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Prevention of MDR Development in Leukemia Cells by Micelle-Forming Polymeric Surfactant

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Abstract

Doxorubicin (Dox) incorporated in nanosized polymeric micelles, SP1049C, has shown promise as monotherapy in patients with advanced esophageal carcinoma. The formulation contains amphiphilic block copolymers, Pluronics, that exhibit unique ability to chemosensitize multidrug resistant (MDR) tumors by inhibiting P-glycoprotein (Pgp) drug efflux system and enhancing pro-apoptotic signaling in cancer cells. This work evaluates whether a representative block copolymer, Pluronic P85 (P85) can also prevent development of Dox-induced MDR in leukemia cells. For *in vitro* studies murine lymphocytic leukemia cells (P388) were exposed to increasing concentrations of Dox with/without P85. For *in vivo* studies, BDF1 mice bearing P388 ascite were treated with Dox or Dox/P85. The selected P388 cell sublines and ascitic tumor-derived cells were characterized for Pgp expression and functional activity (RT-PCR, Western Blot, rhodamine 123 accumulation) as well as Dox resistance (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay). The global gene expression was determined by oligonucleotide gene microarrays. We demonstrated that P85 prevented development of MDR1 phenotype in leukemia cells *in vitro* and *in vivo* as determined by Pgp expression and functional assays of the selected cells. Cells selected with Dox in the presence of P85 *in vitro* and *in vivo* exhibited some increases in IC_{50} values compared to parental cells, but these values were much less than IC_{50} in respective cells selected with the drug alone. In addition to mdr1, P85 abolished alterations of genes implicated in apoptosis, drug metabolism, stress response, molecular transport and tumorigenesis. In conclusion, Pluronic formulation can prevent development of MDR in leukemia cells *in vitro* and *in vivo*.

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Keywords

Multidrug Resistance; P-glycoprotein; Pluronic; Poloxamer; Doxorubicin

Introduction

Nanotechnology holds a tremendous potential for diagnosis and therapy of cancer [1,2]. Furthermore, recent developments indicate that select polymer nanomaterials can implement more than only inert carrier functions by being biological response modifiers [3–7]. One promising nanomedicine-based technology is polymeric micelles, which have been evaluated in several clinical trials as carriers for anticancer drugs [8–10]. In particular, doxorubicin (Dox) incorporated in mixed micelles of Pluronic block copolymers, SP1049C, has shown promise in Phase II study in patients with advanced esophageal carcinoma [11]. Pluronics are amphiphilic synthetic polymers containing hydrophilic poly(ethylene oxide) (PEO) blocks and hydrophobic poly(propylene oxide) (PPO) blocks arranged in a triblock structure: PEO-PPO-PEO [12]. These block copolymers were shown to sensitize multidrug resistant (MDR) tumors refractory to anthracyclines and other chemotherapeutic agents [13–15]. Ability of cancer cells to become simultaneously resistant to different drugs is a serious impediment to chemotherapy [16,17]. The key mechanisms of development of MDR in cancer cells include decreased uptake of drugs in cells, alteration of cellular targets that reduce the capacity of cytotoxic drugs to kill the cells and increased energy-dependent efflux of drugs that can enter the cells by diffusion through the plasma membrane [18]. The most commonly observed mechanism of MDR involves efflux of cytotoxic drugs mediated by P-glycoprotein (Pgp), an ATP-dependent drug efflux transporter [19]. The promoter of the mdr1 gene can be induced by anticancer agents including vincristine, daunomycin, adriamycin and colchicines [20]. An emerging strategy to enhance cytotoxicity of anticancer drugs is their formulation with inhibitors of Pgp [21]. However, as of today most small molecule inhibitors of Pgp have failed during pre-clinical or clinical development because of low efficiency and substantial side effects [22,23]. A nanomedicine technology based on polymeric materials may provide a more successful option for treatment of tumors [11]. Such novel modalities may also be effective in treatment of diseases in which Pgp expression is an unfavorable prognostic factor such as leukemia [24–26]. Therefore, in this work we evaluated effects of a representative block copolymer, Pluronic P85 (P85) on the development of MDR in response to Dox treatment of murine lymphocytic leukemia cells, P388, *in vitro* and *in vivo*. For the first time this study demonstrates that Pluronic block copolymers can prevent induction of MDR in leukemia cells.

Materials and Methods

Cells and Reagents

Murine lymphocytic leukemia cells (P388) were received from the laboratory of Dr. Brian Leyland-Jones at McGill University (Montreal, Canada). Cells were cultured at 37°C in RPMI-1640 medium in an humidified atmosphere of 5% CO_2. Cell culture medium and fetal bovine serum (FBS) were from Gibco Life Technologies, Inc. (Grand Island, NY, USA). Culture flasks and dishes were from Corning Inc. (Corning, NY, USA). Dox was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Pluronic P85 (P85) block copolymer (lot # WPOP-587A) was provided by BASF Corp. (Parispany, NJ, USA). The molecular mass of the PPO block in this sample was approximately 2,500 Daltons, and the content of the PEO blocks was approximately 50 % wt.

In vitro Selection of P388 Cells

Cells were suspended (10^5 cells/ml) in RPMI 1640 media with 10% FBS and incubated at 37° C in a humidified, 5% CO_2 atmosphere. The culture media was supplemented with either Dox alone; Dox formulated with 0.001% P85; or 0.001% P85 alone. When cells grew to a density of ca. 5×10^6 cells/ml, they were resuspended in a 75-cm^2 tissue culture flask and the Dox dose was increased. At different selection points cell sublines were stored at −80°C for further analysis.

Animals

Four-week-old female BDF1 mice (Charles River, Wilmington, MA, USA) were used in this study. Mice were kept four per cage with an air filter cover under light (12-h light/dark cycle) and temperature-controlled (22±1 °C) environment with food and water *ad libitum*. All manipulations with the animals were performed under a sterilized laminar hood. The animals were treated in accordance to the Principles of Animal Care by National Institutes of Health, and Protocol #95-079-07-FC approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee.

Development of Resistant Cells *In Vivo*

Half a million P388 cells suspended in 200 µl phosphate buffered saline (PBS), pH 7.4 were inoculated into peritoneal cavities (*i.p.*) of the four-week-old female BDF1 mice. The abdominal area was carefully checked to detect palpable ascites. After three days, the animals were treated via the tail vein (i.v.) with Dox and/or P85 (all in PBS, 100 µl per mouse), and the treatments were repeated every third day. There were two animals for each control and treatment groups. The animals were monitored for body weight and food intake twice every week and euthanized at signs of becoming moribund. The day of euthanasia of a host animal was designated as its lifespan [27]. Ascite fluids were collected and tumor cells harvested for further selection cycles with new mouse hosts.

Cytotoxicity Assay

Cells were resuspended in 96-well plates (5000 cells/well), grown overnight and treated with Dox. The cytotoxic activity was determined after 72 hours using a standard 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [28]. The absorbency at λ = 450nm was determined using a microKinetics Reader BT 2000. Each concentration point was determined from samplings from eight separate wells. SEM values were less than 10%.

RNA isolation and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated using TRI Reagent® (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's protocol. RNA samples showing two distinct bands (18S and 28S) in 1.2% agarose/formaldehyde gels, and 260/280 nm absorbance ratio of 1.7 to 1.9, were used for RT-PCR and microarray analyses (see below). Reaction mixtures (50 µl) containing 100 ng of RNA, 25 µl 2X Access quick master mix, 1µM sense and antisense primers (Table 1) and 5 Units of AMV reverse transcriptase were prepared using AccessQuick™ RT-PCR system (Promega, Madison, WI, USA, Cat#A1702). PCR amplifications were carried out with PCR Sprint Cycler (Thermo Electron Corp., Milford, MA) as follows: (*i*) reverse transcription (48°C/45 min), (*ii*) initial denaturation (94°C/2 min), (*iii*) 30 amplification cycles (94°C/60s denaturation, 55°C/45s annealing, 70°C/30s extension), and (*iv*) final extension (72°C/5 min). PCR products were size-fractionated against a DNA ladder (Promega, cat. # G2101) on 2% agarose gels and stained with ethidium bromide (0.5 µg/ml). The gel images were captured with GEL DOC 2000 (Bio-Rad, Hercules, CA, USA) and analyzed using Molecular Dynamics Image QuaNT version 5.1 Software (GE Healthcare, Piscataway, NJ, USA).

Western Blot Analysis

Pgp protein levels were determined as previously reported [29] using (*i*) monoclonal antibodies to Pgp, C219 (Dako Corp., Carpinteria, CA, USA) (1:100), (*ii*) monoclonal antibodies to β-actin, anti-β-1-chicken integrin (Sigma Chemical Co.) (1:200), and (*iii*) secondary horseradish peroxide anti-mouse Ig antibodies (Amersham Life Sciences, Cleveland, OH, USA) (1:1500). Specific bands were visualized using a chemiluminescence kit (Pierce, Rockford, IL, USA).

Rhodamine 123 (R123) Accumulation Studies

Cellular accumulation of Pgp substrate, R123 was examined as previously described [30]. Cells (2×10^6) were preincubated for 30 min at 37°C in assay buffer (122 mM sodium chloride, 25 mM sodium bicarbonate, 10 mM glucose, 10 mM HEPES, 3mM potassium chloride, 1.2 mM magnesium sulfate, 1.4 mM calcium chloride, and 0.4 mM potassium phosphate dibasic, pH 7.4), exposed to 3.2 μM R123 in fresh assay buffer for 60 min, washed three times with ice-cold PBS and solubilized in Triton X-100 (1.0 %). R123 content was determined using a Shimadzu RF5000 fluorescent spectrophotometer (λ_{ex} = 505 nm, λ_{em} = 540 nm) and normalized for cellular protein levels as determined by Pierce BCA assay.

Global Gene Expression Assay

All DNA microarray gene expression studies used mouse oligonucleotide arrays custom-printed by a dedicated core facility within the Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, NE, as described previously [31]. Arrays were constructed from a set of 10,178 sense oligonucleotide (65-mers) probes designed for each mouse target gene by Compugen Inc. (Rockville, MD, USA), and manufactured by Sigma-Genosys, Inc. (The Woodlands, TX, USA). Individual arrays contain 10,202 spot features, including 10,154 different genes, 24 replications of GAPDH, and negative controls. Each oligonucleotide was resuspended (30 μM) in 3X SSC and 1.5 picoliters spotted onto poly-L-lysine coated glass slides using a MagnaSpotter robot (BioAutomation Corp., Dallas, TX, USA) with a 12-pin (Telechem, Sunnyvale, CA, USA) configuration in a humidified, HEPA-filtered hood. After spotting, the DNA was cross-linked to the slides by UV irradiation (350 mJ/cm^2) with a Stratalinker UV Crosslinker (Stratagene, Inc., La Jolla, CA, USA) and the printed arrays stored in the dark in a dehumidified atmosphere at room temperature. Before use, arrays were blocked by succinic anhydride treatment (165 mM succinic anhydride in a 24:1 ratio of 1-methyl-2-pyrrolidinone: 1M sodium borate, pH 8.0), and rinsed in ethanol. Array quality was evaluated both upon initial synthesis, as well as over time, by hybridizing with previously characterized stock RNA, and comparing current with prior gene expression profiles using array median centering and Coorelation Coefficients Mapping (CCM) programmed in MATLAB (The Mathworks, Inc., Natick, MA, USA).

For each gene expression comparison, fluorescent targets were generated using an indirect or “two-step” labeling procedure [31]. Typically, cDNA synthesis was performed on total RNA (40μg) from experimental and reference samples by priming with 2μg of anchored oligodeoxythymidylate primer in a total volume of 30μl at 70°C for 10 min and chilled on ice. To each sample 10X first strand buffer (6μl), 0.1M DTT (6μl), 20X aminoallyl-dNTP mix (3μl; 10mM dATP, 10mM dCTP, 10mM dGTP, 6mM dTTP, and 4mM aminoallyl-dUTP), RNase inhibitor (0.5μl) (RNasin, Promega), and StrataScript reverse transcriptase (3μl; 50U/μl) was added. After incubation at 48°C for 60 min, an additional 50 units of StrataScript was added to the samples and incubated for an additional 60 min. The reaction was stopped by adding 12 μl of 0.5M EDTA (pH 8). Residual RNA was hydrolyzed by adding 12 μl of 1M NaOH to the mixture followed by incubation at 65°C for 15 min and cooled to room temperature. The reaction was neutralized with 16.8μl of 1N HCl. First-strand cDNA was purified from unincorporated amino allyl-dUTPs on QIAquick PCR purification columns (Qiagen) according to manufacuter’s protocols, except that QIAquick wash buffer was replaced

with 5 mM potassium phosphate buffer (pH 8.5) containing 80% ethanol, and cDNA was eluted with 4 mM potassium phosphate buffer (pH 8.5) and vacuum-dried. cDNA was resuspended in 10 μl 0.05 M Na_2CO_3 buffer (pH 9), mixed with either Cy3 or Cy5 monofunctional NHS-ester (Amersham Pharmacia), and incubated for 90 min in the dark at room temperature. Cy3- and Cy5-conjugated cDNA targets were purified by QIAquick PCR purification columns, combined, vacuum-concentrated, and diluted to 55 μl with hybridization solution containing final concentrations of 50% formamide, 4.1X Denhardts, and 4.4X SSC. To reduce nonspecific hybridization, the hybridization solution also contained final concentrations of 15 μg human Cot1 DNA (Invitrogen), 12μg poly-deoxyadenylate, and 5 μg of yeast tRNA (Sigma). After clarification by centrifugation, the cDNA/hybridization solution was applied to DNA microarrays and incubated at 42°C for 16–20 h. After hybridization, microarray slides were washed by immersion in 2X SSC, 0.1%SDS for 5 min at room temperature, 1X SSC, 0.01% SDS for 5 min at room temperature, and 0.2X SSC for 2 min at room temperature. The microarrays were dried by centrifugation for 5 min at 1000 rpm and scanned immediately with a ScanArray 4000 confocal laser system (Perkin-Elmer). Fluorescent intensities were background subtracted, and normalization and filtering of the data performed using the QuantArray software package (Perkin-Elmer). After normalization, expression ratios were calculated for each feature [32]. Microarrays were repeated three times using a new RNA extraction from each cell subline except for P85 and DOX (250 ng), which were repeated twice. The means of expression ratios were calculated, submitted to GEO (http://www.ncbi.nlm.nih.gov/geo/), and used in further analysis.

Microarray Data Analysis

Expression ratio values in each sample were $\log_2$ transformed and analyzed using the bivariate scatter plots. For analysis of global gene expression, the Gene expression data containing gene identifiers and corresponding mean expression values was uploaded into the Ingenuity Pathways Analysis application (Ingenuity® Systems, http://www.ingenuity.com, Mountain View, CA, USA). A significance cutoff of 1.8-fold was set to determine differentially regulated genes which were mapped to corresponding gene objects in the Ingenuity Pathways Knowledge Base to identify global gene expression patterns. To identify the canonical signaling pathways affected, pathway prediction analysis of differentially expressed genes was performed.

Results

In vitro Selection of P388 Cells with Dox and Dox-P85

Murine leukemia P388 cells were cultured with increasing concentrations of Dox or Dox formulated with 0.001% P85. Cells selected with Dox alone (P388/Dox) showed stable growth in the presence of 1000 ng/ml Dox after 150 days (Fig. 1A). In contrast, cells selected with Dox and P85 (P388/Dox-P85) could not tolerate more than 15 ng/ml of Dox in culture media. Cells were harvested and frozen at different time points of the selection as shown in Fig. 1A. All together, eleven different cell sublines were characterized for drug resistance. These sublines included parental P388 cells and selected P388 cells (grown at 5 ng/ml Dox with/without P85; 15 ng/ml Dox with/without P85; 125 ng/ml Dox; 250 ng/ml Dox; 500 ng/ml Dox; 750 ng/ml Dox and 1,000 ng/ml Dox). In addition, P388 cells were also cultured in drug-free medium containing 0.001% P85. These cells (P388/P85) were grown stably for 150 days and then used for further analysis.

In vivo Selection of P388 Cells with Dox and Dox-P85

Female BDF1 mice inoculated i.p. with P388 cells were treated i.v. every 3rd day with (*i*) PBS (control), (*ii*) 0.02% P85 (P85), (*iii*) DOX 2.5 mg/kg body weight (Dox) or (*iv*) DOX 2.5 mg/kg body weight with 0.02% P85 (Dox/P85). Mice undergoing chemotherapy showed normal food uptake, general mobility, and overall normal behavior during the course of treatment.

Once animals became moribund (see supplementary Table S-1) peritoneal fluid from ascites was collected and cancer cells were harvested. The cells were inoculated i.p. into a new host mice for the next treatment cycles, and the remaining cells were stored at −80°C for further characterization. Animals receiving Dox and Dox/P85 were injected 32 to 35 times over a period of 115 to 118 days. Animals receiving PBS and P85 were injected 24 to 25 times a period of 85 to 95 days (Table S-1). Kruskal-Wallis non-parametric statistical test shows significant difference in the life span of animals treated in different groups (*p*-value 0.00001). Mann-Whitney non-parametric statistical test used for pair wise comparison shows that Dox/P85 and Dox treatments increased the animal lifespan over PBS control (*p*-value 0.0006 and 0.0004). Furthermore, Dox/P85 treatment increased the animal lifespan over Dox (*p*-value 0.0086). No effect on lifespan observed for P85 and PBS control (*p*-value 1.0000) (Fig. 3A).

Cytotoxicity of Dox in Cells Selected *In Vitro* and *In Vivo*

The IC_{50} of Dox in cell sublines selected *in vitro* revealed the same trend as their tolerance to the drug during selection: IC_{50} increased as the drug exposure increased (Fig. 1B). All cells selected with Dox alone (5 ng/ml and above) showed significantly higher IC_{50} compared to the parental P388 cells. However, drug resistance appeared to develop in two distinct phases: 1) at 5 to 75 ng/ml Dox IC_{50} increased only 3.2- to 5.7-fold, while 2) at 125 ng/ml and above IC_{50} increased by several dozen and hundred folds (see also supplementary Table S-2). Furthermore, it took about 77 days to pass through the first phase, while development of resistance at the second phase was much faster (Fig. 1A). The effects of drug exposure were different in the presence of the copolymer (Fig. 1B). There was no change in IC_{50} in cells selected in the presence of P85 at 5 ng/ml Dox. At 15 ng/ml Dox the IC_{50} increased by ca. 17-fold compared to IC_{50} in parental cells. However, the copolymer prevented cell growth at drug concentrations higher than 15 ng/ml. Furthermore, in cells grown at 15 ng/ml Dox with P85 for a period of 150 days, the IC_{50} was only 9-fold higher than in parental cells (Table S-2). No change in IC_{50} was seen in cells grown with the copolymer alone. Similarly, P85 also appears to suppress development of resistance *in vivo* (Fig. 3B). In the animal model, IC_{50} increased by 2- and 13-fold in the Dox/P85 and Dox treatment regimens, respectively (Table S-2). No changes in drug resistance were observed in either the control or P85 treatment regimens.

Characterization of MDR1 in Cells Selected *In Vitro* and *In Vivo*

Using RT-PCR analysis, no significant change in mdr1 mRNA transcript levels was observed in cells selected with Dox in the presence of P85 *in vitro* (5 and 15 ng/ml Dox) (Fig. 2A) or *in vivo* (Fig. 4A). Moreover, in these cells the level of Pgp was undetectable by Western blot analysis (Fig. 2B and Fig 4B) and no increases in Pgp efflux function were observed by R123 accumulation (Fig. 2C and Fig 4C). In contrast, cells selected with Dox alone *in vitro* or *in vivo* revealed a significant increase in both mdr1 transcript and Pgp protein expression levels as well as a marked response in the functional assay. Specifically, during *in vitro* selection mdr1 mRNA expression significantly increased between 15 to 250 ng/ml Dox, and then remained practically constant (Fig. 2A). Furthermore, once the drug concentration reached 125 ng/ml Dox, Pgp expression was considerably enhanced and R123 uptake reduced (Figs. 2B and 2C). Cells selected with Dox *in vivo* also showed a significant increase in mdr1 expression and a reduced ability for R123 uptake when compared not only to parental cells but also to cells selected with Dox/P85 (Figs. 4A and C). Consistent with this observation, Pgp was detected only in the Dox treatment group and not in any other group (Fig. 4B). Notably, no changes in transcript level, Pgp expression, or R123 accumulation were seen in cells cultured with P85 alone *in vitro* or in cells isolated from animals injected with PBS or P85. Taken together, these results indicate that P85 prevents the development of a drug-induced MDR1 phenotype in leukemia cells *in vitro* and *in vivo*.

Analysis of Gene Expression in Cells

Global gene expression profiles in selected cells were evaluated by oligonucleotide gene microarray, which included approximately 10,000 genes. The entire set of gene expression profiles for each cell selected *in vitro* can be found in the GEO (http://www.ncbi.nlm.nih.gov/geo/). The functional analysis for all genes altered during selection is presented in supplement Table S-3. Canonical signaling pathways analysis is shown in Table S-4. In cells selected with Dox alone there were twenty genes that were progressively up- or downregulated as the drug exposure increased (Table 2). None of these genes were affected in cells selected with Dox in the presence of P85 or cultured with P85. Consistent with the MDR1 characterization data presented above, there was a drastic increase in the mdr1 transcript in cells selected with Dox alone as drug concentration reached 125 ng/ml or more. Notably, mdr1 was the only gene affected by over 15-fold in more than one selected subline. Other genes listed in Table 2 were implicated in cell cycle (APC, BCL3, GATA4), apoptosis (APC, BCL3, CD5, CHRNA1, GATA4, KLF13, PRDX1, FUT8), energy production (SLC25A10, Cox6A2), cell-to-cell signaling (CD5, ENG), drug metabolism (APC, LCN5, UPP1), stress response (PRDX1, GPX5), molecular transport (APC, BCL3, CD5, GC,GATA4, SLC25A10, UPP1) and other functions (Table 3). Some of these genes are directly involved in tumor morphology (APC, ENG) and tumorigenesis (ABCB1, APC, BCL3, ENG, GATA4, GC, S100A6, FUT8). All of these genes interact with each other as presented in supplementary data (Fig. S-1). However, most of these interactions (147) accounted for only six genes, of which mdr1 was the most prominent: ABCB1 (31) > GATA4 (29) > APC (27) > BCL3 (26) > ENG (18) > PRDX1 (16). All together, the microarray data reinforce the conclusion that the block copolymer prevents alterations of multiple genes that may be implicated in progression of drug resistance in tumors.

Discussion

Development of drug resistance to anticancer agents remains one of the major obstacles to cancer chemotherapy [33]. One of the main players in MDR mechanisms is a drug efflux transport protein, Pgp that transports drugs out of tumor cells [16]. Increased expression of Pgp in many types of cancers is an adverse prognostic indicator for chemotherapy [19,34]. Moreover, high levels of Pgp were found more frequently in recurrent or relapsed cancers as well as induced after initial chemotherapy treatments [35]. Therefore, the discovery and use of agents that can prevent the development of drug resistance may result in improved therapeutic outcomes. Pluronic block copolymers are a novel class of polymeric inhibitors of Pgp that sensitize MDR tumors to Dox, paclitaxel, vinblastine, and other anticancer agents *in vitro* [13,15] and *in vivo* [36,37]. Dox incorporated in Pluronic micelles has been successfully evaluated in Phase II clinical trial in patients with advanced esophageal carcinoma, which is known to a have high incidence of MDR [11]. Polymeric micelles represent a novel type of nanomedicines that can deliver and release drugs at the target sites [12]. In addition, they can also release individual block copolymer molecules, which are shown to inhibit Pgp and to sensitize MDR cells [12]. Hence, this work evaluated the ability of a Pluronic block copolymer to prevent development of MDR in leukemia cells *in vitro* and *in vivo*. We chose P85 as representative molecule because it readily forms micelles of nanoscale size and is among the most potent block copolymer sensitizers of MDR [38].

Murine lymphocytic leukemia cells (P388) cultured with increasing concentrations of Dox for 150 days develop a strong MDR phenotype and are able to tolerate several hundred times higher drug concentrations than the parental cells. Characterization of the selected cells by RT-PCR, Western blot, and R123 accumulation assay clearly demonstrates the overexpression of functional Pgp. Furthermore, the development of the MDR phenotype in these cells is reinforced by global gene expression analysis. In contrast, cells cultured with Dox in the

presence of P85 did not develop MDR, as supported by RT-PCR, Western blot, R123 accumulation assay and microarray. These findings confirm that a Pluronic block copolymer can suppress MDR development in leukemia cells.

It is interesting to note that some increase in mdr1 mRNA was detected by RT-PCR in cell sublines grown with relatively low concentrations of Dox alone at 15 ng/ml, whereas Pgp expression was undetectable by Western blot or R123 accumulation. This finding is consistent with previous studies reporting that mdr1 mRNA in sensitive cells (not exposed to drugs) has a short half-life and can be stabilized following exposure to low concentrations of drugs [39, 40]. Recently Baker *et al.* also showed that short term exposure of acute T–cell leukemia cell line to various drugs resulted in increased expression of mdr1 mRNA, but was not associated with increased functional Pgp expression [41]. Thus, the stabilized mdr1 mRNA cannot translate to functional Pgp [42,43]. Notably, in the presence of P85, cells selected with 15 ng/ml Dox (the highest tolerated drug concentration) revealed no detectable increases in mdr1 mRNA compared to parental cells.

We also evaluated the effect of Pluronic on the MDR development in P388 leukemia cells *in vivo*. P388 and Ehrlich ascites cancer cells were shown to develop resistance after multiple passages *in* mice treated with suboptimal doses of mitoxantrone [44,45]. The resistant cell lines showed cross-resistance to daunorubicin and etoposide, and were moderately sensitized by Pgp inhibitors, verapamil and cyclosporin A [46–48]. These animal models have two distinctive advantages. First, *in vivo* development of MDR recapitulates conditions of chemotherapy including pharmacokinetics and biodistribution of the therapeutic agents. Pharmacokinetics and biodistribution of P85 in a mouse model has been recently reported [49]. Second, development of MDR proceeds in an immunocompetent syngeneic host. Based on these observations we used P388 ascites in BDF1 mice for *in vivo* study. This study confirmed that P85 prevents development of Pgp-mediated MDR in cancer cells *in vivo* as determined by RT-PCR, Western blot, and R123 accumulation assay of the selected cells.

To assess the level of drug resistance irrespective to type of resistance, we estimated IC_{50} values in selected cell sublines. As one might expect, these values revealed the same trend as that associated with the amount of Dox tolerated by the cells. It is interesting that cells selected with Dox in the presence of Pluronic *in vitro* and *in vivo* exhibited some increase in IC_{50} compared to parental cells, even though the MDR phenotype was not observed. This finding indicates that the block copolymer may not prevent development of some drug resistant mechanisms not mediated by Pgp. Such resistance mechanisms will need to be evaluated in future studies. They may include altered expression and mutations of topoisomerase II and I [50], activation of metabolic enzymes such as cellular retinoic binding protein, epoxide hydrolase and thioredoxin [51,52], or inhibition of apoptotic signal transduction pathways [51,53,54]. Still, IC_{50} values in cells selected with Dox in the presence of Pluronic were less than IC_{50} values in cells selected with the drug alone. Even more importantly, in addition to mdr1 Pluronic abolished alterations of multiple genes implicated in apoptosis, drug metabolism, stress response, molecular transport and tumorigenesis. In conclusion, this study proves that P85 prevents development of MDR *in vitro* and *in vivo*. Coupled with previous studies demonstrating selective chemosensitization in MDR cells by Pluronics, these findings hold promise to improve the chemotherapy of tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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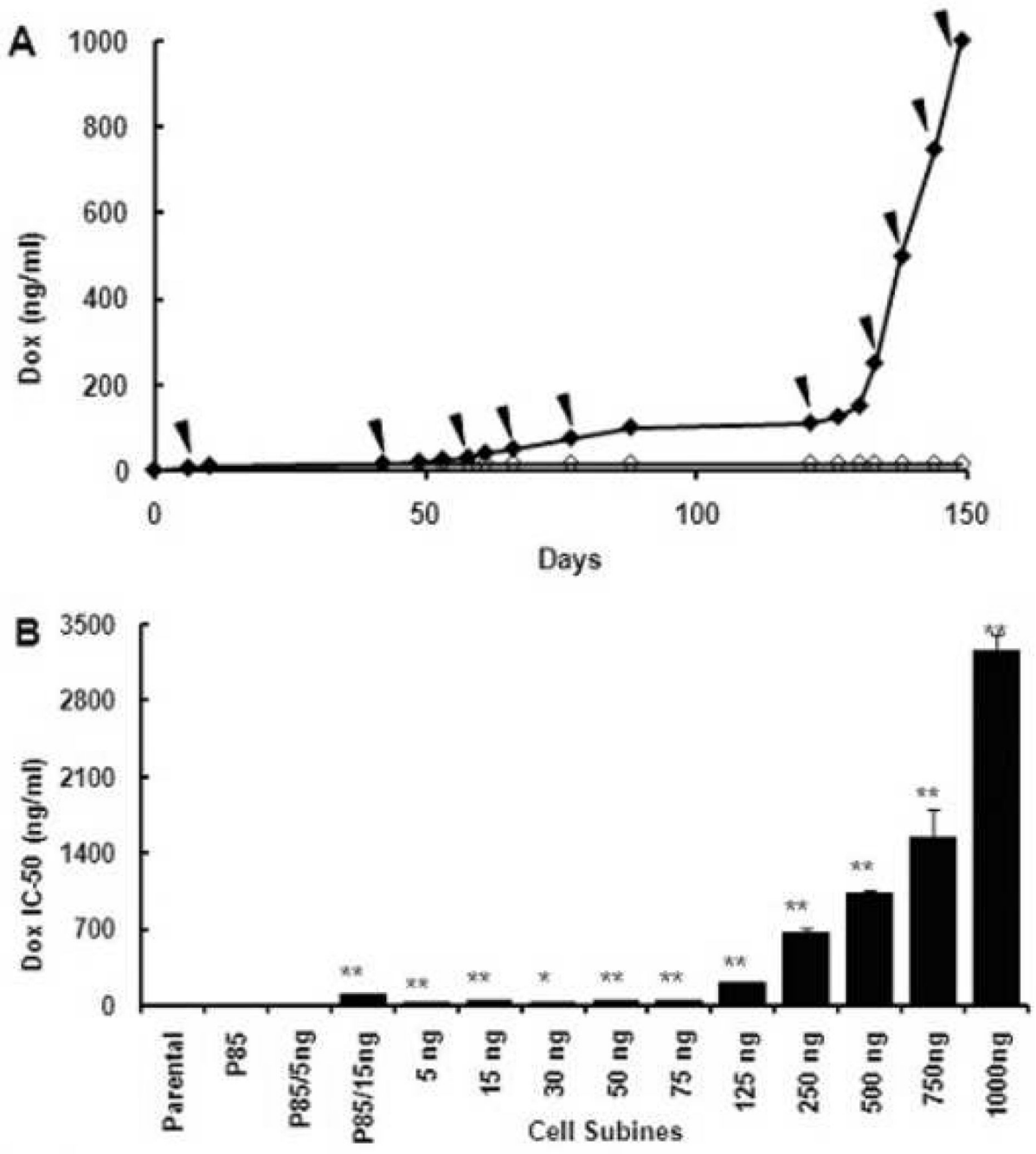

Fig. 1.

Development of drug resistance in P388 cells during *in vitro* selection. **A)** Time course of drug exposure of cells selected with Dox alone (filled diamonds) or Dox with P85 (empty diamonds). Different cell sublines selected with Dox alone are indicated by arrows. **B)** IC_{50} of Dox in parental and selected cells. The labels correspond to parental P388 cells (parental); cells cultured with 0.001% P85 without Dox (P85); cells selected with 0.001% P85 at different Dox concentrations: 5ng/ml (P85+5 ng) and 15ng/ml Dox (P85+15ng); and cells selected without P85 at different Dox concentrations: 5ng/ml (5 ng); 15ng/ml Dox (15 ng); 125ng/ml Dox (125 ng); 250ng/ml Dox (250 ng); 500ng/ml Dox (500 ng); 750ng/ml Dox (750 ng); and 1,000ng/ml Dox (1000 ng). Data are presented as means ± SEM (n = 3) and analyzed using Student's

t-test (two tail, unequal variance). Statistical comparisons between selected and parental cell groups are presented: *$p < 0.05$, ** $p < 0.005$.

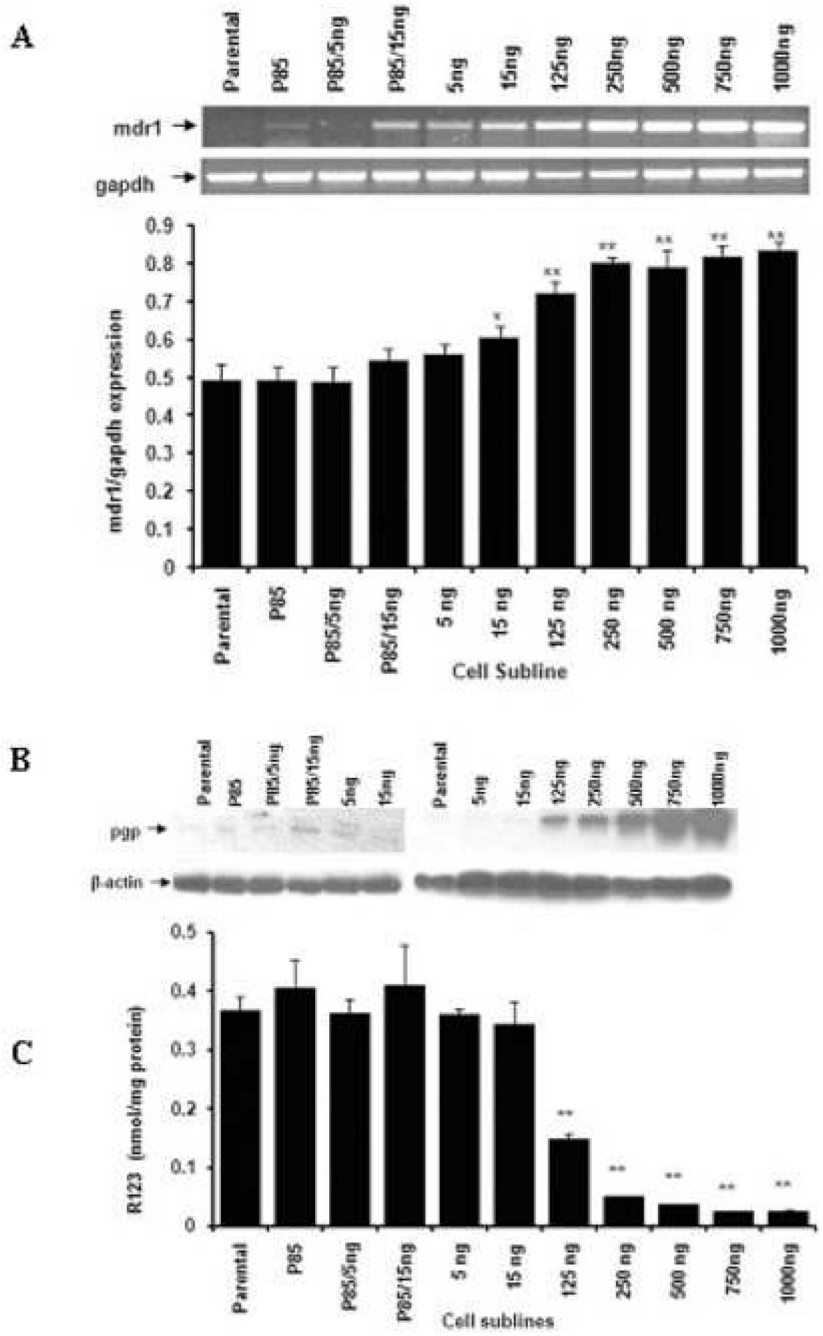

Fig.2.

Characterization of the developed *in vitro* cell sublines. **A)** Expression of mdr1 by RT-PCR analysis shown by a representative gel and mean densitometry data. **B)** Expression of Pgp by Western blot analysis. **C)** R123 accumulation in parental and selected P388 cells. Data are presented as means ± SEM (n = 3) and analyzed using Student's t-test (two tail, unequal variance). Statistical comparisons between selected and parental cell groups are presented: *p < 0.05, ** p < 0.005.

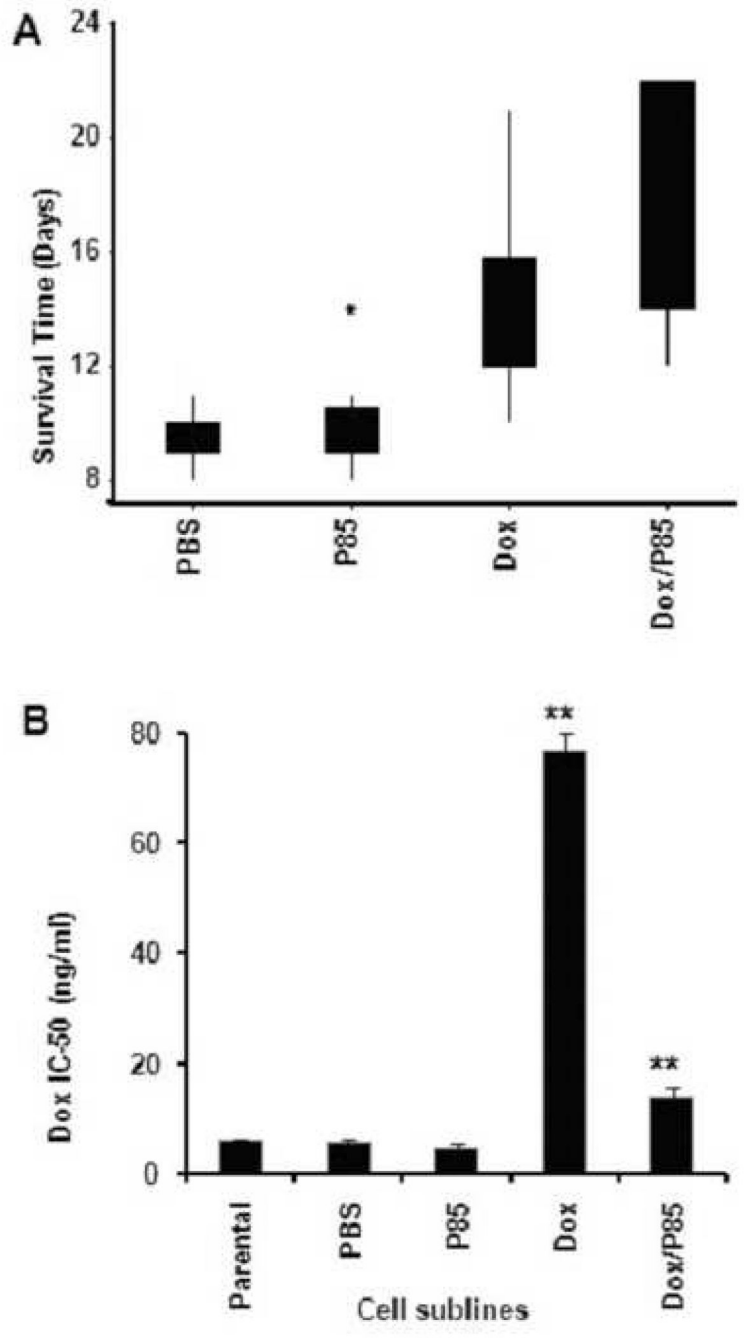

Fig. 3.

Development of drug resistance in P388 cells during *in vivo* selection. **A)** Average lifespan of animals in different treatment regimens. Average lifespan of host animals was calculated for each treatment regimen using data in supplementary Table S-1. Statistical comparisons between different regimens were made using Kruskal-Wallis and Mann-Whitney nonparametric statistical tests. **B)** IC_{50} of Dox in parental and selected cells. The labels correspond to parental P388 cells (parental); PBS control group (PBS); P85 group (P85); Dox group (Dox) and Dox/P85 group (Dox/P85). Data are presented as means ± SEM (n = 3) and analyzed using Student's t-test (two tail, unequal variance). Statistical comparisons between selected and parental cells are presented: *$p < 0.05$, ** $p < 0.005$.

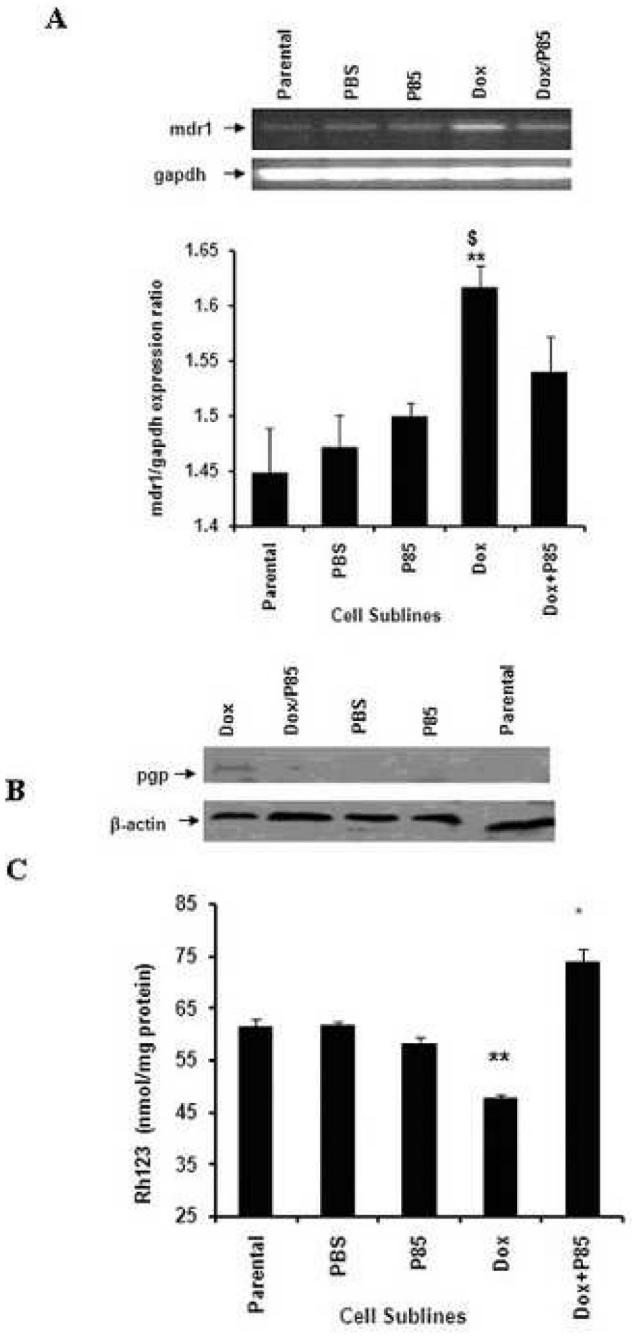

Fig.4.

Characterization of the developed *in vivo* cell sublines. **A)** Expression of mdr1 by RT-PCR analysis shown by a representative gel and mean densitometry data. Data are presented as means ± SEM (n = 3) and analyzed using Student's t-test (two tail, unequal variance). Statistical comparisons between cells selected in Dox and Dox/P85 groups is shown as $ $p < 0.05$. **B)** Expression of Pgp by Western blot analysis. **C)** R123 accumulation in parental and selected P388 cells. Statistical comparisons between selected and parental cells are presented: *$p < 0.05$, ** $p < 0.005$.

Table 1

RT-PCR primers

Gene	GenBank Accession No.	Primers	Sequence	Position (nt)	Amplicon Size (bp)
gapdh	NM_001001303	Forward Reverse	AAGTTGTCATGGATGACCTTGG AAGGTGAAGGTCGGAGTCAACG	534–555 1009 – 1030	497
mdr 1	NM-011075	Forward Reverse	ACTCGGGAGCAGAAGTTTGA GCACCAAAGACAACAGCAGA	2725–2744 2929–2948	224

Table 2

Genes altered during *in vitro* selection

Name and GenBank number	Gene expression change in different cell sublines[a]									
	P85	P85/5 ng	P85/15 ng	5 ng	15 ng	125 ng	250 ng	500 ng	750 ng	1000 ng
ATP-binding cassette, sub-family B member 1 (ABCB1), NM_011076	BT	BT	BT	BT	BT	9.8	16.1	21.1	19.2	31.3
Cholinergic receptor, nicotinic, alpha 1 (CHRNA1), NM_007389	BT	BT	BT	BT	BT	7.8	12.6	9.4	10.4	13.2
Group-specific component (vitamin D binding protein) (GC), M55413	BT	BT	BT	BT	BT	3.4	3.1	5.4	6.7	10.3
Peroxiredoxin 1 (PRDX1) NM_011034	BT	BT	BT	BT	BT	2.5	4.6	2.8	2.6	1.8
Adenomatosis polyposis coli (APC), L19609	BT	BT	BT	BT	BT	BT	BT	1.9	2.4	2.7
CD5 molecule (CD5), NM_007650	BT	BT	BT	BT	BT	BT	BT	BT	1.9	2.3
Glutathione peroxidase 5 (GPX5), NM_010343	BT	BT	BT	BT	BT	BT	BT	BT	2.0	2.6
Lipocalin 5 (LCN5), NM_007947	BT	BT	BT	BT	BT	BT	BT	BT	3.4	2.4
Proline-serine-threonine phosphatase interacting protein 2 (PSTPIP2), NM_013831	BT	BT	BT	BT	BT	BT	BT	BT	2.0	2.7
S100 calcium binding protein A6 (S100A6), NM_011313	BT	BT	BT	BT	BT	BT	BT	BT	3.6	2.0
Uridine phosphorylase 1 (UPP1), NM_009477	BT	BT	BT	BT	BT	BT	BT	BT	4.0	25.7
Fucosyltransferase 8 (FUT8), NM_016893	BT	BT	BT	BT	BT	−2.3	−1.8	−2.0	−1.8	−1.8
Cytochrome c oxidase subunit VIa polypeptide 2 (COX6A2), NM_009943	BT	BT	BT	BT	BT	−3.4	−1.7	−2.6	−2.1	−2.3
Kruppel-like factor 13 (KLF13), NM_021366	BT	BT	BT	BT	BT	−2.6		−2.2	−2.4	−2.1
GATA binding protein 4 (GATA4), NM_008092	BT	BT	BT	BT	BT	BT	BT	−2.6	−2.0	−1.9
Troponin T type 1 (TNNT1), NM_011618	BT	BT	BT	BT	BT	BT	BT	−2.1	−1.8	−3.2
B-cell CLL/lymphoma 3 (BCL3), AF067774	BT	BT	BT	BT	BT	BT	BT	−2.0	−2.2	−3.9
Endoglin, (ENG), NM_007932	BT	BT	BT	BT	BT	BT	BT	BT	−3.5	−2.9
Paroxysmal nonkinesiogenic dyskinesia (PNKD), NM_019999	BT	BT	BT	BT	BT	BT	BT	BT	−2.1	−2.0
Solute carrier family 25, member 10 (SLC25A10), NM_013770	BT	BT	BT	BT	BT	BT	BT	BT	−1.9	−2.0

[a]Same cell labels are used as in Fig. 2. Positive and negative values correspond to expression increase and decrease respectively. BT – below threshold of (+/−) 1.8.

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Table 3

Functions of genes up-regulated or down-regulated in Leukemia cells in response to exposure with Dox and P85.

Function	Genes
Cell Cycle	APC, BCL3, GATA4
Cell Death/Apoptosis	ABCB1, APC, BCL3, CD5, CHRNA1, GATA4, KLF13, PRDX1, FUT8
Cell Morphology	GATA4,TNNT1, APC, BCL3, PRDX1
Cell-To-Cell Signaling and Interaction	CD5, ENG
Cellular Assembly and Organization	APC, ENG, GATA4, PSTPIP2
Cellular Development	ABCB1, ENG, GATA4, APC, BCL3, GATA4, S100A6, FUT8
Cellular Function and Maintenance	APC, ENG, GATA4
Cellular Growth and Proliferation	ABCB1, APC, BCL3, CD5, CHRNA1, ENG, GATA4, PRDX1, S100A6
DNA Replication, Recombination, and Repair	ABCB1, APC, GATA4, UPP1
Drug Metabolism	ABCB1, APC, LCN5,UPP1
Energy Production	SLC25A10, Cox6A2
Free Radical Scavenging/Stress response	PRDX1, GPX5
Gene Expression	APC, BCL3, GATA4, PRDX1
Hematological Disease	BCL3, CD5, KLF13, ABCB1,
Lipid Metabolism	ABCB1, APC, GC, GPX5, LCN5
Molecular Transport	ABCB1, APC, BCL3,CD5, GC,GATA4,SLC25A10,UPP1
Post-Translational Modification	CD5, PRDX1
Protein Trafficking	GATA4, BCL3
Tumor Morphology	APC, ENG
Tumorigenesis	ABCB1, APC, BCL3, ENG, GATA4, GC, S100A6, FUT8