Impact of the Cyclooxygenase System on Doxorubicin-Induced Functional Multidrug Resistance 1 Overexpression and Doxorubicin Sensitivity in Acute Myeloid Leukemic HL-60 Cells

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ABSTRACT

Multidrug resistance (MDR), a challenge in treating childhood acute myeloid leukemia (AML), is frequently associated with decreased drug accumulation caused by multidrug transporter MDR1. Doxorubicin, an important anti-AML drug, is a known MDR1 substrate and inducer. Its cytostatic efficacy is thus limited by MDR1 overexpression. A recent study demonstrated cyclooxygenase-2-dependent, prostaglandin E2 (PGE2)-mediated regulation of mdr1b expression in primary rat hepatocyte cultures. Cyclooxygenase-2 expression is increased in several malignancies and considered a negative prognostic factor. Our study focused on cyclooxygenase system’s impact on drug-induced MDR1 overexpression in AML cells. As a prerequisite, coexpression of MDR1 and cyclooxygenase-2 mRNA in HL-60 cells and primary AML blasts was demonstrated by Northern blot. Interestingly, incubation of AML cells with doxorubicin not only induced functionally active MDR1 overexpression but also mediated increased cyclooxygenase-2 mRNA and protein expressions with subsequent PGE2 release (determined by flow cytometry, rhodamine123 efflux assay, reverse transcription-polymerase chain reaction, and enzyme-linked immunosorbent assay). After preincubation and subsequent parallel treatment with the cyclooxygenase-2-preferential inhibitor meloxicam, doxorubicin-induced MDR1 overexpression and function were reduced (maximally at 0.1–0.5 μM meloxicam), whereas cytostatic efficacy of doxorubicin in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assays was significantly increased by up to 78 (HL-60) and 30% (AML blasts) after 72 h of doxorubicin treatment. In HL-60 cells, meloxicam-dependent effect on doxorubicin cytotoxicity was neutralized by PGE2 preincubation. In conclusion, the cyclooxygenase system, especially the cyclooxygenase-2 isoform, might be involved in regulating doxorubicin-induced MDR1 overexpression in AML cells, with PGE2 seeming to be a mediating factor. Cyclooxygenase inhibitors thus bear promise to overcome MDR in AML and improve therapy.

Over the past three decades, cytostatic and supportive therapies have improved outcome in childhood acute myeloid leukemia (AML); nevertheless, 6% of patients fail to respond to treatment and another 30 to 35% relapse after chemotherapy (Creutzig and Reinhardt, 2002).

This is partly due to the development of a drug-induced “multidrug resistance” (MDR) phenotype of AML blasts. MDR is known to be a serious problem in AML treatment and has been identified as a negative prognostic factor in hematological malignancies (Kaspers and Veerman, 2003). MDR is frequently associated with decreased drug accumulation in cancer cells, caused by multidrug transporters such as MDR1-type P-glycoproteins (Fardel et al., 1996). Multidrug resistance-related proteins, lung resistance protein, and breast cancer-related protein are also discussed as contributing to MDR, but human MDR1 protein in particular and its role in MDR development have been investigated exten-
sively. Human MDR1 protein is encoded by the MDR1 gene and belongs to the large ATP-binding cassette protein superfamily. Generally, MDR1 proteins act as membrane-bound ATP-dependent export “pumps” for a wide range of structurally and functionally unrelated hydrophobic xenobiotics, including various drugs and antineoplastic compounds and some endogenous substrates.

MDR1 proteins are physiologically expressed in cells of excretory organs, such as hepatocytes and renal tubule cells, and in tissues with barrier function like the blood-brain barrier (Cordon-Cardo et al., 1990), implicating a critical role in the elimination of potentially toxic substrates and thus in tissue protection. Moreover, CD34+ stem cells, embryonic cells, and T-killer cells display physiological expression of MDR1 proteins (Chaudhary and Roninson, 1991; Chaudhary et al., 1992; Kobayashi et al., 1994). The wide expression of MDR1 proteins in normal cells and tissues combined with their strong evolutionary conservation suggest additional physiological functions such as apoptosis regulation, cell differentiation (Smyth et al., 1998; Johnstone et al., 2000) and participation in immunological processes (Gupta et al., 1992).

MDR1 overexpression and its induction by chemotherapy have been demonstrated in solid tumors and myeloid leukemias (Chaudhary and Roninson, 1993). Several drugs, such as verapamil or cyclosporine A, have been demonstrated in vitro to inhibit MDR1-dependent transport activity and circumvent the MDR phenotype of tumor cells. Clinical studies have confirmed this MDR-reversing effect in vivo, however, the need for high concentrations of the so-called “chemosensitizers” resulted in an unacceptable increase in toxicity and adverse side effects, impairing treatment efficacy (Chauncey et al., 2000). Therefore, new concepts for resistance intervention are needed.

A recent study has now demonstrated down-regulation of intrinsic, time-dependent mdr1b overexpression in primary rat hepatocyte cultures by cyclooxygenase (COX) inhibitors, especially inhibitors of the inducible COX-2 isoform, such as meloxicam and NS-398 (Ziemann et al., 2002). On the other hand, mdr1b mRNA and functional mdr1b protein expressions were induced by prostaglandin E₂ (PGE₂) as a product of the COX-dependent arachidonic acid metabolism. In addition, other studies demonstrated further induction of intrinsic mdr1b overexpression in rat hepatocytes by stimuli also known to induce COX-2 expression in several cell types, such as reactive oxygen species or some cytokines (Ziemann et al., 1999), suggesting a role of the COX system in regulating mdr1-type P-glycoprotein expression. COX-2 and the more constitutively expressed COX-1 isoform both catalyze the rate-limiting step in prostaglandin synthesis (Smith et al., 2000). Unlike COX-1, COX-2 has been characterized as inducible “immediate early response gene”. Recent studies indicated COX-2 overexpression in several malignancies such as colorectal and breast cancers (Denkert et al., 2003) and in tissues with barrier function like the blood-brain barrier (Sheng et al., 2001). COX-2 overexpression in tumor cells seems to be associated with increased angiogenesis, tumor invasion and suppression of host immunity. Recent studies pointed to inhibitory effects of COX-2 inhibitors on tumor development and proliferation. As relevant mechanisms, induction of apoptosis and anti-angiogenesis was postulated (Masferrer et al., 2000).

In the present study, we addressed the question as to whether a regulatory link exists between the COX system, in particular the COX-2 isoform, and drug-induced functional MDR1 overexpression in AML cells. If MDR1 expression could be modulated by COX inhibition in AML blasts, COX inhibitors might serve as a new tool to enhance antitumor activity of therapeutic agents in hematological malignancies, perhaps without inadequately increasing overall toxicity.

Materials and Methods

Cell Lines and Primary AML Blasts. The human AML cell line HL-60 and the neuroblastoma cell line SHEP were kindly provided by Dr. Claudia Lanvers-Kaminsky (Department of Pediatric Hematology/Oncology, University Children’s Hospital, Muenster, Germany), the neuroblastoma cell line GIMEN by Dr. Andreas Klein-Franke (Department of Pediatric Hematology/Oncology, University Children’s Hospital, Innsbruck, Austria). Cell lines were cultured in RPMI 1640 medium, supplemented with 10% fetal calf serum (FCS), in a humidified atmosphere of 5% CO₂ and 95% air. In designated experiments, the medium contained doxorubicin (synonymous Adriamycin), hydroxydaunorubicin, hydrochloride; adriamycin, Pharmacia GmbH, Erlangen, Germany), PGE₂, arachidonic acid, or meloxicam [4-hydroxy-2-methyl-5-(5-methyl-2-thiazoyl)-2H-1,2-benzothiazine-3-carboxamide-1,1-dioxide] (Sigma Chemie, Deisenhofen, Germany), as indicated.

CD34+ stem cells were kindly provided by Dr. U. Cassens (Department of Transfusion Medicine, University Hospital, Muenster, Germany). Peripheral blood and bone marrow samples were obtained from patients of the AML-BFM 98 trial at the time of diagnosis. Primary blasts were separated by Ficoll density gradient centrifugation, washed in RPMI 1640 medium, frozen with 40% RPMI 1640 medium/40% FCS (Invitrogen, Karlsruhe, Germany)/20% dimethyl sulfoxide (Serva, Heidelberg, Germany), and stored in liquid nitrogen until analysis. In culture experiments, AML blasts were cultured for up to 4 d in RPMI 1640 medium, supplemented with 20% FCS, in a humidified atmosphere of 5% CO₂ and 95% air.

RNA Isolation and Northern Blot Analysis. Total cellular RNA was isolated from guanidinium thiocyanate-phenol-chloroform extraction as described previously (Ziemann et al., 1999). For Northern blot analyses, up to 20 µg of total RNA per lane was separated electrophoretically on 1% formaldehyde/agarose gels. RNA was subsequently blotted onto Hybond N nylon membranes (Amersham Biosciences Inc.). The blots were pre-hybridized with 5× standard saline citrate (3 M NaCl and 0.3 M trisodium citrate) as transfer buffer. Blots were hybridized to oligonucleotides specific for the human MDR1 (5′-CCA CGG ACA CTC CTA CGA GTT GAT C-3′) and COX-2 genes (5′-CAG ATT GTG GCA TAC ATC ATC AGA C-3′). The oligonucleotides had been 5′-end labeled with T4-poly nucleotide kinase (Boehringer Ingelheim GmbH, Ingelheim, Germany) using [γ-³²P]ATP (Amersham Biosciences Inc.). The blots were prehybridized for 3 h and hybridized for 16 h at 38°C, as described previously (Ziemann et al., 1999). They were subsequently washed up to a stringency of 1× standard saline citrate/0.1% SDS (w/v) at 38°C. Expression of specific mRNAs was quantified by a BAS 1500 bio-imaging analyzer (Fuji, Tokyo, Japan).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). COX-2 mRNA was additionally detected by RT-PCR. Amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA served as quality control for RNA preparation. Total cellular RNA was isolated from HL-60 and SHEP cells using TRIzol reagent (Invitrogen, Paisley, UK). A total of 2 µg RNA was used for reverse transcription. The components in 20 µl of total reaction volume were 1 µl of oligo-dT₁₅ primer (Invitrogen, Karlsruhe, Germany), RT-Mix (4 µl of 5× first-strand buffer; Invitrogen), 2 µl of 0.1 M dithiothreitol.
(Invitrogen), 1 μM of 10 mM dNTPs (Roche Applied Science, Indianapolis, IN), and 1 μl of Superscript II RNase H-RT (Invitrogen). Samples were incubated for 10 min at 70°C with an oligo-dT 18 primer, and after addition of RT-Mix, a Superscript RT reverse transcription was performed for 60 min at 42°C. The PCR reactions were prepared in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.1 mg/ml bovine serum albumin, 0.05% Triton X-100, 0.2 μM upper and lower primers, 200 μM dNTPs, and 1 U of Taq polymerase. The following primer pairs were used for PCR reactions: 5′-GAC TCT GGA TGG CCC TCG GGG-3′ as the sense primer and 3′-AGG TGG AGG AGT GGG TGT CGC-5′ as the antisense primer for GAPDH. 5′-TTC TCC TCT CTA CGA GAA GC-3′ as the sense primer and 3′-GAC TCT TTT CTC CGC AAC AG-5′ as the antisense primer for COX-2. For GAPDH, conditions for amplification consist in 3 min at 94°C followed by 30 PCR cycles (30 s at 94°C, 45 s at 66°C, and 60 s at 72°C). After inactivation of the reverse transcriptase for 5 min at 94°C, COX-2 samples were subjected to 40 cycles, each consisting in 30 s at 94°C, 30 s at 94°C, and 90 s at 72°C. Both reactions were terminated by a final extension step of 5 min at 72°C after the last cycle and storage at 4°C. PCR products were analyzed by gel electrophoresis using 0.8% agarose gels and visualized by etidium bromide staining. Molecular weight was calculated by a 1-kilobase standard DNA ladder (Invitrogen). The COX-2 primer set yielded a 340-base pair product, and the GAPDH primer set a 325-base pair PCR product.

**Antibody Staining.** Defrosted cells were washed once with serum-free RPMI 1640 medium; 250 μl of the cell suspension was incubated with 15 μl of antibody mixtures at room temperature for 15 min, avoiding light exposure. Cells were washed twice in ice-cold phosphate-buffered saline and run on a FACSscan flow cytometer (BD Biosciences, Franklin Lakes, NJ). Data acquisition and analysis were performed on at least 10,000 viable cells with Cell Quest software (BD Biosciences). Phycocyanin-labeled glycopephorine A served as a negative control for MDR1 protein analysis, which was performed with the monoclonal also phycoerythrin-labeled MDR1-antibody MRK-16 (BD Biosciences). MRK-16 reacts with surface epitopes of the MDR1 protein. Viable cells were identified by syto 16 (Molecular Probes, Leiden, The Netherlands), which detects intact DNA. MDR1 expression was expressed as percentage of MDR1-positive cells of the viable cell population.

**Flow Cytometry Analysis of Cyclooxygenase-2 Expression.** COX-2 protein expression was determined by flow cytometry (Weber et al., 2002) using an FITC-conjugated monoclonal anti-COX-2 antibody (catalog no.160113; Cayman Chemical, Ann Arbor, MI). FITC-conjugated mouse-IgG1 was used as isotypic control. Data acquisition and analysis were performed on at least 10,000 viable cells with the Cell Quest software (BD Biosciences).

**Determination of Rhodamine123 (2-(6-Amino-3-imino-3H-xanthen-9-yl)benzoic acid methyl ester; Rhod) Efflux.** MDR1-dependent transport activity was determined according to Huet et al. (1998). Viable blasts were identified using syto 16. Efflux of the MDR1 substrate Rhod123 was expressed as percentage of initial accumulation in viable blasts. To investigate the impact of meloxicam on MDR1-dependent transport activity, cells were preincubated for 24 h with different meloxicam concentrations. After 24 h, 0.01 or 0.05 μg/ml doxorubicin (DOX) was added, without medium change, and cells were incubated for additional 3 d. Rhod123 efflux was subsequently analyzed by flow cytometry. Data acquisition and analysis were performed on at least 10,000 viable cells with the Cell Quest software (BD Biosciences).

**3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium Bromide (MTT) Assay.** In vitro drug resistance of HL-60 cells and primary AML blasts was assessed using a 3-d cell culture assay, based on MTT reduction by viable cells to a colored formazan product. Cells were seeded in 96-well microculture plates and preincubated for 24 h with or without different concentrations of the COX-2-preferential inhibitor meloxicam. After 24 h, DOX was added, as indicated, without medium change. The colored formazan product was determined photometrically at 562 nm in an ELISA reader after 24 and 48 h.

**Apoptosis Assay.** Early and late stages of apoptosis were flow cytometrically identified using Annexin V-FITC apoptosis detection kit (BD Biosciences) and the monoclonal antibody Apo 2.7 (BD Biosciences), respectively. Apoptosis was expressed as percentage of Annexin V-positive or Apo 2.7-positive cells of the viable cell population. Data acquisition and analysis were performed on at least 10,000 cells with the Cell Quest software (BD Biosciences).

**Enzyme-Linked Immunoassay for Determination of PGE2.** Using a highly sensitive and specific competitive enzyme-linked immunosorbent assay (ELISA) with a monoclonal antibody from clone E2R1 (Schafer et al., 1996), PGE2 was detected in culture supernatants of HL-60 cells and primary AML blasts with or without 0.15 μg/ml DOX or 1 μM arachidonic acid as a positive control. The detection limit for PGE2 was 3 pg/ml of a 96-well microculture plate. Results were calculated from the standard curve by cubic spline interpolation.

**Statistical Analysis.** Statistical analyses were performed using the Winstat program (Microsoft, Redmond, WA) and Statistical Analysis System version 6.12 (SAS; SAS Institute, Cary, NC). All values were expressed as mean ± S.E. or mean ± S.D., respectively. The statistical significance of the results was calculated using Student’s t test for unpaired values. For the comparison of the single drug experiment (apoptosis assay; different levels of meloxicam and 0.1 μg/ml DOX versus untreated control) analysis of variance and Dunnett’s test for multiple comparison versus control was used. If necessary, data were log-transformed. SAS procedure GLM was used to fit linear models to the data of the two factorial experiments (MTT assays). On the basis of the hypothesis to be tested, the following variables were included in the models: dose of DOX, meloxicam (0 versus >0), dose of meloxicam, and effect of meloxicam at the highest dose level of DOX (test for interaction).

**Results.**

**Induction of Functionally Active MDR1 and COX-2 Overexpression by Doxorubicin.** To examine impact of the COX system on drug-induced MDR1 overexpression in AML cells, we used the anthracyclin DOX, a relevant drug in AML therapy, which is known to be an inducer of MDR1 expression (Hu et al., 1995). The AML cell line HL-60 served as a good model for induction experiments due to its low basal MDR1 expression. Untreated HL-60 cells exhibited low but detectable MDR1 mRNA (44% compared to GIMEN cells) and MDR1 protein expression (Fig. 1A). In presence of 0.01 μg/ml DOX, however, relative expression increased on an average of more than 40-fold after 72 h of incubation (Fig. 1A). Concurrently, DOX-treated HL-60 cells (Fig. 1B) and primary blasts of AML patients (Table 1) exhibited strongly enhanced efflux (HL-60, efflux increased between 2- and 3-fold; patient 1, efflux increased 4-fold; patient 2, efflux increased 2-fold) of the MDR1 substrate Rhod123, which was dose-dependently inhibited by verapamil (50 and 100 μM) as specific inhibitor of MDR1-dependent transport activity (data not shown). Enhanced Rhod123 efflux in the presence of DOX was thus due to an up-regulation of functionally active MDR1 expression. Changes in both MDR1 and COX-2 expression after DOX treatment would be suggestive of involvement of the COX-2 system in DOX-dependent MDR1 regulation. COX-2 expression and activity were investigated in HL-60 cells and primary blasts. In untreated HL-60 cells, COX-2 mRNA expression, as determined by RT-PCR, was low, but inducible by incubation of cells for 72 h with 0.02 μg/ml DOX (Fig. 2A).
DOX-mediated induction of COX-2 mRNA expression resulted in enhanced COX-2 protein levels, as determined by flow cytometry, amounting to a 26% increase (Fig. 2B). PGE$_2$ accumulation in culture supernatants was subsequently evaluated to investigate COX activity. In untreated HL-60 cells, PGE$_2$ release was very low, ranging below the linearity limit of the assay (3 pg/ml), but PGE$_2$ release significantly increased ~8-fold after 72 h in the presence of 0.15 µg/ml DOX (Fig. 3). DOX-induced PGE$_2$ release was comparable with PGE$_2$ levels observed in the presence of the COX substrate arachidonic acid (1 µM) as positive control (Fig. 3). In the present study, DOX also mediated 2- to 7-fold induction of PGE$_2$ release in primary AML blasts of three patients (Table 1), which exhibited significantly higher basal PGE$_2$ levels than HL-60 cells (Table 2).

**Modulation of DOX-Induced MDR1 Overexpression by the COX-2-Preferential Inhibitor Meloxicam**

**MDR1 Protein Expression and Rho123 Efflux.** Assuming a regulatory link between DOX-mediated induction of COX-2 expression and activity and induction of MDR1 overexpression in AML cells, COX-2 preferential inhibitors should reduce DOX-induced MDR1 overexpression. HL-60 cells and primary AML blasts of patient 1 (Table 1), treated for 24 h with the COX-2 preferential inhibitor meloxicam (0.01, 0.1, 0.5, and 1 µM) and subsequently exposed to DOX without medium change, exhibited strong reduction (linearly dose-dependent for meloxicam concentrations 0.01 and 0.1 µM) in DOX-induced MDR1 protein overexpression. The effect was in both cases maximal at 0.1 to 0.5 µM meloxicam, with reductions to 4% (Fig. 1A) and 20% (primary blasts; data not shown) of control cells treated with DOX alone. Interestingly, the effect of meloxicam showed biphasic characteristics. Higher meloxicam concentrations (≥1 µM) seem to induce rather than inhibiting functional MDR1 expression. In the absence of DOX, meloxicam exhibited no significant effect on MDR1 protein levels.

As mentioned above, Rho123 efflux increased markedly in HL-60 cells treated with DOX (Fig. 1B). DOX-induced up-regulation of MDR1-dependent transport activity was markedly inhibited by preincubation for 24 h and parallel treatment of cells with meloxicam. Maximal inhibition was observed with 0.5 µM meloxicam, amounting to a 93% reduction (Fig. 1B, representative experiment). Taking into account three independent experiments performed with 0.01 µg/ml DOX, the inhibiting effect was always maximal at 0.5 µM meloxicam with a resulting mean relative Rho123 efflux of 44 ± 19.9% (mean ± S.E.), corresponding to a mean reduction of DOX-induced efflux by 56%, compared with controls treated with DOX alone. Similar modulation of DOX-induced Rho123 efflux was observed in primary AML blasts of patient 1 (Table 1). With 0.01 µg/ml DOX alone, Rho123 efflux was increased about 4-fold over controls, whereas preincubation for 24 h and parallel treatment with 0.1 or 0.5 µM meloxicam was associated with reductions in DOX-induced Rho123 efflux by 38 and 58%, respectively (data not shown).

**In Vitro Cytotoxicity.** Cytostatic efficacy of DOX was determined in MTT assays in the presence or absence of meloxicam. MTT assays with HL-60 cells demonstrated a significant, dose-dependent increase in cytostatic efficacy of DOX (0.1 and 0.15 µg/ml) by preincubation for 24 h and parallel treatment of cells with meloxicam (0.01, 0.1, 0.5, and 1 µM) (Fig. 4A). After 48 h of DOX incubation, there was a significant difference (**p < 0.001) between cells incubated with DOX alone and DOX combined with previous addition of meloxicam. The amount of increase was significantly different for the two DOX doses (**p < 0.01). After 48 h of incubation with 0.1 µg/ml DOX, the meloxicam-mediated additional cytostatic effect significantly exceeded the cytostatic effect in cells treated with DOX alone by 22 to 48%. Cells treated with 0.15 µg/ml DOX even revealed enhancement of DOX-mediated cytotoxicity of 62 to 78% by meloxicam pre-/parallel treatment (Fig. 4A). Results of SAS procedure GLM showed statistical significance concerning comparison + meloxicam/−meloxicam (**p < 0.01) and dose dependence of the meloxicam effect (**p < 0.01). Comparable MTT assays with primary AML blasts of patient 1 yielded similar results (Fig. 4B). Experiments were repeated with blast samples from three other patients (not listed in Table 1) with comparable results (data not shown). Meloxicam alone exhibited no significant effect on cell viability and cell proliferation in any experiment, indicating that the improved cytostatic efficacy of DOX in cells preincubated/incubated with meloxicam was not due to an additional cytotoxic effect of meloxicam.
product PGE2 might be expected to neutralize the observed enhancement of cytostatic efficacy of DOX in normal cells was evaluated. Neither in lymphocytes from a healthy donor treated with 0.025 or 0.1 μg/ml DOX (p = n.s. comparing + meloxicam/−meloxicam) nor in CD34+ stem cells treated with 0.1 or 0.15 μg/ml DOX (p = n.s. comparing + meloxicam/−meloxicam) was there evidence of a synergistic cytostatic effect of DOX and meloxicam (Fig. 4C). The cytostatic effect of DOX and its enhancement by meloxicam seemed to be restricted to malignant cells with high cell division rates.

**PGE2-Dependent Neutralization of Enhancing Effect of Meloxicam on Cytostatic Efficacy of DOX.** If the enhancing effect of meloxicam on cytostatic efficacy of DOX is attributable to MDR1 reduction via COX inhibition, the COX product PGE2 might be expected to neutralize the observed effect. In additional MTT assays, treatment of cells for 24 to 72 h with PGE2 alone (3 μg/ml) revealed no significant effect on HL-60 cell proliferation (data not shown), but PGE2 pretreatment (24 h)/parallel treatment impaired cytostatic efficacy of DOX (0.1 μg/ml). After 24 h in the presence of DOX, proliferation of PGE2-treated cells significantly exceeded (**p = 0.01**; Fig. 5). After 72 h of incubation with PGE2 (3 μg/ml) added every 24 h, Rho123 efflux increased from 2% in untreated controls up to 99% in PGE2-treated cells (Fig. 5).

The cytostatic efficacy of DOX (0.1 μg/ml) in HL-60 cells pretreated/treated with PGE2 (3 μg/ml) and meloxicam (0.01–1 μM) versus cells pretreated/treated with meloxicam alone was subsequently analyzed. After 72 h, as expected, cells treated with DOX alone demonstrated a decrease in cell viability compared with control cells, but this cytostatic effect was significantly increased (**p < 0.01**) by preincubation/incubation with meloxicam. Parallel preincubation/incubation of cells with PGE2 and meloxicam, compared with cells pretreated/treated with meloxicam alone, significantly enhanced cell viability (**p < 0.01**) by 24% (Fig. 6).

**Apoptosis.** Potential of meloxicam to induce apoptosis was investigated to clarify whether improved cytostatic efficacy of DOX in the presence of meloxicam was due to an additional apoptosis-inducing effect of meloxicam. Annexin V staining and staining with the monoclonal antibody Apo 2.7 were used as endpoints of flow cytometrical analyses, detecting cells in early and late stages of apoptosis, respectively. In the present study, HL-60 cells were incubated with meloxicam (0.01–1 μM) for 96 h and subsequently subjected to flow cytometry. In spite of cells incubated with DOX as positive control, which exhibited significant increase in Annexin V-positive cells (**p < 0.001**), meloxicam (0.01–1 μM)-treated cells did not demonstrate increase in both Annexin V- (with p = n.s. not different from untreated controls) (Fig. 7) and Apo 2.7 (data not shown because of extremely low number of positive cells)-positive cells, compared with untreated controls. In addition, propidium iodide nucleic acid stain was used as endpoints of flow cytometrical analyses, detecting cells in early and late stages of apoptosis, respectively.
used as a control to identify necrotic cells, but meloxicam-treated HL-60 cells showed no enhanced necrosis. The observed effect of meloxicam in the presence of DOX thus seems to be synergistic rather than additive.

Discussion

Recent studies indicated that combination of COX-2-specific NSAIDs with cytostatic drugs possess potential to inhibit tumor development (Hida et al., 2002). The present study pointed for the first time to inhibition of MDR1 expression to be one relevant factor in enhancing cytostatic efficacy of DOX by NSAID treatment. DOX-induced MDR1 overexpression was down-regulated by the COX-2-preferential inhibitor meloxicam in both HL-60 cells and primary AML blasts with subsequent improvement of cytostatic efficacy of DOX.

In our study, DOX significantly up-regulated MDR1 expression and function in HL-60 cells, but the extent of DOX-induced MDR1 protein overexpression was by far higher than enhancement of MDR1-dependent transport activity (Fig. 1). Nevertheless, concerning dose dependence there was clear tendency toward correlation. Taking into account, energy dependence of MDR1-mediated transport, minor increase in MDR1 transport activity, compared with MDR1 protein expression, might be due to DOX-induced disturbance of ATP production. In addition, minor increase might reflect competitive interactions between DOX and Rho123 at the substrate binding side of MDR1.

Observe meloxicam-mediated inhibition of DOX-induced MDR1 overexpression could be due to either inhibition of COX-2 expression and function with subsequent decrease in prostaglandin synthesis and/or COX-independent modification of transcription factors and thus direct inhibition of MDR1 gene transcription.

![Fig. 3. DOX-mediated induction of PGE2 release in HL-60 cells. HL-60 cells were incubated for 72 h with or without 0.15 μg/ml DOX or the COX-substrate arachidonic acid 1 μM (positive control). PGE2 concentrations were subsequently determined in culture supernatants by a PGE2-specific, competitive ELISA. Data represent mean values ± S.E. of three independent culture bottles per culture condition, each measured in duplicate. Significantly different from untreated cells, ***p < 0.001; analysis of variance procedure.](image)

![Fig. 4. Cytostatic efficacy of DOX in meloxicam-treated or untreated HL-60 cells and primary blasts of patient 1. Cells were seeded into 96-well microculture plates, and DOX was added after 24 h of preincubation with or without meloxicam (0.01, 0.1, 0.5, and 1.0 μM) without medium change. Cell viability was measured 48 h later. Four wells per culture condition (eight wells per incubation with meloxicam alone) were determined per experiment. Data represent mean values ± S.E. of three independent experiments (HL-60) or of representative experiments out of two (lymphocytes of healthy donors) and four (primary AML blasts), respectively. A, HL-60 cells (***p < 0.001 comparing meloxicam-treated and untreated cells; ***, p < 0.001 concerning dose dependence of meloxicam effect; concerning the meloxicam effect at different DOX doses there is a significant difference with ***, p < 0.01). B, primary AML blasts (***p < 0.01 comparing meloxicam-treated and untreated cells; ***, p < 0.01 concerning dose dependence of meloxicam effect; no significant interaction between the meloxicam effect and DOX doses). C, healthy lymphocytes (there was no significant difference between meloxicam-treated and untreated cells).](image)
of COX-2 expression via a feedback loop mediated by peroxisome proliferator-activated receptor-γ, which was postulated by Dobbie et al. (2002) for colorectal adenomas after meloxicam treatment.

Beside hypothesis of direct inhibition of MDR1 gene expression by meloxicam-dependent modulation of transcription factors, the present study more likely offers strong evidence that inhibition of COX-2 expression and activity is involved in down-regulation of DOX-induced MDR1 overexpression. Like MDR1, COX-2 expression is increased in several malignancies and has been identified as negative prognostic factor in tumor patients (Denkert et al., 2003). Our study demonstrated for the first time functionally active coexpression of COX-2 and MDR1 in AML cells. MDR1 and COX-2 expression were concurrently induced by DOX treatment, with induction of COX-2 expression and PGE2 release by DOX to be a new aspect in cytostatic AML therapy. Induction of COX-2 might be due to reactive oxygen species formed during DOX metabolism (Bauch, 1989) because reactive oxygen species are known to up-regulate COX-2 expression. Parallel with meloxicam-mediated inhibition of DOX-induced increase in COX-2 expression and PGE2 release (preliminary data, not shown), we could demonstrate dose-dependent down-regulation of DOX-induced MDR1 overexpression in HL-60 cells and primary AML blasts by preincubation/incubation with meloxicam. Maximal inhibition of MDR1 overexpression was achieved at meloxicam concentrations of 0.1 to 0.5 μM. Concentrations resembled IC50 value of 0.70 μM for COX-2 in a human whole blood assay (Chan et al., 1999), thus pointing to involvement of COX inhibition in meloxicam-mediated MDR1 down-regulation. Results are in line with previous studies. Using primary rat hepatocyte cultures, Ziemann et al. (2002) demonstrated inhibition of time-dependent, intrinsic mdr1b overexpression by COX-2 inhibitors, including meloxicam, and the COX-2-specific inhibitor N-[2-(cyclohexyloxy)-4-nitro-phenyl]methanesulfonamide (NS398). In addition, studies of Patel et al. (2002) and Ratnasinghe et al. (2001) pointed to COX-2-dependent regulation of mdr1 genes. Patel et al. (2002) demonstrated induc-

MTT assays in presence of DOX, meloxicam, and PGE2 demonstrated that DOX alone was not as cytotoxic in PGE2-preincubated cells as in PGE2-ununtreated cells, suggesting a role of PGE2 and thus COX activity in the regulation of MDR1 expression in AML cells. This was further supported by results of Rho123 efflux assays, indicating significant induction of MDR1 transport activity by PGE2 in HL-60 cells. Ziemann et al. (2002) already demonstrated induction of functionally active mdr1b overexpression in primary rat hepatocytes by PGE2 treatment. In addition, the enhancing effect of meloxicam on DOX cytotoxicity was neutralized by PGE2. MDR1 expression in AML cells might thus be regulated by prostaglandins. PGE2-mediated regulation of MDR1 expression might be transduced through specific prostaglandin E (EP) receptors. The EP2 receptor seems to be the predominant prostaglandin receptor in HL-60 cells (Ishiguro et al., 1998). In addition, in preliminary MTT assays with the EP1/EP2 receptor antagonist 6-isopropoxy-9-oxoanthene-2-carboxylic acid (AH6809), potency of AH6809 to enhance DOX sensitivity of HL-60 cells could be demonstrated (data not shown), thus pointing to the EP2 receptor to participate in DOX-mediated MDR1 induction. The EP2 receptor is coupled to stimulating G proteins and mediates an increase in cAMP levels. Interestingly, CAMP-dependent induction of MDR1 expression has already been demonstrated (Rohlf and Glazer, 1998). Further studies are needed to resolve receptors and signal transduction pathways involved in PGE2-dependent regulation of MDR1 expression in AML cells.

Based on the present data, meloxicam might be a promising tool for reversal of MDR1-dependent MDR in AML cells, but specificity and adverse effects are to be taken into account when combining meloxicam and cytostatic drugs such as DOX. Our MTT assays with normal lymphocytes and CD34+ stem cells demonstrated selectivity of the meloxicam-dependent increase in cytostatic efficacy of DOX. The effect was restricted to malignant AML cells with high division rate. Nevertheless, there might be adverse effects in other target organs than the blood. DOX treatment has been linked to severe cardiac injury. As shown in rat neonatal cardiomyocytes, cell injury is limited by DOX-dependent induction of COX-2 activity and subsequent release of cytoprotective prostacyclin. DOX-induced cardiac injury was thus aggravated by coadministration of a COX-2 inhibitor due to reduced prostacyclin release. Injury was attenuated by prior administration of the prostacyclin analog iloprost (Dowd et al., 2001). Potential aggravation of DOX-dependent cardiac injury must be kept in mind when combining DOX and meloxicam, even though meloxicam concentrations needed for MDR1 downregulation seem to be rather low, if compared with anti-inflammatory doses. A plasma concentration of 0.5 μM meloxicam (concentration which was most effective in our in vitro experiments) would be achieved in vivo by application of ~2 mg compared with 15 mg indicated for rheumatic diseases. In addition, the observed biphasic effect of meloxicam on MDR1 expression necessitates optimal dosing of meloxicam.

Nevertheless, our results indicate that application of COX-2-preferential or -specific inhibitors as MDR1 modulators might bear promise. As shown for other indications, meloxicam can be controlled relatively well, because plasma levels after i.m. injection are directly proportional to applied doses and meloxicam is well tolerated, especially at low dosage. Concerning reversal of MDR phenotype in tumor cells, meloxicam seems to be more selective and thus less toxic than inhibitors affecting MDR1-dependent transport activity. Previous studies demonstrated potential of NSAIDs to restore normal apoptosis, to inhibit angiogenesis and tumor invasiveness (Masferrer et al., 2000), and to attenuate tumor-mediated immune suppression (Plescia et al., 1975). These aspects together with the potential of meloxicam to inhibit drug-induced MDR1 expression might offer remarkable benefit of NSAIDs in cytostatic therapy of childhood AML. Concerning future studies maybe it would be interesting if regulation of other relevant drug transport proteins may also be linked to COX-2 expression or function. The present study was the first to demonstrate inhibition of drug-induced MDR1 overexpression in AML cells by the COX-2 preferential inhibitor meloxicam, pointing to a regulative link between COX-2 activity, PGE2 release, and DOX-induced MDR1 expression in AML blasts. COX-2 inhibition might thus be a new, promising option to prevent and/or reverse MDR1-mediated multidrug resistance in children suffering from AML and thus to improve treatment outcome.

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