Abstract. It has been shown previously that a novel nitro-oxy derivative of celecoxib exerts antiproliferative and pro-apoptotic effects in human colon cancer cells. The aim of this study was to elucidate whether these biological properties depend on COX-2 inhibition and/or NO release. Therefore, the derivative was decomposed into the parent compound celecoxib and the NO donor benzyl nitrate and the biological role of each was tested in COX-2-positive (HT-29) and -negative (SW-480) colon cancer cells. The main findings were that the nitro-oxy derivative behaved like celecoxib in HT-29 cells in terms of COX-2 and ERK/MAPK inhibition, as well as induction of apoptosis, while the benzyl nitrate had no such effects. Interestingly, the β-catenin system was activated by the nitro-oxy derivative as well as by benzyl nitrate alone more potently than by the parent compound celecoxib, suggesting a possible regulatory role for NO. In SW480 cells, these activities were substantially less pronounced, suggesting the presence of COX-2-dependent mechanisms in the modulation of these parameters.

Epidemiological, animal and cell culture studies have widely shown chemopreventive and tumour-regressive effects of non-steroidal anti-inflammatory drugs (NSAIDs) on colorectal cancer and several other tumour types (1, 2). The antiproliferative effects of NSAIDs are, at least partially, attributed to their cyclooxygenase (COX) inhibitory activity (3). Two isoforms of COX are recognized, of which COX-1 is expressed constitutively, while COX-2 is inducible at sites of inflammation and tissue repair. Furthermore, COX-2 expression is high in many types of cancer and increases early on during colon carcinogenesis (4), and COX-2 derived prostaglandins are able to promote tumour growth by accelerating cellular proliferation, inhibiting apoptosis and enhancing metastasis and angiogenesis (5).

Thus, selective inhibition of the COX-2 isoenzyme offers a useful drug design concept that results in the development of effective anti-inflammatory drugs devoid of the adverse gastrointestinal and/or cardiovascular toxicity associated with the use of traditional NSAIDs (6). Unfortunately, selective COX-2 inhibitors such as celecoxib and rofecoxib alter the natural balance in the COX pathway (7), decreasing the amount of the desirable vasodilatory and anti-aggregatory prostacyclin while simultaneously increasing the level of the undesirable prothrombotic thromboxane A2 (8-9).

Based on the observation that nitric oxide (NO) possesses some of the same useful pharmacological properties as prostaglandins within the gastric mucosa, such as vascular relaxation and inhibition of platelet activation, a new class of coxibs able to release NO has been synthesized (10). NO donor coxib may thus represent a novel approach to reducing the side-effects of coxibs, especially their gastric and cardiovascular toxicity (10).

NO-coxibs consist of a known coxib molecule and an NO-releasing group (typically -NO₂) linked to it via a chemical spacer. This coupling might deliver NO to the site of coxib-induced damage and thus reduce gastric toxicity. Existing data, mostly from animal studies, indicate that this hypothesis may be true (11).

Among NO-donor coxibs, a series of nitro-oxy methyl substituted derivatives of celecoxib that present one or two nitro-oxy functions on the phenyl rings have been studied. These new compounds are able to inhibit the COX-2 enzyme and are capable of dilating rat aorta strips precontracted with phenylephrine in an NO-dependent manner (10). In a previous study, it was demonstrated that in HT-29 colon cancer cells celecoxib and its nitro-oxy derivatives induced a significant reduction of cell growth; a reduction that was accompanied by the inhibition of the ERK-MAPK cascade, the induction of apoptosis and the modulation of the

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E-cadherin/β-catenin system. Among these derivatives, the compound with two nitro-oxy functions was the most effective in affecting the parameters that were studied, raising the question of whether NO may be required for this modulation (12).

Therefore, given the structural complexity of this molecule, this study decomposes the compound’s two nitro-oxy functions into the parent compound celecoxib and the NO donor benzyl nitrate in order to gain insight into the contribution of the NO donor to the known activities of celecoxib (Figure 1). The effects of celecoxib, a nitro-oxy derivative of celecoxib and benzyl nitrate on a number of cellular responses including proliferation, apoptosis, activation of ERK and PI3K pathways, invasion and translocation of β-catenin from the nucleus to the plasma membrane were compared on HT-29 and SW-480 human colon cancer cell lines.

Materials and Methods

Reagents. Celecoxib, a nitro-oxy derivative of celecoxib and benzyl nitrate were a gift from the laboratory of Professor Gasco, Department of Scienza e Tecnologia del Farmaco, University of Torino, Italy (10). The drugs were solubilized in dimethylsulfoxide (DMSO) (Sigma Chemical Co, Saint-Louis, USA) and freshly diluted in culture medium before each experiment. The final DMSO concentration never exceeded 0.1% and this condition was used as a control in each experiment.

Rabbit polyclonal antibody specific for pAkt, ERK1, gp91-phox, bak and procaspase-3, mouse monoclonal antibody specific for β-catenin, caspase-3, PI3-kinase p85α, Akt and pERK1/2, goat anti-rabbit and goat anti-mouse secondary antibodies were obtained from Santa Cruz Biotechnology, Inc (CA, USA). Rabbit polyclonal antibody specific for COX-2 was from Cayman Chemical (Michigan, USA). Enhanced chemiluminescence detection system was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Mouse monoclonal antibody specific for β-actin and all other reagents were purchased from Sigma Chemical Co (Saint Louis, MO, USA).

Cell culture. HT-29 and SW-480 human colon cancer cell lines were obtained from the American Type Cell Collection (Manassas, VA, USA). The cells were grown and maintained in McCoy’s 5A (HT-29) and DMEM (SW-480) medium supplemented with 10% foetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 25 μg/ml amphotericin B. Cells were cultured at 37˚C in a humidified incubator with 5% CO2 and 95% air, and examined regularly using an inverted microscope.

For the experiments, cells were seeded at a density of 3x10⁴ cells/cm² and cultured for 24 h to allow them to adhere to the substratum and then treated with the drugs or DMSO.

Viability assay. Cell viability was assessed by the trypan blue exclusion assay. Aliquots of cell suspension were incubated with trypan blue solution for 5 min. Finally, cells were transferred to a Bürker chamber and counted by light microscope. Dead cells were defined as those stained with the dye.

Protein extraction. Total extracts: Collected cells were suspended in lysis buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.05% aprotinin, 0.1% Igepal) and then incubated for 30 min at 4°C. The suspension was centrifuged for 25 min at 12000 rpm and the supernatant from this centrifugation was saved as the total extracts.

Nuclear extracts: Collected cells were suspended in lysis buffer (10 mM Hepes-NaOH pH 7.8, 10 mM KCl, 2 mM MgCl₂, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, and 0.1 mM PMSF) and then incubated for 15 min at 4°C. After addition of 10% (v/v) Nonidet P-40, the cell suspension was mixed, incubated for 30 min at 4°C,
and centrifuged for 15 min at 3000 rpm. The pellet was resuspended in lysis buffer (50 mM Hepes-NaOH pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF and 10% v/v glycerol). The suspension was mixed for 30 min at 4°C and centrifuged for 15 min at 3000 rpm; the supernatant from this centrifugation was saved as the nuclear extract.

**Membrane-associated fractions:** Collected cells were suspended in hypotonic buffer (10 mM Tris pH 7.4, 0.2 mM MgCl₂, 2 μg/ml pepstatin A, 2 μg/ml leupeptin and 100 μg/ml PMSF) for 10 min on ice and then homogenised with a Dounce homogeniser. The homogenates were centrifuged for 37 min at 20750 rpm; the resulting precipitate was analysed as a membrane-associated fraction.

**Western blotting.** Protein contents in the supernatants were measured using a commercially available assay (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard. Extracts were then subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis on 12, 10 or 7.5% acrylamide gels. The blots were incubated with desired primary antibodies and then incubated with peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulins in Tris-buffered saline-Tween containing 2% (wt/vol) non-fat dry milk, and developed with the enhanced chemiluminescence reagents. Band intensities were quantified by densitometry and the expression of proteins was reported as a proportion of β-actin, ERK1, Akt or gp91-phox protein expression to monitor any discrepancies in gel loading (VersaDoc Imaging System 3000; Bio-Rad).

**Fluorescence microscopy.** The cells were fixed and permeabilized for 20 min at –20°C with methanol/acetone (1:1) and were incubated with anti-β-catenin primary antibody followed by anti-mouse Cy3-conjugated secondary antibody. Then the cells were stained with 4',6-diamidino-2-phenylindole (DAPI, 1 mg/ml in methanol) for 30 min at 37°C to detect nuclei. After washing with PBS, slides were mounted with H₂O/glycerol (1:1) and viewed under a fluorescence microscope equipped with a UV light filter (Dialux 20; Leitz, Wetzlar, Germany).

**Invasion assay.** Invasion was evaluated with Boyden chambers equipped with 8-μm porosity polyvinylpyrrolidone-free polycarbonate filters that were coated with 50 μg/ml of Matrigel solution. Invasiveness was quantified by counting crystal violet-stained cells that invaded Matrigel using a Zeiss microscope (Oberkochen, Germany) equipped with bright-field optics (×40). For each filter/Matrigel, the number of cells in ten randomly chosen fields was counted, and the counts were averaged (means±SD). Results are expressed as the number of migrated cells per high-power field.

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**Figure 2.** Effect on cell growth, ERK phosphorylation state, PI3K/Akt pathway and apoptotic features. HT-29 cells were incubated for 24 h with 50 μM celecoxib, 50 μM nitro-oxy derivative, 100 μM benzyl nitrate, alone or in combination with 50 μM celecoxib. Cell viability was determined by the trypan blue exclusion test (A). Values are represented as a percentage of the control and bars are the means±S.D. from 3 independent experiments. Statistical significance: *p<0.001 vs untreated control; §p<0.001 vs. d; no statistical significance between all groups. The cells were processed for Western blot analysis with anti-pERK1/2 (B), -PI3K, -pAkt (C), -bak, -caspase-3 and -procaspase-3 (D) antibodies. Protein content was normalized with anti-β-actin, ERK1 or Akt antibody and analysed by densitometry. The blots shown are representative of 3 independent experiments.
Gelatinolytic zymography. Conditioned media from the cell cultures were analysed for gelatin degradation activity by SDS-PAGE under non-reducing conditions. Samples for analysis were diluted in 1:1 non-reducing buffer (12.5% 0.5M Tris-HCl pH 6.8, 10% glycerol, 4% SDS, and 0.05% bromophenol blue) and then fractionated by electrophoresis on sodium dodecyl sulfate polyacrylamide gel electrophoresis co-polymerized with 0.2% gelatin A. After separation, the gels were washed twice in 2.5% Triton-X 100 for 15 min and incubated in buffer (Tris-HCl 5 mM, CaCl2 10 mM, NaCl 50 mM, pH 7.6) at 37°C over night. The gelatinolytic activities of matrix metallopeptidase 2 (72 kDa type IV collagenase, MMP-2) were visualised by staining the gel with 0.1% Coomassie brilliant blue and destained with 45% methanol, 10% v/v acetic acid until clear bands suggestive of gelatin digestion were present. The gels were washed out with distilled water and scanned for densitometry (VersaDoc Imaging System 3000; Bio-Rad).

Statistical analysis. Differences between means were analysed for significance using the one-way ANOVA test with the Bonferroni post hoc multiple comparisons being used to assess the differences between independent groups. All values are expressed as means±SD, and differences were considered significant at \( p<0.05 \).

Results

The contribution of the NO-releasing moiety (-ONO₂) to the biological effects of nitro-oxy derivative of celecoxib in HT-29 colon cancer cells was analysed. The cells were treated for 24 h with appropriate concentrations of NO donor benzyl nitrate alone or in combination with celecoxib (IC₅₀: 51 μM) to reconstitute the molecular structure of nitro-oxy derivative (IC₅₀: 40 μM).

For this study, a concentration of 100 μM benzyl nitrate was chosen since it is comparable to the amount of NO that is generated from nitro-oxy derivative as confirmed by Griess reaction (data not shown).
Benzyl nitrate alone at 100 μM concentration did not influence the cell viability rate, while celecoxib, its nitro-oxy derivative and co-administration of celecoxib (50 μM) and benzyl nitrate (100 μM) caused a remarkable decrease of the survival of HT-29 cells (Figure 1A).

Based on the reported ability of celecoxib to inhibit ERK/MAPK, the effect of nitro-oxy derivative and benzyl nitrate alone or in combination with celecoxib on the phosphorylation state of ERK was evaluated. Benzyl nitrate had no effect on the active form of ERK as the reduction of ERK phosphorylation occurred only if the NO donor was in association with celecoxib (Figure 1B).

The PI3-kinase pathway is involved in the regulation of cell survival and apoptosis, and its activation is associated with tumour growth and progression (14). In line with the data regarding ERK phosphorylation, celecoxib, its nitro-oxy derivative and benzyl nitrate in combination with celecoxib reduced the expression of both p85α, the regulatory subunit of PI3-kinase pathway, and phospho-Akt, the downstream effector of the cascade. Again, the NO donor benzyl nitrate alone had no effect (Figure 1C).

As regards the regulation of apoptosis, a significant reduction of procaspase-3 and a considerable increase of caspase-3 and bak was seen only when the NO donor was in association with celecoxib (Figure 1D).

Previous studies evidenced that celecoxib and its nitro-oxy derivative are able to induce the membrane fraction of β-catenin/E-cadherin complex (12). To evaluate whether NO was involved in the regulation of this effect, the expression of β-catenin protein in HT-29 cells treated with benzyl
nitrate was also analysed. Immunofluorescence and Western blot analysis indicated that benzyl nitrate alone was able to increase the expression of beta-catenin in the membrane fraction to a greater extent than celecoxib alone. In addition, the increase was markedly higher in cells exposed to the NO donor in combination with celecoxib with respect to nitro-oxy derivative, (Figure 2A, B), suggesting that the NO-releasing group amplifies the celecoxib effect. In all cases, the increase of membranous β-catenin levels corresponded to the reduction of nuclear β-catenin levels (Figure 2A).

An increase of beta-catenin in the membrane fraction after HT-29 cell treatment with celecoxib and its nitro-oxy derivative may lead to an increase of cell-cell adhesion and to a reduction of invasiveness. Thus the ability of these compounds to reduce colon cancer cell migration was determined. The classic Boyden chamber assay showed that all the compounds significantly decreased the ability of HT-29 cells to invade Matrigel. In addition, the nitro-oxy derivative showed the highest migration inhibition (Figure 3A).

To better determine the ability of celecoxib and its derivative to reduce cell migration, the activity of matrix metalloproteinase-2 (MMP-2), which is involved in the breakdown of the basement membrane by extracellular matrix degradation was also evaluated. The zymogram showed that celecoxib, its nitro-oxy derivative and benzyl nitrate in combination with celecoxib reduced the activity of MMP-2 in the medium supernatant of HT-29 cells. Also in this case, benzyl nitrate alone was less effective than the parent compounds (Figure 3B).

Since it has previously been demonstrated that the nitro-oxy derivative is able to decrease the expression of COX-2, a classical target of celecoxib, the possible role of the NO-releasing moiety was evaluated for this effect. Benzyl nitrate alone did not determine any detectable variation of COX-2 expression, while its co-administration with celecoxib reduced COX-2 protein levels, thus indicating that the NO-releasing group is not implicated in COX-2 expression (Figure 3C).

To verify the involvement of COX-2 and the influence of the NO-releasing group on the effect of the nitro-oxy
derivative the effect of each constituent in a COX-2 nonexpressing SW-480 cell line was also examined.

In these cells, all the compounds that were tested induced a small decrease in SW-480 cell growth (Figure 4A). In accordance with the modest effect on cell viability, the treatment with celecoxib, its nitro-oxy derivative and benzyl nitrate in combination with celecoxib caused only a weak decrease of phospho-ERK expression, while benzyl nitrate alone was ineffective (Figure 4B). The ability to inhibit p85α and Akt expression was maintained in response to celecoxib, nitro-oxy derivative and benzyl nitrate in combination with celecoxib, while NO donor alone did not have any effect (Figure 4C). In contrast, none of the compounds were able to interfere with caspase-3 activation, suggesting that the inhibition of cell growth is not dependent on apoptosis induction (Figure 4D).

Discussion

Data from a previous study (12) demonstrated that a nitrooxy derivative of celecoxib with two NO-releasing moieties effectively inhibits HT-29 human colon cancer cell growth. The biological activity seen with this compound can possibly be ascribed to the presence of a NO releasing moiety in addition to the COX-2 inhibitory effect. The goal of this study was to determine the contribution of the NO donor to the known activities of celecoxib. To evaluate this question this study assessed the effects of each structural component of the nitro-oxy derivative on HT-29 human colon carcinoma cells, which express COX-2, and on SW-480 human colon carcinoma cells which, in contrast, do not express the enzyme.

In accordance with literature data (12, 15, 16), the current data clearly indicate that celecoxib and the nitro-oxy derivative influence the expression level of cell growth regulating proteins and apoptosis markers. For instance, Akt kinase, which is frequently overexpressed in tumour cells, has been found to be inhibited by celecoxib (17). It has been reported that the blockade of Akt activation depends on inhibition of the upstream 3-phosphoinositide-dependent kinase-1 (16) and represents a major molecular mechanism by which celecoxib induces apoptosis. In line with these findings, the nitro-oxy derivative was able to inhibit p85α and Akt kinase with greater potency with respect to benzyl nitrate alone. In a similar manner, the nitro-oxy derivative reduced the phosphorylation state of ERK and modulates the expression of apoptosis regulating proteins.

Several authors have reported that COX-2 expression is regulated through the activation of ERK, JNK, and p38 in transformed cells, and that the amount of COX-2 is significantly decreased by their inhibitors (18-20). In addition, COX-2 overexpression is reported to be associated significantly with activated AKT expression, suggesting an interaction between COX-2 activity and PI3-Kinase/Akt pathway (21). In accordance with these findings, in this study it was observed that celecoxib and its derivative decreased ERK and Akt activation and concomitantly reduced COX-2 expression. Taken together, these findings do not evidence a correlation between the presence of the NO-donating moiety and the ability to inhibit ERK/MAPK and PI3-kinase/Akt pathways or to induce apoptosis, which seem rather to be regulated by COX-2-in dependent mechanisms. This study also showed that treatment of HT-29 human colon carcinoma cells with celecoxib led to an increment of the membrane-bound β-catenin and to a reduction of the nuclear fraction of β-catenin. Interestingly, both benzyl nitrate alone and the nitro-oxy derivative induced beta-catenin protein levels more potently than did celecoxib, indicating that the NO-releasing moiety is crucial for the modulation of this pathway.

The up-regulation of the β-catenin system mediated by celecoxib and its nitro-oxy derivative may suggest chemopreventive effects of these drugs on colon cancer. These compounds may reduce the ability of tumour cells to invade local tissues and to spread to distant sites, either by reducing the secretion of MMP-2 or by inhibiting the adhesiveness of colon cancer cells. In this regard, it has been reported that the inhibition of MMPs exerts a protective effect on tumour promotion through the regulation of proinflammatory enzymes, including COX-2 (22). COX-2 is rapidly induced by growth factors, tumour promoters and hormones, thus playing an important role in inflammation and wound healing (23). Overexpression of COX-2 contributes to carcinogenesis by stimulation of cell survival and angiogenesis (24). From the current results it is, therefore, conceivable that the NO-releasing moiety is not crucial for the inhibition of cell invasion, as the NO donor benzyl nitrate did not influence cell migration and MMP-2 release.

It is not possible to absolutely predict the targets of celecoxib and the derivative responsible for their antiproliferative/antitumour activity. However, it was observed that the cells lacking COX-2 (SW-480) responded to nitro-oxy derivative in a different manner from HT-29 cells.

In particular, in SW-480 cells the antiproliferative effect and the inhibition of ERK/MAPK and PI3-kinase/Akt pathways of nitro-oxy derivative were less effective than those detected in HT-29 cells. These findings indicate that the presence of COX-2 is required above all for the growth inhibitory and pro-apoptotic effects of celecoxib and derivative on colon cancer cells. On the contrary, the fact that celecoxib and nitro-oxy derivative were not able to induce β-catenin expression in SW-480 cells (data not shown) suggests that this effect may involve both COX-2- and NO-dependent mechanisms.
These preliminary data indicate that celecoxib and nitro-oxy derivative do not block prostaglandin synthesis, at least at concentrations that inhibit cell growth (data not shown). This suggests that the effect of nitro-oxy derivative occurs independently of the cellular COX-2 expression, the classical target of the selective COX-2 inhibitors.

In conclusion, the current data suggest that celecoxib and the novel nitro-oxy derivative of celecoxib, which promises to be less toxic than the parent compound, are equally effective in HT-29 and SW-480 colon cancer cells. The structural modifications of celecoxib seem to modify its antiproliferative activity that in the NO derivative is exerted through mechanisms independently of (or in addition to) its COX-2 inhibitory effect.

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