 787–788.

Dannenberg AJ & Howe LR 2003 The role of COX-2 in breast and cervical cancer. *Progress in Experimental Tumor Research* **37** 90–106.

Gasparini G, Longo R, Sarmiento R & Morabito A 2003 COX-2 inhibitors (Coxibs): a new class of anticancer agents? *Lancet Oncology* **4** 605–615.

Gasparini G, Longo R, Torino F & Morabito A 2005 Therapy of breast cancer with molecular targeting agents. *Annals of Oncology* **16** 28–36.

Gottesman MM 2002 Mechanisms of cancer drug resistance. *Annual Review of Medicine* **53** 615–627.

Khuder SA & Mutgi AB 2001 Breast cancer and NSAID use: a meta-analysis. *British Journal of Cancer* **84** 1188–1192.

Levenson AS & Jordan CV 1997 MCF7: the first hormone-responsive breast cancer cell line. *Cancer Research* **57** 3071–3078.

McFadden DW, Riggs DR, Jackson BJ & Cunningham C 2006 Additive effects of Cox-1 and Cox-2 inhibition on breast cancer *in vitro*. *International Journal of Oncology* **29** 1019–1023.

Nussmeier NA, Whelton AA, Brown MT, Langford RM, Hoeft A, Parlow JL, Boyce SW & Verburg KM 2005 Complications of the COX-2 inhibitors parecoxib and valdecoxib after cardiac surgery. *New England Journal of Medicine* **352** 1081–1091.

Patel VA, Dunn MJ & Sorokin A 2002 Regulation of MDR-1 (P-glycoprotein) by cyclooxygenase-2. *Journal of Biological Chemistry* **277** 38915–38920.

Pon YL, Auersperg N & Wong AS 2005 Gonadotropins regulate *N*-cadherin-mediated human ovarian surface epithelial cell survival at both post-translational and transcriptional levels through a cyclic AMP/protein kinase A pathway. *Journal of Biological Chemistry* **280** 15438–15448.

Roberti A, La Sala D & Cinti C 2006 Multiple genetic and epigenetic interacting mechanisms contribute to clonally selection of drug-resistant tumors: current views and new therapeutic prospective. *Journal of Celluar Physiology* **207** 571–581.

Saltz L 2006 Planned treatment interruptions and chemotherapy-free intervals in the treatment of metastatic colorectal cancer: time to start stopping? *Seminars in Oncology* **33** S28–S32.

Sarkadi B, Price EM, Boucher RC, Germann UA & Scarborough GA 1992 Expression of the human multi-drug resistance cDNA in insect cells generates a high activity drug-stimulated membrane ATPase. *Journal of Biological Chemistry* **267** 4854–4858.

Simstein R, Burow M, Parker A, Weldon C & Beckman B 2003 Apoptosis, chemoresistance, and breast cancer: insights from the MCF7 cell model system. *Experimental Biology and Medicine* **228** 995–1003.

Solomon S, McMurray JJV, Pfeffer MA, Wittes J, Fowler R, Finn P, Anderson WF, Zauber A, Hawk E, Bertagnolli M *et al.* 2005 Cardiovascular risk associated with celecoxib in a clinical trial for colorectal adenoma prevention. *New England Journal of Medicine* **352** 1071–1080.

Soule HD, Vazquez J, Long A, Albert S & Brennan MA 1973 human cell line from a pleural effusion derived from a breast carcinoma. *Journal of National Cancer Institute* **51** 1409–1416.

Tagliati F, Zatelli MC, Bottoni A, Piccin D, Luchin A, Culler MD & degli Uberti EC 2006 Role of complex Cyclin D1/Cdk4 in somatostatin subtype 2 receptor-mediated inhibition of cell proliferation of a medullary thyroid carcinoma cell line *in vitro*. *Endocrinology* **147** 3530–3538.

van Wijngaarden J, van Beek E, van Rossum G, van der Bent C, Hoekman K, van der Pluijm K, van der Pol MA, Broxterman HJ, van Hinsbergh VWM & Lowik CGWM 2007 Celecoxib enhances doxorubicin-induced cytotoxicity in MDA-MB231 cells by NF-kB-mediated increase of intracellular doxorubicin accumulation. *European Journal of Cancer* **43** 433–442.

Yasui K, Mihara S, Zhao C, Okamoto H, Saito-Ohara F, Tomida A, Funato T, Yokomizo A, Naito S, Imoto I *et al.* 2004 Alteration in copy numbers of genes as a mechanism for acquired drug resistance. *Cancer Research* **64** 1403–1410.

Zatelli MC, Tagliati F, Piccin D, Taylor JE, Culler MD, Bondanelli M & degli Uberti EC 2002 Somatostatin receptor subtype 1 selective activation reduces cell growth and calcitonin secretion in a human medullary thyroid carcinoma cell line. *Biochemical and Biophysical Research Communications* **297** 828–834.

Zatelli MC, Piccin D, Tagliati F, Ambrosio MR, Margutti A, Padovani R, Scanarini M, Culler MD & degli Uberti EC 2003 Somatostatin receptor subtype 1 selective activation in human growth hormone- and prolactin-secreting pituitary adenomas: effects on cell viability, growth hormone and prolactin secretion. *Journal of Clinical Endocrinology and Metabolism* **88** 2797–2802.

Zatelli MC, Luchin A, Piccin D, Tagliati F, Bottoni A, Vignali C, Bondanelli M & degli Uberti EC 2005*a* Cyclooxigenase-2 inhibitors reverse chemoresistance phenotype in medullary thyroid carcinoma by a P-gp mediated mechanism. *Journal of Clinical Endocrinology and Metabolism* **90** 5754–5760.

Zatelli MC, Piccin D, Tagliati F, Bottoni A, Luchin A & degli Uberti EC 2005*b* SHP-1 restrains cell proliferation in human medullary thyroid carcinoma. *Endocrinology* **146** 2692–2698.