Calcium-activated endoplasmic reticulum stress as a major component of tumor cell death induced by 2,5-dimethyl-celecoxib, a non-coxib analogue of celecoxib


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Abstract

A drawback of extensive coxib use for antitumor purposes is the risk of life-threatening side effects that are thought to be a class effect and probably due to the resulting imbalance of eicosanoid levels. 2,5-Dimethyl-celecoxib (DMC) is a close structural analogue of the selective cyclooxygenase-2 inhibitor celecoxib that lacks cyclooxygenase-2–inhibitory function but that nonetheless is able to potently mimic the antitumor effects of celecoxib in vitro and in vivo. To further establish the potential usefulness of DMC as an anticancer agent, we compared DMC and various coxibs and nonsteroidal anti-inflammatory drugs with regard to their ability to stimulate the endoplasmic reticulum (ER) stress response (ESR) and subsequent apoptotic cell death. We show that DMC increases intracellular free calcium levels and potently triggers the ESR in various tumor cell lines, as indicated by transient intracellular free calcium levels and potent apoptosis at comparable concentrations. The effects of DMC are not restricted to in vitro conditions, as this drug also generates ER stress in xenografted tumor cells in vivo, concomitant with increased apoptosis and reduced tumor growth. We propose that it might be worthwhile to further evaluate the potential of DMC as a non-coxib alternative to celecoxib for anticancer purposes.

Introduction

Nonsteroidal anti-inflammatory drugs (NSAID) play a promising role in the treatment and prevention of various types of cancer (1–3). The biochemical mechanism underlying their effectiveness is thought to be the inhibition of cyclooxygenase (COX) enzymes, which catalyze the initial step in prostaglandin synthesis (4). The traditional NSAIDs, such as flurbiprofen, indomethacin, or sulindac, are able to inhibit both COX-1 and COX-2 enzymes, whereas new generation drugs, such as celecoxib (Celebrex), valdecoxib (Bextra), or rofecoxib (Vioxx), primarily inhibit COX-2. Due to their more selective function, these latter drugs, referred to as coxibs (i.e., COX-2 inhibitors), initially had promised to offer the therapeutic benefit of traditional NSAIDs with less of the associated side effects (5–8); however, this expectation has come under intense scrutiny and has generated considerable controversy in the recent past (9–12).

Celecoxib is widely prescribed under the trade name Celebrex for relief of symptoms of osteoarthritis and rheumatoid arthritis and was also approved as an adjunct to standard care for patients with familial adenomatous polyposis. It is suspected that this drug might be useful for the prevention and treatment of colorectal and possibly other types of cancer, and several clinical trials are ongoing to confirm this expectation. In addition, celecoxib has shown potent anticancer activity in various animal tumor models in the laboratory (13–19). Despite these promising results, however, the underlying molecular mechanisms by which celecoxib exerts its antitumor potential are not completely understood. Particularly intriguing are several reports describing potent antiproliferative and proapoptotic effects of this drug in the absence of any apparent involvement of COX-2 (20–26).

To investigate the COX-2–independent antitumor mechanisms of celecoxib in greater detail, we and others have generated close structural analogues of this compound that

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lack the ability to inhibit COX-2 activity (27–30). One such analogue is 2,5-dimethyl-celecoxib (DMC; ref. 31), a compound that was first described by the group of Ching-Shih Chen at Ohio State University (28, 30). Intriguingly, despite its inability to inhibit COX-2, DMC is able to faithfully mimic all of the numerous antitumor effects of celecoxib that have been investigated thus far, including the reduction of neovascularization and the inhibition of experimental tumor growth in various in vivo tumor models (23, 27–29, 32–36). Therefore, DMC seems to be well suited for studies intended to illuminate the COX-2–independent antitumor effects of celecoxib. As well, DMC might have the potential to become a useful antitumor drug in its own right (31); as a non-coxib, it is conceivable that this drug might not bring about those life-threatening side effects of coxibs that are thought to be due to the inhibition of COX-2 and the ensuing imbalance of eicosanoid levels.

It has been described recently that treatment of cultured cells with various NSAIDs, including celecoxib, generated increased levels of intracellular calcium ([Ca 2+]i) with subsequent activation of the endoplasmic reticulum (ER) stress response (ESR; refs. 37–42). These observations suggested that components of the ESR might participate in NSAID-induced apoptosis, which has been observed at relatively high (0.1 to >1.0 mmol/L) concentrations of these drugs in vitro.

The ESR consists of a set of adaptive pathways that can be triggered by disparate perturbations in normal ER function, such as accumulation of unfolded proteins, lipid or glycolipid imbalances, or changes in the ionic conditions of the ER lumen (see refs. 43, 44 for reviews). The primary purpose of the ESR is to alleviate the stressful disturbance and restore proper ER homeostasis; however, for intense or persistent ER stress, these pathways will trigger programmed cell death/apoptosis. One of the central prosurvival regulators of the ESR is glucose-regulated protein 78 (GRP78/BiP), which has important roles in protein folding and assembly, in targeting misfolded proteins for degradation, in ER Ca 2+ binding, and in controlling the activation of transmembrane ER stress sensors (45). On the other hand, CCAAT/enhancer binding protein homologous transcription factor (CHOP/GADD153) and caspase-4 are critical executioners of the proapoptotic arm of the ESR (46, 47).

Because celecoxib and several other NSAIDs have been shown to stimulate the ESR and cause activation of GRP78 and CHOP, we decided to analyze these events with the non-coxib DMC. We report here that DMC very potently (at 0.05 mmol/L) triggered the ESR in various tumor cell lines in vitro and in vivo. Apparently, this effect of the drug effectively overwhelmed the protective function of GRP78 by very strongly inducing the proapoptotic protein CHOP, which led to activation of caspase-4 and efficient cell death. In comparison, the effects of celecoxib were noticeably weaker, and none of the other coxibs or NSAIDs were able to trigger the ESR or cause cell death at comparable concentrations. Together, our results show that celecoxib and DMC are unique among the group of NSAIDs in their unmatched potency to trigger calcium-induced ER stress, with concomitant tumor cell apoptosis, at moderate concentrations and without the involvement of COX-2. As DMC is even more effective than celecoxib, it might be worthwhile to pursue the development of this non-coxib as an antitumor agent.

Materials and Methods

Materials

Celecoxib is 4-[5-(4-methylphenyl)-3-(trifluoromethyl)1H-pyrazol-1-yl]benzenesulfonamide (48). DMC is a close structural analogue, where the 5-arly moiety has been altered by replacing 4-methylphenyl with 2,5-dimethylphenyl, resulting in 4-[5-(2,5-dimethylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide (23, 27). Both compounds were synthesized in our laboratory according to previously published procedures (see ref. 48 for celecoxib and ref. 27 for DMC). Each drug was dissolved in DMSO at 100 mmol/L (stock solution). For valdecoxib (49) and rofecoxib (50), commercial caplets of Bextra (Pfizer, New York, NY) and Vioxx (Merck, Whitehouse Station, NJ), respectively, were suspended in H2O to disintegrate the excipient, and the active ingredient was dissolved in DMSO at 25 mmol/L. In addition, we used pure rofecoxib powder that was synthesized in our laboratory according to established procedures (51). All traditional NSAIDs were purchased from Sigma (St. Louis, MO) in powdered form and dissolved in DMSO at 100 mmol/L. Thapsigargin and BAPTA-AM were obtained from Sigma and dissolved in DMSO. All drugs were added to the cell culture medium in a manner that kept the final concentration of solvent (DMSO) <0.5%.

Cell Lines and Culture Conditions

Most cell lines were obtained from the American Type Culture Collection (Manassas, VA) and were propagated in DMEM or RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 0.1 mg/mL streptomycin in a humidified incubator at 37°C and a 5% CO2 atmosphere. The glioblastoma cell lines U251 and LN229 were provided by John G. H. Todd, University of Washington (Seattle, WA), and the other cell lines were purchased from ATCC, Inc. (Manassas, VA). These cell lines were maintained in a humidified incubator at 37°C. The cell lines were serum-starved for 24 h before treatment and were disintegrated with trypsin/EDTA before use. Total cell lysates were prepared by lysis of cells with protein lysis buffer (52) and protein concentrations were determined using the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL).

Immunobots and Antibodies

Total cell lysates were prepared by lysis of cells with radioimmunoprecipitation assay buffer (52), and protein concentrations were determined using the bicinchoninic acid protein assay reagent (52), and protein concentrations were determined using the bicinchoninic acid protein assay reagent (52). For Western blot analysis, 50 μg of each sample were processed as described (53). The primary antibodies were purchased from Cell Signaling Technologies (Beverly, MA), Cayman Chemical (Ann Arbor, MI), or Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and used according to the manufacturer’s recommendations. The secondary antibodies were coupled to horseradish peroxidase and detected by chemiluminescence using the SuperSignal West substrate from Pierce. All immunoblots were repeated at least once to confirm the results.
**Induction of ER Stress and Apoptosis by DMC**

**Immunohistochemistry**

Immunohistochemical analysis of protein expression in tumor tissues was done with the use of the Vectastain avidin-biotin complex method kit (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. This procedure uses biotinylated secondary antibodies and a preformed avidin: biotinylated enzyme complex that has been termed the avidin-biotin complex method technique. As the primary antibody, we used anti-CHOP antibody (Santa Cruz Biotechnology) diluted 1:100 in 2% normal goat blocking serum.

**Apoptosis Measurements**

Apoptosis in tumor sections was measured quantitatively with the use of the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay (54). All components for this procedure were from the ApopTag In situ Apoptosis Detection kit (Chemicon, Temecula, CA), which was used according to the manufacturer’s instructions.

Apoptosis in cell cultures in vitro was determined by using the Cell Death Detection ELISA kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer’s instructions. This immunoassay specifically detects the histone region (H1, H2A, H2B, H3, and H4) of mono-nucleosomes and oligonucleosomes that are released during apoptosis. Ninety-six-well plates were seeded with 1,000 cells per well and read at 405 nm in a Microplate Autoreader (Model EL 311SX; Bio-Tek Instruments, Inc., Winooski, VT).

**Colony Formation Assay**

Twenty-four hours after transfection with small interfering RNA (siRNA), the cells were seeded into six-well plates at 200 cells per well. After complete cell adherence, the cells were exposed to drug treatment for 48 h. Thereafter, the drug was removed, fresh growth medium was added, and the cells were kept in culture undisturbed for 12 to 14 days, during which time the surviving cells spawned a colony of proliferating cells. Colonies were visualized by staining for 4 h with 1% methylene blue (in methanol) and then counted.

**Transfections with SiRNA**

Cells were transfected in six-well plates with the use of LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The different siRNAs were synthesized at the microchemical core laboratory of the University of Southern California/K. Norris Jr. Comprehensive Cancer Center, and their sequences were as follows: siRNA targeted at green fluorescent protein (sense); si-GFP, 5′-CAACUGACCCUGAAGUUCCTT-3′ (sense) and 5′-GAACUUGGCGUGCCGUUGTT-3′ (antisense); si-GRP78, 5′-GGAGCGCAUUGAUACUAGATT-3′ (sense) and 5′-UCUAUGCAUAUGCCGUCCTT-3′ (antisense); and si-caspase-4, 5′-AAGUUGCCCUUCUACAGUCATUTT-3′ (sense) and 5′-AAAUGACUGUAAGAGCCACATT-3′ (antisense).

**Cytoplasmic Calcium Imaging**

The cells were loaded by incubating them with 4 μmol/L Fura-2/AM (Invitrogen) for 30 min at room temperature in external solution containing 138 mmol/L NaCl, 5.6 mmol/L KCl, 1.2 mmol/L MgCl₂, 2.6 mmol/L CaCl₂, 10 mmol/L HEPES, and 4 mmol/L glucose (pH 7.4). After loading, the cells were rinsed and transferred to the imaging setup. The cells were treated with individual drugs for 10 s, whereas fluorescence was elicited with the excitation wavelength alternating between 350 and 380 nm, using a Polychromator V (TILL Photonics GmbH, Graftenberg, Germany) to provide illumination via a Zeiss Axiovert 100 microscope with a Zeiss Fluar 40× oil objective (Carl Zeiss, Jena, Germany). Images were captured using a Cascade 512B CCD camera (Photometrics, Tucson, AZ) controlled with MetaFluor software (Molecular Devices, Sunnyvale, CA) at 0.5 Hz acquisition frequency. Ratios of the images obtained at 350 and 380 nm excitation were used to show changes in the cytoplasmic calcium concentration, according to the principles developed by Grynkiewicz et al. (55).

**Drug Treatment of Nude Mice**

Four- to 6-week-old male athymic nu/nu mice were obtained from Harlan (Indianapolis, IN) and implanted s.c. with 5 × 10⁵ U87 glioblastoma cells as described in detail elsewhere (36). For the determination of tumor growth during continuous drug treatment for several weeks, DMC or rofecoxib was mixed with the daily chow (150 mg/kg for DMC; 40 mg/kg for rofecoxib), and tumor growth was monitored and recorded as described (36). For the analysis of short-term effects of drugs on CHOP expression and tumor cell death in vivo, as well as for the determination of drug concentrations in plasma and tumor tissue, tumor-bearing animals were treated with 30, 90, 150, or 180 mg/kg of drug per day for 50 h; each animal received one half of the daily dose of the respective drug every 12 h via direct administration into the stomach with a stainless steel ball-head feeding needle (Popper and Sons, Inc., New Hyde Park, NY). All animals were sacrificed 2 h after the final application of drug, and tumors and blood were collected for analysis. In all experiments, the animals were closely monitored with regard to body weight, food consumption, and clinical signs of toxicity; no differences between non-drug–treated control animals and drug-treated animals were detected.

**Extraction of Plasma for Liquid Chromatography Mass Spectrometry Analysis**

Blood was collected in heparinized syringes using cardiac puncture of nude mice. The blood was allowed to settle at room temperature for 30 min followed by centrifugation at 2,000 rpm for 5 min at 4°C. The plasma was separated from the cells and transferred to a fresh tube. To establish a standard reference, 25 μL of 1.0 μg/mL DMC or celecoxib were added to 50 μL plasma from untreated control animals. For the test samples, the same amount of DMC was added as an internal standard to plasma from animals that had been treated with celecoxib, whereas the same amount of celecoxib was added as an internal standard to plasma from animals that had been treated with DMC. After thorough vortexing, plasma proteins were precipitated using 425 μL acetonitrile and vortexing for 1 min. The entire mixture was centrifuged at 4,500 rpm for 5 min to separate the protein precipitate, and 400 μL of the supernatant were transferred to a fresh tube. The sample
was evaporated using a steady stream of air, and the dried residue was reconstituted using 150 mL of mobile phase consisting of 80:20 (v/v) methanol:10 mmol/L ammonium acetate (pH 4.5). The flow rate to separate the analytes was 350 mL/min, where the retention time for DMC and celecoxib were 3.50 and 3.10 min, respectively. The analytes were then introduced into the Sciex API 3000, which was set in the negative ion mode. The level of DMC and celecoxib used the transition ions 394.0 → 330.2 and 380.0 → 316.2, respectively. The lower level of quantification of this assay was established at 5 ng/mL.

Results
DMC is a close structural analogue of celecoxib that lacks the ability to inhibit COX-2. To investigate whether this compound would be able to induce the ESR, we treated various tumor cell lines with DMC, or in parallel with celecoxib, and determined the expression level of CHOP protein. CHOP is a proapoptotic component of the ESR and is critically involved in the initiation of cell death after ER stress; therefore, we used it as a well-established indicator of ESR in our experimental system. As shown in Fig. 1, both DMC and celecoxib were able to potently induce CHOP in glioblastoma, breast carcinoma, pancreatic carcinoma, Burkitt’s lymphoma, and multiple myeloma cell lines. Thus, both drugs seemed to stimulate the ESR, although celecoxib seemed to be somewhat less potent than DMC in glioblastoma and Burkitt’s lymphoma cell lines.

To evaluate the extent of the ESR after DMC treatment, we analyzed additional indicators of the ESR and compared the effects with those obtained with the use of thapsigargin, an inhibitor of sarcoplasmic/ER Ca2+-ATPases that is frequently used as a strong model inducer of the ESR. Cells were treated with either DMC or thapsigargin for various times in parallel, and the expression levels of CHOP, GRP78, and caspase-4, an ESR-specific caspase, were analyzed. Figure 2 shows that DMC and thapsigargin stimulated the three selected ESR indicators in a similar fashion. Both CHOP and GRP78 were substantially elevated, with a prominent increase noted at the 6-h time point and thereafter. Activation of caspase-4, which was indicated by the appearance of the cleaved (activated) form of this enzyme, was first noted at ~24 h of drug treatment and persisted until the later (36 h) time point. Thus, the stimulation of the ESR was remarkably similar between

**Figure 1.** Celecoxib and DMC induce CHOP protein levels in various cancer cell lines. Several different cancer cell lines (left) were cultured in the presence of celecoxib (Cxb; 40 and 60 μmol/L) or DMC (30 and 50 μmol/L) for 48 h (control (Ctr), nontreated cells). Total cellular lysates were prepared and analyzed by Western blot analysis with specific antibodies to CHOP. As a control for equal loading, all blots were also analyzed with antibodies to actin (only one of these control blots is shown at the bottom). Right, tumor type of each cell line.

**Figure 2.** ER stress indicators are induced similarly by DMC and thapsigargin. U251 glioblastoma cells were cultured in the presence of 1 μmol/L thapsigargin or 60 μmol/L DMC for various times as indicated. Total cell lysates were prepared and analyzed by Western blot with specific antibodies to the ER stress proteins GRP78, CHOP, and caspase-4 (Casp 4). Actin was used as a loading control. Pro-caspase-4 denotes the inactive caspase-4 proenzyme, whereas cleaved caspase-4 is indicative of the activated form of this enzyme. *, faster-migrating band that is inconsistently observed in these Western blots.

80:20 (v/v) methanol:10 mmol/L ammonium acetate (pH 4.5). The flow rate to separate the analytes was 350 mL/min, where the retention time for DMC and celecoxib were 3.50 and 3.10 min, respectively. The analytes were then introduced into the Sciex API 3000, which was set in the negative ion mode. The level of DMC and celecoxib used the transition ions 394.0 → 330.2 and 380.0 → 316.2, respectively. The lower level of quantification of this assay was established at 5 ng/mL.
DMC and the model inducer thapsigargin, suggesting that the effects of DMC were quite potent in this context.

A prominent feature of the ESR is a general, transient down-regulation of overall protein synthesis, in combination with selectively increased translation of ER stress proteins, such as GRP78 (56, 57). We therefore investigated whether DMC and celecoxib would impair cellular translation by determining the incorporation of $^{35}$S-methionine into newly translated proteins. As shown in Fig. 3, both drugs severely diminished the rate of translation in a concentration-dependent manner, with DMC being noticeably more potent. At 2 h of treatment, 60 μM/L DMC and 80 μM/L celecoxib were as effective as thapsigargin, and nearly as effective as the potent translational inhibitor cycloheximide, and reduced ongoing translation by ~90%. This inhibitory effect was transient, as cells returned to unrestricted, fully active protein synthesis by 18 h, despite the continuous presence of DMC or celecoxib (Fig. 3; data not shown for celecoxib). In addition, greatly increased translation of GRP78 could be detected in DMC- and celecoxib-treated cells (Fig. 3; data not shown for celecoxib). Taken together, these results show that DMC and celecoxib cause the typical features of ESR in drug-treated cells.

Due to the striking similarities between the effects of DMC/celecoxib and those of thapsigargin, which is known to leak calcium from the ER and generate a calcium spike in the cytoplasm, we next determined whether DMC, and several coxibs and NSAIDs in comparison, would induce such a response as well. For this purpose, cells were loaded with Fura-2/AM, exposed to 100 μM/L of each drug, and the increase in cytoplasmic calcium levels was measured. As shown in Fig. 4, DMC and celecoxib caused a pronounced calcium spike, which could be observed in each and every cell tested. In contrast, none of the other coxibs (rofecoxib and valdecoxib) or traditional NSAIDs (flurbiprofen, indomethacin, and sulindac) were able to elicit an elevation of cytoplasmic calcium levels. Thus, DMC and celecoxib seemed to be uniquely able to mimic this aspect of thapsigargin, and the potent elevation of intracytoplasmic calcium levels by these drugs is entirely consistent with the generation of ESR, as documented in Figs. 1–3 above. The average maximum calcium peak (Fig. 4B) caused by DMC was somewhat greater than what was measured for celecoxib, but this difference was not statistically significant; however, overall calcium release (Fig. 4A; area under the curve) was consistently 30% to 50% larger with DMC.

To further substantiate the uniqueness of DMC and celecoxib compared with other coxibs and NSAIDs, we next investigated how the observed effects of these two drugs would compare with those of other coxibs and traditional NSAIDs. First, we treated cells with DMC and the three coxibs celecoxib, rofecoxib, and valdecoxib and determined the expression levels of the ER stress indicator protein CHOP. At 50 μM/L, DMC generated a pronounced induction of CHOP, which was detectable as early as 4 h after the onset of drug treatment and continued to increase up to 24 h (Fig. 5A). Treatment of cells with 50 μM/L celecoxib resulted in a similar kinetic of CHOP induction, although the overall levels were noticeable lower compared with DMC. In contrast, neither rofecoxib nor valdecoxib at the same concentration resulted in any detectable CHOP expression (Fig. 5A).

To investigate whether ESR induction could possibly be achieved at higher concentrations of rofecoxib and valdecoxib, the cells were treated with further increased concentrations of each drug. However, as shown in Fig. 5B, even at concentrations of 75 or 100 μM/L, neither rofecoxib nor valdecoxib was able to stimulate any detectable increase in either CHOP or GRP78 protein. In comparison, DMC and celecoxib potently induced the expression of both of these proteins, and once again, DMC
was noticeable more potent than celecoxib; the effects of 50 \( \mu \text{mol} / \text{L} \) DMC were comparable with those of 75 \( \mu \text{mol} / \text{L} \) celecoxib (100 \( \mu \text{mol} / \text{L} \) DMC could not be analyzed because this concentration effectively killed the whole cell population). In addition, the induction of CHOP and GRP78 by DMC and celecoxib could also be observed at the mRNA level (data not shown). Furthermore, the inclusion of calcium chelators (BAPTA-AM and EGTA) effectively prevented the induction of CHOP by DMC (Fig. 5C), indicating that the above described elevation of cytosolic calcium levels were critical to ER stress induction by DMC.

We next investigated the relationship of ER stress induction and cell death. Figure 6A shows that 30 and 50 \( \mu \text{mol} / \text{L} \) DMC potently stimulated the ESR, as indicated by the pronounced induction of CHOP, GRP78, and caspase-4 cleavage/activation. In parallel, three variables of cell growth and cell death were investigated. First, a colony-forming assay was done; this is an indicator of long-term survival that reveals the percentage of individual cells that are able to survive and spawn a colony of new cell growth. Second, the traditional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to determine short-term growth and survival, indicated primarily by the metabolic activity of the entire cell population. Third, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay was done to quantify the fraction of cells undergoing apoptosis. As shown in Fig. 6A, the induction of ESR by DMC closely correlated with greatly reduced survival in colony-forming assays, with reduced cellular activity in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays, and with substantially increased apoptosis.

Celecoxib as well was able to induce cell death/apoptosis (Fig. 6B) and reduced the viability of the cell culture (Fig. 6C), although its potency was clearly less than that of DMC (as indicated by the requirement of higher concentrations). However, none of the other coxibs (rofecoxib and valdecoxib) or traditional NSAIDs (flurbiprofen, indomethacin, and sulindac) had any detectable effect on cell growth and survival and did not induce apoptosis even at concentrations of up to 100 \( \mu \text{mol} / \text{L} \) (Fig. 6B and C). Thus, taken together, these results indicate that celecoxib is unique among these coxibs/NSAIDs because of its superior potency to stimulate the ESR and initiate tumor cell death; in addition, its derivative DMC seems even more effective, clearly arguing that the inhibition of COX-2 is not required to achieve these effects.

We next investigated the contribution of the ESR to reduced tumor cell growth in response to treatment with DMC or celecoxib. For this purpose, we applied specific siRNAs to knock down the expression of either GRP78 or GRP78-siRNA, the cells became more sensitive and there was less cell survival when GRP78 level was reduced (Fig. 7A). As a control, a si-GFP was included in these experiments; furthermore, the efficiency of target knock-down by each specific siRNA was confirmed by Western blot analysis of caspase-4 and GRP78 protein (Fig. 7B). For GRP78-siRNA, the cells became more sensitive and there was less cell survival when GRP78 levels were reduced. For caspase-4 cleavage/activation, the opposite was observed: we found that the sensitivity of cells to drug treatment was significantly reduced (i.e., cell survival after treatment with celecoxib or DMC was increased when caspase-4 expression was diminished by siRNA). Thus, these results agree with the current model of ESR, where GRP78 represents the protective arm, whereas caspase-4 is proapoptotic and necessary for the execution of cell death after ER stress; our results indicate that DMC and celecoxib overpower the protective effort by GRP78 and induce cell death via the stimulation of caspase-4 activity.
In an effort to determine whether ER stress might be relevant during the in vivo antitumor activity of DMC, we used a xenograft nude mouse tumor model and investigated the expression of CHOP protein in tumor tissue from animals treated with DMC or rofecoxib. As shown in Fig. 8, CHOP protein was barely detectable in tumor tissue from control animals (i.e., in the absence of any drug treatment). In contrast, when animals were fed with DMC for 50 h, there was a large increase in CHOP protein expression in their tumor tissue. In comparison, when animals received rofecoxib, no such increase was observed (Fig. 8). In addition, the highly elevated amount of CHOP protein after DMC treatment correlated with significantly increased apoptosis in the tumor tissue, whereas tumors from rofecoxib-treated animals did not display elevated levels of apoptosis (Fig. 8). Furthermore, on longer-term therapy of tumor-bearing animals with either DMC or rofecoxib, it became apparent that only DMC caused significantly reduced tumor growth (Fig. 9), indicating that the induction of ESR and apoptosis by DMC indeed translated into overall reduced tumor growth in this xenograft model. Finally, as an extension of earlier pharmacokinetic/pharmacodynamic determinations of DMC and celecoxib in nude mice (23), we measured the concentration of drug (DMC and celecoxib) in blood and tumor tissues from our experimental animals. Tumor-bearing animals were treated for 2 days with daily dosages ranging from 30 to 180 mg/kg DMC or celecoxib, and the absolute levels ($C_{\text{max}}$) of each drug were determined by liquid chromatography mass spectrometry. As presented in the Table 1, maximal drug concentrations in plasma and tumor tissue increased as daily dosages increased and reached peak levels of 45 $\mu\text{mol/L}$ in the plasma from animals treated with the highest dose of 180 mg/kg. Intriguingly, however, the concentrations in tumor tissues were two orders of magnitude lower than the corresponding plasma concentrations; in animals receiving the highest daily dose of 180 mg/kg, tumor tissue concentrations approximately equivalent to only 0.25 $\mu\text{mol/L}$ were reached. Nonetheless, in all of these tumor tissues, increased levels of CHOP expression were observed, whereas tumor tissues from non-drug treated or rofecoxib treated (30–180 mg/kg) animals were consistently negative for this ER stress indicator protein (see Fig. 8). In general, tumor tissues from animals treated with the lowest dosages of DMC or celecoxib were positive for CHOP, although tumors from those animals exposed to much higher concentrations stained more intensely for this protein. Importantly, these results show that despite the huge difference between the drug concentrations used in vitro and those measured in vivo, in both cases, ER stress and subsequent tumor cell death were achieved.

**Discussion**

The selective COX-2 inhibitor celecoxib seems to hold promise for the treatment and prevention of colorectal cancer and possibly for other cancers as well. Because COX-2 is an oncogene (58) and overexpressed in a large number of tumors, it is generally thought that the COX-2–inhibitory function of celecoxib is critical for its antitumor property (5, 59–61). However, several recent studies (21, 23–26, 29, 62), including from our laboratory (22, 27), have...
indicated that celecoxib might be unique among the class of coxibs because this particular compound seems to be able to also suppress tumor formation in the absence of COX-2 involvement. For example, all coxibs completely inhibit COX-2 at very low micromolar concentrations in cell culture, yet only celecoxib causes efficient growth arrest and induction of apoptosis at low-to-moderate concentrations, an effect that is furthermore independent of the amount, or even the presence, of intracellular COX-2 (22, 25, 28, 32, 34–36).

The observation that DMC is able to potently mimic essentially all of the anticancer effects of celecoxib that have been investigated thus far has far-reaching implications. For instance, it is well-recognized that the chronic use of traditional NSAIDs is troubled by serious side effects; and even the coxibs, which initially were deemed safer, have displayed a risk for life-threatening complications during long-term use, such as cardiovascular events, renal injury, and gastrointestinal toxicity (10, 66–69). This has been a major reason for concern, in particular in view of the fact that for purposes of cancer prevention or therapy, such vascularization and the inhibition of experimental tumor growth in prostate carcinoma, glioblastoma, and Burkitt’s lymphoma xenograft mouse tumor models (23, 27, 28, 30, 32, 34–36).

Figure 6. Induction of CHOP and GRP78 correlates with increased apoptosis and reduced cell growth and survival. U251 glioblastoma cells were treated with different drugs, and various variables of cell growth and cell death were comparatively analyzed. As controls, cells remained either nontreated or were treated with the solvent DMSO alone. A, cells were treated with 30 or 50 μmol/L DMC for 48 h and the effects on cell growth/survival and on cell death were determined by various assays. Top, the results from a colony-forming assay, where the absolute number of surviving cells able to spawn a colony of newly grown cells was determined; middle, the results of conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays done at the end of the 48-h drug treatment period; bottom, the percentage of cells undergoing apoptosis as revealed by the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay after 48-h drug treatment. The expression levels of the ER stress indicators CHOP, GRP78, and caspase-4 at the end of the 48-h drug treatment, as determined by Western blot analysis with specific antibodies (actin served as a loading control). B, cells were treated with various concentrations of different drugs for 48 h, as indicated, and cell death was measured with the cell death ELISA kit. C, cells were treated with DMC or various coxibs and traditional NSAIDs for 48 h, as indicated, and cell growth and survival was determined with the conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (control, nontreated cells were set at 100%). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were done in 96-well plates with the use of 3.0 × 10^4 to 8.0 × 10^4 cells per well as described in detail elsewhere (34). In parallel, the expression levels of CHOP and GRP78 protein were determined by Western blot analysis. Note that DMC is the most potent drug, celecoxib is substantially weaker, and none of the other coxibs or traditional NSAIDs are active under these conditions.
Drugs would have to be administered for extended periods and likely at substantially elevated concentrations—which is known to further increase their risks. Considering that these side effects are believed to be a class effect due to the inhibition of COX-1 and/or COX-2, and presumably are mediated via the resulting imbalance of eicosanoid levels (9, 70, 71), it is tempting to speculate that the clinical use of DMC, which lacks COX inhibitory function, perhaps might avoid many of these unwanted side effects while at the same time maintain the antitumor potency. Naturally, before DMC can be applied in the clinic, the above claims will have to be substantiated by evidence; therefore, it is critical to establish what the underlying molecular events are that mediate the anticancer effects of this drug.

Previous reports have indicated that celecoxib is unique among the class of coxibs because this drug seems to have a second function in addition to its well-known ability to selectively inhibit COX-2; this second function is the capacity to induce tumor cell death/apoptosis at considerably lower concentrations than any of the other coxibs (30, 33, 36, 63-65, 72). When compared side by side in our cell culture system, celecoxib is the only NSAID that substantially reduces tumor cell viability at concentrations well <100 μmol/L. This is even more impressive when compared with, for example, rofecoxib, a highly selective COX-2 inhibitor that has much higher bioavailability due to substantially lower binding to serum proteins and therefore is administered at severalfold lower dosages to patients. For instance, a single p.o. dose of 200 mg celecoxib or 50 mg rofecoxib in healthy volunteers resulted in similar total plasma concentrations; however, the maximum unbound plasma concentrations were 1.4 ng/mL for celecoxib and 42 ng/mL for rofecoxib (73). Comparable conditions have been reported for cell culture conditions as well (74). From this, it becomes clear that the unique ability of celecoxib to stimulate apoptosis at rather low concentrations is not due to differential bioavailability because this particular characteristic is, in fact, significantly inferior to rofecoxib and some other NSAIDs as well (73).

Importantly, the two functions of celecoxib (inhibition of COX-2 and induction of apoptosis) seem to reside in different domains of its molecule and can be separated. Exactly this has been accomplished with DMC, where the first function has been deleted, and yet the second function has been maintained (27, 30). Because DMC potently mimics all of the antitumor properties of celecoxib that have been investigated thus far, not only in vitro but also in xenograft mouse tumor models in vivo, it seems that this second function alone, as displayed by DMC, might suffice for at least some antitumor purposes (31).

In the present report, we introduce evidence that DMC is able to potently stimulate the ESR, which is followed by apoptotic tumor cell death. This effect takes place in the absence of any involvement of COX-2, as indicated by the following aspects: (a) DMC itself has been shown to lack COX-2–inhibitory activity (27, 31, 32), and it is extremely unlikely that its known metabolic products are able to acquire and exert such activity (23); (b) the induction of ESR and cell death by DMC (and celecoxib) also takes place in tumor cells that entirely lack COX-2 protein expression, such as the Mia-PaCa-2 (75), Raji (76), or RPMI/8226 (34) cell lines (shown in Fig. 1); and (c) rofecoxib and valdecoxib, which inhibit COX-2 at least as effectively as celecoxib (even under cell culture conditions; ref. 74), are not able to mimic the ESR-stimulatory and apoptosis-inducing effects of celecoxib and DMC (Figs. 4–6). Thus,
taken together, these observations reveal that DMC does not require COX-2 to exert its antitumor effects and, in extension, that the apoptosis-inducing part of celecoxib (which has been described before; refs. 30, 33, 72, 77) is sufficient to achieve this effect as well.

Our study might seem to contrast with earlier reports that most, if not all, NSAIDs seem to be able to increase intracellular calcium levels and cause ESR (39–41, 78). However, as shown in those studies, compared with celecoxib, NSAIDs generally require concentrations that are one or even two orders of magnitude higher (generally well into the millimolar range) to substantially affect calcium levels and ESR. Our data clearly support the uniqueness of celecoxib and its non-coxib analogue DMC among these drugs because none of the other coxibs or NSAIDs tested was able to increase cytosolic calcium levels or stimulate any of the ESR indicators at concentrations well <100 μmol/L.

ESR during treatment with DMC or celecoxib is quite likely triggered by elevated levels of cytosolic calcium, due to leakage from the ER. An earlier study (37) indicated that celecoxib is able to inhibit sarcoplasmic/ER Ca^{2+}-ATPase activity with an IC_{50} of 35 μmol/L. We have measured sarcoplasmic/ER Ca^{2+}-ATPase activity in microsomal
fractions from U251 glioblastoma cells and detected sarcoplasmic/ER Ca\(^{2+}\)-ATPase inhibition by DMC and celecoxib as well, although with slightly different IC\textsubscript{50}s of \(45\) and \(53\) \(\mu\)mol/L, respectively (data not shown). In addition, increased cytosolic calcium levels after drug treatment could also be detected in calcium-free culture medium (data not shown), pointing to the release of this cation from intracellular stores. Thus, the inhibition of sarcoplasmic/ER Ca\(^{2+}\)-ATPase activity by these drugs could reasonably explain their effects on cytosolic calcium levels. Two additional pieces of evidence support our view that ESR induced by these two drugs is triggered by elevated levels of calcium. First, the inclusion of calcium chelators completely abrogated the drug-induced increase in CHOP (Fig. 5C) and GRP78 (data not shown) protein levels. Second, both DMC and celecoxib were able to early completely block cellular protein synthesis (Fig. 3), which is a characteristic of calcium-induced ER stress (44, 57) and which is entirely consistent with a large body of evidence that has linked the depletion of ER-sequestered calcium to the inhibition of translation in a variety of cell types (79). From these observations, we conclude that elevated calcium levels play an important role in the initiation of ESR by DMC and celecoxib.

How do the above mentioned components of ESR relate to previously reported targets of DMC and celecoxib? There are a few other molecular targets that have been found to be uniquely affected by DMC and celecoxib, but not by other coxibs or NSAIDs; examples are 3-phosphoinositide-dependent kinase 1 (20, 23), cyclins (22, 27, 80), and survivin (36). Compared with these targets, it should be noted that the induction of ESR seems to take place earlier. For instance, whereas the efficient down-regulation of cyclin A, cyclin B, or survivin by celecoxib or DMC generally takes 24 to 36 h to become apparent, the induction of CHOP is detectable within 4 h (Fig. 5) and the increase in intracytoplasmic calcium levels is detectable within seconds (Fig. 4). It is therefore tempting to speculate that these early events (i.e., the ESR) might represent the decisive processes that determine the eventual outcome of drug treatment (i.e., cell death). This view is indeed supported by several observations: (a) knockdown of GRP78 (the protective component of ESR) enhanced cell death by DMC and celecoxib (Fig. 7), which is consistent with a recent report that linked GRP78 expression levels in gastric carcinoma cells to their sensitivity to celecoxib (41); (b) knockdown of caspase-4 (which is a proapoptotic component of the ESR) significantly increased cell survival after treatment with DMC or celecoxib (Fig. 7); and (c) among the various drugs tested, only those that caused a cytosolic calcium spike and subsequent ESR (DMC and celecoxib, but not other coxibs or traditional NSAIDs) were cytotoxic (i.e., the ESR closely correlated with subsequent cell death by apoptosis; Figs. 3–5).

In the past, studies investigating the COX-2–independent effects of celecoxib \textit{in vitro} have been received with reservations, due to the relatively high concentrations of

Table 1. Drug concentrations in plasma and tumor tissue

<table>
<thead>
<tr>
<th>Animal no.</th>
<th>Drug and dosage, mg/kg/d</th>
<th>Maximum plasma levels*, (\mu)g/L ((\mu)mol/L)</th>
<th>Tumor tissue levels*, ng/mg (approximately (\mu)mol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No drug treatment</td>
<td>(&lt;0.5^*)</td>
<td>(&lt;0.2^*)</td>
</tr>
<tr>
<td>2</td>
<td>Cxb 30</td>
<td>6,950 (18.2)</td>
<td>17.53 (0.046)</td>
</tr>
<tr>
<td>3</td>
<td>Cxb 90</td>
<td>9,450 (24.8)</td>
<td>44.03 (0.116)</td>
</tr>
<tr>
<td>4</td>
<td>Cxb 150</td>
<td>14,000 (36.8)</td>
<td>61.23 (0.161)</td>
</tr>
<tr>
<td>5</td>
<td>Cxb 180</td>
<td>17,000 (44.6)</td>
<td>94.76 (0.249)</td>
</tr>
<tr>
<td>6</td>
<td>DMC 30</td>
<td>3,035 (7.6)</td>
<td>8.27 (0.021)</td>
</tr>
<tr>
<td>7</td>
<td>DMC 90</td>
<td>3,190 (8.0)</td>
<td>14.44 (0.037)</td>
</tr>
<tr>
<td>8</td>
<td>DMC 150</td>
<td>9,500 (23.8)</td>
<td>39.04 (0.099)</td>
</tr>
<tr>
<td>9</td>
<td>DMC 180</td>
<td>18,200 (45.5)</td>
<td>110.42 (0.280)</td>
</tr>
</tbody>
</table>

Abbreviation: Cxb, celecoxib.

\(^*\)Average of two measurements.

\(^*\)Detection limit in blood was \(\sim 5\) \(\mu\)g/L.

\(^*\)Detection limit in tumor tissue was \(\sim 5\) ng/mg.
drugs that were required to generate such effects. Whereas drug concentrations between 10 to 80 μmol/L are generally needed to produce antiproliferative and apoptosis-inducing effects in cell culture in vitro, the average celecoxib concentrations (C_{average}) measured in the serum of patients or animals were in the range of 2 to 10 μmol/L (81–83). Thus, this discrepancy has led to the suggestion (19, 84) that in vitro effects of celecoxib (and perhaps DMC) might be an artifact and not reflective of the mechanisms taking place in vivo. It was therefore imperative for us to show whether the induction of ESR could be recapitulated in an in vivo model. As convincingly shown by our results, DMC and celecoxib were able to stimulate the ESR indicator protein CHOP in tumors of a xenograft mouse tumor model (Fig. 8). Even more so, similar to the events in our in vitro system, the number of apoptotic cells in tumors from these drug-treated animals was substantially elevated, and tumor growth was reduced (Fig. 9). We therefore believe that those drug-induced events that we documented under elevated drug concentrations in in vitro do not represent artifacts of the cell culture system but rather are reflective of events that also take place in vivo in drug-treated animals.

The peak concentrations of drug (C_{max}) we measured in the blood of those animals that received the highest daily dosages of DMC or celecoxib were 45 μmol/L (Table 1) and were thus more than double the C_{max} that was reported earlier (23). We do not know the reason for this difference, although we note that Kulp et al. (23) treated their animals for 35 days, whereas we did our measurements after 50 h of treatment. It should be mentioned, however, that lower dosages of drug, in particular of DMC, yielded much lower values for C_{max} (Table 1) but still were able to weakly induce CHOP protein expression in tumor tissues from these animals (data not shown). Thus, the discrepancy between effective in vitro and in vivo concentrations remains, although we present study, as well as earlier reports from us (27, 36) and others (23), provide clear evidence that specific in vitro effects of DMC and celecoxib can also detected in tumor tissue in vivo.

In summary, our results establish the ESR as a crucial component of COX-2–independent cellular responses to DMC and celecoxib. It seems that these two drugs are able to overwhelm the cytoprotective feature of this response and initiate cell death via the potent induction of its proapoptotic modules CHOP and caspase-4. Whereas the celecoxib molecule displays two separate functions (i.e., inhibition of COX-2 and induction of apoptosis), the DMC molecule is a “pure” apoptosis inducer that lacks coxib activity. Our finding that DMC quite potently mimics the ability of celecoxib to stimulate the ESR and subsequent cell death clearly indicates that the ESR-inducing function of these molecules is congruent with their ability to induce apoptosis, not with the ability to inhibit COX-2 (which is further supported by our observation that none of the other COX inhibitors was able to stimulate ESR or cell death in our experimental system). Although it shall remain undisputed that the inhibition of COX-2, as exerted by celecoxib, has clinically relevant antineoplastic applications, we propose that DMC might have antitumor effects in its own right, perhaps under conditions where the carcinogenic process is not dominated by elevated levels of prostaglandins. In this case, the absence of COX-2–inhibitory potential in DMC might turn out to be advantageous, as it is conceivable that the antitumor results might be achieved with less of the coxib-associated side effects.

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References

Induction of ER Stress and Apoptosis by DMC


Molecular Cancer Therapeutics

Calcium-activated endoplasmic reticulum stress as a major component of tumor cell death induced by 2,5-dimethyl-celecoxib, a non-coxib analogue of celecoxib

Peter Pyrko, Adel Kardosh, Yen-Ting Liu, et al.


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