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ORIGINAL ARTICLE

Diverse cross-resistance phenotype to ET-743 and PM00104 in multi-drug resistant cell lines

Zhenfeng Duan · Edwin Choy · Jose Maria Jimeno · Carmen Del Maria Cuevas · Henry J. Mankin · Francis J. Hornicek

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Abstract
Purpose ET-743 (Yondelis®, trabectedin) and PM00104 (Zalypsis®) are marine derived compounds which demonstrate anti-tumor activity. The present study was performed to elucidate the relationship between the expression of ABCB1/MDR1 and ABCC1/MRP1 with resistance to either ET-743 or PM00104.
Methods We evaluate the association between expression of Pgp1, MRP1, and BCRP proteins and ET-743 or PM00104 resistance in a large panel of multi-drug resistant cell lines derived from histologically unrelated human tumors that were selected with paclitaxel, doxorubicin, cisplatin, mitoxantrane, or gemcitibine.
Results Paclitaxel selected resistantcell lines expressed high levels of ABCB1 (but not ABCC1 or ABCG2/BCRP) did not demonstrate cross-resistance to either ET-743 or PM00104. In contrast, the doxorubicin selected resistant cell lines also expressed high level of ABCB1 (but not ABCC1 or ABCG2) but did demonstrate significant cross-resistance to both ET-743 and PM00104. The paclitaxel selected cell lines demonstrated cross-resistance to doxorubicin, vincristine, and mitoxantrane, while most of the above doxorubicin selected cell lines demonstrated cross-resistance to paclitaxel and vincristine, but not to mitoxantrane. On the contrary, cisplatin and gemcitabine selected cell lines demonstrated no cross-resistance to paclitaxel, doxorubicin, ET-743, or PM00104. siRNA down-regulation of ABCB1 expression in doxorubicin selected cell lines caused partial sensitization to both doxorubicin and paclitaxel but not to either ET-743 or PM00104.
Conclusions These results indicate that cell lines selected for resistance to either paclitaxel or doxorubicin are cross-resistant to many other drugs and that, for these cell lines, ABCB1 over-expression is not necessary to confer resistance to either ET-743 or PM00104. Diversity of cross-resistance observed in these multi-drug resistant cell lines are associated with the initial drug used for in vitro selection, but not to ABCB1 expression. This study suggests that a common molecular pathway other than ABCB1 may be involved in the mechanism of resistance to ET-743 or PM00104.

Keywords ET-743 · Yondelis · PM00104 · Zalypsis · ABCB1/MDR1 · Multi-drug resistance gene 1

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Introduction

Cancer cells, even when initially sensitive to cytotoxic drugs, have the propensity to develop resistance over time [8, 29]. As a result, drug resistance is a major problem in cancer therapy. Often, a tumor exposed to one chemotherapeutic agent will develop resistance to several other agents that are unrelated structurally and functionally (referred in the text below as "multi-drug resistance") [24, 29].

The mechanisms of multi-drug resistance are incompletely understood and likely polygenic. Drug resistant cell lines can be selected in vitro using a wide range of

chemotherapeutic agents by serial exposure to gradually increasing drug concentrations [8]. One mechanism that has been well described is the over-expression of ABCB1/MDR1, which encodes for the protein P-glycoprotein 1 (Pgp1) [29]. The over-expression is mediated by ABCB1 gene amplification or by other regulatory mechanisms that lead to increased levels of ABCB1 mRNA and/or transcribed Pgp1 protein. Although drug-selected cell lines usually show the highest levels of resistance to the agent used in the selection process, their pattern of cross-resistance to other drugs can be extremely variable, suggesting a multifactorial basis for cross-resistance.

ET-743 (Yondelis®; Trabectedin) are marine alkaloid derivatives isolated from the Caribbean sea squirt Ecteinascidia turbinate [20, 21]. ET-743 has a broad spectrum of activity toward tumor cell lines at pM and low nM concentrations, and it also has clinical activity toward histologically unrelated solid tumors including ovarian cancer, breast cancer, and sarcomas [1, 27]. In particular, ET-743 treatment is associated with a remarkable, long-term response in a subset of patients with soft tissue sarcomas [23]. More recently, ET-743 has been approved by the EMEA/EU for patients with advanced sarcoma resistant/relapsed to anthracyclines and ifosfamide or in those cases not suitable to receive such conventional agents. Structurally, ET-743 is composed of three tetrahydroisoquinoline subunits containing a central carbinolamine moiety enabling it to covalently bind to DNA. ET-743 binds to the minor groove of the DNA helix with sequence-specific binding preference for GC-rich triplets and subsequently forms covalent adducts with the N2-position of guanine. As a result, the minor groove is opened up and bent toward the major groove. When such binding occurs, DNA strands are cross-linked and cannot be replicated, causing the cell to die [14, 26]. The direction of bending is a novel feature among DNA minor groove-interactive agents, thus making ET-743 unique. There were reports that cell lines deficient in DNA mismatch repair are partially resistant to cisplatin, where they are sensitive to ET-743. The cell lines deficient in DNA nucleotide excision repair (NER) are hypersensitive to cisplatin and are partially resistant to ET-743 [6, 26].

PM00104 is a novel chemical entity related to Jorumycin, a marine natural compounds and in the family of Renieramycins, obtained from molluscs and sponges, respectively [10, 28]. PM00104 also has in the in vitro and in vivo anti-tumor activity in a wide variety of solid and hematological tumors. PM00104 also binds to DNA and is cytotoxic; however, it does not activate the "DNA damage checkpoint" response. Thus, PM00104 has cytotoxic effects dependent on DNA binding that are not associated with DNA repair mechanisms [10]. Like other chemotherapeutic drugs, ET-743 and PM00104 exposure over sustained periods of treatment will result in the development of drug resistance, but the mechanisms are not yet understood.

Different studies have reported conflicting descriptions of the relationship between ABCB1 expression and ET-743 resistance in human cancer cell lines [15]. In the human ovarian cell line IGROV-1 selected for ET-743 resistance, ABCB1 is overexpressed [9]. Sensitivity could be restored by the addition of the Pgp1 inhibitor PSC-833, indicating that ET-743 may be a Pgp substrate. Another study however, observed that two Pgp over-expressing human epidermoid cancer cell lines (KB-8-5 and KB-C-2) were not resistant to ET-743 [15]. Additionally, sub-lethal concentrations of ET-743 could reverse resistance to doxorubicin and vincristine in these cell lines. Currently, there are no reports describing the mechanism of PM00104 resistance in tumor cells.

In this study, we first profile the expression of Pgp1, MRP1, and BCRP proteins in a large panel of multi-drug resistant cell lines derived from histologically unrelated human tumors that were selected with paclitaxel, doxorubicin, cisplatin, mitoxantrane, or gemcitibine. We then identify a group of cross-resistant drugs for each of these cell lines and associate drug resistance with expression of ABC-family proteins. Finally, we characterize drug resistance profiles for doxorubicin selected cell line after inhibiting ABCB1 mRNA expression using siRNA. We find that cell lines selected for resistance to paclitaxel and doxorubicin (but not to cisplatin, mitoxantrane, or gemcitibine) are associated with the over-expression of ABCB1. We also find that acquired multi-drug resistance is associated with distinct patterns of resistance to ET-743 and PM00104 depending on the agents used to select cell lines for resistance. While siRNA inhibition of ABCB1 reverses resistance to paclitaxel and doxorubin, it does not reverse resistance to ET-743 and PM00104. These results suggest that ET-743 and PM00104 resistance is a complex phenotype that can arise through more than one mechanism.

Materials and methods

Cell lines, antibodies and drugs

The human osteosarcoma cell line U-2OS, KHOS, human uterin sarcoma cell line MESSA and it is doxorubicin selected drug resistant cell line MESSA/Dx5, human ovarian cancer cell line SKOV-3, human breast cancer cell line MCF-7, human colon cancer cell line SW480, human non-small cell lung cancer cell line H-69 and it is doxorubicin selected drug resistant cell line H-69AR were obtained from the American Type Tissue Collection (Rockville, MD). The paclitaxel-resistant U-2OS$_{TR}$, SKOV-3$_{TR}$, OVCAR8$_{TR}$, MCF-7$_{TR}$, and SW480$_{TR}$ lines as well as doxorubicin resistant MCF-7$_{DR}$ and germcitbine resistant OVCAR5$_{GR}$ cell lines were established as previously

reported [7, 8]. Briefly, the paclitaxel, doxorubicin, or germcitbine resistant cell lines were selected over a period of 6–10 months by continuous culture in media containing step-wise increases in paclitaxel or doxorubicin. Dr. Patricia Donahoe (Massachusetts General Hospital, Boston, MA) provided the human OVCAR5, OVCAR8 ovarian cancer cell lines. Dr. Efstathios Gonos (Institute of Biological Research and Biotechnology, Athens, Greece) provided the doxorubicin resistant U-2OS R2 (referred in the text below as U-2OS$_{DR}$), KHOS R2 (referred in the text below as KHOS$_{DR}$) cell lines [18]. Dr. Stephen B Howell (The University of California Medical Center, San Diego, CA) provided the cisplatin-resistant ovarian cancer IGROV1cp and 2008cp70 cell lines [5]. Dr. Erasmus Schneider (Wadsworth Center, Albany, NY) provided the mitoxantrane resistant breast cancer MCF-7/MX cell line [22]. Dr. Katia Scotlandi (Institute Orthopedics Rizzoli, Italy) provided ET-743 resistant TC-ET 6 nM and TC-ET 12 nM cell lines [19]. Paclitaxel, Doxorubicin, and Cisplatin were obtained through unused residual clinical material provided by the pharmacy at the Massachusetts General Hospital. ET-743 and PM00104 were supplied by PharmaMar (Spain). The stock solution of drugs were prepared according to the drug specifications and stored at −20°C.

The Pgp1 monoclonal antibody C219 was purchased from Signet (Dedham, MA). The mouse monoclonal antibody to MRP1 and MTT reagent were purchased from Sigma-Aldrich (St. Louis, MO). The monoclonal antibody to BCRP antibody was purchased from Chemicon (Temecula, CA). The Goat anti-rabbit-HRP and goat anti-mouse-HRP were purchased from Bio-Rad (Hercules, CA). SuperSignal® West Pico Chemiluminescent Substrate was purchased from PIERCE (Rockford, IL).

Cell culture

All the cell lines were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100-units/ml penicillin and 100 μg/ml streptomycin (Invitrogen). Cells were incubated at 37°C in 5% CO_2-95% air atmosphere and passaged when near confluent monolayers were achieved using trypsin–EDTA solution. Drug-resistant cell lines were periodically cultured in the respective drug to confirm their drug resistance characteristics. Cells were free on mycoplasma contamination as tested by MycoAlert® Mycoplasma Detection Kit from Cambrex (Rockland, ME).

Cytotoxicity assay

Drug cytotoxicity was assessed in vitro using the MTT assay as previously described [3]. Briefly, 2×10^3 cells per well were plated in 96-well plates in culture medium (RPMI 1640 supplemented with 10% fetal bovine serum and penicillin/streptomycin) containing increasing concentrations of drug. After 7 days of culture, 10 μl MTT (5 mg/ml in PBS, obtained from Sigma) were added to each well and the plates were incubated for 4 h. The resulting formazan product was dissolved with acid-isopropanol and the absorbance at a wavelength of 490 nm (A_{490}) was read on a SPECTRAmax® Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA). The absorbance values were normalized by assigning the value of the control line in the medium without drug to 1.0 and the value of the no cell control to 0. Experiments were performed in triplicate.

Western blotting

Pgp1, MRP1, and BCRP proteins were analyzed in total cell lysates. Total cell lysates were prepared, and Western blot analysis was performed as previously described. Briefly, the cells were lysed in 1× RIPA lysis buffer (Upstate Biotechnology, Charlottesville, VA) and protein concentration was determined by the DC Protein Assay (Bio-Rad). Twenty-five micrograms of total protein were resolved on NuPage™ 4–12% Bis–Tris gels (Invitrogen) and immunoblotted with specific antibodies. Primary antibodies were incubated in TBS (pH 7.4) with 0.1% Tween-20 with gentle agitation overnight at 4°C. Horseradish peroxidase (HRP)-conjugated secondary antibodies (Bio-Rad) were incubated in TBS (pH 7.4) with 5% nonfat milk (Bio-Rad) and 0.1% Tween-20, at a 1:2,000 dilution for 1 h at room temperature with gentle agitation. Positive immunoreactions were detected by using SuperSingal® West Pico Chemiluminescent Substrate.

Drug efflux assay

TheVybrant™ multi-drug resistance assay kit (Invitrogen/Molecular Probes) was used to measure drug efflux properties of different resistant cell lines. This assay utilizes the fluorogenic dye calcein acetoxymethyl ester (calcein AM) as a substrate for efflux activity of Pgp1 or other membrane pump ABC proteins. Calcein AM is taken up by cells and hydrolyzed by cytoplasmic esterases to fluorescent calcein. Calcein AM is well retained in the cytosol. However, multi-drug resistant cells expressing high levels of Pgp1 rapidly extrude non-fluorescent calcein AM from the plasma membrane, reducing accumulation of fluorescent calcein in the cytosol. Drug sensitive and resistant cells (3×10^5) were cultured in 96-well plate for 24 h and then incubated in 0.25 μM calcein AM in 150 μl total volume. After 30 min, the cells in plate were washed and centrifuged twice with 200 μl cold RPMI1640 culture medium, and cell fluorescence was measured at a wavelength of 490 nm (A_{490}) on a SPECTRAmax® Microplate Spectrofluorometer (Molecular Devices).

ABCB1 siRNA assay

The human ABCB1 On-Targetplus SMARTpool siRNA was purchased from Dharmacon, Inc. (Chicago, IL) and was used according to the manufacturer's instructions. For transfection, cells were either plated on 96 well plates for MTT assays or plated on dishes for Western blot protein detection. Transfections were performed with siPORT™ *NeoFX*™ siRNA transfection reagents (Ambion. Inc, Austin, TX) as directed by the manufacturer. The Silencer™ EGFP siRNA (Ambion) and siControl® reagent (Dharmacon) were used as positive and negative controls in all experiments. For ABCB1 inhibition, the final concentration of siRNA was 100 nM. Media was replaced with RPMI1640 supplemented with 10% FBS 24 h after transfection. Total protein was isolated after 48 h of ABCB1 siRNA transfection.

Results

Analysis of Pgp1, MRP1, and BCRP expression in multi-drug resistant cell lines

All resistant cell lines were generated in vitro by exposing parental cell lines to increasing levels of drug concentrations (see "Materials and methods"). To measure the expression of Pgp1, MRP1, and BCRP, we analyzed these proteins in a large panel of resistant cell lines derived from histologically unrelated tumor types. Western blot analysis demonstrated that Pgp1, which was not detected in the parental cell lines, was overexpressed in paclitaxel selected cell lines U-2OS$_{TR}$, SKOV-3$_{TR}$, OVCAR8$_{TR}$, MCF-7$_{TR}$, SW480$_{TR}$, and the doxorubicin selected cell lines U-2OS$_{DR}$, KHOS$_{DR}$, MESSA/Dx5, MCF-7$_{DR}$ but not in the mitoxantrane selected cell line MCF-7/MX (Fig. 1). Cisplatin and gemcitabine selected cell lines IGROV-1cp, 2008cp70 and OVCAR5GR did not overexpress Pgp1 (data not shown). In addition, we found neither MRP1 nor BCRP is overexpressed in paclitaxel or cisplatin selected cell lines. We confirmed that BCRP is highly expressed in MCF-7/MX and MRP1 is highly expressed in H-69AR (Fig. 1). Expression of MRP1 or BCRP was not observed in other parental or drug resistant cell lines (Fig. 1). These studies confirm that Pgp1 (but not MRP1 or BCRP) play an important role in multi-drug resistant cell lines selected by paclitaxel and doxorubicin but not mitoxantrane, cisplatin, or gemcitabine.

Examination of ET-743 and PM00104 resistance in cell lines selected by different anti-cancer agents

We next examined the cross-resistance profiles in our panel of cell lines. Most of these cell lines revealed a well-described multi-drug resistance pattern besides resistance to the drug used for selection (Table 1). Doxorubicin

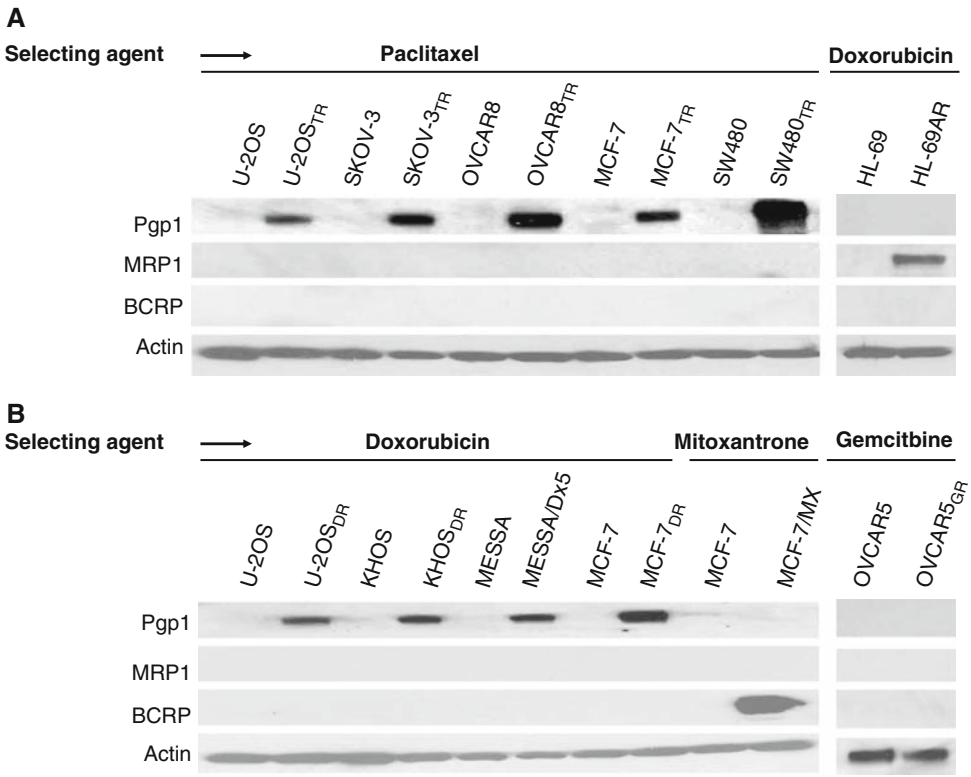

Fig. 1 Western blot analysis of Pgp1, MRP1, and BCRP in drug sensitive and resistant cell lines. Expression of Pgp1, MRP1, and BCRP was assessed with total cellular protein isolated from the indicated cell lines and immunoblotted with specific antibodies as described in "Materials and methods". The blots were also probed with an anti-actin monoclonal antibody to assess relative protein levels in the sample lanes. **a** Cell lines selected with paclitaxel, doxorubicin. **b** Cell lines selected with doxorubicin, mitoxantrane, and gemcitabine

Table 1 Diverse cross-resistance phenoptype in multidrug resistant cell lines

Cell line	Tumor origin	Selecting drug	ET-743 resistance	PM00104 resistance	Paclitaxel resistance	Doxorubicin resistance
U-2OS$_{TR}$	Bone	Paclitaxel	S	S	R	R
SKOV-3$_{TR}$	Ovarian	Paclitaxel	S	S	R	R
OVCAR8$_{TR}$	Ovarian	Paclitaxel	S	S	R	R
MCF-7$_{TR}$	Breast	Paclitaxel	S	S	R	R
SW480$_{TR}$	Colon	Paclitaxel	S	S	R	R
U-2OS$_{DR}$	Bone	Doxorubicin	R	R	R	R
KHOS$_{DR}$	Bone	Doxorubicin	R	R	R	R
MESSA/Dx5	Uterine	Doxorubicin	R	R	R	R
MCF-7$_{DR}$	Breast	Doxorubicin	R	R	R	R
IGROV1cp	Ovarian	Cisplatin	S	S	S	S
2008cp70	Ovarian	Cisplatin	S	S	S	S
OVCAR5$_{GR}$	Ovarian	Gemcitabine	S	S	S	S
TC-ET 6 nM	Bone	ET-743	R	R	R	R
TC-ET 12 nM	Bone	ET-743	R	R	R	R
MCF-7$_{MX}$	Breast	Mitoxantrane	S	S	S	S

S same sensitivity as wild-type cells, *R* resistance

selected cell lines (U-2OS$_{DR}$, KHOS$_{DR}$, MESSA/Dx5, MCF-7$_{DR}$) displayed cross-resistance to both ET-743 and PM00104 (Table 1; Fig. 2). On the other hand, paclitaxel selected cell lines (U-2OS$_{TR}$, SKOV-3$_{TR}$, OVCAR8$_{TR}$, MCF-7$_{TR}$, SW480$_{TR}$), did not display cross-resistance to either ET-743 or PM00104 (Table 1; Fig. 2). While most of the above doxorubicin resistant cell lines demonstrated cross-resistance to paclitaxel and vincristine but not to mitoxantrane, the above paclitaxel resistant cell lines demonstrated cross-resistance to doxorubicin, vincristine, and mitoxantrane. For example, the paclitaxel selected osteosarcoma cell line U-2OS$_{TR}$ revealed 60-fold increased resistance to mitoxantrane while doxorubicin selected cell line U-2OS$_{DR}$ did not show any significant cross-resistance to mitoxantrane (Fig. 3a). We further studied the calcein AM accumulation in these drug resistant cells. Calcein AM is a nonfluorescent hydrophobic molecule, rapidly penetrates mammalian cell membranes and converts into fluorescent calcein by cytoplasmic esterases. In the ABCB1 or MRP1-expressing cells, calcein AM is extruded by Pgp1 or MRP1 before its intracellular conversion [11]. We found both of U-2OS$_{TR}$ and U-2OS$_{DR}$ revealed decreasing intracellular accumulation of calcein AM as compared with U-2OS (Fig. 3b). Higher calcein AM efflux were found in all drug resistant cell lines overexpressing ABCB1, MRP1, or BCRP (data not shown). Indicate these genes overexpression were responsible for the efflux of calcein AM, paclitaxel, doxorubicin, or mitoxantrane. We also observed a distinct pattern of cross-resistance when comparing doxorubicin, paclitaxel, ET-743 selected cell lines to cisplatin, gemcitabine, and mitoxantrane selected cell lines. For example, ET-743 selected Ewing's sarcoma cell lines (TC-ET 6 nM and TC-ET 12 nM) revealed cross-resistance to both paclitaxel and doxorubicin. In contrast, cisplatin, gemcitabine or mitoxantrane selected cell lines (IGROV1cp, 2008cp70, OVCAR5$_{GR,}$ and MCF-7/MX) demonstrated no cross-resistance to paclitaxel, doxorubicin, ET-743, or PM00104 (Table 1).

Comparison of relative levels of drug resistance in paclitaxel and doxorubicin selected cell lines

Specifically, we compared the relative levels of cross-resistance between the paclitaxel selected and doxorubicin selected breast cancer cell lines, MCF-7$_{TR}$ and MCF-7$_{DR}$. Although both cell lines displayed cross-resistance to paclitaxel, doxorubicin, and vincristine, they displayed strikingly different levels of relative cross-resistance to mitoxantrane, ET-743, and PM00104 (Fig. 4). In addition, sub-lethal concentrations of ET-743 (5 nM) or PM00104 (8 nM) could not reverse resistance to doxorubicin and paclitaxel in MCF-7$_{TR}$, MCF-7$_{DR}$ cell lines (data not shown).

Effects of inhibition of ABCB1 expression on drug sensitivities

To evaluate the contribution of the ABCB1 mRNA expression levels in multi-drug resistance, the ABCB1 gene in KHOS$_{DR}$ cells was downregulated with siRNA, and relative drug sensitivities were evaluated by comparison of the IC$_{50}$ values determined by MTT in siRNA-treated and control multi-drug resistant cell lines. Western blot revealed Pgp1 (ABCB1) expression was significantly decreased after the cells treated with siRNA (Fig. 5a). Cytotoxicity was measured 6 days after treatment with siRNA (see "Materials and methods"). For paclitaxel and doxorubicin sensitivities, the

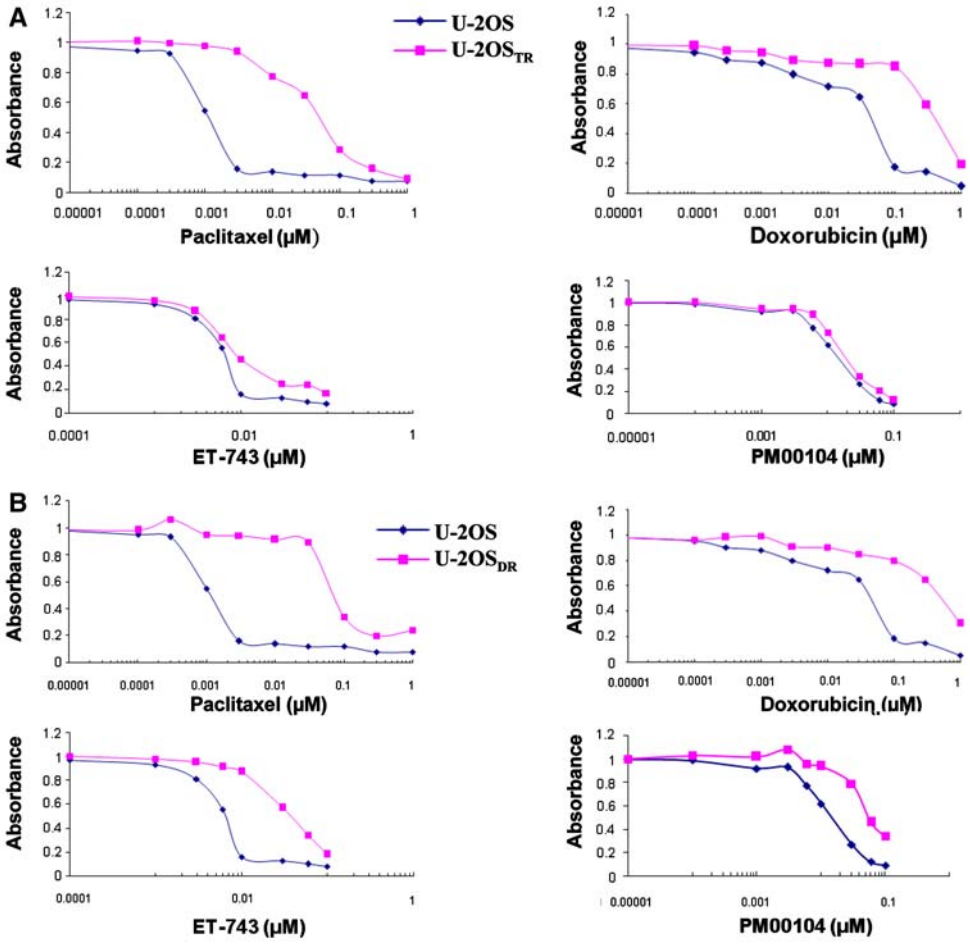

Fig. 2 Comparison of drug resistant phenotype in paclitaxel or doxorubicin selected cell lines. **a** Drug resistance in paclitaxel selected cells. **b** Drug resistance in doxorubicin selected cells. U-2OS, U-2OS$_{TR}$, and U-2OS$_{DR}$ cells were exposed to varying concentrations of paclitaxel, doxorubicin, ET-743, or PM00104 for 6 days. Growth inhibition was determined by incubation with the tetrazolium dye MTT and by absorbance measurement at 490 nm. Data are means of three replicates at each concentration

IC_{50} values of ABCB1 down-regulation KHOS$_{DR}$ were lower than in untreated cell lines, suggesting that ABCB1 is necessary for resistance to paclitaxel and doxorubicin. In contrast, the ABCB1 down-regulation did not recover sensitivity to ET-743 or PM00104 (Fig. 5b), suggesting that ABCB1 expression is not necessary for resistance to ET-743 or PM00104 in these doxorubicin selected cells.

Discussion

Patients with advanced ovarian, breast carcinomas as well as bone or soft tissue sarcomas typically undergo several lines of single or multi-agent chemotherapeutic regimens before ultimately succumbing to their disease [4, 29]. Multi-drug resistance is thought to play a major role in the inevitable failure of tumors to respond to each successive line of chemotherapy. Therefore, understanding the patterns and mechanisms of cross-resistance and finding ways to overcome it is an important goal. In this study, we profiled cross-resistance in a large panel of multi-drug resistant cell lines derived from wide array of human tumors. Daughter cell lines were selected for resistance to different chemotherapeutic agents: paclitaxel, doxorubicin, cisplatin, gemcitabine, and ET-743.

The best-described mechanism for multi-drug resistance is the drug efflux gene ABCB1/MDR1 via its transporter protein, Pgp1 [29]. Pgp1 expression is induced by exposure to one of several chemotherapeutic agents, and sustained Pgp1 expression leads to immediate cross-resistance to a second chemotherapeutic agent. We know that Pgp1, however, is not the sole mechanism for multi-drug resistance [8, 29]. In preclinical models, cancer cell lines that are selected for resistance using graduated exposure to various chemotherapeutic agents develop distinct patterns of cross-resistant drugs. Though previous groups have characterized multi-drug resistant cell lines for Pgp1 overexpression, we are the first to preclinically compare cross-resistance profiles of ET-743 and PM00104 and assess the role of Pgp1 across a large collection of cell lines generated by selection from a panel of chemotherapeutic agents using parental cell lines derived from histologically unrelated tumors.

In this study, we examined the preclinical role for ABCB1 in cross-resistance, with particular focus on ET-743 and PM00104. We were initially intrigued that, although ABCB1 is over-expressed in all paclitaxel and

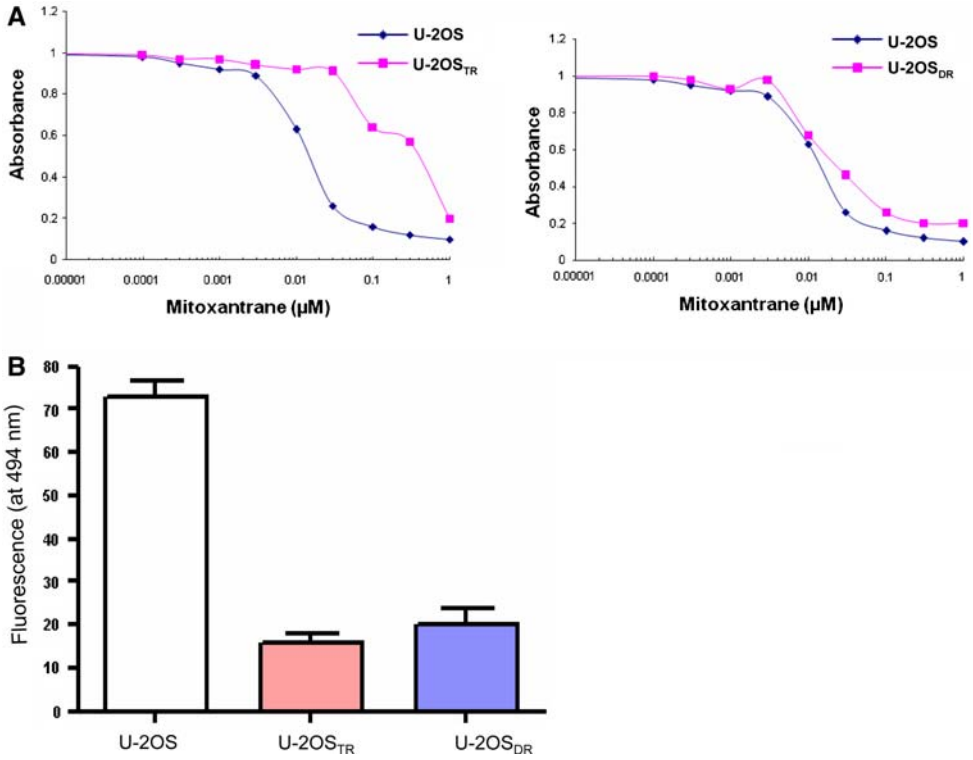

Fig. 3 Different mitoxantrane resistance and Calcein AM efflux in paclitaxel or doxorubicin selected cell lines. **a** U-2OS, U-2OS$_{TR}$, and U-2OS$_{DR}$ cells were exposed to varying concentrations of mitoxantrane for 6 days. Growth inhibition was determined by incubation with the tetrazolium dye MTT and by absorbance measurement at 490 nm. Data are means of three replicates at each concentration. **b** Calcein AM efflux in different cells. Triplicated cultures of U-2OS, U-2OS$_{TR}$, and U-2OS$_{DR}$ cells were cultured for 24 h and then incubated in calcein AM for 30 min, and the cell fluorescence was measured by SPECTRAmax Microplate Spectrofluorometer reader

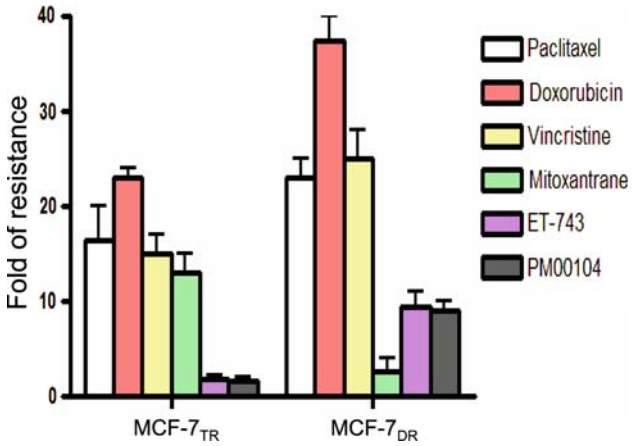

Fig. 4 Comparison of level of drug resistance in paclitaxel and doxorubicin selected cell lines. MCF-7, MCF-7$_{TR}$, and MCF-7$_{DR}$ cells were exposed to varying concentrations of paclitaxel, doxorubicin, vincristine, mitoxantrane, ET-743, or PM00104 for 6 days. Growth inhibition was determined by incubation with the tetrazolium dye MTT and by absorbance measurement at 490 nm. The *Y* axis shows the relative resistance fold index expressed for each particular drug as the ratio of the IC_{50} value of the drug-resistant cells to the IC_{50} value of the drug-sensitive parental cell line

doxorubicin selected cell lines, only doxorubicin selected cell lines are cross-resistant to ET-743 and PM00104. In fact, several publications have also reported Pgp1 overexpression in cell lines that are not resistant to ET-743 [2, 15]. One of these reports demonstrated that treatment with ET-743 was actually associated with ABCB1 downregulation [15]. In our group, we have previously reported that there was no significant difference in the levels of ABCB1 or MRP1, but we did see differences in collagen and heat shock proteins expression in ET-743 selected chondrosarcoma cell lines [25]. Therefore, we directly tested the role of Pgp1 in cross-resistance by using siRNA to down regulate Pgp1. Here, we show that Pgp1 down-regulation can indeed reverse resistance to both paclitaxel and doxorubicin. However, despite Pgp1 down-regulation, ET-743 and PM00104 resistance remained. These results, taken together, strongly suggest that Pgp1 over-expression is neither sufficient nor necessary for cross-resistance to ET-743 or PM00104 in doxorubicin selected cell lines.

When we looked at multi-drug resistant profiles, we were intrigued to see remarkable similarity in preclinical cross-resistance patterns among cell lines selected by the same drugs despite being derived from histologically unrelated tumors. Cell lines selected for resistance by doxorubicin, regardless of histology, became cross-resistant to ET-743 and PM00104; in contrast, cell lines selected for resistance by gemcitabine, or paclitaxel mostly remained sensitive to ET-743 and PM00104. This suggests that multi-drug resistance mechanisms are more a result of the initial drug used for selection rather than being a histologically based phenomenon.

This observation raises several intriguing clinical hypothesis. First, it suggests that ET-743 and PM00104 would be less likely to be effective after development of chemoresistance. In fact, this is observed clinically. Even though ET-743 demonstrated a 17.1% overall response rate in

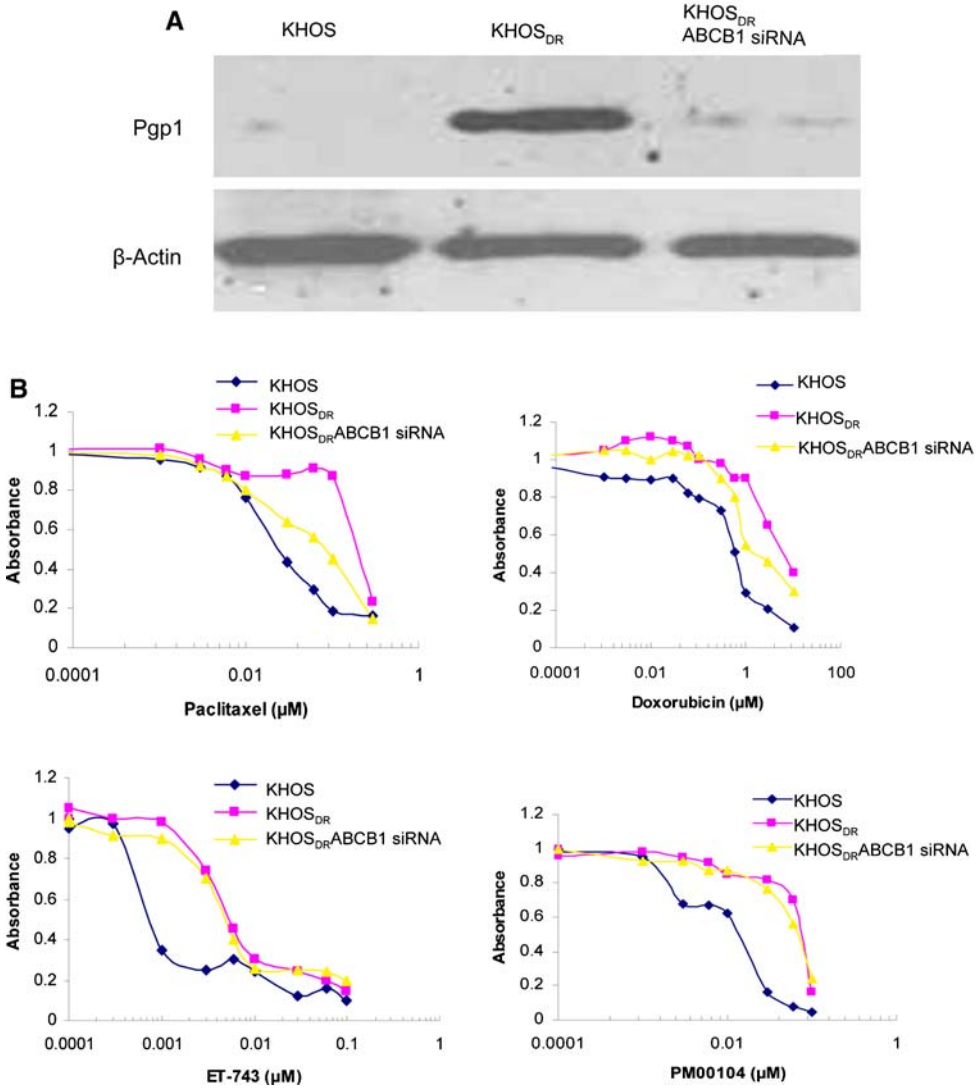

Fig. 5 Effect of ABCB1 inhibition on drug sensitivity in doxorubicin selected drug resistant cells. **a** Confirmation of protein Pgp1 knockdown by Western blot. Total protein was isolated 48 h post-transfection and Pgp1 expression was analyzed by Western blotting with anti Pgp1 or anti actin antibodies. **b** The doxorubicin selected drug resistant cell line KHOS$_{DR}$ was transfected with ABCB1 On-Targetplus SMARTpool siRNA. The relative sensitivity of each line to paclitaxel, doxorubicin, ET-743 and PM00104 was determined by MTT analysis 96 h post-transfection

chemotherapy-naïve patients with advanced soft tissue sarcomas [12], among previously treated patients ET-743 demonstrated an 8% overall response rate [17]. When comparing ET-743 response rates in platinum-sensitive versus platinum-resistant patient cohorts of patients with recurrent ovarian cancer, ET-743 demonstrated a 29% overall response rate in the platinum-sensitive cohort while showing only a 6.3% response rate in the platinum-resistant cohorts [16]. Second, our results suggest that ET-743 is particularly unlikely to be effective after development of chemoresistance to doxorubicin. In the above study by Le Cesne et al. [17], though the first-line agent was not described, doxorubicin is typically accepted as first-line treatment for advanced sarcomas and may possibly explain the low response rate to subsequent treatment with ET-743 [17]. Third, we can hypothesize that ET-743 may be more active in a subset of sarcoma patients who are doxorubicin-naïve. This subset has actually increased in population size in the United States after reports that the combination of gemcitabine and docetaxel offers a significant overall response rate (53%) in patients with leiomyosarcomas, a major subset of soft tissue sarcomas [13]. Therefore, our observations would lend support to the design of a clinical trial that tests the hypothesis that there might be a higher clinical response rate in subsets of sarcoma patients initially treated with gemcitabine and docetaxel compared to those initially treated with a doxorubicin-based regimen.

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