Selective Energy Depletion and Sensitization of Multiple Drug-Resistant Cancer Cells by Pluronic Block Copolymer

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SUMMARY: This work demonstrates that exposure of cells to a poly(ethylene oxide)-poly(propylene oxide) block copolymer, Pluronic P85, results in substantial decrease in ATP levels selectively in MDR cells. Cells expressing high levels of P-glycoprotein are highly responsive to the Pluronic treatment, while those with low levels of expression are much less responsive. Cytotoxicity studies suggest that Pluronic acts as a chemosensitizer and potentiates cytotoxic effects of doxorubicin in MDR cells. Because many mechanisms of drug resistance are energy-dependent, a successful strategy for treating MDR cancer could be based on selective energy depletion in MDR cells. Therefore, the finding of energy-depleting effects of Pluronic P85, in combination with its sensitization effects is of considerable theoretical and practical significance.

Introduction

Poly(ethylene oxide)-poly(propylene oxide) block copolymers (Pluronic® or "poloxamer") have recently been used in formulations for treatment of drug-resistant cancers[1)]. A formulation that contains doxorubicin, SP1049C, is currently undergoing Phase I clinical trials. Experimental studies have demonstrated that Pluronic block copolymers sensitize multidrug-resistant (MDR) cells, resulting in an increase in the cytotoxic activity of anthracyclines and other cytotoxic drugs by 2 - 3 orders of magnitude[2,3)]. This finding is important in the light of the major problem of drug resistance to antineoplastic agents, which severely limits the chemotherapy of many cancer tumors[4)]. Tumors with the MDR phenotype have been widely recognized as one of the most difficult types to treat. MDR cells overexpress efflux transporters belonging to a superfamily of ATP-binding cassette (ABC) proteins, such as Pgp and MRP (multidrug resistance-associated protein), which pump drugs out of a cell[4,5)]. The glutathione/glutathione S-transferase detoxification system is frequently activated in MDR cells contributing to drug resistance[6)]. MRP acts in concert with this system, providing for the efflux of glutathione conjugates of xenobiotics from the cells[5)].

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Another impediment to treatment, which is present in MDR cells, involves the sequestration of drugs within cytoplasmic vesicles, followed by extrusion of the drug from the cell[7]. Drug sequestration in MDR cells is achieved through the maintenance of abnormally elevated pH gradients across organelle membranes by the activity of H^+-ATPase, an ATP-dependent pump[8]. Energy-dependent processes associated with the above drug resistance mechanisms impose higher energy requirements upon the MDR cells, which might render these cells more sensitive to energy-depleting agents as opposed to the wild-type cells which have lower energy requirements[9]. This paper, for the first time, demonstrates that Pluronic P85 induces a dramatic reduction in ATP levels selectively in MDR cells, while non-MDR cells are not responsive to this block copolymer in this manner. Energy depletion induced by P85 correlates with its observed sensitization effect in MDR cells with respect to doxorubicin. This provides new insight regarding the mechanism of potentiation of cytotoxicity of drugs by Pluronic in MDR cells.

Materials and Methods

Doxorubicin was purchased from Sigma Chemical Co. (St. Louis, MO) and Pluronic P85 (lot # WPOP-587A) was provided by BASF Corp. (Parispany, NJ). Doxorubicin/P85 was prepared by adding doxorubicin to the culture medium containing various concentrations of P85. Cell lines were cultured as suggested by providers. BBMEC cells were isolated and cultured as described previously[10]. The cells were seeded at a density of 25 000 cells/cm^2 into 24-well plates and were used for accumulation studies after reaching confluency (typically within 6-7 days). Following exposure of the cells to the block copolymer, ATP levels were determined in the cell lysates using a luciferin/luciferase assay[11]. Each data point represented the mean $\pm$ SEM of a minimum of 4 replicates. The cytotoxic activity of doxorubicin was then evaluated using a standard MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay[12]. Each concentration point was repeated in eight wells. SEM values were less than 10%.

Results and Discussion

To examine effects of P85 on intracellular ATP levels, resistant (MCF-7/ADR) and sensitive (MCF-7), cells were exposed to this block copolymer at various concentrations (without drug) for 2 h. After that, cells were lysed and ATP was quantified as described above. Figure 1a presents the ATP levels observed in resistant and sensitive cells following the treatment with P85. In the resistant cells, exposure to low concentrations of the Pluronic (ca. 0.05 % wt.)

resulted in a decrease in the ATP level to 3.8 % of the initial value. In contrast, in the sensitive cells, ATP levels did not drop until much higher concentrations of the block copolymer (ca. 5 % wt.) were reached. At these P85 concentrations, the ATP levels in the sensitive cells decreased to 15 % of the initial value. Therefore, exposure to low concentrations of P85 causes a selective decrease in ATP levels (energy depletion) in MDR cells but not in the sensitive cells.

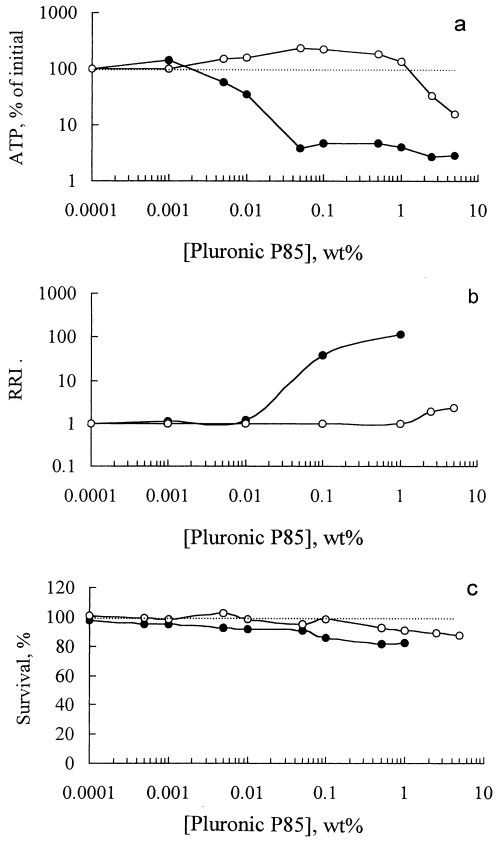

Fig. 1: Effects of Pluronic P85 on (a) intracellular ATP levels, (b) doxorubicin resistance reversion index (RRI) and (c) cell survival in resistant MCR7/ADR (filled circles) and sensitive MCF-7 (empty circles) cells.

The effect of P85 on the ATP levels in MDR cells correlates well with the sensitization effect of this block copolymer in MDR cells. For this study, cells were exposed to doxorubicin alone or doxorubicin/P85 solutions (0.0001 - 5 % P85) for 2 h and the cytotoxic activity of doxorubicin was then evaluated. Figure 1b presents the resistance reversion indexes (RRI) for MCF-7/ADR cells upon co-administration of doxorubicin with various doses of P85. (Here and below RRI is

defined as the ratio of the IC_{50} of the drug in the control medium and P85 solution of various concentrations.) The data show that P85 potentiates the cytotoxic activity of doxorubicin with respect to MDR cells. This effect was registered at approximately the same doses of P85 that cause energy depletion in MDR cells. In contrast, treatment with the same doses of P85 did not result in a similar potentiation of cytotoxicity in the non-MDR line, MCF-7 (Fig. 1b).

To examine whether the Pluronic affects the viability of cells, both resistant and sensitive cells were exposed to various doses of P85 (without drug) for 2 h and cytotoxicity was evaluated as described above. As is seen in Fig. 1c, P85 did not induce significant cytotoxic effects in either resistant or sensitive cells over the range of concentrations tested (0.0001 - 5 %). The ability of the cells to restore ATP levels following removal of P85 from the culture medium was tested in the following experiment. In this experiment, MCF-7/ADR cells were exposed to 0.1 % P85 for 2 h to induce reduced ATP levels, then the P85 containing supernatants were removed and cells were cultured in the copolymer-free media for various time periods. Measurements of the ATP levels at various times after removal of P85 suggested that ATP levels restored after 15 h and then maintained at the pretreatment levels for at least 70 h (Table 1). In a similar experiment, sensitive MCF-7 cells were exposed to 5 % P85 (a dose that causes energy depletion in these cells) and then cultured in the absence of P85 for various time periods to examine energy restoration. In this case, ATP levels were also restored after the 15-h interval (Table 1).

Table 1. Intracellular ATP levels before and after treatment of MCF-7/ADR or MCF-7 cells with Pluronic P85[a]

Time of measurement of ATP level	ATP, nmol/mg protein[b]	
	MCF-7/ADR	MCF-7
Before P85 treatment	302 ± 21	29.0 ± 1.4
Immediately after P85 removal	15.3 ± 2.3	2.4 ± 0.34
15 h after P85 removal	402 ± 13	40.0 ± 1.9
70 h after P85 removal	301 ± 28	28.5 ± 1.5

[a] Cells were exposed to 0.1 % (MCF-7/ADR) or 5 % (MCF-7) P85 and then incubated for various time periods in the copolymer free culture media. [b] Mean ± SEM (n=4).

Therefore, exposure of resistant and sensitive cells to different doses of P85 resulted in a transient energy depletion, which was reversed when the block copolymer was removed. The resistant cells were much more responsive to P85, exhibiting profound decreases in ATP levels at a hundred time lower concentration of the block copolymer compared to the sensitive cells. The observed transient energy depletion in the absence of antineoplastic agent was not

accompanied by observable toxicity in the cells since Pluronic alone did not exert any significant cytotoxic effect in either resistant or sensitive cells. However, if the antineoplastic agent, doxorubicin, was introduced concurrently with the block copolymer, the cytotoxic effect of doxorubicin significantly increased compared with the drug alone.

To examine correlation of the P85-induced energy depletion with the Pgp expression in the cells, this study compared the effects of the block copolymer on ATP levels in several mammalian cell types which either express or do not express Pgp. First, the cell panel used in this study included pairs of resistant and sensitive cancer cell lines: MCF-7/ADR and MCF-7 (human breast carcinoma cells) as well as KBv and KB (human epithelial cells). Second, the panel included cells with intrinsic Pgp expression, Caco-2 (human colon epithelium cells) and BBMEC (bovine brain microvessel endothelial cells), as well as Pgp-negative cells, HUVEC (human umbilical vein endothelial cells) and C2C12 (murine myoblast cells). Third, this panel also included porcine kidney epithelial cells, LLC-MDR1, transfected with the human MDR1 gene, and their non-transfected counterparts, LLC-PK1[13]. The cells were exposed for 2 h to P85 at various concentrations and then ATP levels were determined as described above. The effective concentrations of P85 that induce 50% decrease in ATP levels in the cells (EC_{50}) were determined from the dose-response curves. EC_{50} values characterizing the responsiveness of various cell types to P85 are presented in Table 2.

Table 2. Concentrations of Pluronic P85 that induce 50% decrease in ATP levels (EC_{50}) in the Pgp-expressing and non-Pgp cells following 2 h exposure to the block copolymer (in the absence of the drug).

Cells	Pgp expression	EC_{50}, wt. %	$(EC_{50})_{Pgp(+)} / (EC_{50})_{Pgp(-)}$ [a]
MCF-7	–	2.25	-
MCF-7/ADR	+	0.009	250[b]
KB	–	0.675	-
KBv	+	0.036	18.75[c]
C2C12	–	4.5	-
HUVEC	–	0.0675	-
Caco-2	+	0.000675	6670[d]
BBMEC	+	0.018	250[d]
LLC-PK1	–	0.45	-
LLC-MDR1	+	0.0045	100[e]

[a] Relative responsiveness of cells to P85 is calculated as the ratio of EC_{50} of Pgp-expressing cells to EC_{50} of corresponding non-Pgp cells. [b] Compared with MCF-7 cells. [c] Compared with KB cells. [d] Compared with C2C12 cells. [e] Compared with LLC-PK1 cells.

As can be seen in Table 2, the responsiveness of cells to P85 correlated well with expression of Pgp in these cells. Specifically, energy depletion was observed in the resistant cell lines MCF-7/ADR and KBv, but not in the sensitive parental cell lines MCF-7 and KB. Likewise, Pgp-expressing Caco-2 and BBMEC cells responded to P85 at lower concentrations of the block copolymer than the Pgp-negative HUVEC and C2C12 cells. Finally, transfected LLC-MDR1 cells, overexpressing Pgp, were highly responsive to P85 treatment, while their non-Pgp counterparts were much less responsive. On the basis of results of this study, it appears that Pgp expression is a factor that renders cellular metabolism responsive to treatment with the block copolymer. Among Pgp-negative cells, the highest responsiveness to P85 treatment was observed for HUVEC, which exhibited an EC_{50} value of 0.0675 % (Table 2). Although these cells were characterized as Pgp-negative[14], they might express some other transport systems that could play a similar role as Pgp, resulting in the response to the block copolymer.

The relationship between P85-induced changes in ATP levels and the sensitization effect in the resistant cells was further examined using KBv cells. This cell line was exposed for 2 h to doxorubicin alone or doxorubicin formulated with 0.1% P85. In an attempt to bypass Pluronic-induced energy depletion, one more treatment group was also included in this study, in which doxorubicin/P85 was supplemented with 50 μM ATP and 10^{-5} M dodecylamine, as a permeabilizing agent. As previously reported[15], treatment of the cells with dodecylamine in combination with P85 allows transport of ATP into the cells from the extracellular media. Following exposure of the cells to doxorubicin solutions, drug-induced cytotoxicity was determined. In a parallel study, the KBv cells were exposed for 2 h to the same treatment as solutions without doxorubicin and intracellular ATP levels were determined. As can be seen in Fig. 2a, in the absence of ATP in the extracellular media, P85 induced a substantial decrease in ATP in KBv cells. The cytotoxic activity of doxorubicin was also enhanced in this case resulting in ca. 83-fold decrease in IC_{50} for doxorubicin/P85 formulation compared to the IC_{50} of the free drug. However, when the external media were supplemented with ATP and dodecylamine, both the energy depletion and sensitization effects of P85 were considerably reduced. In this case, the IC_{50} value of doxorubicin formulated with Pluronic was only 5.5 times less than the IC_{50} of free doxorubicin. No effect from the addition of extracellular ATP and dodecylamine on the IC_{50} of the drug was observed in the absence of P85 (data not shown). It appears that there is a direct relationship between the levels of ATP and sensitization of resistant cells to doxorubicin by P85.

This relationship was further validated by comparing the effect of P85 with that of a combination of metabolic inhibitors, 2-deoxy-D-glucose and sodium azide, in KBv cells.

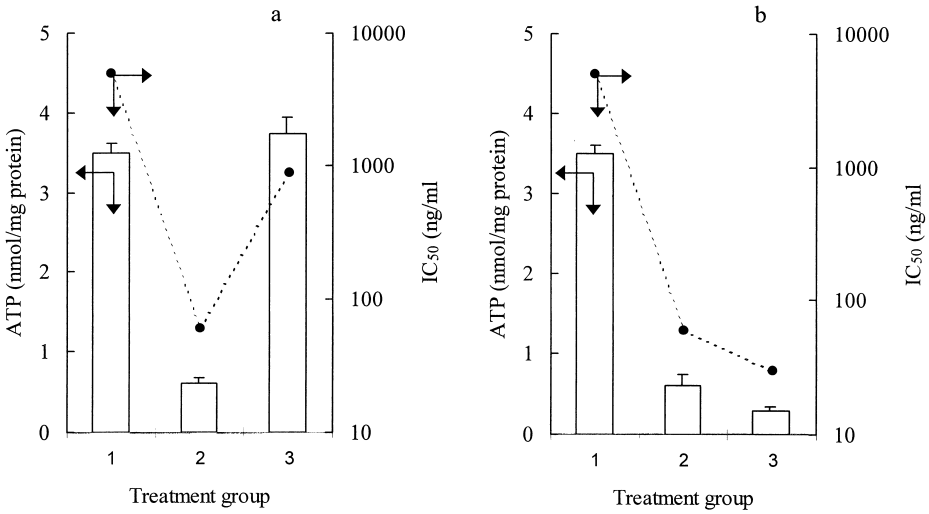

Fig. 2: Intracellular ATP levels (bars) and doxorubicin cytotoxicity (IC_{50}, filled circles) modulated by various agents in KBv cells. ATP levels (means ±SEM, n=4) were determined after exposure of the cells for 2 h to the following treatment solutions:
(a) (1) copolymer-free media, (2) P85 (0.1%), (3) P85 (0.1%) in the presence of extracellular ATP (50 μM) and dodecylamine (10^{-5} M); (b) (1) copolymer-free media, (2) P85 (0.1%), (3) sodium azide (150 μM) and 2-deoxy-D-glucose (50 mM). (For cytotoxicity studies, the corresponding treatment solutions also contained various concentrations of doxorubicin, and IC_{50} values were determined using a standard MTT assay following three-day culturing of the cells in the drug-free medium.)

As in the previous section, the resistant cells were exposed for 2 h to doxorubicin in various formulations and the cytotoxicity was determined as described above. In this experiment, however, along with the control medium and P85 treatment, the cells were also treated by exposure to a mixture of 2-deoxy-D-glucose (50 mM) and sodium azide (150 μM). In a parallel study, intracellular ATP levels were also determined following exposure of the KBv cells to the corresponding treatment solutions (without doxorubicin). As is seen in Fig. 2b, exposure of the cells to the 2-deoxy-D-glucose and sodium azide mixture resulted in a considerable decrease in ATP, which was comparable in magnitude with the effect of P85 observed in these cells. Furthermore, the decrease in ATP levels induced by the metabolic inhibitors was also accompanied by an increase in the sensitivity of the cells to the drug, which again was similar to the effect of P85 (Fig. 2b). Therefore, inhibition of metabolism in these resistant cells leads to the sensitization of the cells to cytotoxic action of the antineoplastic agent.

Overall, on the basis of this study, the sensitization effect of the block copolymer in resistant cells appears to be related to energy depletion. First, both ATP decreases and sensitization are observed upon treatment of the resistant cells with the same doses of the block copolymer.

Second, the study of the time course of the energy depletion demonstrated that ATP levels decrease during the first two hours of exposure of the resistant cells to Pluronic, which coincides with the time course of the sensitization of the resistant cells (data not presented in the figures). Finally, this work demonstrates that treatment of the cells with the energy-supplementation system substantially decreases the sensitization effect of Pluronic P85 and reduces the resistance reversion index.

The apparent relationship between energy depletion and enhanced cytotoxicity in cancer cells is supported by literature. For example, metabolic modulators alone, such as 2-deoxy-D-glucose, iodoacetic acid, fluoroacetic acid, oligomycin, azide, antimycin and rotenone, which can inhibit energy conservation at various levels (i.e., glucose uptake, glycolysis, citric acid cycle, oxidative phosphorylation), have been shown to induce apoptotic cell death in cancer cells[16]. Furthermore, a three-drug combination, PMA (*N*-(phosphonoacetyl)aspartate, 6-(methylsulfanyl)purine, 6-aminonicotinamide), which contains inhibitors of pyrimidine and purine pools, can potentiate drug activity and enhance drug-induced apoptosis in cancer tumors[17].

Previous reports have suggested that certain Pluronic block copolymers, including the P85 studied in this work, can affect metabolism in cells. For example, decreased ATP levels were observed, following exposure to P85, in the Jurkat T-cell lymphoma[15]. Furthermore, a study by Kirillova *et al.* suggested that P85 inhibits respiration both in isolated mitochondria and in whole cells[18]. An important observation made in the present work is that cells expressing Pgp are much more responsive to Pluronic than cells that do not express Pgp. It is possible that high rates of energy consumption by the drug efflux pump, combined with Pluronic-induced inhibition of respiration, determine the responsiveness of the resistant cells to the block copolymer. Under the conditions of inhibition of respiration necessary for ATP synthesis, the high rates of ATPase activity due to the presence of drug efflux pumps (and, possibly, some other energy-dependent mechanisms) could result in a rapid exhaustion of the intracellular ATP pools in the resistant cells. Alternatively, cells that do not exhibit these resistance mechanisms would appear to be less responsive to inhibition of respiration and would not exhibit energy depletion, at least to the extent observed in the resistant cells. Such a hypothesis is in line with the earlier observation that resistant cells have an increased glucose utilization rate compared to sensitive cells[19]. Furthermore, the toxicity of 2-deoxy-D-glucose, a glucose antimetabolite, was found to be consistently higher in MDR cells than in the parental drug-sensitive lines[9,19].

Previous work has demonstrated that Pluronic affects multiple mechanisms of drug resistance in MDR cells. This includes inhibition of drug efflux proteins resulting in increased drug accumulation and abolishment of sequestration of drugs in the cytoplasmic compartments[2,3]. Both the drug efflux systems and the drug sequestration mechanisms are ATP-dependent. While it is not clear at present whether Pluronic can directly affect these mechanisms (e.g., by changing the properties of the cell membranes), it cannot be excluded that energy depletion serves at least as an additional factor for the ultimate shutdown of resistance systems. In any case, it appears that energy depletion is an Achilles' heel of MDR cells, turning the high energy requirements imposed on these cells by drug resistance mechanisms into a vulnerability resulting in enhanced cytotoxic effects. While a number of experimental and clinical approaches have been studied to overcome MDR, including the use of MDR chemosensitizers, the appearance of several distinct transporters in resistant cells may limit the success of those agents, which target a single drug efflux pump. Furthermore, a combination of several independent mechanisms of drug resistance might complicate chemotherapy and reinforces the need for development of novel drugs and drug formulations effective against drug-resistant cancers. It has long been suggested that a broadly successful strategy for killing drug-resistant cancer cells could be based on selective energy depletion in these cells, since many mechanisms of drug resistance are energy-dependent[20]. Therefore, the finding of energy-depleting effects of Pluronic block copolymers, in combination with their very high sensitization effects and ability to inhibit multiple mechanisms of drug resistance in MDR cells, is of considerable theoretical and practical significance.

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