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ORIGINAL ARTICLE

Increased cellular accumulation and distribution of amrubicin contribute to its activity in anthracycline-resistant cancer cells

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Abstract
Purpose Multi-drug resistance and cumulative cardiotoxicity are major limitations for the clinical use of anthracyclines. Here, we evaluated and compared the cross-resistance of amrubicin, a third-generation synthetic anthracycline and potent topoisomerase (topo)-II inhibitor with little or no observed cardiotoxicity to other anthracyclines and the topo-II inhibitor etoposide in drug-resistant tumor models in order to elucidate its potential mechanisms of action.
Methods Amrubicin activity was assessed in multi-drug-resistant cell lines and human tumor explants using cytotoxicity assays, confocal microscopy, fluorescence time-lapse imaging, flow cytometry, immunoblotting, and gene expression profiling techniques.
Results We demonstrate that both doxorubicin-resistant tumor cell lines and several drug-resistant human ovarian and breast tumor explants retain sensitivity to amrubicin. In addition, we observed similar levels of amrubicin uptake and accumulation in doxorubicin-sensitive versus doxorubicin-resistant cell lines. Although amrubicin is a weak P-glycoprotein substrate, transport and retention of amrubicin were not solely modulated by P-glycoprotein in the resistant cell lines overexpressing drug efflux pumps. The cellular retention of amrubicin is likely to be a result of rapid influx due to its high intrinsic permeability and lipophilic properties, and this may explain why amrubicin overcomes pleiotropic drug resistance. Consistent with drug accumulation studies, amrubicin induced DNA damage, G_2–M cell cycle arrest, and apoptosis in both doxorubicin-sensitive and doxorubicin-resistant lines. Using gene expression profiling studies, several classes of genes were significantly and uniquely regulated following amrubicin, but not doxorubicin or etoposide, treatment.
Conclusions Amrubicin appears to have a distinct mode of action that overcomes typical anthracycline resistance mechanisms. Therefore, amrubicin may be useful in the treatment of anthracycline-refractory or anthracycline-resistant tumors.

Keywords Amrubicin · Anthracycline · Multi-drug resistance · Topoisomerase-II inhibitor

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Introduction

Anthracyclines, such as doxorubicin, daunorubicin, and epirubicin, are widely used in a broad range of human cancers, including leukemias, lymphomas, sarcomas, and carcinomas [1]. However, there are major limitations associated with the clinical use of anthracyclines, including

cumulative cardiotoxicity and development of resistance resulting in treatment failure.

Amrubicin is a synthetic 9-aminoanthracycline agent approved in Japan for the treatment of non-small cell and small cell lung cancer (SCLC) [2]. In preclinical studies, amrubicin has shown a broad spectrum of antitumor activity in a variety of tumor types, including hematologic malignancies and SCLC [3–5]. In addition, it has been demonstrated to be less cardiotoxic compared with doxorubicin. Myocardial damage, typically observed following treatment with an anthracycline, was minimal in rabbits and dogs treated with amrubicin and did not further exacerbate low-grade doxorubicin-induced cardiomyopathy in dogs [4, 6]. Amrubicin is metabolized to amrubicinol, which is approximately 100-fold more potent than amrubicin and has enhanced topoisomerase (topo)-II inhibitory activity. Clinically achievable plasma levels of amrubicin and amrubicinol are 20 and 2 μM, respectively, with half-life values of 17.6–24.5 h and 4–7 h, respectively [7, 8].

In recent European and US-based phase II studies, amrubicin was shown to be efficacious and safe to use in patients with SCLC refractory to first-line platinum-containing chemotherapy [9]. The overall response rate was 21.3%, and median overall survival was 6 months. Safety and efficacy were also demonstrated in a randomized phase II study comparing amrubicin to topotecan in chemosensitive patients [10]. The results of these studies were consistent with results from a randomized phase II Japanese trial, which compared amrubicin with topotecan as second-line therapy in patients with sensitive or refractory SCLC [11].

Anthracyclines are known to exert cytotoxicity through three mechanisms: (a) inhibition of DNA and RNA synthesis by intercalating between base pairs of the DNA/RNA strand; (b) inhibition of the topo-II enzyme, preventing the relaxation of supercoiled DNA and thus blocking DNA transcription and replication; and (c) the generation of iron-mediated, free oxygen radicals that damage the DNA and cell membranes [12, 13]. Similar to etoposide, a topo-II inhibitor, amrubicin, induces covalent DNA protein complexes (stabilized DNA–topo-II complexes) and double-stranded DNA breaks [14]; however, it is a more potent inducer of double-stranded DNA breaks than doxorubicin. It has also been suggested that amrubicin may produce less reactive oxygen species than doxorubicin [15].

Cellular resistance to anthracyclines and topo-II inhibitors is often mediated by the overexpression of efflux pump(s) in the cell membrane, resulting in lower steady-state concentrations of the drug at the target site. The most common efflux pump in the cell membrane is P-glycoprotein, which is encoded by the human multi-drug resistance (MDR) gene 1 (*MDR1*) and belongs to a larger family of proteins referred to as the ATP-binding cassette (ABC) family [16]. Overexpression of efflux pumps reduces intracellular accumulation and retention of antitumor drugs via active outward transport, leading to decreased cytotoxicity and a failure of tumor cells to undergo apoptosis [17]. In addition, anthracycline resistance can be mediated by downregulation of the target topo-II gene or protein [17], resulting in decreased activity of and sensitivity to drugs.

The objective of the current study is to examine the cross-resistance of amrubicin with other anthracyclines (doxorubicin and epirubicin) and the topo-II inhibitor etoposide in drug-resistant cell lines and human tumor explants, and to evaluate potential mechanisms of action of amrubicin at clinically achievable concentrations.

Materials and methods

Cell lines

Human SCLC (H69) cells, ovarian cancer (OVCAR8) cells, and their doxorubicin-selected MDR variants H69-AR [18] and NCI-ADR/RES were cultured in Roswell Park Memorial Institute 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD). A human uterine sarcoma cell line (MES-SA) and its MDR variant (MES-SA-DX5 [19]) were maintained in McCoy's 5A-supplemented media with 10% FBS. All cell lines were obtained from American Type Culture Collection (Rockville, MD). We confirmed that the doxorubicin-sensitive and doxorubicin-resistant cells had different gene expression profiles, including expression of drug transporters [20–22]. ABCB1 (MDR1/P-glycoprotein) was one of the major genes overexpressed in NCI-ADR/RES (200-fold) and MES-SA-DX5 (140-fold) cells compared with their parental cell lines. As previously reported, ABCC1 (MRP1; multi-drug-resistant protein 1) was upregulated in H69-AR (123-fold) compared with H69 cells [23].

Reagents

Amrubicin was synthesized by Dainippon Sumitomo Pharma (Osaka, Japan), and etoposide, doxorubicin, and epirubicin were purchased from Sigma-Aldrich (St. Louis, MO). GF120918 (elacridar; Sequoia Research Products Ltd, Pangbourne, UK) and verapamil (Invitrogen, Carlsbad, CA) were used in some experiments as indicated. Anti-MRP1 and P-glycoprotein antibodies were purchased from Abcam Inc. (Cambridge, MA). Anti-cleaved caspase-3 and anti-γH2AX antibodies were purchased from Cell Signaling (Beverly, MA). Propidium iodide, Alexa 488-conjugated anti-mouse immunoglobulin (IgG), and Alexa 405-conjugated anti-rabbit IgG were purchased from Molecular Probes (Eugene, OR).

Western blotting

Cells were harvested in RIPA buffer. Proteins were separated by SDS–PAGE, immunoblotted with their respective antibodies, and visualized by enhanced chemiluminescence on film.

Cell viability assay

Overall, 2,000–6,000 cells per well in a 96-well plate were incubated for 24 h and then treated for 72 h with cytotoxic agents (doses ranging from 0.07–5 μM for H69 and H69-AR cells, and 0.03–30 μM for the other cell lines). The CellTiter-Glo® assay (Promega Corp., Madison, WI) was used to quantify viable cells. EC_{50} values were determined using the GraphPad Prism software.

In tumor explant studies, cancer specimens were obtained from surgical biopsies that were sent for routine evaluation in the Oncotech EDR® (Extreme drug resistance) Assay. Sensitivity to DOX and ETO was previously determined using the EDR assay. Disaggregated cells from the viable tumor tissue were plated on soft agar in the presence of the drug, and if the cancer cells grow in the presence of a very high (extreme) dosage of the drug, studies have shown that the cancer is unlikely to respond to that drug in the patient's body. The cryopreserved tumor explants were then used in the CellTiter-Glo® assay. A M14 melanoma cell line was also included on each day of performing CellTiter-Glo® testing in order to ensure potency of each drug during the course of the study.

Immunofluorescence/confocal microscopy

Cells were fixed in 3.7% formaldehyde and permeabilized in 0.1% Triton X-100/PBS, washed, blocked in 3% bovine serum albumin/PBS for 1 h, and incubated with primary antibody overnight at 4°C. Following 1-h incubation with secondary antibody, cells were incubated either with 0.2 μg/mL of 4′,6′-diamidino-2-phenylindole (DAPI) or with propidium iodide. Images were captured on a Nikon fluorescence microscope or laser scanning confocal microscope (Nikon C1si Spectral Imaging System) and analyzed using the NIS-elements imaging software. For intracellular localization of amrubicin and doxorubicin, cells were incubated with drugs.

Time-lapse video microscopy

Cells were maintained in an incubation system, with CO_2 and humidity control, on a heated microscope stage during acquisition of video time-lapse images. Images were acquired every 60 s using a 40 × 1.4 N.A. Plan Apo objective lens with 1.5× auxiliary magnification using an automated shutter and video camera (Kappa Optronics Inc., Monrovia, CA) and analyzed using the NIS-elements imaging software. For some experiments, the same area was fixed in 4% paraformaldehyde and immunostained for epifluorescence microscopy. The outline of the cell was traced manually, and the integrated fluorescence intensity over the entire area of the cell was graphed over time.

Flow cytometric detection of functional drug efflux

A fluorogenic dye, calcein acetoxymethyl ester (calcein-AM), is a substrate for efflux activity of P-glycoprotein 1 and other membrane pump ABC proteins; it was used as a positive control in the drug efflux assay. P-glycoprotein modulators (elacridar or verapamil) were added to cells immediately before the addition of calcein-AM (0.25 μM), doxorubicin, or amrubicin and incubated for 2 h at 37°C. Drug accumulation/efflux was stopped by pelleting the cells and adding ice-cold medium. Cells were washed and the fluorescence of calcein, amrubicin, or doxorubicin from 5,000 cells was analyzed by a FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA) equipped with an argon laser source. Data were analyzed using FlowJo version 7.2.5, and fluorescence was measured as the medium channel value using arbitrary units.

Gene expression profiling

Cells were treated with cytotoxic EC_{50} and EC_{10} concentrations of amrubicin or doxorubicin for 24 h. Total RNA was isolated, and gene expression was evaluated using Affymetrix U133 Plus 2.0 chips (Affymetrix; Santa Clara, CA). All statistical analyses were carried out using the limma package in R software [24]. Gene filtering was done by using limma package [25] with Benjamini, and Hochberg-adjusted P values of 0.05 and fold changes of 1.5 were used as cutoffs for gene filtering. NextBio (www.nextbio.com) was used to identify the enriched biogroups in the filtered gene lists.

Results

Amrubicin is effective in doxorubicin-resistant cell lines and tumor explants

To evaluate the cross-resistance of amrubicin with other anthracyclines (doxorubicin and epirubicin) and the topo-II inhibitor etoposide, cell lines resistant to doxorubicin (H69-AR, MES-SA-DX5, and NCI-ADR/RES) or their parental anthracycline-sensitive lines (H69, MES-SA, and OVCAR8) were incubated with drug for 72 h. In the parental lines, doxorubicin and epirubicin were the most

potent inhibitors of cell growth, whereas etoposide and amrubicin had similar EC_{50} values (Fig. 1a; Table 1). Doxorubicin sensitivity was 21- to 45-fold lower (mean 32-fold), and epirubicin sensitivity was 7- to 59-fold lower (mean 22-fold) in the anthracycline-resistant cell lines compared with the parental cell lines; etoposide sensitivity was fourfold to ninefold lower (mean sixfold) in the resistant cell lines. By contrast, amrubicin sensitivity was unchanged in the anthracycline-resistant cell line (NCI-ADR/RES) and only threefold to fourfold lower (mean 2.7-fold) in the other two lines (H69-AR and MES-SA-DX5). In all anthracycline-resistant cell lines, sensitivity to amrubicin was 2.5- to 6-fold (mean fourfold) higher than sensitivity to etoposide (Fig. 1a; Table 1). These results indicate that although the resistant lines (H69-AR, MES-SA-DX5, and NCI-ADR/RES) were clearly resistant to doxorubicin and epirubicin, the resistance was lower or absent for amrubicin. To further evaluate amrubicin in a more clinically relevant model, we also tested the activity of amrubicin in human breast cancer and ovarian cancer explants that had been previously determined to be either sensitive or resistant to doxorubicin and etoposide (Online Resource 1). Doxorubicin and etoposide concentrations used in these assays were established based on clinically achievable concentrations (the maximum concentration tested was twice the maximal plasma concentration). In all tested models, amrubicin dose dependently inhibited growth, and notably, many tumors that were resistant to doxorubicin and/or etoposide retained sensitivity to amrubicin (representative doxorubicin-sensitive and doxorubicin-resistant tumors are shown in Fig. 1b, suggesting that the amrubicin mechanism of action could be distinct from that of doxorubicin and etoposide, and suggesting that amrubicin may be effective in treating tumors refractory to these drugs.

Amrubicin accumulates in both doxorubicin-sensitive and doxorubicin-resistant cell lines

MDR cells usually transport antitumor drugs out of the cell through active efflux pumps, thereby decreasing the intracellular concentration of drugs and diminishing their damage to the cell. To better understand how doxorubicin-resistant lines retain sensitivity to amrubicin, we compared the intracellular accumulation and distribution of amrubicin to that of doxorubicin. Subcellular distribution by laser scanning confocal microscopy of parental OVCAR8 cells showed that doxorubicin was localized to the nucleus (with distinct spots of intense fluorescence indicative of chromosomal DNA intercalation), whereas amrubicin was predominantly localized to the cytoplasm (in 20–30% of cells, amrubicin was also observed in the nuclei; Fig. 2a). Accumulation of doxorubicin but not amrubicin was significantly lower in NCI-ADR/RES cells compared with parental OVCAR8 cells. Similar results were found in MES-SA-DX5 (resistant) and MESA-SA (sensitive) cells (Online Resource 2). In addition, flow cytometry studies showed similar or at most slightly higher amrubicin accumulation in H69AR compared to H69 cells (Online Resource 3).

Live image analysis of amrubicin and doxorubicin transport in OVCAR8 and NCI-ADR/RES cell lines

The kinetics of accumulation for amrubicin and doxorubicin in doxorubicin-sensitive and doxorubicin-resistant cells was evaluated using time-lapse video imaging. Representative images of amrubicin and doxorubicin in OVCAR8 cells show that within 2 min, amrubicin accumulated in the cytoplasm and in vesicles at the cell margins; notably, the drug remained detectable throughout the 30-min incubation (Fig. 2b, top panels). In contrast, doxorubicin showed an initial burst in accumulation in the nucleus and unlike amrubicin, and its fluorescence decreased notably over the 30-min time period (Fig. 2b, bottom panels). Time-lapse microscopy of drug (amrubicin or doxorubicin) alone without cells showed no detectable photo-quenching. Amrubicin accumulation in OVCAR8 and NCI-ADR/RES cells was virtually indistinguishable (Fig. 2c, left panel); however, consistent with our previous results, no significant doxorubicin fluorescence was observed in the resistant NCI-ADR/RES cells (Fig. 2c, right panel).

P-glycoprotein inhibitors do not modulate amrubicin accumulation in doxorubicin-resistant cell lines

The resistance of MDR-overexpressing tumors to antineoplastic chemotherapy is often associated with reduced cellular drug accumulation through the overexpression of transport molecules (drug efflux pumps). As anthracyclines are known substrates for ABC-type efflux transporters, we explored the potential involvement of P-glycoprotein in the accumulation of amrubicin and doxorubicin. The ability of doxorubicin-sensitive (OVCAR8) and doxorubicin-resistant (NCI-ADR/RES) cells (overexpressing P-glycoprotein) to accumulate drugs in the absence or presence of the P-glycoprotein modulators verapamil and elacridar was evaluated using flow cytometry (Fig. 3). Calcein-AM was used as a positive control (data not shown). Merged histograms show that amrubicin accumulates in both OVCAR8 and NCI-ADR/RES cells, and the accumulation is similar with or without P-glycoprotein inhibitors (Fig. 3a). P-glycoprotein inhibitors had no effect on doxorubicin accumulation in OVCAR8 sensitive cells; however, the reduced accumulation of doxorubicin in the resistant cells (NCI-ADR/RES) was completely reversed by verapamil

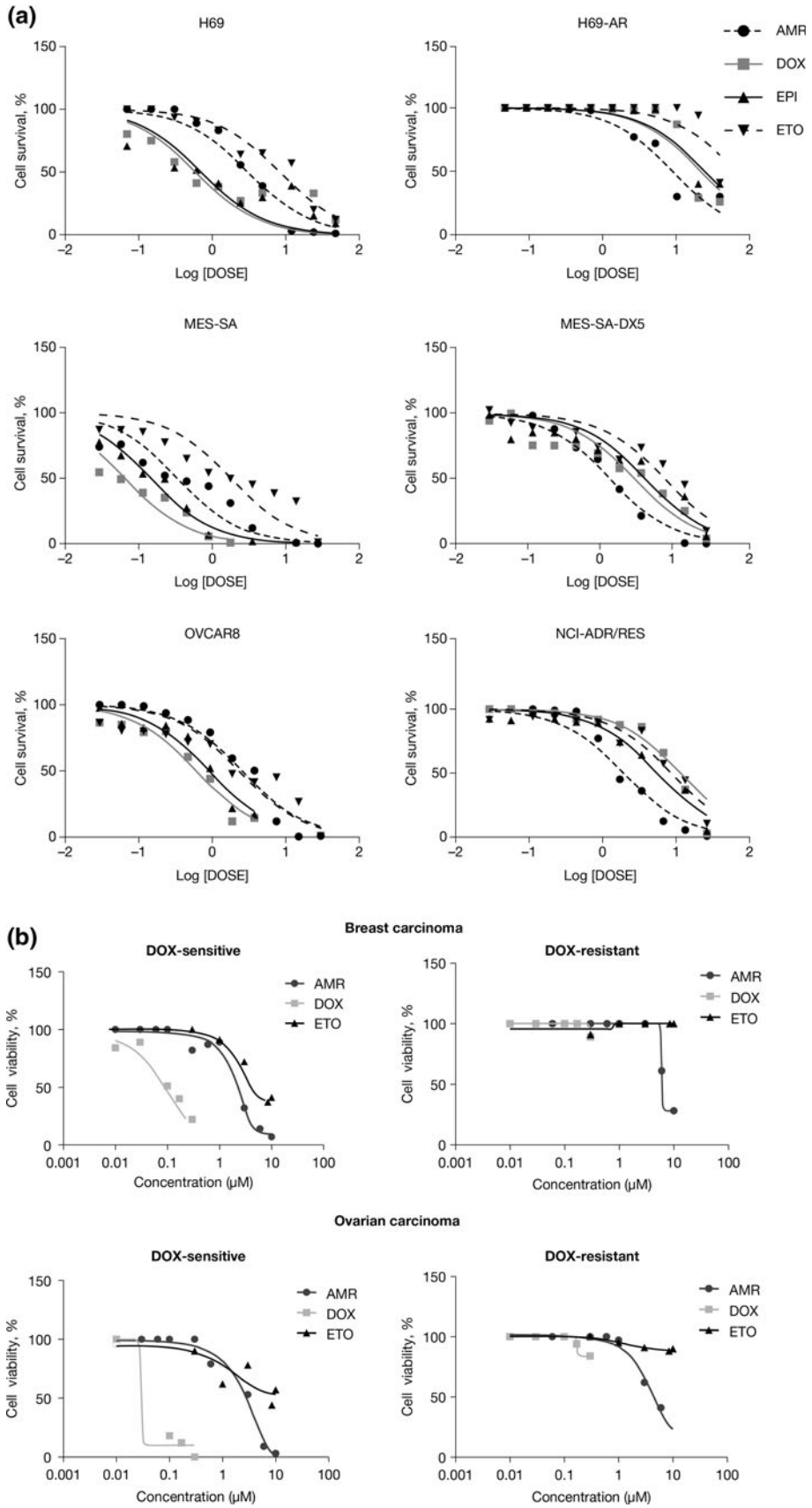

Fig. 1 Amrubicin (AMR) inhibits the growth of drug-sensitive and drug-resistant cell lines and primary human tumor explants. **a** Doxorubicin (DOX)-resistant cell lines have lower or no cross-resistance to AMR. CellTiter-Glo® analysis (cell viability analysis) showing the percentage of viable cells following 72-h drug exposure. Cells were treated with AMR, etoposide (ETO), DOX, and epirubicin (EPI) at concentrations shown in the figures. **b** Cell viability curves for representative DOX-sensitive and DOX-resistant human breast and ovarian carcinoma explants. Cell viability was assessed using CellTiter-Glo®, and explants were treated with drug for 96 h. DOX concentrations ranged from 0.01 to 0.3 μM, ETO concentrations ranged from 0.3 to 10 μM, and AMR concentrations ranged from 0.01 to 10 μM

Table 1 DOX-resistant cell lines have lower or no cross-resistance to amrubicin

Cell lines	EC_{50} values (μM) in DOX-resistant and DOX-sensitive cell lines			
	Amrubicin	DOX	ETO	EPI
H69	3	0.6	8.6	1
H69-AR	10	27	80	31
MES-SA	0.4	0.1	1.9	0.2
MES-SA-DX5	1.4	3	7.8	4.3
OVCAR8	2.6	0.6	2.3	0.9
NCI-ADR/RES	2.2	12.7	9.4	6.1
Relative resistance factors[a] for the cell lines to the drugs				
H69-AR	3×	45×	9×	31×
MES-SA-DX5	4×	30×	4×	22×
NCI-ADR/RES	<1×	21×	4×	7×

DOX doxorubicin, *EPI* epirubicin, *ETO* etoposide

[a] Obtained by dividing the EC_{50} values of the resistant cells by those of the parental cells. Values obtained from 3 experiments

and elacridar (Fig. 3b). Similar results were observed in NCI-ADR/RES and MES-SA-DX5 using fluorescence microscopy (Online Resource 4). These results indicate that unlike doxorubicin, which is actively exported from cells overexpressing P-glycoprotein, amrubicin can accumulate in the drug-resistant cells.

Amrubicin induces DNA damage in doxorubicin-sensitive and doxorubicin-resistant cell lines

Anthracyclines and topo-II inhibitors are known to induce cell death by a mechanism involving double-strand DNA damage. As the doxorubicin-resistant cell lines have little or no cross-resistance to amrubicin, we evaluated DNA damage in these sensitive and resistant cells by determining the extent of histone H2AX phosphorylation on serine 139 [26]. Amrubicin induced γH2AX in both doxorubicin-sensitive (OVCAR8) and doxorubicin-resistant (NCI-ADR/RES) cells with equal potency by 2 h as shown by confocal microscopy (Fig. 4a). At equimolar drug concentrations (2 μM), amrubicin was less potent than doxorubicin in inducing γH2AX in OVCAR8 cells. However, in contrast to amrubicin, no DNA damage was detected with doxorubicin treatment in the drug-resistant NCI-ADR/RES cells (Fig. 4b). Pretreatment of NCI-ADR/RES cells with the P-glycoprotein modulators elacridar and verapamil permitted doxorubicin to accumulate in the NCI-ADR/RES cells, and as a result, significant phosphorylation of histone H2AX was observed (Fig. 4b, lower panels).

Amrubicin induces G_2–M cell cycle arrest and caspase 3 cleavage in doxorubicin-sensitive and doxorubicin-resistant cell lines

The cytotoxic effects of amrubicin and doxorubicin were further examined by surveying cell cycle arrest and apoptosis. Amrubicin induced G_2–M arrest in both OVCAR8 and NCI-ADR/RES cells (Fig. 5a). However, relatively higher concentrations of amrubicin were needed to induce equivalent levels of arrest in resistant cells. In contrast, treatment of OVCAR8 cells with a low doxorubicin concentration (0.02 μM) for 24 h induced a marked cell arrest at G_2–M phase (G_2-phase decreased from ~19% in the control to ~69% in the treated cells). However, doxorubicin concentrations of 1.8 μM (Fig. 5b) and higher (5 μM, data not shown) failed to arrest the NCI-ADR/RES cell cycling.

Furthermore, amrubicin, but not doxorubicin, induced apoptosis as determined by caspase 3 cleavage in both the doxorubicin-sensitive and doxorubicin-resistant cells (Fig. 5c). Following 2.5 μM amrubicin treatment, 55% of OVCAR8 cells and 50% of NCI-ADR/RES cells stained positive for cleaved caspase 3. In contrast, only ~5% of NCI-ADR/RES cells underwent apoptosis following doxorubicin treatment (2.5 μM).

Overall, the results presented here demonstrate that amrubicin at or below the clinically achievable concentrations induces DNA damage, cell cycle arrest, and apoptosis in chemoresistant cell lines.

Amrubicin and doxorubicin differentially regulate gene expression

To further explore genes that may be uniquely regulated by amrubicin-resistant cell lines, gene expression profiling was evaluated in OVCAR8 and NCI-ADR/RES cells treated for 24 h with vehicle, amrubicin, or doxorubicin at EC_{10} and EC_{50} doses (established following 72-h drug treatment).

A Venn diagram summarizing the number of genes that are distinctly and/or commonly regulated by amrubicin in OVCAR8 and NCI-ADR/RES cells is shown in Online Resource 5. Overall, 1,631 genes were commonly regulated by amrubicin in both cell lines, and these were further analyzed using NextBio to identify the enriched biogroups. The pathways that were significantly regulated by amrubicin included those related to focal adhesion, extracellular matrix–receptor interaction, SKP2-mediated E2F1 degradation pathway, p27 pathway, tumor necrosis factor pathway, ubiquinone biosynthesis, and cell cycle checkpoint (Online Resource 6).

To further understand the specific pathways that allow for amrubicin sensitivity in doxorubicin-resistant cells,

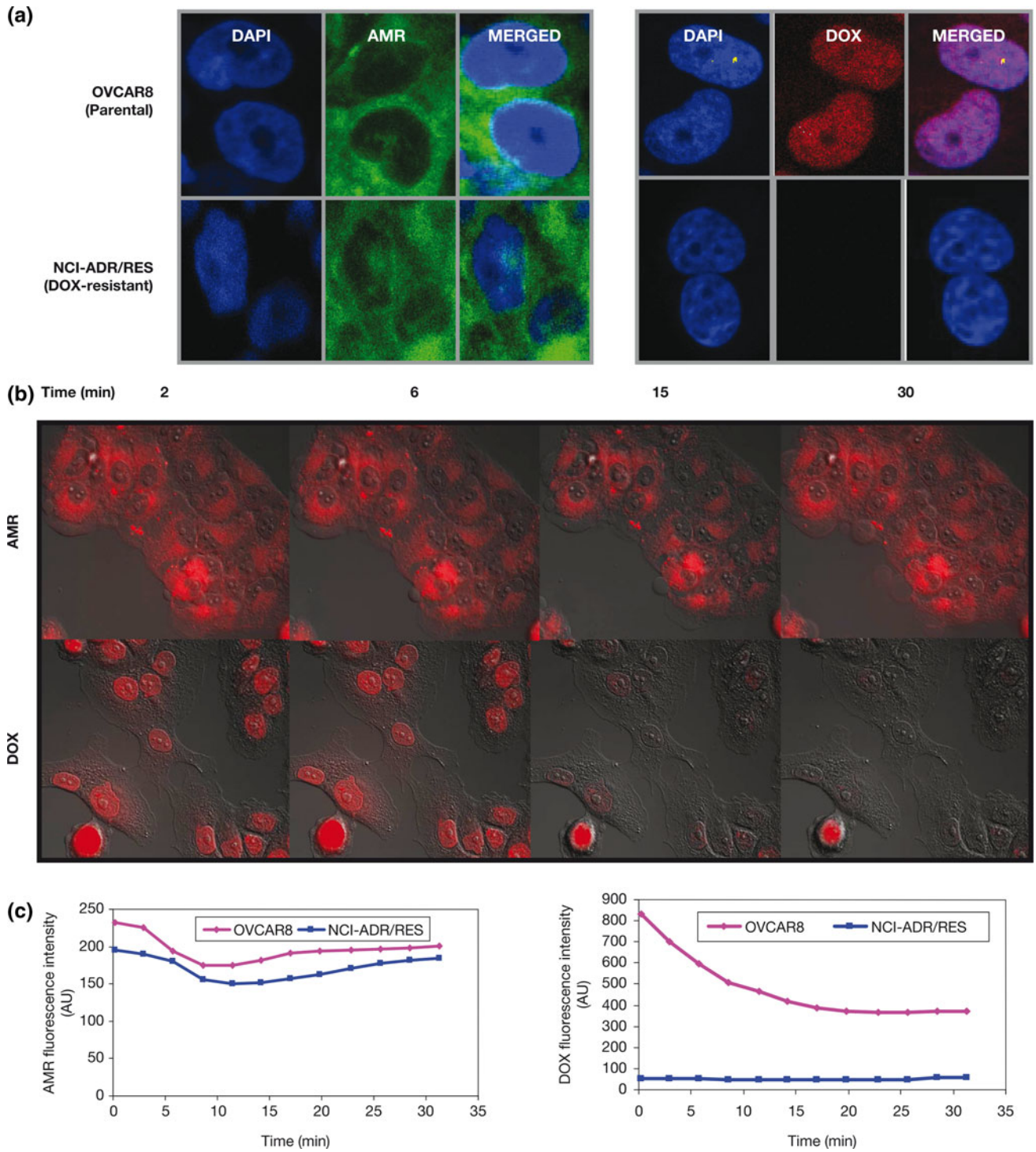

Fig. 2 **a** Accumulation of doxorubicin (DOX), but not amrubicin (AMR), is lower in DOX-resistant compared with DOX-sensitive cell lines. Confocal microscopy images of AMR autofluorescence (*green, left panel*) and DOX autofluorescence (*red, right panel*) in OVCAR8 and NCI-ADR/RES cells treated with 2 μM of drug for 2 h at 60× magnification. **b** Representative time-lapse images (merged differential interface contrast and AMR fluorescence). AMR (*top panel*) and DOX (*bottom panel*) localization in OVCAR8 cells following addition of 2.5 μM of drug. **c** AMR and DOX fluorescence intensity over time. About 10–15 cells were tracked over time for quantitation of drug fluorescence. Cells were counterstained with DAPI (*blue*) for the nucleus

genes that were regulated by amrubicin, but not by doxorubicin and etoposide in the doxorubicin-resistant cell lines, were assessed. The overlap between genes regulated by amrubicin, doxorubicin, and etoposide at EC_{50} doses in NCI-ADR/RES cells is shown in Online Resource 5; 3,011 genes were uniquely regulated by amrubicin. As shown in

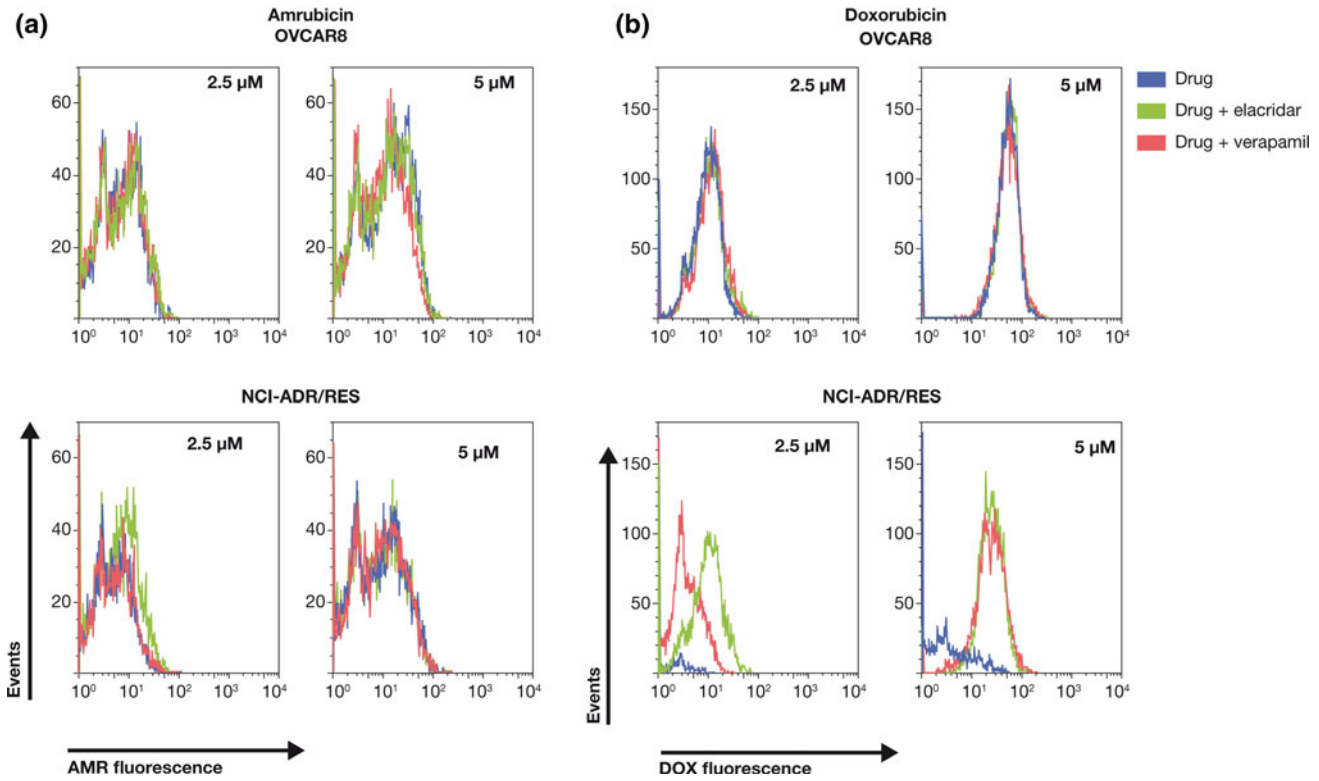

Fig. 3 Amrubicin accumulation is not modulated by P-glycoprotein inhibitors. Flow cytometric analysis of **a** amrubicin and **b** doxorubicin accumulation in OVCAR8 (*top panel*) and NCI-ADR/RES (*bottom panel*) cell lines. Cells were incubated with 2.5 or 5 μM drug ± P-glycoprotein inhibitors elacridar (100 nM) and verapamil (50 μM) for 2 h at 20× magnification. Note elacridar is 100-fold more potent than verapamil

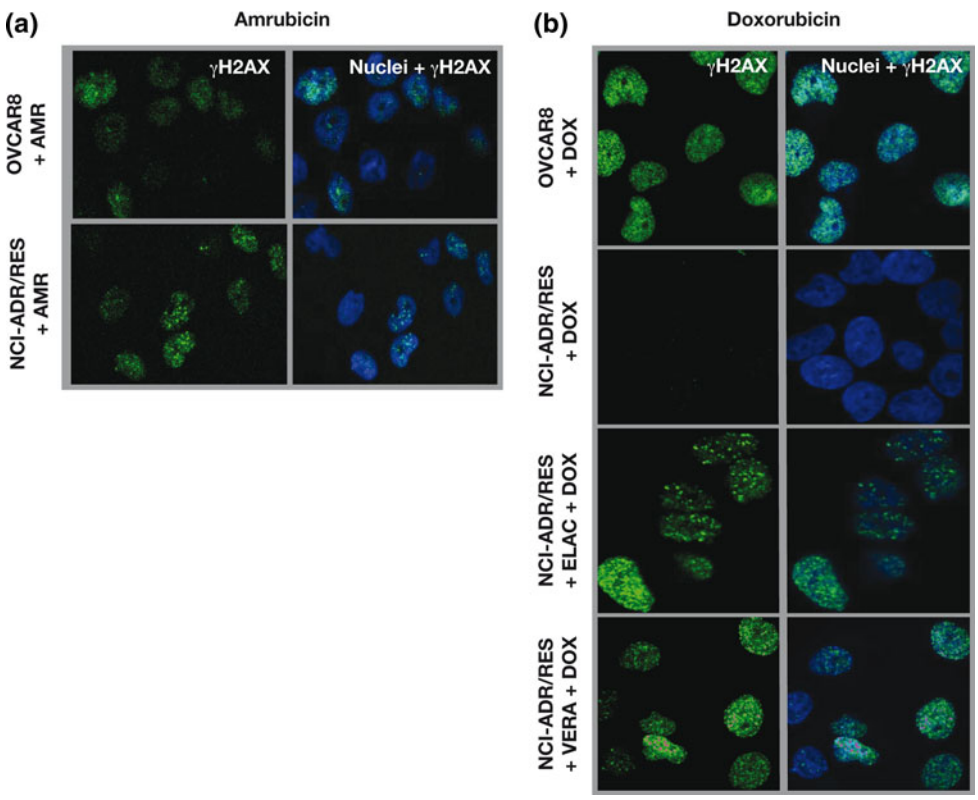

Fig. 4 Amrubicin (AMR) but not doxorubicin (DOX) induces DNA damage in both DOX-sensitive and DOX-resistant cell lines. Confocal microscopic images of γH2AX in **a** AMR- and **b** DOX-treated OVCAR8 and NCI-ADR/RES cell lines. Cells were treated with 2 μM of drug for 2 h. NCI-ADR/RES cells were treated with 2 μM DOX alone, DOX + 100 nM elacridar (ELAC), or DOX + 50 nM verapamil (VERA) as indicated (B, *lower panels*). γH2AX staining is shown in green, and counterstaining with DAPI is shown in blue. Merged images show the co-localization of γH2AX with DAPI (60× magnification)

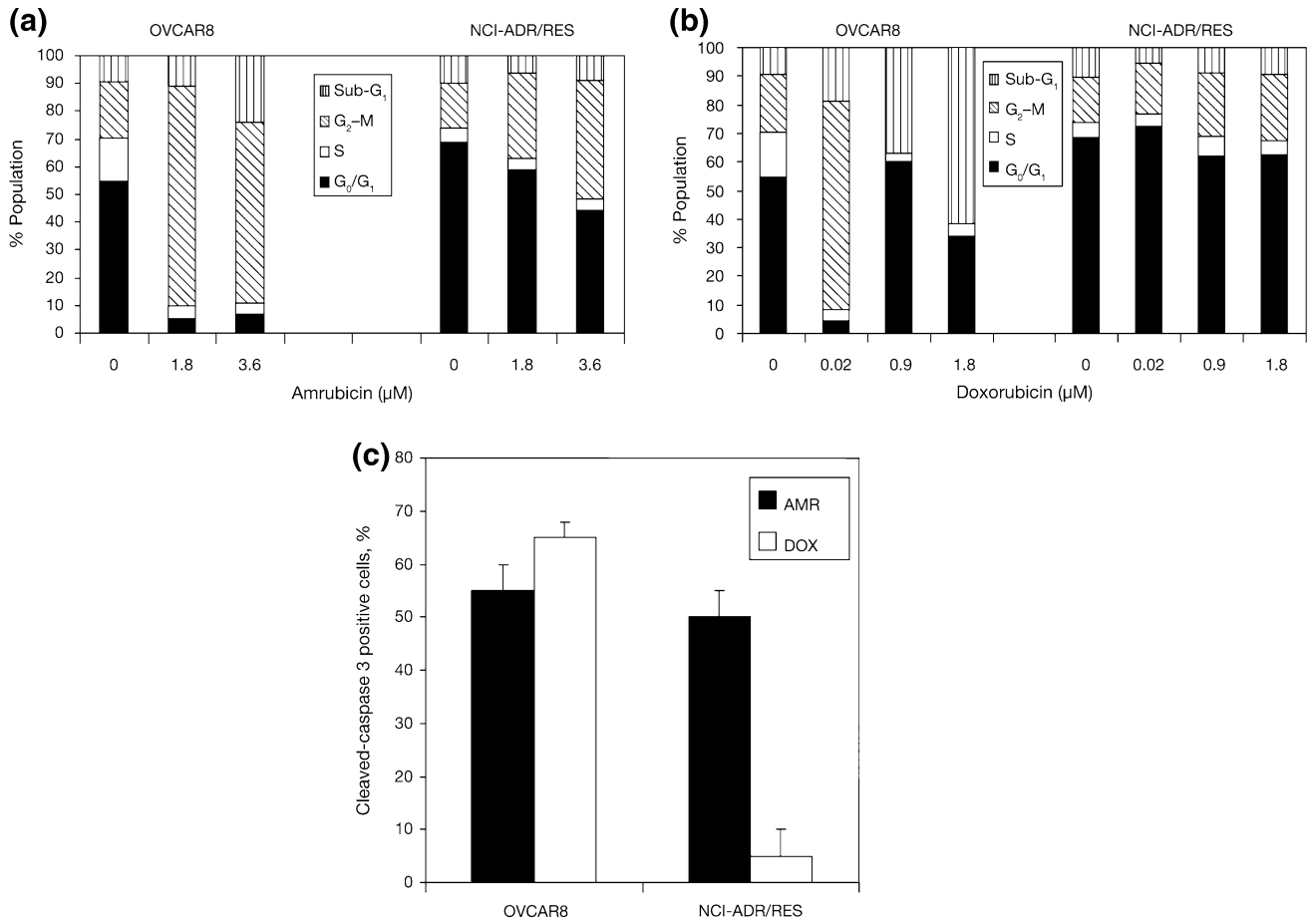

Fig. 5 Amrubicin (AMR) but not doxorubicin (DOX) induces G_2–M cell cycle arrest and caspase 3 cleavage in both DOX-sensitive and DOX-resistant cells. Cell cycle progression was assessed by staining with DAPI and quantitation by flow cytometry. Percentage of cells in sub-G_1, G_0/G_1, S, and G_2–M cell cycle phases after treatment with **a** AMR and **b** DOX for 24 h are shown. **c** Apoptosis was detected by immunostaining for cleaved caspase 3, 24 h following treatment with AMR or DOX at 2.5 μM. Graph shows percentage of cleaved caspase 3–positive cells relative to control. Reported values are mean ± standard deviation of 3 separate repeated experiments

Online Resource 7, genes essential to DNA damage repair (BRCA1, BRCA2, and ATR pathways) were significantly and uniquely upregulated following amrubicin treatment. Upregulation of these genes may be in response to DNA damage induced by amrubicin in the doxorubicin-resistant NCI-ADR/RES cells. For example, *RAD50*, *TP53*, *CHEK1*, *BRCA2*, *RAD1*, *RAD51*, *FANCD2*, and *RAD17* were among the genes regulated by amrubicin, but not by doxorubicin or etoposide in the resistant cell line.

Discussion

Anthracyclines have potent antitumor activity and have been used widely in the treatment of acute leukemia, malignant lymphoma, and solid tumors. However, emergence of resistant tumor cells during treatment occurs frequently, resulting in failure of cancer chemotherapy. The data presented here indicate that amrubicin, a third-generation synthetic anthracycline, is potent and active in human MDR cell lines and primary tumor explants, which are insensitive to other anthracyclines. MDR is associated with decreased intracellular retention of chemotherapeutic drugs which can be attributed to reduced rates of drug influx [27, 28], altered intracellular binding [27], and active efflux of the drugs [29]. Over the past couple of decades, drug resistance research has focused on the ABC-superfamily of exporters, in particular the P-glycoproteins. Although amrubicin is a weak P-glycoprotein substrate, our research focuses on elucidating the additional mechanisms that exist to allow amrubicin to be efficacious even in P-glycoprotein-overexpressing tumor cells.

Studies reported here demonstrate that cell lines and tumor explants resistant to doxorubicin and etoposide (H69-AR, MES-SA-DX5, and NCI-ADR/RES) retain sensitivity to amrubicin. Doxorubicin accumulation in drug-resistant NCI-ADR/RES and MES-SA-DX5 cells was significantly less compared with amrubicin in the same

cells. Similarly, low doxorubicin accumulation has been reported in other MDR cell lines [30]. Interestingly, we found no evidence for decreased rates of amrubicin accumulation in the doxorubicin-resistant cell lines compared with the parental cell lines. Our data indicate that in both doxorubicin-sensitive and doxorubicin-resistant cells, amrubicin is predominantly localized in the cytoplasm (with 20–30% distribution in the nuclei). Based on this nucleocytoplasmic distribution, we hypothesize that amrubicin exerts cytotoxicity by interaction with the cell membrane and cytoplasmic compartments, as well as with DNA and topo-II in the nucleus. Interestingly, previous reports have suggested that other anthracyclines may also act through regulating both internal and external membrane structures in the cell, such as binding to the phospholipid cardiolipin on the mitochondrial membrane and peroxidation of membrane-unsaturated lipids in the cytoplasmic compartment [31].

Our data are similar to those reported by Yamaoka et al. [32] who observed that amrubicin remained active and cytotoxic in doxorubicin-resistant P388 murine leukemia cells. Although amrubicin could be a weak P-glycoprotein substrate, our experiments demonstrate that P-glycoprotein inhibitors do not affect the accumulation of amrubicin in doxorubicin-resistant cells. This may be due to the high intrinsic permeability (Caco-2 transporter assay, permeability coefficient [Papp A-to-B = 17]; unpublished data) and lipophilic properties of amrubicin. Thus, one mechanism to explain amrubicin sensitivity in MDR tumor cells may be that drug efflux via P-glycoprotein is not a rate-limiting step for amrubicin as it is for doxorubicin and other anthracyclines. We observed a similar lack of effect of an MRP1 inhibitor (MK571) on the accumulation of amrubicin in the H69-AR cells that overexpress MRP1 (Online Resource 3).

Mammalian cells possess two genetically distinct topo-II isoforms, both of which are targeted by anthracyclines. Several studies point to the expression and activity of the topo-IIα isoform as the isoform involved in resistance to topo-II inhibitors. Interestingly, a recent study demonstrated an absence of correlation between the chemosensitivity of amrubicin and mRNA expression levels of the RanBP2, topo-IIα, and topo-IIβ genes [33]. RanBP2 is a small ubiquitin-like modiWer (SUMO) ligase for DNA topo-II and plays an important role in targeting topo-II to centromeres during mitosis and in maintaining chromosome stability. Although we observed lower levels of topo-IIα in doxorubicin-resistant NCI-ADR/RES cells compared with its drug-sensitive parental line (OVCAR8; data not shown), both cell lines were equally sensitive to amrubicin, therefore suggesting that topo-II expression is not a factor in sensitivity to amrubicin.

Topo-IIα inhibitors induce enzyme-linked DNA breaks, and the resulting DNA damage can lead to cell cycle arrest, induction of apoptosis, and mitotic cell death [34, 35]. The data presented here demonstrate that amrubicin, unlike doxorubicin, is active in classical anthracycline-resistant tumor cell lines and induces DNA damage, G_2–M cell cycle arrest, and apoptosis. Amrubicin also inhibits growth of drug-resistant primary human tumor explants. Thus, we confirm that amrubicin is more efficacious than doxorubicin in doxorubicin-resistant cells. Consistent with these functional data, gene expression profiling demonstrated that amrubicin upregulated several genes known to promote cell cycle arrest, DNA repair (e.g., *CDKN2B* and *GADD45*), or apoptosis (e.g., tumor necrosis factor α-induced protein 3, Fas, HRK, Bim, PARP family protein, and caspases) in doxorubicin-resistant NCI-ADR/RES cells. *CDKN2B* and *GADD45* are p53-dependent genes that control G_1–S or G_2–M cell cycle arrest following DNA damage and permit DNA repair before replication or mitosis [36, 37].

Loss of DNA repair function is also believed to result in resistance to anthracyclines [38–40]. Amrubicin cytotoxicity in doxorubicin-resistant cell lines significantly correlated with its ability to induce DNA damage repair genes. For example, *TP53* is a DNA repair gene that is distinctly regulated by amrubicin, but not by doxorubicin or etoposide in NCI-ADR/RES cells. *TP53* is a major player in DNA damage response and apoptosis, and several studies have shown *TP53* upregulation following treatment with an anthracycline [39, 41].

In summary, our data indicate that amrubicin is capable of potent antitumour effects even in doxorubicin-resistant tumor models by inducing cell cycle arrest, DNA damage, and apoptosis. Therefore, amrubicin, although similar in structure to doxorubicin, is likely to have a different cellular mode of action and may be useful in treating patients who are resistant or refractory to classical anthracyclines.

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