Nuclear Trapping through Inhibition of Exosomal Export by Indomethacin Increases Cytostatic Efficacy of Doxorubicin and Pixantrone

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Abstract

Purpose: Although R-CHOP–based immunochemotherapy cures significant proportions of patients with aggressive B-cell lymphoma, tumor cell susceptibility to chemotherapy varies, with mostly fatal outcome in cases of resistant disease. We and others have shown before that export of cytostatic drugs contributes to drug resistance. Now we provide a novel approach to overcome exosome-mediated drug resistance in aggressive B-cell lymphomas.

Experimental Design: We used well-established centrifugation protocols to purify exosomes from DLBCL cell lines and detected anthracyclines using FACS and HPLC. We used shRNA knockdown of ABCA3 to determine ABCA3 dependence of chemotherapy susceptibility and monitored ABCA3 expression after indomethacin treatment using qPCR. Finally, we established an in vitro assay using a chorioallantoic membrane (CAM) assay to determine the synergy of anthracycline and indomethacin treatment.

Results: We show increased efficacy of the anthracycline doxorubicin and the anthracenedione pixantrone by suppression of exosomal drug resistance with indomethacin. B-cell lymphoma cells in vitro efficiently extruded doxorubicin and pixantrone, in part compacted in exosomes. Exosomal biogenesis was critically dependent on the expression of the ATP-transporter A3 (ABCA3). Genetic or chemical depletion of ABCA3 augmented intracellular retention of both drugs and shifted the subcellular drug accumulation to prolonged nuclear retention. Indomethacin increased the cytostatic efficacy of both drugs against DLBCL cell lines in vitro and in vivo in a CAM assay.

Conclusions: We propose pretreatment with indomethacin toward enhanced antitumor efficacy of anthracyclines and anthracenediones. Clin Cancer Res; 1–10. © 2015 AACR.

Introduction

The majority of patients with diffuse large B-cell lymphomas (DLBCL) can be cured with current immunochemotherapy regimens (1). The combination of rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisolone (R-CHOP) represents the mainstay backbone of treatment, and anthracyclines such as doxorubicin essentially contribute to its cytostatic efficacy by inhibiting topoimerase II in the tumor cell nucleus (2, 3).

Other anthracyclines and anthracenediones (4) have shown cytostatic efficacy against aggressive lymphoma, with reduced cardiac toxicity compared with doxorubicin, vincristine, and prednisolone (R-CHOP) represents the mainstay backbone of treatment, and anthracyclines such as doxorubicin essentially contribute to its cytostatic efficacy by inhibiting topoimerase II in the tumor cell nucleus (2, 3).

However, a significant proportion of DLBCL patients experience primary progression or relapse of the disease, with a mostly fatal outcome (4). The novel anthracenedione-type topoimeterase II inhibitor pixantrone has shown cytostatic efficacy against aggressive lymphoma, with reduced cardiac toxicity compared with other anthracyclines and anthracenediones (5, 6).

Exosomes are extracellular vesicles of characteristic morphology in electron microscopy and a density of 1.13 to 1.19 g/mL in a sucrose gradient (7). They originate from multivesicular bodies (MVB) in the late endocytic compartment and they are released upon fusion of MVBs with the plasma membrane (8). Exosome secretion occurs in a variety of different cell types, especially tumor cells and hematopoietic cells, including B and T lymphocytes (9). Exosomes carry multiple proteins and functional RNA molecules like mRNA and miRNAs depending on their cellular origin (10). The biologic functions attributed to tumor-derived exosomes include immunosuppression, stimulation of angiogenesis, and modulation of the tumor stroma (11, 12). Exosome secretion may yet also contribute to drug resistance. Following initial cell and nuclear penetration tumor cells rapidly sequester drugs, in particular anthracyclines, into subcellular compartments, and from there vesicular transport leads to export of cytostatic drugs via exosomes (13–15). Thus in leukemia and lymphoma, we found that high levels of ATP-binding cassette (ABC) transporter A3 (ABCA3) are crucial for exosome biogenesis and modulate drug resistance (13, 14, 16, 17).
Translational Relevance

Patients suffering from diffuse large B-cell lymphomas are treated in curative intention using immunochemotherapy regimens. But still tumor cell susceptibility to chemotherapy varies, with a mostly fatal outcome in cases of resistant disease. Export of cytostatic drugs has been well recognized to contribute to tumor cell drug resistance. We here discovered that lymphoma cells extrude anthracyclines compacted in exosomes, that the biogenesis of exosomes can be suppressed by silencing the ABC transporter A3, and that this pathway is targetable by the cyclooxygenase inhibitor indomethacin. ABCA3 inhibition through indomethacin increases tumor cell susceptibility to doxorubicin and the novel anthracenedione pixantrone. Importantly, pixantrone exerts significantly less toxicity in cardiomyocytes, widening the therapeutic window to augment nuclear drugs levels by pretreatment with indomethacin. Interference with exosome biogenesis emerges as a promising approach to overcome drug resistance, and we suggest indomethacin as a clinically feasible prime candidate for this purpose.

Here, we show that depletion of ABCA3 using shRNA or the COX inhibitor indomethacin is effective to reduce exosome secretion and to increase drug susceptibility. We established an in vivo assay on the chorioallantoic membrane (CAM) of chicken eggs as a model for lymphoma treatment, and we found enhanced cytotoxicity of doxorubicin and pixantrone in a cotreatment setting with indomethacin.

Materials and Methods

Cells, antibodies, drugs, and vectors

DLBCL cell lines SU-DHL-4 and Balm3 were obtained from a public depository (DSMZ), OCI-Ly1 and OCI-Ly3 were provided by the Ontario Cancer Institutes (Toronto, ON Canada M5G 2M9); the cell lines were propagated in RPMI-1640 (SU-DHL-4 and Balm3) or IMDM (OCI-Ly1 and OCI-Ly3) supplemented with 25 mmol/L Hepes, GlutaMAX I (Gibco-BRL), 1 x penicillin/ streptomycin (Sigma and Biochrom), and 10% heat-inactivated FCS (Gibco-BRL), authenticity was proved by short tandem repeat (STR) DNA typing.

Cell viability was determined using the CellTiter 96 AQueous One Solution Reagent as previously published (18). EC50 was defined as the concentration of drug causing a 50% inhibition of cell growth compared with untreated control and was determined using the curve-fitting function (sigmoidal dose response, variable slope) of GraphPad Prism version 4.03 for Windows (GraphPad Software, http://www.graphpad.com).

The primary antibodies used in this study for the applications were as indicated: anti–flotillin-2 (clone 29, BD Biosciences), anti-CD9 (clone M-L13, BD Biosciences), anti-CD63 (clone H5C6, BD Biosciences), anti-CD81 (clone JS81, BD Biosciences), anti-ADAM10 (ab84595, abcam). Secondary antibodies against mouse or rabbit Ig were obtained from Santa Cruz Biotechnology. The COX inhibitor indomethacin (Sigma) was dissolved in DMSO and used at the concentrations indicated.

For silencing ABCA3, two validated specific shRNA sequences [RNAiConsortium (TRC), http://www.broadinstitute.org/maimail/trc: TRC clone ID TRCN0000059338, here referred to as shABCA3.forward 5'-CCGG(GCCCA-GCTTATGGAGAATTT)CTCGAG (AAATTTCCCAATGA-GCTTGCGC)TITTTG-3'; reverse 5'-AATTCGAAA(AATTT-CCCAATGACGTTGCCG)CTCGAG(GCACACGTCT- ATGT-GGAAATTT)-3'], were cloned into pLKO.1-eGF (Addgene), and lentiviral particles were produced in the HEK293T producer cell line with the plasmids pCMV-ΔR8.91 (containing gag, pol, and rev genes) and pMD.G (VSV-G–expressing plasmid) following standard protocols (19).

Exosome preparation and quantification

Exosomes were prepared by differential centrifugation according to standard protocols (10). Exosomes were quantified by measuring whole protein (Bio-Rad DC Protein Assay), Western blot analysis detecting flotillin-2 and CD63 in comparison with whole cells or control exosome preparations as previously described (20).

Flow cytometry

Detection of membrane-bound fluorescence and intracellular fluorescence was performed using standard protocols for flow cytometry. FACS analysis of exosomal surface proteins was carried out after exosomes had been coupled to 4-μm aldehyde/sulfate to latex beads (life technologies) as previously published (21). Antibody staining followed standard protocols for antibody staining of cells in suspension. For fluorescence analysis of CAM lymphoma cells, CAM lymphomas were homogenized, washed in PBS, and stained with a FITC-labeled anti human CD20 monoclonal antibody (abcam ab46895). CD20-positive cells were gated and analyzed for doxorubicin or pixantrone fluorescence. The fluorescence emitted from doxorubicin after excitation at 351 nm was detected at 560 nm, whereas that from pixantrone was detected at 670 nm after excitation at 650 nm.

Microscopy

For fluorescence microscopy, fresh CAM lymphomas were frozen in liquid nitrogen and cryodissected. CD20 antibody staining was performed according to standard protocols. All samples were mounted in Fluoromount (DAKO) and analyzed with the TCS-2 AOBS confocal laser scanning microscope (Leica) with a 40 x inversion objective (Leica). The data were exported as TIFF files and arranged using Adobe Photoshop without further modification of the primary image.

Total Internal Reflection Fluorescence microscopy (TIRFM) was performed with a Leica AM TIRF MC set up on a Leica DM6000 B with a HCX PL APO 100 x objective, NA 1.47. A culture dish with a glass bottom (WillCo dish, 0, 17 mm thin) was placed on the sample stage of the microscope. To get the cells into close contact to the culture dish, the probes were weighed with a coverslip. The system was run by Leica LAS AF software.

Fixation of exosomes for electron microscopy was performed according to the Tokuyasu method (22). Ultrathin cryosections were cut from the frozen samples and labeled with primary antibodies detected with protein A conjugated to gold (PAG). Sections were contrasted with uranyl acetate methyl cellulose on ice for 10 minutes, embedded in the same solution, and examined with a Phillips CM120 electron microscope.

LDS-PAGE, Western blot analysis, and qPCR

LDS-PAGE, protein transfer, and antigen detection were performed according to standard protocols. Secondary HRP-
conjugated antibodies against anti-rabbit or anti-mouse were purchased from Santa Cruz Biotechnology. For chemoluminescence detection, standard ECL (Pierce) was used.

For quantification of mRNA, qRT-PCR of hABCA3 and β-actin transcripts was performed in triplicates on a TaqMan cycling machine (ABI Prism 7900HT Sequence Detection System, Applied Biosystems) following previously published protocols (13). Briefly, the SYBR green kit (Qiagen) was used according to the manufacturer’s protocols. Analysis was performed using the analysis of absolute gene expression data using qRT-PCR and the 2(-ΔΔCT) Method as previously described (23). The hABCA3 primer (us 5'-TTTTCATACCATGACCCCATC-3'; ds 5'-CCCTTGCCCT-CAAATTTCCC-3') yielded an amplicon of 139 bp (24), and the β-actin primer (us 5'-CACACTGTCGCCATCTACGA-3'; ds 5'-TCAGGATCTCCTGAGCTTGGTACG-3') yielded an amplicon of 99 bp. A dilution series of eGFP-N1 (1 × 10^−3 to 1 × 10^−1 mol/L; ref. 25) was run in parallel with all reactions to allow comparison.

**CAM Assays**

For CAM Assays, fertilized chicken eggs were incubated at 37°C and 80% relative humidity. On day 3, the eggshells were opened, and Matrigel and incubated at 37°C in 50% cell culture medium were mixed with 50 μL precooled Matrigel and incubated at 37°C for 15 minutes to form a gel, and grafted on top of the CAM as described previously (26). On day 17, the lymphomas were incubated with 1 ml of 10 μmol/L indomethacin or 1 mL of PBS. On day 18, either 50 μg of doxorubicin or pixantrone were injected into a blood vessel crossing the CAM using a 32-gauge needle (see Supplementary Fig. S2B). Exposure to indomethacin or PBS was repeated on day 10, lymphoma cells SU-DHL-4, OCI-Ly1, or OCI-Ly3 were inoculated: 2 × 10^6 lymphoma cells in 50 μL cell culture medium were mixed with 50 μL precooled Matrigel and incubated at 37°C for 15 minutes to form a gel, and grafted on top of the CAM as described previously (26). On day 17, the lymphomas were incubated with 1 ml of 10 μmol/L indomethacin or 1 mL of PBS. On day 18, either 50 μg of doxorubicin or pixantrone were injected into a blood vessel crossing the CAM using a 32-gauge needle (see Supplementary Fig. S2B). Exposure to indomethacin or PBS was repeated on day 18. For lymphoma size analysis, lymphomas were harvested on day 20 and weighted. For fluorescence analysis, lymphomas were harvested on day 19 and processed for further studies.

**Statistical analysis**

The indicated statistical tests were performed using GraphPad Prism version 4.03 for Windows (GraphPad Software, http://www.graphpad.com), and differences with P < 0.05 were considered significant, as marked by asterisks in the respective figures. Error bars represent SDs of samples.

**Results**

**Trapping of anthracyclines in exosomes**

We purified extracellular vesicles of the DLBCL cell lines SU-DHL-4, OCI-Ly1 OCI-Ly3 by differential centrifugation of cell culture supernatants and identified exosomes with typical cup-shaped morphology with a diameter of 50 to 100 nm in electron microscopy (Fig. 1A). After coupling purified exosomes to aldehyde/sulfate latex beads, we detected the exosomal marker proteins ADAM10, CD9, and CD63 on the exosome covered beads (Fig. 1B and C). Applying this technique to exosomes that were purified from the supernatant of DLBCL cell lines after exposure to 1 μmol/L doxorubicin, we discovered an intense red fluorescent doxorubicin signal in the exosomes (Fig. 1D and E and Supplementary Fig. S3B). Confirming these results we detected high concentrations of doxorubicin in purified exosomes compared with the vesicle-free supernatant using high-performance liquid chromatography (HPLC; Supplementary Fig. S1A). Finally, Total Internal Reflection Fluorescence (TIRF) microscopy of SU-DHL-4 cells treated with doxorubicin revealed, in parallel to the intense nuclear drug accumulation early directly after drug exposure, vesicular cytoplasmic doxorubicin-sequestration and budding of doxorubicin-containing vesicles from the plasma membrane to the extracellular space (Supplementary Fig. S1B).

**ABCA3 is critical for exosomal drug resistance and indomethacin reduces ABCA3 expression**

We previously described ABCA3 expression as crucial for MV8- and exosome biogenesis, contributing to a multidrug-resistant phenotype in acute myeloid leukemia (13, 14, 16). Silencing ABCA3 in lymphoma cells strongly impaired exosome biogenesis, associated with increased susceptibility of lymphoma cells to humoral immunotherapy (16). Here, we found that exposure to doxorubicin enhanced the expression levels of ABCA3 in OCI-Ly1 and SU-DHL-4 lymphoma cell lines (Fig. 2A), associated with increased exosome release into the cell supernatant (Fig. 2B). Using a lentiviral shRNA knockdown of ABCA3 in both SU-DHL-4 and OCI-Ly1 cells, we found an augmented susceptibility of both cell lines to doxorubicin, compared with a scramble control (Fig. 3A). We and others recently identified the cyclooxygenase inhibitor indomethacin as a drug resistance modulator (16, 27–29) with suppression of ABCA3 expression on the transcriptional level (16, 28, 30). Importantly, indomethacin cotreatment almost completely diminished the doxorubicin-induced increase in ABCA3 expression after doxorubicin (Fig. 2A). In consequence, indomethacin also efficiently reduced lymphoma exosome release elicited by chemotherapy. We pulsed equal numbers of SU-DHL-4 cells with or without 1 μmol/L doxorubicin for 3 hours with or without 10 μmol/L indomethacin cotreatment and harvested the cell supernatant after 24 hours. As quantified by immunodetection of exosomal marker proteins, indomethacin cotreatment diminished the increase in exosome release upon doxorubicin treatment (Fig. 2B).

**Indomethacin pretreatment augments nuclear accumulation of doxorubicin and pixantrone and increase drug susceptibility of lymphoma cells in vitro and in vivo**

Given the efficient reduction in ABCA3 expression and exosome release, we rationalized that coincubation of doxorubicin and pixantrone with indomethacin should increase intracellular cytostatic drug levels. Exploiting the specific fluorescence of the drug by flow cytometry, we analyzed SU-DHL-4 and OCI-Ly1 cells pulsed with pixantrone with or without indomethacin pretreatment. We found that pretreatment with 10 μmol/L indomethacin for 24 hours significantly enhanced doxorubicin and pixantrone retention inside the lymphoma cells (Fig. 2C). We then tested in vitro the cytotoxicity of doxorubicin and pixantrone with or without indomethacin cotreatment. The DLBCL cell lines SU-DHL-4, OCI-Ly1, and OCI-Ly3 were pulsed with 1 to 20 μmol/L doxorubicin or 10 to 40 μmol/L pixantrone with or without 10 μmol/L indomethacin 24 hours pretreatment. Although incubation with indomethacin alone did not affect cell viability up to a concentration of 10 μmol/L, both doxorubicin and pixantrone proved to be highly cytotoxic. Importantly, pretreatment with indomethacin significantly increased cytotoxicity of both doxorubicin and pixantrone (SU-DHL-4 and OCI-Ly1: Fig. 3B, OCI-Ly3: Supplementary Fig. S3A).

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In order to relate the effects of indomethacin to further agents interfering with subcellular drug sequestration, we also evaluated celecoxib, a selective COX 2 inhibitor, in this experimental setting. Noteworthy, celecoxib did not consistently alter susceptibility of SU-DHL-4 or OCI-Ly1 cells to doxorubicin (Supplementary Fig. S4A). Likewise, while other groups have documented inhibition of V-H(+)-ATPase activity and in consequence increased lysosomal pH by omeprazole sensitize tumor cells toward cytostatics (31), we did not detect altered cytotoxicity of doxorubicin after pretreatment omeprazole in SU-DHL-4 or OCI-Ly1 cells (Supplementary Fig. S4B).

Finally, we established a CAM assay to evaluate the effects of indomethacin on doxorubicin and pixantrone efficacy in vivo (Supplementary Fig. S2). SU-DHL-4, OCI-Ly1, and OCI-Ly3 lymphoma cells were implanted onto CAMs of fertilized chicken eggs. After pretreatment with indomethacin or PBS, lymphomas were treated with doxorubicin or pixantrone via intravenous injection, and finally explanted and weighted (Supplementary Fig. S2C). Although indomethacin alone did not affect lymphoma growth, both doxorubicin and pixantrone led to significant tumor shrinkage, strongly enhanced by indomethacin pretreatment for both chemotherapy drugs (Fig. 4). These findings were also confirmed in the ABC-type DLBCL cell line OCI-Ly3 (Supplementary Fig. S3C). Correspondingly, we analyzed the tumors 24 hours after drug exposure. By immunohistology, the tumors exposed to indomethacin and cytostatic drugs displayed a significantly distorted architecture and reduced tumor cell density (Figs. 5A and 6A). FACS analysis of SU-DHL-4 and OCI-Ly1 CAM lymphomas cell preparations showed a significantly enhanced pixantrone fluorescence in indomethacin-pretreated lymphomas compared with pixantrone treatment alone (Fig. 6C). Importantly, confocal fluorescence microscopy also revealed a significantly stronger nuclear drug signals in the lymphomas pretreated with indomethacin (Figs. 5B and 6B). Although the nuclei of tumor cells without indomethacin were devoid of doxorubicin or pixantrone and some drug was detectable in the cytoplasm, the tumor cell nuclei of indomethacin-pretreated lymphomas displayed significant drug accumulation (Figs. 5B and 6B). Thus

**Figure 1.** Trapping of doxorubicin in exosomes. A, exosomes of SU-DHL-4 cells were purified by differential centrifugation and showed a typical cup-shaped morphology with a diameter of 50 to 100 nm in electron microscopy. B and C, purified exosomes of SU-DHL-4 cells were coupled to 4 μm aldehyde/sulfate latex beads, stained with exosomal marker proteins ADAM10, CD9, and CD63, and analyzed by flow cytometry (examples in B, C: unpaired t test. *, P < 0.05; ***, P < 0.001). D and E, cell lines SU-DHL-4, OCI-Ly1, and Balm3 were cultured in exosome-free medium with or without 1 μmol/L doxorubicin (DXR) for 3 hours, washed and cultured in fresh exosome-free medium for another 20 hours. Exosomes were prepared and mean fluorescence quantified by flow cytometry after excitation at 560 nm. Exosomes from doxorubicin-treated cells emitted significant fluorescence compared with control exosome preparations from cells w/o doxorubicin exposure (example for SU-DHL-4 in D; E: paired t test, SU-DHL-4 n = 10, OCI-Ly1 n = 6, Balm3 n = 8; *, P < 0.05; ***, P < 0.001).
indomethacin pretreatment of lymphomas results in a significant prolongation of cytostatic drug retention in the tumor cell nuclei early after initial exposure, associated with increased cytotoxic efficacy.

**Discussion**

We found that lymphoma cells extrude and detoxify doxorubicin and pixantrone through extracellular vesicles featuring the characteristics of exosomes. Importantly, this pathway can be interrupted by inhibiting the expression and function of the ABC transporter A3, increasing the susceptibility of the tumor cells toward cytostatic drugs.

Anthracyclines and anthracenedione have a central role in treatment of malignancy, and doxorubicin represents a well-established, pivotal constituent of the R-CHOP drug backbone in aggressive lymphoma treatment (2). However, primary progression or relapse of lymphoma disease occurs once the cancer cells develop resistance against the cytostatic effects (4). As a major resistance mechanism, ATP-binding cassette (ABC) transporter proteins were discovered to shuttle anthracyclines across the plasma membrane into the extracellular space (13, 32). In particular the ABC transporter B1, also known as p-glycoprotein or MDR1, transports a broad spectrum of cytostatic drugs, and its high expression was found associated with unfavorable treatment outcome in several clinical chemotherapy trials (33). These findings prompted clinical trials to exploit the inhibition of ABC transporter activity in order to improve cytostatic efficacy (34, 35). Such trials have met variable success, due to incomplete inhibitor efficacy as well as the abundance of transporter proteins, of which the human ABC transporter family only comprises more than 46 different proteins (34, 36).

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Figure 2. Indomethacin suppresses ABCA3 expression and enhances cellular doxorubicin and pixantrone retention. A, qPCR measurement revealed augmentation of ABCA3 expression in SU-DHL-4 and OCI-Ly1 cells after treatment with 1 μmol/L doxorubicin for 3 hours while pretreatment with 10 μmol/L indomethacin for 24 hours suppressed ABCA3 expression (n = 3, *P < 0.05). B, SU-DHL-4 cells treated with a sublethal dose of 1 μmol/L doxorubicin for 3 hours showed increased exosome production, whereas pretreatment with 10 μmol/L indomethacin led to a suppression of exosomal biogenesis. C, the cell lines SU-DHL-4 and OCI-Ly1 were incubated with or without drug for 3 hours with or without a 24-hour pretreatment with 10 μmol/L indomethacin, and drug-associated fluorescence was repetitively analyzed by flow cytometry up to 24 hours after excitation at 560 or 670 nm, respectively. Pretreatment with 10 μmol/L indomethacin for 24 hours led to increased doxorubicin or pixantrone fluorescence indicating augmented drug retention (two-way ANOVA and Bonferroni test; ***, P < 0.001).
may also converge: the ABC transporter A3 contributes to drug sequestration in late endosomal organelles, that is, multivesicular bodies and lysosomes, as described by us and others for acute myeloid leukemia, chronic myeloid leukemia, and lung cancer cells (13, 14, 17, 24, 30, 37, 38). Here, we extend the evidence for vesicular export of anthracycline type drugs to aggressive lymphoma cells, and document the compaction of such drugs in exosomes. Exosomal drug exports start directly after drug exposure, when nuclear drug concentrations are maximal. Interference with the vesicular export mechanism increases the levels of intracellular drug, associated with augmented cytotoxic efficacy.

Indomethacin, an inhibitor of COX 1 and 2, represses the expression of ABCA3 at the transcriptional level, sufficient to counteract the increased ABC transporter expression triggered by drug exposure of the tumor cells (Fig. 2A). Of note, the functional effects of indomethacin appeared specific for the transcriptional effects of this drug, rather than the inhibition of COX. Cotreatment of cells with the COX 2 inhibitor celecoxib did not increase the cytotoxic effects of doxorubicin against the lymphoma cell lines in vitro (Supplementary Fig. S4A). Also, omeprazole, a proton pump inhibitor, was used to inhibit V-H(+)-ATPase activity and increase the pH of lysosomal organelles. However, we did not find increased cytotoxicity of doxorubicin after pretreatment with omeprazole (Supplementary Fig. S4B). These data argue for the transcriptional repression of ABCA3 as the main mechanism by which indomethacin increases the cytotoxic efficacy of doxorubicin and pixantrone.

By inhibiting ABCA3-supported exosome biosynthesis, indomethacin increased doxorubicin and pixantrone retention in the tumor cells. Importantly, beyond global intracellular drug levels, indomethacin shifted the pattern of cytostatic drug retention. Although the nuclei were free of drugs at 24 hours after drug exposure in the controls, indomethacin cotreatment retained the drugs in tumor cell nuclei (Figs. 2A). Of note, the functional effects of indomethacin appeared specific for the transcriptional effects of this drug, rather than the inhibition of COX. Cotreatment of cells with the COX 2 inhibitor celecoxib did not increase the cytotoxic effects of doxorubicin against the lymphoma cell lines in vitro (Supplementary Fig. S4A). Also, omeprazole, a proton pump inhibitor, was used to inhibit V-H(+)-ATPase activity and increase the pH of lysosomal organelles. However, we did not find increased cytotoxicity of doxorubicin after pretreatment with omeprazole (Supplementary Fig. S4B). These data argue for the transcriptional repression of ABCA3 as the main mechanism by which indomethacin increases the cytotoxic efficacy of doxorubicin and pixantrone.

However, elevating intracellular anthracycline levels might have harmful side effects. The therapeutic window of conventional...
anthracyclines and anthraquinones is limited by the toxicity the drug exerts in normal cells, for example, in cardiomyocytes. Both topoimerase II inhibition and generation of reactive oxygen species (ROS) are known mechanisms of sarcolemma damage (40), and significant numbers of patients experience clinically relevant cardiac failure following anthracycline treatment. Pixantrone is significantly less cardiotoxic compared with conventional anthracyclines, due to lower ROS induction and/or a different mode of topoimerase II inhibition (41). Thus we surmise that elevating intracellular drug levels in the cardiomyocytes as well as other

Figure 4. Indomethacin increases efficacy of doxorubicin and pixantrone in vivo. SU-DHL-4 and OCI-Ly1 lymphoma cells were seeded on the chorioallantoic membrane of chicken eggs. After 7 days, lymphomas were treated with 50 ng doxorubicin or pixantrone intravenously with or without a topical pretreatment with 1 mL of 10 μmol/L indomethacin for 24 hours. Doxorubicin and pixantrone led lymphoma regression, significantly enhanced by indomethacin pretreatment for both doxorubicin and pixantrone (Student’s t test, a) n = 6, b) n = 6, c) n = 8, d) n = 6; *, P < 0.05; **, P < 0.01; ***, P < 0.001).

Figure 5. Indomethacin pretreatment enhances doxorubicin retention in the tumor, drug-induced tumor tissue distortion and shifts doxorubicin (DXR) from the cytoplasm into the nucleus. SU-DHL-4 CAM tumors were explanted 24 hours after doxorubicin treatment with and without indomethacin pretreatment and analyzed by confocal microscopy visualizing DXR by fluorescence after excitation at 560 nm with DAPI counterstain. Doxorubicin treatment distorted the lymphoma tissue, significantly enhanced after indomethacin pretreatment (open arrowhead in A). Indomethacin pretreatment was associated with increased DXR retention in the tumor cells (A), and with a shift of doxorubicin fluorescence from the cytoplasm to the nucleus (B).
normal cells by indomethacin might be less toxic, and that indomethacin may be given to patients in parallel to pixantrone without endangering the heart. Clearly, the in ovo assay is of limited value for the prediction of a pixantrone/indomethacin toxicity to be expected in the human setting, when primarily the cardiac situation of an adult heart and long-term side effects have to be considered. However, we calculated the amounts of indomethacin given in our experimental setting to yield in a chicken plasma concentration of 0.075 μg/mL, far below the peak plasma concentrations of 1.14 ± 0.58 μg/mL achieved by a single dose of 50 mg indomethacin in the adult human being (42). Thus we extrapolate that the doses of indomethacin sufficient to modulate doxorubicin and pixantrone in the CAM assay setting may reflect clinically achievable concentrations.

The combination of COX inhibition and chemotherapy has been applied to patients with non–small cell lung cancer with promising results, yet only with schedules containing taxanes and platinum (43). To the best of our knowledge and in contrast with some in vitro evidence presented here and elsewhere (28, 29, 44–46), the clinical outcome of an anthracycline/COX inhibitor combination has not yet been reported, not as a designated clinical trial nor as retrospective series summarizing the inadverently coincidental use of both classes of drugs. While we share the concerns of combining a conventional anthracycline with indomethacin, we propose, taking into account the low cardiac toxicity of pixantrone, the cotreatment of indomethacin and pixantrone as an effective and feasible regimen to be tested in a clinical trial.

In conclusion, we found that exosomal trapping/secretion extrudes doxorubicin and pixantrone compacted in exosomes into the extracellular space. Indomethacin interrupts this mechanism, shifts the drugs into the nucleus and augments tumor cytotoxicity, suggesting the combination of pixantrone and indomethacin for clinical evaluation.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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