2,5-Dimethyl-Celecoxib Inhibits Cell Cycle Progression and Induces Apoptosis in Human Leukemia Cells

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Received April 6, 2015; accepted August 28, 2015

ABSTRACT

Cyclooxygenase-2 (COX-2) is an essential regulator of cancer promotion and progression. Extensive efforts to target this enzyme have been developed to reduce growth of cancer cells for chemopreventive and therapeutic reasons. In this context, cyclooxygenase-2 inhibitors present interesting antitumor effects. However, inhibition of COX-2 by anti-COX-2 compounds such as celecoxib was recently associated with detrimental cardiovascular side effects limiting their clinical use. As many anticancer effects of celecoxib are COX-2 independent, analogs such as 2,5-dimethyl-celecoxib (DMC), which lacks COX-2-inhibitory activity, represent a promising alternative strategy. In this study, we investigated the effect of this molecule on growth of hematologic cancer cell lines (U937, Jurkat, Hel, Raji, and K562). We found that this molecule is able to reduce the growth and induces apoptosis more efficiently than celecoxib in all the leukemic cell lines tested. Cell death was associated with downregulation of Mcl-1 protein expression. We also found that DMC induces endoplasmic reticulum stress, which is associated with a decreased of GRP78 protein expression and an alteration of cell cycle progression at the G1/S transition in U937 cells. Accordingly, typical downregulation of c-Myc and cyclin D1 and an upregulation of p27 were observed. Interestingly, for shorter time points, an alteration of mitotic progression, associated with the downregulation of survivin protein expression was observed. Altogether, our data provide new evidence about the mode of action of this compound on hematologic malignancies.

Introduction

Hematologic disorders are among the most frequent cancers, and despite the existing therapies, mortality rate remains very high due to development of chemoresistance (Stankovic and Marston, 2008). Therefore, there is an urgent need to discover new therapeutic approaches for the treatment of hematologic malignancies. Cyclooxygenase-2 (COX-2), a key inflammatory regulator (Chandrasekharan and Simmons, 2004; Ristimaki, 2004; Howe, 2007; Sobolewski et al., 2010), is overexpressed in many cancer types and contributes to tumor aggressiveness by favoring cancer cell proliferation and survival (Ristimaki, 2004; Wun et al., 2004; Secchiero et al., 2005; Wang et al., 2005). In this context, specific COX-2 inhibitors, such as the "coxib" family, have been tested as good candidates for cancer therapy (Sobolewski et al., 2010). Most of these molecules are strong inducers of apoptosis or slow down cell cycle in many cancer models, including hematologic malignancies (Jendrossek et al., 2003; Arunasree et al., 2008; Liu et al., 2008; Lau et al., 2009; Mutter et al., 2009; Park et al., 2010). Moreover, COX-2 inhibitors are able to sensitize tumors to chemotherapy, radiotherapy, or photodynamic therapy (Cao and Prescott, 2002; Sobolewski et al., 2010). Despite these beneficial effects, chronic use of COX-2 specific inhibitors and non-steroidal anti-inflammatory drugs were recently associated with severe side effects, including gastric ulcers or increased risks of stroke or myocardial infarction (Ortiz, 2004; Hudson et al., 2007). Moreover, antitumor properties of these molecules are not necessarily depending on COX-2 inhibition (Schonthal, 2006; Ryan et al., 2008). Altogether, these data suggest

ABBREVIATIONS: ATF4, activating transcription factor 4; COX, cyclooxygenase; CHOP, C/EBP homologous protein; DMC, 2,5-dimethyl-celecoxib; ER, endoplasmic reticulum; FACS, fluorescence-activated cell sorter; GRP78, 78 kDa glucose-regulated protein; MGG, May-Grünewald Giemsa; MMP, mitochondrial membrane potential; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SERCA, sarco/endoplasmic reticulum Ca2+ -ATPase; UPR, unfolded protein response.
DMC Induces Apoptosis and Cell Cycle Alterations in Leukemia

DMC (dimethyl-celecoxib) and its mother compound, celecoxib, induce apoptosis through different mechanisms. Notably, DMC downregulates critical factors involved in proliferation such as Mcl-1 and c-Myc, showing that these molecules induce apoptosis through different mechanisms. Apoptosis is an organized process, where Bcl-2 family members play an important role in mitochondria. The mitochondrial events occurring during apoptosis (i.e., loss of mitochondrial membrane potential, release of cytochrome c) depend on the activation of the proapoptotic proteins Bax and Bak, which can be inhibited through heterodimerization with antiapoptotic proteins such as Bcl-2, Bcl-xL, or Mcl-1. OSU-03012 can decrease Bcl-2 expression (Liu et al., 2013), but the effect of DMC on the expression of these proteins is unknown.

Noncoxib celecoxib analogs affect also tumor growth through alteration of cell cycle progression (Pyrko et al., 2006). Very few studies have been performed to clearly understand this effect, but it appeared that OSU-03012 and DMC led to cyclin A and B downregulation (Kardosh et al., 2005; Zhang et al., 2007), which are important regulators of G2/M transition.

In this study, we investigated the effect of DMC on growth of hematologic cells in comparison with its mother compound, celecoxib. We observed that DMC reduces cancer cell growth more efficiently. Our findings reveal that the growth inhibitory properties of DMC are due to an induction of apoptosis, ER stress, and cell cycle alterations. Our findings revealed the ability of DMC to downregulate critical factors involved in these processes such as Mcl-1 and c-Myc.

Materials and Methods

Cell Culture and Reagents. U937 (human histiocytic lymphoma), Jurkat (acute lymphoid leukemia T cells), K562 (chronic myeloid leukemia), Raji (Burkitt’s lymphoma), and Hel (human megakaryocytic acute myeloid leukemia) cells came from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) (Braunschweig, Germany). Cell lines were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 1% (v/v) antibiotic-antimycotic solution, and 2 mM L-glutamine (all from Bio-Whittaker, Verviers, Belgium) in a humidified atmosphere at 37°C and 5% CO2. Similarly, the 293T cell line (human embryonic kidney) was cultured in Dulbecco’s modified Eagle’s medium (Sigma, Bornem, Belgium) supplemented with 10% (v/v) fetal calf serum (Brunschwig, Basel, Switzerland) and 1% (v/v) of penicillin/streptomycin from Bio-Whittaker. Peripheral blood mononuclear cells from healthy adult human donors were isolated by standard density separation method using Ficoll-Hypaque from GE Healthcare (Rosendaal, The Netherlands) as previously described (Ferrario et al., 2011). Purified peripheral blood mononuclear cells (PBMCs) were washed twice with phosphate-buffered saline (PBS)-1X and resuspended in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum and 1% antibiotic-antimycotic solution at a cell density of 2.0 × 10^6 cells/ml. PBMCs were kept in a humidified atmosphere at 37°C and 5% CO2.

Celecoxib and 2,5 dimethyl celecoxib (Fig. 1) were purchased from Sigma-Aldrich and dissolved at 20 mM in 100% DMSO; necrostatin-1 was from Sigma-Aldrich. Cycloheximide, MG132, thapsigargin, ethylenglycol-bis(beta-aminoethyl-ether) (EGTA), and Z-Val-FMK were purchased from Calbiochem (Belgium).

Cell Proliferation Assay. The amount of viable cells was assessed by counting trypan blue (Lonza, Verviers, Belgium)-excluded cells with a Cedex cell counter (Innovatis AG, Howald, Luxembourg). At time 0, cells were plated in 6-well plates (20,000 cells/ml).

Cell Cycle Analysis. Cells were washed twice in PBS-1X and then fixed and permeabilized with 70% ethanol in PBS-1X and resuspended in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum and 1% antibiotic-antimycotic solution at a cell density of 2.0 × 10^6 cells/ml. PBMCs were kept in a humidified atmosphere at 37°C and 5% CO2.

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Cell Cycle Analysis. Cells were washed twice in PBS-1X and then fixed and permeabilized with 70% ethanol in PBS-1X as previously described (Sobolewska et al., 2011). Then, cell cycle distribution was analyzed by flow cytometry (FACSCalibur, Becton Dickinson Biosciences, San Jose, CA) by DNA staining with propidium iodide (1 µg/ml, Sigma-Aldrich) and RNase A (100 µg/ml; Roche, Luxembourg, Luxembourg) in PBS. Events were recorded (10,000 events/sample) using the CellQuest software (http://www.bdbiosciences.com/features/products/display_product.php?keyID=92); data were analyzed with the FlowJo 8.8.7 software (Tree Star, Inc., Ashland, OR).

May-Grünwald Giemsa Histochemical Analysis. Cells (2 × 10^6) were washed twice with phosphate buffer solution (PBS) and then resuspended in PBS and fixed on a slide using the cytocentrifuge.
The slides were sequentially incubated in a pure modified May-Grünwald eosin-methylene blue solution (Merck, Leuven, Belgium) for 5 minutes in May-Grünwald solution diluted in water (1:5, v/v) and, finally, in Giemsa solution (Merck) diluted in water (1:20, v/v) for 20 minutes. The slides were washed in water and observed with an optical microscope (Leica, DM 2000, Lecuit, Luxembourg).

Immunofluorescence Analysis of Phospho-Histone H3. U937 cells were fixed and permeabilized as previously described (Sobolewski et al., 2011) and immunostained with an antibody against histone H3 phosphorylated on serine 10 (BD Biosciences Pharmingen, Erembodegem, Belgium) (1:50). After a 1-hour incubation at room temperature, cells were washed twice in PBS and incubated with a fluorophore-conjugated antimouse secondary antibody (Alexa Fluor 568, Molecular Probes/Invitrogen, Merelbeke, Belgium) for 1 hour at room temperature. After two washes with PBS, the cells were analyzed by flow cytometry as described above.

Analysis and Quantification of Calcium Content and Calcium Release by the ER. After treatment with DMC for 8 hours, U937 cells were washed twice in Hanks’ balanced salt solution supplemented with 10 mg/ml glucose and 65 mM Ca$^{2+}$ (2 x 10$^{-9}$M). Cells were finally kept at room temperature for 20 minutes to allow de-esterification of the dye. The fluorescence intensity was monitored by flow cytometry at 488 nm.

To observe the release of calcium by the ER, U937 cells were stained with Fluo4-AM as described above and then treated with EGTA (65 mM) to chelate extracellular calcium and then treated with 300 nM thapsigargin as previously described (Cerella et al., 2011a). The fluorescence intensity was monitored by flow cytometry at 488 nm during 250 seconds.

Analysis and Quantification of Apoptosis/Necrosis. The percentage of apoptotic cells was evaluated by counting the number of cells displaying apoptotic features, such as fragmented and condensed nuclei (Juncker et al., 2011). At least 100 cells in three random fields were counted by fluorescence microscopy (Leica-DM IRB microscope) after staining the nuclei with Hoechst 33342 (1 μg/ml Sigma). Apoptosis was also confirmed by annexin V/propidium iodide staining and fluorescence-activated cell sorter (FACS) analysis according to the manufacturer protocol (BD Biosciences). For the quantification of necrosis/secondary necrosis, a propidium iodide staining was performed in addition to the Hoechst-33342 staining, and the number of propidium iodide positive cells was counted similarly to apoptotic cells.

Analysis of Mitochondrial Membrane Potential. The loss of mitochondrial membrane potential was analyzed by MitoTracker Red system (StatSpin, Aartselaar, Belgium). The slides were sequentially incubated in a pure modified May-Grünwald eosin-methylene blue solution (Merck, Leuven, Belgium) for 5 minutes in May-Grünwald solution diluted in water (1:5, v/v) and, finally, in Giemsa solution (Merck) diluted in water (1:20, v/v) for 20 minutes. The slides were washed in water and observed with an optical microscope (Leica, DM 2000, Lecuit, Luxembourg).

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Analysis of Mitochondrial Membrane Potential. The loss of mitochondrial membrane potential was analyzed by MitoTracker Red

Fig. 2. DMC decreases the growth of different hematologic cancer cell models more efficiently than celecoxib. U937 and K562 cells were seeded at 2 x 10$^5$ cells/ml and then treated with 0, 10, 20, and 40 μM of celecoxib or DMC for 48 hours (A). The cell concentration was estimated by trypan blue exclusion assay. The percentage of growth inhibition for U937 cells with celecoxib or DMC (40 μM) after 48 hours of treatment are represented in the table below the graphs. The pictures corresponding to U937 cells treated with DMC (40 μM) for 24 hours are represented in (B). The experiments are representative of three independent experiments (± S.D.). *P < 0.05, **P < 0.001 compared with controls.
staining (Molecular Probes/Invitrogen) as previously described (Cerella et al., 2011b). Briefly, 1 × 10^6 cells were incubated for 20 minutes at 37°C with 50 nM of MitoTracker Red. Fluorescence intensity was measured by flow cytometry. Events were recorded (10,000 events/sample) using the CellQuest software. Data were further analyzed with the FlowJo 8.8.7 software.

Caspase-3/-7 Activity Assay. The activity of caspases-3/-7 was assessed with a luminescent-based assay (Caspase-3/-7 Glo assay, Promega, Leiden, The Netherlands). Briefly, cells were seeded in 24-well plates at a density of 200,000 cells/well. After treatment, cells were harvested according to the manufacturer’s protocol. The activity of caspases was revealed by luminescence using a Centro LB 960 Microplate Luminometer (Berthold, Bad Wildbad, Germany).

Real-Time Polymerase Chain Reaction. Total RNA from U937 cells was extracted with the Nucleospin RNA II kit (Macherey Nagel, Oensingen, Switzerland) according to the manufacturer’s protocol. cDNA was synthesized from 1 μg of total RNA by using Oligotex primers and the Superscript First strand Synthesis System (Invitrogen). The real-time polymerase chain reaction (PCR) reaction was performed with the Platinum® High Fidelity Tag DNA Polymerase and specific primers (Eurogentec, Seraing, Belgium) for c-Myc sense: 5’-TGC TCC ATG AGG AGA CAC C-3’; antisense: 5’-TCG ATT TCT TCC TCA TCT TG-3’; cyclin D1 (sense: 5’-CGT GGC CTC TAA GAT GAA GG-3’; antisense: 5’-CCA TCT GAG CTT GAT CAC CA-3’), p27 (sense: 5’-AAT AAG GAG GAC ACC TGC AA-3’; antisense: 5’-CTG GAG TCT GAA ACAAT AG-3’), cyclin B1 (sense: 5’-ATG CTCT TCC TCT GC-3’; antisense: 5’-AGA AGG CCA AAG GCT CAC-3’), and β-actin (sense: 5’-CTG GAG TCT GAA ACAAT AG-3’; antisense: 5’-AAG GAG TCT GAA ACAAT AG-3’), ATF4 (sense: 5’-AAT GGT TCT CAC GAG CCAACAAG-3’; antisense: 5’-GGC ACTTCCTTCCAAGGTTACA-3’), GRP78 (sense: 5’-CGT GGAGATCATCGCCAAC-3’; antisense: 5’-ACATAGGACGGCGTTGACA-3’), CHOP (sense: 5’-CA CTCTTCCAAGGTTACA-3’; antisense: 5’-AGC TCTTCCAAGGTTACA-3’), and ATF4 (sense: 5’-AAT GGT TCT CAC GAG CCAACAAG-3’; antisense: 5’-GGC ACTTCCTTCCAAGGTTACA-3’), GRP78 (sense: 5’-CGT GGAGATCATCGCCAAC-3’; antisense: 5’-ACATAGGACGGCGTTGACA-3’)). The PCR amplification was performed for 40 cycles with the following settings: 94°C for 2 minutes, 60°C for 1 minute, 68°C for 2 minutes. Results were expressed as a ratio between mRNA of target gene and β-actin.

Western Blot Analysis. Total protein lysates were obtained by resuspending the cells in mammalian protein extraction reagent (M-PER, Thermo-Fisher Scientific, Erembodegem, Belgium) supplemented with protease inhibitors (Complete, Roche). For protein stability analysis, cells were treated with cycloheximide (10 μg/ml) for 0, 15, 30, 60, 90, and 120 minutes before cell lysis. Protein concentrations were calculated using the Bradford method as previously
described (Bradford, 1976). Cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10%) using 20–40 μg of total protein extract. Then proteins were transferred to PVDF membranes and blocked for 1 hour with 5% nonfat milk in PBS-Tween. Membranes were incubated with 0.5–1 μg/ml of the following primary antibodies: overnight for anticaspase-8, -9, Bcl-xL, Mcl-1, survivin, phospho-AKT (Thr308), AKT (Cell Signaling, Leiden, The Netherlands), or anticaspase-3, -7 anti-cyclin B1, anti-cyclin D1, anti-p27, anti-GRP78 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Bcl-2 (Calbiochem), anti-c-Myc (BD Biosciences) and 1 hour for anti-β-actin (Sigma-Aldrich). Membranes were washed three times with PBS-Tween, and then incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 hour at room temperature. The different antibodies were diluted in a PBS-1% Tween solution containing 5% of milk. The protein bands were revealed using the ECL Plus Western Blotting Detection System Kit (GE Healthcare). β-Actin was used as a loading control. A densitometric analysis of the Western blots was performed using ImageJ 1.43u software, with β-actin for normalization.

Zebrafish Toxicity Assay. Zebrafish (Danio rerio) were obtained from the Zebrafish International Resource Center (ZIRC, Eugene, OR). Zebrafish were grown at 28.5°C in a standard fish aquarium with a 14:10 hour light/dark cycle and fed 3 times/day; 14 hours before the toxicity assay, embryos were treated with 0.003% of phenylthiourea to remove pigmentation. Two hours before the assay, the embryo’s shell was eliminated and then treated with up to 48 hours with celecoxib or DMC (0–100 μM) in 24-well plates. Viability and abnormal development were assessed after 24 and 48 hours of treatment under light microscopy (Carl Zeiss Stereo microscope DV4, Seoul, Korea). Pictures were taken by fixing zebrafish embryos onto a glass slide with 3% methyl-cellulose (Sigma-Aldrich, Seoul, Korea).

Statistical Analysis. Statistical analyses were performed using Student’s t test for unpaired data. For the zebrafish toxicity assay, statistical analysis was performed using a one-way analysis of variance together with a Dunnett’s post hoc analysis. P values ≤ 0.05 (*), 0.01 (**), or 0.001 (***) were considered significant.

Results

DMC Reduces Growth of Leukemic and Lymphoblastic Cell Lines. The effect of celecoxib and 2,5 dimethyl celecoxib (0, 10, 20, and 40 μM) on the growth of U937 and K562 cells was assessed by trypan blue exclusion assay for up to 48 hours of treatment (Fig. 2, A and B). Our findings revealed that celecoxib and DMC were able to reduce the
growth of both cell lines, but DMC was more efficient than celecoxib (59.45 ± 12.38% of growth inhibition after 48 hours for celecoxib versus 85.67 ± 2.49% for DMC). To generalize our results, we demonstrated inhibition of cell proliferation by DMC in other leukemic cell models including Hel, Jurkat, and Raji (Supplemental Fig. 1). U937 cells appeared to be the most sensitive cell line, with a cytostatic effect from 20 μM.

**DMC Induces Apoptosis in Hematologic Cell Lines.** Inhibition of cell growth by DMC can be due to an induction of apoptosis. We investigated this possibility by analyzing alteration of nuclear morphology by Hoechst 33342 staining and fluorescence microscopy (Fig. 3, A and B). A significant accumulation of apoptotic cells was observed in Hel, Jurkat, K562, Raji, and U937 cell lines with 40 μM after 24 hours. No significant apoptosis induction was observed with celecoxib at the same concentration (40 μM) in Jurkat, K562, and U937 cells (Fig. 3A). In Hel cells, celecoxib induced apoptosis but less efficiently than DMC. As observed in U937 cells, nuclei of DMC-treated cells display characteristic apoptotic morphologies, with nuclei shrinkage and cleavage (Fig. 3B). We confirmed this apoptotic effect by annexin V/propidium iodide staining and FACS analysis (Fig. 3C). This analysis showed 5.44 ± 0.21% of early apoptosis (annexin V positive/propidium iodide negative cells) in untreated cells versus 20.5 ± 1.36% in DMC-treated cells (40 μM). Moreover, the percentage of cells in late apoptosis (annexin V positive/propidium iodide positive cells) also increased, with 0.87 ± 0.19% in control cells versus 3.38 ± 0.07% in DMC-treated cells. Apoptosis is even stronger after 48 hours of treatment and becomes significant from 20 μM in U937 cell lines, whereas celecoxib induces apoptosis only from 60 μM after 48 hours in U937 cells (Fig. 4, A and B). Apoptosis is a complex process, which can lead to the activation of caspases. We were able to see the cleavage of caspase-3, -7, and -9 and -8 upon DMC treatment (from 20 μM) after 24 hours, suggesting caspase-dependent apoptosis (Fig. 4C) and confirming the nuclear morphology analysis (Fig. 4A). To assess caspase dependency, we further used the pan-caspase inhibitor Z-Vad (Fig. 4D and Supplemental Fig. 2). Pretreatment of U937 cells with Z-Vad partially prevented DMC-induced apoptosis as well as DMC-induced caspase-3/7.
activity. The prevention of apoptosis was 69.16 ± 2.6% after 4 hours of treatment and 42.74 ± 14.71% after 8 hours of treatment with DMC. These data indicate that DMC induced apoptosis in caspase-dependent and -independent manners. Necroptosis represents one of these caspase-independent types of cell death, which could be triggered by DMC. Therefore, we also assessed the possibility that DMC could induce necroptosis in U937 cells by using necrostatin-1 to inhibit necroptosis. However, pretreatment of U937 cells with necrostatin-1 did not affect DMC-induced cell death and DMC-induced caspases activation (Fig. 4, D and E, and Supplemental Fig. 2). These results indicate that necroptosis is not involved in DMC-induced cell death.

**DMC Reduces Expression of Antiapoptotic Proteins.**

Induction of apoptosis can be the consequence of mitochondrial events, such as the loss of mitochondrial membrane potential (Burlacu, 2003). We compared the abilities of the two drugs to induce the loss of mitochondrial membrane potential by using Mitotracker staining (Fig. 5A). We found that DMC increases by 87.6 ± 14.44% the amount of cells with a low mitochondrial membrane potential (MMP), whereas no significant effect was observed with celecoxib at the same concentration. Noteworthy, when looking at the population of cells with high MMP, we observed a slight increase of the fluorescence intensity in DMC and celecoxib-treated cells compared with control. This phenomenon suggests a hyperpolarization of the mitochondria, which can occur before apoptosis induction (Sanchez-Alcazar et al., 2000). To determine whether the loss of MMP occurs before apoptosis commitment, we first performed a kinetic analysis in U937 cells to determine the minimum time required for DMC to induce apoptosis. A significant induction of apoptosis was already observed after 4 hours of treatment with 40 μM (Fig. 5B). Next, we analyzed MMP in a nonapoptogenic conditions i.e., U937 cells treated for 2 hours with 40 μM (Fig. 5C). Our data revealed a loss of MMP in DMC treated cells (5.56 ± 1.17% in control cells versus 33.7 ± 7.49% in DMC-treated cells). Interestingly, the percentage of cells with low MMP corresponds to the percentage of apoptotic cells at later time points (18.66 ± 5.51% of apoptosis with 40 μM after...
These data indicate that the loss of MMP is preceding apoptosis induction.

The downregulation of antiapoptotic Bcl-2 family members (Bcl-2, Bcl-xL, or Mcl-1) plays an important role in the regulation of mitochondrial events occurring during apoptosis (Burlacu, 2003). Accordingly, we investigated the effect of DMC on Bcl-2, Bcl-xL, and Mcl-1 protein expression in U937 cells (Fig. 6A). Our data revealed a modest downregulation of Bcl-2 and a strong downregulation of Mcl-1 proteins with 20 μM of DMC after 8 hours, before apoptosis induction, thus suggesting a causative link between these events. The downregulation of Mcl-1 was even stronger after 24 hours of treatment (Fig. 6B). A downregulation of Mcl-1 was also observed with celecoxib, after 48 hours with 40 μM, but the effect is lower than DMC (Fig. 6C). In addition, a kinetic analysis of Bcl-2, Bcl-xL, and Mcl-1 protein expression revealed that Mcl-1 downregulation is an early event after DMC treatment, occurring already after 15 minutes of treatment, before apoptosis induction (Fig. 6D). Interestingly, this downregulation is not a consequence of a modulation of protein stability (Fig. 7A). Indeed, by using cycloheximide as a protein synthesis inhibitor, we found that the half-life of Mcl-1 protein is not significantly affected by DMC treatment (74.7 ± 12.5 minutes for untreated cells versus 75.3 ± 9.5 minutes for DMC-treated cells). Moreover, the proteasome inhibitor MG132 was unable to revert DMC-induced Mcl-1 downregulation as well as DMC-induced apoptosis, thus suggesting a proteasome-independent effect (Fig. 7B). In addition, no alterations of Mcl-1 mRNA expression were observed after 2 or 24 hours of treatment (Fig. 7C). Our results show that DMC-induced
apoptosis is associated with the downregulation of antiapoptotic Bcl-2 family members and suggest that DMC downregulates Mcl-1 expression through a translation inhibition.

**DMC Induces Endoplasmic Reticulum Stress in U937 Cells.** The antitumor properties of DMC or OSU-03012 have been associated with their abilities to induce ER stress (Pyrko et al., 2007), which in turn inhibit protein translation (Teske et al., 2011). Such effect could explain the downregulation of Mcl-1 observed with DMC treatment. We found that DMC was able to increase the mRNA expression of several well-known ER stress markers, including ATF4 (activating transcription factor 4), CHOP (C/EBP homologous protein), and GRP78 (78 kDa glucose-regulated protein) (Lee and Ozcan, 2014), after 8 hours of treatment, before apoptosis induction (Fig. 8A).

As a positive control, we used 293T cells treated for 12 hours with thapsigargin and similar modulations were observed. The expression of these markers is tightly regulated at the transcriptional level by PERK and ATF6 signaling (Lee, 2014), which are activated during the unfolded protein response (UPR) (Lee, 2014). Accordingly, our data suggest that DMC induces UPR in U937 cells. Interestingly, GRP78 is a negative regulator of PERK, IRE1, and ATF6 (Lee, 2014), thus showing that there is a negative feedback loop controlling UPR. We analyzed the expression of GRP78 at the protein level (Fig. 8A, right panel), and, interestingly, we found a dose-dependent downregulation of GRP78 after 8 hours of treatment with DMC. Interestingly, it is know that there is a tight connection between GRP78 and the PI3K/AKT pathway. Indeed, AKT can be a downstream target of GRP78 and on another hand AKT controls also GRP78 levels (Dai et al., 2010; Lin et al., 2011). We analyzed the phosphorylation status of AKT upon DMC treatment (phosphorylation at Thr308) (Supplemental Fig. 3A). Our data revealed that DMC did not affect the phosphorylation of AKT in U937 cells. Moreover, the pretreatment of U937 cells with the PI3K inhibitor LY294002 did not sensitize the cells to DMC-induced apoptosis (Supplemental Fig. 3B). Altogether, these data suggest that AKT is not involved in DMC-induced ER stress/apoptosis in our cell model. It is known that DMC induces ER stress through an inhibition of SERCA (sarco/endoplasmic reticulum...
Ca\textsuperscript{2+}-ATPase) and thus the release of calcium from the ER (Johnson et al., 2002; Pyrko et al., 2007) to the cytosol (Deniaud et al., 2008). Accordingly, we observed an increase of 120.75 ± 48.85% of the calcium amount in U937 cells treated for 8 hours with DMC (Fig. 8B). Then, to determine the origin of this calcium level, we analyzed the amount of calcium in the ER by treating control or DMC-treated cells (8 hours of treatment with 10 and 20 \textit{mM}) with thapsigargin (SERCA inhibitor) to elicit calcium release by the ER (Fig. 8, C and D). We found that in control cells, thapsigargin elicited efficiently the release of calcium from the ER. However, we found that DMC-treated cells (20 \textit{mM}) did not respond to thapsigargin and no calcium release was observed. Our results indicate that in DMC-treated cells the calcium stores in the ER are empty. These data are a further indication that DMC induces ER stress in U937 cells.

**DMC Induces Cell Cycle Alterations at the G1/S Transition and in Mitosis in U937 Cells.** ER stress induction is frequently associated with cell cycle alterations, especially at the G1/S phase (Han et al., 2013). Moreover, our previous results (Sobolewski et al., 2011) already demonstrated that celecoxib affects G1/S transition in U937 cells. The structural similarities between celecoxib and DMC might be associated with common biologic effects. We analyzed the effect of nonlethal doses of DMC on cell cycle distribution after 24 hours of treatment (Fig. 9A). Our data show that DMC induces an accumulation of U937 cells in G0/G1 phase of cell cycle (45.3 ± 3.76% in DMC-treated cells versus 37.7 ± 2.33% for untreated cells), thus showing that DMC shares some common characteristics with celecoxib (Sobolewski et al., 2011). Next, we analyzed the expression of factors playing an important role in the G1/S transition, especially proteins involved in the early steps of this transition such as c-Myc. In U937-treated cells, a downregulation of c-Myc protein could be observed with 20 \textit{mM} of DMC (Fig. 9B). This effect is associated with the modulation of different c-Myc target genes, especially the downregulation of cyclin D1, cyclin B1, and an upregulation of p27 at the mRNA level (Fig. 9B). We confirmed the modulation of cyclin D1 and cyclin B1 at the protein level but for p27, no significant increase was observed.
Interestingly, the downregulation of cyclin B1 correlates with the decrease in the number of cells in G2/M phase of cell cycle (Fig. 9A). We also found that DMC does not affect c-Myc mRNA level and that at a higher concentration (40 μM) DMC downregulates c-Myc expression at the protein level already within 1 hour of treatment (Fig. 9C). A similar but less important accumulation in G0/G1 phase of cell cycle was also observed in K562 cells treated with 40 μM of DMC after 48 hours (Supplemental Fig. 4A). We also found that c-Myc is downregulated and p27 is upregulated in this cell line (Supplemental Fig. 4B), thus showing that this effect is not restricted only to U937 cells. In the other cell lines, Hel, Jurkat, and Raji cells, only an increase of the sub-G1 population could be observed (Supplemental Fig. 4C), thus confirming the induction of apoptosis observed in Fig. 3, but no specific alteration of cell cycle was found. Our data show for the first time the ability of DMC to inhibit the expression of c-Myc in leukemic cell models.

Recently, it was suggested that DMC could induce an alteration of mitosis in an ER-stress-dependent manner (Stankovic and Marston, 2008). These pieces of evidence prompted us to clarify this point. To this purpose, a detailed cell morphology analysis with May-Grünwald Giemsa (MGG) staining was performed after 12 hour of treatment (Fig. 10A). We used concentrations of DMC below 40 μM to stay in a range of concentration unable to affect cell viability. The analysis demonstrated an increase in the percentage of mitotic cells (18.67 ± 3.06%) with mainly prophase-like features. This effect has also been observed earlier (4 hours) before induction of apoptosis, thus suggesting a link between the two events (Fig. 10B). After 8 hours of treatment, the increased number of cells in prophase was not further increased compared with 4 hours of treatment, thus suggesting that DMC induces a delay in mitotic progression. In contrary, a decrease of the mitotic index was observed with celecoxib, thus showing another difference between the compounds despite their structural similarities. In K562 cells, no accumulation of mitotic cells has been observed, even after 48 hours of treatment, thus suggesting a correlation between apoptosis and mitotic alteration (Fig. 10C). In U937 cells, we confirmed this accumulation in early mitosis by the analysis of histone H3 phosphorylation (on serine 10).
by FACS and fluorescence microscopy after 12 hours (Fig. 10D). This effect was accompanied by a modest accumulation of the cells in G2/M (Fig. 10E).

The accumulation of mitotic cells can be the consequence of a spindle defect. We verified this aspect with alpha-tubulin/phospho-histone H3 (Ser10) immunostaining (Fig. 10F). The analysis revealed an accumulation of cells with bipolar spindles, characteristic of cells in prophase (Nigg and Stearns, 2011). Thus, data suggest a possible effect on chromosome segregation. Analysis of the expression of different cell cycle regulators involved in mitosis progression (cyclin B1, survivin) revealed a modest accumulation of cyclin B1, in line with the accumulation of phospho-histone H3 observed previously. Interestingly, a downregulation of survivin has been observed with DMC (Fig. 10G). As survivin is a key component for chromosome segregation during mitosis (Mita et al., 2008), its downregulation may contribute to the accumulation of mitotic cells in prophase upon DMC treatment. Moreover, survivin is also an inhibitor of caspase cleavage, and thus its downregulation may also favor the induction of apoptosis.

Altogether, our data also demonstrate that DMC reduces growth of U937 cells by affecting cell cycle differently. First, we observed an immediate effect on mitosis, especially a delay of the prophase/metaphase transition followed by an arrest of cells in G0/G1 phase of cell cycle.

**Effect of DMC on Healthy Cells Models.** The growth inhibitory properties of DMC suggest possible beneficial effects for the treatment of hematologic malignancies. However, to assess the toxicity of DMC on healthy analogs of our cancer models, we treated peripheral blood mononuclear cells (PBMCs) with celecoxib or DMC (0–40 μM) for up to 48 hours. Our nuclear morphology analysis shows that celecoxib did not induce significantly apoptosis in this range of concentration. However, DMC significantly induced apoptosis of PBMCs from 40 μM (Fig. 11A). This result was confirmed by caspase-3 cleavage analysis (Fig. 11B). Noteworthy, at 40 μM, DMC appeared to be more toxic for PBMCs than for K562 and Raji cells (Fig. 3A). These data suggest that DMC should be used at concentrations between 10 and 20 μM, where an inhibition of cell proliferation was observed in all our cancer cell models.
whereas no significant induction of apoptosis was observed on healthy cells. We further investigated the toxicity of DMC on zebrafish development. The zebrafish represents a suitable and sensitive model for the assessment of drug toxicity (Raldua and Pina, 2014). Accordingly, zebrafish embryos were treated with DMC or celecoxib (0–100 μM), and viability and morphology were assessed after 24 and 48 hours (Fig. 11, C and D, and Supplemental Fig. 5). Our data indicate that for up to 20 μM of DMC and up to 40 μM of celecoxib, no significant effects on viability or development were observed after 24 and 48 hours of treatment. In contrary, from 40 μM of DMC, a significant decrease of the viability of developing zebrafish was observed after 48 hours (11 zebrafish embryos alive among 40 in the whole experiment). Moreover, at this concentration, typical alterations of the development of the fishes (Raldua and Pina, 2014) were observed, especially yolk sac edema and tail shortening. For concentrations higher than 40 μM (100 μM) of DMC, none of the zebrafish embryos survived. With celecoxib treatment, the viability was impacted only from 100 μM. At this concentration, we observed that 26/40 zebrafish embryos were alive after 24 hours of treatment versus 36/40 in the control condition. After 48 hours, only 16/40 embryos were alive. These data indicate that celecoxib is less toxic than DMC in this model. Altogether these results are consistent with our toxicity assay performed on PBMCs and suggest that DMC possess the most interesting antitumor properties in a range of concentrations between 10 and 20 μM.

Discussion

In our study, we demonstrated that DMC induces apoptosis in different hematologic cancer cells. Comparing DMC and celecoxib, we found that DMC induces apoptosis in a range of concentrations where celecoxib is not yet toxic for the cells. These data show that DMC is more efficient than its mother compound to induce apoptosis. In U937 cells, we demonstrated that DMC was able to induce caspases cleavage and Z-Vad could partially revert DMC-induced apoptosis. These data suggest also that DMC induces caspase-independent cell death mechanism(s). In this study, we excluded the possibility
that necroptosis could be one of these mechanisms. However, other death pathways could be involved, such as apoptosis-inducing factor-induced apoptosis (Cande et al., 2002) or autophagic cell death. Interestingly, OSU-03012, which is structurally and mechanistically similar to DMC can induce autophagic cell death in hepatocellular carcinoma (Gao et al., 2008). In this study, we focused mainly on the caspase-dependent cell death and we found that apoptosis was preceded by an early downregulation of the antiapoptotic protein Mcl-1. The fact that all cell lines are affected by the treatment independently of their level of expression of COX-2 (Sobolewski et al., 2011) suggests a COX-2-independent effect. Moreover, this effect is p53 independent, because U937 cells do not express this protein (Sobolewski et al., 2011). Antiapoptotic proteins of the Bcl-2 family mediate their effects by inhibiting proapoptotic proteins such as Bax and Bak, which activate the loss of mitochondrial potential and the release of cytochrome c (Burlacu, 2003). Our findings revealed the ability of DMC to downregulate Mcl-1 protein expression and to induce the loss of MMP. These findings confirm the results obtained in a study of Schonthal (2007) in adherent tumor cell models. Our study shows that this effect can be observed also in leukemic cell models. Interestingly, we also found a slight mitochondria hyperpolarization in DMC and celecoxib-treated cells. This effect has been observed in other models before apoptosis induction, such as camptothecin-induced apoptosis in Jurkat cells (Sanchez-Alcazar et al., 2000). Mcl-1 represents an important therapeutic target in solid and hematologic malignancies (Akgul, 2009). Therefore, the ability of DMC to downregulate its expression efficiently suggests an interesting therapeutic property.

DMC-induced apoptosis has been associated with an induction of endoplasmic reticulum stress usually associated with an arrest of protein translation, which can lead to the rapid downregulation of short-lived proteins, such as Mcl-1 or c-Myc (Pyrko et al., 2007; Trivigno et al., 2013). Accordingly, we also found that DMC induces E stress in U937 cells, and our data suggest an inhibition of Mcl-1 translation after DMC exposure. These data are in agreement with previous published studies and suggest that Mcl-1 downregulation is an important intermediate in the cytostatic and/or apoptotic effect of DMC. Moreover, Mcl-1 downregulation may represent
the link between DMC-induced ER stress and DMC-induced mitochondrial alteration(s). The ability of DMC as well as celecoxib to induce ER stress has been associated mainly with its ability to inhibit SERCA, thus leading to an important leakage of calcium from the ER (Johnson et al., 2002; Pyrko et al., 2007; Coca et al., 2014). We also found that upon DMC treatment, the amount of calcium in the ER is strongly decreased, thus suggesting a common mechanism in our model. Noteworthy, the calcium signaling behind ER stress may explain the ability of DMC to induce apoptosis in a caspase-independent manner. Indeed, calcium overload can activate other death-related proteases such as apoptosis-inducing factor-induced apoptosis (Norberg et al., 2010), calpains (Orrenius et al., 2015), or can directly affect mitochondrial membrane potential through the uptake of calcium by the mitochondria (Orrenius et al., 2015). In addition to the release of calcium by the ER, we found that DMC down-regulates GRP78 protein expression. Because GRP78 is a critical component of protein folding, its loss is usually responsible for the activation of UPR (Wang et al., 2009; Pi et al., 2014). In other models, it has been shown that the loss of GRP78 protein expression leads to the activation of PERK and ATF6, which in turn activates the transcription of ATF4, GRP78, and CHOP. These data are consistent with our PCR analysis showing an increase of GRP78, ATF4, and CHOP at the mRNA level. The expression of GRP78 is increased in most cancer types compared with their healthy counterparts (Wang et al., 2009; Zhang and Zhang, 2010), thus giving an advantage to cancer cells to resist to ER stress-induced cell death. Several studies have suggested GRP78 as a therapeutic target. In this context, DMC may represent an interesting therapeutic molecule. The mechanism by which DMC decreases GRP78 protein expression was not investigated in our study. GRP78 is regulated by protein degradation through the proteasome but also cathepsins (Shi et al., 2009; Bertrand et al., 2013). In our study, we could not revert Mcl-1 downregulation and DMC-induced apoptosis with the proteasome inhibitor MG132, thus suggesting a proteasome-independent effect.
The ability of DMC and OSU-03012 to inhibit AKT activity (Wang et al., 2008; Fan et al., 2011) may give part of the answer. Indeed, PI3K/AKT pathway can prevent ER stress through an accumulation of GRP78 (Dai et al., 2010), and, on the other hand, AKT is also activated by GRP78 (Lin et al., 2011). Therefore, the inhibition of AKT by DMC or OSU-03012 may account for the induction of ER stress in cancer cells. However, in our study, we did not see any effect on AKT phosphorylation (Thr308), despite the strong downregulation of GRP78 protein expression.

ER stress is associated with cell cycle alteration, especially at the G1/S transition and at the G2/M phases (Stankovic and Marston, 2008; Han et al., 2013). We found in U937 cells that DMC treatment induces an accumulation of the cells in G0/G1 phase of cell cycle, which was associated with a downregulation of c-Myc protein expression. As a consequence, c-Myc target genes were also modulated and we observed the downregulation of cyclin D1 mRNA and an induction of p27 mRNA. Moreover, we found a downregulation of cyclin B1 expression.

We previously published that celecoxib could affect cell cycle progression at the G1/S transition through similar mechanisms and independently of COX-2 (Sobolewski et al., 2011), thus showing that DMC and celecoxib share common properties. Some studies have documented an effect of the celecoxib analog OSU-03012 at G2/M transition through a downregulation of cyclin A and B (Zhang et al., 2007). Similar results have been observed with DMC together with an upregulation of p27 in a Burkitt’s lymphoma model in vitro and in vivo (Kardosh et al., 2005). Moreover, inhibition of CDK1 and CDK2 was observed. These pieces of data prompted us also to investigate mitosis for shorter times of treatment. Our findings revealed that DMC induces an early accumulation of U937 cells in mitosis, particularly in prophase/metaphase phases. This effect was associated with cyclin B1 accumulation and is not enhanced at longer time points (8 hours). Interestingly this effect was not observed anymore after 24 hours, where an accumulation of the cells in G0/G1 was observed. These discrepancies could be explained by a delay in mitotic...
progression but not an arrest, which occurs quickly after DMC treatment. Consequently cells are still able to re-enter in G1 phase of cell cycle but remain in this phase because of an arrest of the G1/S transition. Therefore, after 24 hours the amount of cells in the other phases is strongly decreased. The ability of DMC to induce ER stress may account also for this early effect on mitosis. Indeed, alterations of calcium homeostasis have been associated with abnormal proliferation and mitosis dysfunction (Whitaker, 1997). DMC-induced ER-stress is associated with the leakage of calcium from the ER, thus leading to a substantial increase of cytosolic calcium amount, which could affect mitotic spindle. Interestingly, an accumulation of multiple myeloma cells in G2/M has been observed upon OSU-03012 treatment (Zhang et al., 2007), suggesting common mechanism between the two analogs. However this effect was never described with DMC. Moreover, mitotic defects are frequently associated with an induction of p53 family members, which affect mitosis through different mechanisms, like the activation of checkpoint kinases or the induction of p21 expression, a well-known inhibitor of CDK1-cyclin B complex (Cobrinik, 2005). However, U937 cells do not express p53 or p73 (Sobolewski et al., 2011). This effect on mitosis was not observed with celecoxib, thus showing major differences in their mechanisms despite their similar molecular structure. This difference might explain also why DMC is a stronger antiproliferative agent. In parallel, the fact that there is no significant increase of mitotic index in K562 cell line, suggests a good correlation between apoptosis and the mitotic alteration. An accumulation of cells in prophase may be the consequence of an alteration in prophase/metaphase transition. One mechanism implicated in this transition is the alignment and the correct attachment of microtubules to chromosomes for cytokinesis. This mechanism is driven by the chromosomal passenger complex, a complex of proteins containing Aurora kinase A, Borealin, INCENP (inner centromer protein), and survivin (Carmena et al., 2012). Many studies have discovered that any alterations of chromosomal passenger complex components lead to mitosis defects, including perturbations of chromosome alignment, multipolar spindles, and abnormal cytokinesis (Lamers et al., 2011). In our study, we observed a downregulation of survivin. Thus it is conceivable that this effect might contribute to the alteration of mitotic progression. This protein belongs to the inhibitors of apoptosis family, which inhibit the activation of caspases (Kelly et al., 2011). Thus its downregulation may also contribute to DMC-induced apoptosis.
Downregulation of survivin has already been observed with DMC in other cancer cells models (Pyrko et al., 2006) in a p53-independent manner. In these studies, the consequence on mitosis was not demonstrated. Our study provides this first observation, which is consistent with this previous study, because U937 cells do not express p53 (Sobolewski et al., 2011).

COX-2 inhibitors induce apoptosis or reduce cell cycle progression in many different cancer cell types (Sobolewski et al., 2010). These effects involve various mechanisms, which are associated with the specific inhibition of COX-2 enzyme. However, for some inhibitors, like celecoxib, several off-target mechanisms have been discovered (Ryan et al., 2008). Moreover, the chronic and specific inhibition of COX-2 can be associated with severe side effects (Antman et al., 2005), thus limiting their use for therapeutic purposes. Nonactive COX-2 inhibitors analogs such as DMC seem to be good candidates to separate the antitumor properties of celecoxib and their detrimental effects. In this paper, we demonstrated that DMC possess stronger antitumor properties than celecoxib.

Some studies demonstrated synergistic or additive effect between DMC and chemotherapy (Kardosh et al., 2008) or photodynamic therapy (Ferrario et al., 2011). In U937 cells, we already published that a combination of celecoxib or DMC with etoposide (VP16) does not generate any beneficial effects (Cerella et al., 2011b). However, we did not try yet to combine DMC with other agents, especially those currently used or for the treatment of hematologic malignancies, such as anthracyclines (e.g., cytarabine) (Roboz, 2012). Moreover, other molecules such as targeted therapies like FMS-like tyrosine kinase 3 inhibitors (Sudhindra and Smith, 2014) or JAK2 inhibitors (Geyer and Mesa, 2014) and also histone deacetylase inhibitors (Vesci et al., 2014) and hypomethylating agents (Ishikawa, 2014) (Fenaux, 2005) represent potential therapeutic strategies for the treatment of hematologic malignancies, and thus combining these molecules with DMC may enhance their antitumor properties. The combination of DMC and bortezomib seems to be beneficial, as evidenced by a study on glioblastoma cells (Kardosh et al., 2008). Moreover, some studies have reported that the downregulation of Mcl-1 can sensitize
different kinds of tumor cells to extrinsic apoptosis, induced by tumor necrosis factor–related apoptosis ligand (Kim et al., 2008; Jacquemin et al., 2012). DMC may sensitize cancer cells to these apoptotic stimuli more efficiently than COX-2 inhibitors. Such effect might be useful to improve the prognosis of many cancers (Kuijlen et al., 2010).

In summary (Fig. 12), our study provides new insights about the effect of DMC on leukemic cell models and provides a kinetic of alterations induced by DMC, especially on ER stress, apoptosis, and cell cycle progression. Our data show that DMC is a more potent growth inhibitor compared with celecoxib. DMC also modulates the expression of proteins of major concern in oncology, such as Mcl-1, GRP78, c-Myc, and survivin, thus suggesting that DMC may represent a promising agent for the treatment of hematologic malignancies.

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