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Effects of pluronic and doxorubicin on drug uptake, cellular metabolism, apoptosis and tumor inhibition in animal models of MDR cancers

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ABSTRACT

Cancer chemotherapy is believed to be impeded by multidrug resistance (MDR). Pluronic (triblock copolymers of poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO), PEO-*b*-PPO-*b*-PEO) were previously shown to sensitize MDR tumors to antineoplastic agents. This study uses animal models of Lewis lung carcinoma (3LL-M27) and T-lymphocytic leukemia (P388/ADR and P388) derived solid tumors to delineate mechanisms of sensitization of MDR tumors by Pluronic P85 (P85) *in vivo*. First, non-invasive single photon emission computed tomography (SPECT) and tumor tissue radioactivity sampling demonstrate that intravenous co-administration of P85 with a Pgp substrate, ^{99}Tc-sestamibi, greatly increases the tumor uptake of this substrate in the MDR tumors. Second, ^{31}P magnetic resonance spectroscopy (^{31}P-MRS) in live animals and tumor tissue sampling for ATP suggest that P85 and doxorubicin (Dox) formulations induce pronounced ATP depletion in MDR tumors. Third, these formulations are shown to increase tumor apoptosis *in vivo* by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and reverse transcription polymerase chain reaction (RT-PCR) for caspases 8 and 9. Altogether, formulation of Dox with P85 results in increased inhibition of the growth solid tumors in mice and represents novel and promising strategy for therapy of drug resistant cancers.

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1. Introduction

Appearance of MDR is a serious problem [1–5] that at least in some cases is believed to impede outcomes of chemotherapeutic regimens in treatment of cancer [6]. New antineoplastic agents and combination chemotherapies have produced limited success for MDR tumors [3]. Dose-intensified and high-dose regimens are active in certain cases but such regimens are accompanied with increased treatment-related morbidity and mortality [3,4]. Limited success was also achieved with chemosensitizing agents that inhibit the drug efflux transporter P-glycoprotein (Pgp/ABCB1) [7,8]. Several generations of Pgp-inhibitors were developed and evaluated in clinical trials [8–10]. The early agents such as cyclosporine A and verapamil had relatively low affinity to Pgp and were toxic. The more potent and less toxic second generation agents (valspodar, biricodar and others) have shown success, but their use has been impeded by their effects on non-targeted proteins. Notably, they also inhibit cytochrome P450 resulting in increased blood drug levels [10]. Thus, new agents are currently in development that would improve drug pharmacokinetics [7,8].

Apart from these approaches using low molecular mass compounds to modulate Pgp is the use of triblock copolymers of poly (ethylene oxide) (PEO) and poly(propylene oxide) (PPO), (PEO-*b*-PPO-*b*-PEO) also known as Pluronics or poloxamers. Pluronic block copolymers are listed in the U.S. and British Pharmacopoeia under the name “poloxamers” as excipients and are widely used in a variety of clinical applications [11]. One formulation containing doxorubicin (Dox) and a mixture of Pluronic L61 and F127, SP1049C, that is particularly relevant for the present study, has successfully completed Phase II human trials in advanced esophageal adenocarcinoma [12]. Contrary to most low molecular mass inhibitors of Pgp, that are tailored to interact specifically with the transport system protein, Pluronics have a broad spectrum of activities. First, they inhibit Pgp drug efflux pump [12], which involves interaction of Pluronic

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molecules with MDR cell membranes, decrease in membrane microviscosity and inhibition of Pgp ATPase activity [13,14]. Second, they inhibit respiratory chain complexes in mitochondria of MDR cells and thus deplete ATP that deprives the MDR cells of the energy source [14]. Third, they promote generation of reactive oxygen species (ROS) and simultaneously inhibit the glutathione/glutathione *S*-transferase (GSH/GST) detoxification by decreasing GSH and inhibiting GST activity [13]. Fourth, they attenuate drug sequestration in acidic vesicles, which may increase drug bioavailability within the cancer cell [15]. Finally, they decrease membrane potential in mitochondria of MDR cells, promote release of cytochrome C and overall enhance pro-apoptotic signaling and mitigate anti-apoptotic cellular defense of MDR cells [16]. It is also remarkable that despite rather simple structure and lack of precise spatial arrangement of pharmacophoric groups, Pluronics appear to be selective with respect to the MDR cell phenotype [14]. This is most noticeably seen in ATP depletion by Pluronic, which correlates with the level of Pgp expression in the cancer cells [17].

The current study investigates the effects of Pluronic P85 (P85) in mouse models of MDR solid tumors. Formulation of Dox with P85 resulted in increased inhibition of Lewis lung carcinoma (3LL-M27) and T-lymphocytic leukemia (P388/ADR and P388) tumors in mice. Three major effects of Pluronic formulations observed in MDR tumors *in vivo* include 1) significant increase of tumor accumulation of a Pgp substrate, ^{99}Tc-sestamibi (shown by non-invasive single photon emission computed tomography (SPECT) and tumor tissue radioactivity sampling), 2) ATP depletion (shown by ^{31}P magnetic resonance spectroscopy (^{31}P-MRS) in live animals and tumor tissue sampling for ATP) and 3) enhanced apoptosis (shown by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and reverse transcription polymerase chain reaction (RT-PCR) for caspases 8 and 9). Thus present *in vivo* studies confirmed major pathways for P85 chemosensitization as they affect MDR cancer cells and support the notion that formulation of drugs with Pluronic represents a novel and promising strategy for drug resistant cancers. At the same time significant difference in dose dependence of inhibition of tumor growth in immunocompetent and immunodefficient mice is noted, which for the first time suggests involvement of the immunological component(s) in the antitumor activities of drug and Pluronic formulations. Overall, such formulations display superior antitumor activity in MDR and non-MDR tumors, and their clinical application may be broader than it was initially suggested for Pgp overexpressing cancers.

2. Materials and methods

2.1. Drugs and chemicals

The present study used P85 (lot # WPOP-587A) provided by BASF Corp. (Parispany, NJ). The molecular mass of the polypropylene-oxide (PO) segment in this copolymer sample was approximately 2500 Da, and the content of the polyethylene-oxide (EO) chains was ~50% (w/w). The physicochemical characteristics of Pluronic copolymers have been previously reported [18]. Dox was purchased from Sigma Chemical Co. (St. Louis, MO, USA). ^{99}Tc-Sestamibi (Cardiolite) was received from Cardinal Health (Omaha, NE, USA). The tritium labeled copolymer (^{3}H-P85) was obtained by exposure of P85 to tritium gas (NEN Life Science Products, Boston, MA).

2.2. Cell culture

Lewis lung carcinoma 3LL-M27 cells were cultured in DMEM with 10% FBS, 10 mM HEPES and 1% penicillin/streptomycin. The murine leukemia P388 and P388/ADR cells were cultured in RPMI 1640 with 10% FBS (fetal bovine serum), 10 mM HEPES and 1% penicillin/streptomycin. All other tissue culture reagents were obtained from Gibco Life Technologies, Inc. (Grand Island, NY, USA). Cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2.

2.3. Animals

The experiments were performed with female C57BL/6 or BDF1 mice at 11–12 weeks of age (Taconic Laboratories, Germantown, NY). The animals were kept at 4–5 per cage with a filter cover under light (12 h light/dark cycle) and handled according to institutional guidelines. All manipulations with the animals were performed under a sterilized laminar hood. Food and water were given *ad libitum*. Homozygous B6.CB17-Prkdc^{scid}/SZJ mice with the severe combined immune deficiency spontaneous mutation characterized by absence of functional T-cells and B-cells were employed to evaluate involvement of immune system. All procedures involving animals were carried out under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Nebraska Medical Center (UNMC).

2.4. Preparation of Dox/Pluronic formulations

P85 was dissolved at various concentrations (0.0002–1 wt.%) in saline at 4 °C and then sterilized by filtration through a 0.2 μm filter. Dox/P85 compositions were obtained by addition of a sterile isotonic solution of Doxorubicin HCl (2 mg/ml) to the copolymer solutions. These compositions were incubated at 37 °C for at least 1 h prior to their use in the experiments.

2.5. Evaluation of mdr1 levels by reverse transcription polymerase chain reaction (RT-PCR)

To assess the level of mdr1 gene in solid tumors, C57BL/6 mice or BDF1 mice were injected subcutaneously (s.c.) with 3LL-M27 (C57BL/6), P388/ADR (BDF1) or P388 (BDF1) cells (10^6 cells/mouse in 50 μl saline), and tumors were allowed to grow for 7–10 days. When solid tumors reached ca. 300 mm^2, tumor tissues were dissected and RNA was extracted using Reagent® (Molecular Research Center, Inc. Cincinnati, OH) according to the manufacturer's protocol. RNA quality was determined via ethidium bromide staining following agarose/formaldehyde (1.2%) gel electrophoresis. Quantity of extracted RNA was determined by ratio of absorbance at 260 and 280 nm recorded in spectrophotometer. 100 ng of total RNA was reverse-transcribed with AccessQuick RT-PCR system (Promega, Madison, WI, USA). For PCR amplification, 100 ng of RNA was included in a total of 50 μl reaction mixture, containing 1× Access Quick Master Mix, sense and antisense primers, 1 μM each (the sequences are shown in Table 1) and 5 U AMV reverse transcriptase. PCR amplification was performed at 48 °C for 45 min (reverse transcription), 94 °C for 2 min (initial denaturation) followed by 30 cycles at 94 °C for 60 s (denaturation), 55 °C for 45 s, (annealing) 70 °C for 30 s (extension) and lastly 72 °C for 5 min (final extension). PCR products were run along with a DNA ladder (Promega)

Table 1
Summary of primer sequences for mdr1, gadph, caspases 8 and 9, and b-actin.

No	Gene	GenBank #	Primer for RT-PCR	Band size
1	mdr1	NM-011075	Sense ACTCGGGAGCAGAAGTTTGA Antisense GCACCAAAGACAACAGCAGA	224 bp
2	gadph	NM-001001303	Sense AAGTTGTCATGGATGACCTTGG Antisense AAGGTGAAGGTCGGAGTCAACG	497 bp
3	b-actin	NM-007393	Sense AGCCATGTACGTAGCCATCC Antisense CTCTCAGCTGTGGTGGTGAA	228 bp
4	caspase 8	NM-009812	Sense GGCCTCCATCTATGACCTGA Antisense GCAGAAAGTCTGCCTCATCC	212 bp
5	caspase 9	NM-015733	Sense GATGCTGTCCCCTATCAGGA Antisense GGGACTGCAGGTCTTCAGAG	205 bp