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Pegylated liposomal formulation of doxorubicin overcomes drug resistance in a genetically engineered mouse model of breast cancer

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ABSTRACT

Success of cancer treatment is often hampered by the emergence of multidrug resistance (MDR) mediated by P-glycoprotein (ABCB1/Pgp). Doxorubicin (DOX) is recognized by Pgp and therefore it can induce therapy resistance in breast cancer patients. In this study our aim was to evaluate the susceptibility of the pegylated liposomal formulation of doxorubicin (PLD/Doxil®/Caelyx®) to MDR. We show that cells selected to be resistant to DOX are cross-resistant to PLD and PLD is also ineffective in an allograft model of doxorubicin-resistant mouse B-cell leukemia. In contrast, PLD was far more efficient than DOX as reflected by a significant increase of both relapse-free and overall survival of *Brca1*$^{-/-}$;*p53*$^{-/-}$ mammary tumor bearing mice. Increased survival could be explained by the delayed onset of drug resistance. Consistent with the higher Pgp levels needed to confer resistance, PLD administration was able to overcome doxorubicin insensitivity of the mouse mammary tumors. Our results indicate that the favorable pharmacokinetics achieved with PLD can effectively overcome Pgp-mediated resistance, suggesting that PLD therapy could be a promising strategy for the treatment of therapy-resistant breast cancer patients.

1. Introduction

Chemotherapy remains the principal therapeutic modality in cancer treatment. Despite recent successes, such as the discovery of the BCR-ABL tyrosine kinase inhibitors in chronic myeloid leukemia (CML), all-trans retinoic acid (ATRA) in acute promyelocytic leukemia (APL) and other advances in testicular cancer, pediatric leukemias and Hodgkin's lymphomas, which have demonstrated striking effects on patients survival [1], resistance and relapse remains a major obstacle [2].

Breast cancer is the most common malignancy in women. Approximately 60% of the breast cancer patients diagnosed at an early stage of the disease receive chemotherapy, but only a minor fraction of patients actually benefit from it [3]. 30% of women diagnosed with early stage breast cancer will progress to metastatic disease where there are only few treatment options [4]. Response to anthracycline- or taxane-based treatment regimens is overall weak and not long-lasting [5]. A study concluded that 50–70% of relapsing tumors from surgically removed adenocarcinomas were already drug resistant [6].

Resistance to anticancer agents is based on several mechanisms. Cancer cells can downregulate the drug target, tune down pathways leading to apoptosis, upregulate DNA repair mechanisms or increase the metabolism of drug molecules [7]. One of the most frequent and most investigated mechanisms of cellular drug resistance relies on the active efflux of the chemotherapeutical compounds from the cells. P-glycoprotein (ABCB1/Pgp), a member of the ATP Binding Cassette (ABC) transporter family was shown to extrude numerous, structurally unrelated chemotherapeutic drugs from resistant cancer cells [8]. There is ample evidence to prove the link between the activity of Pgp and clinical anticancer drug resistance. Pgp expression is an independent prognostic factor in acute myeloid leukemia (AML) [9,10] and acute nonlymphoblastic leukemia (ANLL) [11]. Pgp function in tumor cells shows negative correlation with response to the treatment and reliably predicts therapy response in AML [12].

Recently, genetically engineered mouse models (GEMMs) have been introduced to the study of drug resistance mechanisms. GEMMs closely mimic cancer in human patients and therefore offer a unique

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opportunity to study the evolution of drug resistance. In particular, conditional deletion of the Brca1 and p53 genes was shown to give rise to mammary carcinomas that mimic many aspects of the human disease [13]. In contrast to the xenograft models, these spontaneous tumors become drug resistant as a result of the treatment [14]. Like most human cancers, *Brca1*$^{-/-}$*;p53*$^{-/-}$ tumors show initial sensitivity to doxorubicin, topotecan, cisplatin [15], and the poly(ADP-ribose)-polymerase (PARP) inhibitor olaparib, which induce synthetic lethality in BRCA1- or BRCA2-deficient cells [16]. However, the tumors always acquire resistance to docetaxel, doxorubicin, topotecan or olaparib [17,18], based on the increased expression of the Abcb1 or Abcg2 genes. Inhibition of ABCB1 using tariquidar successfully reversed drug resistance [19], and the relevance of the efflux-based drug resistance was also confirmed in ABC transporter-deficient tumors [18].

While there is a constant need for finding new targets, the efficacy of existing drugs could also be restored by eliminating resistance mechanisms. Unfortunately, attempts to circumvent reduced drug accumulation by inhibiting drug efflux have failed in clinical trials, because inhibition of Pgp altered the pharmacokinetic properties of the coadministered cytotoxic compounds [20]. Because selective modulation of Pgp in cancer cells remains difficult to achieve, attempts to circumvent MDR rely on further strategies such as targeting the paradoxical hypersensitivity of MDR cancer [21–24]. Another possibility is to develop drugs that bypass efflux either through the chemical modification of the cytotoxic compounds or through novel formulations of existing therapeutics [25].

Doxorubicin (DOX) is still one of the most effective chemotherapeutic agents used in lung, breast, ovarian, uterine cancers and in lymphomas and leukemias [26–28]. The pegylated liposomal formulation of doxorubicin (PLD/Doxil®/Caelyx®) was developed to overcome DOX's dose limiting cardiotoxicity and myelosuppression [29]. PLD has the ability to avoid the reticuloendothelial system (“stealthness”) [30], and as a result of the enhanced permeability and retention (EPR) effect, PLD is “passively” targeted to tumors [31,32]. PLD's efficacy was evaluated in several allo- and xenograft models of colon [33], breast [34], ovarian [35], lung [36], leukemia [37], lymphoma [38], bladder [39] and prostate cancer [40]. These studies have convincingly demonstrated that PLD has an equal or even better performance than DOX (reviewed in [41]). Clinical trials comparing PLD to DOX in patients with metastatic breast cancer (MBC) proved that both treatments are comparably efficient [42].

DOX is recognized by Pgp and therefore it can induce therapy resistance in breast cancer patients [43]. In this study our aim was to evaluate the susceptibility of PLD to MDR. We show that cells selected to be resistant to DOX are cross-resistant to PLD. However, in contrast to doxorubicin, PLD treatment results in a durable response of BRCA1-deficient mammary tumors, and PLD remains effective in DOX-resistant ABCB1-expressing tumors.

2. Materials and methods

2.1. Drugs

Cytotoxic drugs doxorubicin (dox, teva), pegylated liposomal doxorubicin Caelyx® (PLD, Janssen) and cisplatin (CDDP, Accord Healthcare) were purchased directly from the manufacturers. The compounds used in the DT40 cytotoxicity assays were purchased from Selleckchem (olaparib), Sigma-Aldrich (paclitaxel, SN-38, doxorubicin) or Accord Healthcare (PLD, 5-FU) or TEVA (etoposide). Daunorubicin was a kind gift from Dr. Gábor Mező (ELTE, Hungary).

2.2. Cell lines

The human uterine sarcoma cell lines MES-SA and the doxorubicin selected MES-SA/Dx5 were obtained from ATCC (MES-SA: No. CRL-1976™, MES-SA/Dx5: No. CRL-1977™). The human mammary carcinoma cell lines MCF7, T47D, MDA-MB-231, MDA-MB-468, Hs578T, BT-549, the mouse leukemia cell line P388 and its doxorubicin selected subline P388/ADR were obtained from the National Cancer Institute's Developmental Therapeutics Program (National Institutes of Health). P388/ADR and Dx5 cells were maintained in 800 and 500 nM doxorubicin (Adriamycin), respectively. The human breast cancer and the mouse leukemia cell lines were cultured in RPMI media (Life Technologies) supplemented with 10% fetal bovine serum, 5 mM glutamine, and 50 units/ml penicillin and streptomycin (Life Technologies). MES-SA and MES-SA/Dx5 cells were cultured in supplemented DMEM media (Life Technologies). The chicken B-cell line DT40 was grown in RPMI-1640 medium supplemented with 7% fetal bovine serum, 3% chicken serum, 50 μM 2-mercaptoethanol and penicillin/streptomycin. Wild-type DT40 clone18 cells [44] and a BRCA1 null mutant line [45] were used. All cell lines were cultured at 37 °C, 5% CO_2.

2.3. In vitro cytotoxicity assay

Viability was assessed by the PrestoBlue® assay (Life Technologies), according to the manufacturer's instructions. Briefly, cells were plated in 96- or 384-well plates, treated in the given concentration range with the indicated compounds for 120 h or 72 h in case of DT40 cells. Viability of the cells was measured spectrophotometrically using an EnSpire microplate reader (Perkin Elmer). Data were normalized to untreated cells; curves were fitted by the Graph Pad Prism 5 software using the sigmoidal dose–response model. Curve fit statistics were used to determine IC_{50} values.

2.4. Immunohistochemistry

Snap-frozen tissues were cut with a cryostat into 5 μm sections. The tissue slices were transferred onto microscope slides and fixed with ice-cold methanol for 10 min. Tissue sections were then washed in PBS, blocked with 3% bovine serum in PBS (1 h), stained with hematoxylin and eosin and then the sections were mounted with ProLong Gold (Life Technologies). Immunohistochemistry images were examined by an Eclipse TS100 microscope (Nikon).

2.5. RNA isolation and RT-PCR

Snap-frozen tumor samples were pulverized under liquid nitrogen and were homogenized in TRIzol™ Reagent (Life Technologies). Total RNA was isolated from tissue samples using Direct-zol® MiniPrep kit (Zymo Research) according to the manufacturer's guidelines. In-column DNAse I treatment was applied to prevent DNA contamination. cDNA samples were prepared from 300 ng total RNA using the Promega Reverse Transcription System Kit. The Pre-Developed TaqMan® assay Actin β (Actβ) (Life Technologies) was used as endogenous control in real-time PCR (RT-PCR) experiments; for quantifying Abcb1a and Abcb1b mRNA levels the respective TaqMan® primers were used. RT-PCR analyses were carried out using the StepOne™ Real-Time PCR System (Life Technologies); mRNA fold changes were determined using the $2^{-\Delta\Delta Ct}$ method. Relative mRNA levels were presented as mean values ± S.E.M. of 3 independent experiments.

2.6. Animal experiments

All animal protocols were approved by the Hungarian Animal Health and Animal Welfare Directorate according to the EU's most recent directives. All surgical procedures were performed according to the Committee on the Care and Use of Laboratory Animals of the Council on Animal Care at the Institute of Enzymology, RCNS in Budapest, Hungary (22.1/2291/3/2010).

P388 and P388/ADR cells (1×10^6/animal) were injected into the intraperitoneal cavity of 6–8 weeks old male BDF1 mice and 48 h later a