

Overcoming acquired doxorubicin resistance of ovarian carcinoma cells by verapamil-mediated promotion of DNA damage-driven cytotoxicity

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Abstract. The efficacy of anticancer therapeutics is limited by acquired drug resistance of tumor cells. The present study aimed to characterize and overcome resistance mechanisms to the anthracycline derivative doxorubicin (Doxo). To this end, comparative analyses of Doxo-induced stress responses of parental A2780 ovarian carcinoma cells and Doxo-resistant A2780ADR variants were performed. A2780ADR cells revealed cross-resistance to multiple compounds, including anticancer drugs [cisplatin (CisPt) and etoposide (Eto)] and DNA repair/DNA damage response (DDR) inhibitors (olaparib, niraparib, entinostat, prexasertib and rabusertib). A2780ADR cells formed markedly fewer DNA double-strand breaks (DSB) following Doxo exposure compared with parental A2780 cells, resulting in a mitigated DDR, reduced proliferation inhibition and attenuated apoptosis. Potential resistance mechanisms identified to contribute to Doxo resistance of A2780ADR cells include increased Doxo efflux due to increased multi-drug resistance gene 1 (MDR1) expression and reduced

topoisomerase II α protein expression. Substantial resensitization of A2780ADR cells to Doxo was achieved by both the RAC1 GTPase inhibitor EHT1864, the histone deacetylase inhibitor entinostat (EST) and, most effectively, the calcium channel blocker verapamil (Ver). Notably, Ver-mediated sensitization also pertains to Eto and CisPt. The synergistic effect of Ver in combination with Doxo, which is reflected by low combination index (CI<0.8), probably involves inhibition of MDR1-mediated drug export, increased intracellular steady state levels of Doxo and elevated DSB formation, eventually promoting pro-toxic mechanisms of the DDR. However, combination treatment with Doxo and Ver also increased the cytotoxic response of non-malignant murine cardiomyocytes, murine embryonic stem cells and human induced pluripotent stem cells. Taken together, the present study suggested inhibition of MDR1-mediated Doxo efflux by Ver a useful approach to overcome acquired drug resistance of A2780ADR cells by stimulating DDR-related cytotoxicity, yet at the price of a potentially increased risk of normal tissue toxicity.

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Abbreviations: ATM, Ataxia telangiectasia mutated; ATR, ATM- and Rad-3 related; cAT, conventional anticancer therapeutics; Chk1/2, checkpoint kinase 1/2; CisPt, cisplatin; DDR, DNA damage response; Doxo, doxorubicin; DSB, DNA double-strand breaks; EHT, Rac1 inhibitor EHT1864; Eto, etoposide; EST, entinostat; ERK2, extracellular regulated kinase 2; γ H2AX, Ser139 phosphorylated histone H2AX; HDACi, histone deacetylase inhibitor; hiPSC, human induced pluripotent stem cells; MDR1, multi-drug resistance gene 1; mESC, mouse embryonic stem cells; PARPi, poly (ADP-ribose) polymerase inhibitor; Ver, verapamil.

Key words: doxorubicin, anticancer drug resistance, DNA damage response, drug transport, verapamil

Introduction

The anticancer efficacy of tumor therapeutics is impaired by inherent or acquired tumor cell resistance. In addition, adverse effects on normal tissue limit the maximum possible cumulative dose of the anticancer drug that can be applied. Against this background, alternative and well-tolerated therapeutic options are needed. Anthracyclines are conventional (that is, genotoxic) anticancer therapeutics (cAT) which are used for the treatment of numerous malignancies, including hematological disorders, sarcomas and breast cancer (1). They impair the genetic integrity and thus the malignancy of tumor cells by inhibiting topoisomerase II (Topo II), which is essential for DNA replication. As a consequence of Topo II poisoning, DNA double-strand breaks (DSB) are formed, which effectively trigger mechanisms of cell death (2,3). DNA intercalation, inhibition of DNA helicases, disruption of mitochondrial functions and formation of reactive oxygen species (ROS) (4) also contribute to the antitumor effect of anthracyclines. Tumor cell resistance mechanisms are often agent-specific and were classified into pre-, on- and post-target mechanisms (5).

Pre-target resistance mechanisms, such as mechanisms of transport or detoxification, eventually reduce the level of drug-induced primary DNA damage and, in consequence, DNA damage-triggered cell death. With Doxo, overexpression of the drug exporter protein p-glycoprotein (P-gp/MDR1) is considered as an important mechanism of acquired Doxo resistance (6,7). However, as cells express a variety of different transporters (importers and exporters) for Doxo and other cAT (7,8), the outcome of anticancer drug treatment is ultimately determined by the combined activity/expression of multiple importers and exporters. Against this background and having in mind that cellular mechanisms contributing to acquired drug resistance in a genetically heterogeneous tumor cell population are probably manifold, it would be desirable to effectively target transport dependent (that is, pre-target mechanisms) and/or transport-independent (that is, on- or post-target) mechanisms that contribute to drug resistance. Since cAT-induced DNA damage induces a complex stress response termed DNA damage response (DDR), which regulates mechanisms of cell cycle progression, DNA repair and, finally, survival- and death-related pathways (3,9), factors of the DDR are considered as particular promising pharmacological targets to overcome inherent or acquired tumor cell resistance (10-12).

The DDR becomes fine-tuned by the PI3-like kinase Ataxia telangiectasia mutated (ATM) and the ATM and Rad3-related kinase (ATR) (13-15), with ATM being of particular relevance for the regulation of DSB-induced stress responses and ATR for replicative stress responses (16-18). By coordinating the activation of cell cycle checkpoints, DNA repair and cell death-related pathways, the ATM/ATR-regulated network represents the major molecular switch that defines the balance between survival and death (3). In line with this, ATM- and ATR-regulated pathways contribute to tumor cell resistance (19) and DDR modulating compounds have been proved as useful to improve anticancer therapy (20-23). In the case of oncogene-driven replicative stress, tumor cells are often particularly sensitive to compounds that impair a coordinated replicative stress response, thereby eventually enforcing replication fork collapse and death (21,22,24-27). Alterations in DNA repair provides another Achilles' heel for personalized anticancer therapy as reflected by synthetic lethality (28-30). Here, defective DSB repair by homologous recombination, for example due to hereditary breast cancer associated gene 1/2 deficiency (BRCAness), predicts the hypersensitivity of malignant cells to inhibition of PARP-related backup DNA repair pathways by PARP inhibitors (such as olaparib or niraparib) (31,32).

The present study used ovarian carcinoma cells (A2780ADR) as *in vitro* model of acquired Doxo resistance (33). It comparatively characterized i) the stress responses of wild-type A2780 and drug resistant A2780ADR cells to Doxo treatment, ii) the cross-resistance of A2780ADR variants to other anticancer drugs (Eto and CisPt) as well as to a set of candidate compounds interfering with DDR/DNA repair, RAC1 GTPase signaling or drug transport and iii) the outcome of a combined treatment of A2780ADR cells with Doxo plus the aforementioned inhibitors. Thereby, the present study aimed to identify compounds that are particularly effective to overcome acquired Doxo resistance of malignant cells.

Materials and methods

Materials. Chemicals were obtained from the following providers: Entinostat (MS-275) was obtained from Selleck Chemicals, Doxo from STADA Consumer Health & STADAPHARM GmbH, etoposide, Etoposide, Etoposide, Etoposide (AZD-7762) and dexamethasone were from MilliporeSigma, cisplatin from Accord Healthcare GmbH, olaparib from APeXBIO Technology LLC, niraparib from MedChemExpress, EHT1864 was purchased from Tocris Bioscience, rabeprazole (LY2603618) and ricolinostat (ACY-1215) from MedChemExpress and verapamil (Ver) from Thermo Fisher Scientific, Inc. The following primary antibodies were used: Copper transporting ATPase (ATP7A), extracellular regulated kinase 2 (ERK2), phosphorylated (p)-histone H3 (Ser10) from Thermo Fisher Scientific Inc., cleaved caspase-7 (Asp198), p-Chk1 (Ser 345), cyclin B1, galactosidase β (E2U2I), GAPDH (14C10), MDR1/ABCB1 (D3H1Q), p-P53 (S15), PARP, TopBP1(D8G4L), topoisomerase IIa (D10G9), 53BP1 and Ki67 were from Cell Signaling Technology Inc., pChk2 (T68) [Y171], copper uptake protein 1 (CTR1/SLC31A1) [EPR7936] and Rad51 from Abcam, γ H2AX (Ser 139) clone JBW301, p-KAP-1 (S824) and p-RPA32 (S4/S8) from Bethyl Laboratories Inc., organic cation transporter-2 (OCT2) from Biozol Diagnostics Vertrieb GmbH, p16 (F-12) and p21 (C-19) from Santa Cruz Biotechnology, Inc. As secondary antibodies, horseradish peroxidase-conjugated secondary antibodies goat anti-mouse IgG and mouse anti-rabbit IgG were used (Rockland Immunochemicals Inc.).

Cell culture and treatment of cells. Parental A2780 ovarian carcinoma cells (A2780) as well as a doxorubicin resistant variant (A2780ADR) were from the European Collection of Authenticated Cell Cultures and were cultured in RPMI-1640 medium (MilliporeSigma) containing 10% fetal calf serum, 1% glutamine and 1% Pen/Strep at 37°C in a humidified atmosphere containing 5% CO₂. Cells were authenticated by STR profiling during the last three years. All experiments were performed with mycoplasma free cells. Immortalized HL-1 cardiomyocytes were provided by Professor W.C. Claycomb (Louisiana State University, New Orleans, LA, USA) (34) and were grown on gelatin (2 mg/ml)/fibronectin (1 mg/ml) (Sigma Aldrich; Merck KGaA) coated dishes and maintained in Claycomb medium, supplemented with 10% FBS and 100 μ M norepinephrine (Sigma Aldrich; Merck KGaA). Mouse embryonic stem cells (ESC; LF2) were isolated from the mouse strain 129J (35) and were from Professor A. Smith (University of Oxford, UK). They were cultivated under feeder-free conditions on 0.1% gelatin-coating using knock-out Dulbecco's Modified Eagle's Medium (KO-DMEM) (Gibco; Thermo Fisher Scientific, Inc.) supplemented with knock-out serum replacement (15%) (Gibco; Thermo Fisher Scientific, Inc.), penicillin/streptomycin (1%), glutamax (1%), β -mercaptoethanol (5×10^{-5} M) (Invitrogen; Thermo Fisher Scientific, Inc.) and leukemia inhibitory factor (LIF; MilliporeSigma) (1,000 U/ml) at 37°C in an atmosphere containing 5% CO₂. b4-hiPSC were generated from human foreskin fibroblasts as previously described (36). Cells were cultured on plates coated with reduced growth factor basement membrane matrix (Gibco; Thermo Fisher Scientific, Inc.) in

StemMacs medium (Miltenyi Biotec GmbH) supplemented with 10 mM Y-27632 dihydrochloride (MilliporeSigma).

Determination of cell viability. Cell viability was determined using the Alamar blue assay (37). Viable cells are characterized by an effective mitochondrial metabolism of the non-fluorescent dye resazurin (Sigma-Aldrich; Merck KGaA) to fluorescent resorufin (excitation: 535 nm, emission: 590 nm). Relative viability in the untreated control was set to 100%. If not stated otherwise, data are shown as the mean $\pm$ standard deviation (SD) of ≥ 3 independent experiments, each performed in biological quadruplicates. The combination index (CI) was determined (38) for the calculation of additive ($CI > 0.8 < 1.2$), synergistic ($CI \leq 0.8$) or antagonistic ($CI \geq 1.2$) drug interactions in the co-treatment experiments.

Analysis of doxorubicin import and export. Doxo import and export were measured by exploiting the inherent red fluorescence of Doxo as previously described (39). Briefly, following 2 h of pulse-treatment with different Doxo concentrations, the fluorescence of the cells was measured as a surrogate marker of drug uptake by flow cytometry (excitation: 450 nm, emission: 560 nm). After a post-incubation period of up to 6 h in the absence of Doxo, the residual intracellular fluorescence was again measured by flow cytometry. The time dependent decrease in Doxo fluorescence was calculated as a surrogate marker of Doxo export.

Cell cycle analysis by flow cytometry. For flow cytometry-based analysis of cell cycle distribution, cells were trypsinized and combined with floating cells present in the medium. Cells were pelleted (1,000 $\times$ g, 10 min, 4°C) and suspended in PBS. DNase-free RNase A (SERVA Electrophoresis GmbH) was added (2 μ g/ml, 1 h at room temperature). DNA was stained with propidium iodide (PI) (Sigma-Aldrich; Merck KGaA) for 20 min in the dark. Cell number was adjusted to 10^6 cells/ml with PBS and analysis was performed using BD Accuri C6 flow cytometer (BD Biosciences).

Analysis of apoptosis and senescence. Cell death by apoptosis was monitored by flow cytometry-based quantitation of the subG₁ fraction, which represents the apoptotic cell fraction. In addition, cleavage of PARP protein and pro-caspase-7 was monitored by western blotting. To monitor senescence β -GAL expression was analyzed by western blotting.

Analysis of proliferation. To monitor proliferation, the incorporation of the nucleoside analogue 5-ethynyl-2'-deoxyuridine (EdU) into S-phase cells as well as the percentage of pH3 (Ser10) positive cells (mitotic index) and Ki-67 positive cells were determined. To this end, cells were seeded on cover slips and cultivated for the indicated time period. EdU-incorporation was analyzed using the EdU-Click 488 Kit (baseclick GmbH), which is based on a pulse-labeling of S-phase cells with 10 μ M EdU according to the manufacturer's protocol. To determine the mitotic index, cells were fixed with 4% formaldehyde/PBS followed by incubation PBS -0.3% TritonX-100 (5 min; room temperature). After blockage of unspecific binding (5% BSA in 0.3% Triton X-100/PBS (1 h; room temperature), anti-Ser10 phosphorylated histone H3

antibody (pH3; Thermo Fisher Scientific Inc.; dilution 1:1,000; 16 h; 4°C) and Ki-67 antibody (Cell Signaling Technology Inc.; dilution 1:500; 16 h; 4°C) were added. Incubation with Alexa Fluor® 488 labeled goat anti-rabbit and Alexa Fluor 555 labeled goat anti-mouse secondary antibody was performed for 120 min at room temperature. pH3- and Ki-67-positive cells were counterstained with DAPI-containing Vectashield (Vector Laboratories, Inc.) and analyzed by Olympus BX43 microscope (40x objective) (Olympus Corporation).

Immunocytochemistry-based analysis of DSB formation. The frequency of nuclear foci formed by S139 phosphorylated H2AX (γ H2AX foci) is a commonly used surrogate marker of DSBs (40,41) and was assayed by immunocytochemistry-based method. The appearance of nuclear 53BP1 foci, which is another marker of DSBs (42,43), was also determined by immunocytochemistry. Upon treatment of cells grown on cover slips, cells were fixed with 4% formaldehyde in phosphate-buffered saline (PBS; Merck KGaA; 15 min; room temperature) followed by permeabilization by incubation in PBS -0.3% TritonX-100 (5 min; room temperature). After blocking [1 h; room temperature; blocking solution: 5% BSA (Merck KGaA) in PBS/0.3% Triton X-100 (Sigma-Aldrich; Merck KGaA)], incubation with primary γ H2AX antibody (1:2,000; cat. no. 07-727; MilliporeSigma) and 53BP1 antibody (1:500; cat. no. 4937S; Invitrogen; Thermo Fisher Scientific, Inc.) was performed overnight (4°C), followed by incubation with the secondary fluorescence-labelled antibody [Alexa Fluor 488 goat-anti-rabbit IgG (H+L); cat. no. A11008; Invitrogen; Thermo Fisher Scientific, Inc.] (1:500, 2 h; room temperature, in the dark). Cells were mounted in Vectashield Mounting medium (anti-fading; Vector Laboratories, Inc.) containing the blue fluorescent DNA stain 4',6-Diamidin-2-phenylindol (DAPI; cat. no. H-1200; Biozol Diagnostics Vertrieb GmbH) at room temperature and the number of nuclear γ H2AX foci and 53BP1 foci was scored using an Olympus BX43 fluorescence microscope (Olympus Corporation). Only nuclei with distinct foci were evaluated. γ H2AX foci pan-stained nuclei, which are indicative of apoptotic cells, were excluded from the analyses. If not stated otherwise, data are shown as the mean $\pm$ SD from three independent experiments with each ≥ 50 nuclei analyzed per experimental condition.

Western blotting. The activation of the DDR was investigated by western blotting using total cell extracts obtained by lysing an equal number of cells in 150 μ l RIPA buffer (20 min on ice). RCD-CD-Protein Assay (cat. no. 500-0120; Bio-Rad Laboratories, Inc.) was used for protein determination. After sonication (6 KHz; 5x2 sec; on ice) (EpiShear Probe sonicator; Active Motif, Inc.) and centrifugation (10,000 $\times$ g; 4°C; 10 min), Roti®-Load buffer (5 min; room temperature) was added to the supernatant and proteins were denatured by heating (5 min, 95°C). Afterwards, 20 μ g protein was loaded per lane and proteins were separated by SDS-PAGE (6 or 12.5% gel) and transferred onto a nitrocellulose membrane (Cytiva) via the Protean Mini Cell System. After blocking [5% non-fat milk in TBS/0.1% Tween 20 (Merck KGaA; 2 h; room temperature)], the membrane was incubated with the corresponding primary antibody (1:1,000; overnight; 4°C). The following primary antibodies were used: ATP7A (cat. no. PA5-103110; Invitrogen;

Thermo Fisher Scientific; Inc.), Caspase-7 cleaved (Asp198; cat. no. 9491S; Cell Signaling Technology, Inc.), Chk1phospho (Ser345; cat. no. 2341, Cell Signaling Technology, Inc.), Chk2phosphoT68 (Y171; cat. no. ab32148; Abcam), CTR1/SLC31A1 (EPR7936; cat. no. ab129067; Abcam), Cyclin B1 (cat. no. 4138, Cell Signaling Technology, Inc.), ERK2 [PA5-32396, Invitrogen/Thermo Fisher Scientific; Carlsbad, USA], Galactosidase beta (E2U21) (cat. no. 27198, Cell Signaling Technology, Inc.), GAPDH (14C10; cat. no. 2118S; Cell Signaling Technology, Inc.), H2AX phospho (Ser139, cloneJBW301; cat. no. 05-636, Merck KGaA), MDR1/ABCB1 (D3H1Q; cat. no. 12683; Cell Signaling Technology, Inc.), OCT2 (cat. no. MBS9600162, Biozol Diagnostics Vertrieb GmbH), P16 (F-12; cat. no. sc-1661; Santa Cruz Biotechnology, Inc.), p21 (C-19; cat. no. sc-397; Santa Cruz Biotechnology, Inc.), P53 phospho (S15; cat. no. 9284S; Cell Signaling Technology, Inc.), PARP (cat. no. 9542S, Cell Signaling Technology, Inc.), Rad51 (cat. no. ab63801; Abcam), RPA32 phospho (S4/S8; cat. no. ICH-00422; Bethyl Laboratories Inc.), TopBP1 (D8G4L; cat. no. 14342, Cell Signaling Technology, Inc.), Topoisomerase II alpha (D10G9; cat. no. 12286, Cell Signaling Technology, Inc.). After washing (TBS/0.1% Tween 20), the secondary (peroxidase-conjugated) antibody was added (1:2,000; 2 h; room temperature) [Anti-mouse IgG (H&L; goat) Antibody Peroxidase Conjugated (cat. no. 610-1302, Rockland Immunochemicals, Inc.) or Anti-rabbit IgG (H&L; goat) Antibody Peroxidase Conjugated (cat. no. 611-1302, Rockland Immunochemicals, Inc.)]. The ChemiDoc Touch Imaging System (Bio-Rad Laboratories, Inc.) was used for visualization of the bound antibodies. Image Lab 6.1.0 build 7 (Bio-Rad Laboratories, Inc.) was used for densitometrical analyses. Changes in protein expression of factors of interest were identified by normalization to the expression of a housekeeping protein (such as ERK2).

Reverse transcription-quantitative (RT-q) PCR. Total RNA was purified from up to 5×10^6 cells per preparation using the RNeasy Mini Kit (Qiagen, Hilden, Germany), followed by reverse transcriptase reaction with High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Inc.). RNA extraction, cDNA synthesis and qPCR were performed according the manufacturer's protocols. For each PCR reaction 40 ng of cDNA and 0.25 μ M of the corresponding primers (Eurofins MWG Synthesis GmbH) were used. Quantitative real-time PCR analysis was performed in triplicates using the SensiMix SYBR Hi-ROX Kit (Meridian Bioscience, Inc.) and a CFX96 Real-Time System (Bio-Rad Laboratories, Inc.) with the Bio-Rad CFX Manager 3.1 software. PCR runs (35–40 cycles) were performed as follows: 95°C, 10 min; 95°C, 15 sec; 60°C, 30 sec; 72°C, 40 sec; 72°C, 10 min. Melting curves were recorded to ensure the specificity of the amplification reaction. mRNA levels were normalized by geometric averaging using β -actin and GAPDH as internal control genes as reported (44). Unless stated otherwise, relative mRNA expression of untreated control cells was set to 1.0. Primer sequences used for RT-qPCR analyses are depicted in Table S1.

Statistical analyses. Student's t-test and the One-way ANOVA with Dunnett's post-hoc test were employed to confirm statistically significant differences between different experimental

groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Doxorubicin-resistant (A2780ADR) ovarian cancer cells are cross-resistant to the anticancer drugs etoposide and cisplatin. The present study employed parental ovarian cancer cells (A2780) and thereof derived Doxo resistant variants (A2780ADR). Comparative analysis of their viability following Doxo treatment for 72 h revealed IC_{50} of 0.04 and 0.38 μ M for A2780 and A2780ADR, respectively (Fig. 1A). A2780ADR cells revealed a profound cross-resistance to the Topo II inhibitor etoposide (Eto; IC_{50} A2780: 0.14 μ M; IC_{50} A2780ADR: 0.92 μ M; Fig. 1B), while being only weakly cross-resistant to the platinating agent cisplatin (CisPt; IC_{50} A2780: 0.62 μ M; IC_{50} A2780ADR: 1.91 μ M; Fig. 1C). Based on their corresponding IC_{50} , A2780ADR cells are characterized by a ~ 10 , ~ 7 and ~ 3 -fold higher resistance to Doxo, Eto and CisPt, respectively, as compared with the parental A2780 cells. Analyzing viability following a 24 h treatment period, no major differences were observed as concluded from the calculation of the corresponding IC_{50} values (Fig. S1).

Doxorubicin-resistant (A2780ADR) ovarian cancer cells are characterized by altered expression of drug transporters and enhanced doxorubicin export. Altered drug transport, as mediated for instance by p-glycoprotein, is one possible mechanism contributing to acquired drug resistance of cancer cells (45,46). Analyzing the mRNA expression of various drug importers and exporters, an elevated mRNA expression of the importer CTR2 and a reduced mRNA expression of the exporter BCRP was observed in Doxo resistant A2780ADR cells as compared with the wild-type cells (Fig. 1D). Notably, while the mRNA expression of MDR1 was clearly detectable in A2780ADR cells, it was below detection limit in A2780 cells (Fig. 1D). Lack of MDR1 expression in the parental cells and high expression in A2780ADR was confirmed on the protein level (Figs. 1E and S2). To monitor the cells' activity of drug import and export, the present study took advantage of the inherent reddish fluorescence of Doxo and comparatively analyzed alterations in the intracellular fluorescence of A2780 and A2780ADR cells following Doxo treatment. The data show a similar increase in fluorescence in both cell lines after 2 h of Doxo pulse-treatment (Fig. 1F), indicating that both cell lines have comparable Doxo uptake capacity (i.e. import). However, analyzing the remaining intracellular Doxo concentration after a subsequent 6 h post-incubation period in the absence of Doxo, residual fluorescence was markedly lower ($\sim 50\%$) in A2780ADR as compared with parental A2780 cells (Fig. 1F). This finding showed that A2780ADR cells were characterized by an about twice as fast drug export as A2780 parental cells, which is very probably due to their elevated MDR1 expression as concluded from the results of the mRNA and protein expression analyses. Thus, increased drug export probably contributes to the high Doxo resistance of A2780ADR cells. However, having in mind the ~ 10 -fold higher Doxo resistance of A2780ADR cells as compared with A2780 cells, it was hypothesized that mechanisms other than just drug export additionally contributed to their pronounced

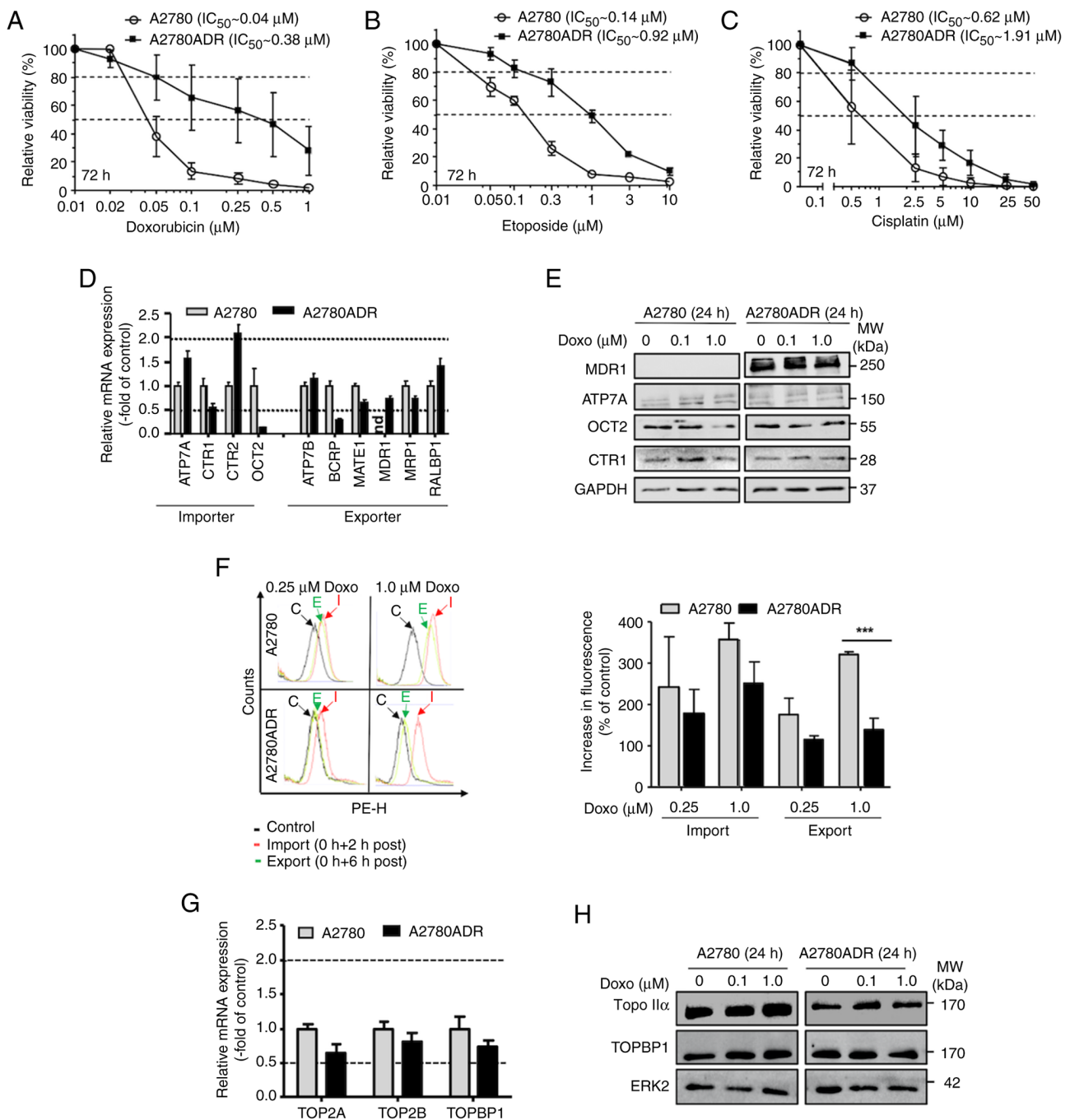


Figure 1. Comparative analysis of the response of A2780 and A2780ADR cells to treatment with anticancer drugs (Doxo, Eto and CisPt) and effect of mechanisms of drug transport. Logarithmically growing parental A2780 and A2780ADR variant cells were treated with the anticancer drugs (A) Doxo, (B) Eto and (C) CisPt at the indicated concentrations. At 72 h after drug addition, viability was monitored by use of the AlamarBlue assay as described in methods. Data shown are the mean $\pm$ SD from three independent experiments each performed in biological quadruplicates (n=3; n=4). Dashed lines indicate inhibitory concentrations (IC₂₀ and IC₅₀). For viability data (IC₅₀) after 24 and 72 h of treatment also see Fig. S1 and Table SII. (D) Comparative analysis of the mRNA expression of selected transporters in A2780 and A2780ADR. Data shown are the mean $\pm$ SD from triplicate determinations. mRNA expression of transporters was normalized to GAPDH mRNA levels and set to 1.0 in the parental A2780 cells. The dashed lines indicate changes in mRNA levels of ≥ 2.0 and ≤ 0.5 , which are considered as biologically relevant. (E) Comparative analysis of the protein expression of representative drug transporters under basal situation and after 24 h treatment with Doxo (0.1 and 1.0 μM). Data shown are from a representative western blotting using ERK2 protein levels as loading control. Data obtained after 72 h are presented in Fig. S2 (left panel). (F) Intracellular Doxo fluorescence was measured by flow cytometry-based methods after 2 h Doxo pulse-treatment (0.25 and 1.0 μM) and was taken as indicative of drug import. To measure drug export, Doxo pulse-treated cells were post-incubated for 6 h in the absence of the drug before fluorescence was monitored. Data shown in the left panel are representative results obtained from flow cytometry analyses. C, control; I, import; E, export. The histogram in the right panel depicts quantitative data obtained from n=3 independent experiments each performed in biological triplicates (n=3). ***P≤0.001. (G) Analysis of basal mRNA expression of topoisomerase II isoforms TOP2A, TOP2B and TopBP1. Data shown are the mean $\pm$ SD from triplicate determinations. Relative mRNA level in A2780 cells was set to 1.0. The dashed lines indicate changes in mRNA levels of ≥ 2.0 and ≤ 0.5 , which are considered as biologically relevant. (H) Comparative analysis of the protein expression of TOP2A and TopBP1 under basal situation and after 24 h treatment with Doxo (0.1 μM, 1.0 μM). Data shown are from a representative western blotting using ERK2 as protein loading control. Data obtained after 72 h Dox treatment are presented in Fig. S2 (right panel). Doxo, doxorubicin; Eto, etoposide; CisPt, cisplatin; SD, standard deviation; Nd, not detectable TOP2A, topoisomerase IIα; TOP2B, topoisomerase IIβ; TOPBP1, topoisomerase binding protein 1; MDR1, multi-drug resistance gene 1; ATP7A, copper transporting ATPase; OCT2, organic cation transporter-2; CTR1, copper uptake protein 1; Topo IIa, topoisomerase IIα; ERK2, extracellular regulated kinase 2.

drug resistant phenotype. Since Topo II α and II β are relevant primary targets for the anticancer efficacy of Doxo, we additionally investigated their mRNA and protein expression. A2780 and A2780ADR cells revealed similar mRNA levels of Topo II α , Topo II β and Topoisomerase II β binding protein (TopBP1; Fig. 1G). On the protein level, A2780ADR were characterized by a reduced expression of Topo II α protein under basal situation and 24-72 h following Doxo treatment (Figs. 1H and S2). Overall, these data indicated that both alterations in drug transport catalyzed by MDR1 and the protein expression of Topo II α contributed to the strongly enhanced Doxo resistance of A2780ADR cells as well as to their profound cross-resistance to etoposide.

Analyses of proliferation and cell cycle progression of A2780 and A2780ADR cells following treatment with topoisomerase II inhibitors. To further analyze the outcome of altered drug export in A2780 vs. A2780ADR cells, cell cycle progression was analyzed after 24-72 h of Doxo and Eto treatment. At early time point of analysis (that is, 24 h), A2780 revealed a more pronounced accumulation of cells in G₂/M phase compared with A2780ADR (Fig. 2A). This effect was seen both upon both Doxo and Eto treatment (Fig. 2A). Notably, SubG₁ fraction was not yet enhanced following Doxo or Eto treatment in A2780 cells at this early time point of analysis. Under basal situation, the percentage of SubG₁ phase cells was slightly higher at the 24 h time point of analysis as compared with 72 h. It was hypothesized that this minor effect reflected stress that resulted from the reseeding procedure. After an extended treatment period of 72 h, parental cells revealed a clear increase in the percentage of Doxo- and Eto-treated cells present in the SubG₁ fraction, which was not observed in the drug resistant A2780ADR variants (Fig. 2B). Taken together, these data showed that parental A2780 cells were hypersensitive to G₂/M blocking activity and apoptosis induction triggered by Topo II poisoning anticancer drugs.

To monitor proliferative activity, the percentage of Ki-67 positive cells was analyzed in both cell variants. In addition, mitotic index was calculated by determining the percentage of pH3 positive cells. The data obtained show a marked decrease in the percentage of Ki-67 positive A2780 parental cells following treatment with Doxo but not Eto (Fig. 2C). By contrast, measuring the mitotic index, a marked decrease in the frequency of pH3 positive cells was found in both parental cells and resistant variants following Doxo or Eto treatment (Fig. 2C). Measuring S-phase activity by analyzing the incorporation of EdU, a very strong reduction in the percentage of EdU positive parental cells upon both Doxo and Eto treatment it was again observed, which was much weaker in the resistant A2780ADR cells compared with the wild-type cells (Fig. 2D). Summarizing, the data show that A2780ADR cells are highly resistant to the antiproliferative activity of Topo II inhibitory compounds.

Comparative analyses of Doxo-induced formation of DSB and activation of DDR-related mechanisms in A2780 and A2780ADR cells. Inhibition of Topo II isoforms leading to the formation of DSB is considered as a major molecular mechanism underlying the anticancer activity of

Doxo (47,48). Measuring the formation of nuclear γ H2AX foci as well as 53BP1 foci and co-localized γ H2AX/53BP1 foci as surrogate markers of DNA damage (that is, DSB), a markedly lower number of DNA damage-associated foci in the A2780ADR cells as compared with the parental A2780 cells was observed, both after treatment with Doxo and Eto (Fig. 3A). Following high-dose treatment with Doxo, a high percentage of γ H2AX pan-stained A2780 cells was detectable, which was not observed in A2780ADR cells (Fig. 3A). These data show that the level of Doxo- and Eto-induced DSB was substantially reduced in the drug resistant variant compared with the parental cells. As DSBs are a potent trigger of mechanisms of the DDR, the present study next investigated the activation status of prototypical markers of the DDR by western blotting. An excessive increase in the DDR-related protein levels of γ H2AX, p-Kap1, p-Chk2 and p-p53 in Doxo treated A2780 parental cells only, both after Doxo treatment period of 24 and 72 h. By contrast activation of these DDR-related factors was not found in A2780ADR cells (Fig. 3B). Notably, parental cells were also characterized by a great increase in the protein expression of the senescence marker p21, which was not detectable in the Doxo resistant A2780ADR cells (Fig. 3B). Eto treatment of A2780 cells also caused a distinct increase in γ H2AX, p-P53 and p21 protein expression (Fig. S3).

Cross-sensitivity of parental (A2780) and doxorubicin-resistant (A2780ADR) ovarian carcinoma cells to various anticancer drugs and inhibitors of DNA repair and DDR. Aiming to overcome the Doxo resistance of A2780ADR cells, the present study investigated their response to selected inhibitors of DDR- and DNA repair-related mechanisms, which are considered as promising targets to improve anticancer therapy and to overcome acquired drug resistance of tumor cells (49-52). To this end, the present study comparatively investigated the outcome of a 72 h treatment period of A2780 parental and A2780ADR cells with Poly (ADP-ribose) polymerase (PARP) inhibitors (olaparib and niraparib), checkpoint kinase 1/2 (Chk1/2) inhibitors (prexasertib and rabusertib) and histone deacetylase (HDAC) inhibitors (entinostat and ricolinostat). In addition, the calcium channel blocker Ver, which is reported to interfere with drug transport (53), the catalytic topo II inhibitor dexrazoxane, which is able to protect the heart from Doxo-induced cardiotoxicity by depleting both topo II isoforms independent of metal chelation (54-56) and Rac1 GTPase inhibitors (EHT1864 and Ehop16), which are reported to interfere with Doxo-induced DNA damage formation and DDR activation (57,58), were included into the study. From the IC₅₀ it was concluded that the data revealed a clear cross-resistance of A2780ADR cells to the poly (ADP-ribose) polymerase inhibitor (PARPi) olaparib (>10-fold) and niraparib (>5-fold), the Chk1/2i prexasertib (~4-fold) and rabusertib (~2.5-fold) and the HDAC class I inhibitor entinostat (~3-fold; Fig. 4). A2780ADR cells were neither appreciably cross-sensitive nor hypersensitive to any of the other pharmacological modulators (such as ricolinostat, Ver, EHT1864, EHOP16 and dexrazoxane) employed. Measuring cell viability 24 h after drug treatment, no major differences were observed (Fig. S4). The IC₅₀ and cross-sensitivities are summarized in Table SII.

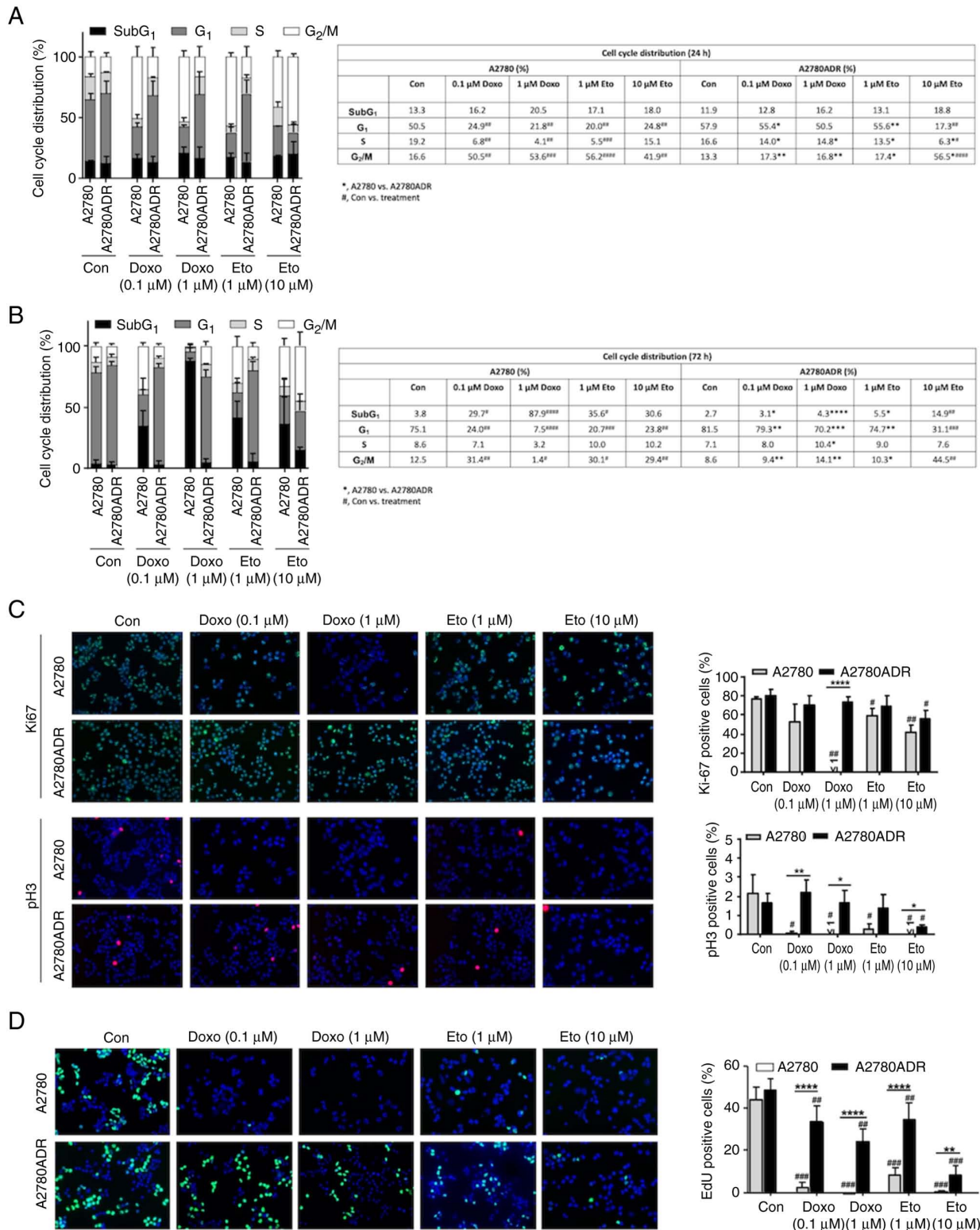


Figure 2. Cell cycle progression and proliferation following treatment of A2780 and A2780ADR cells with Topo II inhibitors. Logarithmically growing cells were treated with the indicated concentrations of Doxo or etoposide Eto for (A) 24 or (B) 72 h. Afterwards, cell cycle distribution was analyzed by flow cytometry and the percentage of cells present in different phases of the cell cycle (SubG₁-, G₁-, S- and G₂/M-phase) was quantified. Data shown in the histogram (left panel) are the mean $\pm$ SD from n=3 independent experiments each performed in biological triplicates. The table on the right panel summarizes the mean values and indicates statistical differences between the individual groups. *P $\leq$ 0.05; **P $\leq$ 0.01 (A2780 vs. A2780ADR); #P $\leq$ 0.05; ##P $\leq$ 0.01 (Con vs. treated group). (C) Logarithmically growing parental A2780 and Doxo resistant A2780ADR cells were treated with the indicated concentrations of Doxo (0.1 and 1.0 μ M) or Eto (1.0 and 10 μ M). At 24 h later the percentage of Ki-67 positive or pH3 positive cells was determined as described in methods. Total magnification, x400. Quantitative data shown in the histogram are the mean $\pm$ SD from n=3 independent experiments, each performed with n=5 biological replicates. *P $\leq$ 0.05; **P $\leq$ 0.01; ***P $\leq$ 0.0001 (A2780 vs. A2780ADR). Con vs. treatment: #P $\leq$ 0.05; ##P $\leq$ 0.01; ###P $\leq$ 0.001. (D) Logarithmically growing parental A2780 and Doxo resistant A2780ADR cells were treated with the indicated concentrations of Doxo (0.1 and 1.0 μ M) or Eto (1.0 and 10 μ M). At 24 h later cells were pulse-labeled with EdU for 2 h as described in Methods and the percentage of EdU positive cells was determined microscopically (total magnification, x400). Quantitative data shown in the histogram are the mean $\pm$ SD from five biological replicates. A2780 as compared with A2780ADR: **P $\leq$ 0.05; ***P $\leq$ 0.0001. Con vs. treatment: ##P $\leq$ 0.01; ###P $\leq$ 0.001. Doxo, doxorubicin; Eto, etoposide; SD, standard deviation.

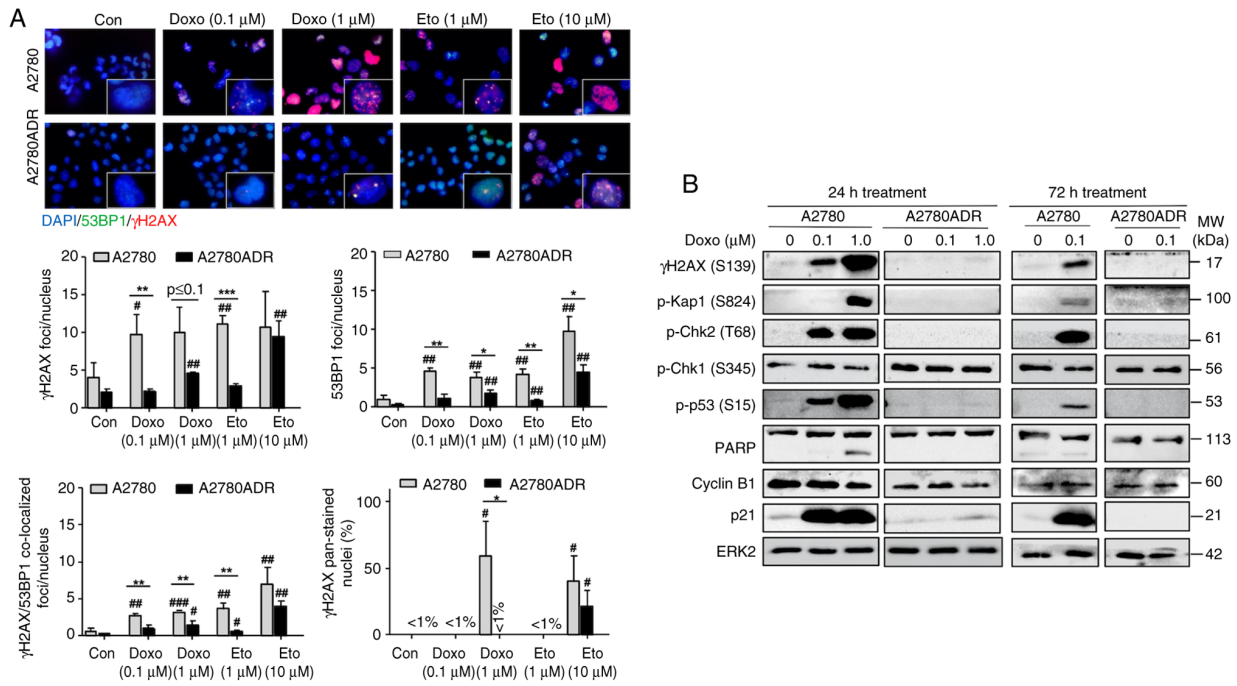


Figure 3. Effect of Topo II inhibitors on DNA damage formation and activation of DDR-related mechanisms in A2780 and A2780ADR cells. (A) At 24 h after treatment of logarithmically growing cells with the indicated concentrations of Doxo or Eto, the number of nuclear γH2AX-foci, 53BP1-foci, γH2AX/53BP1 co-localized foci and γH2AX pan-stained cells was analyzed. The upper part of the figure shows representative images (total magnification, x1,000). Quantitative data depicted in the histogram are the mean ± SD from n=3 independent experiments with each five images being analyzed per experimental condition. *P≤0.05; **P≤0.01; ***P≤0.001 (A2780 vs. A2780ADR); #P≤0.05; ##P≤0.01; ###P≤0.001 (treated vs. untreated control). Control experiments performed by use of 1st or 2nd antibody only or no antibody at all did not interfere with the signal of main interest (that is, nuclear foci; data not shown). (B) Logarithmically growing cells were treated with the indicated concentrations of Doxo for 24 or 72 h. Afterwards, the protein expression of DDR-related factors was analyzed by western blotting using EKR2 protein expression as loading control. Doxo, doxorubicin; Eto, etoposide; p-, phosphorylated; Chk1/2, checkpoint kinase 1/2; ERK2, extracellular regulated kinase; γH2AX, Ser139 phosphorylated histone H2AX; 2; Kap1, KRAB-associated protein 1; PARP, poly (ADP-ribose) polymerase; p21, cyclin-dependent kinase inhibitor 1; p53, tumor suppressor p53.

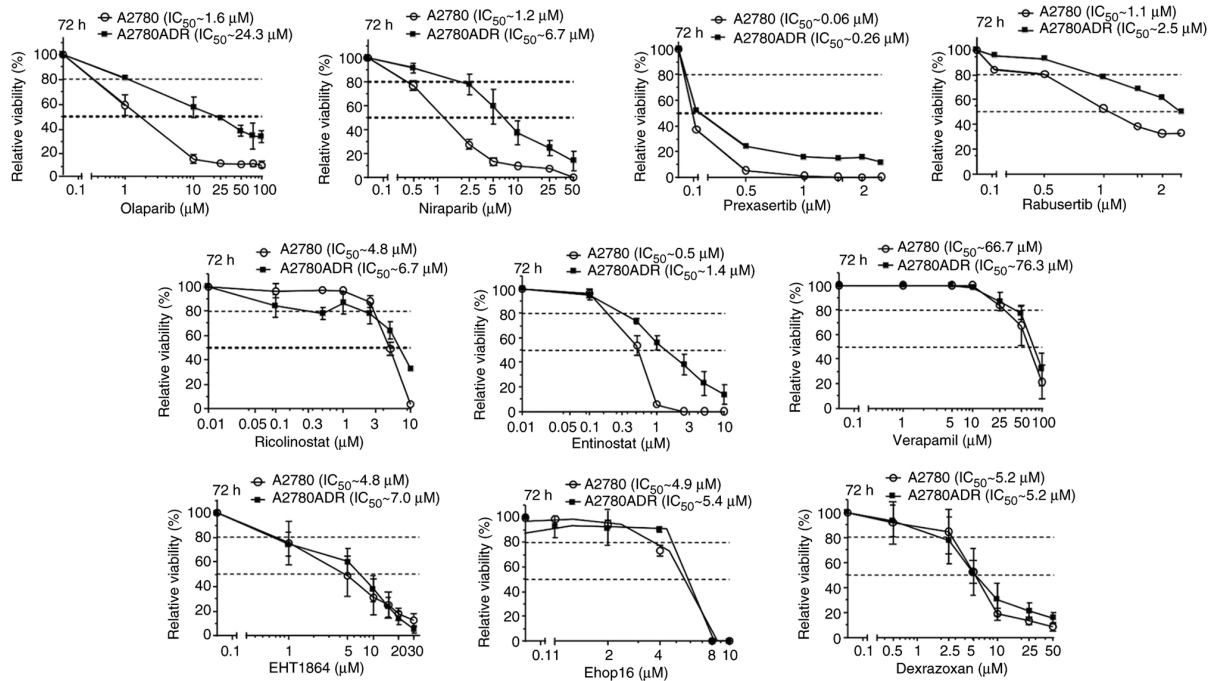


Figure 4. Analysis of cross-sensitivity of parental A2780 and Doxo-resistant A2780ADR cells to selected inhibitors of DDR- and DNA repair-related mechanisms. Logarithmically growing parental A2780 and A2780ADR variant cells were treated with selected pharmacological inhibitors of DNA repair (olaparib and niraparib), DDR (prexasertib and rabusertib), HDAC (ricolinostat and entinostat), Rac1 GTPase (EHT1864 and Ehop16), drug transport (verapamil) and Topo II (dexrazoxane) at the indicated concentrations. At 72 h after drug addition, viability was monitored by use of the AlamarBlue assay as described in methods. Data shown are the mean ± SD from three independent experiments each performed in biological quadruplicates (n=3; n=4). Dashed lines indicate inhibitory concentrations (IC₂₀ and IC₅₀). Data obtained from treatment period of 24 h are presented in Fig. S4. For IC₅₀ after 24 h and 72 h see Table SII. Doxo, doxorubicin; DDR, DNA damage response; HDAC, histone deacetylase; SD, standard deviation; EHT, Rac1 inhibitor EHT1864.

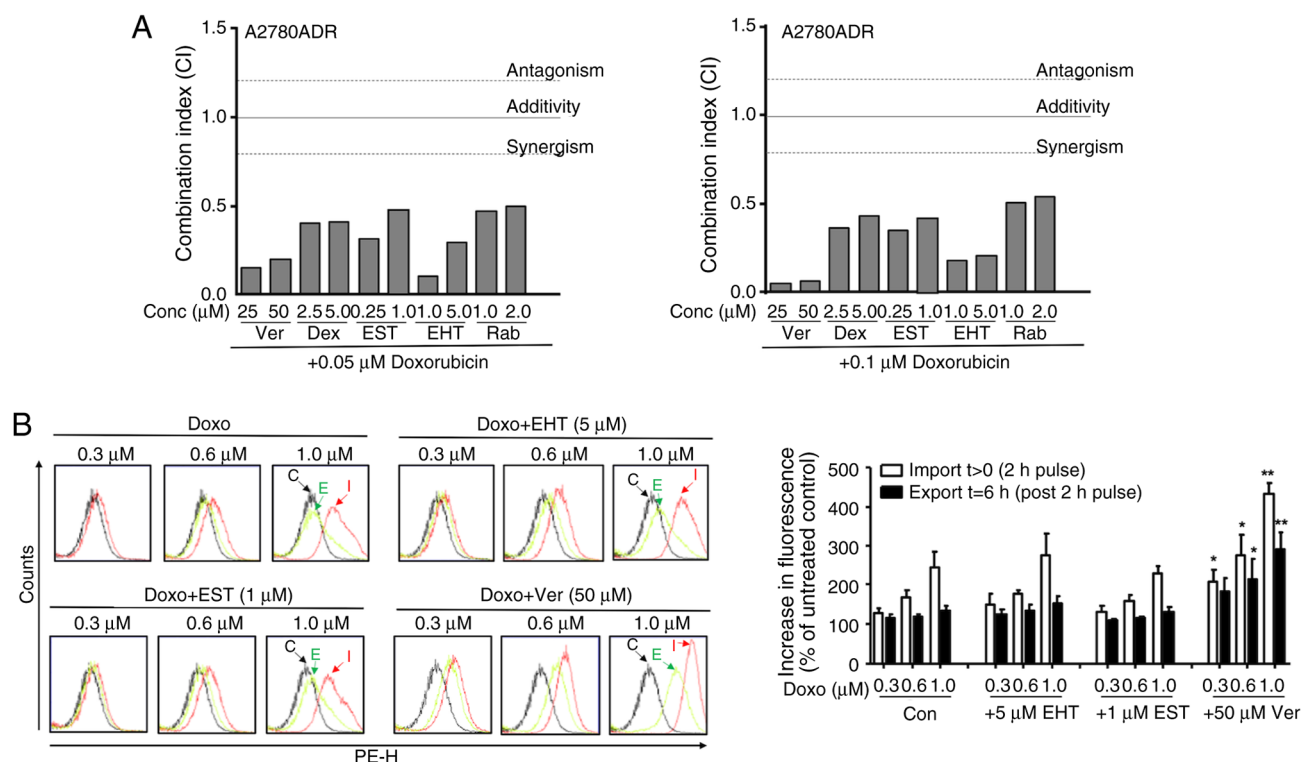


Figure 5. Combined treatment of A2780ADR with Doxo and selected inhibitors causes synergistic toxicity. (A) Logarithmically growing Doxo resistant A2780ADR cells were co-treated with Doxo and selected pharmacological inhibitors at the indicated concentrations. At 72 h after drug addition, viability was monitored by use of the AlamarBlue assay and CI was calculated as described in methods. Data shown are the mean $\pm$ SD from three independent experiments each performed in biological quadruplicates (n=3; n=4). (B) Intracellular Doxo fluorescence was measured by flow cytometry-based method after co-treatment with Doxo and selected pharmacological inhibitors as described in methods. To measure drug export, Doxo pulse-treated cells were post-incubated for 6 h in the absence of the drug before fluorescence was monitored. Data shown in the left panel are representative results obtained from flow cytometry analyses. C, control; I, import, E, export. The histogram in the right panel depicts quantitative data obtained from n=3 independent experiments each performed in biological triplicates (n=3). Statistical significance: *P $\leq$ 0.05; **P $\leq$ 0.001. Doxo, doxorubicin; CI, combination index; SD, standard deviation; EST, entinostat; EHT, Rac1 inhibitor EHT1864; Dex, dexrazoxane; Rab, rabeprazole; Ver, verapamil.

Co-treatment of doxorubicin resistant A2780ADR with DDR modifiers overcomes Doxo resistance by inducing synergistic toxicity. Based on the results of these extensive studies, the present study addressed the question whether the pharmacological inhibitors under investigation were able to overcome the acquired Doxo resistance of A2780ADR cells. To this end, entinostat, EHT1864, rabeprazole, dexrazoxane and Ver were selected for Doxo co-treatment analyses. Each of the selected inhibitors synergistically increased the cytotoxicity of Doxo in the drug resistant A2780ADR cells as concluded from the calculated CI (Fig. 5A). Based on the CI, the most pronounced synergistic toxicity was observed when Doxo was combined with the transport inhibitor Ver or the Rac1 inhibitor EHT1864 (Fig. 5A). The high efficacy of Ver was associated with increased intracellular steady state levels of Doxo under situation of co-treatment (Fig. 5B). It was not found if EHT or EST were used for Doxo co-treatment (Fig. 5B). These data indicated that synergistic toxicity resulting from combined treatment of A2780ADR cells with Doxo and Ver was at least partially due to Ver-mediated inhibition of drug export mechanisms leading to higher concentration of intracellular Doxo. By contrast, synergism observed upon use of EST and EHT1864 is suggested to be independent of drug transport. Notably, the data were in line with published data showing that Rac1 is involved in chemoresistance (59,60) and class I HDACi are useful of

overcome acquired anticancer drug resistance of malignant cells (61-64).

Notably, Ver also synergistically increased the cytotoxicity of Doxo in A2780 parental cells (Fig. S5A), showing that the Ver effect is not restricted to cells with acquired Doxo resistance but also pertains to parental tumor cells. The pharmacological inhibitors entinostat (EST), EHT1864 and rabeprazole (Rab) were also effective in combination with Doxo whereas dexrazoxane (Dex) was not (Fig. S5A). In addition, verapamil (Ver) also conferred synergistic toxicity in A2780ADR cells in combination with etoposide (Fig. S5B), showing that the Ver effect is not limited to Doxo but also comprises other anticancer drugs. Other inhibitors also conferred synergistic toxicity in combination with Eto, with EHT being most effective (Fig. S5B). To further investigate whether Ver is also able to overcome acquired resistance to anticancer drugs others than Topo II inhibitors, the present study investigated the influence of Ver on the CisPt sensitivity of CisPt-resistant A2780CisR cells, including EHT and EST for control. Data obtained show that all three modulators promoted CisPt-induced cytotoxicity in A2780CisR cells, with Ver and EST being most effective (Fig. S6).

In order to investigate whether the synergistic cytotoxicity evoked by combined treatment with Ver and Doxo was related to an elevated DNA damage induction, the present study measured the outcome of the co-treatments on the formation

of DSB. To this end, the number of nuclear γ H2AX and 53BP1 foci, which are indicative of DSB, was analyzed. Data obtained show that Ver most markedly increased the number of both nuclear γ H2AX and 53BP1 foci as well as γ H2AX/53BP1 co-localized foci compared with Doxo mono-treatment (Fig. 6A). In addition, the percentage of γ H2AX pan-stained cells was also markedly enhanced upon co-treatment with Ver and Doxo as compared with the mono-treatments (Fig. 6A). Apparently, Ver was able to markedly stimulate the formation of DSB if used in combination with Doxo, demonstrating that Ver potentiates the genotoxic effects of Doxo in A2780ADR cells. Analyzing proliferation by monitoring the percentage of EdU positive cells, the most substantial antiproliferative effects were also observed upon combining Doxo with Ver (Fig. 6B). In addition, Ver also markedly increased the percentage of PI positive cells if used in combination with Doxo (Fig. 6C), showing that Ver potentiates Doxo-induced toxicity.

Influence of combined treatment of A2780 and A2780ADR cells with Doxo and selected inhibitors on mechanisms of the DDR and mRNA expression of selected susceptibility-related genes. Aiming to further elucidate the molecular mechanisms underlying the observed synergistic toxicity and to substantiate the assumed increase in DSB formation under situation of Ver + Doxo co-treatment, the present study investigated the outcome of combined treatments on the activation status of a subset of DDR-related factors that are regulating replicative stress responses and cell death by western blotting. The data obtained showed that Ver especially was able to potentiate Doxo-mediated phosphorylation of the DDR-related factors p53, RPA32, Chk2 and H2AX, especially following 72 h of co-treatment (Fig. 7A). Moreover, if Doxo was combined with Ver, PAPR cleavage and cleavage of pro-caspase 7, which are indicative of the activation of apoptotic pathways, was observed (Fig. 7A). Thus, it was hypothesized that specific inhibition of drug transport by Ver conferred a broad re-sensitization of A2780ADR cells by fostering Doxo-induced formation of DSB and, in consequence, by activating DDR-dependent mechanisms that stimulated pro-apoptotic pathways. In addition, co-treatment of A2780ADR cells with Doxo plus Ver increased the mean mRNA expression of pre-selected factors involved in the regulation of oxidative stress response, DNA repair and cell cycle regulation compared with Doxo or Ver mono-treated control (Fig. 7B).

Influence of combined treatment on the viability of non-malignant cells. Irreversible cardiotoxicity is a dose-limiting adverse effect of anthracyclines (65,66). Hence, potentiating the anticancer efficacy of Doxo if combined with pharmacological modifiers brings up the concern of elevated side effects of the co-treatment, especially regarding heart damage. To address this aspect, the present study investigated the effect of combination treatments using immortalized murine HL-1 cardiomyocyte cells as an *in vitro* model. The results revealed more than additive cytotoxicity if Doxo was combined with Ver (Fig. 8A). By contrast, tendentially antagonistic effects were observed if Doxo was combined with the Rac1 inhibitor EHT1864 (Fig. 8A). This is in line with previous studies showing that pharmacological inhibition of Rac1 is able to protect cardiac cell types from Doxo-induced injury *in vitro*

and *in vivo* (67-70). Moreover, the present study investigated the cytotoxicity of Doxo in combination with Ver, EHT1864 and EST using stem cell lines of murine (mESC) and human (hiPSC) origin. We found that Ver promotes the Doxo sensitivity of both mESC and hiPSC (Fig. 8B and C). By contrast Rac1 inhibition by EHT1864 again did not evoke synergistic toxicity in combination with Doxo in these non-malignant cells (Fig. 8A-C).

Discussion

In order to characterize the molecular mechanisms contributing to the Doxo resistant phenotype of A2780ADR cells and, furthermore, to identify bioactive compounds to overcome their acquired platin-resistance, extensive cross-resistance analyses were performed. The results obtained show profound cross-resistance of A2780ADR cells to Eto and a rather moderate cross-resistance to CisPt. Having in mind that inhibition of Topo II is a common mechanism underlying the anticancer efficacy of Dox and Eto, we assume that the acquired Doxo resistance of A2780ADR cells is related to Topo II-associated mechanisms, although the contribution of other mechanisms to Doxo resistance of A2780ADR cells, such as ROS formation, intercalation or inhibition of helicases (4,71,72), cannot be ruled out. The weak cross-resistance to CisPt might be based on overlapping drug transporters for Doxo, Eto and CisPt (7,8) or DSB as a common type of DNA damage that result from both Doxo-/Eto-mediated Topo II inhibition and DNA interstrand cross-links caused by CisPt. Data obtained from the subsequent analysis of drug export supported the hypothesis that drug export contributes to the Doxo resistance of A2780ADR cells. Notably, the export activity of parental A2780 cells is only moderately (~20%) higher than that of A2780 cells. By contrast, the drug resistant A2780ADR variants reveal an ~10-fold higher Doxo resistance than the parental cells as concluded from their IC₅₀. Therefore, it was assumed that mechanisms others than drug export additionally contributed to the profound Doxo resistant phenotype of A2780 cells. One of this hypothesized Doxo-related additional resistance mechanism of A2780 cells might be a reduced protein expression of the main Doxo target protein Topo II α (73).

Other feasible mechanisms of acquired Doxo resistance may be alteration in DDR and DNA repair, since such mechanisms are well-known to define the balance between cell death and survival signaling (3) by regulating cell cycle progression, cell death and DNA repair. Analyzing Doxo- and Eto-induced alterations in the activation of cell cycle checkpoints, the present study observed a pronounced resistance of A2780ADR cells to both Doxo- and Eto-induced G₂/M arrest as well as apoptosis as reflected by the percentage of cells present in the SubG₁ fraction. Notably, while Doxo caused a great increase in the SubG₁ fraction of A2780 cells, such an effect was not found following Eto treatment. It is hypothesized that this may be related to the not fully identical mode of action of both drugs. While both drugs are poisoning Topo II, Doxo has additional cytotoxic activities, such as intercalation into DNA, inhibition of DNA helicases and ROS formation via mitochondrial toxicity (5). Hence, it is feasible that, apart from TOP2 dependent mechanism, A2780ADR cells also benefit

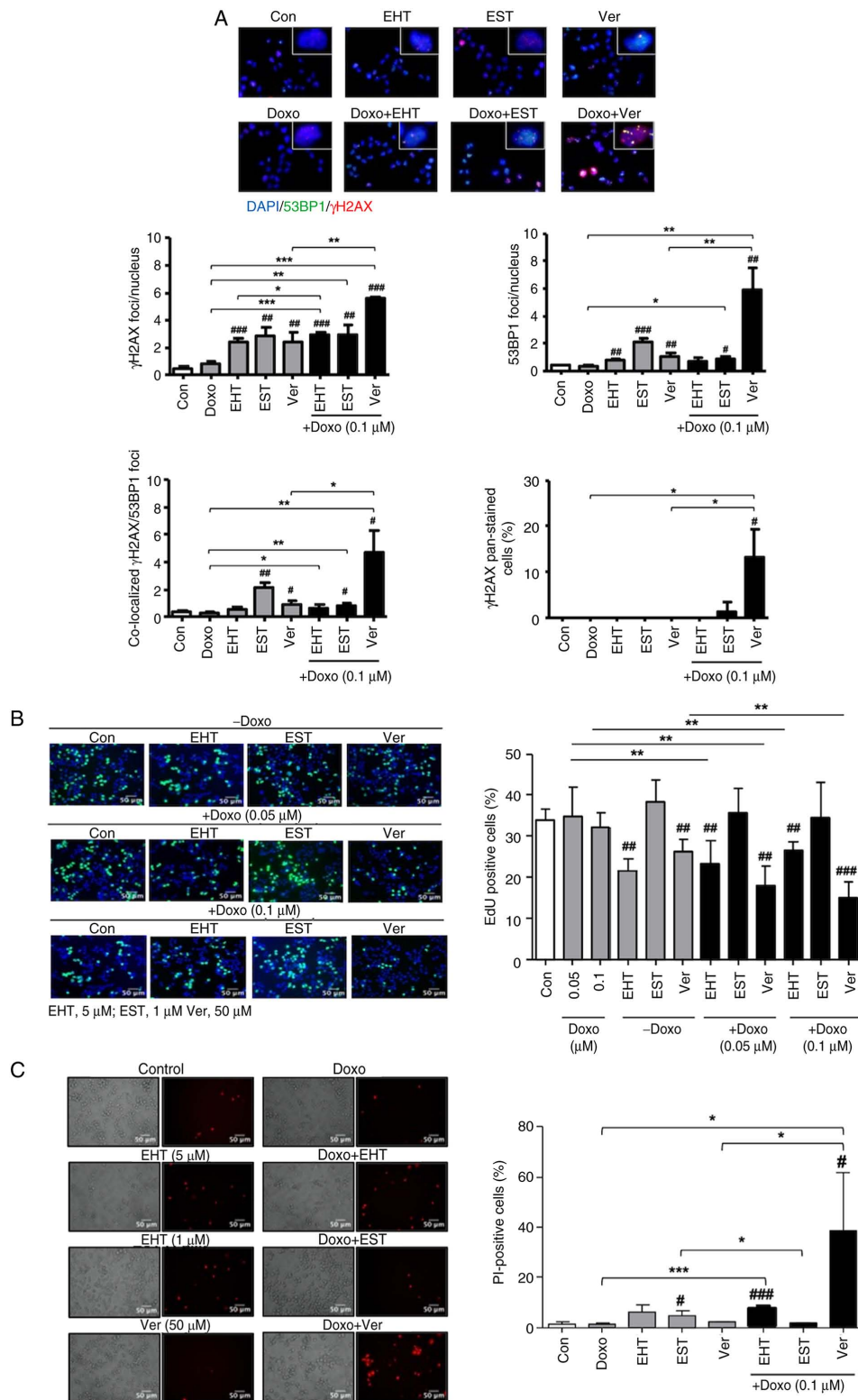


Figure 6. Influence of combined treatment of A2780ADR with Doxo and selected inhibitors on DNA damage formation, proliferation and cell death. (A) At 24 h after treatment of logarithmically growing cells with the indicated concentrations of Doxo and inhibitors (EHT, 5 μM; EST, 1 μM; Ver, 50 μM), the number of nuclear γH2AX-foci, 53BP1-foci, γH2AX/53BP1 co-localized foci and γH2AX pan-stained cells was analyzed as described in methods. The upper part of the figure shows representative images (total magnification, x1,000). Quantitative data depicted in the histogram are the mean ± SD from n=5 microscopical images analyzed per experimental condition. *P≤0.05, **P≤0.01, ***P≤0.001 mono-treatment vs. co-treatment; #P≤0.05, ##P≤0.01, ###P≤0.001 untreated vs. treated group). (B) Logarithmically growing Doxo resistant A2780ADR cells were co-treated with the indicated concentrations of Doxo and selected pharmacological inhibitors (EHT, 5 μM; EST, 1 μM; Ver, 50 μM). At 24 h later, cells were pulse-labeled with EdU to monitor proliferation as described in methods and the percentage of EdU positive cells was determined microscopically (total magnification, x400). Quantitative data shown in the histogram are the mean ± SD from five replicates. **P≤0.05; ##P≤0.01; ###P≤0.001 (vs. untreated control). (C) PI staining of mono- and co-treated A2780ADR cells 72 h after treatment with Doxo (0.1 μM) and pharmacological inhibitors (EHT, 5 μM; EST, 1 μM; Ver, 50 μM). Left panel: representative images; right panel: percentage of PI positive cells (mean ± SD from n=5 microscopical images analyzed per experimental condition) (40x microscope objective). *P≤0.05; **P≤0.01. mono-treatment vs. co-treatment; #P≤0.05; ##P≤0.01, Con vs treated group. Doxo, doxorubicin; EST, entinostat; EHT, Rac1 inhibitor EHT1864; Ver, verapamil; SD, standard deviation; PI, propidium iodide.

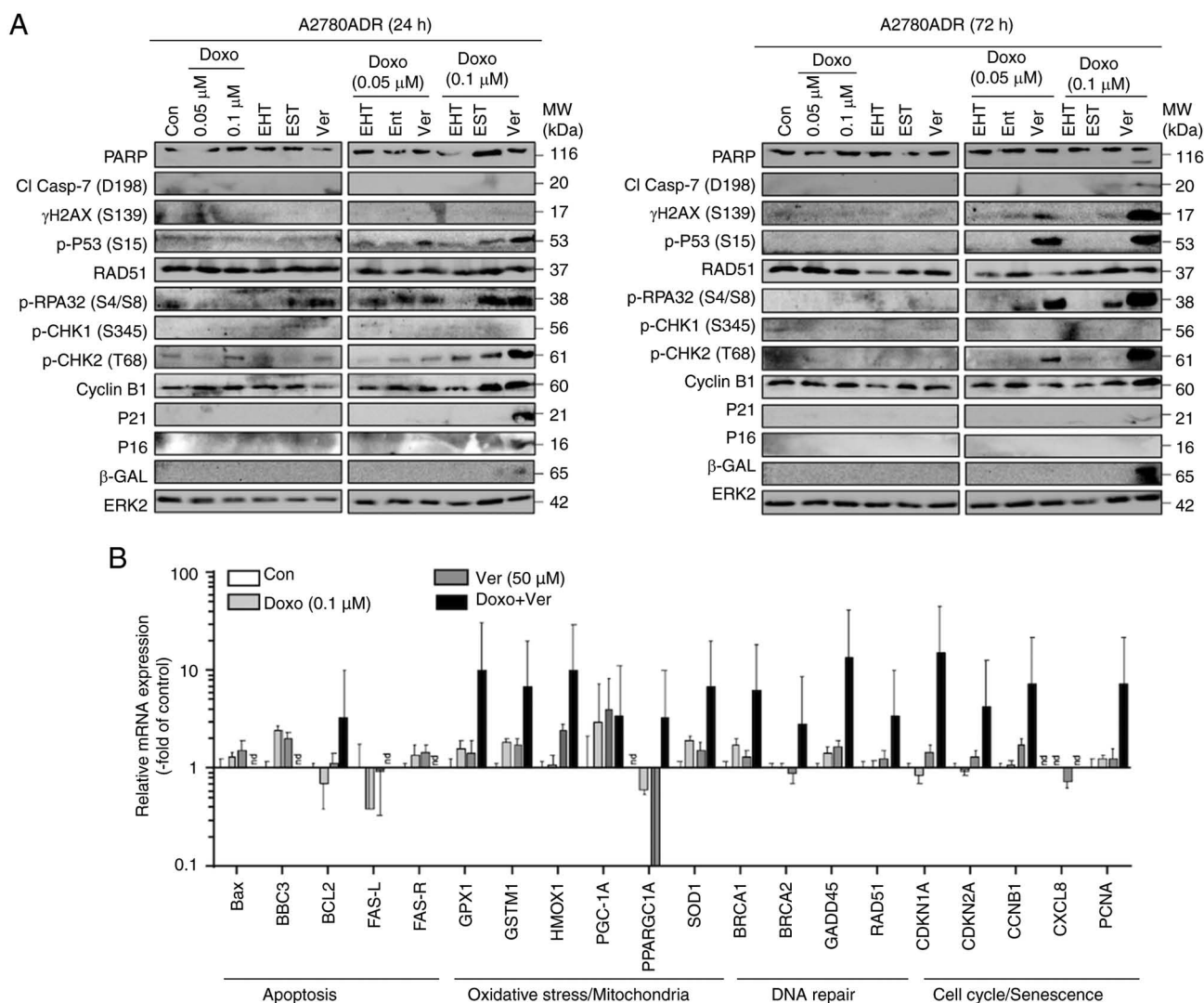


Figure 7. Influence of combined treatment of A2780ADR cells with Doxo and selected inhibitors on mechanism of the DDR and mRNA expression of selected susceptibility-related genes. (A) Logarithmically growing A2780ADR cells were co-treated with the indicated concentrations of Doxo and selected pharmacological inhibitors (concentrations see Fig. 5) for 24 or 72 h. Afterwards, the protein expression of DDR-related factors was analyzed by western blotting. For loading control, blots were re-probed with ERK2 antibody. (B) Reverse transcription-quantitative PCR of the mRNA expression of selected factors known to contribute to different mechanisms of drug sensitivity. Data shown are mean $\pm$ SD from triplicate determinations as described in methods. Relative mRNA level in untreated A2780ADR cells was set to 1.0. Doxo, doxorubicin; DDR, DNA damage response; p-, phosphorylated; nd, not detectable; Bax, Bcl-2 associated protein X; Bcl-2, B-cell lymphoma; BBC3, Bcl-2 binding component 2; BRCA1, 2, breast cancer associated gene 1,2; Cl casp-7, cleaved caspase 7; Chk, checkpoint kinase; CXCL8, chemokine ligand 8 (interleukin 8); p21, CDK inhibitor 1; p16, CDK inhibitor 2; CDKN1A/2A, cyclin dependent kinase inhibitor 1A/2A; CCNB1, Cyclin B1; b-Gal, beta-galactosidase; FASL, FAS ligand; FASR, FAS receptor; GADD, growth arrest and DNA damage inducible GPX1, glutathione peroxidase 1; GSTM1, glutathione S-transferase 1; HMOX1, heme oxygenase 1; γ H2AX, Ser139 phosphorylated histone H2AX; p53, tumor suppressor p53; PARP, poly (ADP-ribose) polymerase; PCNA-proliferating cell nuclear antigen; PGC1A, PPARG coactivator 1; PPARGC1A, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; RAD51, radiation damage gene 51; RPA replication protein A; SOD1, superoxide dismutase 1; Ver, verapamil.

from additional cytoprotective mechanisms against Doxo, including mechanisms related to oxidative stress defense. Moreover, the activation of S-phase checkpoint by Doxo is also mitigated in A2780ADR as compared with wild-type cells. This is concluded from their very weak S-phase block following Doxo treatment as measured by EdU incorporation analyses as well as the analyses of Ki67 and pH3 positive cells. Summarizing, it is tempting to hypothesize that acquired drug resistance of A2780ADR cells to Doxo is due to alterations in their drug-stimulated activation of cell cycle checkpoints, including S-phase related checkpoints, and apoptosis-related mechanisms of the DDR. Having in mind that DSB are potent trigger of the DDR, the present study next assayed the steady

state levels of DSB after 24 h of drug treatment by monitoring the number of nuclear γ H2AX foci, which are well-accepted surrogate markers of DSB (40). Indeed, both Doxo- and Eto induced formation of DSB was largely reduced in the A2780ADR cells as compared with the wild-type. These findings indicated that lower steady-state levels of drug-induced DSB in the Doxo resistant variant majorly account for their substantially increased drug resistance. This hypothesis gains further support by almost complete lack of activation of DDR-related factors (such as KRAB-associated protein 1, Chk2, p53 and p21) in A2780ADR compared with parental A2780 cells following Doxo treatment. As with Doxo, treatment of A2780 cells with Eto also caused a distinct increase

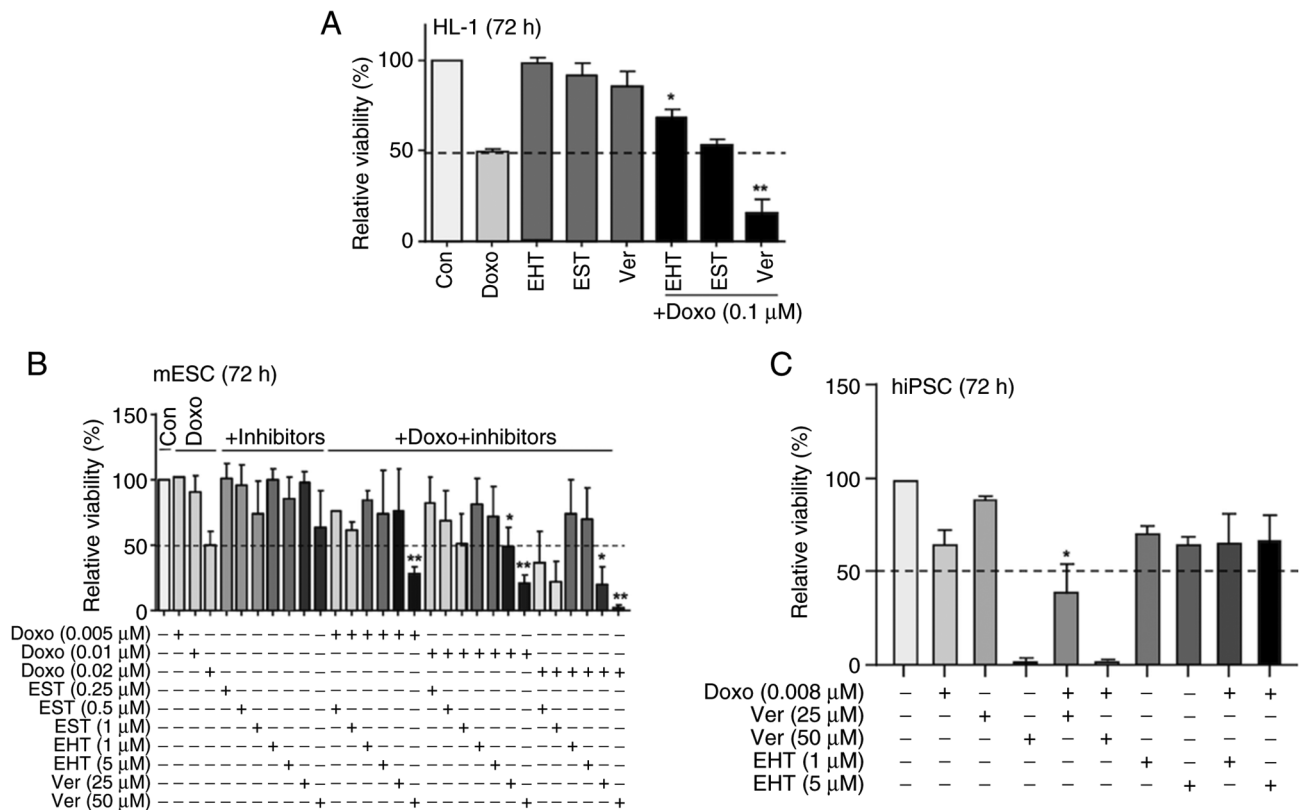


Figure 8. Effects of co-treatment of Doxo with selected pharmacological inhibitors on non-malignant cells. Logarithmically growing (A) non-malignant murine HL-1 cardiomyocyte cells, (B) mESC and (C) hiPSC were treated with the indicated concentrations of Doxo and selected pharmacological inhibitors for 72 h. Afterwards, cell viability was analyzed by the use of the AlamarBlue assay as described in methods. Data shown are the mean $\pm$ SD from n=1-3 independent experiments each performed in biological quadruplicates. * $P \leq 0.05$; ** $P \leq 0.001$ (mono- vs. co-treated group). Dashed lines indicate 50% viability. Doxo, doxorubicin; mESC, murine embryonic stem cells; hiPSC, human induced pluripotent stem cells; EHT, Rac1 inhibitor EHT1864; EST, entinostat; Ver, verapamil.

in the protein expression of γ H2AX, p53 and p21. This is noteworthy, since the pronounced p21 induction following Doxo treatment was associated with a strong decrease in the percentage of Ki67 positive cells, while Eto treatment rather also caused strong p21 induction but no major reduction in Ki67 positive cells. So, it appears feasible that the Doxo-induced p21 induction reflects an early stress response that is associated with senescence while the response to Eto is not. Discussing the response of cells regarding Ki67, it should be noted that Ki67 is considered more than just a proliferation marker (74). It is related to chromatin and subject to ubiquitination-related mechanisms (75) as well as complex transcriptional control mechanisms (76). Hence, it was hypothesized that Doxo and Eto differently interfere with mechanisms of Ki67 regulation. Overall, the present study supported the hypothesis that the immense Doxo resistance of A2780ADR cells (that is, ~10-fold as compared with A2780 cells) is also attributable to an attenuated Doxo-induced formation of DNA damage (that is, DSB) and largely reduced activation of DDR-related pro-death mechanisms (51,52,77).

DDR and DNA repair-associated factors are promising targets to improve anticancer therapy and to overcome acquired drug resistance of tumor cells (49-52). Therefore, the present study analyzed the influence of a set of pharmacological inhibitors interfering with these mechanisms on the viability of A2780ADR cells. The data showed

cross-resistance of the Doxo resistant cells to PARPi (that is, Olaparib and niraparib) as well as Chk1/2 inhibitors (that is, prexasertib and rabusertib) and the HDACi entinostat. This cross-resistance data further supported the hypothesis that mechanisms of DNA repair/DDR contribute to the Doxo-resistant phenotype of A2780ADR cells. However, the A2780ADR cells were not hypersensitive to any of the other inhibitors tested (that is, ricolinostat, Ver, EHT1864, Etoposide and dexamethasone), showing the specificity of the effects observed with PARPi and Chk1/2i. These data clearly demonstrated that mono-treatment with DDR modifying drugs is not particularly useful for preferential killing of drug resistant cells. Therefore, the present study next investigated whether co-treatment with a selected subset of inhibitors (that is, entinostat, EHT1864, rabusertib, dexamethasone and Ver) was useful to re-sensitize A2780ADR cells to Doxo treatment. The data obtained showed that EHT and Ver evoked the most noticeable synergistic toxicity in combination with Doxo. Notably, Ver and EHT also caused synergistic toxicity in combination with Eto and, moreover, both Ver and EST were highly efficient at overcoming acquired CisPt resistance of A2780CisR cells. In summary, the data demonstrated that Ver is particular useful to re-sensitize both Doxo-, Eto- and CisPt-resistant A2780 cells, indicating that Ver is able to overcome acquired drug resistance towards multiple anticancer agents.

As to the molecular mechanisms involved, the present study found that part of the Ver effect was related to a reduced drug export, leading to higher intracellular steady-state concentration of Doxo. Notably, synergistic toxicity observed if EHT or EST are combined with Doxo is independent of drug transport. To investigate the influence of the pharmacological inhibitors on Doxo-induced DNA damage formation, the steady state level of DSB was monitored after a 24 h treatment period. This experimental setup was considered as particular appropriate because it takes into account the complex kinetic processes that define the level of Doxo-induced DNA damage that is detectable at a certain time point of analysis and, furthermore, allows the detection of the net outcome of the pharmacological modifiers on these processes. In line with the toxicity data, co-treatment with Ver resulted in a significant increase in Doxo-induced formation of DSB, as measured by the number of nuclear γ H2AX and 53BP1 foci, which was not observed for EHT and EST. In addition, the percentage of γ H2AX pan-stained and PI positive cells also largely increased under situation of co-treatment. Surprisingly, mono-treatment with both EHT, EST and Ver caused a weak increase in DNA damage as reflected on the level of nuclear γ H2AX foci. As to EHT, we have previously demonstrated DSB-inducing activity also in non-malignant cells (78). Moreover, Rac1 has been reported to be expressed in the nucleus (79) and is involved in the regulation of DDR- and DNA repair-related mechanisms (80,81). These pleiotropic functions of Rac1 are a feasible explanation of a moderate induction of DNA damage under situation of EHT mono-treatment. Regarding EST, it was hypothesized that DNA damage resulting from mono-treatment is due to the well-known interference of this class I HDACi with mechanisms of DNA repair and DDR (82-84). With respect to Ver it is important to bear in mind that this Ca^{2+} channel blocker is considered to preferentially induce apoptosis in multi-drug resistant cells via ROS-dependent mechanisms (85). Thus, it is feasible that induction of oxidative DNA damage is responsible for the induction of DSB under the situation of Ver mono-treatment. Moreover, in line with the data of the present study, the reversal of Doxo resistance by Ver has been associated with drug accumulation and DNA damage formation (86,87). Taken together, the present study showed that Ver caused a distinct potentiation of the genotoxic, antiproliferative and cell-death inducing effects of Doxo. Thus, it is hypothesized that Ver is able to overcome acquired Doxo resistance of A2780ADR cells by fostering the Doxo-induced toxicity via increased formation of DSB, leading to potentiated cytotoxicity by pronounced inhibition of proliferation and induction of cell death.

To further characterize the molecular mechanisms underlying the observed synergistic toxicity and to substantiate the increase in DSB formation under situation of Ver plus Doxo co-treatment, the outcome of combined treatments on the activation status of a subset of DDR-related factors were analyzed by western blotting. The data demonstrated that specifically Ver, but not EHT or EST, potentiated the Doxo-stimulated activation of multiple DDR-related factors (that is, p53, RPA32 and Chk2) as well as cleavage of PARP and pro-caspase 7, which is indicative of apoptotic cell death. In addition, complex alterations in the mRNA expression of susceptibility-related factors involved in the regulation of oxidative stress responses, cell cycle regulation and senescence as well as DNA repair were observed. These results

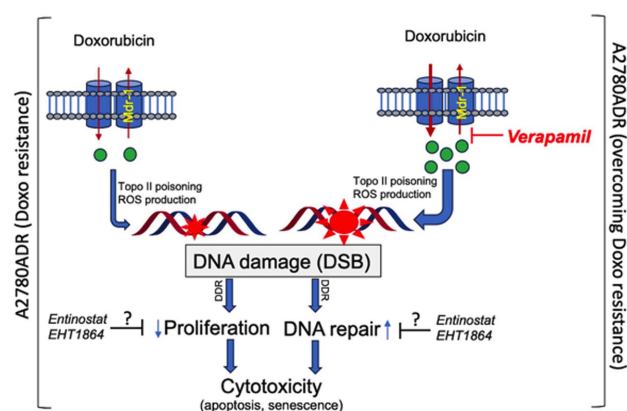


Figure 9. Hypothetical model of Ver-mediated resensitization of Doxo-resistant tumor cells. It was hypothesized that Ver increased the anticancer efficacy of Doxo in a synergistic manner in anticancer drug resistant ovarian A2780ADR cells. This is probably due to inhibition of MDR1-mediated drug export, leading to higher intracellular steady-state concentrations of Doxo. In consequence, Doxo-mediated Topo II poisoning is promoted, eventually causing increased DNA damage (that is, DSB) formation and activation of DSB-related pro-toxic signaling mechanism which impair cell proliferation and stimulate cell death- and senescence-related pathways. Apart from verapamil, inhibition of Rac1 GTPase-regulated signaling by EHT1864 and inhibition of HDAC class I by entinostat are also useful to overcome acquired Doxo resistance of A2780ADR cells, yet with the exact molecular mechanisms involved being unclear. Ver, verapamil; Doxo, doxorubicin; DSB, DNA double-strand breaks.

indicated that the synergistic activity of Ver in combination with Doxo probably relies on an amplification of multiple DDR-related pro-toxic stress responses of A2780ADR cells. To understand whether combined treatment may also increase adverse effects of Doxo on normal cells, combination experiments were performed using different types of non-malignant cells (that is, HL-1 cardiomyocytes, murine and human stem cells). Unfortunately, data obtained indicated that Doxo-induced toxicity was also elevated in non-malignant cells if used in combination with Ver. This finding raised the concern of a possible increase in adverse effects resulting from co-treatment regimen *in vivo*. Thus, while Doxo plus Ver co-treatment is evoking substantial synergistic toxicity in Doxo resistant malignant cells, the therapeutic window of this combination treatment might be narrow. Notably in this context, Rac1 inhibition by EHT1864 did not cause any synergistic toxicity in combination with Doxo in non-malignant cells. This finding is in line with *in vitro* and *in vivo* data (80,88,89) and points to a potentially favorable therapeutic window of EHT1864 if used in combination with Doxo.

In summary, acquired Doxo resistance of A2780ADR was associated with a reduced drug uptake, which is probably due to high MDR-1 expression and a diminished Topo II α expression. Furthermore, it was accompanied by an attenuated drug-stimulated S-phase block, mitigated formation of DSB and reduced DDR activation as compared with A2780 parental cells. Notably, acquired Doxo resistance of A2780 cells can be overcome most effectively by inhibition of drug exporters using Ver (Fig. 9). In consequence of Ver co-treatment, the intracellular steady-state concentration of Doxo increased, promoting the formation of DSB, thereby in turn triggering pro-toxic mechanisms of the DDR that promoted inhibition of cell proliferation and stimulation of senescence- and cell death-associated mechanisms. Notably,

targeting of Rac1- and/or HDAC class I-related mechanisms was also highly efficient to overcome acquired Doxo resistance, yet independent of drug transport. However, the molecular mechanisms involved here are still unclear and, hence, will be subject of forthcoming studies. In conclusion, the present study suggested that Ver is highly effective to re-sensitize Doxo-resistant tumor cells by promoting DNA damage-triggered cytotoxicity related to proliferation-, cell death- and, possibly, senescence-associated mechanisms. However, combined treatment with Ver may also amplify Doxo-stimulated adverse outcome pathways in non-malignant cells. Accordingly, forthcoming pre-clinical *in vivo* studies are needed for a meaningful definition of the therapeutic range of a Doxo plus Ver co-treatment regimen.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

EM was responsible for conceptualization, methodology, performing experiments, data analysis and interpretation, visualization and writing. SF and LM were responsible for performing experiments, data analysis and visualization. MS was responsible for methodology, data analysis and supervision. JM was responsible for methodology and data analysis. LA was responsible for performing experiments, data analysis and visualization. MK was responsible for resources, funding acquisition and writing original draft. GF was responsible for conceptualization, data interpretation, funding acquisition, writing original draft, resources and supervision. EM, LA, SF, LM and GF confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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