BIOCHEMICAL PHARMACOLOGY 76 (2008) 62–69

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

Dimethylcelecoxib inhibits prostaglandin E_2 production

Ivonne Wobst, Susanne Schiffmann, Kerstin Birod, Thorsten J. Maier, Ronald Schmidt, Carlo Angioni, Gerd Geisslinger, Sabine Grösch *

pharmazentrum frankfurt/ZAFES, Institute for Clinical Pharmacology, Johann Wolfgang Goethe-University Frankfurt, Theodor Stern Kai 7, 60590 Frankfurt/Main, Germany

ARTICLE INFO

Article history:
Received 13 March 2008
Accepted 16 April 2008

Keywords:
Dimethylcelecoxib
Cancer
COX
mPGES-1
PGE_2

ABSTRACT

Dimethylcelecoxib (DMC), a derivative of celecoxib, has been developed to distinguish between the COX-dependent and COX-independent anti-carcinogenic effects of celecoxib. Although DMC has been shown to have no COX-inhibitory activity, it is important to ensure that DMC has no other influence on prostaglandin production. Interestingly, in this study we show that DMC inhibits PGE_2 production *in vitro* in the low micromolar range in different cancer cell lines. This effect can be at least partly explained by our findings that DMC inhibits microsomal prostaglandin E synthase-1 (mPGES-1) activity in a cell-free assay. Moreover, it prevents mPGES-1 up-regulation after stimulation of HeLa cells with IL-1β and TNFα. Conversely, DMC has no effect on the expression levels of COX-1, COX-2, cytosolic PGES (cPGES) or mPGES-2 in these cells. However, in the cell-free assay DMC inhibits mPGES-1 to a maximum of 65% only and concentrations needed for inhibition of mPGES-1 activity are about 10-fold higher than needed for inhibition of PGE_2 production in cell culture. This suggests that DMC also has an impact on other proteins involved in PGE_2 production. In cell culture experiments the anti-proliferative effect of DMC, measured by the WST-1 assay, seems not to be dependent on PGE_2 inhibition, as DMC was equally effective in unstimulated HeLa cells as well as in stimulated HeLa cells, and the addition of external PGE_2 did not reverse the anti-proliferative effect of DMC in HCA-7 cells. We conclude that DMC is not a suitable non-prostaglandin-inhibiting control substance for research purposes.

1. Introduction

Celecoxib, a selective cyclooxygenase-2 (COX-2) inhibitor, exhibits exceptional anti-proliferative properties in comparison to other COX-2 selective inhibitors (coxibs). As a consequence celecoxib is the only non-steroidal anti-inflammatory drug (NSAIDs) approved for the treatment of patients with familial adenomatous polyposis, which is a precancerous disease. Coxibs were primarily developed to spare gastrointestinal site effects as compared to traditional NSAIDs. Both, traditional NSAIDs and coxibs are used for treatment of rheumatoid arthritis and have anti-inflammatory, analgesic and anti-pyretic effects, which can be mainly attributed to their COX-2-inhibitory potency. With respect to their anti-

* *Corresponding author*. Tel.: +49 69 6301 7820; fax: +49 69 6301 7636.
E-mail address: groesch@em.uni-frankfurt.de (S. Grösch).

Abbreviations: BSA, bovine serum albumin; COX-1/2, cyclooxygenase-1/2; DMC, dimethylcelecoxib; DMSO, dimethyl sulfoxide; DTT, dithiotreitol; EDTA, ethylenediaminetetraacetate; FCS, fetal calf serum; IL-1β, interleukin-1 beta; LC/MS–MS, liquid chromatography tandem mass spectrometry; NSAID, non-steroidal-anti-inflammatory drug; PGES, prostaglandin E synthase; PBS, phosphate buffered saline; PMSF, phenyl-methylsulfonylfluoride; SDS-PAGE, sodiumdodecylsulfate-polyacrylamide gel electrophoresis; TNFα, tumor necrosis factor alpha; WST-1, water soluble tetrazolium-1.

0006-2952/$ – see front matter
doi:10.1016/j.bcp.2008.04.008

carcinogenic activity the molecular mechanism is not as clear. Even though it is known that COX-inhibition plays a crucial role in the anti-carcinogenic effect of NSAIDs (reviewed in [1–4]), several COX-independent mechanisms have been ascertained to be also of importance (reviewed in [5–7]). COX-2 independent mechanisms have been identified on the one hand by the use of cancer cell lines which do not express COX-2 but were also sensitive against the anti-proliferative effect of COX-2 inhibitors [8]. On the other hand when non-COX-inhibitors, with high structural similarities to COX-inhibitors, were applied they also demonstrated anti-proliferative effects [9,10]. One of these substances is DMC (dimethylcelecoxib = {4-[5-(2,5-dimethylphenyl)-3(trifluoro-methyl)-1*H*-pyrazol-1-yl]benzenesulfonamide}) which is a close derivative of celecoxib. The synthesis of DMC was first published by Song et al., who investigated the anti-tumor activity of celecoxib and various celecoxib derivatives with the aim to dissociate between the apoptosis-inducing activity from the COX-2-inhibitory activity of celecoxib [11]. Seven compounds were synthesized with various COX-2 inhibitory activities. Compound 7 (later known as DMC) was described to have no COX-2 inhibitory activity but was a highly potent inducer of apoptosis and therefore used as control substance to investigate COX/PGE_2-independent mechanisms involved in the anti-carcinogenic effect of coxibs. The indicated IC_{50} value of DMC for COX-2 inhibition was taken from a publication of Penning et al., however, in this paper DMC was not experimentally tested [12]. Thus, the first evidence, showing that DMC is not a COX-2 inhibitor, was published by Schönthal recently [13]. In this paper COX-2 inhibition of DMC was measured by performing the cell-free COX inhibitor screening assay demonstrating that DMC up to 100 μM has only marginal effects on COX-2 activity. Nevertheless, to ascertain if DMC is useful as a non-COX-2-inhibiting control substance it has to be shown that it has no influence not only on COX-activity, but on prostaglandin production in general. In this regard PGE_2 is especially important because PGE_2 is the most prevalent prostaglandin up-regulated in several cancer entities [14–17] and contributes to an increase in angiogenesis, inhibition of apoptosis and promotion of cell proliferation [18–20].

2. Materials and methods

2.1. Cells and reagents

HCA-7 (human colon carcinoma) cells were purchased from European Collection of Cell Cultures (ECC, Salisbury, UK), HeLa (human cervix carcinoma) cells from Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) and A-549 (human lung carcinoma) cells from Cell Bank of the Japanese Collection of Research Bioresources (JCRB)/Human Science Research Resources Bank (HSRRB). HCA-7 and A-549 cells were cultured in DMEM (Dulbecco's modified Eagle's medium), and HeLa cells in RPMI medium 1640. All media contained high glucose, GlutaMAX, 10% FCS, and were purchased from Invitrogen (Germany) as well as 100 units/ml penicillin G and 100 μg/ml streptomycin. Cells were cultured at 37 °C in an atmosphere containing 5% CO_2. Celecoxib, etoricoxib, dimethylcelecoxib, rofecoxib, valdecoxib and SC-560 (a selective COX-1-inhibitor) 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 liquid chromatography tandem mass spectrometry (LC/MS–MS) as described previously [21,22] and was >99%. Recombinant human IL-1 beta (IL-1β) and recombinant human tumor necrosis factor alpha (TNFα) were purchased from PeproTech (London, UK).

2.2. COX inhibitor screening assay

Inhibition of COX-1 (ovine) and COX-2 (human recombinant) activity by DMC was measured using a COX Inhibitor Screening Assay Kit (Cayman Chemicals, Ann Arbor, MI, USA), according to the manufacturer's protocol. SC-560, a selective COX-1 inhibitor, and celecoxib, a selective COX-2 inhibitor, were used as positive controls. The COX inhibitor screening assay measures directly the amount of PGE_2, PGD_2 and $PGF_{2\alpha}$ produced by $SnCl_2$ reduction of COX-derived PGH_2. Additionally to this protocol the amounts of prostaglandins were quantified by LC–MS/MS analysis as described previously [23].

2.3. Prostaglandin measurement

Cells were incubated for 24 h at 37 °C in medium containing 10% FCS. The medium was replaced with fresh media with addition of either 0.1% DMSO (control) or DMC (0.05–50 μM) and, simultaneously, IL-1β (2 ng/ml) for stimulation of A-549 cells or IL-1β (1 ng/ml) + TNFα (5 ng/ml) for stimulation of HeLa cells. HCA-7 cells produce high amounts of PGE_2 without stimulation, therefore, we pre-incubated HCA-7 cells with media containing either DMSO (0.1%) or DMC (0.05–50 μM) for 1 h and replaced this media thereafter with fresh media and the same additions. This procedure ensures that all PGE_2 was removed that was produced when DMC has not displayed its full activity. After incubation for 16 h supernatant was collected and briefly centrifuged. The amount of PGE_2 in supernatant was determined using the PGE_2 Correlate EIA™-Kit (Assay Designs Inc., Ann Arbor, USA) according to the manufactures protocol. Additionally to this protocol the amounts of prostaglandins (PGE_2, PGD_2, $PGF_{2\alpha}$) were quantified by LC–MS/MS analysis as described previously [23].

2.4. mPGES-1 activity assay

In order to investigate the impact of DMC on microsomal prostaglandin E synthase-1 (mPGES-1) activity in vitro the microsomal fraction of HeLa cells was prepared. Approximately 4×10^6 cells were incubated for 24 h at 37 °C in medium containing 10% FCS. The medium was removed and cells were stimulated with IL-1β (1 ng/ml) + TNFα (5 ng/ml) for 16 h. After washing with 10 ml PBS, cells were scraped in 2 ml PBS and centrifuged at 2500 × *g* for 2 min at 4 °C. Cell pellets were resuspended in 600 μl potassium phosphate buffer (Kpi-buffer; 0.1 M; pH 7.4), containing 1× Complete™ protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), sucrose (0.25 M) and reduced glutathione (GSH; 1 mM). Samples were sonicated and centrifuged at 170,000 × *g* for

1 h at 4 °C. The microsomal fraction (pellet) was resuspended in 50 μl Kpi-buffer (0.1 M, pH 7.4), containing 1× Complete™ and reduced GSH (2.5 mM) and total protein content was measured using the Bradford method.

The mPGES-1 activity assay was performed as described by Thoren and Jakobsson [24]. Briefly, 0.1 mg/ml protein was incubated with different concentrations of DMC (0.1–1000 μM), MK-886 (0.1–500 μM) (Sigma, St. Louis, MO, USA), celecoxib (1–200 μM), lumiracoxib or valdecoxib (5–200 μM) for 30 min on ice. The reaction was initiated with 20 μM PGH_2 and terminated after 1 min by adding a stop solution containing 40 mM iron chloride ($FeCl_2$) and 80 mM citric acid. After solid phase extraction the amount of produced PGE_2 was measured by LC–MS/MS analysis as described previously [23].

2.5. Western blot assay

HeLa cells were seeded at a density of 1.8×10^6 cells per dish, respectively, in medium containing 10% FCS and incubated for 24 h at 37 °C. Cells were then stimulated with TNFα (5 ng/ml) + IL-1β (1 ng/ml) and simultaneously treated with increasing concentrations of DMC (5–40 μM) for 16 h. mPGES-1 and mPGES-2 protein was analysed in the microsomal fraction as described above. Cytosolic PGES (cPGES) and mPGES-2 protein was detected in the cytosolic fraction and COX-1 and COX-2 protein in whole cell lysates. Immunoblotting was performed as described previously [25]. Briefly, the protein content of lysates was estimated using the Bradford method (Biorad, Germany) and aliquots of 50 μg protein were separated onto a 10–15% SDS-polyacrylamide gel and transferred to nitrocellulose membranes (Hybond-C, Amersham). Blocked membranes were incubated with the respective primary antibody directed against mPGES-1 (rabbit polyclonal antiserum, a kind gift from Per-Johan Jakobsson (Sweden)), COX-1 (mouse monoclonal), COX-2, mPGES-2, cPGES (rabbit polyclonal; Caymann, Ann Arbor, MI, USA). Anti-β-actin antibody (mouse monoclonal; Sigma; St. Louis, MO, USA) was used as loading control.

2.6. Proliferation assay

The water soluble tetrazolium-1 (WST-1) assay (Roche) was used to determine the viability and proliferation rate of the cells after treatment with DMC. HeLa and HCA-7 cells were seeded at a density of 3×10^3 and 6×10^3 cells/well, respectively, in 100-μl culture medium containing 10% FCS into 96-well microplates and incubated for 24 h at 37 °C. Medium was removed and HeLa cells were either stimulated with IL-1β (1 ng/ml) + TNFα (5 ng/ml) or not and simultaneously treated with increasing concentrations of DMC (5–100 μM). HCA-7 cells were treated with increasing concentrations of DMC (20–100 μM) with or without addition of PGE_2 (10 nM–10 μM) (Cayman Chemicals, Ann Arbor, MI, USA). After 20 h, 10 μl of WST-1 reagent were added to each well and the cells were incubated for further 90 min. The formation of the dye was measured at 450 nm against a reference wavelength of 620 nm using a 96-well spectrophotometric plate reader (SpectraFluor Plus, Tecan, Crailsheim, Germany).

2.7. RT-PCR

For extraction of total RNA about 5×10^6 HeLa cells stimulated with IL-1β (1 ng/ml) + TNFα (5 ng/ml) and treated with 20 μM DMC or 30 μM MK-886 for 0, 2, 4 or 6 h were harvested in PBS and centrifuged at 2500 × *g* for 2 min. RNA was isolated from cells using the RNeasy Mini Kit (Quiagen, Hilden, Germany) according to the manufactures protocol. RNA concentration was assessed using a Nanodrop UV spectrophotometer. cDNA was synthesized using the 1st Strand cDNA synthesis Kit for RT-PCR (Roche).

Quantification was done by RT-qPCR using absolute QPCR Sybr® Green Rox Mix and absolute QPCR Rox Mix (ABgene, Epsom, UK). mPGES-1 specific primers (forward: 5′-ACG-CTG-CTG-GTC-ATC-AAG-AT-3′; reverse: 5′-CCG-TGT-CTC-AGG-GCA-TCC-T-3′) were designed by using the primer express software (Applied Biosystems, Weiterstadt, Germany). Human β-actin LUX Primer Set (Invitrogen, Karlsruhe, Germany) was used as control. The PCR was performed using a standard protocol recommended by manufacture and analysis was performed using the ABI PRISM® 7700 Sequence detection system (Applied Biosystems). For data analysis, the fit point method was employed. The relative amount of mPGES-1 mRNA was standardized against β-actin using the deltaC_t method as described previously [25]. Afterwards, the relative mPGES-1 mRNA amount of stimulated HeLa cells treated with DMC or MK-886 were related to control cells.

2.8. Statistics

Proliferation data are presented as mean ± standard error of the mean (S.E.M.). 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. DMC is not a COX-inhibitor

Firstly, we determined the COX-inhibitory activity of DMC by performing a commercially available COX-inhibitory screening assay using purified COX-1 (ovine) and COX-2 (human recombinant) enzymes. As shown in Fig. 1 DMC inhibits COX-1- and COX-2-activity only marginally at concentrations up to 100 μM. In comparison the COX-1 inhibitor (SC-560) and COX-2 inhibitor (celecoxib) show clear inhibitory effects on COX-1 or COX-2 enzyme activities at concentrations of only 0.1 and 0.5 μM, respectively.

3.2. Inhibition of PGE_2-production by DMC

To date, DMC is used as a non-COX-2-inhibiting control substance in comparison to celecoxib. In order to use DMC for this purpose it must be ensured that it has no impact on prostaglandin production. Therefore, we investigated the effect of DMC on PGE_2 production in three different cancer

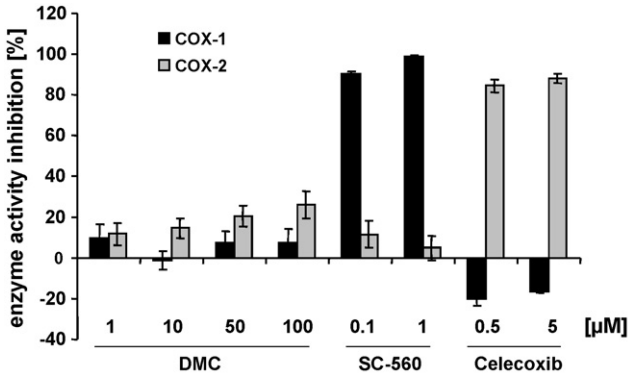

Fig. 1 – Bar graph demonstrating inhibition of COX-1 and COX-2 enzyme activity measured by the COX inhibitor screening assay. As shown, 0.1 μM SC-560 (a selective COX-1 inhibitor) and 0.5 μM celecoxib (a selective COX-2 inhibitor) inhibits COX-1 or COX-2 activity by about 90%, respectively, whereas DMC has only weak effects on both enzymes, even at substantially higher concentrations. All experiments were carried out in quadruplicate; plotted values represent mean ± S.E.M.

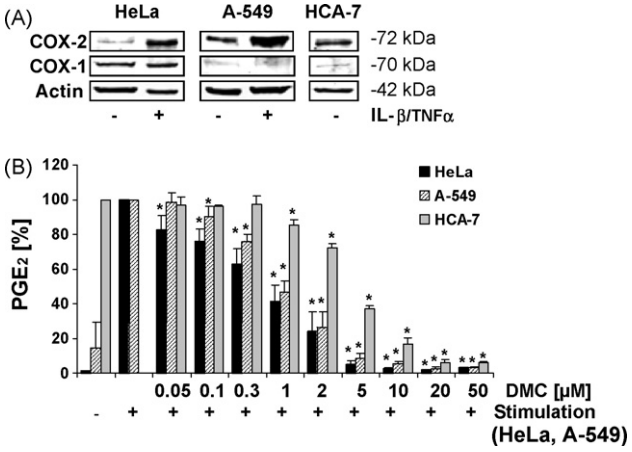

Fig. 2 – (A) Western blot analysis of COX-1 and COX-2 in human cancer cell lines. Protein expression was detected in HeLa and A-549 cells which are either stimulated with 5 ng/ml TNFα + 1 ng/ml IL-1β or 2 ng/ml IL-1β alone, respectively, or are non-stimulated as HCA-7 cells. 50 μg of total protein extract was separated on a 12% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. The expressions of COX-1 and COX-2 were detected using a specific mouse monoclonal anti-COX-1 and a rabbit polyclonal anti-COX-2 antibody. The expression of β-actin was used as a loading control. A representative experiment out of three is shown for each antibody treatment. (B) Percentage of PGE_2 in supernatants of stimulated HeLa and A-549 cells and unstimulated HCA-7 cells after treatment with increasing concentrations of DMC. Cells were incubated with media with addition of either 0.1% DMSO (control) or DMC (0.05–50 μM) and, simultaneously, IL-1β for stimulation of A-549 cells or IL-1β + TNFα for stimulation of HeLa cells. After an incubation time of 16 h supernatant was collected and the amount of PGE_2 was determined using the PGE_2 Correlate EIA™-Kit. Presented data are the mean ± S.E.M. of four to six independent experiments. Statistical significant differences in PGE_2 levels after DMC treatment were indicated with an asterisk. *$p < 0.05$.

cell lines (HeLa, a cervix carcinoma cell line; A-549, a non-small cell lung cancer cell line; HCA-7, a colon carcinoma cell line). Each cell line expresses different amounts of COX-2 and PGE_2. In HeLa and A-549 cells COX-2 expression and hence PGE_2 production has to be stimulated either with 5 ng/ml TNFα + 1 ng/ml IL-1β or 2 ng/ml IL-1β alone, respectively. HCA-7 cells express high amounts of COX-2 and produce PGE_2 without stimulation (Fig. 2A and B). PGE_2 production was determined by the use of a commercially available PGE_2 ELISA kit. Fig. 2B shows that DMC inhibits PGE_2 production in HeLa, A-549 and HCA-7 cells in a concentration-dependent manner. The IC_{50} values for PGE_2 inhibition are 0.64 ± 0.15 μM (HeLa), 0.83 ± 0.11 μM (A-549), and 3.08 ± 0.05 μM (HCA-7), respectively. These data are surprising, as DMC has been shown to inhibit neither COX-1 nor COX-2 activity at these concentrations. Therefore, we looked for alternative targets of DMC within the pathway from arachidonic acid to PGE_2 to explain its PGE_2 inhibitory effects.

3.3. *DMC inhibits mPGES-1-activity*

We investigated the effect of DMC on prostaglandin E synthases (PGES). Three PGESs are described; the microsomal prostaglandin E synthase-1, the mPGES-2 and the cytosolic PGES. Due to the fact that basal PGE_2-level in HeLa cells is only marginal but highly inducible after IL-1β and TNFα stimulation (Fig. 2B) we used this cell line for further studies and determined the expression levels of all prostaglandin E synthases in these cells. cPGES and mPGES-2 are constitutively expressed in HeLa cells whereas mPGES-1 is highly inducible after stimulation with TNFα and IL-1β (Fig. 3).

As it is already known that mPGES-1 is coupled to COX-2 and mainly responsible for PGE_2 production after stimulation of cells with IL-1β and TNFα [26] we tested the influence of DMC on mPGES-1 activity using microsomal fractions of HeLa cells. As shown in Fig. 4A, DMC inhibits HeLa mPGES-1 activity with an IC_{50} value of 15.60 ± 2.25 μM. Its efficacy, however, only reached 65% of the maximum effect of mPGES-1 activity. In contrast, the unspecific mPGES-1 inhibitor MK-886 [27] reduces mPGES-1 activity up to 100% with an IC_{50} value of 3.98 ± 0.36 μM. We then tested the ability of other coxibs such as celecoxib, lumiracoxib, valdecoxib, etoricoxib, and rofecoxib to inhibit mPGES-1 activity. As shown in Fig. 4B celecoxib and lumiracoxib also inhibit mPGES-1 activity with a maximum efficacy of up to about 70 and 80%, respectively, but these substances were less potent than DMC (IC_{50} values: 21.89 ± 3.26 μM, and 33.01 ± 4.15 μM for celecoxib and lumiracoxib, respectively). Valdecoxib inhibited mPGES-1 activity with an IC_{50} value of 75.08 ± 18.7 μM, whereas all the other coxibs used did not inhibit mPGES-1 activity even when used at a concentration of 200 μM.

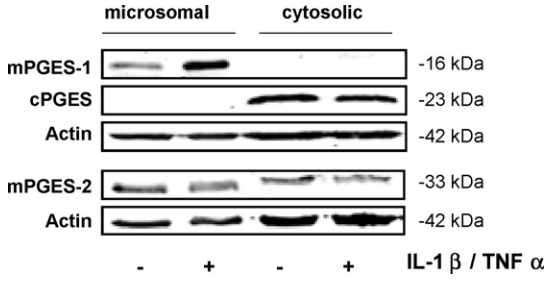

Fig. 3 – Western blot analysis of prostaglandin E synthases in non-stimulated and stimulated HeLa cells. Microsomal and cytosolic fractions of HeLa cells were prepared and 50 μg protein were separated onto a 15% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. mPGES-1, mPGES-2 and cPGES were detected using specific rabbit polyclonal antibodies. The expression of β-actin was used as loading control. mPGES-1 is only present in the microsomal fraction and highly inducible after stimulation of HeLa cells, whereas cPGES could be only detected in the cytosolic fraction and is not inducible. mPGES-2 is detectable both in the microsomal and cytosolic fraction but also not inducible by treatment of HeLa cells with IL-1β and TNFα. A representative experiment out of three is shown for each antibody treatment.

3.4. *DMC prevents from mPGES-1 induction after stimulation of HeLa cells with cytokines*

As mPGES-1 activity was only inhibited at DMC concentrations 10-fold higher than the IC_{50} value for PGE_2 inhibition we tested the influence of DMC on PGES expression levels. As shown in Fig. 3, cPGES and mPGES-2 are constitutively expressed in HeLa cells, whereas mPGES-1 increases 8–24 h after stimulation of HeLa cells reaching a maximum at 16 h (data not shown). Therefore, all further experiments have been conducted after an incubation period of 16 h. Induction of mPGES-1 protein expression after treatment of HeLa cells with IL-1β and TNFα is prevented by DMC concentrations ≥15 μM whereas mPGES-2 and cPGES expression remains constant (Fig. 5A). In addition, DMC (30 μM) prevented mPGES-1 mRNA induction in HeLa cells treated with IL-1β and TNFα (Fig. 5B).

3.5. *The anti-proliferative effect of DMC is independent of PGE_2 inhibition*

In the literature the anti-proliferative activity of DMC in various cancer cells is well documented [13,28,29]. Due to the lack of its COX-inhibitory activity this anti-proliferative effect has been described to be COX/PGE_2-independent. In light of the data presented here this assumption has to be re-evaluated. Therefore, we treated stimulated and unstimulated HeLa cells with increasing concentrations of DMC and measured cell viability by a commercially available cell proliferation kit. Fig. 6A demonstrates that both stimulated and unstimulated HeLa cells were equally sensitive against DMC ($IC_{50\ HeLa\ unstimulated}$, 49.99 ± 1.62 μM; $IC_{50\ HeLa\ stimulated}$, 47.66 ± 5.82 μM). Furthermore, we also compared proliferation rates of HCA-7 cells

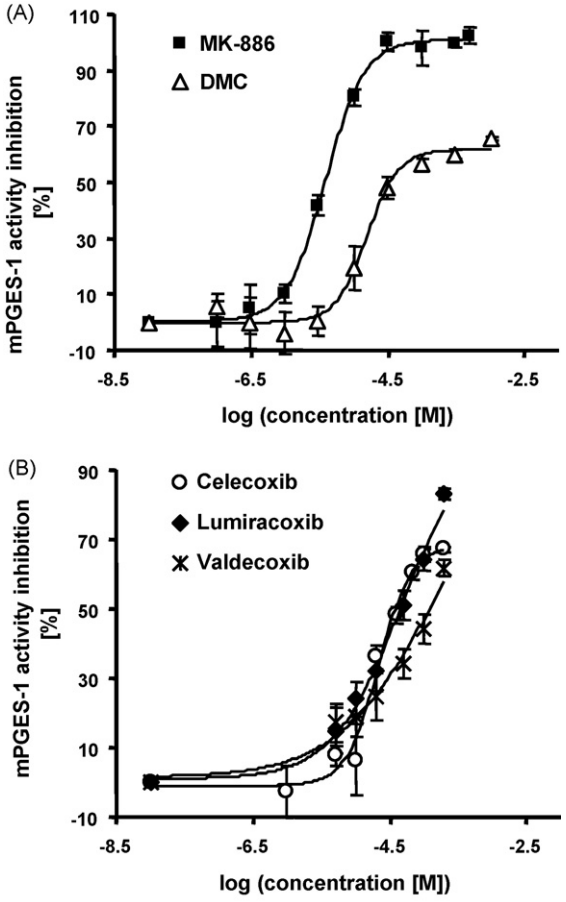

Fig. 4 – Relative mPGES-1 enzyme activity after DMC treatment using microsomal fractions of stimulated HeLa cells. 0.1 mg/ml microsomal protein was incubated with different concentrations of DMC (0.1–1000 μM) or MK-886 (0.1–500 μM) (A) or increasing concentrations of celecoxib (1–200 μM), lumiracoxib (5–200 μM), and valdecoxib (5–200 μM), respectively (B) for 30 min on ice. The reaction was initiated by addition of 20 μM PGH_2 and terminated after 1 min by adding a stop solution containing 40 mM iron chloride ($FeCl_2$) and 80 mM citric acid. After solid phase extraction the amount of produced PGE_2 was measured by LC–MS/MS analysis. For calculation of the IC_{50} values for mPGES-1 inhibition we used the non-linear regression analysis and a sigmoid Emax model. All experiments were carried out in triplicate; data are mean ± S.E.M.

(which express high levels of PGE_2 constitutively) treated with DMC with and without exogenous addition of PGE_2 (10 nM–10 μM). Additionally, no significant differences were detected regarding the sensitivity against DMC ($IC_{50\ HCA\text{-}7\ without\ PGE2}$, 57.81 ± 0.94 μM; $IC_{50\ HCA\text{-}7\ with\ 0.01\ \mu M\ PGE2}$, 64.41 ± 1.98 μM; $IC_{50\ HCA\text{-}7\ with\ 0.1\ \mu M\ PGE2}$, 54.31 ± 6.67 μM; $IC_{50\ HCA\text{-}7\ with\ 1\ \mu M\ PGE2}$, 60.07 ± 3.53 μM; $IC_{50\ HCA\text{-}7\ with\ 10\ \mu M\ PGE2}$, 58.76 ± 2.45 μM) (Fig. 6B). DMC also has anti-proliferative activity in the human colon carcinoma cell line HCT-116, which does not express COX-1 or COX-2 and produces no PGE_2 ($IC_{50\ HCT\text{-}116}$: 63.28 ± 9.55 μM, data not shown).

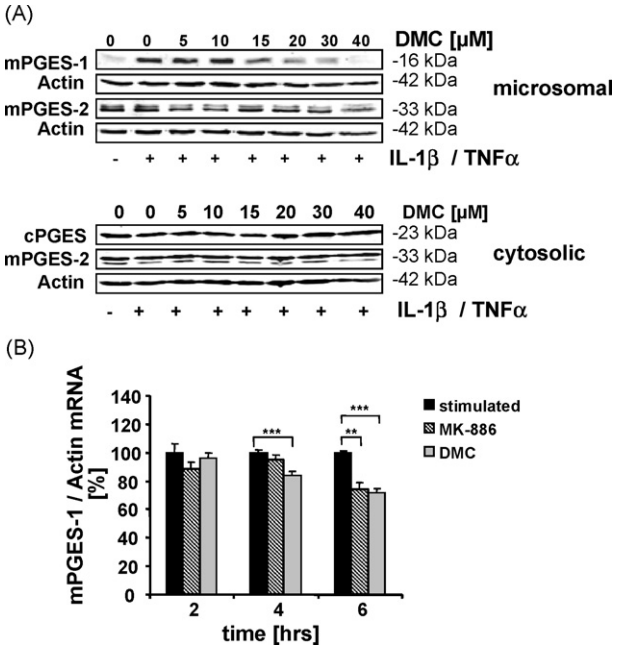

Fig. 5 – (A) Western blot analysis of mPGES-1, mPGES-2 and cPGES in microsomal and cytosolic fractions of stimulated HeLa cells after treatment with increasing concentrations of DMC. Up-regulation of mPGES-1 expression after stimulation of HeLa cells with IL-1β and TNFα is impeded by simultaneous treatment of cells with DMC at concentration ≥15 μM whereas no changes in expression levels of mPGES-2 or cPGES could be detected after DMC treatment. A representative experiment out of three is shown for each antibody treatment. (B) Determination of the relative amount of mPGES-1 mRNA in stimulated HeLa cells with and without simultaneous treatment of MK-886 (20 μM) or DMC (30 μM) using Taqman® PCR. The content of mPGES-1 mRNA in HeLa cells was standardized against actin-mRNA and the amount of mPGES-1 mRNA in stimulated HeLa cells treated either with MK-886 or DMC is expressed as a ratio of that of stimulated HeLa cells. The mean ± S.E.M. of three independent experiments is shown. Significant differences in the relative amount of mPGES-1 mRNA between stimulated HeLa cells treated with MK-886 or DMC vs. stimulated HeLa cells without co-treatment is indicated with an asterisk, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

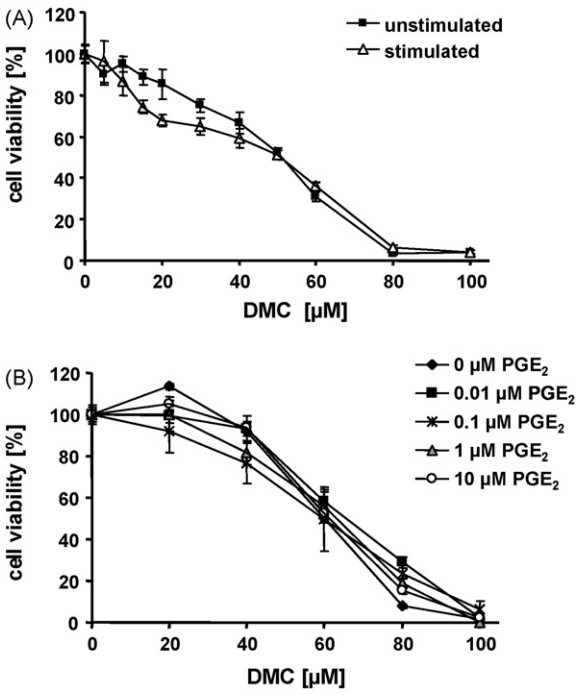

Fig. 6 – Determination of cell viability using the WST-1 assay after treatment of (A) stimulated or non-stimulated HeLa cells with DMC or (B) unstimulated HCA-7 cells with DMC or DMC and PGE_2. HeLa and HCA-7 cells were seeded in a 96-well microplate and incubated for 24 h at 37 °C. Medium was removed and HeLa cells were either stimulated with IL-1β plus TNFα or not and simultaneously treated with increasing concentrations of DMC (5–100 μM). HCA-7 cells were treated with increasing concentrations of DMC (20–100 μM) with or without addition of PGE_2 (10 nM–10 μM). After 20 h 10 μl of WST-1 reagent were added to each well and absorption was measured after 90 min at 450 nm using a spectrophotometric plate reader. The mean ± S.E.M. of three independent experiments is shown.

4. Discussion

Dimethylcelecoxib, a close structural analog of celecoxib, has been used as negative control substance for COX- and consequently also for PGE_2-inhibition in different studies investigating COX-independent anti-carcinogenic mechanisms [29–31]. Here, however, we clearly demonstrated that DMC potently inhibits PGE_2 production *in vitro*. The IC_{50} values in different cancer cell lines are in the range of 0.6 and 3 μM. Our data are in line with previously published data from Backhus et al. showing a reduction of PGE_2 in IL-1β stimulated A-549 cells after DMC treatment. Unfortunately, Backhus used only two concentrations of DMC (10 and 50 μM) that did not allow calculating an IC_{50} for PGE_2 inhibition. He presented the data of PGE_2-inhibition in a logarithmic scale plot so it is not obvious that 50 μM DMC inhibits PGE_2 production by more than 98% in these cells. Furthermore, Backhus and co-workers described DMC as a non-PGE_2-inhibitor (Fig. 2 of Backhus et al. [32]). Another paper from Kardosh et al. shows that DMC has no impact on the PGE_2-level in COX-2 overexpressing U87-glioblastoma cells, expressing high amounts of PGE_2 without stimulation [29]. These results could be explained by differences in the PGE_2-assay conditions. In our experiments HCA-7 cells (which produce also high amounts of PGE_2 without stimulation) were pre-incubated with media containing DMC (0.05–50 μM) for 1 h and this media was replaced with fresh media containing DMC (0.05–50 μM). This procedure ensures that all PGE_2, that was produced when DMC has not displayed its full activity, was removed. In the paper from Kardosh et al. cells were not pre-incubated, thus PGE_2 production could take place until DMC displays its full activity [29]. In line with published data [13], however, we confirmed that DMC inhibits

neither COX-1 nor COX-2 activity in a cell-free assay. DMC has also no effect on COX-1 protein expression and increases COX-2 expression slightly at concentrations $\geq$30 μM (data not shown), indicating that DMC must have an impact on other enzymes of the PGE_2 production pathway. Both COX-2 and mPGES-1 are up-regulated in different cancer cells or are co-regulated by several cytokines and play a distinctive role in cancer development [20,33–35]. Therefore, we investigated the effect of DMC on prostaglandin E synthases, catalysing the conversion of PGH_2 to PGE_2. The cytosolic PGES uses PGH_2 produced by COX-1 whereas the microsomal PGES-1 uses COX-2-derived endoperoxide. mPGES-2 can use both sources of PGH_2 (reviewed in [36]). In HeLa cells PGE_2 production by COX-1/cPGEs or mPGES-1 is negligible as shown by the minimal PGE_2 level in unstimulated cells (Fig. 2B). Following stimulation of HeLa cells with TNFα and IL-1β an up-regulation of PGE_2 can be ascribed to COX-2 and mPGES-1 or -2. As we did not see inhibition of PGE_2 production in the cytosolic fraction (location of active mPGES-2 and cPGES; data not shown) we assume that mPGES-1 is the main enzyme responsible for PGE_2 production in HeLa cells. Interestingly, mPGES-1 activity could be inhibited by DMC in a cell-free assay, but the maximum effect reached was only 65% with an IC_{50} of 15 μM. In contrast, MK-886, which is a MAPEG (membrane-associated proteins involved in eicosanoid and glutathione metabolism) inhibitor, inhibiting also FLAP (5-lipoxygenase-activating protein) and LTC4 (leukotriene C4) synthase [27,37], represses mPGES-1 activity by 100%. The loss of efficacy of DMC in the cell-free assay could be explained last but not least by: (1) DMC is not a direct inhibitor of mPGES-1 but rather represses mPGES-1 activity by indirect mechanisms perhaps by inducing conformational changes of the enzyme or allosteric inhibition; (2) in the performed mPGES-1 assay we used 20 μM PGH_2 as substrate which is probably much higher than the substrate concentration in the intact cell assay. If DMC acts as a competitive inhibitor, the substrate concentration used in our mPGES-1 activity assay might be too high to compete against them. Both hypotheses, however, have to be further investigated. In contrast to COX-1 and COX-2, DMC also diminished mPGES-1 protein and mRNA expression but only at concentrations greater than 15 μM. We can conclude that DMC has a clear impact on mPGES-1 activity and expression but only at 10-fold higher concentrations than needed for PGE_2 inhibition.

Interestingly, proliferation assays revealed that *in vitro* PGE_2 inhibition seems not to be crucial for the anti-proliferative effect of DMC (Fig. 6A and B). Additionally, *in vivo*, in the nude mice model, DMC inhibits tumor growth of COX-2/PGE_2 depleted human colon cancer xenografts [28], but we cannot exclude that inhibition of PGE_2 production in the tumor surrounding mouse tissue contributes to the anti-carcinogenic effect of DMC.

In conclusion, we clearly demonstrated that the non-COX-2-inhibitor, DMC, inhibits PGE_2 production in the low micromolar range. Neither DMC's impact on mPGES-1 activity nor on the mPGES-1 expression level could be solely responsible for this effect. Furthermore, after stimulation of HeLa cells with IL-1β and TNFα also other negligible detectable prostaglandins (PGD_2, $PGF_{2\alpha}$) are slightly diminished by DMC treatment (see supplement 1) so that also other enzymes, important for prostaglandin production, have to be investigated as targets of DMC. This study has demonstrated that DMC is not suitable as a non-prostaglandin-inhibiting control substance.

Acknowledgments

We thank Prof. Per Johan Jakobsson for providing us with the anti-mPGES-1 antibody. The work was supported by the European Graduate School ''Roles of Eicosanoids in Biology and Medicine'' (DFG GRK 757/1), the DFG Forschergruppe ''Signalling durch Fettsäuremetabolite und Sphingolipide'' (FOG 784), and in part by DFG GE 695/2-3.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2008.04.008.

REFERENCES

[1] Peek Jr RM. Prevention of colorectal cancer through the use of COX-2 selective inhibitors. Cancer Chemother Pharmacol 2004;54(Suppl. 1):S50–6.
[2] Rao CV, Reddy BS. NSAIDS and chemoprevention. Curr Cancer Drug Targets 2004;4(1):29–42.
[3] Thun MJ, Henley SJ, Patrono C. Nonsteroidal anti-inflammatory drugs as anticancer agents: mechanistic, pharmacologic, and clinical issues. J Natl Cancer Inst 2002;94(4):252–66.
[4] Evans JF, Kargman SL. Cancer and cyclooxygenase-2 (COX-2) inhibition. Curr Pharm Des 2004;10(6):627–34.
[5] Grosch S, Maier TJ, Schiffmann S, Geisslinger G. Cyclooxygenase-2 (COX-2)-independent anticarcinogenic effects of selective COX-2 inhibitors. J Natl Cancer Inst 2006;98(11):736–47.
[6] Soh JW, Weinstein IB. Role of COX-independent targets of NSAIDs and related compounds in cancer prevention and treatment. Prog Exp Tumor Res 2003;37:261–85.
[7] Tegeder I, Pfeilschifter J, Geisslinger G. Cyclooxygenase-independent actions of cyclooxygenase inhibitors. FASEB J 2001;15(12):2057–72.
[8] Grosch S, Tegeder I, Niederberger E, Brautigam L, Geisslinger G. COX-2 independent induction of cell cycle arrest and apoptosis in colon cancer cells by the selective COX-2 inhibitor celecoxib. FASEB J 2001;15(14):2742–4.
[9] Grosch S, Tegeder I, Schilling K, Maier TJ, Niederberger E, Geisslinger G. Activation of c-Jun-N-terminal-kinase is crucial for the induction of a cell cycle arrest in human colon carcinoma cells caused by flurbiprofen enantiomers. FASEB J 2003;17(10):1316–8.
[10] Janssen A, Maier TJ, Schiffmann S, Coste O, Seegel M, Geisslinger G, et al. Evidence of COX-2 independent induction of apoptosis and cell cycle block in human colon carcinoma cells after S- or R-ibuprofen treatment. Eur J Pharmacol 2006;540(1–3):24–33.
[11] Song X, Lin HP, Johnson AJ, Tseng PH, Yang YT, Kulp SK, et al. Cyclooxygenase-2, player or spectator in cyclooxygenase-2 inhibitor-induced apoptosis in prostate cancer cells. J Natl Cancer Inst 2002;94(8):585–91.

[12] Penning TD, Talley JJ, Bertenshaw SR, Carter JS, Collins PW, Docter S, et al. Synthesis and biological evaluation of the 1,5-diarylpyrazole class of cyclooxygenase-2 inhibitors: identification of 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benze nesulfonamide (SC-58635, celecoxib). J Med Chem 1997;40(9):1347–65.
[13] Schonthal AH. Antitumor properties of dimethyl-celecoxib, a derivative of celecoxib that does not inhibit cyclooxygenase-2: implications for glioma therapy. Neurosurg Focus 2006;20(4):E21.
[14] McLemore TL, Hubbard WC, Litterst CL, Liu MC, Miller S, McMahon NA, et al. Profiles of prostaglandin biosynthesis in normal lung and tumor tissue from lung cancer patients. Cancer Res 1988;48(11):3140–7.
[15] Rigas B, Goldman IS, Levine L. Altered eicosanoid levels in human colon cancer. J Lab Clin Med 1993;122(5):518–23.
[16] Vanderveen EE, Grekin RC, Swanson NA, Kragballe K. Arachidonic acid metabolites in cutaneous carcinomas. Evidence suggesting that elevated levels of prostaglandins in basal cell carcinomas are associated with an aggressive growth pattern. Arch Dermatol 1986;122(4):407–12.
[17] Jung TT, Berlinger NT, Juhn SK. Prostaglandins in squamous cell carcinoma of the head and neck: a preliminary study. Laryngoscope 1985;95(3):307–12.
[18] Sheng H, Shao J, Morrow JD, Beauchamp RD, DuBois RN. Modulation of apoptosis and Bcl-2 expression by prostaglandin E2 in human colon cancer cells. Cancer Res 1998;58(2):362–6.
[19] Gallo O, Franchi A, Magnelli L, Sardi I, Vannacci A, Boddi V, et al. Cyclooxygenase-2 pathway correlates with VEGF expression in head and neck cancer. Implications for tumor angiogenesis and metastasis. Neoplasia 2001;3(1):53–61.
[20] Kamei D, Murakami M, Nakatani Y, Ishikawa Y, Ishii T, Kudo I. Potential role of microsomal prostaglandin E synthase-1 in tumorigenesis. J Biol Chem 2003;278(21):19396–405 [Epub March 7, 2003].
[21] Brautigam L, Nefflen JU, Geisslinger G. Determination of etoricoxib in human plasma by liquid chromatography-tandem mass spectrometry with electrospray ionization. J Chromatogr B Analyt Technol Biomed Life Sci 2003;788(2):309–15.
[22] Franzen R, Pautz A, Brautigam L, Geisslinger G, Pfeilschifter J, Huwiler A. Interleukin-1beta induces chronic activation and de Novo synthesis of neutral ceramidase in renal mesangial cells. J Biol Chem 2001;276(38):35382–9.
[23] Schmidt R, Coste O, Geisslinger G. LC–MS/MS-analysis of prostaglandin E2 and D2 in microdialysis samples of rats. J Chromatogr B Analyt Technol Biomed Life Sci 2005;826(1/2):188–97.
[24] Thoren S, Jakobsson PJ. Coordinate up- and down-regulation of glutathione-dependent prostaglandin E synthase and cyclooxygenase-2 in A549 cells. Inhibition by NS-398 and leukotriene C4. Eur J Biochem 2000;267(21):6428–34.
[25] Maier TJ, Schilling K, Schmidt R, Geisslinger G, Grosch S. Cyclooxygenase (COX-2)-dependent and -independent anticarcinogenic effects of celecoxib in human colon carcinoma cells. Biochem Pharmacol 2004;67(8):1469–78.
[26] Bage T, Modeer T, Kawakami T, Quezada HC, Yucel-Lindberg T. Regulation of prostaglandin E synthases: effects of siRNA-mediated inhibition of microsomal prostaglandin E synthase-1. Biochim Biophys Acta 2007;1773(10):1589–98.
[27] Mancini JA, Blood K, Guay J, Gordon R, Claveau D, Chan CC, et al. Expression, and up-regulation of inducible rat prostaglandin e synthase during lipopolysaccharide-induced pyresis and adjuvant-induced arthritis. J Biol Chem 2001;276(6):4469–75.
[28] Schiffmann S, Maier T, Wobst I, Janssen A, Corban-Wilhelm H, et al. The anti-proliferative potency of celecoxib is not a class effect of coxibs. Biochem Pharmacol, doi:10.1016/j.bcp.2008.04.017.
[29] Kardosh A, Wang W, Uddin J, Petasis NA, Hofman FM, Chen TC, et al. Dimethyl-celecoxib (DMC), a derivative of celecoxib that lacks cyclooxygenase-2-inhibitory function, potently mimics the anti-tumor effects of celecoxib on Burkitt's lymphoma in vitro and in vivo. Cancer Biol Ther 2005;4(5):571–82.
[30] Lin HP, Kulp SK, Tseng PH, Yang YT, Yang CC, Chen CS. Growth inhibitory effects of celecoxib in human umbilical vein endothelial cells are mediated through G1 arrest via multiple signaling mechanisms. Mol Cancer Ther 2004;3(12):1671–80.
[31] Kardosh A, Soriano N, Liu YT, Uddin J, Petasis NA, Hofman FM, et al. Multitarget inhibition of drug-resistant multiple myeloma cell lines by dimethyl-celecoxib (DMC), a non-COX-2 inhibitory analog of celecoxib. Blood 2005;106(13):4330–8.
[32] Backhus LM, Petasis NA, Uddin J, Schonthal AH, Bart RD, Lin Y, et al. Dimethyl celecoxib as a novel non-cyclooxygenase 2 therapy in the treatment of non-small cell lung cancer. J Thorac Cardiovasc Surg 2005;130(5):1406–12.
[33] Dempke W, Rie C, Grothey A, Schmoll HJ. Cyclooxygenase-2: a novel target for cancer chemotherapy? J Cancer Res Clin Oncol 2001;127(7):411–7.
[34] Fosslien E. Molecular pathology of cyclooxygenase-2 in neoplasia. Ann Clin Lab Sci 2000;30(1):3–21.
[35] Kawamori T, Wakabayashi K. COX-2 and prostanoid receptors: good targets for chemoprevention. J Environ Pathol Toxicol Oncol 2002;21(2):149–53.
[36] Samuelsson B, Morgenstern R, Jakobsson PJ. Membrane prostaglandin e synthase-1: a novel therapeutic target. Pharmacol Rev 2007;59(3):207–24.
[37] Riendeau D, Aspiotis R, Ethier D, Gareau Y, Grimm EL, Guay J, et al. Inhibitors of the inducible microsomal prostaglandin E2 synthase (mPGES-1) derived from MK-886. Bioorg Med Chem Lett 2005;15(14):3352–5.