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Novel nitro-oxy derivatives of celecoxib for the regulation of colon cancer cell growth

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Abstract

Celecoxib is a non-steroidal anti-inflammatory drug (NSAID) developed as a selective inhibitor of cyclooxygenase-2 (COX-2). Despite the associated cardiovascular toxicity risk, celecoxib has been found to be effective in reducing cancer risk in animal and human studies. In the present study the antiproliferative activity of novel nitro-oxy-methyl substituted analogues of celecoxib (NO-cel), potentially less cardiotoxic, has been investigated *in vitro* on human colon cancer cells and compared with action of the parent drug. Moreover, experiments were performed in order to evaluate whether COX-2 pharmacological inhibition may affect β -catenin/E-cadherin signalling pathway. All the tested analogues of celecoxib exerted a significant antiproliferative activity on COX-2 positive HT-29 human colon cancer cells, being less effective on the COX-2 negative SW-480 human colon cancer cell line. In particular, the analogue displaying two nitro-oxy functions fully mimicked the known inhibitory properties of celecoxib, including inhibition of COX-2, as well as of ERK/MAPK and β -catenin signalling pathways. Interestingly, the latter compound also elicited a strong reorganization of the β -catenin/E-cadherin complex, which has been suggested to be relevant for colon carcinogenesis. On these premises, NO-cel analogues of celecoxib can represent promising colon cancer chemopreventive agents potentially able to affect colon cancer development.

Keywords

- Colon cancer;
 - NO-celecoxib;
 - Cyclooxygenase-2;
 - Proliferation;
 - β -Catenin/E-cadherin
-

1. Introduction

Colon cancer represents one of the most common human malignancies in either developing or industrialized countries. Tumour progression in most colon cancers is characterized by cyclooxygenase-2 (COX-2) overexpression [1] and aberrant activation of the WNT/ β -catenin pathway [2]. Along these lines, it has been suggested that the oncogenic potential of COX-2 may be mediated through the involvement of the recently discovered peroxisome proliferator-activated receptor δ (PPAR δ) factor, which is again overexpressed in neoplastic tissue [3].

Overexpression of COX-2 seems consistent with the suggested major role of prostaglandin E₂ (PGE₂), a potent COX-2-derived mediator that has been shown to affect several tumour-related processes, including cell proliferation, motility and apoptosis [4]. As a matter of fact, up-regulation or depletion of COX-2 has been reported to promote and inhibit, respectively, with PGE₂ increased generation resulting in the nuclear accumulation of β -catenin [5].

β -Catenin is a binding partner for the product of the adenomatous polyposis coli (APC) tumour suppressor gene, which acts as a negative regulator of β -catenin and WNT signalling [6]. In normal cells β -catenin usually binds to APC and, following phosphorylation by glycogen synthase kinase-3 β (GSK-3 β), is then targeted to be degraded by the ubiquitin proteasome pathway.

Following activation of the WNT signalling pathway, which operates through inhibition of GSK-3 β , β -catenin remains unphosphorylated and proteasome degradation is prevented. Stabilized β -catenin is then released from the complex with APC and translocates into the nucleus acting as a transcriptional factor, leading to the increased expression of WNT target genes including c-myc and PPAR β/δ , which are known to play a relevant role in tumourigenesis [3] and [7]. Alternatively, β -catenin can form a complex with E-cadherin at adherens junctions located to promote cell–cell adhesion. Indeed, a decrease in cell–cell adhesion has been shown to be associated with changes in the structure or in the expression of the E-cadherin/ β -catenin complex [8], potentially resulting in a loss of contact inhibition of cell proliferation and then favouring invasion and metastasis [9]. E-cadherin loss and abnormal E-cadherin expression have been observed in tumour development [10], and correlate with colorectal carcinogenesis [11].

At present, despite the recent significant advances in terms of therapeutic strategies, it is unknown whether COX-2/PGE₂ and WNT/ β -catenin signalling pathways may converge during colon cancer development. Along these lines, literature in the field is focussing the attention on the anti-colorectal cancer activity of conventional non-steroidal anti-inflammatory drugs (NSAIDs), which are known to represent potent but non-selective COX-inhibitors [12] and [13].

Although the anticancer action of NSAIDs was originally believed to rely on the inhibition of COX-1 and COX-2 activities and the related production of prostaglandins [14], at present the real mechanism of action of the individual NSAIDs is disputed, with is increasing evidence suggesting that specific NSAIDs may even act, at least in part, in a COX-independent manner [15].

In any case, the potential relevance of NSAIDs and their use as chemopreventive or anticancer agents has been greatly limited by their well-known gastro-toxicity intrinsically related to the gastro-protective effects attributed mainly to the activity of COX-1 isoform.

In order to circumvent this problem, selective inhibitors of COX-2 with a lower risk of gastrointestinal side effects, designated as coxibs, have been designed as an effective alternative to conventional NSAIDs. However, the clinical use of coxibs revealed consistent risks of cardiovascular toxicity [16], [17], [18] and [19] that was attributed to the ability to reduce the biosynthesis of prostacycline (PGI₂) in favour of thromboxanes [20] and [21]. Currently, among the group of selective COX-2 inhibitors only celecoxib has been approved by Food and Drug Administration (FDA), for adjuvant treatment of patients with familial adenomatous polyposis (FAP), a precancerous disease.

Some years ago, a novel class of pharmaceutical agents, nitric oxide non-steroidal anti-inflammatory drugs (NO-NSAIDs), was found to display reduced gastro-toxicity, likely through several NO-mediated mechanisms [22]. NO-NSAIDs consist of a conventional NSAID molecule to which a NO-releasing group (typically ONO₂) is linked covalently via an appropriate spacer. These drugs may potentially exert also an anti-tumour activity, since they are several-fold more potent than conventional NSAIDs in inhibiting colon cancer cell growth, *in vitro* and in animal models [23]. Among them, NO-donating aspirin (NO-ASA) NCX4040 and NCX4016 can be considered as promising anticancer agents [24] that, interestingly, is likely to be due to the formation of quinone methide from the spacers [25] and [26], with neither aspirin nor NO moieties being able to contribute to the overall antitumour effect.

More recently a new class of coxibs able to release NO (NO-coxibs) has been developed in order to both implement gastric tolerance and to reduce cardiotoxicity of the parent drug [27] and [28]. To this class of compounds also belong NO-donor analogues of celecoxib (NO-cel) that present one or two nitro-oxy functions on the phenyl rings [29], [30], [31] and [32] that have been reported to inhibit COX-enzymes with a preference for COX-1 or COX-2 isoform, depending on the position of ONO₂ function. In addition, they have reported to be able to dilate rat aorta strips pre-contracted with phenylephrine in a NO-dependent manner.

The present study was then performed in order to investigate the effect of these new NO-donor analogues of celecoxib on colon cancer cell growth and survival, as well as their impact on the E-cadherin/ β -catenin system.

2. Materials and methods

2.1. Reagents

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

Cell culture plasticware were from TPP (Trasadingen, Switzerland). Rabbit polyclonal antibody specific for PPAR β , Bak, procaspase-3, c-myc and for ERK1, mouse monoclonal antibody specific for E-cadherin, β -catenin and phospho-ERK1/2, 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 and COX-2 purified protein was from Cayman Chemical (Michigan, USA). Mouse Cy3-conjugated secondary antibody and enhanced chemiluminescence (ECL) detection system was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Cell culture

The human HT-29 and SW-480 colon cancer cell lines were obtained from American Type Cell Culture (Manassas, VA, USA). The different human colon cancer cell lines 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 regularly examined using inverted microscope.

For the experiments, cells were seeded at a density of 3×10^4 cells/cm² and cultured for 24 h to allow to adhere to the substratum and then treated with the drugs or DMSO.

2.3. Proliferation assay

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

2.4. Protein extraction

2.4.1. Total extracts

Cells were seeded in 75 cm² plates and properly treated. Collected cells were suspended in lysis buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM phenylmethyl-sulfonyl 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 12,000 rpm and the supernatant from this centrifugation was saved as the total extracts.

2.4.2. Nuclear extracts

Cells were seeded in 75 cm² plates and properly treated. Collected cells were suspended in lysis buffer containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (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 resulting pellet was resuspended in lysis buffer containing 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.

2.4.3. Membrane-associated fractions

Cells were seeded in 75 cm² plates and properly treated. Collected cells were suspended in hypotonic buffer containing 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 homogenized with a Dounce homogenizer. The homogenates were centrifuged for 37 min at 20,750 rpm; the resulting precipitate was analysed as the membrane-associated fraction.

2.5. Western blot analysis

Protein content was determined using the Protein Assay Kit 2 (Bio-Rad Laboratory, Hercules, CA).

Equal amounts of proteins were mixed with solubilization buffer containing 250 mM Tris (pH 8.8), 4% sodium dodecyl sulfate (SDS), 16% glycerol, 8% 2-mercaptoethanol and 0.1% bromophenol blue, and then fractionated by electrophoresis on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto nitrocellulose for 2 h in a Biorad electroblotting device. Nitrocellulose matrices were blocked with 5% milk in TBST (1 M Tris buffer saline, pH 7.4, 5 M NaCl, 0.1% Tween-20) for 1 h at room temperature. For immunodetection, matrices were incubated overnight at 4 °C with primary antibody. The matrices were then detected by incubation for 1 h at room temperature with the corresponding horseradish peroxidase-conjugated secondary antibody. The immunoreactive bands were visualized using the ECL system. Band intensities were quantified by densitometry and expression of proteins was reported as a proportion of β-actin or ERK1 protein expression to control for any discrepancies in gel loading. Fold change versus control values has been calculated by normalizing densitometric values obtained from the various proteins with those obtained for β-actin or ERK1 (VersaDoc Imaging System 3000, Biorad).

2.6. DAPI staining

Cells were plated onto glass slides in 12-well plates and properly treated. The cells were fixed with 95% ice-cold ethanol for 5 min and stained with 4',6-diamidino-2-phenylindole (DAPI, 1 mg/ml in methanol) for 30 min at 37 °C in the dark. After washings with phosphate-buffered saline (PBS), slides were mounted with H₂O/glycerol (1:1) and viewed under a fluorescence microscope equipped with a UV light filter (Dialux 20, Leitz).

2.7. Fluorescence microscopy

Cells were seeded on 6-well culture plates, allowed to adhere for 24 h and then treated. After treatment, the cells were fixed and permeabilized for 20 min at -20 °C with methanol/acetone (1:1). Cells were then incubated with antibody anti-E-cadherin and anti-β-catenin (1:250 in PBS containing 0.5% Triton X-100 and 0.05% NaN₃), overnight at 4 °C. After washings with PBS containing 0.5% Triton X-100 and 0.05% NaN₃, cells were incubated with anti-mouse Cy3-conjugated secondary antibody (1:1000 in PBS containing 0.5% Triton X-100 and 0.05% NaN₃) for 60 min at room temperature. After washings with PBS, the cells were stained with DAPI for 30 min at 37 °C to detect nuclei. After washings with PBS, slides were mounted with H₂O/glycerol (1:1) and viewed under a fluorescence microscope equipped with a UV light filter (Dialux 20, Leitz).

2.8. Real-time polymerase chain reaction (PCR) analysis

Total RNA was extracted using NucleoSpin[®] RNA II kit (Macherey-Nagel, Germany). Real-time PCR was performed using single-stranded cDNA prepared from total RNA (1 µg) with a High Capacity cDNA Archive kit (Applied-Biosystems, CA). Forward (FW) and reverse (RV) primers were designed using Beacon Designer software (Bio-Rad, Hercules, CA).

Gene access number	Sequence: FW: forward primer; RV: reverse primer	Annealing (°C)	Cycles
GAPDH	FW-5'-GTC GGA GTC AAC GGA TTT GG-3'	52	30
NM_002046	RV-5'-GGG TGG AAT CAT ATT GGA ACA TG-3'		
COX-2	FW-5'-TGG TCT GGT GCC TGG TC-3'	58	35
NM_000963	RV-5'-AGT ATT AGC CTG CTT GTC TGG-3'		

[Table options](#)

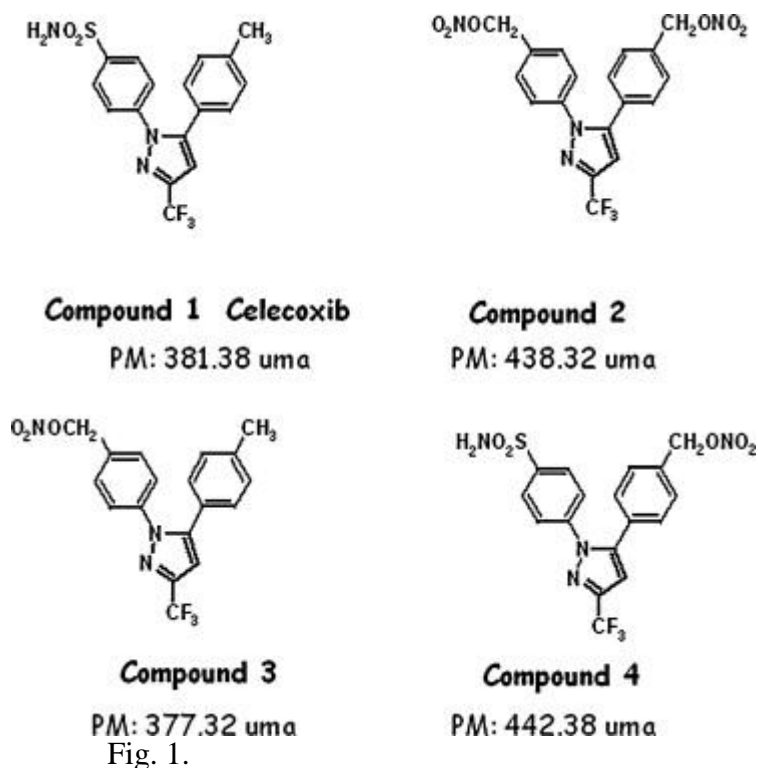
25 µl of a PCR mixture, containing cDNA template equivalent to 80 ng of total RNA, 5 pmol each of the forward and reverse primers and 2 × iQ[™] SYBR[®] Green SuperMix, were amplified using an iCycler PCR (Bio-Rad, Hercules, CA). Each sample was tested six times and the threshold cycle (Ct) values were the corresponding mean. The fold change was defined as the relative expression compared to that at time 0 (time of seeding cells), calculated as $2^{-\Delta\Delta Ct}$, where $\Delta Ct = Ct_{\text{sample}} - Ct_{\text{GAPDH}}$ and $\Delta\Delta Ct = \Delta Ct_{\text{sample}} - \Delta Ct_{\text{time 0}}$.

2.9. Statistical analysis

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

3. Results

A number of ONO₂-positional isomers (NO-cel) of celecoxib was generated by substituting the methyl and/or the amino-sulfonyl groups of celecoxib with a -CH₂ONO₂ group ([Fig. 1](#)), as reported by Boschi et al. [\[32\]](#).



Molecular structure of celecoxib and of its nitro-oxy derivatives.

[Figure options](#)

The dose- and time-dependent effects of NO-cel on the growth of both cell lines were compared with that displayed by celecoxib. All tested drugs resulted in an antiproliferative effect, characterized by a significant decrease of total and living cells as compared to control conditions, without induction of necrotic cell death, that never exceeded 10% of total cells. In both cell lines growth inhibition was dose-dependent and the number of living cell was found to be significantly reduced after a 72 h exposure.

Each compound caused a strong (40–60%) reduction of viable COX-2 positive HT-29 cell numbers when administered at 50 μ M concentration, with compound **2** being the most effective ([Fig. 2](#)). By contrast, COX-2 negative SW-480 cells were barely sensitive to these compounds, as shown by the fact that treatment with celecoxib and NO-cel induced only a modest decrease of cell growth ([Fig. 3](#)). The decrease in cell number following the treatment of HT-29 cells with NO-cel was operated either through inactivation of the mitogenic ERK/MAPK signal transduction pathway and/or the induction of apoptosis, with compound **2** and celecoxib being the most effective drugs, as shown by the following evidence: (a) induction of a decrease in pERK levels ([Fig. 4A](#)); (b) induction of significant changes in the intracellular levels of the apoptosis-regulating protein bak and of procaspase-3 ([Fig. 4B](#)); (c) induction of nuclear condensation and of apoptosis-related features in cells stained with DAPI ([Fig. 4C](#)).

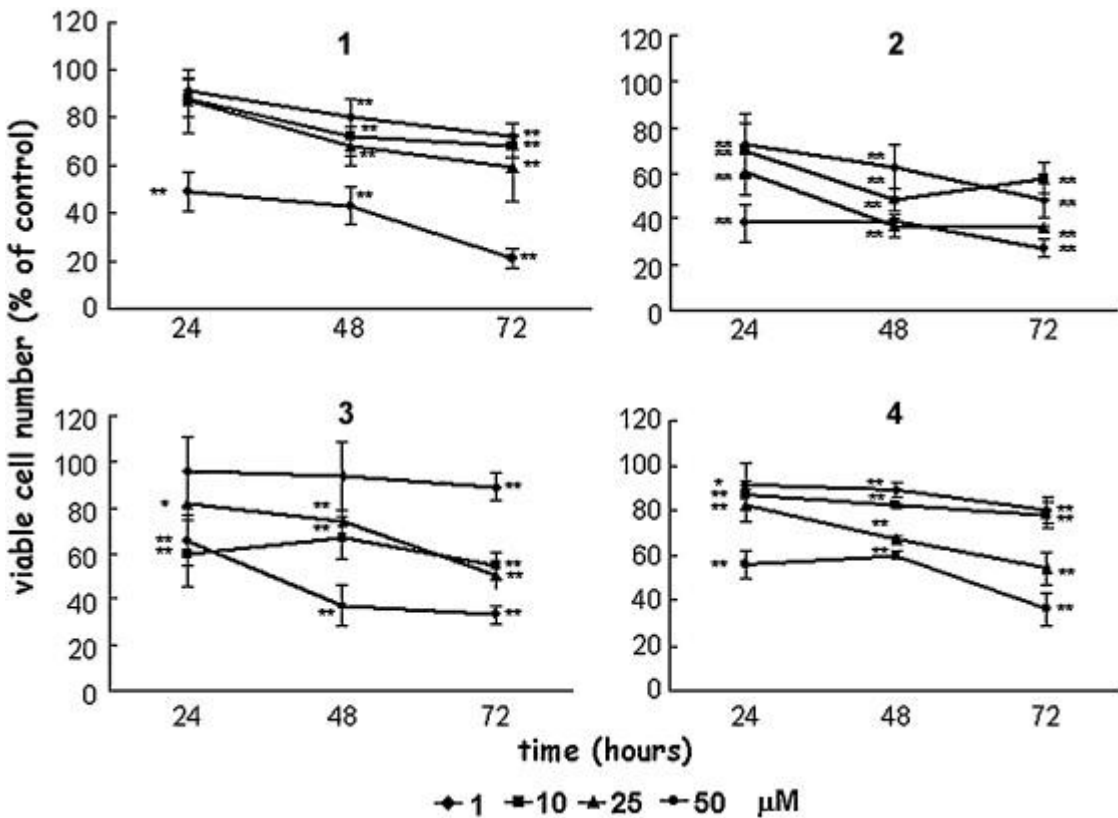


Fig. 2.

Effect of celecoxib and its derivatives on the growth of COX-2 positive HT-29 colon cancer cells. The cells were exposed for 24, 48 and 72 h to different concentrations of compounds 1–4 as indicated. Untreated cells incubated in the presence of vehicle (0.1% DMSO) were used as controls. Cell viability was determined by the trypan blue exclusion test and calculated by standardizing viable untreated cells to 100%. The values represent the mean of 3 independent experiments each performed in triplicate (bars, SD). Statistical significance compared with untreated control: * $P < 0.01$, ** $P < 0.001$, by one-way ANOVA test with the Bonferroni.

[Figure options](#)

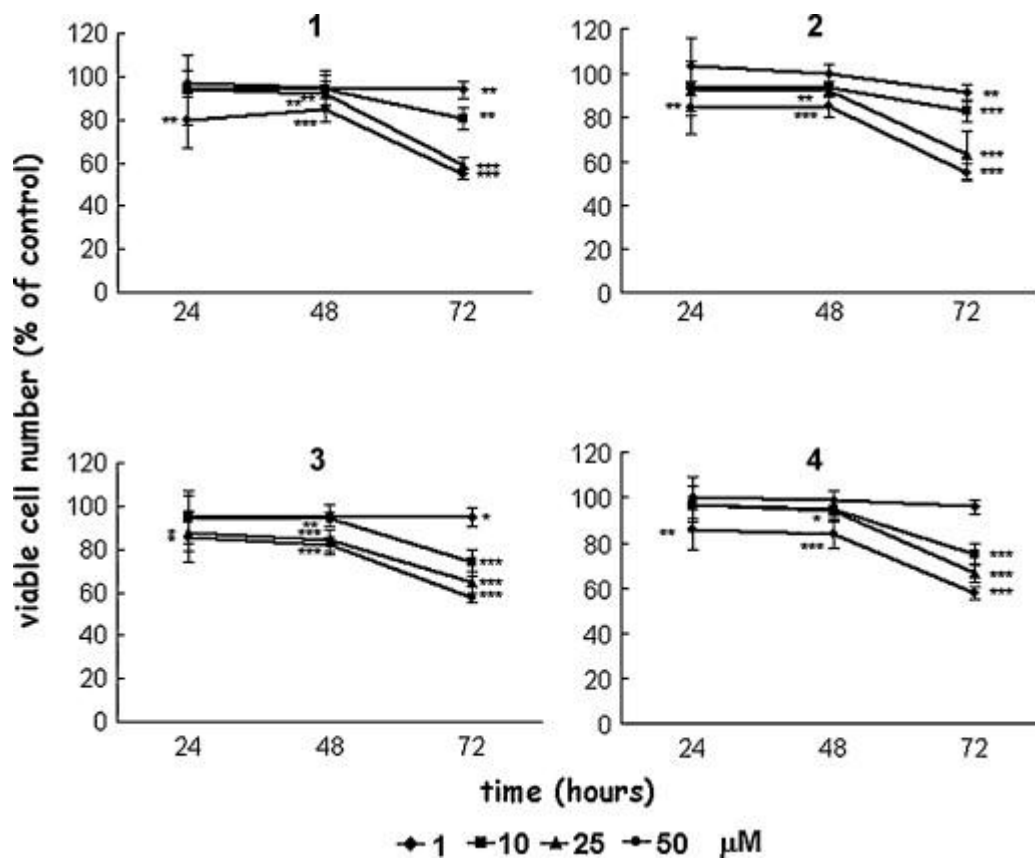


Fig. 3.

Effect of celecoxib and its derivatives on the growth of COX-2 negative SW-480 colon cancer cells. The cells were exposed for 24, 48 and 72 h to different concentrations of compounds 1–4 as indicated. Untreated cells incubated in the presence of vehicle (0.1% DMSO) were used as controls. Cell viability was determined by the trypan blue exclusion test and calculated by standardizing viable untreated cells to 100%. The values represent the mean of 3 independent experiments each performed in triplicate (bars, SD). Statistical significance compared with untreated control: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, by one-way ANOVA test with the Bonferroni.

[Figure options](#)



Fig. 4.

Effect of celecoxib and its derivatives on ERK1/2 phosphorylation state and on apoptotic features in HT-29 cells. Cells were exposed for 24 h to compounds **1–4** (50 μ M) and processed for western blot analysis with anti-pERK1/2 (A), anti-bak or anti-procaspase-3 (B). Protein contents were normalized with anti-ERK1 or anti- β -actin and quantified by densitometry. To detect nuclei untreated or treated (compound **2**, 50 μ M) cells were stained with DAPI (C).

[Figure options](#)

The role of COX-2 was then further investigated in order to analyse its involvement in growth inhibition. As expected, treatment with celecoxib and compound **2** decreased the expression of intracellular COX-2 protein levels (approx. 40% inhibition) to a very similar and significant extent, whereas compounds **3** and **4** were much less effective ([Fig. 5A](#)). COX-2 mRNA levels were found to be essentially unchanged following a 24 h incubation with the mentioned drugs (data not shown), with only compound **2** ([Fig. 5B](#)) being able to exert a modest decrease in COX-2 expression, as detected at an earlier time point (i.e., 6 h). According to literature data [[33](#)], no significant change in COX-2 protein levels was observed in SW-480 cells (which are COX-2 negative ([Fig. 5C](#))).

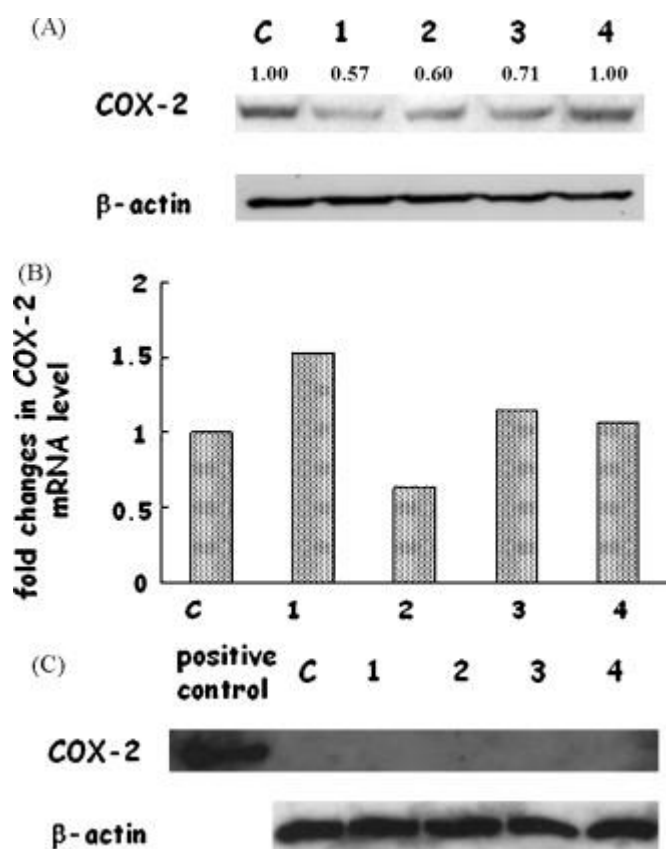


Fig. 5.

Effect of celecoxib and its derivatives on COX-2 protein and mRNA levels. (A) HT-29 cells, treated for 24 h with compounds 1-4 (50 μM), were probed with anti-COX-2 antibody, normalized with anti-β-actin and quantified by densitometry. (B) HT-29 cells were treated for 6 h with compounds 1-4 (50 μM) and COX-2 mRNA levels were determined by RT-PCR. Each sample was tested six times and data are reported as variation calculated taking the values of control cells as 1. (C) SW-480 cells were treated for 24 h with compounds 1-4 (50 μM) and processed for Western blot analysis with anti-COX-2 antibody. COX-2 purified protein was used as positive control.

[Figure options](#)

In order to establish whether the E-cadherin/β-catenin system was involved in the antiproliferative effect of nitro-oxy derivatives, both expression and intracellular distribution of these adhesion proteins were investigated. When HT-29 cells were treated with celecoxib and its derivatives (50 μM) a very significant increase in the expression of E-cadherin proteins was detected, with celecoxib being the most effective drug (Fig. 6A). In treated cells E-cadherin staining was found to be increased as compared to control cells and to be almost exclusively localized at cell membrane (Fig. 6B, compounds 3 and 4 not shown). Since β-catenin is known to bind to the cytoplasmic tail of E-cadherin, then regulating cell-cell adhesion, we examined whether the increase in E-cadherin may reflect an increase in membrane β-catenin protein levels. Immunofluorescence analysis showed an increase in β-catenin localized at the cell membrane, once again particularly in HT-29 cells treated with compound 2 (Fig. 7) that led to the maximal enhancement of β-catenin protein levels (approx. 5-fold higher vs. control values) (Fig. 8A).

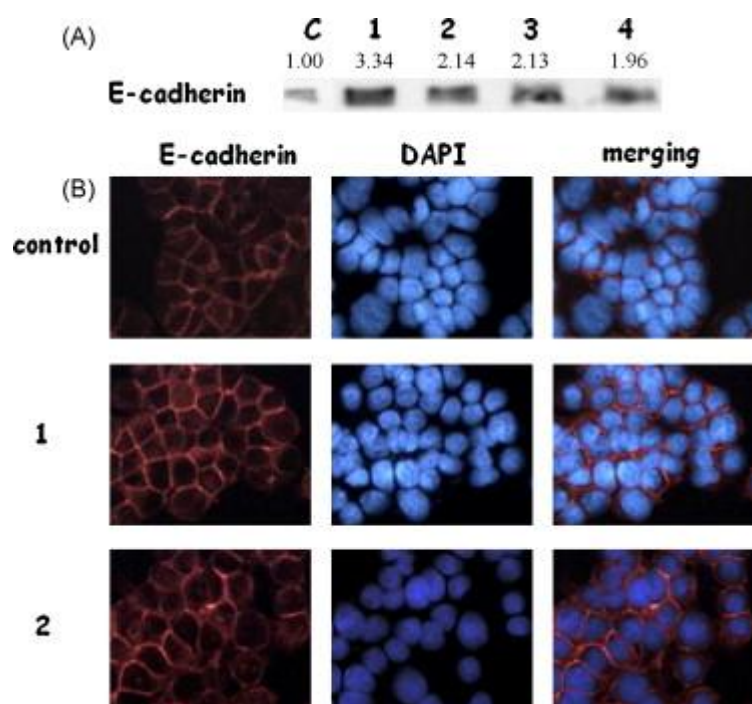


Fig. 6.

Effects of celecoxib and its derivatives on expression levels (A) and intracellular localization (B) of E-cadherin in HT-29 cells. (A) Membrane-associated fraction was obtained from cells treated for 24 h with compounds **1–4** (50 μ M). The fraction was probed with anti-E-cadherin antibody and protein content was routinely stained with Ponceau Red dye and quantified by densitometry. (B) Cells were treated for 24 h with celecoxib and compound **2** (50 μ M). At the end, cells were exposed to anti-E-cadherin antibody followed by anti-mouse Cy3-conjugated secondary antibody. To detect nuclei cells were stained with DAPI. The right panels show the overlaid pictures (400 \times final magnification).

[Figure options](#)

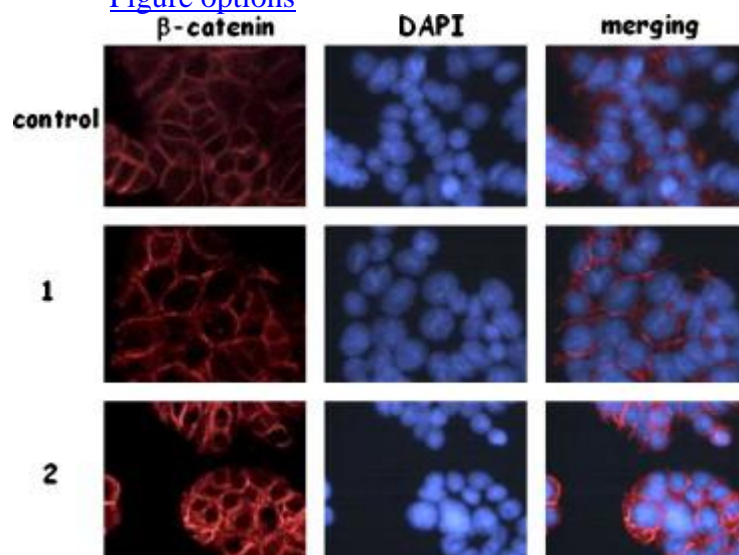


Fig. 7.

Effects of celecoxib and compound **2** on intracellular localization of β -catenin in HT-29 cells. After a 24 h exposure to celecoxib and compound **2** (50 μ M) cells were incubated with anti- β -catenin antibody followed by anti-mouse Cy3-conjugated secondary antibody. To detect nuclei the cells were stained with DAPI. The right panels show the overlaid pictures (400 \times final magnification).

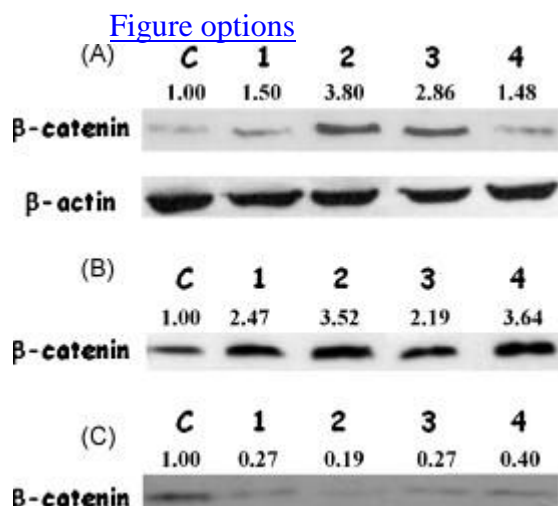


Fig. 8.

Effects of celecoxib and its derivatives on β -catenin expression in HT-29 cells. Total (A), membrane-associated (B) and nuclear cell extracts (C) after a 24-h exposure to compounds **1–4** (50 μ M) were probed with anti- β -catenin antibody, normalized with anti- β -actin or Ponceau Red dye and quantified by densitometry.

[Figure options](#)

Next we decided to evaluate whether the treatment with nitro-oxy-coxibs was able to affect β -catenin/E-cadherin interactions at plasma membrane level. As expected, subcellular fractionation studies showed a strong increase in membrane bound β -catenin, in particular after exposure to compounds **2** and **4** ([Fig. 8B](#)). This was paralleled by a consistent reduction of nuclear β -catenin, as shown by the weaker β -catenin nuclear staining in treated versus untreated cells ([Fig. 8C](#)).

Expression of β -catenin target genes c-myc and PPAR β/δ , which are known to play a relevant role in tumourigenesis [3], was then investigated. Western blot analysis revealed that protein levels of both target genes were markedly reduced at the time of most significant changes in β -catenin reorganization ([Fig. 9](#)), once again with celecoxib and compound **2** being the most effective drugs, suggesting a putative role for c-myc and PPAR β/δ in drug-induced inhibition of cell proliferation.



Fig. 9.

Effects of celecoxib and compound **2** on expression of c-myc and PPAR β/δ in HT-29 cells. Cells were treated for 24 h with celecoxib or compound **2** (50 μ M); total lysates were probed with anti-c-myc or anti-PPAR β/δ antibody, normalized with anti- β -actin and quantified by densitometry.

[Figure options](#)

Finally, in order to further analyse the involvement of COX-2 in mediating the biological effects of celecoxib and NO-cel, pERK and membrane E-cadherin/ β -catenin interactions were evaluated in COX-2 negative SW-480 cell line. Both celecoxib and compound **2** caused a modest decrease of pERK levels according to their reported effects on cell proliferation, whereas no change in the levels of membrane bound β -catenin and E-cadherin was detected ([Fig. 10](#)).



Fig. 10.

Effects of celecoxib and compound **2** on expression of pERK, β -catenin and E-cadherin in SW-480 cells. Cells were treated for 24 h with celecoxib or compound **2** (50 μ M). Total lysates were probed with anti-pERK1/2 antibody and normalized with anti- β -actin; membrane-associated fraction was probed with anti- β -catenin or anti-E-cadherin antibody and protein content was routinely stained using Ponceau Red dye. Protein contents were quantified by densitometry.

[Figure options](#)

4. Discussion

Celecoxib has been proposed as a putative chemopreventive agent [34] based on the findings that colon cancer progression is related to up-regulation of COX-2 and increased content of its main product PGE₂[35]. The use of celecoxib has been approved for the treatment of patients with FAP [36], but the major problem in using this drug remains an increased risk cardiotoxicity [20].

Celecoxib has already been reported to reduce survival and proliferation in HT-29 cells through inhibition of different MAPK [37], [38] and [39]. In this study, *in vitro* treatment of COX-2 positive HT-29 colon cancer cells with celecoxib and NO-cel was characterized by reduction of cell growth, accompanied by inhibition of ERK/MAPK and induction of apoptosis. These

findings are consistent with studies showing ERK activation to occur in cancer cell proliferation [40], with down-regulation being more likely associated with growth inhibition [41] and [42]. Our results showed that reduction in pERK and changes in the levels of apoptosis-related proteins can occur in parallel with the reduction of COX-2 protein levels. Only the detected decrease of COX-2 protein levels induced by compound **2** can be related to a down-regulation of COX-2 mRNA expression, suggesting that reduction in cell proliferation by this compound may then follow COX-2 inhibition at transcriptional level.

As expected, COX-2 is likely to play a significant role in the antiproliferative effects of NO-cel, since COX-2 positive colon cancer cells (HT-29) exhibited a higher susceptibility to these compounds as compared to COX-2 negative cancer cells.

Other COX-2 inhibitors have been reported to negatively affect tumour growth and invasion through alternative molecular mechanisms, involving c-Met kinase and WNT activity [43]. Most colorectal tumours show overexpression of COX-2 and β -catenin, which are known to be involved in both cell proliferation and cell–cell adhesion [9]. Accordingly, some authors [44] have suggested that the anticarcinogenic effects of celecoxib in human colon cancer Caco-2 cells might be related, at least in part, to the accumulation of β -catenin in the cytoplasm. More recently, celecoxib has been demonstrated to repress β -catenin-related oncogenic signal transduction [43], through an increase in glycogen synthase kinase 3 β kinase activity associated with a rapid increase of β -catenin phosphorylation.

In the present study, a reduction in nuclear β -catenin followed the treatment with celecoxib and NO-cel and occurred concomitantly with a relevant increase in either total or membranous fraction of β -catenin. β -Catenin redistribution throughout the cell may be then closely related to a reduction in invasiveness, in agreement with the common observation that an increased expression of nuclear β -catenin in malignant tumours is frequently associated with a more aggressive behaviour of cancer cells [45], [46] and [47].

Moreover, up-regulation of membranous β -catenin induced by celecoxib and NO-cel was accompanied by increased expression of E-cadherin, suggesting that celecoxib and related analogues increased the amount of β -catenin available to form complexes with E-cadherin. This is potentially relevant since down-regulation of E-cadherin is currently believed to play a role in tumour invasion, which requires the ability of cells to spread to distant sites and is associated with reduced cell–cell adhesion [9]. Overexpression of E-cadherin and β -catenin caused by celecoxib and NO-cel can contribute to decrease cell migration, cell adhesion, and tumour invasiveness, as already shown for other COX-2 inhibitors [48] and [49].

These results may account for a strong association between β -catenin reorganization and decreased cell proliferation, which is also sustained by evidence for decreased amounts of β -catenin-related gene products c-myc and PPAR β/δ . As a matter of fact, down-regulation of β -catenin target genes has been shown to attenuate colon carcinogenesis [3] and, therefore, it is conceivable that reduction in β -catenin transcriptional activity may result in inhibition of cell proliferation.

At present, the real role of PPAR β/δ in colon carcinogenesis is still controversial. Anti-tumour effect of PPAR β/δ has been suggested by reduced tumourigenicity obtained through its genetic disruption [50], and other authors have suggested that colon carcinogenesis may be attenuated by

ligand activation of PPAR β/δ [51]. Conversely, some authors proposed that the growth of colorectal adenoma may be promoted via PPAR β/δ activation [52] and [53]. In our study both celecoxib and compound **2** reduced the expression of PPAR β/δ , therefore inhibition of PPAR β/δ may represent an additional mechanism by which NO-cel exert their antiproliferative effect.

In conclusion, our results show that these novel chemical entities, NO-cel, have potential anticancer properties, as the inhibition of proliferation of HT-29 and SW-480 colon cancer cells seems to suggest. In HT-29 cell line growth inhibition occurred probably through COX-2-related pathways, induction of apoptosis and involvement of the E-cadherin/ β -catenin system. Among NO-cel, the compound with two nitro-oxy functions (**2**) was highly effective in negatively modulating transcriptional activity of β -catenin, raising the question of whether NO may be required for this modulation [54] and [55]. Thus, the results presented here support the inverse relationship reported by several laboratories between β -catenin-dependent cell proliferation and intercellular adhesion mediated by adherens junctions [56] and [57]. Further experimental work would provide additional details on the mechanism of the antiproliferative effect of NO-cel, to support their role for treatment or chemoprevention of colon cancer.

Conflict of interest

The authors declare that there are no conflicts of interest.

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