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Impact of the cyclooxygenase system on doxorubicin-induced functional MDR1 overexpression and doxorubicin sensitivity in acute myeloid leukemic HL-60 cells

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AML	acute myeloid leukemia
COX-2	cyclooxygenase-2
DOX	doxorubicin
ELISA	Enzyme-linked immuno-sorbent assay
EP	prostaglandin E receptor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
MDR	multidrug resistance
NSAID	nonsteroidal anti-inflammatory drug
PGE_2	prostaglandin E_2
Rho123	rhodamine 123

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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 E_2 (PGE_2)-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, co-expression 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 PGE_2 release (determined by flow cytometry, rhodamine123 efflux assay, RT-PCR, ELISA). After pre-incubation 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 MTT 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 PGE_2 pre-incubation. In conclusion, the cyclooxygenase system, especially the cyclooxygenase-2 isoform, might be involved in regulating doxorubicin-induced MDR1 overexpression in AML cells, with PGE_2 appearing to be a mediating factor. Cyclooxygenase inhibitors thus bear promise to overcome MDR in AML and improve therapy.

Introduction

Over the past three decades, cytostatic and supportive therapy have improved outcome in childhood acute myeloid leukemia (AML); nevertheless, 6% of patients fail to respond to treatment and another 30-35% 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 extensively. Human MDR1 protein is encoded by the *MDR1* gene and belongs to the large ATP-binding cassette (ABC) protein super-family. 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 anti-neoplastic 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 (Johnstone et al., 2000; Smyth et al., 1998) 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

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± 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 downregulation 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 E_2 (PGE_2) 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 also leukemia (Nakanishi et al., 2001). High levels of prostaglandins, possibly reflecting overexpression of the COX-2 enzyme and playing important roles in proliferation and differentiation of various cancer cells, have been detected in different tumor types (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 were postulated (Masferrer et al., 2000).

In the present study, we addressed the question as to whether a regulative 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 anti-tumor 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 (Muenster, Germany), the neuroblastoma cell line GIMEN by Dr. Andreas Klein-Franke (Goettingen, Germany). Cell lines were cultured in RPMI 1640, supplemented with 10% fetal calf serum (FCS), in a humidified atmosphere of 5% CO_2 and 95% air. In designated experiments, the medium contained doxorubicin (synonymous adriamycin hydrochloride and hydroxydaunorubicin hydrochloride; Adriblastin®, Pharmacia GmbH, Erlangen, Germany), PGE_2, arachidonic acid, or meloxicam [4-hydroxy-2-methyl-N-(5-methyl-2-thiazolyl)-2H-1,2-benzothiazine-3-carboxamide-1,1-dioxide] (Sigma, Deisenhofen, Germany), as indicated. CD34+ stem cells were kindly provided by Dr. U. Cassens (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 1640/40% FCS (Gibco, Karlsruhe, Germany)/20% dimethylsulfoxide (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, supplemented with 20% FCS, in a humidified atmosphere of 5% CO_2 and 95% air.

RNA Isolation and Northern Blot Analysis

Total cellular RNA was isolated by guanidinium thiocyanate-phenol-chloroform extraction as decribed previously (Ziemann et al., 1999). For Northern Blot analyses, up to 20 µg of total RNA per lane were separated electrophoretically on 1% formaldehyde/agarose gels. RNA was subsequently blotted onto Hybond N nylon membranes (Amersham Pharmacia Biotech, Braunschweig, Germany) by capillary transfer using 20xSSC (3M NaCl, 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-labelled with T4-polynucleotide kinase (Boehringer) using [γ-^{32}P] ATP (Amersham Pharmacia Biotech). The blots

were pre-hybridized 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 1xSSC/0.1% SDS (w/v) at 38 °C. Expression of specific mRNAs was quantified by a BAS 1500 Bio-Imaging Analyzer (Fujix, Tokyo, Japan).

Reverse Transcription Polymerase Chain Reaction

COX-2 mRNA was additionally detected by reverse transcription polymerase chain reaction (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 (Gibco BRL, 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 oligo-dT_{18} primer (Invitrogen, Karlsruhe, Germany), RT-Mix (4 µl 5 x First-Strand Buffer (Invitrogen)), 2 µl 0.1 M dithiothreitol (Invitrogen), 1 µl 10 mM dNTPs (Roche Applied Science), and 1 µl 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 and 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 $MgCl_2$, 0.1 mg/ml BSA, 0.05% Triton X-100, 0.2 µM of upper and lower primers, 200 µM dNTPs, and 1 U Taq polymerase (Invitrogen). The following primer pairs were used for PCR reactions: 5`-GAC TGT GGA TGG CCC CTC CGG – 3´ as the sense primer and 3´- AGG TGG AGG AGT GGG TGT CGC – 5´ as the anti-sense primer for GAPDH, 5´- TTC TCC TGC CTA CTG GAA GC – 3´ as the sense primer and 3´- GAC TCC TTT CTC CGC AAC AG – 5´ as the anti-sense primer for COX-2. For GAPDH, conditions for amplification consist in 3 min at 94 °C followed by 30 PCR cycles (30 seconds at 94 °C, 45 seconds at 66 °C, 60 seconds 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 seconds at 94 °C, 30 seconds at 53 °C, and 90 seconds 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 ethidium bromide staining. Molecular weight was calculated by a 1-kb standard

DNA ladder (Invitrogen). The COX-2 primer set yielded a 340 bp, the GAPDH primer set a 325 bp PCR product.

Antibody Staining

Defrosted cells were washed once with serum free RPMI 1640; 250 µl of the cell suspension were incubated with 15 µl of antibody mixtures at room temperature (RT) for 15 min, avoiding light exposure. Cells were washed twice in ice-cold phosphate-buffered saline and run on a FACScan flow-cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Data acquisition and analysis were performed on at least 10 000 viable cells with the Cell Quest software (Becton Dickinson). Phycoerythrin-labelled glycophorine A served as a negative control for MDR1 protein analysis, which was performed with the monoclonal also phycoerythrin-labelled MDR1-antibody MRK-16 (Beckton Dickinson). MRK-16 reacts with surface epitopes of the MDR1-protein. Viable cells were identified by syto 16 (Molecular Probes, Leiden, Netherlands) which detects intact DNA. MDR1 expression was expressed as percent 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 a FITC-conjugated monoclonal anti-COX-2 antibody (Cayman; Cat. No.160113). 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 (Becton Dickinson).

Determination of Rhodamine123 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 rhodamine123 (Rho123; 2-(6-Amino-3-imino-3H-xanthen-9-yl)benzoic acid methyl ester) was expressed as percent of initial accumulation in viable blasts. To investigate the impact of meloxicam on MDR1-dependent transport activity, cells were pre-incubated for 24 h with different meloxicam concentrations. After

24 h, 0.01 µg/ml or 0.05 µg/ml doxorubicin (DOX) were added, without medium change, and cells were incubated for additional 3 d. Rho123 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 (Becton Dickinson).

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 3-(4, 5-dimethylthiazol-2, 5-diphenyl) tetrazolium bromide (MTT) reduction by viable cells to a colored formazan product. Cells were seeded in 96-well micro-culture plates and pre-incubated 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 photo-metrically at 562 nm in an ELISA reader after 24 h and 48 h.

Apoptosis Assay

Early and late stages of apoptosis were flow-cytometrically identified using Annexin V-FITC Apoptosis Detection Kit (Becton Dickinson Biosciences) and the monoclonal antibody Apo 2.7 (Beckton Dickinson), respectively. Apoptosis was expressed as percent 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 (Becton Dickinson).

Enzyme-Linked Immunoassay for Determination of PGE_2

Using a highly sensitive and specific competitive enzyme-linked immunoassay (ELISA) with a monoclonal antibody from clone E2R1 (Schafer et al., 1996), PGE_2 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 PGE_2 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, USA) and SAS (Statistical Analysis System Version 6.12, SAS Institute Inc, Cary, NC). All values were expressed as mean ± standard error (SE) or mean ± standard deviation (SD), 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 vs. untreated control) analysis of variance and Dunnett's test for multiple comparison vs 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 vs > 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 doxorubicin (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% as compared to GIMEN cells) and MDR1 protein expression (Figure 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 (Figure 1A). Concurrently, DOX-treated HL-60 cells (Figure 1B) and primary blasts of AML patients (listed in 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 Rho123, which was dose-dependently inhibited by ± verapamil (50 µM and 100 µM) as specific inhibitor of MDR1-dependent transport activity (data not shown). Enhanced Rho123 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 following DOX treatment would be suggestive of involvement of the COX-2 system in DOX-dependent MDR1 regulation. Though 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 (Figure 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 (Figure 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 (Figure 3). DOX-induced PGE_2 release was comparable to PGE_2 levels observed in the presence of the COX substrate arachidonic acid (1 µM) as positive control (Figure 3). In the present study, DOX also mediated 2- to 7-fold induction of PGE_2 release

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in primary AML blasts of 3 patients (listed in 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 (listed in Table 1), treated for 24 h with the COX-2 preferential inhibitor meloxicam (0.01 µM, 0.1 µM, 0.5 µM, and 1 µM) and subsequently exposed to DOX without medium change, exhibited strong reduction (linearly dose-dependent for meloxicam concentrations 0.01 µM and 0.1 µM) in DOX-induced MDR1 protein overexpression. The effect was in both cases maximal at 0.1 - 0.5 µM meloxicam, with reductions to 4% (Figure 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 (Figure 1B). DOX-induced up-regulation of MDR1-dependent transport activity was markedly inhibited by pre-incubation for 24 h and parallel treatment of cells with meloxicam. Maximal inhibition was observed with 0.5 µM meloxicam, amounting to a 93% reduction (Figure 1B, representative experiment). Taking into account 3 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 ± SE), corresponding to a mean reduction of DOX-induced efflux by 56%, compared to controls treated with DOX alone. Similar modulation of DOX-induced Rho123 efflux was observed in primary AML blasts of patient 1 (listed in Table 1). With 0.01 µg/ml DOX alone, Rho123 efflux was increased about 4-fold over controls, whereas pre-incubation 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 pre-incubation for 24 h and parallel treatment of cells with meloxicam (0.01 µM, 0.1 µM, 0.5 µM, 1 µM) (Figure 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 (Figure 4A). Results of SAS procedure GLM showed statistical significance concerning comparison +meloxicam/-meloxicam (**$p < 0.01$) and dose dependency of the meloxicam effect (**$p < 0.01$). Comparable MTT assays with primary AML blasts of patient 1 yielded similar result (Figure 4B). Experiments were repeated with blast samples from 3 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 pre-incubated/incubated with meloxicam was not due to an additional cytotoxic effect of meloxicam.

To investigate tumor cell selectivity, influence of pre-treatment/treatment with meloxicam on 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) there was evidence of a synergistic cytostatic effect of DOX and meloxicam (Figure

4C). The cytostatic effect of DOX and its enhancement by meloxicam seemed to be restricted to malignant cells with high cell division rates.

PGE_2-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 PGE_2 might be expected to neutralize the observed effect. In additional MTT assays, treatment of cells for 24 to 72 h with PGE_2 alone (3 µg/ml) revealed no significant effect on HL-60 cell proliferation (data not shown), but PGE_2 pre-treatment (24 h)/parallel treatment impaired cytostatic efficacy of DOX (0.1 µg/ml). After 24 h in the presence of DOX, proliferation of PGE_2-treated cells significantly exceeded (** $p_{t\text{-test}} < 0.01$) that of cells incubated with DOX alone by 30%, possibly due to PGE_2-mediated induction of MDR1 expression (data not shown). This assumption is supported by the fact that MDR1-dependent transport activity in HL-60 cells could be induced by PGE_2. After 72 h of incubation with PGE_2 (3 µg/ml) added every 24 h, Rho123 efflux increased from ~2% in untreated controls up to ~99% in PGE_2-treated cells (Figure 5).

The cytostatic efficacy of DOX (0.1 µg/ml) in HL-60 cells pre-treated/treated with PGE_2 (3 µg/ml) and meloxicam (0.01 µM) versus cells pre-treated/treated with meloxicam alone was subsequently analyzed. After 72 h as expected, cells treated with DOX alone demonstrated a decrease in cell viability as compared to control cells, but this cytostatic effect was significantly increased (** $p < 0.01$) by pre-incubation/incubation with meloxicam. Parallel pre-incubation/incubation of cells with PGE_2 and meloxicam, as compared to cells pre-treated/treated with meloxicam alone, significantly enhanced cell viability (** $p < 0.01$) by 24% (Figure 6).

Apoptosis

Potential of meloxicam to induce apoptosis was investigated to clarify whether or not 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) (Figure 7) and Apo 2.7 (data not shown because of extremely low number of positive cells) positive cells, as compared to untreated controls. In addition, propidium iodide nucleic acid stain was 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 NSAID 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 downregulated 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 upregulated MDR1 expression and function in HL-60 cells, but extent of DOX-induced MDR1 protein overexpression was by far higher than enhancement of MDR1-dependent transport activity (Figure 1). Nevertheless, concerning dose dependency there was clear tendency towards correlation. Taking into account, energy dependence of MDR1-mediated transport, minor increase in MDR1 transport activity, as compared to 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.

Observed 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.

There exists some evidence that certain NSAID like ibuprofen exert COX-independent activity against inflammatory processes by direct inhibition of transcription factors such as nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1) (Tegeder et al., 2001) with subsequent downregulation of inflammation-related genes such as *COX-2* or *TNF-α*. The *MDR1* gene promoter also contains putative binding sites for AP-1 and NF-κB, which seem to be relevant for *MDR1* gene induction (Bentires-Alj et al., 2003). Interestingly, high meloxicam concentrations (≥ 1 µM) induced rather than inhibited MDR1 expression in this study, being in line with a previous study on COX-dependent regulation of mdr1b expression in primary rat hepatocytes (Ziemann et al., 2002). Biphasic dose response of meloxicam might be explained by dose-dependent, COX-

independent modulation of NF-κB, as inhibition of NF-κB at low and activation of NF-κB at high concentrations has been demonstrated for the COX-2 specific inhibitors celecoxib and rofecoxib (Niederberger et al., 2003). Phenomenon of biphasic effect of meloxicam might also be explained by upregulation of COX-2 expression via a feedback loop mediated by peroxisome proliferator-activated receptor (PPAR) gamma, which was postulated by Dobbie et al. (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 downregulation of DOX-induced MDR1 overexpression. Like MDR1, COX-2 expression is increased in several malignancies and has been identified as negative prognostic factor in tumour patients (Denkert et al., 2003). Our study demonstrated for the first time functionally active co-expression 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 PGE_2 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) as reactive oxygen species are known to upregulate COX-2 expression. Parallel with meloxicam-mediated inhibition of DOX-induced increase in COX-2 expression and PGE_2 release (preliminary data, not shown), we could demonstrate dose-dependent downregulation of DOX-induced MDR1 overexpression in HL-60 cells and primary AML blasts by pre-incubation/incubation with meloxicam. Maximal inhibition of MDR1 overexpression was achieved at meloxicam concentrations of 0.1 to 0.5 µM. Concentrations resembled IC50 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 downregulation. Results are in line with previous studies. Using primary rat hepatocyte cultures, Ziemann et al. (Ziemann et al., 2002) demonstrated inhibition of time-dependent, intrinsic mdr1b overexpression by COX-2 inhibitors including meloxicam and the COX-2-specific inhibitor NS398 (N-[2-(Cyclohexyloxy)-4-nitro-phenyl]methanesulfonamide). In addition, studies of Patel et al. (Patel et al., 2002) and Ratnasinghe et al. (Ratnasinghe et al., 2001) pointed to COX-2 dependent regulation of *mdr1*

genes. Patel et al. demonstrated induction of functionally active mdr1 expression in COX-2-overexpressing rat mesangial cells, transfected with rat COX-2 cDNA. Overexpression could be blocked by NS398 treatment. Ratnasinghe et al. noticed parallel MDR1 and COX-2 expression in breast cancer cells and postulated modulation of MDR1 by prostaglandins via induction of phosphokinase C and subsequent expression of c-jun, a subunit of AP1.

MTT assays in presence of DOX, meloxicam and PGE_2 demonstrated that DOX alone was not as cytotoxic in PGE_2-pre-incubated cells as in PGE_2-untreated cells, suggesting a role of PGE_2 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 PGE_2 in HL-60 cells. Ziemann et al. (2002) already demonstrated induction of functionally active mdr1b overexpression in primary rat hepatocytes by PGE_2 treatment. In addition, the enhancing effect of meloxicam on DOX cytotoxicity was neutralized by PGE_2. MDR1 expression in AML cells might thus be regulated by prostaglandins. PGE_2-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 AH6809 (6-Isopropoxy-9-oxoxanthene-2-carboxylic acid) 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 (Rohlff and Glazer, 1998). Further studies are needed to resolve receptors and signal transduction pathways involved in PGE_2-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 like 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

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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 co-administration of a COX-2 inhibitor due to reduced prostacyclin release. Injury was attenuated by prior administration of the prostacyclin analogue 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 to 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 tumour cells, meloxicam seems to be more selective and thus less toxic than inhibitors affecting MDR1-dependent transport activity. Previous studies demonstrated potential of NSAID to restore normal apoptosis, to inhibit angiogenesis and tumor invasiveness (Masferrer et al., 2000), and to attenuate tumour-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 NSAID 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, PGE_2 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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Figure Legends

Fig. 1. Meloxicam-mediated reduction of DOX-induced MDR1 overexpression in HL-60 cells and of Rho123 efflux in HL-60 cells. HL-60 cells were pre-incubated for 24 h with or without different meloxicam concentrations prior to incubation with 0.01 µg/ml DOX without medium change. After 72 h of DOX treatment, MDR1 protein expression and Rho123 efflux were determined flow-cytometrically. DOX-induced MDR1 overexpression and Rho123 efflux without meloxicam were set to 100%. **(A)** Relative MDR1 protein expression. Expression was measured as percent MDR1-positive blasts of total viable blast population. **(B)** Rho123 efflux assay. Efflux is expressed as percent of initial Rho123 accumulation. Representative experiments out of three.

Fig. 2. Induction of COX-2 mRNA and COX-2 protein expression in HL-60 cells by DOX treatment. **(A)** Representative RT-PCR. Total RNA was extracted from SHEP cells (positive control), untreated HL-60 cells, and HL-60 cells treated with 0.02 µg/ml DOX for 72 h. COX-2 and GAPDH mRNA were reversely transcribed and amplified as described in the methods section. PCR products were run on 0.8% agarose gels and visualized by ethidium bromide staining. **Lane 1,** SHEP cells; **lane 2**, untreated HL-60 cells; **lane 3**, HL-60 cells treated with DOX **(B)** Flow-cytometry analysis. Expression of COX-2 protein was determined by intracellular staining with a monoclonal anti-COX-2 antibody. FITC conjugated mouse IgG1 served as negative control. Representative experiment out of three.

Fig. 3. DOX-mediated induction of PGE_2 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). PGE_2 concentrations were subsequently determined in culture supernatants by a PGE_2 specific competitive ELISA. Data represent mean values ± SE of 3 independent culture bottles per culture condition, each measured in duplicate. Significantly different from untreated cells, ***p< 0.001; Analysis of Variance Procedure.

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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 pre-incubation 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 (8 wells per incubation with meloxicam alone) were determind per experiment. Data represent mean values ± SE of 3 independent experiments (HL-60) or of representative experiments out of 2 (lymphocytes of healthy donors) and 4 (primary AML blasts), respectively. **(A)** HL-60 cells; (***p<0.001 comparing meloxicam-treated and -untreated cells; ***p<0.001 concerning dose dependency 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 dependency 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).

Fig. 5. Induction of Rho123 efflux by PGE_2 in HL-60 cells. HL-60 cells were treated with 3 µg/ml PGE_2, which was added to the culture medium every 24 h. Rho123 efflux of vital PGE_2-treated and vital untreated controls was determined after 24, 48, and 72 h. Representative experiment. Data represent mean values ± SD of a duplicate determination.

Fig. 6. Neutralization of the cytotoxicity-enhancing effect of meloxicam by simultaneous application of PGE_2 in HL-60 cells. Cells were pre-incubated for 24 h simultaneously with PGE_2 (3 µg/ml) and meloxicam (0.01, 0.1, 0.5, 1.0 µM) or with meloxicam alone, respectively. Subsequently DOX (0.1 µg/ml) was added without medium change. After 72 h of DOX incubation, viability was determined by MTT assay with 4 wells per culture condition (12 wells per untreated control). Viability of cells treated in parallel with meloxicam and DOX was significantly lower than that of cells treated with DOX alone, at 0.01 µM meloxicam cell viability of PGE_2 pre-treated cells was significantly higher, **p< 0.01; Analysis of Variance Procedure for treated groups.

Fig. 7. Annexin V apoptosis assay with meloxicam-treated HL-60 cells. Cells were incubated with or without meloxicam (0.01, 0.1, 0.5 and 1 µM) for 96 h. Untreated cells served as negative and HL-60 cells pre-incubated for 24 h without DOX and subsequently treated with 0.1 µg/ml DOX for 72 h served as positive control. Induction of apoptosis was investigated by flow-cytometrical determination of Annexin V positive cells. Data represent mean values ± SE of 3 independent experiments. DOX-treated cells are significantly different from meloxicam-treated cells and untreated controls (***p<0.001); meloxicam-treated cells are not significantly different from untreated controls (p = n.s.); Analysis of Variance Procedure.

Collection ID	Gender	Age (years)	FAB-Subtype	Sample type	Estimated blasts, %	relative MDR1 mRNA expression, %	relative COX-2 mRNA expression, %
1	m	5,5	M5	Pheresis	92	65	138
2	f	0,5	M1	PB	93	47	188
3	m	14,4	M4	BM	87	82	118

m, male.

f, female.

PB, peripheral blood

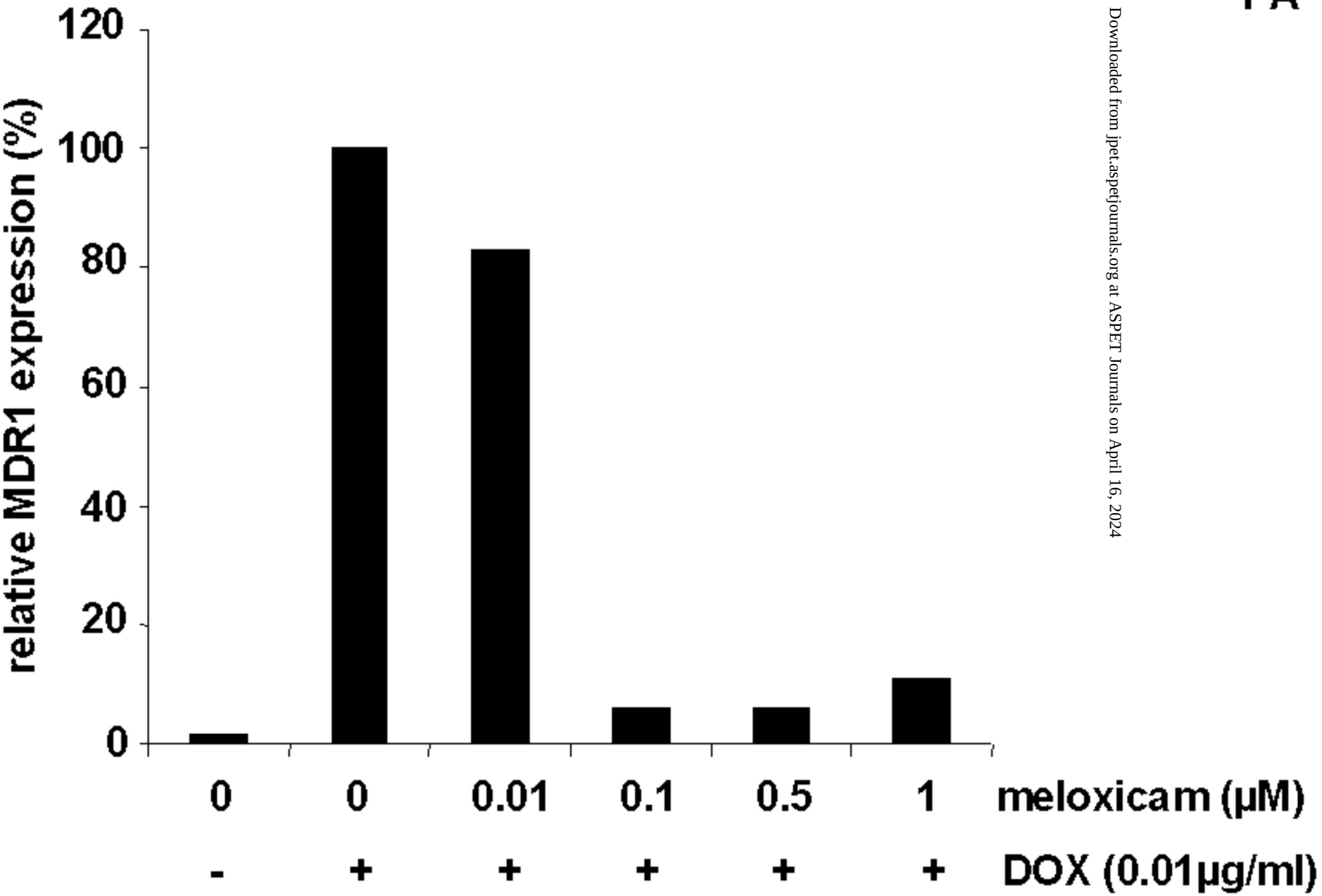
BM, bone marrow.

Table 1 Patients data. MDR1 and COX-2 mRNA expression, determined by Northern Blot and related to GIMEN neuroblastoma cells used as a standard because of marked co-expression of MDR1 and COX-2 mRNA. Expressions in GIMEN cells were set to 100%.

PGE_2 release after 72h (pg/ml)		
Collection ID	**DOX = 0 µg/ml**	**DOX = 0.01 µg/ml**
1	447	853
2	415	886
3	1187	8044

Table 2 DOX-mediated induction of PGE_2 release in 3 patient samples. Cells were incubated for 72 h with or without 0.01 µg/ml DOX. PGE_2 concentrations were subsequently determined in culture supernatants by a PGE_2 specific competitive ELISA. Data represent mean values of 3 independent culture bottles per culture condition, each measured in duplicate. Each DOX-treated sample significantly different from untreated controls, ***p< 0.001; Student t test for unpaired values.

1 A



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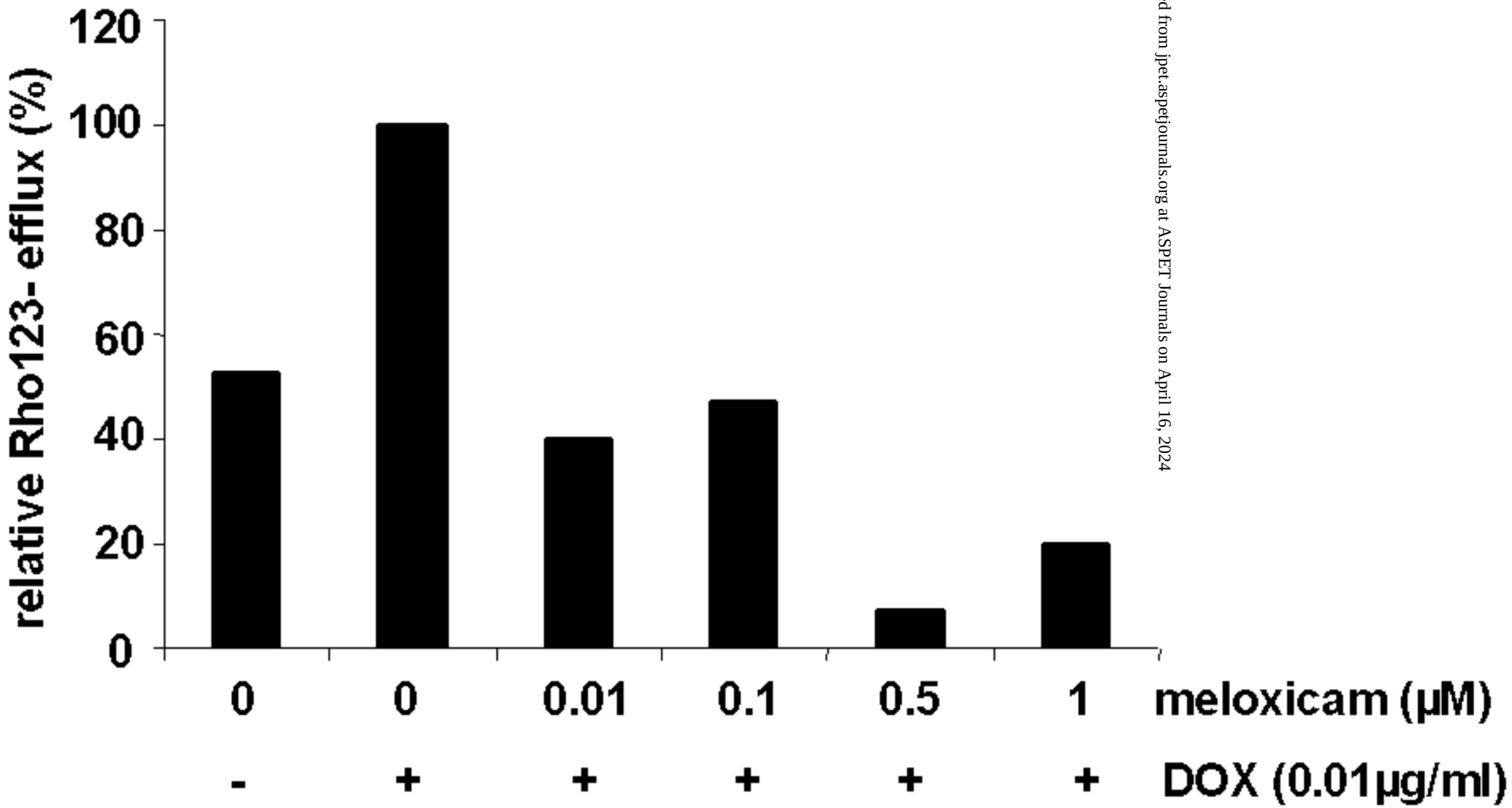

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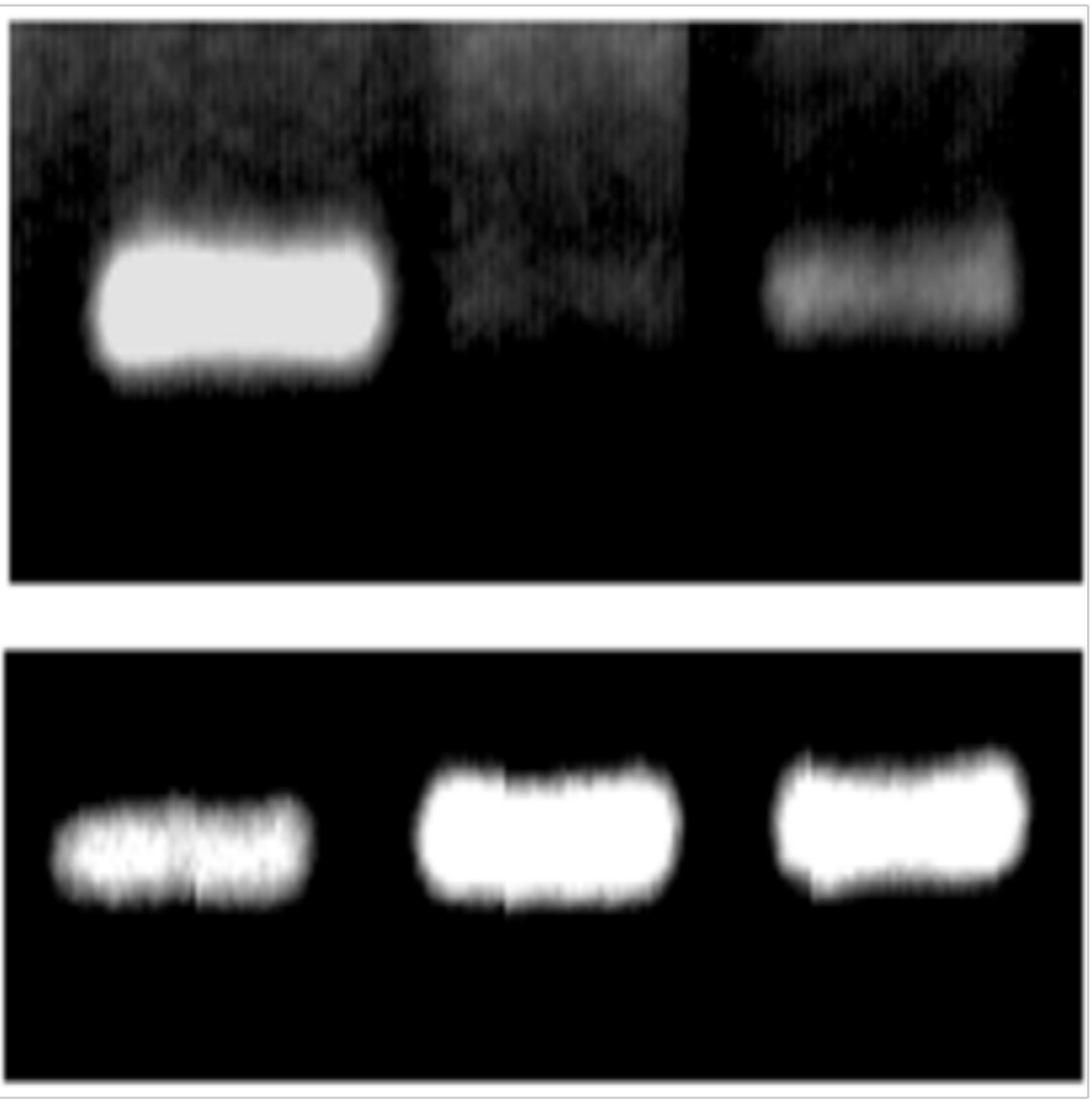

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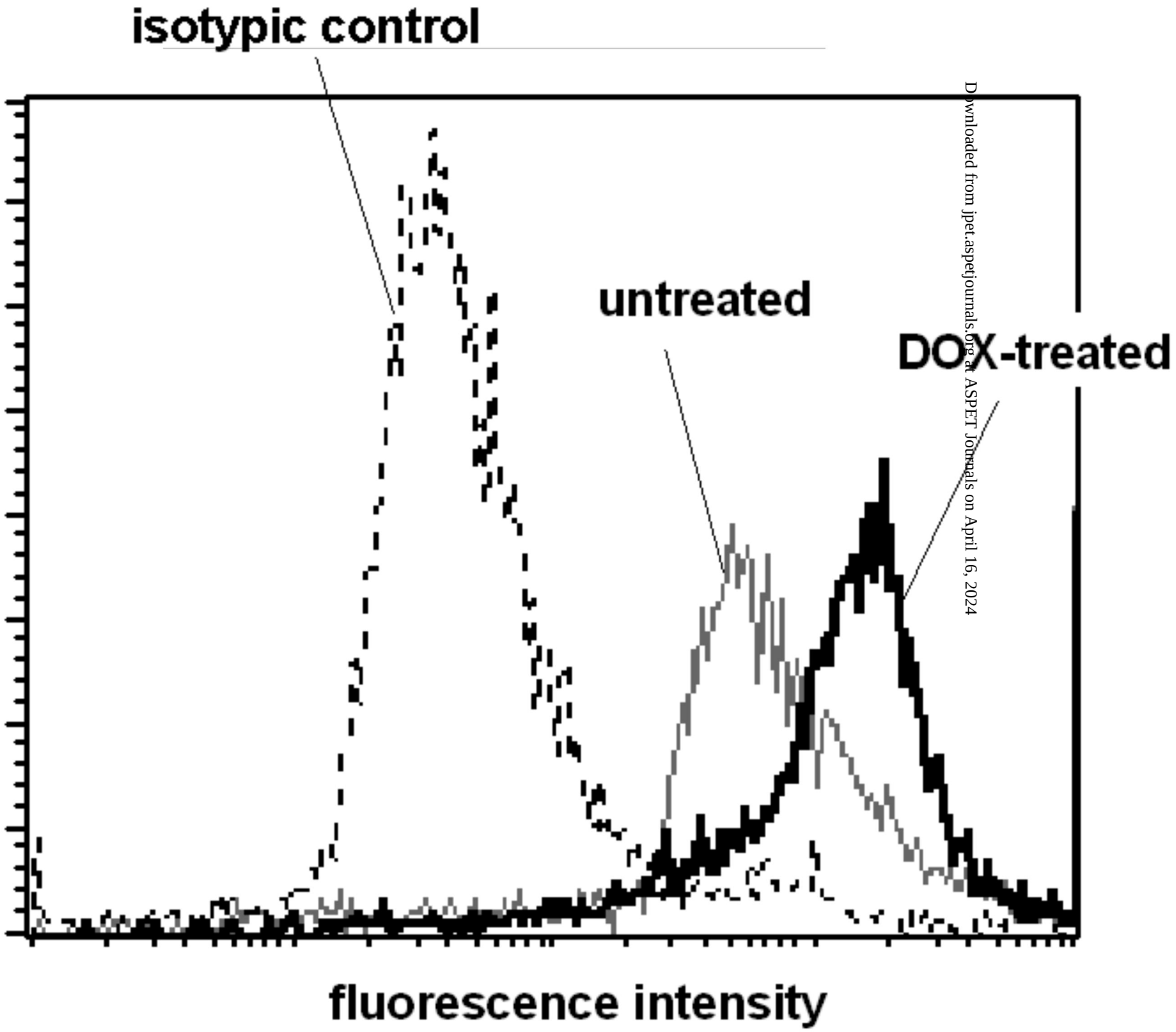

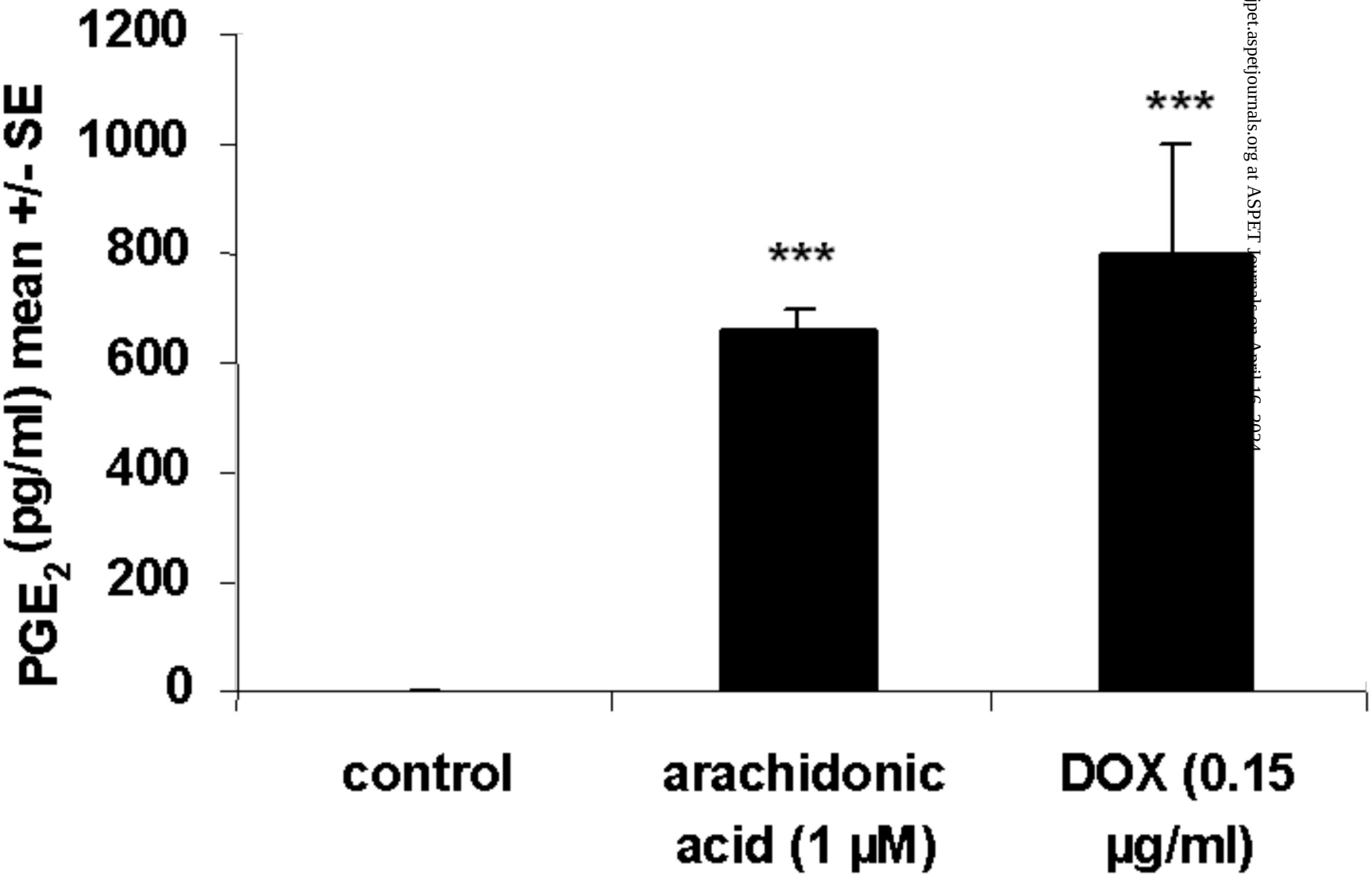

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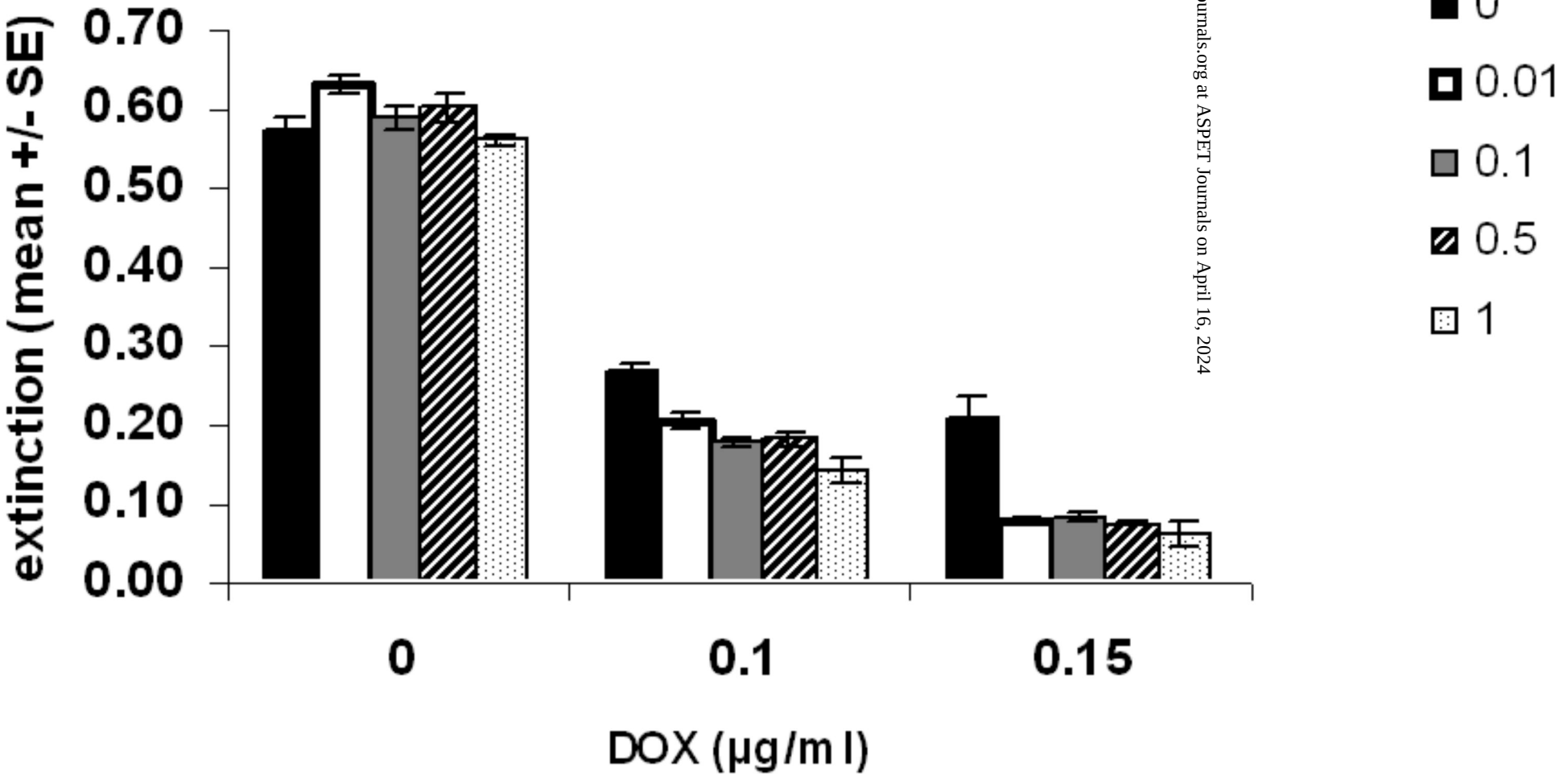

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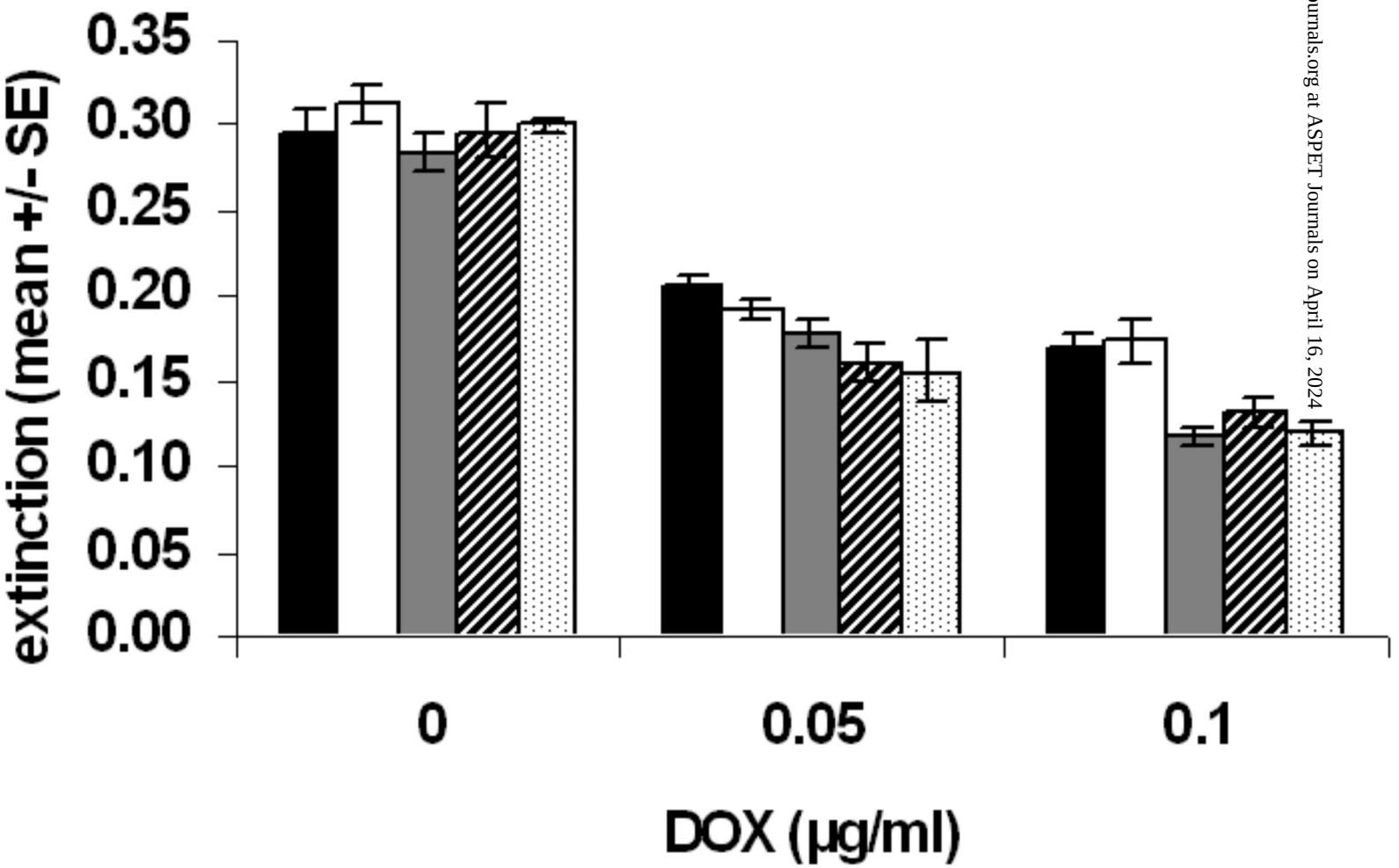

4 C