



Published in final edited form as:

Mol Pharm. 2014 August 4; 11(8): 2566–2578. doi:10.1021/mp500298q.

Pluronics and MDR Reversal: An Update

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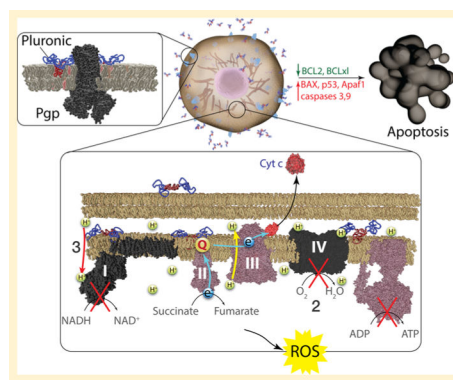
Abstract

Multidrug resistance (MDR) remains one of the biggest obstacles for effective cancer therapy. Currently there are only few methods that are available clinically that are used to bypass MDR with very limited success. In this review we describe how MDR can be overcome by a simple yet effective approach of using amphiphilic block copolymers. Triblock copolymers of poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO), arranged in a triblock structure PEOPPO-PEO, Pluronics or “poloxamers”, raised a considerable interest in the drug delivery field. Previous studies demonstrated that Pluronics sensitize MDR cancer cells resulting in increased cytotoxic activity of Dox, paclitaxel, and other drugs by 2–3 orders of magnitude. Pluronics can also prevent the development of MDR *in vitro* and *in vivo*. Additionally, promising results of clinical studies of Dox/Pluronic formulation reinforced the need to ascertain a thorough understanding of Pluronic effects in tumors. These effects are extremely comprehensive and appear on the level of plasma membranes, mitochondria, and regulation of gene expression selectively in MDR cancer cells. Moreover, it has been demonstrated recently that Pluronics can effectively deplete tumorigenic intrinsically drug-resistant cancer stem cells (CSC). Interestingly, sensitization of MDR and inhibition of drug efflux transporters is not specific or selective to Pluronics. Other amphiphilic polymers have shown similar activities in various experimental models. This review summarizes recent advances of understanding the Pluronic effects in sensitization and prevention of MDR.

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Notes The authors declare no competing financial interest.



Keywords

Pluronic; cancer drug resistance; mitochondria; Pgp; cancer stem cells; lipid rafts; plasma membrane

1. INTRODUCTION

Chemotherapy remains the main treatment option for most cancers despite of its limitations, such as systemic toxicity, severe side effects, and limited efficacy. The major reason for chemotherapy failure is poor delivery of drug to cancer cells and/or intracellular targets. There are a number of barriers that have to be overcome for successful treatment, and multidrug resistance (MDR) is one of them. Tumors of different origin have different susceptibility to chemotherapy, and frequently cancers are intrinsically resistant. On the other hand, even though many primary tumors and metastatic lesions, for example breast, ovarian, and small cell lung carcinomas initially respond well to the chemotherapeutic treatment, cancers often relapse and develop drug resistance. Moreover, cancer cells simultaneously acquire resistance not only to the drug the patient was treated with but also to the broad spectrum of drugs that are structurally and functionally unrelated to each other. Initially MDR was attributed to the expression of drug efflux transporters on the cell membrane that actively pump the drugs out of the cells.¹ Now it is generally recognized that MDR is a complex phenomenon and usually is governed by one or more of the following mechanisms: (1) active drug removal by drug efflux transporters of the ATP-binding cassette (ABC) superfamily, such as P-glycoprotein (Pgp, ABCB1), multidrug resistance-associated protein 1 (MRP1, ABCC1), and breast cancer resistance protein (BCRP, ABCG2); (2) loss of cell surface receptors or drug transporters or alterations in membrane lipid composition that limit diffusion of the drug into the cells; (3) compartmentalization of the drug in cellular vesicles; (4) altered/increased drug targets; (5) increased drug metabolism; (6) alterations in cell cycle; (7) active damage repair; and (8) inhibition of apoptosis (Figure 1).

Despite much effort contributed to overcoming MDR, the success is still very limited in clinical settings. This effort mainly centered on the following approaches.²⁻⁶ First, the modification of treatment regimens by increasing the dose of the administered drug(s) or using non-cross-resistant drugs. Second, use of small molecule inhibitors of drug efflux

transporters to increase the drug uptake in MDR tumors.^{7–9} Third, use of antibodies and antibody fragments to target and inhibit drug efflux transporters.^{10–12} Fourth, silencing of the gene expression of the drug efflux transporters^{13–15} or antiapoptotic proteins, such as BCL2^{13,16} using antisense oligonucleotides, siRNA, or micro RNA. Fifth, use of small molecules to suppress non-ABC transporter-mediated resistance.^{17,18} Finally, use of nanotechnology-based carriers to bypass drug efflux transporters in MDR cancer cells.² Of these approaches the first two were evaluated in clinics. Unfortunately, a simple dose increase has been associated with increased risks of systemic toxicity and severe side effects, while finding a proper combination of non-cross-resistant drugs in many cases is complicated. As far as the use of the Pgp inhibitors is concerned, the outcomes were often poor, and many such inhibitors failed due to toxicity or drug metabolism associated issues.^{8,9} Moreover, most of the approaches under development face traditional drug delivery issues, which are especially severe in the cases of nucleic acid or protein therapeutics.

Nanotechnology offers several advantages both for the delivery of the chemotherapeutic agents, allowing them to bypass drug efflux transporters, and for the delivery of agents that could inhibit drug resistance mechanisms to increase efficacy of the chemotherapy. First, it allows improving pharmacokinetic parameters of administered compounds. Nanomedicines have longer circulation times and can passively accumulate in the tumors with leaky vasculature and poor lymphatic drainage by the enhanced permeability and retention (EPR) effect.^{19,20} Attaching specific tumor-targeting antibodies, antibody fragments, or other targeting moieties (receptor ligands, peptides, etc.) can result in active targeting of the nanomedicines to the tumor cells, which can further improve drug delivery. Second, two or more active compounds can be incorporated into a single carrier allowing simultaneous delivery of several cytotoxic drugs for combination therapy and/or a cytotoxic drug with a MDR modulator, such as small molecule inhibitor, antibody, or nucleic acid. Third, a nanocarrier can be designed in such a way that it will release its cargo at the tumor site in response to specific tumor conditions, such as pH or presence of particular enzymes, therefore limiting other organs and tissues to the exposure to free drug and reducing systemic toxicity. Finally, in contrast to small molecules that mainly utilize diffusion to penetrate the cells, nanocarriers are taken up by either “passive” endocytosis or receptor-mediated endocytosis and, therefore, can bypass drug efflux transporters on the plasma membrane. In the latter case the endocytosis is triggered by interaction of targeting ligand with its receptor on plasma membrane, which accelerates the uptake compared to “passive” endocytosis. If the receptor is predominantly expressed on cancer cells, in addition to faster uptake this allows selective targeting of the nanocarrier to cancer cells.

Additionally, polymeric carriers can have a biological activity of their own. One such example is represented by a class of copolymers, called Pluronic block copolymers or poloxamers, that are widely used in various drug delivery systems^{21–32} and in tissue engineering.^{33–36} Pluronics are triblock copolymers of poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO), arranged in PEO-PPO-PEO structure. Depending on the length of the blocks the hydrophilic–lipophilic balance (HLB) of the copolymers changes. In the solution Pluronics spontaneously form micelles above the critical micelle concentration (CMC). The core of the micelles contains PPO blocks and allows incorporation of

hydrophobic drugs. Previously thought to be “inert”, Pluronics display a unique set of biological activities and have been shown to be potent sensitizers of MDR cancer cells *in vitro* and *in vivo*.^{21,23,37–40} Moreover, Pluronics were shown to prevent the development of MDR upon selection with an anthracycline antibiotic, doxorubicin (Dox), both *in vitro* and *in vivo*.^{41,42} We have also recently demonstrated that Pluronics in combination with Dox can deplete tumorigenic cell subpopulations and decrease cancer cells’ tumorigenicity and tumor aggressiveness upon treatment *in vivo*.²² In this review we will discuss each of these mechanisms in more details.

2. REVERSAL OF ABC TRANSPORTER-MEDIATED RESISTANCE BY PLURONICS

2.1. Structure and Function of ABC Transporters

The first drug efflux transporter in cancer cells was described by Juliano and Ling in 1976.¹ They have shown that drug-resistant Chinese hamster ovary cells express a 170 kDa membrane glycoprotein, now known as P-glycoprotein (Pgp, ABCB1), that was unique to the drug-resistant cells.¹ The cells were selected for resistance to colchicine and showed cross-resistance to a wide range of different compounds. The degree of drug resistance correlated with the amount of Pgp on the cell surface. Later, in early 1990s a second drug efflux transporter, called multidrug resistance-associated protein (MRP1 or ABCC1), was discovered in a drug-resistant lung cancer cell line.⁴³ Pgp and MRP1 show a partial overlap in substrate specificity. Normally MRP1 plays a major role in cell detoxifying mechanism by transport of exogenous and endogenous compounds conjugated to glutathione (GSH), which for some substrates is required as a cofactor for MRP1 activity. In contrast, Pgp does not require a cofactor and can efflux a wide variety of functionally and structurally diverse but commonly hydrophobic drugs.⁴⁴ Another important drug efflux transporter, named breast cancer resistance protein (BCRP, ABCG2), was identified in 1998 by Doyle et al. in human breast cancer cell line selected for Dox resistance.⁴⁵ Its expression is associated with resistance to number of drugs, such as mitoxantrone, camptothecins, anthracyclines, etc.⁴⁶ Pgp, MRP1, and BCRP belong to the large superfamily of ATP-binding cassette (ABC) membrane transporters with 48 members of the superfamily that are divided into 7 subgroups (A–G). They have conserved structures and ubiquitously expressed in all forms of living organisms, from bacteria to humans. Pgp is the most studied ABC transporter (Figure 2). It is a product of *mdr1* gene and can be found in many normal tissues, like epithelial cells of gastrointestinal tract,⁴⁷ liver, the luminal membrane of proximal tubular epithelial cells in kidney,^{48,49} cornea,⁵⁰ and the luminal membrane of the endothelial cells in the blood–brain barrier.⁵¹ Overall, Pgp is mostly expressed in tissues with barrier functions and its main role is to protect the organism from toxic compounds. It has a typical structure for ABC transporters and comprises two transmembrane domains (TMDs), each of which has 6 membrane-spanning α helices, and two intracellular nucleotide-binding domains (NBDs), which bind and hydrolyze ATP providing energy for transmembrane movement of the drugs (Figure 2). Pgp substrates are mostly hydrophobic (but structurally unrelated) and partition into a lipid bilayer.⁵² Among these substrates are important anticancer drugs including several anthracyclines (Dox, daunorubicin, mitoxantrone), vincristine, taxanes, etoposide, teniposide, actinomycin D, and others. Understanding the mechanism of Pgp function is

critical for the design of novel effective MDR modulators. Several models for Pgp-mediated drug transport have been proposed.^{53–56} Recently the crystal structure of mouse Pgp, which has 87% sequence identity to human Pgp, was described⁵⁶ (Figure 2). By analyzing the costructures of Pgp complexes with two cyclopeptide inhibitors the authors elucidated the mechanism of drug efflux by Pgp and provided insight into the transporter's broad substrate specificity. The drug-binding pocket of Pgp is localized in the TM domain of the protein. The inward-open conformation of Pgp allows the substrate access both from cytoplasm and from the inner leaflet of the membrane but not from the upper leaflet or extracellular space. The upper part of the drug-binding pocket contains predominantly hydrophobic and aromatic amino acid residues, and the lower half of the chamber has more polar and charged residues. The drug-binding pocket in Pgp is very large and in inward-facing conformation is accessible through two portals that are wide enough to fit hydrophobic drugs and phospholipids and allow Pgp to “scan” the inner leaflet to select and bind specific lipids and hydrophobic drugs before transport.⁵⁶ Overall, the authors proposed, that Pgp has broad flexibility and can sample widely open conformations to accommodate large substrates, explaining the broad substrate specificity of the transporter. Usually the drug enters Pgp's binding site from the inner leaflet of the membrane, which stimulates the binding of two molecules of ATP by NBDs followed by their dimerization. The dimerization of NBDs causes the major conformational change in the protein and formation of the outward-facing structure, open to the extracellular space. The drug is released due to the change of the affinity of the protein to it or is facilitated by ATP hydrolysis, which brings the protein back to the initial state.⁵⁶

2.2. Inhibition of Pgp Activity by Pluronic: Role of Pluronic–Membrane Interactions

As was mentioned above, Pluronic block copolymers are potent sensitizers of MDR cells. The sensitization mechanism is complex and involves multiple events happening at different levels in the cell. The polymer–cell interaction starts in the cell membrane, where drug efflux transporters are localized. Pluronics were shown to be strong inhibitors of ABC transporters, specifically Pgp, MRP, and BCRP.^{39,57–59} They suppress the transporters' ATPase activity and their interaction with the drug. The inhibition might be in part due to the alterations of lipid microenvironment of the transporters by Pluronic. Due to their amphiphilic structure, Pluronic block copolymers can interact with cell membrane and change its properties,⁶⁰ which are critical for proper function of ABC transporters.

2.2.1. Role of Lipid Microenvironment for Pgp Function—

Membrane structure and composition play a crucial role in cell physiology, function, and signaling. Plasma membrane is a heterogeneous structure composed of various domains with different lipid composition and packing.⁶¹ In particular, so-called “lipid rafts” are compact membrane microdomains containing predominantly cholesterol and sphingolipids (mainly sphingomyelin) with long and saturated fatty acids, that are “floating” in more fluid membrane phase that contains glycerophospholipids with shorter and unsaturated acyl chains (Figure 2).⁶² These domains are resistant to low temperature solubilization by some detergents, like Triton X100 or Brij 96, and this is used for their isolation. Depending on the cell line and the method used for membrane fractionation Pgp can be found either mostly in detergent-resistant membrane fractions or distributed between the detergent-resistant and

detergent-soluble fractions.^{63–66} Furthermore, it was found that Pgp distribution between different membrane fractions depends on the transporter's expression level: the lower the expression of Pgp is, the greater portion of Pgp is localized in detergent-resistant cholesterol-rich membrane domains.⁶⁷ It is well-known that the function of most membrane proteins is directly linked to the composition and viscosity of their lipid microenvironment. Pgp is a lipid flippase⁶⁸ and requires interaction with phospholipids for continuous display drug-mediated ATPase activity⁶⁹ and interaction with the substrate.⁷⁰ Moreover, an increasing number of studies report that Pgp localization in lipid rafts and precise properties of rafts are essential for the transporter's proper function.⁶² For example depletion of cholesterol with methyl- β -cyclodextrin in drug-resistant VLB human T-cell lymphoblastic leukemia cells led to disassembly of the lipid rafts, redistribution of Pgp from lipid rafts to other microdomains of plasma membrane, and inhibition of Pgp transporter activity. On the other hand, enrichment of membranes with cholesterol also resulted in inhibition of Pgp function, although the localization of Pgp did not change compared to control. However, the increase in cholesterol content changed the lipid raft distribution and composition, which most likely accounts for the impairment of the Pgp function.⁷¹ It was also shown recently that caveolin-1 overexpression decreases plasma membrane cholesterol levels (similar to the effect of methyl- β -cyclodextrin that depletes cholesterol from the membrane) and results in the increase of membrane fluidity and inhibition of Pgp function in drug-resistant Hs578T/Dox cells.⁷² Another study by Barakat et al. demonstrated that there are two functionally different populations of Pgp in drug-resistant human CEM lymphoblastic leukemia cells.⁶³ The first population localized in detergent-resistant membrane fraction has higher ATPase activity, which is completely inhibited by orthovanadate and activated by verapamil. The second population localized in soluble membrane fractions has lower ATPase activity and is less sensitive to orthovanadate. Moreover, verapamil, a well-known Pgp activator, inhibits Pgp ATPase activity in this second population.⁶³ The authors conclude that interaction of Pgp with its substrates could be affected by different lipid microenvironment in soluble membrane fractions, specifically by lower content of cholesterol compared to the detergent-resistant membrane fraction.⁶³

2.2.2. Pluronic Interaction with Lipid Membranes—Pluronic binding to the cell membrane depends on Pluronic hydrophobicity and the temperature.⁷³ The binding is driven by hydrophobic interactions of PPO chain blocks with the fatty acid residues in the lipid bilayer and by hydrophilic interactions of PEO chain blocks with the polar groups of the lipids at the membrane surface. This binding may lead to either membrane destabilization⁷⁴ or healing of “injured” membranes.^{75,76} Pluronics also exhibit ionophoric activity and can facilitate transmembrane transport of low molecular drugs, accelerate phospholipid's flip-flop rate, and decrease membrane microviscosity.^{73,77,78} Pluronic effects on the membrane transport depend on the copolymer HLB, concentration, and the exposure time. For example, hydrophobic Pluronic L61 ((EO)₄-(PO)₃₀-(EO)₄, HLB 3, MW 2000 g/mol, EO = ethylene oxide; PO = propylene oxide) depending on the level of its aggregation can act either as a transmembrane carrier of drug molecules or as an ion channel.⁷⁸ Specifically, it was proposed that L61 monomers and dimers can act as the carriers while L61 oligomers are likely to form the channels.⁷⁸ On the other hand hydrophilic Pluronic F68 (Poloxamer 188, (EO)₇₆-(PO)₃₉-(EO)₇₆, HLB 29, MW 8400 g/mol) with 80% PEO content effectively

restores damaged cell membranes after electroporation, heat shock, or intense radiation.^{79–81} Using X-ray reflection (XR) and grazing-incidence X-ray diffraction (GIXD) methods in a model Langmuir lipid monolayer of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (DPPG), Wu et al. have shown that F68 interacts with the damaged membrane areas, but does not affect the ordered membrane phase, and gets excluded when lipid packing density is restored.⁷⁶ Recently it was demonstrated that F68 molecules do not insert into lipid bilayer nor affect the overall lipid packaging, however, they facilitate the membrane sealing activity by diminishing the fluctuation of membrane surface and hydration of the inner part of the bilayer.⁸² However, in another study using giant unilamellar vesicles (GUV) as model membrane system Wang et al. demonstrated that F68 can incorporate in the membranes, disrupt their integrity, and act as a permeabilizer if it is exposed to the membranes for sufficient time.⁸³

Overall, the interaction of Pluronics with lipid membranes proceeds in two steps: (1) the absorption at the membrane and (2) the insertion in the membrane (Figure 3). The first step is common to all Pluronics and does not depend much on the copolymer structure. The second step depends strongly on the hydrophobicity of the copolymer with the more hydrophobic copolymers being more likely to insert.⁸³ Extremely hydrophilic Pluronics absorb on the membrane without penetrating into the lipid bilayer. Pluronics with longer PPO blocks insert into the membrane below the polar head groups, loosen the lipid packaging, and, therefore, act as permeabilizers.⁸² They can translocate through the membrane (depending on their HLB). Furthermore, using molecular dynamics simulations Nawaz and coauthors observed that membrane bends upon insertion of Pluronics.⁸⁴ They have shown that membrane-disruptive activity of Pluronics is due to interaction of hydrophilic blocks with the polar head groups of the lipid molecules and depends on the length of the PEO block. Short PEO blocks drag the polar groups toward the inner part of the membrane, which results in membrane bending and permeabilization. Pluronics with longer PEO blocks can temporarily stabilize the local structure of the membrane.

Pluronic copolymers can significantly increase the antitumor activity of PEGylated liposomal drugs *in vivo*, specifically DOXIL by stimulating the drug release from liposomes at the tumor site.³⁰ One of the main problems of long circulating liposomal drugs is insufficient release of the active compound at the tumor site. We have demonstrated that “post-administration” of Pluronic P85 ((EO)₂₆-(PO)₄₀-(EO)₂₆, HLB 16, MW 4600 g/mol) 48 h after DOXIL results in Dox release and redistribution toward tumor bulk along with a marked improvement of antitumor activity. This effect is time-dependent as it is essential to allow sufficient time for the liposomes to accumulate at the tumor site before administering Pluronic. It is likely that the enhanced antitumor effect at least in part is due to facilitated release of Dox from the liposomes in the tumors induced by Pluronic. Furthermore, in addition to permeabilization effect on liposomal membranes the copolymer could also sensitize the MDR cells and deplete the cancer stem cells (CSCs) (as discussed below).^{22,29}

Another important aspect in Pluronic interactions with lipid membranes is the dependence of these interactions on the cell type and the membrane composition. For example, the membrane microviscosity of murine myeloma SP2/0 cells significantly decreased after

treatment with L61, while the membrane viscosity in normal mouse splenocytes was less affected.⁷³ Moreover, Pluronic P105 ((EO)₃₇-(PO)₅₆-(EO)₃₇, HLB 15, MW 6500 g/mol) was demonstrated to permeabilize the acidic endosomal vesicles in drug-resistant A2780/ADR cells, while the vesicles in sensitive cells were less affected.⁸⁵ These differences may be attributed to differences in membrane lipid compositions. Several studies have reported lower fluidity and higher heterogeneity of plasma membrane in MDR cells compared to sensitive cells.^{86,87} Drug-resistant cells also contain smaller amounts of unsaturated fatty acids and have higher content of esterified cholesterol and triglycerides.^{88,89} Using liposomes of different lipid composition and viscosity it was demonstrated that the L61 effects on lipid flip-flop and membrane permeability toward Dox increase as the membrane viscosity increases.⁹⁰

Pluronic inhibit Pgp and MRP ATPase activities by decreasing maximum reaction rate (V_{max}) and the affinity of the enzyme to ATP as well as to the substrates such as vinblastine (expressed as increase in Michaelis constant, K_m).⁴⁰ Some neutral detergents, such as Tween-20, Nonidet P-40, and Triton X-100, were also shown to inhibit Pgp ATPase activity at concentrations that are required for membrane fluidization.⁹¹ Overall, alterations in membrane structure and fluidity induced by various compounds strongly affect Pgp function. Therefore, it was suggested that inhibition of the transporter's activity by Pluronic is at least partly due to the Pluronic-induced changes in the local membrane environment (Figure 2).

3. EFFECT OF PLURONIC ON CANCER CELLS' METABOLISM

To further understand the mechanism of Pluronic sensitization of MDR cancer cells one needs to focus on the events at the subcellular level, which were characterized in great detail using P85 as an example.²³ This copolymer exhibits evident and profound selectivity with respect to energy metabolism in MDR cancer cells. It is rapidly taken up by the cells via a caveolae-mediated endocytosis pathway⁹² and colocalizes with mitochondria already 15 min after exposure to the cells.³⁸ This results in a drastic depletion of intracellular ATP levels in MDR cancer cells, while non-MDR cells require significantly higher doses of Pluronic to achieve similar depletion. Noteworthy, the ability to deplete cellular ATP levels strongly correlates with the chemosensitization properties of the copolymers in MDR cells.⁹³ The selectivity of Pluronic copolymers toward MDR phenotype is probably attributable to innate metabolic and physiological differences between MDR and non-MDR cells. In contrast to normal cells, that use oxidative phosphorylation for ATP production, cancer cells mostly rely on glycolysis as an adaptation to hypoxic conditions in the early stages of tumor development.⁹⁴ Drug-resistant cells require more ATP to support the drug efflux transporter activity and drug metabolism. Adaptations leading to MDR therefore in part are associated with changes in energy metabolism to meet new energy requirements. It was shown that human breast cancer cells with acquired resistance to Dox exhibit 3-fold higher glycolysis rate than their sensitive counterparts.⁹⁵ Another study by Miccadei et al. found that both respiration and glycolysis rates are increased in drug-resistant Ehrlich cells, resulting in almost 50% higher ATP production compared to the drug sensitive cells.⁹⁶ It was also shown that MDR cells have significantly higher activity of the respiratory chain complexes in mitochondria where nearly 50% of ATP was produced, compared to only 35% of ATP

produced in mitochondria of sensitive cells. Moreover, it was later demonstrated that MDR cancer cells have lower mitochondrial membrane potential, use fatty acids for mitochondrial oxidation when glucose becomes limited, and have high levels of expression of uncoupling protein 2 (UCP2), which results in less efficient ATP synthesis.⁹⁷ Overall, the compromised mitochondrial function in MDR cells may be the Achilles' heel of MDR cells that allows effective and selective inhibition of ATP production in drug-resistant cells.

When Pluronic reaches mitochondria of MDR cells, it inhibits complexes I and IV of the respiratory chain and depletes mitochondrial membrane potential.³⁸ The mechanism of Pluronic inhibition of respiratory chain complexes' activities is not fully understood. In mitochondria Pluronic may undergo chemical reaction and provide peroxides to respiratory chain. In other words Pluronic may act as a prooxidant, which were shown to induce apoptosis in cancer cells.⁹⁸ Noteworthy, the effects of Pluronic on Pgp activity, ATP levels, and cytotoxicity are reversible. Pgp function is restored 1 h after the removal of Pluronic. At the same time, the amount of cell-bound Pluronic rapidly decreases. The sensitization effect of Pluronic is abolished in the same time frame, while it takes about 10 h to restore ATP levels.³⁸ Interestingly, Pgp expression seems to be essential for Pluronic effects on respiration and ATP levels. Inhibition of oxygen consumption as well as ATP depletion by Pluronic was observed not only in drug-selected resistant cells but also in cells stably transfected with *mdr1* gene, encoding Pgp.^{38,39} Inhibition of Pgp with highly specific inhibitor GF120918 abolished the Pluronic-induced ATP depletion, while the inhibitor itself did not affect ATP levels in MDR cells.³⁸

Pluronic effects in MDR cancer cells exhibit remarkably simple and clear structure–functional relationships.⁵⁷ The studies of the concentration dependence of the Pluronic in MDR cells effect suggested that these effects are produced mainly by the copolymer single chains as they leveled up or decreased above the CMC. Hydrophilic Pluronics with HLB 20 and above have little if any sensitization effect in MDR cells. Using Pgp expressing brain microvessel endothelial cells (BMECs) it was demonstrated that such Pluronics do not decrease membrane microviscosity, do not inhibit Pgp ATPase activity, practically do not internalize in the cells, and do not induce ATP depletion.⁹⁹ Of all other Pluronics with HLB fewer than 20 the most active in MDR cells are the copolymers with intermediate lengths of the hydrophobic PPO block from about 30 to about 60 PO units.¹⁰⁰ Such copolymers include L61, P85, and P105 discussed above. These copolymers bind with the cell membranes, decrease membrane microviscosity, and inhibit Pgp ATPase activity.⁹⁹ Moreover, they internalize into cells and produce ATP depleting effects. The copolymers with shorter PPO blocks, fewer than 30 PO units, also internalize in cells. However, they do not decrease membrane microviscosity, do not inhibit Pgp ATPase, and do not deplete ATP. Presumably, they are not sufficiently “disruptive” to the membrane structures to produce all these effects. The copolymers with longer PPO blocks produce strong effects decreasing membrane microviscosity and inhibiting Pgp ATPase. But they do not penetrate inside the cells and do not reach mitochondria remaining stuck in the cell membranes, presumably due to their extreme hydrophobicity. Accordingly, such hydrophobic copolymers do not induce ATP depletion.⁹⁹ Notably, it was demonstrated that both ATP depletion and inhibition of Pgp ATPase activity are essential for the sensitization of Pgp overexpressing cells.^{39,93}

When one of these factors was excluded, the drug efflux pump remained functional in both MDR cancer and Pgp-expressing BMECs.^{39,93}

4. EFFECT OF PLURONIC ON PROAPOPTOTIC SIGNALING

Oxidative stress is a condition in which the balance between the production of reactive oxygen species (ROS) by cells and the ability to detoxify them is impaired. If oxidative stress persists, the formed peroxides and free radicals will damage all components of the cell, including membranes, proteins, and DNA. Accumulation of significant damage, which a cell fails to repair, will lead to apoptosis. Generally, oxidative stress is associated with increased production of ROS and/or decreased ability of the cell to eliminate these species. Glutathione is a major cellular antioxidant that protects the cells against ROS, toxins, and drugs. It is a tripeptide that exists in reduced (GSH) and oxidized (GSSG) states, and normally more than 90% of cellular glutathione is in a reduced state. An accurate ratio between GSH and GSSG is important to maintain the intracellular redox state, with a decrease in GSH/GSSG ratio indicative of oxidative stress. GSH is also a cofactor of glutathione S-transferase (GST), the major cellular detoxifying enzyme. Furthermore, several members of the MRP family of ABC transporters require GSH for transport activity. Pluronic was shown to deplete the GSH levels and inhibit the GST activity in several MDR cell lines.⁵⁷ Inhibition of the GSH/GST detoxifying system in turn decreases the MRP-mediated efflux. The decrease of cellular GSH is also an early sign of apoptosis induced by oxidative stress, death receptor activation, or mitochondrial apoptotic signaling.¹⁰¹

One of the major sources of ROS in the cells is electron transport chain in mitochondria. In normal conditions oxygen is reduced in mitochondria by cytochrome *c* oxidase (complex IV) to produce water. However, a small amount of electrons passing through the electron transfer chain reduce oxygen to produce superoxide radical. The main superoxide radical producing complexes in mitochondria are NADH dehydrogenase (complex I) and cytochrome *bc1* complex (complex III). It is well-known that inhibition of complex I by certain inhibitors like rotenone, piericidin A, and rolliniastatin increases the ROS production. As was mentioned above, Pluronic quickly reaches mitochondria and inhibits complexes I and IV in MDR cells (Figure 4). Moreover, it stimulates the production of ROS and release of cytochrome *c*, which are the early signs of mitochondrial apoptotic pathway.³⁸ If ROS are not neutralized, they induce damage of mitochondrial membrane, proteins, and DNA. This leads to permeabilization of outer mitochondrial membrane, swelling of mitochondria, and release of proapoptotic proteins, like cytochrome *c*, apoptosis inducing factor (AIF),¹⁰² and endonuclease G.¹⁰³ In cytoplasm cytochrome *c* binds to apoptosis protease activating factor (APAF-1) and forms apoptosome. The apoptosome cleaves and activates the procaspase-9 and forms caspase 9. The activated caspase 9 in turn activates the effector caspases, which all together contribute to the completion of apoptosis. Similar to ATP depletion and inhibition of respiration, Pluronic induced the ROS formation and cytochrome *c* release selectively in MDR cells, while non-MDR cells did not respond in that manner.³⁸

In addition to induction of ROS production and cytochrome *c* release in MDR cells, Pluronic promotes drug-induced apoptosis. Treatment of MDR cells with Dox/Pluronic P85

formulation significantly enhanced the proapoptotic signaling compared to the drug alone and inhibited the antiapoptotic defense mechanisms *in vitro*.¹⁰⁴ Similar effects were observed *in vivo*. It was demonstrated that Dox/Pluronic treatment of tumor-bearing mice significantly increased levels of caspases 8 and 9 compared to Dox alone.¹⁰⁵

Overall, Pluronic induces early as well as late stages of proapoptotic signaling in MDR cells *in vitro* and *in vivo*. Inhibition of mitochondria respiratory chain complexes is most likely the main reason for increased ROS production in MDR cells after treatment with Pluronic. Additionally, depletion of major intrinsic cellular antioxidant GSH would increase cell sensitivity to the ROS. It has been shown that drug-induced ROS production may be directly linked to their cytotoxic activity^{106,107} and that detoxification of free radicals by GSH/GST is very important in MDR cells to facilitate drug resistance.¹⁰⁸ Therefore, when combined with Dox, Pluronic not only drastically increases the drug accumulation in the cells but also promotes the apoptosis in the MDR cells. This in combination with the Dox effects results in significantly increased cell death.

5. PLURONICS PREVENT DEVELOPMENT OF MDR AND SUPPRESS CSCS

The mechanism of development of MDR in cancer remains a highly debated subject, and most likely there is no uniform theory that will apply to all cancers.^{109–114} It is now widely accepted that CSCs play an important role in cancer development, metastasis, and development of drug resistance. CSCs comprise a small cell subpopulation within the tumor with distinct functional and phenotypical characteristics. First, CSCs overexpress specific markers. However, these markers differ from cancer to cancer and to date there is no uniform marker that can be used to isolate CSCs from every tumor.¹⁰⁹ Second, CSCs have unlimited ability to divide and produce cells of all other phenotypes in the tumor. Third, CSCs are able to form tumors when transplanted into mice and to form so-called tumorspheres when grown in anchorage independent conditions. Finally, CSCs are intrinsically drug resistant: they overexpress drug efflux transporters, such as Pgp and BCRP, have active antiapoptotic pathways, and spend most of their time in the G₀ nondividing cell cycle state, which makes them insensitive to cytostatic drugs often used in chemotherapy.¹¹⁵ Therefore, CSCs can avoid classical chemotherapy and repopulate the tumor, possibly leading to MDR development. Moreover, there are reports suggesting that CSCs' phenotype is dynamic and can be acquired by non-CSCs under certain conditions.^{109,110} Overall, successful therapy needs to be equally efficient in eliminating both bulk tumor cells and CSCs.

In addition to MDR chemosensitization properties, Pluronic also prevent the development of MDR upon selection with cytotoxic drugs *in vitro* and *in vivo*.^{41,42} Specifically, in one study human breast carcinoma MFC7 cells were selected with Dox for drug resistance in the presence or absence of P85 at concentration below CMC (0.001 wt %).⁴¹ The cells cultured with Dox/P85 were not able to grow at concentrations of the drug exceeding just 10 ng/mL. In contrast, cells cultured with Dox alone eventually developed MDR and could tolerate up to 10,000 ng/mL Dox in the culture media. Further analysis has shown that cells treated with Dox/P85 did not overexpress Pgp and, therefore, remained sensitive to the drug. In contrast, cells exposed to Dox alone exhibited significant overexpression of Pgp. This developed drug

resistance can be resensitized by Pluronic to the initial level of the drug sensitive cells. Interestingly, when the cells were selected with lower concentration of Dox, they were not sensitized by Pluronic, even though they displayed low levels of Pgp expression and detectable levels of *mdr1* mRNA. Functional analysis of Pgp activity using accumulation of Pgp substrate (Rhodamine 123) showed that Pgp in those cells was not or nearly not functional compared to more resistant cells.^{41,42} Even though cells selected with lower concentrations of Dox were not sensitized with Pluronic, they showed strong ATP depletion in response to Pluronic treatment.⁴¹ Moreover, it was demonstrated that selection of cells with Dox and Dox/P85 resulted in very different changes in the gene expression patterns in these cells. P85 alone, however, had little if any effect on the gene expression.⁴¹ Similar results were observed in P388 murine leukemia tumor cells selected for Dox resistance with or without P85 both *in vitro* and *in vivo*.⁴² Overall, this suggests that simple addition of “inert” polymer excipient to the drug drastically changes pharmacogenomic responses of cancer cells to this drug.

However, our understanding of the mechanism behind the prevention of MDR development by Pluronic and alterations in gene expression profiles is very limited. In view of CSC theory a small population of tumor cells is guiding tumor progression, metastasis, and MDR development. Since CSCs share certain characteristics of MDR cells, such as overexpression of drug efflux transporters (Pgp, BCRP) and altered metabolic pathways,^{116–118} we proposed that Pluronics can sensitize CSCs to chemotherapeutic drugs similar to MDR cells. In a recent study using the same P388 leukemia ascitic tumor model as before,⁴² we demonstrated that Dox/Pluronic combination, SP1049C, comprising mixed micelles of Pluronic F127 ((EO)₁₀₀-(PO)₆₅-(EO)₁₀₀, HLB 22, MW 12 600 g/mol) and L61, effectively decreases frequency of tumor initiating cells and, as a result, suppresses tumorigenicity and tumor aggressiveness *in vivo*.²² In agreement with previous findings, SP1049C also prevented the development of MDR by inhibition of BCRP overexpression. In contrast to Dox alone, SP1049C depleted the tumorigenic CD133+ and ALDH+ cell subpopulations. Furthermore, *in vitro* pretreatment of ascitic cells with SP1049C significantly reduced the *in vitro* colony forming potential of the cells already at 10 ng/mL Dox, while Dox alone had the same effect at 10 times higher concentration. As mentioned above, Dox/Pluronic combination drastically changes the gene expression profiles in cancer cells compared to Dox or Pluronic alone upon continuous exposure. In this work we have shown that DNA methylation patterns also change drastically upon *in vivo* treatment of cancer cells with SP1049C compared to saline control, polymers, or Dox alone. It is well-known that misregulation of DNA methylation/demethylation plays an important role in cancer origin, progression, angiogenesis, metastasis, and MDR development.^{119–122} SP1049C not only induced the strongest epigenetic changes but also showed very small overlap of affected genes with other treatment groups. Functional analysis of affected genes done using ingenuity pathway analysis (IPA) has shown that the top affected biological functions and canonical pathways affected by SP1049C treatment relate to cellular function, growth, and maintenance, as well as regulation of stem cell differentiation and pluripotency. Altogether, on top of MDR sensitization, the prevention of MDR development by Pluronics, depletion of tumorigenic cell subpopulations, and decrease of tumorigenicity and tumor

aggressiveness offer significant advantages for the development of new formulations of approved and/or experimental therapeutics.

6. RECENT EXAMPLES OF PLURONIC-BASED AND SIMILAR DRUG DELIVERY SYSTEMS

Pluronic copolymers attracted a lot of attention in drug delivery and tissue engineering applications. Pluronic-based micellar formulation of Dox, SP1049C, was the first in class polymeric micelle drug to advance to clinical stage¹²³ and has successfully completed phase II clinical trial in advanced esophageal cancer patients.¹²⁴ In studies in rodent and nonrodent animal models it has been demonstrated, as well as in patients, that MTD and pharmacokinetic profiles of Dox alone and SP1049C are very similar.³⁷ SP1049C did enhance the tumor accumulation of the drug in tumor bearing mice. Moreover, animal studies using MDR overexpressing tumors have shown that Pluronic formulations *in vivo* exhibit key effects observed in mechanistic studies *in vitro*.¹⁰⁵ First, noninvasive single photon emission computed tomography (SPECT) and tumor tissue radioactivity sampling demonstrated that intravenous coadministration of Pluronic P85 with a Pgp substrate, ⁹⁹Tc-sestamibi, greatly increases the tumor uptake of this substrate in the MDR tumors. Second, ³¹P magnetic resonance spectroscopy (³¹P-MRS) in live animals and tumor tissue sampling for ATP suggest that P85 and Dox formulations induce pronounced ATP depletion in MDR tumors. Finally, these formulations were also 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.

In phase I clinical study of SP1049C in 26 patients, maximum tolerated dose (MTD) and dose-limiting toxicity (DLT) were determined as 70 and 90 mg/m² respectively. SP1049C also showed slower clearance compared to conventional Dox. In phase II study 21 patients (19 evaluable for response) with metastatic or locally advanced unresectable adenocarcinoma of the esophagus and gastroesophageal junction (GEJ) were treated with 75 mg/m² SP1049C every 3 weeks until disease progression or unacceptable toxicity. In this study SP1049C demonstrated prominent single agent antitumor activity (47% objective response rate in the evaluable population, 9 partial responders, 10 month median overall survival, and 6.6 month progression free survival) with toxicity profile similar to that of Dox at equivalent dose and administration schedule.

Unique biological activities of Pluronics in addition to their drug solubilization properties make Pluronics a very attractive platform for drug delivery. For example, in recent work Chen and coauthors used mixed micelles of P105 and F127 to overcome Pgp-mediated MDR to methotrexate (MTX) *in vitro* and *in vivo*.¹²⁵ This system has shown relatively high drug loading and pH-dependent drug release, improved pharmacokinetics, biodistribution and antitumor activity in human lung (A549) and oral epidermoid carcinoma (KBv) MDR xenograft tumor models, and reduced systemic toxicity (Table 1). The same group has also used Pluronic P105/F127 mixed micelles to deliver docetaxel (DTX) to Taxol-resistant non-small cell lung cancer.¹²⁶ While in drug sensitive cells the micelles had similar IC₅₀ to Taxotere, in drug-resistant A549/Taxol cells they demonstrated 10-fold lower IC₅₀ compared to Taxotere control (0.059 µg/mL vs to 0.593 µg/mL). *In vivo* A549/Taxol

drug-resistant tumor model DTX loaded mixed Pluronic micelles showed 69.05% tumor inhibition, versus 34.43% for Taxotere control (Table 1).¹²⁶

In another work Shen et al. developed novel Pluronicpolyethylene imine (PEI)/ α -tocopheryl polyethylene glycol 1000 succinate (TPGS) nanoparticles to overcome paclitaxel (PTX) drug resistance and codeliver survivin shRNA.¹²⁷ TPGS was used to improve micelle stability and drug loading, P85 was used to form micelles and inhibit GST activity, and PEI was used to bind shRNA. These complex nanoparticles have shown a synergistic effect in cytotoxicity experiments in A549/T PTX resistant cells, but not in parental A549 drug sensitive cells, and displayed effective antitumor activity *in vivo* in MDR tumor model. Furthermore, the authors have shown that GST isolated from MDR cells was 3.8 times more active than extracted from sensitive cells and that both P85 and P85–PEI conjugate effectively inhibited only GST of MDR cells but not of non-MDR cells. This is an important observation, since GST plays an important role in PTX metabolism and its inhibition would increase accumulation of PTX in the cells. Other examples that use Pluronic MDR reversal properties for overcoming MDR include poly(caprolactone)-modified Pluronic P105 (P105-CL) PTX loaded micelles developed by Wang et al.¹²⁸ to overcome ovarian cancer PTX drug resistance. These polymers displayed ATP depletion, inhibition of mitochondrial function, and membrane fluidization activities, similar to what was reported before for other Pluronics.^{57,99} A few years earlier the same group developed folate-targeted Pluronic micelles for delivery of PTX and circumvention of MDR.¹²⁹ The authors have shown that folate conjugated Pluronic P105 or L101 PTX loaded micelles better accumulate in MCF7/ADR cells and have significantly higher efficiency compared to nontargeted micelles of PTX alone (Table 1).

The biological response-modifying properties are, however, not unique to Pluronics. A number of other natural and synthetic polymers have been reported to inhibit drug efflux transporters.^{130,131} For example, polymers developed by Cambon and colleagues with similar architecture to Pluronics, but with poly(styrene oxide) (PSO) instead of PPO, also form micelles which have shown efficient drug loading and pH-dependent release, as well as Pgp inhibition activity.¹³⁰ Furthermore, in another study from the same group the authors evaluated the structure–activity relationships of nearly 30 copolymers with structures similar to Pluronics, but containing different hydrophobic blocks, including propylene oxide, lactide, methylene, butylene oxide, valerolactone, caprolactone, styrene oxide, and glycidyl.¹³² Many of the screened copolymers induced increase of Dox accumulation in the Pgp overexpressing MDR cells, as well as inhibition of Pgp ATPase activity. Notably, the most active copolymers had longer hydrophobic chains compared to what is considered optimal for Pluronics,⁹⁹ that is, Pluronics with intermediate length of hydrophobic block and relatively low HLB.

Furthermore, TPGS was also reported to inhibit Pgp.¹³³ TPGS is a common form of vitamin E, and it has been recognized as a potent enhancer of oral absorption of drugs due to inhibition of drug efflux transporters. Collnot et al. compared TPGS with different PEG lengths (200–6000) and have found that commercial TPGS-1000 is one the most potent analogues in the series of polymers. Other pharmaceutical excipients, including some Tweens (PEGylated sorbitanes), Brij (Alkyl-PEO surfactants), and Myrj (PEO-stearates),

also demonstrated Pgp inhibition, that strongly depends on HLB of the polymer,¹³⁴ albeit they generally remain less potent than Pluronics.

Altogether, there are number of polymers that possess the advantageous properties of inhibition of drug efflux transporters and can be used to overcome cancer MDR or to improve oral drug bioavailability. Pluronics, however, represent the most studied group of potent polymers with respect to molecular mechanism of Pgp inhibition and MDR sensitization. Considering similar activities observed in other groups of polymers, it is likely that some general patterns of structure–activity relationships of Pluronics (HLB, architecture, etc.) and spectrum of biological effects can be extrapolated to other amphiphilic polymers.

7. CONCLUSIONS

Intrinsic and acquired drug resistance represents the great obstacle for successful treatment of cancer. Numerous approaches have been utilized in attempts to overcome drug resistance with limited success. In this review we have discussed the biological properties of Pluronic block copolymers and other polymers with similar biological activities, which, in addition to carrier function, make them an attractive platform for drug delivery. The MDR chemosensitization activity of Pluronics (and other surfactants) has been known for a while now, and the mechanisms have been extensively studied (Figure 5). However, we are still far from complete understanding of how exactly Pluronics interact with MDR cells and why these effects are specific to MDR phenotype. Recent studies have shown that combination of chemotherapeutic drug (Dox) with Pluronic effectively depletes tumorigenic cell subpopulation and decreases tumorigenicity and tumor aggressiveness.²² This finding being so simple by nature drastically changes the whole concept from Pluronics being just another MDR modulator to a class of agents that might help to combat cancer at its root by killing CSCs. On the other hand, we now have even more questions regarding the mechanism of action of Pluronic than we had before. We believe that thorough understanding of these mechanisms will allow better design of Pluronic (and similar polymers)-based drug delivery systems for effective cancer therapy.

Acknowledgments

This work was supported in part by the grants from the National Institutes of Health 2RO1 CA89225, UO1 CA151806, an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under grant P20GM103480, as well as *The Carolina Partnership*, a strategic partnership between the UNC Eshelman School of Pharmacy and The University Cancer Research Fund through the Lineberger Comprehensive Cancer Center.

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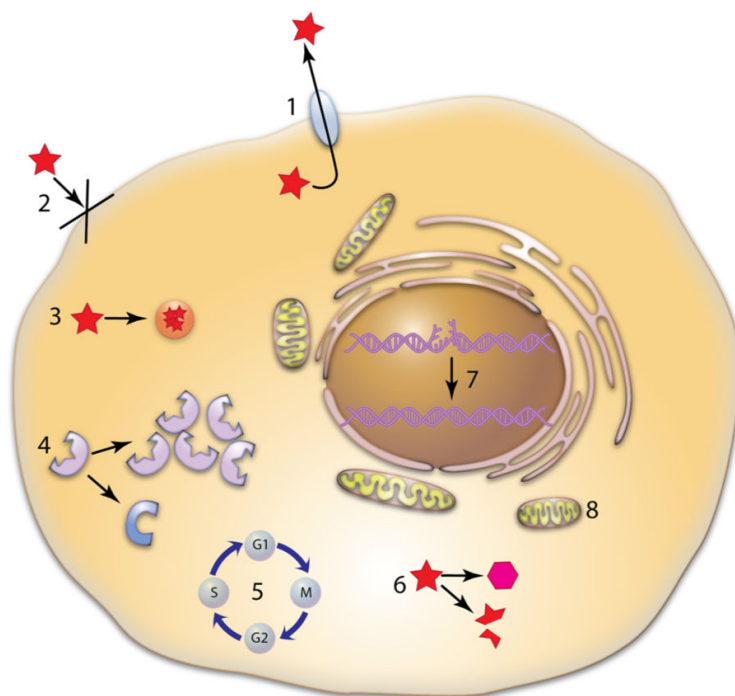


Figure 1. Mechanisms of MDR in cancer cells: (1) active drug efflux by drug transporters, such as Pgp, MRP, and BCRP; (2) loss of cell surface receptors and/or drug transporters or alterations in membrane lipid composition; (3) compartmentalization of the drug in cellular vesicles; (4) altered/increased drug targets; (5) alterations in cell cycle; (6) increased drug metabolism/enzymatic inactivation; (7) active damage repair; and (8) inhibition of apoptosis.

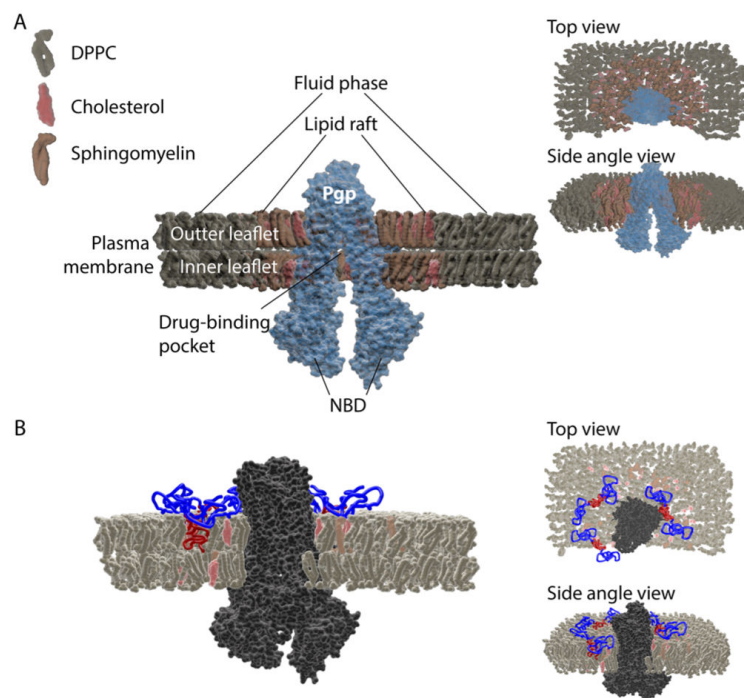


Figure 2. Structure and localization of Pgp in plasma membrane. (A) Pgp is a transmembrane protein with drug-binding pocket localized in the inner leaflet of the plasma membrane, and two NBD localized in cytoplasm. Functional Pgp is localized in cholesterol, sphingomyelin, and GM1 ganglioside-rich membrane microdomains, called lipid rafts, where it is surrounded by fluid phase of the membrane, containing unsaturated fatty acids like DPPC. Pgp is pictured in inward-open (outward closed) conformation ready to bind substrate. The model is based on X-ray analysis⁵⁶ and NMR data from protein data bank (<http://www.rcsb.org/>). (B) Incorporation of Pluronic into lipid bilayer disrupts lipid rafts, possibly causing conformational changes in Pgp, which results in inhibition of Pgp ATPase and transport activities.

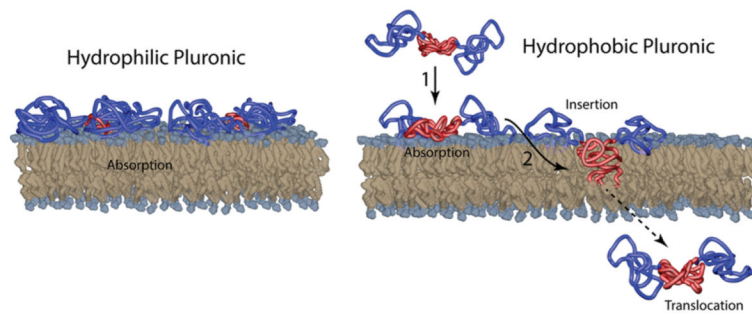


Figure 3. Schematic presentation of interaction of Pluronics with different hydrophobicity with lipid membranes: (1) absorption of Pluronic molecules on the surface of the membrane, (2) insertion into the lipid bilayer, and (3) translocation through the membrane.

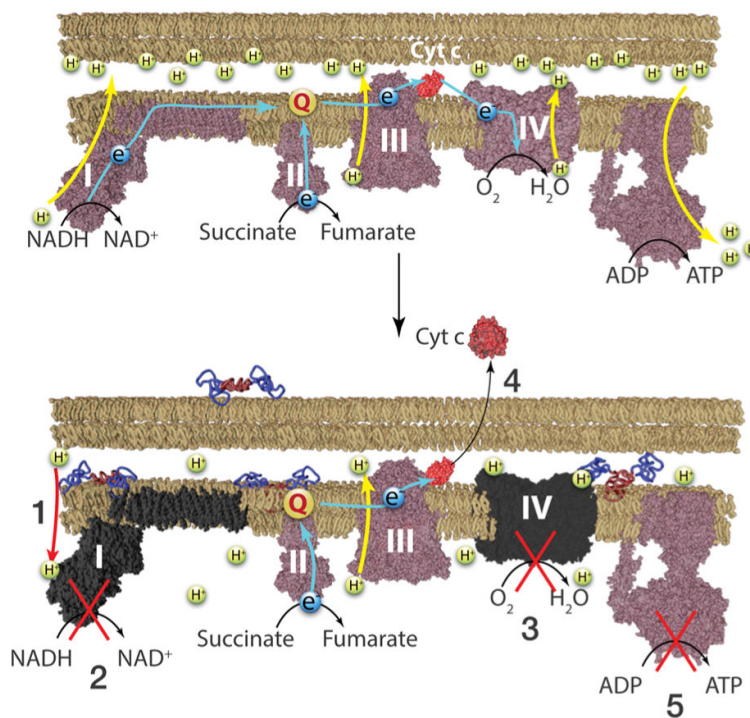


Figure 4. Effect of Pluronic on mitochondrial electron transport chain in MDR cancer cells. Pluronic quickly enters the cells, reaches mitochondria, and induces mitochondrial membrane depolarization (1), inhibition of complexes I (2) and IV (3), release of cytochrome c (4), and ATP depletion (5).

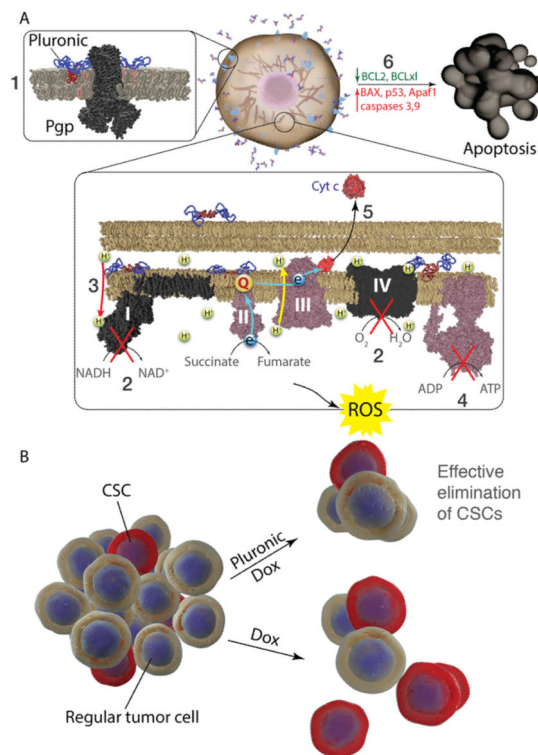


Figure 5. Summary of Pluronic effects in cancer cells. (A) Pluronic binding with plasma membrane of MDR cancer cells (1) induces membrane fluidization, disruption of membrane microdomains, and inhibition of drug efflux transporters' activity (Pgp shown as an example). Pluronic also reaches mitochondria where it (2, 3) inhibits complexes I and IV of mitochondria respiratory chain and (3) induces inner mitochondrial membrane depolarization. This (4) results in ATP depletion and (5) promotes cytochrome *c* release and ROS generation in MDR cells. Altogether, the MDR cells respond to a Dox/Pluronic combination by (6) an increased proapoptotic signaling and decreased antiapoptotic defense. (B) Moreover, Dox/Pluronic combination effectively depletes tumorigenic subpopulation of CSCs, prevents development of MDR, and significantly alters DNA methylation and gene expression profiles.

Table 1

Recent Examples of Pluronic-Based Formulations To Overcome MDR

polymer	drug	name/ company	disease	development stage
Pluronic F127/L61	Dox ^{123,124}	SP1049C/ Supratek Pharma Inc.	GI cancer	phase II completed
Pluronic P105/F127	methotrexate ¹²⁵ or docetaxel ¹²⁶		human carcinoma (KB), human embryonic kidney cell line (HEK-293), human lung adenocarcinoma (A549), human lung carcinoma (H-460)	preclinical
Pluronic-polyethylene imine (PEI)/ <i>D</i> - α - tocopheryl polyethylene glycol 1000 succinate (TPGS)	paclitaxel/survivin shRNA ¹²⁷		human lung adenocarcinoma (A549)	preclinical
poly(ϵ -caprolactone)-modified Pluronic P105 (P105-CL)	PTX ¹²⁸		ovarian cancer	preclinical
folate conjugated Pluronic P105 or L101	PTX ¹²⁹		breast cancer (MCF7/ADR)	preclinical