Abstract. Background: Epidemiological studies have revealed a protective effect of NSAIDs, which principally target cyclooxygenase (COX)-1 and COX-2, on the development of colorectal cancer. Increased expression of COX-2 was shown in colorectal adenocarcinoma. However, some effects were shown to be COX-independent. Here, we compared two selective COX-2 inhibitors for their effect on the growth of colorectal tumour cells in vitro. Materials and Methods: Fifteen tumour cell lines were characterized for COX-1 and COX-2 expression by Western blot and RT-PCR. The effect of celecoxib and rofecoxib on their growth was assessed by staining of DNA with crystal violet. Results: COX-2 expression varied among cell lines, whereas COX-1 was always expressed. Rofecoxib displayed a limited dose-related effect on cell proliferation, whereas celecoxib strongly inhibited cell growth at high concentrations. Both effects appeared COX-2-independent. Conclusion: Rofecoxib, which is devoid of apoptotic effect at high concentration but efficient at pharmacological concentrations, revealed a potential new mechanism of action of NSAIDs towards colorectal cancer.

Cyclooxygenases (COX) are the key enzymes in the conversion of arachidonic acid to prostaglandins (PG) and other eicosanoids (1). Two genes have been identified, although at the protein level several isoforms have been described resulting from alternative splicing (2). COX-1 has been shown to be constitutively expressed in a variety of tissues including gastro-intestinal epithelium and has been shown to play a central role in several physiological processes including platelet aggregation and parturition (3). COX-2 is a highly inducible gene, that is activated by various stimuli including pro-inflammatory cytokines and growth factors (4).

In 1994, Eberhart et al. were the first to describe a significant elevation of COX-2 expression in most human colorectal carcinomas, but also in approximately 50% of adenomas (5). Such findings were later documented by others (6, 7). Moreover, a similar elevated expression has been reported in azoxymethane-induced colonic tumours in rats (8) and in APC mutant mice (9). It is associated with increased PG levels in the tissues, especially PGE2 (10). The fact that such elevated expression was found in human premalignant adenomas and also in polyps in APC mutant mice argues for an impliciation as an early event during the course of carcinogenesis. Indeed, Tsujii and DuBois demonstrated that transfecting rat intestinal epithelial cells with a COX-2 expression vector, leading to its constitutive expression, provokes phenotypic alterations such as modulation of their adhesion properties and inhibition of induced apoptosis (11). These changes enhanced their tumorigenic potential and were reversed by sulindac sulphide. Moreover, Liu et al., using transgenic mice in which COX-2 expression was under a promoter targeting selectively the mammary glands, showed that COX-2 overexpression alone was sufficient to induce mammary carcinomas (12).

Therefore, the targeting of COX-2 with more or less selective inhibitors was proposed as a new therapeutic approach for colorectal cancer. These inhibitors were shown to display chemopreventive effects in different models of colorectal carcinogenesis (for review see 13). Moreover, epidemiological studies revealed that regular consumption of aspirin or other non-steroid anti-inflammatory drugs (NSAIDs) reduces the risk of colorectal cancer (14-16). Finally, it was demonstrated that sulindac administration, and more recently celecoxib, to patients with familial...
adenomatous polyposis resulted in a reduction in the number and size of colorectal polyps (17, 18). Although sulindac was inefficient as a primary chemoprevention agent (19), it was found efficient in the retained rectal segment of FAP patients after prophylactic colectomy (20).

All these findings suggest that COX-2 plays an important part in colorectal carcinogenesis and that NSAIDs, especially COX-2 selective inhibitors, have potential chemopreventive properties in human colorectal cancer. However, the exact mechanisms by which NSAIDs inhibit colon carcinogenesis are still elusive, and some may be independent of COX-2 expression (21). In prostate cancer cells, Song et al. questioned whether COX-2 was a "player" or a "spectator" in COX-2 inhibitor-induced apoptosis (22). Indeed, COX-2 inhibitors have been shown to inhibit cell proliferation, induce apoptosis, inhibit angiogenesis and stimulate the immune system (23-26). Although reduction in prostaglandins synthesis may account for these activities, other mechanisms unrelated to COX-2 may also be involved.

The aim of our study was to compare the effect of two selective COX-2 inhibitors, namely celecoxib (Celebrex™, Pharmacia) and rofecoxib (Vioxx™, Merck), on colorectal adenocarcinoma cell growth. These cells were also characterized for COX-1 and COX-2 expression and some other features of colonic tumours.

Materials and Methods

Cell lines and reagents. Caco-2, Colo-205, HCT-116, HCT-EB, HCT-GEO, HRT-18, HT-29, LS174T, LS180, SW48, SW480, SW620, SW948 and SW1116 human colorectal adenocarcinoma cell lines were characterised previously (27). Cells were cultured in RPMI 1640 supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, 50 U/ml penicillin and 50 mg/ml streptomycin, except for HCT-EB and HCT-GEO, which were cultured in DMEM containing 25 mM glucose instead of RPMI. Caco-2 cells were also cultured in DMEM supplemented with 20% FCS. All tissue culture reagents were from Invitrogen (Cergy Pontoise, France), except FCS which were from Invitrogen (Cergy Pontoise, France). DMEM supplemented with 20% FCS was supplied (Gibco, Paisley, UK). The drugs were dissolved in dimethylsulfoxide (DMSO) to obtain a stock solution of 20 mM.

Western blot analysis. Cells were plated onto 25-cm² flasks at a density of 2×10⁶ cells per flask and allowed to grow for 48 h. After washes with phosphate-buffered saline (PBS), proteins were extracted at 4 °C for 1 h with 500 μl of lysis buffer containing 10 mM Tris-HCl (pH 7.4), 20 mM NaCl, 5 mM MgCl₂, 0.5% Nonidet P-40, 1 mM phenylmethanesulphonyl fluoride, 2.1 μM pepstatin, 1.4 μM leupeptin (Sigma). Homogenates were centrifuged at 10,000 x g for 2 min and supernatant was determined using the DC protein assay (Biorad, Hercules, CA, USA). The reaction mixture contained 2 μl of the supplied 10X Titanium Taq PCR buffer (containing magnesium chloride), 2 μl of a 1/1,000 dilution of SYBR Green I (Roche Molecular Biochemicals, Meylan, France), 1 μl of each primer (0.4 μM each), 0.4 μl of Titanium Taq DNA polymerase (Clontech, Palo Alto, CA, USA). The reaction was done in the linear range of amplification (determined for each primer pair-cDNA combination). Standard PCR reactions were performed with 1 μl of the cDNA solution, 1 μl of each primer solution, 200 μl of each dNTP, 1.5 mM MgCl₂, Goldstar DNA polymerase reaction buffer and 0.5 units of Goldstar DNA polymerase (Eurogentec, Seraing, Belgium). Each PCR cycle consisted of 1 min at 92 °C, 1 min at 58 °C and 1 min at 72 °C.

Primers were:
- β-actin Forward 5'-GGCATCGTGATGACCTGG-3'
- Reverse 5'-GGCTGGAAAGTGACGAGCGA-3'
- COX-1 Forward 5'-GTTCACACCTCTCATGTTTGGAG-3'
- Reverse 5'-TGTTGTGATACCAGCATCTCC-3'
- COX-2 Forward 5'- TCTAAATGGAATTGTTGGAAGAAT-3'
- Reverse 5'-AGATCATTCTCGGTTGATCTTT-3'.

Real-time quantitative RT-PCR. The amplification conditions of COX-2, β-actin and glyceraldehyde-3 phosphate dehydrogenase (GAPDH) templates were optimised for the RotorGene 2000 instrument (Ozyme, Saint Quentin en Yvelines, France). PCR amplifications were performed using Titanium Taq DNA polymerase (Clontech, Palo Alto, CA, USA). The reaction mixture contained 2 μl of the supplied 10X Titanium Taq PCR buffer (containing magnesium chloride), 2 μl of a 1/1,000 dilution of SYBR Green I (Roche Molecular Biochemicals, Meylan, France), 1 μl of each primer (0.4 μM each), 0.4 μl of Titanium Taq DNA polymerase, 0.5 μl of dNTPs (10 mM each) and PCR-grade water to a volume of 15 μl. Microtubes (0.2 ml) were loaded with 15 μl of this master mix and 5 μl of the template (cDNA diluted 1/100) and the run was initiated. The cycling conditions were as follows: denaturation for 5 min at 95 °C, amplification for 45 cycles, with denaturation for 5 sec at 95 °C, annealing for 1 min at 58 °C and extension for 1 min at 72 °C. To exclude primer-dimer artefacts, fluorescence was not measured at the end of the extension step, but at a temperature (83 °C) above the melting point of primer-dimers and below the melting point of the specific PCR product (90 °C).

Primers were chosen on separate exons to amplify cDNA but not genomic DNA. The following primers were used:
- COX-2 Forward 5'-GCCCTTCTTATCCTGTGGG-3'
- Reverse 5'-AATCAGGAAAGCTTGTTTTTAC-3'
- β-actin Forward 5'- GGAGCAATGATCTTGTTGAG-3'
- Reverse 5'-CTCTTCTTATGTTAGCTCCTC-3'
- GAPDH Forward 5'- TGAACGGGAAGCTCACTGG-3'
- Reverse 5'- TTCCTACCTGGTCTGTGA-3'

Cell proliferation assay. Cells were seeded in 24-well plates at 1×10⁴ cells/well, allowed to grow for a day, then exposed in quadruplicate wells to increasing concentrations of celecoxib or rofecoxib. After 6 days in culture with the drugs, the cell number was estimated using the colorimetric crystal violet assay, as described previously (28).

RT-PCR analysis. Total RNA from cells plated onto 25-cm² flasks was isolated using TRIzol Reagent™ (Invitrogen). cDNA was generated on 1 μg of total RNA in a reaction volume of 20 μl using Superscript II M-MLV reverse transcriptase (RT; Invitrogen). PCR was done in the linear range of amplification (determined for each primer pair-cDNA combination). Standard PCR reactions were performed with 1 μl of the cDNA solution, 1 μl of each primer solution, 200 μl of each dNTP, 1.5 mM MgCl₂, Goldstar DNA polymerase reaction buffer and 0.5 units of Goldstar DNA polymerase (Eurogentec, Seraing, Belgium). Each PCR cycle consisted of 1 min at 92 °C, 1 min at 58 °C and 1 min at 72 °C. Primers were:
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- Reverse 5'-GGCTGGAAAGTGACGAGCGA-3'
- COX-1 Forward 5'-GTTCACACCTCTCATGTTTGGAG-3'
- Reverse 5'-TGTTGTGATACCAGCATCTCC-3'
- COX-2 Forward 5'- TCTAAATGGAATTGTTGGAAGAAT-3'
- Reverse 5'-AGATCATTCTCGGTTGATCTTT-3'.

The amplification conditions of COX-2, β-actin and glyceraldehyde-3 phosphate dehydrogenase (GAPDH) templates were optimised for the RotorGene 2000 instrument (Ozyme, Saint Quentin en Yvelines, France). PCR amplifications were performed using Titanium Taq DNA polymerase (Clontech, Palo Alto, CA, USA). The reaction mixture contained 2 μl of the supplied 10X Titanium Taq PCR buffer (containing magnesium chloride), 2 μl of a 1/1,000 dilution of SYBR Green I (Roche Molecular Biochemicals, Meylan, France), 1 μl of each primer (0.4 μM each), 0.4 μl of Titanium Taq DNA polymerase, 0.5 μl of dNTPs (10 mM each) and PCR-grade water to a volume of 15 μl. Microtubes (0.2 ml) were loaded with 15 μl of this master mix and 5 μl of the template (cDNA diluted 1/100) and the run was initiated. The cycling conditions were as follows: denaturation for 5 min at 95 °C, amplification for 45 cycles, with denaturation for 5 sec at 95 °C, annealing for 15 sec at 66 °C and extension for 20 sec at 72 °C. To exclude primer-dimer artefacts, fluorescence was not measured at the end of the extension step, but at a temperature (83 °C) above the melting point of primer-dimers and below the melting point of the specific PCR product (90 °C).

Primers were chosen on separate exons to amplify cDNA but not genomic DNA. The following primers were used:
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- Reverse 5'-AATCAGGAAAGCTTGTTTTTAC-3'
- β-actin Forward 5'- GGAGCAATGATCTTGTTGAG-3'
- Reverse 5'-CTCTTCTTATGTTAGCTCCTC-3'
- GAPDH Forward 5'- TGAACGGGAAGCTCACTGG-3'
- Reverse 5'- TTCCTACCTGGTCTGTGA-3'
An external standard curve was generated with serial 5-fold dilutions (1/40, 1/200 and 1/1000) of a control sample (cDNA from SW1116 cells). The reference curve was constructed by plotting the relative amounts of these dilutions vs the corresponding Ct (threshold cycle) values. The correlation coefficient of these curves was always greater than 0.99. The amount of COX-2, β-actin or GAPDH transcripts was calculated from these standard curves using the RotorGene software. Samples were tested in triplicate and the average values were used for quantification. For each sample, the ratio between the relative amount of COX-2 and the mean between β-actin and GAPDH was calculated to compensate for variations in quantity or quality of starting mRNA as well as for differences in reverse transcriptase efficiency.

After completion of the cycling process, samples were subjected to a temperature ramp from 60°C to 99°C, with continuous fluorescence monitoring for melting curve analysis. For each amplification, apart from primer-dimers, a single narrow peak was obtained at the expected melting temperature (86°C), indicating specific amplification without significant by-products.

Mononucleotide repeat microsatellites analysis. Colorectal cancer cell lines were suspended in 20 mM Tris-HCl pH 7.4, 5 mM EDTA, 0.4% SDS, 1 mg/ml proteinase K, at 37°C overnight for DNA extraction. Genomic DNA was precipitated by ammonium acetate 3M final and ethanol. Finally, DNA was rinsed in 70% ethanol and resuspended in TE buffer (20 mM Tris-HCl pH 7.4, 1mM EDTA).
Primers used to amplify BAT-25 and BAT-26 were as described (29). One of each pair of PCR amplification primers is fluorescently-labelled with either FAM (BAT25, blue), or TET (BAT26, green). The amplification for the two mononucleotide repeats was performed in the same PCR reaction mixture containing 10 mM Tris-HCl, 1.5 mM MgCl2, 0.01% (w/v) gelatin, 50 mM KCl, 200 mM of each deoxynucleoside triphosphate (Pharmacia, Uppsala, Sweden), 10 pmol of each primer, 1.25U of AmpliTaq Gold (Applied Biosystems, Foster City, USA) and 10 ng of DNA in a total volume of 20 ìl. PCR was performed for 35 cycles of 0.5 min at 94ÆC, 0.5 min at 55 and 3 min at 70ÆC. A step of 15 min at 95ÆC was performed before the cycles. The fluorescent PCR products were electrophoresed on an ABI PRISMì 310 Genetic Analyser (Applied Biosystems), and results analysis was performed with Genotyper 2.5.2 software (Applied Biosystems). All replication error negative (RER-) cell lines showed only minor size variation for BAT 26 and BAT 25 alleles not exceeding 2 bp from one cell line to another, as described previously (30). RER+ cell lines showed BAT26 and/or BAT25 alleles shortening from -4 bp to -15 bp compared to RER- cell lines.

Cell cycle analysis. Cells in exponential growth phase were exposed for 24 h to rofecoxib and celecoxib at increasing concentrations or to DMSO as control. The cells were then harvested using a trypsin/EDTA solution and stained with propidium iodide using the DNA-prep Coulter kit (Beckman-Coulter, Villepinte, France), according to the manufacturer’s instructions. Cell DNA content was then analysed by flow cytometry using an EPICS XL (Beckman-Coulter). Raw data for the distribution of DNA content retrieved from the EPICS XL were treated using Multicycle AV software (Phoenix Flow Software, San Diego, CA, USA) to generate DNA content histograms, and curve fitting analysis was performed to obtain the percentage of G0/G1 through G2/M populations. Sub-diploid cells were considered as apoptotic cells.

Results

Expression of cyclooxygenase-1 protein and mRNA. A great discrepancy exists in the literature considering the expression of COX for each particular cell line, thus we first characterised these cells for both COX isoforms. Considering COX-1 expression, all cell lines exhibited COX-1 mRNA expression as assessed by semi-quantitative RT-PCR (Figure 1). At the protein level, we also detected COX-1 in all cell lines, albeit that SW480 presented lower protein level than the others.

Expression of cyclooxygenase-2 protein and mRNA. As opposed to COX-1 expression, COX-2 expression varied among cell lines (Figure 2A). At the protein level, a very strong expression was observed in SW1116 and in Caco-2 cells; a lower expression was found in the HT-29 and in SW48 cell lines. A barely detectable COX-2 signal was also obtained in the LS174T and LS180 lines. We failed to

Table I. Characterisation of the different human colorectal adenocarcinoma cell lines used. Expression of COX-2 mRNA was assessed by real time RT-PCR. Results are expressed as the ratio between the relative amount of COX-2 mRNA and the average of the relative amounts of two normalisation genes β-actin and GAPDH. Three to five determinations performed in triplicate were done. Replication error (RER) status was determined by analysis of BAT-26 and BAT-25 microsatellites, as described in the Materials and Methods section. Differentiation capacities of the cell lines were described in detail by Blottiere et al. (27). It was based on cell polarity, CEA and brush border hydrolase expression, and histopathological features of tumours after graft into Nude mice. (+++): well-differentiated; (++): moderately-differentiated; (+): poorly-differentiated. n.c.: not characterised.

<table>
<thead>
<tr>
<th>COX-2 mRNA</th>
<th>RER status</th>
<th>Differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caco-2</td>
<td>125.8</td>
<td>-</td>
</tr>
<tr>
<td>Colo-205</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>HCT-116</td>
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<td>+</td>
</tr>
<tr>
<td>HCT-EB</td>
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<td>-</td>
</tr>
<tr>
<td>HCT-GEO</td>
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<td>-</td>
</tr>
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<td>HRT-18</td>
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<td>-</td>
</tr>
<tr>
<td>HT-29</td>
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<td>-</td>
</tr>
<tr>
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<td>+</td>
</tr>
<tr>
<td>LS180</td>
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<td>+</td>
</tr>
<tr>
<td>SW48</td>
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<td>-</td>
</tr>
<tr>
<td>SW480</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>SW620</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>SW707</td>
<td>7.6</td>
<td>+</td>
</tr>
<tr>
<td>SW948</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>SW1116</td>
<td>1022.6</td>
<td>-</td>
</tr>
</tbody>
</table>

Table II. Effect of celecoxib and rofecoxib on the proliferation of human colorectal adenocarcinoma cell lines. Results are expressed as the percentage of cell remaining in the presence of 10 ìM and 50 ìM as compared to control. The amount of cells from triplicate wells was determined using crystal violet staining. SEM did not exceed 10%.

<table>
<thead>
<tr>
<th></th>
<th>Celecoxib</th>
<th>Rofecoxib</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 ìM</td>
<td>50 ìM</td>
</tr>
<tr>
<td>Caco-2</td>
<td>69.3</td>
<td>3.9</td>
</tr>
<tr>
<td>Colo-205</td>
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</tr>
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<td>HCT-GEO</td>
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<td>3.3</td>
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<tr>
<td>HRT-18</td>
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<td>5.8</td>
</tr>
<tr>
<td>HT-29</td>
<td>60.6</td>
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</tr>
<tr>
<td>LS174T</td>
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<td>3.4</td>
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<td>LS180</td>
<td>57.7</td>
<td>1.9</td>
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<tr>
<td>SW48</td>
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<td>5.6</td>
</tr>
<tr>
<td>SW480</td>
<td>67.0</td>
<td>9.0</td>
</tr>
<tr>
<td>SW620</td>
<td>79.2</td>
<td>16.0</td>
</tr>
<tr>
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<td>16.3</td>
<td>0.4</td>
</tr>
<tr>
<td>SW948</td>
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<td>5.5</td>
</tr>
<tr>
<td>SW1116</td>
<td>60.4</td>
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detect COX-2 protein in the other cell lines tested. The results of semi-quantitative RT-PCR analysis (Figure 2B) corroborate the Western blot analysis. In order to accurately quantify COX-2 transcripts, real-time RT-PCR were also performed using another pair of primers. The level of COX-2 mRNA was reported to two normalisation genes, β-actin and GAPDH. The high expression in SW1116 was confirmed with almost 10 times more mRNA than in HT-29 or Caco-2 (Table I). Moreover, we were also able to detect COX-2 mRNA in three other cell lines, HCT-116, HCT-EB and SW707.

Relationship between COX-2 expression and the phenotype of cell lines. We attempted to test whether COX-2 expression was linked to one of the two pathways of genomic instability. Thus, we characterised the genetic alteration of these cell lines by studying BAT-25 and BAT-26 microsatellite loci (Table I). Microsatellite stability (MSS, also termed RER-) corresponded to cell lines in which both alleles did not exceed 2bp from one cell line to another. Microsatellite instability (MSI, also termed RER+) displayed alleles shortening from -4 to -15 bp as compared to MSS cell lines. Most of the cell lines tested were RER- (also termed MSS, microsatellite stability), especially the highly COX-2-expressing cells SW1116 and Caco-2. Four cell lines were found unstable; HCT-116, LS-174T, LS180 and SW707. Surprisingly, and as opposed to what has been published elsewhere (30), SW48, which expressed COX-2, was observed stable. Although, low or no COX-2 protein was detected, COX-2 mRNA were detected in the 4 RER+ cell lines. Therefore, no correlation was noticed between microsatellite status and COX-2 expression (p>0.05).

We then addressed the question of whether there was a link between the differentiation capacities of the cells and COX-2 expression. The differentiation status of the cell lines was described in detail previously (27) and summarised in Table I. It was based on several criteria, including polarity, CEA and brush border hydrolase expression, and histopathological features of tumors after graft into Nude Buecher et al: Rofecoxib, Celecoxib and Colorectal Tumour Cell Proliferation

Table III. Effect of celecoxib and rofecoxib on cell cycle distribution on HCT116 cells after 24 hours. Results representative of one experiment of the three performed in duplicate are expressed as mean % of the cells in the different phases of the cell cycle. 20,000 cells were analyzed.

<table>
<thead>
<tr>
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<th>Sub-diploid</th>
<th>G1</th>
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<th>G2/M</th>
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<tbody>
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<td>31.1</td>
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<td>Celecoxib</td>
<td></td>
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</tr>
<tr>
<td>10 μM</td>
<td>2.2</td>
<td>35.9</td>
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</tr>
<tr>
<td>20 μM</td>
<td>1.4</td>
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<td>50 μM</td>
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<tr>
<td>Rofecoxib</td>
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<tr>
<td>10 μM</td>
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<td>20 μM</td>
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<td>44.9</td>
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</tr>
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</table>

Figure 3. Effect of celecoxib and rofecoxib on growth of HT-29 (triangle), SW1116 (lozenge), HCT-116 (square) and SW480 (cross) cells. Cells in 24-well plates were exposed to the drugs for six days. Cell number was determined by DNA staining using crystal violet. The results are given as % ± SEM of values obtained with controls.
mice. Among the 4 well-differentiated cell lines, 2 expressed highly COX-2 protein, and 2 were negative. No correlation was found between the differentiation capacities of a cell line and the expression of COX-2 (p>0.05). Moreover, we studied the expression of COX-2 in SW1116 cells at different differentiation stage, i.e. below confluence (exponentially growing undifferentiated cells), just confluent, and 1 and 2 weeks after confluence (the later being well-differentiated, presenting domes and highly expressing alkaline phosphatase and dipeptidyl aminopeptidase IV). Using SW1116, we observed that the differentiation stage did not influence COX-2 expression (data not shown).

**Sensitivity to celecoxib- and rofecoxib-induced inhibition of cell proliferation.** We examined the effect of two selective COX-2 inhibitors on the proliferation of the different colorectal tumour cell lines in vitro. Cell number was evaluated by crystal violet staining of DNA. The effect of increasing concentrations of the drugs was measured after 6 days. As shown in Figure 3, the two compounds inhibited cell growth. The effect of celecoxib and rofecoxib appeared independent of COX-2 expression, as illustrated for the 4 cell lines: SW1116, which highly expresses COX-2, HT-29 which also expresses COX-2 but at a lower level, HCT-116 which expresses COX-2 mRNA but not the protein, and SW480 which was completely negative. The percentage of inhibition found for two concentrations of celecoxib and rofecoxib, 10 and 50 μM, are reported in Table II. Clearly, there was no correlation between this parameter and COX-2 expression.

Interestingly, the anti-proliferative response differed between the two compounds. Indeed, we observed that, when increasing the concentration of celecoxib from 10 μM to 50 or 100 μM, the percentage of remaining cells fell almost completely, whereas for rofecoxib a plateau was observed. Due to this phenomenon, we were unable to calculate the IC50 for rofecoxib and therefore presented the values obtained with two concentrations of both inhibitors. For example, on SW1116 cells, at 10 μM, rofecoxib inhibited cell growth by almost 60%, however at 50 μM, the inhibition was less than 50%. With celecoxib, at 10 μM a 40% inhibition was noticed, but it reached 85% at 50 μM.

At 50 μM or above, celecoxib was more effective than rofecoxib on all cell lines tested. However, at 10 μM rofecoxib was more efficient than celecoxib on several cell lines e.g. Caco-2, LS174T and SW1116. Others cell lines, including LS180, HCT-116, SW707 and SW620, were identically sensitive to both drugs. Again, this was unrelated to COX-2 expression (p>0.05).

The effect of celecoxib and rofecoxib on cell cycle distribution and on apoptosis are presented in Table III. We observed that celecoxib dose-dependently induced apoptosis as revealed by the presence of sub-diploid cells. This was associated with an arrest in the G1-phase of the cell cycle. At 100 μM, the growth inhibition was maximal, apoptosis became very important and G1 arrest was not observed. With rofecoxib, we did not observed any induction of apoptosis and a mild G1 accumulation was noticed. Similar results were obtained with SW1116 and HT-29 cells (not shown).

**Discussion**

In the present study, we compared the effect of two selective COX-2 inhibitors, celecoxib and rofecoxib, on the growth of a panel of human colorectal cancer cell lines. We characterised the expression of both COX isoforms in all these lines by Western blot and RT-PCR. We clearly found that, at high concentration, celecoxib was the most efficient drug in inhibiting cell proliferation, whereas at lower concentration, both molecules were almost equally efficient. Thus, the two compounds present different mechanisms of action. We also observed that the inhibitory effect appears unrelated to COX-2 status.

First, we performed a full characterisation of COX isoforms expression in all the cell lines. This is an important issue since, depending on the studies, COX status is still controversial. For instance, HCT-116 was described to express none of the COX (31) or to be COX-2-negative and express low COX-1 (32). However, as observed in our study, others found COX-1 protein and mRNA in HCT-116 (4). Considering COX-2, HCT-116 was found COX-2-negative both at the protein and mRNA level (4). In our study, we found a low COX-2 mRNA expression by real-time RT-PCR, but not by semi-quantitative RT-PCR. A similar low COX-2 mRNA expression was also documented in HCT-116 (33). Differences in technical approaches may explain these discrepancies, since the antibodies used and the Western conditions varied from one laboratory to another. Moreover, at the mRNA level, the analysis conditions, Northern blot or RT-PCR and, in the latter, the number of cycles performed are important. In that matter, the use of real-time RT-PCR allowed a gain in quantitative analysis. The finding that COX-1 was expressed in all the cell lines analysed is important to consider in order to analyse the mechanism of action of NSAIDs. Moreover, no study reported any reduced or suppressed COX-1 expression in colorectal tumours in situ.

We then addressed the question of the relationship between COX-2 expression and the phenotype of the cells. First, we found no association between COX-2 expression and the differentiation process. As quoted by Shao et al. (8), the well-differentiated cells including Caco-2, HCA-7 and LS174T expressed COX-2, whereas poorly-differentiated cells such as DLD-1 were negative. In our study, the well-differentiated cells Caco-2 and SW1116 strongly expressed COX-2, however other well differentiated cells like HCT-EB and HCT-GEO were negative (very low COX-2 mRNA
was detected by real-time RT-PCR). On the contrary, the poorly-differentiated cells SW48 were found COX-2-positive. In human, it has been shown that moderately- to well-differentiated carcinomas showed significantly higher COX-2 immunoreactivity than poorly-differentiated tumours (34). However, it was also reported that the transfection of COX-2 RNA antisense in rat intestinal epithelial (RIE) cells resulted in loss of COX-2 expression and increased differentiation, as measured through alkaline phosphatase activity. On the contrary, RIE cells transfected with the sense COX-2 expression vector showed high COX-2 expression and low alkaline phosphatase activity (11).

Finally, inhibition of COX-2 by sulindac also resulted in increased alkaline phosphatase activity. In our hands, high expression of COX-2 in SW1116 cells did not interfere with its differentiation, and two weeks confluent well-differentiated SW1116 cells expressed COX-2 to a similar level as non-confluent undifferentiated SW1116 cells.

We further investigated the microsatellite status of the cell lines by studying BAT-25 and BAT-26 alleles since, in human, reduced COX-2 protein was described in colorectal carcinoma with defective mismatch repair system (7). Alone, BAT-26 was described as a good indicator of replication error phenotype in colorectal cancer cells (30), therefore, the association of the two alleles allowed a reliable phenotypic characterisation, although for clinical studies the actual guidelines recommend the analysis of 5 markers (35). Only 4 cell lines were found to be RER+. Among these 4 lines, none expressed a high level of COX-2 protein, however, the small number analysed did not allow a highly reliable correlation analysis although, based on COX-2 mRNA level, no correlation was observed ($p>0.05$). It is noteworthy that SW48 cells, which expressed COX-2 protein, were found to present microsatellite stability in our hands, whereas it was noted unstable previously (30).

We then compared the anti-proliferative properties of two COX-2 inhibitors, celecoxib and rofecoxib. These two inhibitors were selected because they represent the last generation of NSAIDs which selectively target COX-2. The $IC_{50}$ towards COX-2 were very close, and varied from 0.34 to 0.96 $\mu$M for celecoxib and from 0.31 to 0.84 $\mu$M for rofecoxib, depending on studies (36, 37). In our hands, celecoxib was the most potent COX-2 inhibitor for growth inhibition of colorectal cancer cells whatever their COX-2 status was. These results support previous findings obtained in leukaemia cell lines and epithelial cancer cell lines other than colorectal (38). At the highest celecoxib concentration (100 $\mu$M), an almost complete inhibition of proliferation was seen, whereas at this concentration rofecoxib was much less efficient. On the contrary, at lower concentration (10 $\mu$M) both drugs were effective, some cells being more efficiently growth-inhibited with rofecoxib than with celecoxib. Thus, it appears that a dual mechanisms occurred.

At high concentrations (50 $\mu$M and above), celecoxib was shown to induce apoptosis, whereas rofecoxib did not (39, 40). On HCT-116, SW1116 and HT-29 cells, we also observed that celecoxib used at high concentration rapidly induced apoptosis, while rofecoxib was without apoptotic effect. A recent work, performed in prostate cancer cells, demonstrated that celecoxib-induced apoptosis was independent of COX-2 (22), as suggested in our study. This pro-apoptotic effect is now well characterised and involves the AKT/PKB pathway through PDK1 inhibition (41).

At low concentrations, the two drugs inhibited cell proliferation, again by a mechanism independent of COX-2 inhibition, the effect being observed whatever the COX-2 status was. It is questionable whether COX-1 is involved in such an inhibitory effect. First, COX-1 was expressed in all the cell lines tested. Moreover, a COX-1 selective inhibitor showed growth inhibition capacities, and was almost devoid of apoptotic effect on colorectal cell lines (42). The potential role of COX-1 in neoplasia was also revealed in a murine model of intestinal tumorigenesis, where genetic disruption of COX-1 led to a reduction of intestinal tumorigenesis similarly to what was found for COX-2 (43). However, rofecoxib and celecoxib, used at low concentrations, displayed a similar effect on cell proliferation, but were shown to very differently inhibit COX-1 activity. Indeed, the $IC_{50}$ towards COX-1 was 1.2 $\mu$M for celecoxib and 63 $\mu$M for rofecoxib (36). Thus, it seems unlikely that COX-1 is involved in such an effect and the precise mechanism of action remains to be characterised.

Understanding of this mechanism of action is of importance because, in vivo, both in human and in murine models of carcinogenesis, the apoptotic effect, which was observed at high concentration (above 50 $\mu$M), may not play a major role in celecoxib and other NSAIDs inhibition of tumour development. Indeed, in human plasma, celecoxib concentrations were 10 to 100 times lower, i.e. between 0.5 and 5 $\mu$M. Similarly, in Min mice the serum concentration of celecoxib found to inhibit tumour growth was 2-5 $\mu$M. Moreover, rofecoxib, which has almost no pro-apoptotic effect, also showed a potent anti-tumour effect in vivo (44).

In summary, our results show that rofecoxib and celecoxib differently affected the growth of colorectal cancer cells, both in a COX-2-independent manner. Celecoxib induced apoptosis whereas rofecoxib did not. The implication of COX-1, although unlikely because of the low efficacy of rofecoxib towards COX-1, deserves supplementary studies. Furthermore, our findings suggest that a potentially new mechanism may be involved in the rofecoxib effect on cell growth. This mechanism, which remains to be characterized, seems independent of COX-2 expression, and may also be independent of the PDK1/AKT pathways revealed by celecoxib.
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References


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