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Research Paper

Cyclooxygenase Inhibitors Down Regulate P-glycoprotein in Human Colorectal Caco-2 Cell Line

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Purpose. Elevated expression of the ABC transporters P-glycoprotein (P-gp), and breast cancer resistance protein (BCRP) seems to correlate with multidrug resistance of cancer cells. In this study we investigated the effect of COX inhibitors in modulating P-gp and BCRP expression and P-gp activity in Caco-2 cells.
Methods. mRNA and protein expression of MDR1 and BCRP were evaluated by real time PCR and western blot respectively. The activity of P-gp was measured by intracellular accumulation of rhodamine123 or ^{3}H-Digoxin.
Results. The chronic exposure of Caco-2 to indomethacin heptyl ester (indo HE) (0.4 μM) or nimesulide (10 μM) (selective COX-2 inhibitors) and naproxen (6 μM) (non selective inhibitor COX-1/COX-2) significantly decreased the expression and activity of P-gp. In contrast, the acute treatment by nimesulide and naproxen did not modify these parameters while indo HE treatment (48–72 h) caused a protein decrease and a functional inhibition of P-gp. Unexpectedly, the short-term treatment with naproxen induced an important increase of BCRP expression, but this induction was lost after long-term treatment. No modification of BCRP expression was observed after indo HE or nimesulide treatment.
Conclusion. Our observations suggest a possible down regulation of P-gp by COX inhibitors, which may enhance the accumulation of chemotherapy agents.

KEY WORDS: BCRP; Caco-2; COX-2 inhibitor; MDR1; P-gp.

INTRODUCTION

The major issue in cancer treatment is that prolonged chemotherapy could lead to the selective survival of multidrug resistant (MDR) cells that exhibit simultaneous resistance to a wide spectrum of structurally and functionally unrelated chemotherapeutic agents. One of the better understood mechanisms leading to MDR is the over-expression of membrane efflux proteins including the 170 kDa P-glycoprotein (P-gp).

P-gp is a transmembrane phosphoglycoprotein from the ATP-binding Cassette superfamily (ABC), first identified in drug-resistant Chinese hamster ovary (CHO) by Ling and Thompson in 1974 (1). P-gp is an efflux pump, which transports a wide range of compounds, preferentially hydrophopic cationic compounds, from the inside of the cell back to the extracellular space, leading to a decrease in drug concentration within the cell and a reduced cancer-chemotherapy efficacy. P-gp is also present in normal tissues where it protects the organism from many drugs by decreasing intracellular concentration (2). Indeed, P-gp is widely expressed in circulating lymphocytes, in the epithelial cells of kidney, liver and intestine and in the endothelial cells in brain and placenta (3).

Cyclooxygenases (COX), also known as prostaglandin endoperoxide synthases or prostaglandin H synthases (4), are key enzymes required for the conversion of arachidonic acid to prostaglandins that affect a number of physiological and pathological states (4). Since early 1990s, two cyclooxygenase enzymes, COX-1 and COX-2 have been identified. The expression of the two COX isoenzymes is differently regulated. COX-1 is constitutively expressed in most human tissues (5) and has characteristics of a housekeeping enzyme which supplies tissues with prostaglandins required to maintain physiological organ functions, such as cytoprotection of the gastric mucosa (6) and regulation of renal blood flow (7). On the other hand, COX-2 is found to be highly induced, in response to inflammatory signals, by growth factors and cytokines at sites of inflammation (4,8) and is therefore believed to play an important role in the prostaglandin E2 (PGE2) production involved in pathophysiological processes (9).

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely prescribed as analgesic and anti-inflammatory agents. Their mechanism of action includes the inhibition of both the COX-1 and COX-2 isoenzymes (10). The new class of COX-2 selective inhibitors (COXIBs) that preferentially inhibits the COX-2 enzyme thereby reducing side effects including gastrointestinal ulceration and bleeding, has emerged as important therapeutic tools for treatment of arthritis (11).

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However, the cardiovascular side effects observed with celecoxib and rofecoxib led to the withdrawal of rofecoxib and the publication of precautions concerning the use of celecoxib. In clinical studies (12,13), COX-2 is reported to be expressed not only in inflammatory tissues such as intestinal mucosa of inflammatory bowel disease (IBD) patients, but also in neoplasic lesions of the colon. Numerous studies showed that COX-2 has a central role in the development of colorectal cancer by the induction of antiapoptotic effects (14), the increased invasiveness, and the promotion of angiogenesis (15). Recent epidemiological studies revealed a 40–50% reduction in mortality from colorectal cancer in individuals taking NSAIDs, and evidences suggest that they also affect the incidence and progression of the other types of cancer. Moreover, Arber *et al.* (16) recently showed that the use of 400 mg of celecoxib once daily significantly reduced the occurrence of colorectal adenomas within three years after polypectomy.

Different mechanisms have been suggested to contribute to the antitumor activity of NSAIDs such as the inhibition of cell cycle progression (17), the induction of apoptosis (18,19) and the inhibition of angiogenesis (20,21). Another mechanism by which COX-inhibitors could enhance the action of cytostatics has been proposed: COX-inhibitors could modulate the resistance of tumours to chemotherapeutic drugs by affecting the activity of membrane transporter proteins of the ABC-transporter family (22,23).

In this study, we addressed this issue *in vitro* by studying the effects of short and long term treatment by three selective COX-2 inhibitors, indomethacin heptyl ester, a derivative of indomethacin showing enhanced selectivity for the COX-2 isoform (24), nimesulide and celecoxib, and a non selective COX inhibitor, naproxen, on the expression (mRNA and protein) of P-gp and BCRP and on the activity of P-gp in a human colon cancer Caco-2 cell line.

MATERIAL AND METHODS

Chemicals

All chemicals were of the highest quality available and were obtained from commercial sources. Indomethacin heptyl ester, nimesulide (COX-2 selective inhibitors) and naproxen (nonselective COX inhibitor) were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Celecoxib was from Pfizer (Paris, France). ^{3}H-Digoxin was from Perkin Elmer life science product (Boston, MA, USA) as a solution in ethanol at 1mCi/ml (RAS=23.4 Ci/mmol). Digoxine Nativelle® 0.5 mg/2 ml came from Procter and Gamble Pharmaceuticals (Neuilly sur Seine, France). Rhodamine 123 and verapamil were from Sigma Chemicals (Saint Louis, MO, USA). Cyclosporin (Sandimum®) and valspodar were from Novartis (Novartis Pharma, Basel, Switzerland).

Cells Culture

The human colon adenocarcinoma cell line Caco-2 (used between passage 51 and 63) was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% (*v*/*v*) decomplemented fetal bovine serum (FBS), 1% (*v*/*v*) of non essential amino acids and 1% (*v*/*v*) of penicilline–streptomycine mixture. All these components were purchased from Invitrogen Corporation (Cergy Pontoise, France). Cells grown in culture flasks of 75 cm^2 were trypsinized every week and the medium was changed daily from the third day of culture. They were maintained in a 37°C incubator with a 5% carbon dioxide and 95% humidity in air atmosphere. For transport studies and for protein and mRNA expression experiments, 24, 12 and six well plastic culture dishes were used and cells were seeded at a density of 25×10^3, 50×10^3 and 1×10^5 cells/well respectively. For brush-border membrane proteins preparation, 500,000 cells were seeded in 25 cm^2 plastic flasks and were used at 21 days of culture corresponding to full differentiated Caco-2 cells.

Cell Viability: The MTT Assay

The cell viability in presence of the various products was evaluated using the MTT colorimetric assay. Differentiated Caco-2 cells seeded in 96-well dishes were treated for 24, 48, and 72 h by indomethacin heptyl ester 0.4 μM or nimesulide 10 μM or naproxen 6 μM or celecoxib 100 μM and 10 μM. Thereafter, cell viability was determined by the MTT test according to the procedure described by Mosmann (25). Briefly, 10 μl of MTT solution (Sigma) at 5 mg/ml in phosphate buffered saline (PBS) were added to each well, 0.1% of Triton X-100 was used as positive control (T^+), and plates were incubated at 37°C for 4 h. Medium was removed and 50 μl of acid-isopropanol (0.04 N HCl in isopropanol) were added to each well and mixed thoroughly to completely dissolve the dark blue crystals. The optical density values (OD) were measured at 570 nm using a multiwell-scanning spectrophotometer (MRX II microplate reader, Dynex Technologies). Results were expressed as a percentage of viability (% of viability = [OD (+ studied compound) − OD (T^+)]/[OD(control) − OD(T^+)]) and the product was considered toxic if the percentage of viability was below 90%.

Prostaglandin E2 (PGE2) Quantification

PGE2 concentration was determined in the culture medium of monolayers seeded in 24 well dishes and incubated with indomethacin heptyl ester (0.4 μM), nimesulide (10 μM) and naproxen (6 μM) for 48 h. At the end of the incubation, 250 μl aliquots of the incubation medium were acidified with 1 ml of formic acid (1%) and PGE2 was extracted in 2 ml ethyl acetate. After discarding the aqueous phase, the organic phase was evaporated under a stream of air, the dry residues were dissolved in assay buffer and PGE2 levels were determined with the PGE2 monoclonal enzyme immunoassay kit (Cayman Chemicals) according to the manufacturer's protocol, results are expressed in pg of PGE2/ml of medium.

Cells Treatment Protocols

Caco-2 cells were treated by COX inhibitors diluted in culture medium at 0.4 μM of indomethacin heptyl ester, 10 μM of nimesulide and 6 μM of naproxen which were defined as no toxic doses. Two protocols of treatment were applied:

- Short-term treatment for 24 h, 48 h, 72 h from the 14th day of culture
- Long-term treatment where cells were maintained in contact with COX inhibitors for 4 passages then 14 days necessary for differentiation.

Celecoxib had been excluded from our experiments since 100 or 10 μM treatments caused a marked decrease (20–30%) of cell viability.

RNA Extraction and Reverse Transcription-Real Time Polymerase Chain Reaction (RT-PCR)

At the end of the culture period, the medium was removed and total RNA was extracted from Caco-2 cell monolayers by using RNAble (Eurobio, les Ulis, France) according to the protocol provided by the manufacturer. Integrity of RNA after extraction was checked by ethidium bromide agarose gel electrophoresis. Five micrograms of total RNA was reverse transcribted in cDNA by Superscript II RNA reverse transcriptase (Invitrogen) using oligo-dT as a primer. For PCR amplification of cDNA, 5 μl of the 1:20 diluted RT products were added to 5 μl of solution containing 0.5 μM forward and reverse primer each, 3 mM MgCl2 and 1 X PCR buffer (Roche diagnostics, Meylan, France; containing FastStart Tag DNA polymerase, dNTP and SYBR Green 1 dye). PCRs were carried out using the oligonucleotides primers and conditions listed in Table I.

PCR products were separated by electrophoresis through a 2% agarose gel, followed by ethidium bromide staining to ensure the amplification of an appropriate size product.

Protein Extraction and Western Blot Analysis

The culture medium was removed and cells were washed twice with ice-cold PBS then scraped in 1 ml of ice-cold PBS and centrifuged for 5 min at 3,000×*g*.

For total protein extraction, pellet cells were homogenised at 4°C in lyses buffer [TENTS buffer; Tris–HCl 10 mM at pH 7.4, EDTA 5 mM at pH 8, NaCl 126 mM, Triton X-100 1% (*v*/*v*) and SDS 0.1%] supplemented with 1 mM phenylmethansulfonyl fluorid (PMSF) and 5 μg/ml of pepstatin, aprotinin and leupeptin as protease inhibitors (Sigma). The homogenates were centrifuged at 12,000×*g* for 20 min at 4°C and the supernatants, containing total proteins, were collected for Western blot analysis.

For membrane protein extraction, the pellets were homogenised in a buffer containing 250 mM sucrose, 50 mM Tris–HCl at pH 7.4, the protease inhibitors pepstatin, aprotinin, leupeptin 5 μg/ml and 1 mM PMSF. The homogenates were centrifuged 10 min at 3,000×*g*, and the supernatants were once again centrifuged for 30 min at 15,000×*g*. The pellets containing the membrane proteins were resuspended in a buffer containing 50 mM Mannitol, 50 mM Tris–HCl at pH 7.4, 1 mM PMSF and 5 μg/ml of pepstatin, aprotinin and leupeptin and stored at −80°C until use.

For brush-border protein extraction, the brush-border-rich fraction was isolated using the MgCl2 precipitation procedure as described previously (26). The final pellet containing brush-border membrane proteins was resuspended in a small volume of buffer (300 mM mannitol, 20 mM HEPES/Tris–HCl at pH 7.4, 100 mM KCl) and stored at −80°C until used. Protein concentrations in the lysates were quantified by bicinchoninic acid protein Assay Reagent Kit (Sigma) using bovine serum albumin (BSA) as a standard.

Equal amounts (twenty micrograms) of proteins were solubilized in electrophoresis sample buffer containing β-mercaptoethanol and size-separated on an 8% polyacrylamide gel. Resolved protein were transferred electrophoretically (200 mA, 3 h, 4°C) to a nitrocellulose membrane and subjected to immunoblot analysis. The membranes were blocked 1 h in 10% non-fat dry milk in Tween 20 Tris-buffered saline (TTBS) buffer [containing 20 mM Tris, 200 mM NaCl, 0.1% Tween 20 (*v*/*v*), pH 7.5]. After washing with TTBS the membranes were subsequently incubated with mouse monoclonal primary antibodies diluted in TTBS at 1:100 dilution of antibody C219 anti-P-gp (Dako Corporation, Glostrup, Denmark) overnight at 4°C, at 1:5,000 dilution of antibody anti-β actin (clone AC74, Sigma chemicals) for 1 h at room temperature, or at 1:100 dilution of antibody anti-BCRP (clone BXP-21, Chemicon International, Hampshire, UK) for 2 h at room temperature. After washing four times for 10 min in TTBS, the membranes were further incubated 1 h at room temperature with the secondary rabbit anti-mouse horseradish peroxidase-conjugated antibody diluted at 1:10,000 (Dako). The membranes were washed three times for 10 min in TTBS and one time for 10 min in TBS and then revealed using a chemiluminescence system (ECL, Perkin Elmer, UK) and exposed to auto radiographic film (Kodak®, Sigma). The intensity of the bands was quantified using Scion Image (NIH, Scion Corporation, Bethesda, USA). The level of P-gp or BCRP protein for each lane was normalised to the level of β-actin as an internal loading control.

P-gp Activity Study

Activity experiments were realized by the measurement of intracellular accumulation of Rhodamine123 (Rho123) or ^{3}H-Digoxin (P-gp substrates) which is inversely proportional to P-gp activity. Cellular uptake studies were performed on Caco-2 cell monolayers grown on 24-well dishes. The day of experiment, the culture medium was removed by aspiration

Table I. Primers and PCR conditions for *MDR1, BCRP* and *GAPDH* amplification

Primers(5′–3′)	Denaturation	Annealing	Extension	Cycles	Expected Fragment (pb)
MDR1	95°C, 5 s	60°C, 5 s	72°C, 4 s	40	106
Forward	GCTGGGAAGATCGCTACTGA				
Reverse	GGTACCTGCAAACTCTGAGCA				
BCRP	95 °C, 5 s	65°C, 5 s	72°C, 4 s	50	90
Forward	CAGGTCTGTTGGTCAATCTCACATCCAT				
Reverse	ATCCATATCGTGGAATGCTGAAG				
GAPDH	95°C, 5 s	62°C, 5 s	72°C, 6 s	40	112
Forward	TGCACCACCAACTGCTTAG				
Reverse	GATGCAGGGATGATGTTC				

and cells, treated according to protocols described in section 2.5, were washed twice with HEPES buffer at 37°C (consisting of 5.4 mM KCl, 2.8 mM CaCl2, 1 mM MgSO4, 0.3 mM NaHPO4, 137 mM NaCl, 0.3 KH_2PO_4 and 10 mM de HEPES at pH 7.4). Wells destined for positive control were preincubated for 1 h at 37°C with 10 μM cyclosporin A in HEPES, other wells were stabilised in HEPES alone during this period. Then, HEPES was removed and cells were incubated with 10 μM Rho123 or ^{3}H-Digoxin (0.2 mCi/ml radiolabeled digoxin +0.5 μM digoxin) for 30 min at 37°C and in the presence of cyclosporin A for positive control wells. At the end of the incubation, HEPES was aspirated gently, and cells were washed twice with 1 ml of ice-cold HEPES to remove any extracellular Rho123 or ^{3}H-Digoxin. For substrate uptake measurement, monolayers were lysed in 10% Triton X-100 (in Tris-HCl 1 M) and vortexed vigorously. The concentration of Rho123 in each sample was determined quantitatively by fluorescence spectrophotometry (λ_{ex}=500 nm, λ_{em}=525 nm) (Perkin Elmer LS50B) and standardized by the protein content of each sample. The uptake of radiolabeled compound (^{3}H-Digoxin) was determined by counting the samples in a Beckman LS 6000 TA liquid scintillation counter (Beckman, Ireland).

Ability of COX-inhibitors to Act as Substrates/Competitive Inhibitors of P-gp

Cells were pre-incubated with COX inhibitors diluted in HEPES for 30 min at 37°C (phase of loading) followed by 30 min of incubation with rhodamine123 or ^{3}H-Digoxin in presence of COX inhibitors (phase of competition). The experiment was then completed as described previously.

Statistical Analysis

Results are expressed as mean±SEM. Statistical significance of differences between mean values was assessed with the non-parametric, Mann–Whitney unpaired test. Differences were considered statistically significant when P values were <0.05.

RESULTS

Model Validation

To assess whether the Caco-2 cell line could be a suitable model for evaluating the effects of COX-2 inhibitors on MDR1 and BCRP activity and expression, we first quantified the expression profile of these two proteins by Western blot every 3 days for 21 days after seeding. As shown in Fig. 1, Caco-2 cells expressed P-gp and BCRP as early as day 3 post seeding. The expression of P-gp increased within time to reach the highest level between day 14 and 17 and then stabilized, which could result from the increase of mRNA levels of MDR1 with differentiation as reported by Siissalo *et al.* (27). In contrast, the expression of BCRP was the highest as early as day 3 and then decreased with culture time. The expression of P-gp and BCRP protein was mainly localized at the membrane level especially at the brush-border of Caco-2 cells, which mimics the polarized physiological localization of theses transporters on the enterocytes in the small intestine.

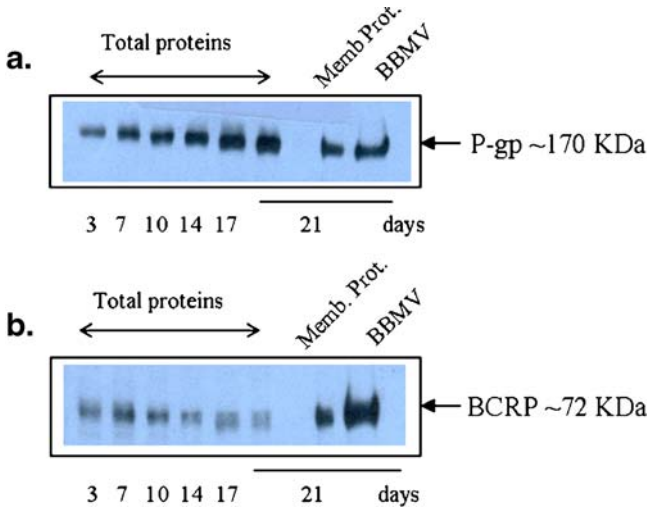

Fig. 1. P-gp (**A**) and BCRP (**B**) expression in Caco-2 cells during culture. Equal amounts (20 μg) of total proteins extracted at day (3, 7, 10, 14, 17 and 21) post seeding, of membrane proteins (*MP*) and of brush-border membrane proteins (*BBMV*) extract at day 21 post seeding, were analysed by Western blot. The immunodetection of transporters was performed using the specific monoclonal antibody C219 for P-gp and BXP-21 for BCRP.

Secondly we verified and characterized the functionality of P-gp in Caco-2 cells using verapamil (100 μM), cyclosporin A (10 μM) and valspodar (10 μM) as well-known P-gp inhibitors with Rho123 and ^{3}H-Digoxin as non specific and more specific P-gp substrate respectively. As shown in Fig. 2, verapamil and valspodar induced respectively a 1.5 and 1.7 increases of Rho123 and ^{3}H-Digoxin cellular accumulation. Cyclosporin A produced a marked increase of Rho123 uptake (×4) and ^{3}H-Digoxin (×2) as compared with untreated cells. In the following experiments cyclosporin A was used as the positive control for the P-gp inhibition.

PGE2 Production

In order to determine the amount of endogenous PGE2 secreted by Caco-2 cells and to test the inhibitory capacity of the used doses of the COX inhibitors, the concentration of PGE2 was measured. As shown in Fig. 3, Caco-2 cells produced a low PGE2 level under standard cell culture conditions, however, neither the two COX-2 selective inhibitors (0.4 μM indomethacin heptyl ester, 10 μM nimesulide) nor the non-selective COX inhibitor (6 μM naproxen) did modify PGE2 secretion to the culture medium. These results indicated that the used doses of these inhibitors were not able to induce a further decrease of the low basal cyclooxygenase activity.

Ability of COX-Inhibitors to Act as Substrates/Competitive Inhibitors of P-gp

This experiment was based on the possible competition between Rho123 and another substrate towards the efflux by P-gp. As shown in Fig. 4, pre-incubation of Caco-2 cells for 30 min with COX inhibitors did not modify Rho123 or ^{3}H-Digoxin intracellular retention which propose that they did not compete with these substrates on the transport by P-gp or BCRP. This suggests that indomethacin heptyl ester,

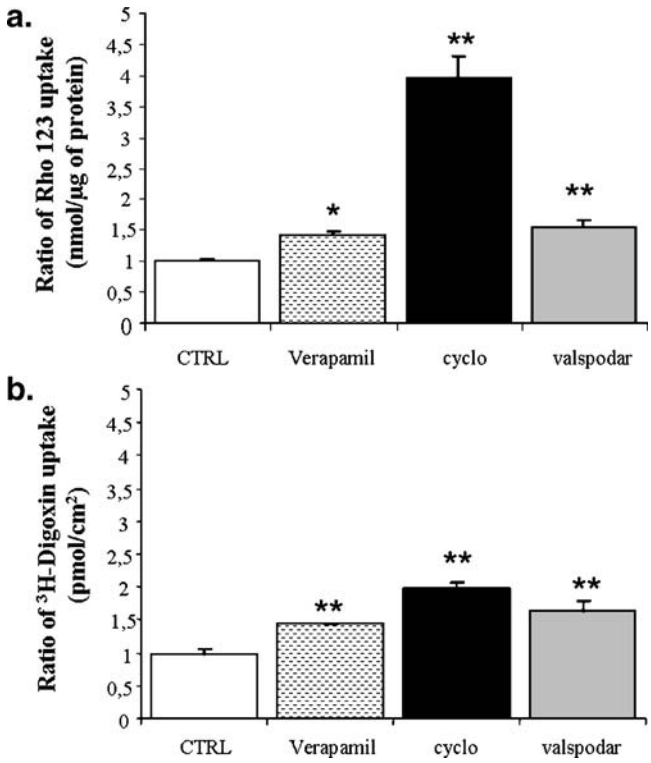

Fig. 2. Intracellular accumulation of Rho123 (**A**) or ^{3}H-Digoxin (**B**) in absence or presence of three inhibitors of P-gp, verapamil (100 µM), cyclosporin A (10 µM) and valspodar (10 µM) for 1 h in Caco-2 monolayers. Rho123 accumulation was normalized per mg of proteins. Values represent the mean±SEM (n=6). *p<0.05, **p<0.01 *vs* control.

nimesulide and naproxen are neither substrates nor competitive inhibitors of P-gp or BCRP.

Effects of COX Inhibitors on P-gp, BCRP in Caco-2 Cells

Effects After Short-Term Treatment

Caco-2 cells were exposed to indomethacin heptyl ester (0.4 µM), nimesulide (10 µM) and naproxen (6 µM) for 24, 48 and 72 h and the effects of this short-term treatment were

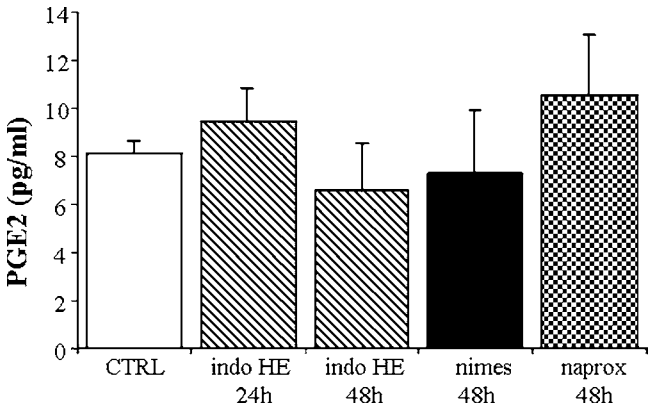

Fig. 3. Secretion of Prostaglandin E2 in the culture medium of differentiated Caco-2 cells. PGE2 in cell supernatant was determined by enzyme immunoassay after various treatments (CTRL, 24 h or 48 h indo HE (0.4 µM), 48 h nimesulide (10 µM) or 48 h naproxen (6 µM). Data are expressed as the mean±SEM (n=4).

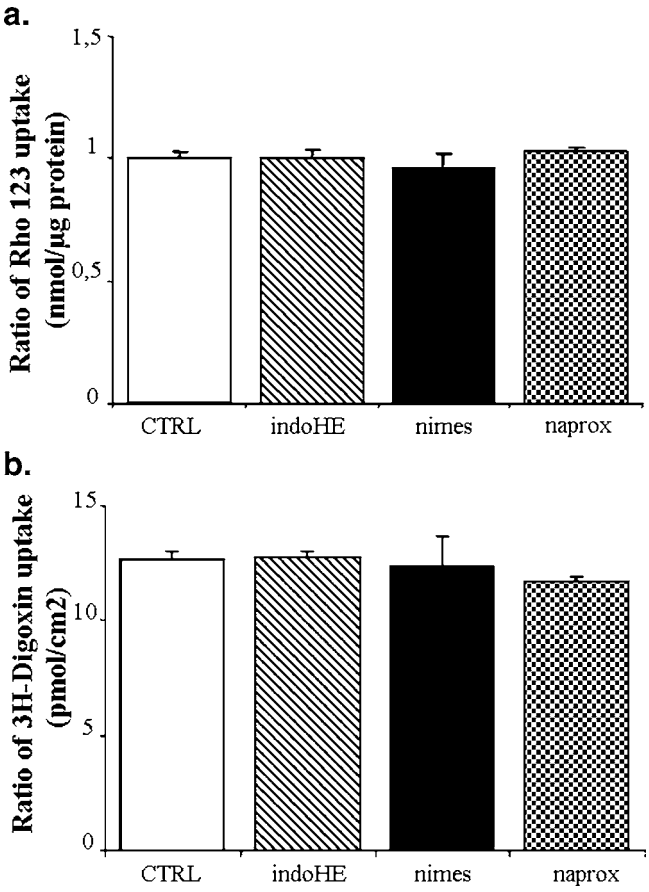

Fig. 4. Character substrate/competitive inhibitor of COX inhibitors towards Pgp studied by the competitive inhibition of Rho123 (**A**) or ^{3}H-Digoxin (**B**) efflux. Caco-2 cells were pre-incubated for 30 min with COX inhibitors suited with 30 min of incubation with Rho123 or ^{3}H-Digoxin in presence of COX inhibitors. Rho123 accumulation was standardized by the protein content. Data are expressed as the mean± SEM (n=4–6).

investigated on *MDR1*, *BCRP* mRNA and protein expression, and on P-gp activity.

The doses of COX-inhibitors (excepted Celecoxib) applied in these experiments did not induce significant mitochondrial toxicity towards Caco-2 cells as monitored by MTT assay (more than 90% cell viability, data not shown).

P-gp activity was evaluated by measuring the intracellular accumulation of Rho123 in Caco-2 cells exposed to the COX inhibitors. As reported in Fig. 5A the short-term treatment with indomethacin heptyl ester induced a significant down regulation of P-gp activity from 48 h (Rho123 uptake ~×1.3, p<0.01 *vs* control) and 72 h of treatment (Rho123 uptake ~×1.2, p<0.05 *vs* control) but no modification was observed after 24 h of treatment. On the other hand, nimesulide did not influence P-gp activity after short-term treatment. However, naproxen significantly decreased the intracellular accumulation of Rho123 after 48 and 72 h of treatment (−20%, p<0.05 *vs* control).

Since Rho123 has been demonstrated to be a substrate of P-gp but also of BCRP, we used ^{3}H-Digoxin as a more specific substrate. As shown in Fig. 5B, the 48 h-indomethacin heptyl ester treatment increased ^{3}H-Digoxin intracellular accumulation in the same pattern to that observed with Rho123 (~×1.3, p<

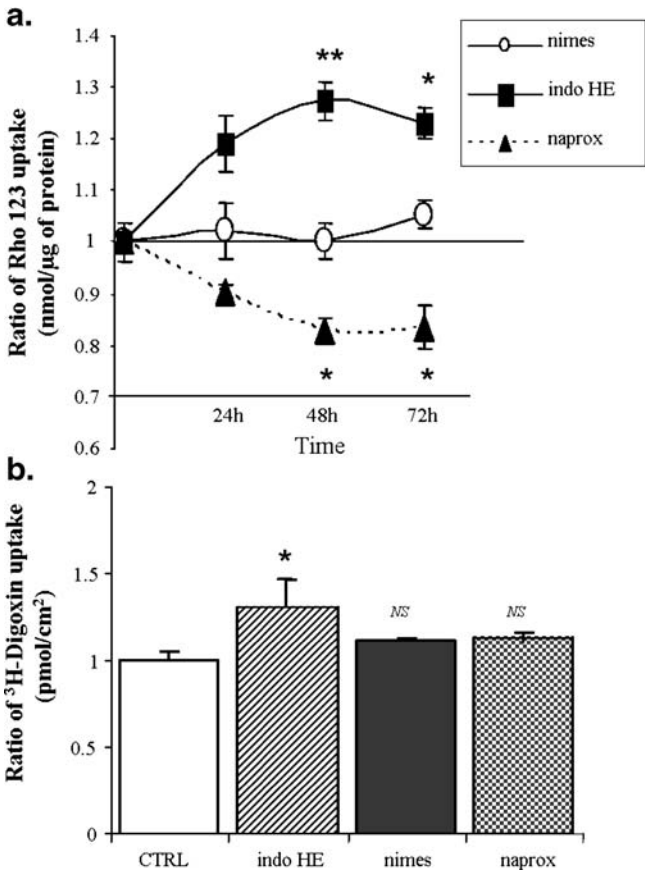

Fig. 5. Effects of COX inhibitors on P-gp activity after short-term treatment. **A**. Intracellular accumulation of Rho123 in Caco-2 cells treated with indomethacin heptyl ester (0.4 µM), nimesulide (10 µM) and naproxen (6 µM) for 24, 48 and 72 h. Sample fluorescence was standarized by the protein content. **B**. Intracellular accumulation of ^{3}H-Digoxin in Caco-2 cells treated with indomethacin heptyl ester (0.4 µM), nimesulide (10 µM) and naproxen (6 µM) for 48 h. Data are expressed as the mean±SEM (n=6). *p<0.05, **p<0.01 *vs* control. *NS* Not different from control.

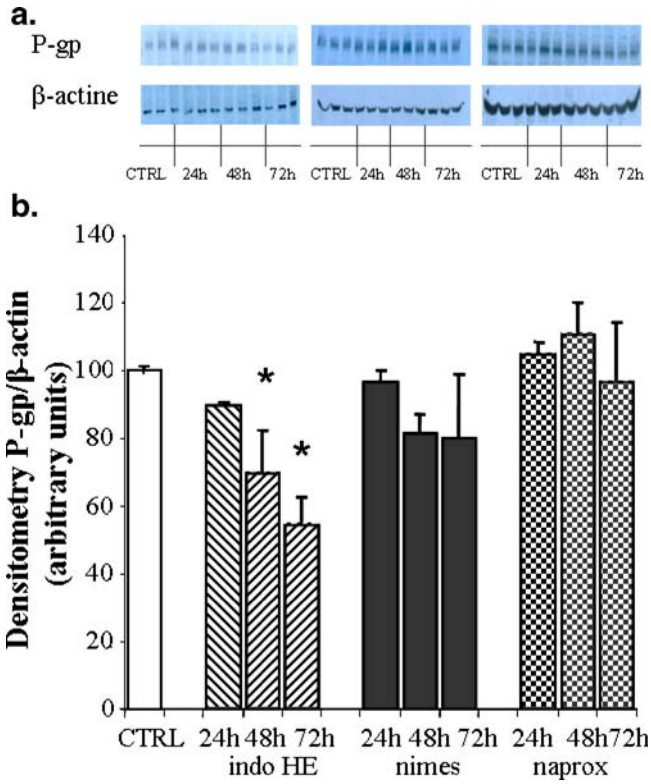

Fig. 6. Effects of COX inhibitors on P-gp protein expression after short-term treatment. **A**. Western blot analysis of P-gp and β-actin in 20 µg of total protein extract from Caco-2 cells treated with indomethacin heptyl ester (0.4 µM), nimesulide (10 µM) and naproxen (6 µM) for 24, 48 and 72 h. The immunodetection of the P-gp transporter was performed using the specific monoclonal antibody C219. **B**. Densitometric analysis of immunoblots was performed using the NIH image analysis and the results were normalized directly by β-actin expression. Data are expressed in arbitrary units and are expressed as the mean±SEM (n=3). *p<0.05 *vs* control.

0.05 *vs* control). Nimesulide still had no effect on ^{3}H-Digoxin uptake alike Rho123 uptake assay. In contrast, ^{3}H-Digoxin uptake was not affected by naproxen.

As shown in Fig. 6, 24 h-indomethacin heptyl ester (0.4 µM) treatment did not modify significantly the P-gp protein amount. However, a significant reduction in P-gp protein expression was observed after 48 and 72 h of treatment (−30%, p<0.05 *vs* control and −50%, p<0.05 *vs* control) respectively. Nimesulide and naproxen did not induce a significant difference of P-gp protein expression compared to control after treatment of 24, 48 or 72 h.

Next we investigated the effect of COX inhibitors on BCRP protein expression. Figure 7 showed that naproxen treatment led to an induction of BCRP expression which became significant at 48 and 72 h (×1.8 and ×2.2, p<0.05 *vs* control respectively) corresponding to the significant decrease of Rho123 retention obtained in activity study. In contrast, short-term treatment with indomethacin heptyl ester or nimesulide did not influence the protein expression level of BCRP.

To investigate whether the level of *MDR1* mRNA in Caco-2 cells was affected by COX inhibitor treatment and could account for the decrease in activity and protein expression, a real-time PCR technique was performed. As reported in Fig. 8, a reduction in *MDR1* expression was evident in 24 h indomethacin heptyl ester treated cells (−50%, p<0.05 *vs* control). Nimesulide induced a decrease of *MDR1* mRNA level after 24 h of treatment, but this response did not reach statistical significance. Naproxen did not show any significant effect on *MDR1* mRNA expression after treatment of 24 h.

In addition, the effects of 24 h treatment with COX inhibitors on the level of *BCRP* mRNA were also studied. As reported in Fig. 9, indomethacin heptyl ester and nimesulide did not modify BCRP mRNA level while a very significant induction was observed after 24 h treatment with naproxen (×2.5 p<0.01 *vs* control).

Effects After Long-term Treatment

To verify whether indomethacin heptyl ester might keep its modulating effects observed on P-gp after short-term treatment and to investigate the possible effects of chronic exposure to nimesulide and naproxen on P-gp, Caco-2 cells were maintained for four passages in contact with mentioned COX inhibitors, then P-gp, BCRP mRNA and protein expression, and P-gp activity were evaluated.

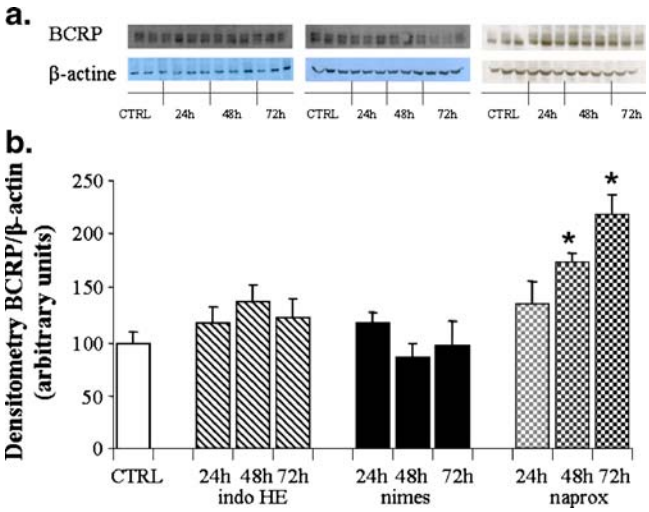

Fig. 7. Effects of COX inhibitors on BCRP protein expression after short-term treatment. **A**. Western blot analysis of BCRP and β-actin in 20 μg of total proteins extracted from Caco-2 cells treated with indomethacin heptyl ester (0.4 μM), nimesulide (10 μM) and naproxen (6 μM) for 24, 48 and 72 h. The immunodetection of the BCRP transporter was performed using the BXP-21 antibody. **B**. Densitometric analysis of immunoblots was performed using the NIH image analysis and the results were normalized directly by β-actin expression. Data are expressed in arbitrary units and are expressed as the mean±SEM (n=3). *p<0.05 *vs* control.

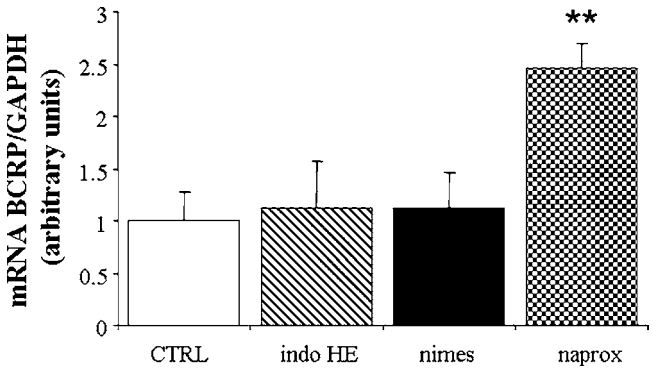

Fig. 9. Effects of COX inhibitors on *BCRP* mRNA expression. Real-time PCR analysis of *BCRP* mRNA expression in Caco-2 cells incubated for 24 h with indomethacin heptyl ester (0.4 μM), nimesulide (10 μM) and naproxen (6 μM). Values were normalized to the *GAPDH* content of samples and expressed as the mean±SEM (n=6). **p<0.01 *vs* control.

The effect of indomethacin heptyl ester and nimesulide on P-gp activity (Fig. 10) showed that the accumulation of Rho123 was greatly affected. The elevated Rho123 intracellular concentrations were nearly equivalent to those observed in the presence of the positive control, cyclosporin A (×2.6 and ×2.5 p<0.01 *vs* control respectively). Additionally, a small but significant increase of Rho123 retention with naproxen treatment was observed (×1.3 p<0.05).

The expression of P-gp in control cells and in COX-inhibitors treated cells determined by Western blot analysis is presented in Fig. 11. A strong down-regulation (−60%) of the P-gp protein level was observed after chronic exposure of 0.4 μM indomethacin heptyl ester. In contrast of the short-term treatment, the chronic exposure of cells to 10 μM nimesulide and 6 μM naproxen significantly reduced the expression of P-gp as revealed by the densitometric analysis of immunoblots (−30 and −20% respectively).

As shown in Fig. 12, comparison of BCRP protein levels showed an increase of 20% in naproxen treated cells over control but without reaching statistical significance. In parallel, the long term treatment with indomethacin heptyl ester or nimesulide was still unable to modify the BCRP protein expression.

To investigate whether levels of *MDR1* mRNA were affected by COX-2 inhibitors long-term treatment, real-time PCR was performed. As shown in Fig. 13, the 4 passages indomethacin heptyl ester and naproxen treatment led to a strong decrease of *MDR1* mRNA expression as compared with untreated cells (−50% p<0.05 and −60% p<0.01 *vs* control respectively). However, nimesulide was ineffective to reduce *MDR1* mRNA level.

Similarly to the absence of effect on BCRP protein expression after long term treatment, the three tested COX inhibitors had no influence on the *BCRP* mRNA level (Fig. 14).

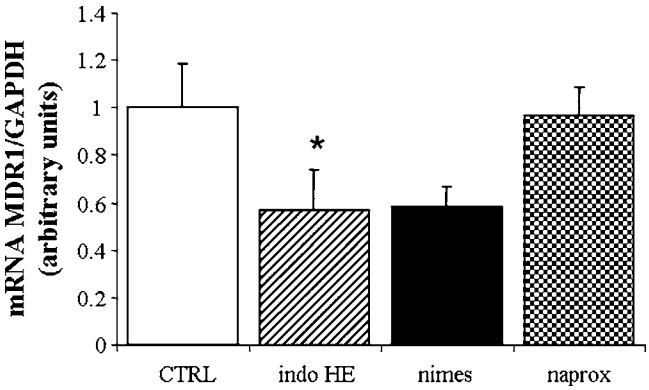

Fig. 8. Effects of COX inhibitors on *MDR1* mRNA expression. Real-time PCR analysis of *MDR1* mRNA expression in Caco-2 cells incubated for 24 h with indomethacin heptyl ester (0.4 μM), nimesulide (10 μM) and naproxen (6 μM). Values were normalized to the *GAPDH* content of samples and expressed as the mean±SEM (n=6–9). *p<0.05 *vs* control.

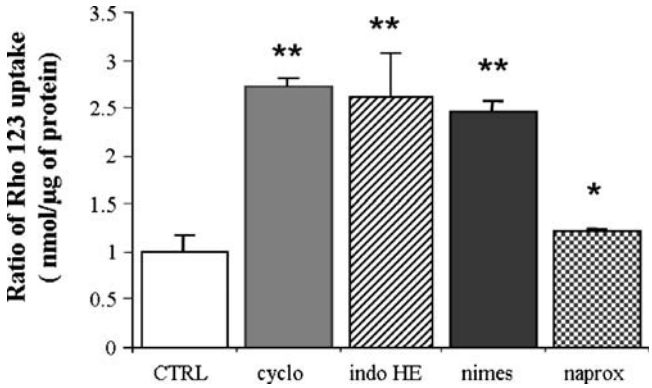

Fig. 10. Effects of COX inhibitors on P-gp activity after long-term treatment. Intracellular accumulation of Rho123 in Caco-2 cells treated with indomethacin heptyl ester (0.4 μM), nimesulide (10 μM) and naproxen (6 μM) for four passages. Sample fluorescence was standarized by the protein content. Data are expressed as the mean±SEM (n=6). *p<0.05, **p<0.01 *vs* control.

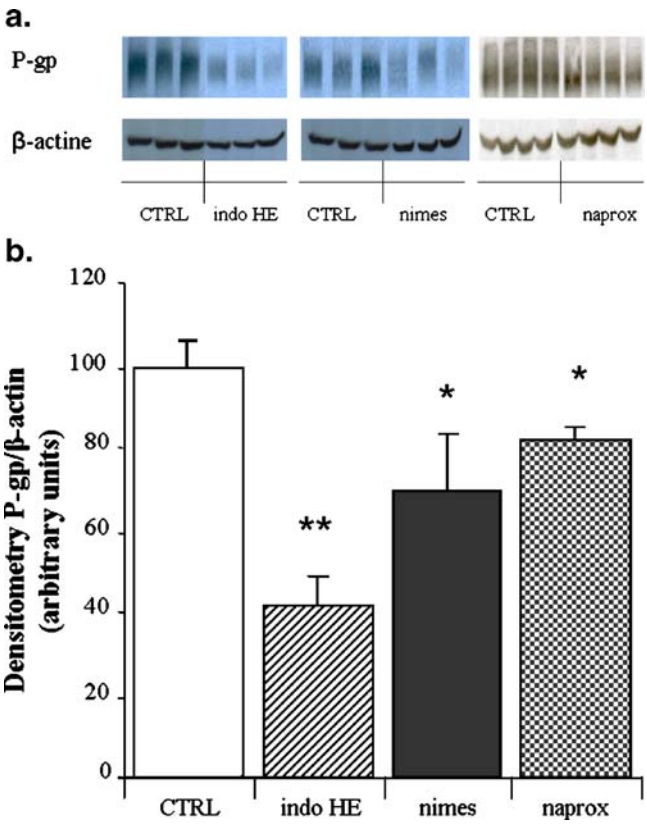

Fig. 11. Effects of COX inhibitors on P-gp protein expression after long-term treatment. **A**. Western blot analysis of P-gp and β-actin in 20 μg of total proteins extracted from Caco-2 cells treated with indomethacin heptyl ester (0.4 μM), nimesulide (10 μM) and naproxen (6 μM) for four passages. The immunodetection of the P-gp transporter was performed using the specific monoclonal antibody C219. **B**. Densitometric analysis of immunoblots was performed using the NIH image analysis and the results were normalized directly by β-actin expression. Data are expressed in arbitrary units and are expressed as the mean±SEM (n=3). *p<0.05, **p<0.01 *vs* control.

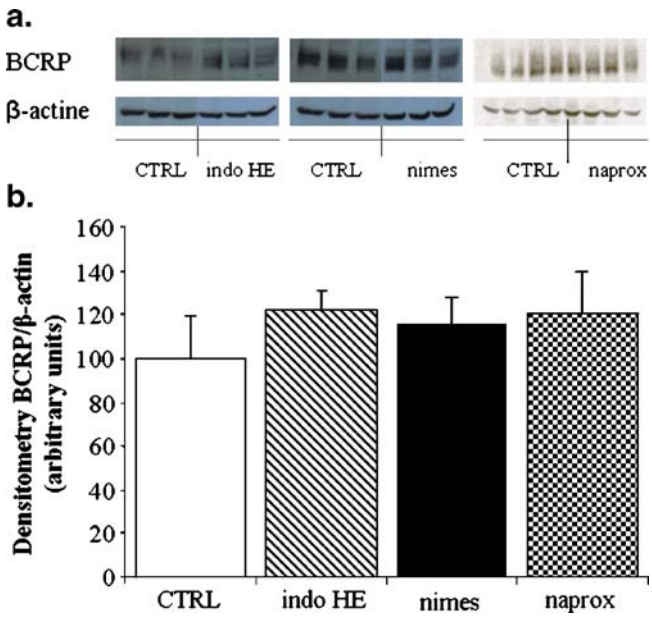

Fig. 12. Effects of COX inhibitors on BCRP protein expression after long-term treatment. **A**. Western blot analysis of BCRP and β-actin in 20 μg of total proteins extracted from Caco-2 cells treated with indomethacin heptyl ester (0.4 μM), nimesulide (10 μM) and naproxen (6 μM) for 4 passages. The immunodetection of the BCRP transporter was performed using the BXP-21 antibody. **B**. Densitometric analysis of immunoblots was performed using the NIH image analysis and the results were normalized directly by β-actin expression. Data are expressed in arbitrary units and are expressed as the mean±SEM (n=3).

DISCUSSION

Caco-2 cell line is a model of value in studying the regulation of P-gp despite the defective regulation of the nuclear factor PXR (pregnane X receptor) (28) which is important for MDR1 induction (29).

In this study, we showed for the first time that the long term treatment with the two specific COX-2 inhibitors, indomethacin heptyl ester and nimesulide, and the COX-1/COX-2 inhibitor, naproxen, down-regulates P-gp expression and activity *in vitro*, in Caco-2 cell line. Moreover, Indomethacin heptyl ester is also able to inhibit P-gp transport activity and expression at both protein and mRNA levels after short term treatment. Our observations suggest a possible downregulation of P-gp by COX inhibitors, which may enhance the accumulation of P-gp substrates. Indeed, Patel *et al.* (22) showed that COX-2 overexpression induces the expression of MDR1 and they suggested that COX-2 inhibition might reduce the chemoresistance phenotype. So nonsteroidal anti-inflammatory drugs (NSAIDs) and COX-2 inhibitors, extensively used as anti-inflammatory agents, have been investigated for cancer chemoprevention and chemotherapy (20,30), and they were proposed as chemosensitiers that could improve chemotherapy in part by inhibiting P-gp. Our results support this orientation.

Taking as objective the research of a putative inhibition of P-gp function by COX inhibitors, we had to validate our model of inhibition. Three substances of reference were tested: cyclosporin A, verapamil and valspodar. The use of Rho123 as a probe to evaluate P-gp activity is well established (31,32). This fluorescent substrate was used for examining the functional pertinence of the regulation of P-gp and/or BCRP

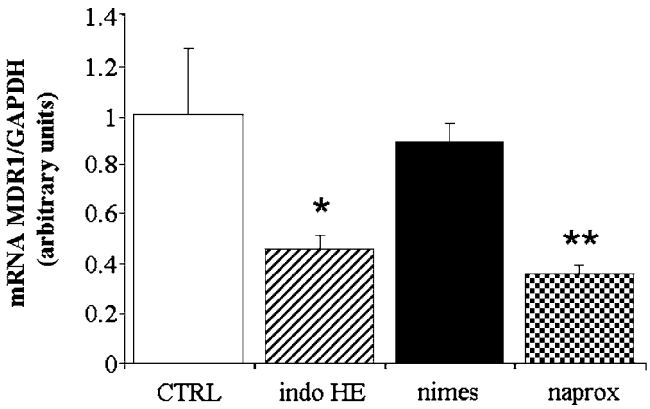

Fig. 13. Effects of COX inhibitors on *MDR1* mRNA expression after long-term treatment. Real-time PCR analysis of *MDR1* mRNA expression in Caco-2 cells incubated for 4 passages with indomethacin heptyl ester (0.4 μM), nimesulide (10 μM) and naproxen (6 μM). Values were normalized to the *GAPDH* content of samples and expressed as the mean±SEM (n=6). *p<0.05 *vs* control.

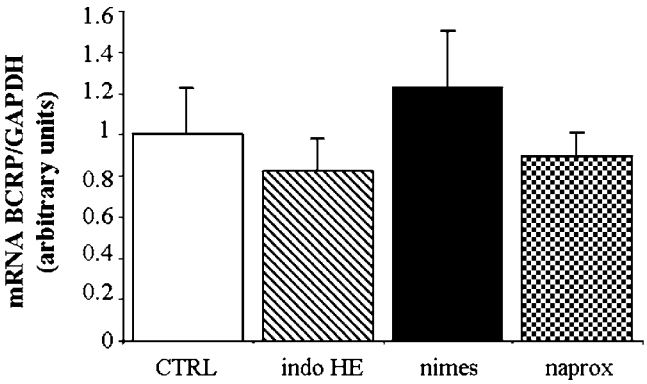

Fig. 14. Effects of COX inhibitors on *BCRP* mRNA expression after long-term treatment. Real-time PCR analysis of *BCRP* mRNA expression in Caco-2 cells incubated for four passages with indomethacin heptyl ester (0.4 μM), nimesulide (10 μM) and naproxen (6 μM). Values were normalized to the *GAPDH* content of samples and expressed as the mean±SEM (n=6).

expressions. The augmentation of intracellular accumulation of this substrate was interpreted as a diminution of P-gp and/or BCRP activity and vice versa. The three tested inhibitors of reference were able to significantly increase the retention of Rho123 in Caco-2 cells reflecting an inhibition of P-gp in Caco-2 cells. ^{3}H-Digoxin, another substrate more specific of P-gp, was used to complete the validation of our model and to dissociate P-gp activity from that of BCRP when that was necessary. Similarly, cyclosporin A, verapamil and valspodar induced an important increase of ^{3}H-Digoxin intracellular accumulation indicating that P-gp is effective in Caco-2 cells. Cyclosporin A which induced the most marked retention with the two substrates, was used as positive control of inhibition. As no information were available about the IC50 of COX-2 inhibitors in Caco-2 cells, we chose arbitrary concentrations which equal to ten times the IC50 obtained using human recombinant COX-2. These concentrations were always less than those needed to inhibit COX-1. For naproxen, a non selective COX-inhibitor, we used a concentration equal to ten times the IC50 for COX-1 which crosses with the IC50 for COX-2. No cytotoxic effect was observed with indomethacin heptyl ester (0.4 μM), with nimesulide (10 μM) or with naproxen (6 μM). In contrast, an inhibitory effect of celecoxib on cell survival was found in our model with the two tested doses (10 and 100 μM). Consequently, celecoxib was excluded of our study.

The activity of P-gp can be inhibited by different mechanisms: (1) the competition at the site of fixation of the substrate, (2) blockade of ATP hydrolysis, (3) diminution of the protein or transcriptional expression or (4) modification of the cellular localization of the protein. We demonstrated that any of our compounds were able to act directly as substrate of P-gp or competitive inhibitor of Rho123 or ^{3}H-Digoxin efflux.

The short-term treatment by indomethacin heptyl ester but neither nimesulide nor naproxen induced an important diminution of P-gp protein and mRNA expression. This negative regulation was translated by a diminution of the activity of this transporter observed by the increase of intracellular accumulation of both Rho123 and ^{3}H-Digoxin. Unexpectedly, naproxen induced a diminution of Rho123 uptake.

The fact that Rho123 is also proposed to be a suitable probe for BCRP raised the question whether this transporter could interfere in the results mentioned above. So we investigated the effects of COX-inhibitors on BCRP at both protein and mRNA levels. Our results showed that modification of these parameters was observed neither after short-term nor long term treatment with indomethacin heptyl ester or nimesulide. Differentially, the short-term exposition to naproxen was able to significantly up-regulate the BCRP protein and mRNA expression which could explain the discrepancy observed between the absence of naproxen effect on ^{3}H-Digoxin uptake and the decrease of Rho123 intracellular accumulation. Several studies have reported that Rho123 is not a substrate for the wild-type form of BCRP however there are mutant forms of BCRP that do transport Rho123 (33–35). In our study we verified that Rho123 was transported by BCRP in our Caco-2 cells as prazosin and mitoxantrone, well know substrates of BCRP (36), were able to compete with the Rho123 efflux and produced an important increase of its uptake (data not shown).

Previously, it has been shown that many drugs that down-regulate P-gp activity lose their inhibitory effect with the augmentation of dosage in term of dose or duration of treatment. For example, in humans it has been shown that the coadministration of verapamil and digoxin for 6 weeks results in an initial increase in the plasma concentrations of digoxin, followed by a gradual decrease (37). For the first time, our results showed that the down-regulation of P-gp persisted after long term-treatment for four passages followed by 2 weeks needed for cell differentiation.

The decreased protein expression and activity resulted from the downregulation of gene expression with indomethacin heptyl ester and naproxen. In contrast, for nimesulide, the regulation was not associated with decreased *MDR1* gene expression. Additional studies are required to resolve this issue.

A finding consistent with our results was reported recently by Puhlmann *et al.* (38) who showed that doxorubicin-induced MDR1 overexpression was down regulated by the COX-2-preferential inhibitor meloxicam in both HL-60 cells and primary AML (Acute Myeloid Leukemic) blasts with subsequent improvement of cytostatic efficacy of doxorubicin.

Different signalling pathways might be proposed to be involved in the negative regulation of *MDR1* by COX-inhibitors. On the one hand, there are some evidence that certain NSAIDS such as ibuprofen exerts COX-independent activity against inflammatory processes by direct inhibition of transcription factors such as nuclear factor-KB (NF-KB) and activator protein-1 (AP-1) (39) with subsequent down-regulation of inflammation-related genes such as COX-2 or tumour necrosis factor-α (TNF-α). The *MDR1* gene promoter also contains putative binding sites for AP-1 and NF- KB, which seem to be relevant for *MDR1* gene induction (40). In consequence, the inhibition of these factors by NSAIDS would induce a negative regulation of *MDR1* gene. Moreover, previous study by Smith *et al.* (41) also demonstrated that COX-inhibitors exert anti-proliferative effects on human colorectal cancer cells by different mechanisms which were not entirely dependent on COX-2 expression. This COX-2 independent mechanism could explain our results since, according to previous study (42), COX-2 mRNA was detected at very low level in differentiated

Caco-2 cells, furthermore we were unable to visualize an immunoreactive protein by western blot analysis (data not shown). This low level of COX-2 expression reported in differentiated Caco-2 could support the COX-2-independent pathway to be involved in the observed results. Besides, the used concentration of COX-inhibitors were demonstrated not to be able to produce further inhibition of the low PGE2 synthesis observed in our cells. In line with our results, Zatelli *et al.* showed that rofecoxib, a selective COX-2 inhibitor, sensitizes MTC (medullary thyroid carcinoma) cells, TT, to the cytotoxic effects of doxorubicin, reducing P-gp expression and function by a COX- independent mechanism (23). On the other hand, Ratnasinghe *et al.* (30) noticed parallel MDR1 and COX-2 expression in breast cancer cells and postulated the modulation of *MDR1* by prostaglandins via the induction of phosphokinase C and subsequent expression of c-Jun, a subunit of AP-1. The inhibition of prostaglandins synthesis by COX-inhibitors could block this cascade resulting in a negative modulation of *MDR1*. This mechanism could be involved when studying the action of COX-inhibitors in Caco-2 cells after COX-2 stimulation, and is the subject of further investigations.

CONCLUSION

In summary, our results showed that selective COX-2 inhibitors, indomethacin heptyl ester and nimesulide, and a non selective COX-2-inhibitor, naproxen, reduced *MDR1* expression, P-gp relative amount and function in Caco-2 cells by a PGE2 independent mechanism. This points to a clinical interest of these compounds to overcome multi-drug resistance and enhance the cytotoxic effects of specific drugs that are substrates of P-gp and used in the treatment of colorectal cancer.

ACKNOWLEDGMENTS

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