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The anti-proliferative potency of celecoxib is not a class effect of coxibs

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ABSTRACT

Celecoxib, a COX-2 (cyclooxygenase-2)-selective inhibitor (coxib), is the only NSAID (non-steroidal anti-inflammatory drug) that has been approved for adjuvant treatment of patients with familial adenomatous polyposis. To investigate if the anti-proliferative effect of celecoxib extends to other coxibs, we compared the anti-proliferative potency of all coxibs currently available (celecoxib, rofecoxib, etoricoxib, valdecoxib, lumiracoxib). Additionally, we used methylcelecoxib (DMC), a close structural analogue of celecoxib lacking COX-2-inhibitory activity. Due to the fact that COX-2 inhibition is the main characteristic of these substances (with exception of methylcelecoxib), we conducted all experiments in COX-2-overexpressing (HCA-7) and COX-2-negative (HCT-116) human colon cancer cells, in order to elucidate whether the observed effects after coxib treatment depend on COX-2 inhibition. Cell survival was assessed using the WST proliferation assay. Apoptosis and cell cycle arrest were determined using flow cytometric and Western blot analysis. The *in vitro* results were confirmed *in vivo* using the nude mouse model. Among all coxibs tested, only celecoxib and methylcelecoxib decreased cell survival by induction of cell cycle arrest and apoptosis and reduced the growth of tumor xenografts in nude mice. None of the other coxibs (rofecoxib, etoricoxib, valdecoxib, lumiracoxib) produced anti-proliferative effects, indicating the lack of a class effect and of a role for COX-2. Our data emphasize again the outstanding anti-proliferative activity of celecoxib and its close structural analogue methylcelecoxib in colon carcinoma models *in vitro* and *in vivo*.

1. Introduction

Epidemiological, animal and cell culture studies have shown chemopreventive and tumor-regressive effects of NSAIDs on colorectal cancer and several other tumor-types [1,2]. The anti-proliferative effects of NSAIDs were, at least partially, attributed to their COX-inhibitory activity [3–5]. COX-2 derived prostaglandins were demonstrated to promote tumor growth

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Abbreviations: APC, adenomatous polyposis coli; cel, celecoxib; COX-2, cyclooxygenase-2; eto, etoricoxib; FAP, familial adenomatous polyposis; FCS, fetal calf serum; HPLC, high performance liquid chromatography; LC–MS/MS, liquid chromatography coupled with tandem mass spectrometry; lum, lumiracoxib; LPS, lipopolysaccharide; DMC, methylcelecoxib; NSAID, non-steroidal anti-inflammatory drug; PARP, poly (ADP)-ribose polymerase; PGE2, prostaglandin E_2; rof, rofecoxib; val, valdecoxib.

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by accelerating the cellular proliferation rate, inhibiting apoptosis and enhancing metastasis and angiogenesis [6–8]. In addition, several COX-independent mechanisms were reported to contribute to the anti-proliferative effects of NSAIDs (for review see [9,10]), whereas each NSAID appeared to have unique COX-independent targets. COX-2-selective inhibitors, such as celecoxib and rofecoxib, were developed to reduce the incidence of gastrointestinal side effects associated with non-selective long-term inhibition of COX-1 and COX-2 by traditional NSAIDs. In recent years, further COX-2-selective NSAIDs with a higher COX-2-selectivity have been synthesized (valdecoxib, etoricoxib and lumiracoxib) and introduced to the market. Unfortunately, randomised clinical trials and observational studies have shown an increased risk of myocardial infarction, stroke, hypertension and heart failure during treatment of patients with coxibs [11], leading to the withdrawal of rofecoxib and valdecoxib from the market in 2004 and 2005. Furthermore, several cases of severe livertoxicity led to a stop of approval of lumiracoxib in the EU in November 2007.

Within the family of coxibs, celecoxib has been most often investigated for its anti-proliferative effects *in vitro* as well as *in vivo*. In line with these experiments, celecoxib is the only NSAID currently approved for adjuvant treatment in patients with familial adenomatous polyposis, a precancerous disease. Several COX-2-independent molecular mechanisms that contribute to its anti-proliferative effects *in vitro* and *in vivo* have been described for celecoxib [10]. Very recently, gene expression profiles of colonic mucosa of patients with hereditary nonpolyposis colon cancer treated either with celecoxib (200, 400 mg) or placebo were analysed. The expression levels of more than 1400 genes were altered after celecoxib treatment. Main response rates were detected in genes of the categories: immune response, inflammatory reaction, cell signaling and cell adhesion, response to stress, transforming growth factor-β signaling, and apoptosis [12].

This study was performed to investigate the anti-proliferative activity of all COX-2-selective inhibitors currently available. Presuming predominant COX-2-dependency, celecoxib's anti-proliferative potency should be true for all other COX-2-selective inhibitors and represent a class effect as proposed for the cardiovascular side effects. To address this issue we compared the anti-proliferative activities of all coxibs *in vitro* using COX-2-overexpressing (HCA-7) and COX-2-negative (HCT-116) human colon cancer cells and *in vivo* using the nude mouse model.

2. Material and methods

2.1. Cells and reagents

The human colon cancer cell line HCA-7 was purchased from the European Collection of Cell Cultures (ECC, Salisbury, UK). HCT-15 and HCT-116 colon carcinoma cells were purchased from Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). HCA-7 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) containing sodium pyruvate supplemented with 8 mM L-glutamine. HCT-116 cells were incubated in McCoy's 5A medium and HCT-15 cells were cultured in RPMI medium. All media contained 10% FCS, 100 units/ml penicillin G and 100 μg/ml streptomycine. Cells were cultured at 37 °C in an atmosphere containing 5% CO_2. FCS, media, penicillin G and streptomycine were purchased from invitrogen GmbH (Karlsruhe, Germany). Lipopolysaccharide (LPS) was purchased from Sigma–Aldrich (Schnelldorf, Germany).

Celecoxib, etoricoxib, methylcelecoxib, rofecoxib and valdecoxib were synthesized by WITEGA Laboratorien Berlin-Adlershof GmbH. Lumiracoxib was provided by Norvartis (Basle, Switzerland). The identity and purity of all coxibs was determined using ^{1H}NMR and HPLC as described previously [13] and was >99%.

2.2. Determination of prostaglandin E_2 level in human HCA-7 colon carcinoma cells

HCA-7 cells were seeded at a concentration of 5×10^5 per dish and incubated for 24 h at 37 °C and 5% CO_2 in medium containing 10% FCS. Cells were then preincubated with the coxibs (0.001–50 μM) for 30 min. Cells were then incubated with fresh medium containing 10% FCS and the coxibs at the concentrations described above for 2 h at 37 °C. After the 2 h incubation, PGE_2 (prostaglandin E_2) concentrations in the supernatant were determined using a PGE_2 Correlate-EIA-Kit (Assay designs, Ann Arbor, USA) according to the protocol of the manufacturer.

2.3. Human COX-2 whole blood assay

DMC and celecoxib (1, 5, 10, 50, 100 μM) or vehicle (DMSO) were added to 500 μl fresh human heparinized whole blood. Then, COX-2-mediated PGE_2 production was started by addition of 100 μg/ml LPS and the samples were further incubated for 24 h at 37 °C. The blood was centrifuged at 12,000 × *g* for 10 min and plasma PGE_2 concentrations were determined using LC/MS-MS as described previously [14]. All PGE_2 concentrations of LPS-stimulated samples were corrected for the respective basal plasma PGE_2 concentrations in unstimulated blood.

2.4. In vitro cell viability assay

The WST-1 assay (Roche Diagnostic GmbH, Mannheim) was used to determine the proliferation rate of HCT-116, HCT-15 and HCA-7 cells, which were seeded at a density of 5×10^4, 3×10^4 and 7×10^4 cells/well, respectively and treated with various coxibs for 20 h. Celecoxib, methylcelecoxib, rofecoxib, etoricoxib, valdecoxib and lumiracoxib were used up to a concentration of 100 μM. Cell viability was assayed according to the distributor's protocol.

2.5. Detection of cell cycle arrest and apoptosis using flow cytometry

HCT-116 and HCA-7 cells were seeded at a density of 4×10^5 and 5×10^5 cells per dish, respectively, in medium containing 10% FCS and incubated for 48 h at 37 °C in an atmosphere containing 5% CO_2. Cells were then treated for 20–48 h with increasing drug concentrations (0, 20, 40, 60, 80, 100 μM for celecoxib, methylcelecoxib, rofecoxib, etoricoxib, valdecoxib, lumiracoxib and 0,

10, 20, 30, 40, 50, 60 μM for methylcelecoxib in HCA-7 cells) in medium containing 10% FCS. The flow cytometric analysis was performed as described previously [15].

2.6. Western blot analysis

HCT-116 and HCA-7 cells were seeded at a density of 3.5×10^5 and 6×10^5 cells per dish, respectively, in medium containing 10% FCS and incubated for 48 h at 37 °C in an atmosphere containing 5% CO_2. Cells were then treated with increasing drug concentrations (0, 20, 40, 60, 80, 100 μM for celecoxib, rofecoxib, etoricoxib, valdecoxib, lumiracoxib; 0, 20, 30, 40, 50, 60 μM for methylcelecoxib) for 20 h. Immunoblotting was performed as described previously [15]. The antibodies used were diluted as follows: primary antibody raised against COX-2 (1:1000, mouse monoclonal), PARP (poly (ADP)-ribose polymerase, 1:100, rabbit polyclonal), cyclin D1 (1:100, rabbit polyclonal), p27 (1:100, rabbit polyclonal), beta-catenin (1:400, goat polyclonal), Erk-2 (1:1000, mouse monoclonal). The antibodies were purchased from Santa Cruz Biotechnology (Heidelberg, Germany).

2.7. Tumor growth in nude mice

Male NMRI nude mice (Harlan Winkelmann GmbH (Borchen)) at the age of 8 weeks were used for all experiments. HCT-116 and HCT-15 cells were injected subcutaneously (1×10^7 cells/tumor) at the right and left dorsal flank. Etoricoxib (25 mg/kg), rofecoxib (25 mg/kg), celecoxib (10 mg/kg) and methylcelecoxib (10 mg/kg) were administered orally using a gavage needle. Therapy started 4 days after the injection of the tumor cells at a frequency of once daily, five days a week, for up to 3 weeks. Control mice received vehicle. The weight of the mice was determined and tumor dimension was assessed using a caliper rule three times a week. The tumor volume V_T was calculated using the following standard formula: $V_T = (\text{width}^2 \times \text{length} \times \pi)/6$. The animals were killed 22 days after the tumor cell injection. In all experiments the ethics guidelines for investigations in conscious animals were obeyed and the local Ethics Committee for Animal Research approved the experiments.

2.8. Statistics

Proliferation, cell cycle and nude mice data are presented as mean ± S.E.M. (standard error of the mean). The SPSS 9.01 computer software was used for statistical analyses. IC_{50} values were analysed using a sigmoid Emax model followed by subsequent submission to univariate analysis of variance (ANOVA) and *t*-tests using a Bonferroni α-correction for multiple comparisons, α was set at 0.05.

3. Results

3.1. Effect of celecoxib and methylcelecoxib on prostaglandin E_2 synthesis in human HCA-7 colon carcinoma cells

Methylcelecoxib (DMC), a close structural analogue of celecoxib was recently reported to lack COX-2-inhibitory activity [16] and was used as a control agent in several studies investigating COX-2-independent anti-proliferative effects of celecoxib and other NSAIDs [16,17]. As a prerequisite for the use of DMC in our cellular assays we first wanted to confirm the lacking COX-2-inhibitory activity of this drug under our cell culture conditions. HCA-7 cells show highly elevated levels of PGE_2 (>1000 pg/ml) in the medium supernatant due to high COX-2 expression and, therefore, represent an appropriate model for studying drugs that interfere with COX-2-mediated PGE_2 synthesis [18]. Surprisingly, the IC_{50} value of methylcelecoxib for PGE_2 production in HCA-7 cells is 3.08 ± 0.05 μM (data not shown). Celecoxib potently suppressed PGE_2 synthesis with an IC_{50} value

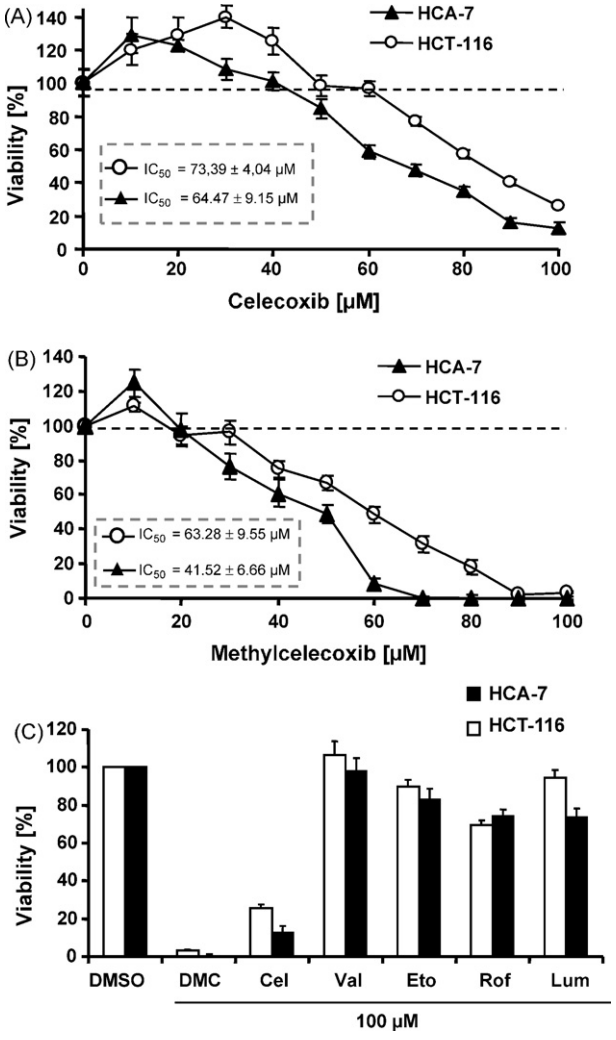

Fig. 1 – Coxib-induced reduction of HCA-7 and HCT-116 cell viability. (A and B) The data of celecoxib and methylcelecoxib used at 0–100 μM for 20 h. (C) The comparison over all coxibs (methylcelecoxib (DMC), celecoxib (Cel), valdecoxib (Val), etoricoxib (Eto), rofecoxib (Rof) and lumiracoxib (Lum)) used at 100 μM for 20 h. The cell viability was determined using the WST-1 proliferation assay. The proliferation rate of DMSO treated cells was set to 100% cell viability. The relative viability rate was then expressed as the ratio of the cell viability of treated versus untreated cells. Data are mean ± S.E.M. of three independent experiments.

<0.01 μM under identical conditions (data not shown). In contrast, DMC only weakly inhibited prostaglandin E_2 synthesis in LPS-stimulated human whole blood (IC_{50} for DMC approximately 100 μM, for celecoxib approximately 1 μM, data not shown). A study that addresses the molecular mechanism responsible for prostaglandin E_2 inhibition in HCA-7 cells by DMC was recently submitted.

3.2. *Effect of coxib treatment on the viability of HCT-116 and HCA-7 colon carcinoma cells*

The sensitivity of the colon carcinoma cells HCT-116 and HCA-7 to the various coxibs was determined using the WST-1 proliferation assay. Methylcelecoxib was a more potent inhibitor of HCA-7 and HCT-116 cell proliferation (IC_{50}(HCA-7) = 41.52 ± 6.66 μM and IC_{50}(HCT-116) = 63.28 ± 9.55 μM) than celecoxib (IC_{50}(HCA-7) = 64.47 ± 9.15 μM and IC_{50}(HCT-116) = 73.39 ± 4.04 μM (Fig. 1A and B)). To exclude that the anti-proliferative effects of DMC are due to a reduction of PGE_2, we investigated whether exogenously added PGE_2 abrogates the anti-proliferative potency of DMC. However, 10 μM PGE_2 did not alter the anti-proliferative effects of DMC (data not shown). In general, HCA-7 cells were more sensitive against the anti-proliferative effect of celecoxib and methylcelecoxib than HCT-116 cells. Fig. 1C presents the viability of HCA-7 and HCT-116 cells after treatment with 100 μM of coxibs. Valdecoxib, etoricoxib, rofecoxibs and lumiracoxib only marginally reduced cell proliferation (10–30% at 100 μM), making it impossible to calculate IC_{50} values.

3.3. *Analysis of cell cycle distribution and induction of apoptosis after treatment of the cells with the various coxibs*

Several studies demonstrated that the anti-proliferative effects of celecoxib are based on induction of apoptosis and a cell cycle block [15]. Therefore, we analysed the coxib-mediated effects on cell cycle distribution or apoptosis using flow cytometry. No effects on cell cycle distribution were seen after treatment of HCT-116 and HCA-7 cells with rofecoxib, valdecoxib, etoricoxib and lumiracoxib up to concentrations of 100 μM for 20 h or 48 h (data not shown, Supplement 1).

Interestingly, after treatment of HCA-7 cells with increasing concentrations of celecoxib or methylcelecoxib, a strong increase of cells in the subG_1-phase, representing the fraction of fragmented apoptotic cells, was observed. This was accompanied by a decline of cells in the G_1-, S- and G_2-phase (Fig. 2A and B). In contrast, celecoxib and methylcelecoxib in HCT-116 cells caused a concentration-dependent significant increase of cells in the G_1-phase and a decrease of cells in the S- and G_2-phase, indicating the induction of G_1 cell cycle arrest (Fig. 2A and B). Only higher concentrations of celecoxib (100 μM) and methylcelecoxib (60 μM) induced a significant increase in the number of cells in subG_1-phase.

3.4. *Assessment of cyclin D1 and p27 expression after coxib treatment of the cells*

To investigate whether the cell cycle block induced by celecoxib and methylcelecoxib in HCT-116 cells could be explained by changes in the expression level of different cell

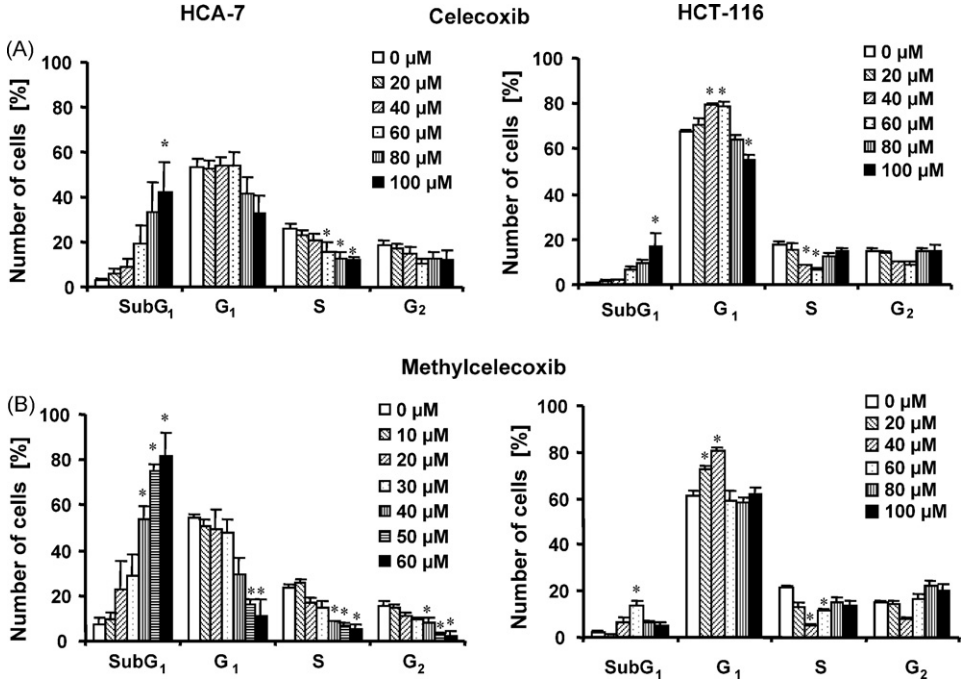

Fig. 2 – Percentage cell cycle distribution of HCA-7 and HCT-116 cells after treatment with increasing concentrations of celecoxib (A) or methylcelecoxib (B) for 20 h. Cells were harvested after trypsinisation, fixed with ethanol and the DNA content assessed using ethidium-bromide staining and fluorescence-activated cell sorter (FACS) analysis. Data are mean ± S.E.M. of three independent experiments. Statistical significant differences are indicated with an asterisk ($p < 0.05$).

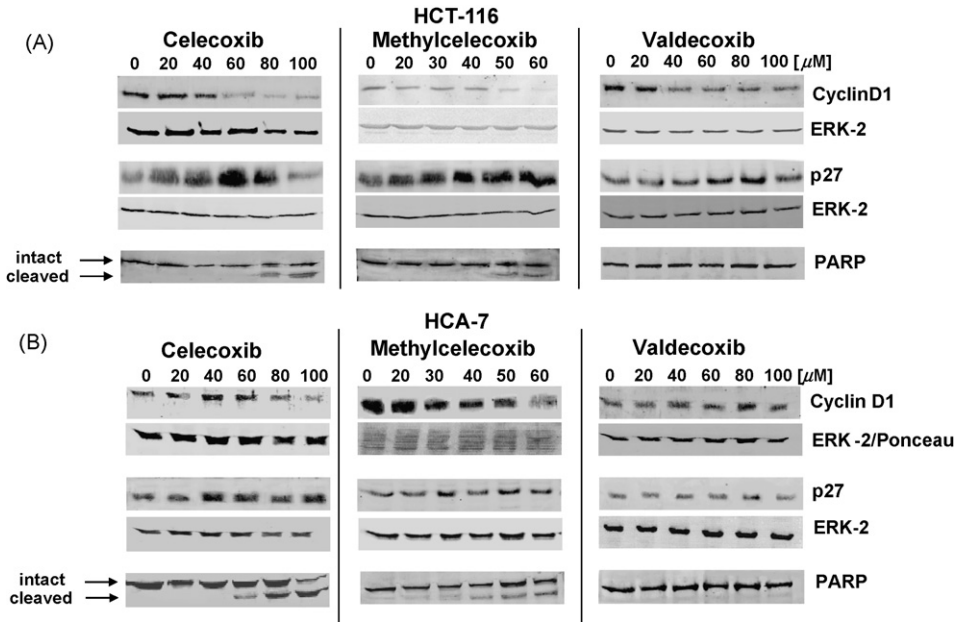

Fig. 3 – Western blot analysis of cyclin D1 and p27 protein expression and PARP cleavage in HCT-116 (A) and HCA-7 (B) cells after treatment with increasing concentrations of celecoxib, valdecoxib and methylcelecoxib for 20 h. Due to the pronounced induction of apoptosis in HCT-116 and HCA-7 cells after treatment with methylcelecoxib, the use of this drug was restricted to concentrations up to 60 μM. For Western blot analysis 50 μg of total protein extract were separated onto a 12% SDS-polyacrylamide gel and electro-blotted onto a nitrocellulose membrane which was then incubated with specific antibodies directed against cyclin D1 (34 kDa), p27 (27 kDa), PARP (116 kDa) and the apoptotic cleavage product of PARP (85 kDa). Equal loading of the gels was checked by staining the membrane with ponceau solution and reprobing the membranes with an ERK-2 antibody. A representative experiment from at least two independent experiments is shown.

cycle regulating proteins, we assessed the expression of cyclin D1, which promotes the G_1–S transition, and p27, which effectively inhibits the G_1–S progression by down-regulating the cyclin D1/CDK (cyclin-dependent kinase) activity [19].

Treatment of COX-2-negative HCT-116 cells with celecoxib, methylcelecoxib and valdecoxib reduced cyclin D1 and induced p27 (Fig. 3A) expression in a concentration-dependent manner. Interestingly, valdecoxib also concentration-dependently reduced cyclin D1 and induced p27 expression (Fig. 3A) in HCT-116 cells although no G_1-block was observed in these cells by flow cytometry analysis. In agreement with the flow cytometry data in HCA-7 cells, celecoxib and methylcelecoxib caused in comparison to HCT-116 cells a weaker degradation of cyclin D1 and weaker induction of p27 expression (Fig. 3B). By contrast, no effects on cyclin D1 and p27 expression were seen after treatment of HCA-7 cells with valdecoxib. Rofecoxib, etoricoxib and lumiracoxib (up to 100 μM) had no effect on the expression of cyclin D1 or p27 in either cell line (data not shown).

3.5. *Analysis of PARP cleavage after coxib treatment of the cells by the Western blotting*

As a second marker of apoptosis we analysed the cleavage of the caspase substrate PARP (poly (ADP)-ribose polymerase). In agreement with the flow cytometry data in HCA-7 cells, PARP cleavage occurred at lower concentrations of celecoxib (≥60 μM) and methylcelecoxib (≥30 μM) than in HCT-116 cells (≥80 μM celecoxib, ≥50 μM methylcelecoxib) (Fig. 3). No PARP cleavage was seen in either cell line following treatment with valdecoxib (Fig. 3) or any other coxib (rofecoxib, etoricoxib and lumiracoxib, data not shown).

3.6. *Effect of orally administered coxibs on tumor growth in nude mice*

To confirm the results obtained from the *in vitro* cell culture experiments *in vivo*, celecoxib (10 mg/kg), methylcelecoxib (10 mg/kg), rofecoxib (25 mg/kg), and etoricoxib (25 mg/kg) were administered orally to tumor-implanted nude mice and the tumor volume was assessed over three weeks. Celecoxib and methylcelecoxib significantly ($p < 0.01$) inhibited the growth of HCT-116 colon cancer xenografts as compared to animals receiving placebo (Fig. 4A). In contrast, rofecoxib and etoricoxib had no statistically significant effect on the growth of COX-2-deficient HCT-15 colon cancer xenografts (Fig. 4B). The animal experiments with rofecoxib and etoricoxib were performed within a previous study that utilized the human colon carcinoma cell line HCT-15. Both HCT-116 and HCT-15 cells represent epithelial-like, COX-2-negative but COX-1-positive, colon carcinoma cells. Previously published data demonstrated that HCT-15 tumor xenografts were, as the HCT-116 cells used in this study, similar sensitive to the anti-tumorigenic effect of celecoxib (Supplement 2) [20]. Nevertheless, we additionally compared the anti-proliferative effect of rofecoxib, etoricoxib, methylcelecoxib and celecoxib on cell

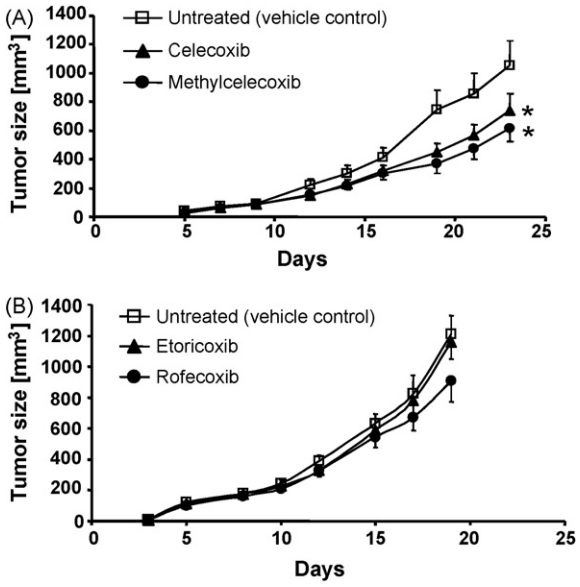

Fig. 4 – Effects of orally administered celecoxib and methylcelecoxib (A) or rofecoxib and etoricoxib (B) on the growth of HCT-116 and HCT-15 xenografts in nude mice. The data of rofecoxib and etoricoxib in nude mice, carring HCT-15 xenografts, were obtained prior to this study. To keep the number of animals low we did not repeat this data with HCT-116 colon carcinoma cells. Above all, HCT-116 cells and HCT-15 cells, both representing epithelial-like COX-2-negative but COX-1-positive colon carcinoma cells, show similar sensitivity to celecoxib [20]. Approximately 1×10^7 HCT-116 or HCT-15 tumor cells were injected subcutaneously into the left and right dorsal flank of nude mice. Mice received daily either vehicle or 10 mg/kg celecoxib or methylcelecoxib and 25 mg/kg rofecoxib or etoricoxib, respectively. Treatment started 4 days post implantation. Tumor dimensions were determined at the time points indicated and tumor volume was calculated as described in the chapter materials and methods. A significant inhibition of tumor growth in celecoxib- or methylcelecoxib-treated mice versus untreated control mice is indicated with an asterisk ($p < 0.01$).

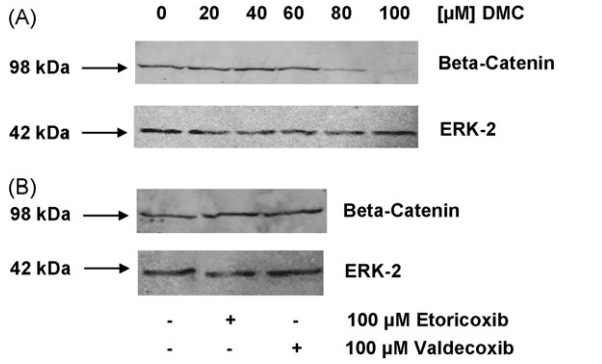

Fig. 5 – Western blot analysis of beta-catenin protein levels in HCA-7 cells after treatment with increasing concentrations of celecoxib (0–100 μM) (A) or 100 μM valdecoxib/etoricoxib (B). For Western blot analysis 50 μg of total protein extract were separated onto a 12% SDS-polyacrylamide gel and electro-blotted onto a nitrocellulose membrane which was then incubated with specific antibodies directed against beta-catenin. Equal loading of the gels was checked by staining the membrane with ponceau solution and reprobing the membranes with an ERK-2 antibody. A representative experiment from three independent experiments is shown. Because we already confirmed the COX-2-independency of beta-catenin degradation in a former study using rofecoxib, the experiment with valdecoxib and etoricoxib was performed once.

viability of HCT-15 cells *in vitro*. Methylcelecoxib was the most effective substance, followed by celecoxib. Rofecoxib and etoricoxib did not reduce cell proliferation up to 100 μM (Supplement 3). The IC_{50} values of methylcelecoxib (IC_{50}(HCT-15) = 57.87 ± 0.47 μM) and celecoxib (IC_{50}(HCT-15) = 90.10 ± 0.96 μM) were comparable to the values calculated for HCT-116 cells (see Section 3.2). Due to these similarities, we did not repeat the nude mice experiments for rofecoxib and etoricoxib with the HCT-116 cell line because of lack of ethical justification.

3.7. *Determination of beta-catenin protein levels in HCA-7 cells treated with coxibs*

Mutations of the APC (adenomatous polyposis coli) tumor suppressor protein are an early event in colorectal carcinogenesis and cause overexpression of beta-catenin in nearly all colorectal carcinoma tissues. Beta-catenin functions as a transcription factor involved in oncogenic signal transduction thereby strongly promoting tumor growth and impairing patient survival. Our studies [21], as well as those of others [22], demonstrated that suppression of beta-catenin-mediated signal transduction appears to be a major mechanism of celecoxib responsible for its chemopreventive efficacy against colorectal cancer. Given the distinct anti-proliferative effects of DMC in cell culture assays as well as its anti-neoplastic efficacy in the nude mice model, we hypothesized that DMC may target the APC/beta-catenin pathway. As can be seen in Fig. 5A, DMC clearly reduced beta-catenin protein levels in HCA-7 cells at drug concentrations as low as 60–80 μM. In line with the data of rofecoxib [21], etoricoxib and valdecoxib had no effect on beta-catenin expression even at high concentrations of 100 μM (Fig. 5B).

4. Discussion

Numerous studies over the last few years were performed to explain the anti-proliferative effects of the COX-2-selective inhibitor celecoxib. However, the molecular mechanisms responsible for the anti-proliferative effects of celecoxib are still not fully understood.

The present study was performed to investigate the extent to which the anti-proliferative potency of celecoxib is a class effect of all coxibs. This is the first study that compared the

anti-proliferative potency of all coxibs currently available (celecoxib, rofecoxib, valdecoxib, etoricoxib, lumiracoxib) and methylcelecoxib (close structural analogue of celecoxib) both in COX-2-positive (HCA-7) and COX-2-negative (HCT-116) colon carcinoma cells. For the first time, we demonstrate that methylcelecoxib significantly reduces the growth of xenografted human colon carcinoma cells in nude mice, implying a possible clinical value of methylcelecoxib for the treatment of colorectal cancer. Although several papers have linked COX-2 inhibition (resulting in PGE_2 inhibition) with apoptosis and induction of a cell cycle block in colon carcinoma cells [23,24], our proliferation and flow cytometric results indicate that this can not be the primary mechanism of celecoxib. Despite used at concentrations that completely inhibit COX-2 (IC_{50} values about 0.1–1.1 μM [25,26]) and higher COX-2-selectivity than celecoxib, valdecoxib, etoricoxib and rofecoxib showed very weak or no anti-proliferative effects *in vitro* and *in vivo*. Methylcelecoxib, the close structural derivative of celecoxib, caused the strongest anti-proliferative effects among all coxibs tested both in cell culture assays and in the nude mice model. Although methylcelecoxib inhibits PGE_2 production in HCA-7 cells, this was not the mechanism responsible for the anti-proliferative effects because exogenously added PGE_2 did not abrogate the drug's anti-proliferative effects. Previously published data emphasize that rofecoxib, in comparison to celecoxib, shows lower anti-proliferative effects *in vitro* and *in vivo* [27,28] and in patients with metastasizing colon cancer [29]. In comparison to structurally different COX-2-selective inhibitors, such as NS-398, nimesulid, meloxicam and etodolac, celecoxib has been shown to exhibit the greatest anti-carcinogenic potency among all NSAIDs tested [30].

Our Western blot data clearly indicate that only celecoxib and methylcelecoxib influence the expression level of cell cycle regulating proteins and of an apoptosis marker, which can be regulated by different COX-independent mechanisms. In a recent review we summarized COX-independent mechanisms of celecoxib that contribute to its strong anti-proliferative activity [10]. For instance, Akt kinase, which is frequently overexpressed in tumor cells, was previously found to be inhibited by celecoxib [31]. Zhu et al. demonstrated that blockade of Akt activation depends on inhibition of the upstream 3-phosphoinositide-dependent kinase-1 (IC_{50} = 48 μM for celecoxib and IC_{50} = 38 μM for methylcelecoxib, recombinant enzymes) and represents a major molecular mechanism by which celecoxib induces apoptosis [32]. In line with these findings, using Akt-inhibiting coxibs (celecoxib, methylcelecoxib) and coxibs lacking Akt-inhibitory activity (such as rofecoxib) Kulp et al. were able to demonstrate in human prostate cancer cells that a clear correlation exists between the anti-proliferative efficacy of these drugs and their potency to inhibit Akt kinase [33]. In similar content, we recently described that treatment of human colon carcinoma cells with 60–100 μM celecoxib led to a degradation of membrane-bound and cytosolic beta-catenin, which may depend on inhibition of Akt and subsequent activation of the glykogensynthase kinase 3β. Similar results could not be observed after treatment with rofecoxib [21]. Interestingly, methylcelecoxib also potently reduced beta-catenin protein levels in HCA-7 cells, indicating that targeting the beta-catenin pathway may contribute to the anti-carcinogenic efficacy of this drug.

However, considering that more than 45 different proteins have been shown to be regulated after celecoxib treatment, it is not possible to predict the celecoxib targets responsible for its anti-proliferative effect. Rather, it may be assumed that a variety of COX-2-independent mechanisms synergistically contribute to the anti-proliferative effect of celecoxib. Our data clearly demonstrate that the anti-proliferative effect of celecoxib is unique and not a class effect of all coxibs.

Also *in vivo*, celecoxib and methylcelecoxib exhibited the strongest anti-tumorigenic effect on tumor xenografts lacking COX-2. *In vivo* we could not exclude that inhibition of prostaglandine E_2 synthesis in the tumor surrounding mouse tissue contributes to the anti-tumorigenic effect of celecoxib and methylcelecoxib. However, since the two COX-inhibitors etoricoxib and rofecoxib showed weak or no anti-tumorigenic effects in the nude mouse model, we conclude that (1) COX-2 inhibition is not the only mechanism underlying the anti-proliferative effect of celecoxib *in vivo* and thus COX-independent mechanisms play a distinct role and (2) the anti-proliferative effect of celecoxib is not shared by other coxibs. Nevertheless, discrepancy still exists between the high celecoxib concentrations needed to affect intracellular COX-2-independent targets (≥30 μM, *in vitro* assays) and the celecoxib concentrations found in the plasma of patients under tumor therapy (3–8 μM). This discrepancy may be due to the contrasting time scales of *in vivo* and *in vitro* experiments. Tumor regression in patients or animals requires weeks or month of drug treatment whereas anti-proliferative effects in cultured cells have to be observed within hours. Accordingly, celecoxib was recently reported to reduce a neointimal hyperplasia in rats through inhibition of Akt-signaling, indicating that modulation of Akt-kinase activity by celecoxib actually plays a distinct role *in vivo* [34]. Moreover, Yamada et al. [35] observed a suppression of beta-catenin-accumulated crypts in rats treated with celecoxib, which confirms the *in vitro* data of Maier et al. [21].

Thus, we can conclude that the anti-proliferative effect of celecoxib is not a class effect of all coxibs and that celecoxib represents a potentially unique drug within the coxib-group due to its anti-proliferative activity. Moreover, our *in vivo* data suggest, that the non-COX-2 inhibitor methylcelecoxib may be of clinical relevance for the treatment of colorectal cancer and, possibly, of other tumor types.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2008.04.017.

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