Published in final edited form as:

J Control Release. 2005 July 20; 105(3): 269–278. doi:10.1016/j.jconrel.2005.03.019.

Pluronic block copolymers alter apoptotic signal transduction of doxorubicin in drug-resistant cancer cells

Tamara Minko^a, Elena V. Batrakova^b, Shu Li^b, Yili Li^b, Refika I. Pakunlu^a, Valery Yu. Alakhov^c, and Alexander V. Kabanov^{b,*}

^aDepartment of Pharmaceutics, Rutgers, The State University of New Jersey, 160 Frelinghuysen Road, Piscataway, NJ 08854, USA

^bCenter for Drug Delivery and Nanomedicine and Department of Pharmaceutical Sciences, College of Pharmacy, University of Nebraska Medical Center, 985830 Nebraska Medical Center, Omaha, Nebraska NE 68198-5830, USA

^cSupratek Pharma Inc., 215 Bvd. Bouchard, Suite 1315, Laval, Quebec, Canada H9S1A9

Abstract

Pluronic block copolymer P85 (P85) sensitizes multidrug resistant (MDR) cancer cells resulting in the increase of cytotoxic activity of antineoplastic agents. This effect is attributed to the inhibition of the most clinically relevant drug efflux transporter, P-glycoprotein (Pgp), through the combined ATP depletion and inhibition of Pgp ATPase activity. The present study elucidates effects of an anticancer agent, doxorubicin (Dox), formulated with P85 on drug-induced apoptosis in MDR cancer cells. Early and late stages of apoptosis were detected by Annexin V and TUNEL methods, respectively. In parallel experiments, the expression of genes related to apoptosis, *BCL2*, *BCLXL*, *BAX*, *P53*, *APAF1*, *Caspase 3*, and *Caspase 9*, was determined by RT-PCR. The obtained data suggest that Dox/P85 formulation induces apoptosis in the resistant cancer cells more efficiently than free Dox. The treatment of the cells with Dox alone simultaneously activated a proapoptotic signal and an antiapoptotic cellular defense. Therefore, the apoptosis induction by Dox was substantially limited. In contrast, the treatment of the cells with Dox/P85 formulation significantly enhanced the proapoptotic activity of the drug and prevented the activation of the antiapoptotic cellular defense. This is likely to result in the stronger cytotoxic response of the resistant cells to the Dox/P85 formulation compared to the free drug.

Keywords

Apoptosis; Multidrug resistance; Pluronic; Polymer

1. Introduction

The A–B–A block copolymers of poly(ethylene oxide) (A) and poly(propylene oxide) (B), poloxamers or Pluronics, were shown to interact with multidrug resistant (MDR) cancer cells resulting in chemosensitization of these cells with respect to antineoplastic agents [1–4]. SP1049C, a formulation of doxorubicin (Dox) with mixed micelles of Pluronics L61 and F127, is being evaluated in clinical trials for the treatment of MDR tumors [5]. The sensitization of MDR cells by Pluronics appears to be related to the inhibition of drug efflux systems,

particularly, P-glycoprotein (Pgp) [3], which actively pumps the drugs out of the cells and thus reduces their cytotoxic effects. The mechanism involves the interaction of the Pluronic molecules with cell membranes, decrease in membrane microviscosity accompanied by significant inhibition of Pgp ATPase activity [6,7]. Of equal importance is strong energy depletion caused by Pluronics in MDR cancer cells [2]. The inhibition of the efflux proteins and ATP depletion combined causes a shut-down of the drug efflux systems, increase in the drug entry in the cells, and as a result, effective sensitization of MDR cells [2,6,7].

It became lately evident that the impact of Pluronic block copolymers in cancer cells is more diverse. A recent study suggested that Pluronic can prevent development of MDR in breast cancer cells selected with doxorubicin [8]. Furthermore, the analysis of the global gene expression profiles in the selected cells suggested that Pluronic can significantly alter the genomic responses to the drug [8]. It is noteworthy that in the absence of a drug Pluronic had little, if any, effects on gene expression profiles. Thus, it is possible that it affected the intracellular signal transduction pathways, which underlie specific genetically controlled responses of the cells to the drugs. It has been demonstrated that synthetic polymers, such as poly(2-hydroxypropylmethacrylate) (pHPMA) and poly(ethyleneglycol) (PEG), conjugated to anticancer drugs can considerably affect the apoptosis signaling pathways [9,10]. However, the effects of polymer excipients with the drugs that are not covalently bound to a polymer have not been reported to the best of our knowledge.

It is significant in the view of the present consideration that the group of genes responsible for apoptosis plays an important role in chemoresistance in cancer cells attributed to a “disabled apoptotic program”. In particular, apoptosis can be restricted in MDR cells by the elevated level of an antiapoptotic gene, *BCL2*, related to the expression of BCL2 protein [11,12]. Moreover, reduced expression of a proapoptotic gene, *P53*, also results in chemoresistance in more than 50% of all human malignancies and clinically has been correlated with the worse prognosis for patients with several types of cancers [11,13,14]. Finally, the intimate relationship between the Pgp overexpression and inhibition of multiple forms of caspase-dependent apoptosis was shown in MDR cells [15–17]. It appears that inhibition of Pgp by chemosensitizing agents can restore the normal apoptotic cascade in the cells with defective signaling pathways. Therefore, ability of Pluronics to enhance proapoptotic signaling in MDR cells demonstrated in the present study is of considerable interest for the potential application of these formulations in clinical therapy of drug resistant tumors. The present study elucidated the effects of Dox formulated with Pluronic P85 (P85) on apoptosis in two different MDR cell lines overexpressing Pgp, human epithelial cell line, KBv, and human breast carcinoma cell line, MCF7/ADR.

2. Materials and methods

2.1. Chemicals and preparation of pluronic solutions

Dox was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Vinblastine was purchased from Faulding Pharmaceutical Co. (Elizabeth NJ, USA). All other reagents were either analytical, or cell culture grade and were purchased from Sigma Chemical Co. (St. Louis, MO, USA). P85 (lot # WPOP-587A) was kindly provided by BASF Corp. (Parsippany, NJ, USA). Drug solutions with the block copolymer were prepared by addition of Dox to the culture medium containing various concentrations of P85.

2.2. Cells and culture conditions

The MDR subline of human epithelial cells, KBv, was derived by selecting of KB (ATCC CCL-17) cells with vinblastine. These cells were cultured in DMEM with 10% FBS, 10 mM HEPES, and penicillin/streptomycin supplemented with 1 Ag/ml vinblastine. The MDR

subline of human breast carcinoma cells, MCF-7/ADR, derived by selection of MCF-7 (ATCC HT-B22) with Dox were kindly presented by Y.L. Lee (William Beaumont Hospital, Royal Oak, MI, USA). These cells were cultured in the same media described above, except HEPES was increased to 1%. MCF7/ADR culture was supplemented with the 10 ng/ml Dox. The cells were shown to overexpress Pgp as reported earlier [2].

2.3. Apoptosis studies with annexin V and tunel methods

KBv cell monolayers were grown in flasks T-75 (Fisher Scientific, Pittsburgh, PA, USA) until confluence. On the day of treatment, the medium was removed and replaced with Dox in the absence or presence of P85 (the concentrations of Dox (1 µg/ml) and P85 (1%) resulting in 50% survival in Dox-P85 treated cells and 100% survival in drug-free treated cells were used). The cells were exposed to these solutions for 2 h, then washed two times with ice-cold PBS, and incubated in P85-free and drug-free DMEM for another 24 h. Translocation of phosphatidylserine to the outer layer of the plasma membrane was detected by Annexin V based flow cytometry [18]. For this purpose the cells ($1 \times 10^6/100 \mu\text{l}$) were stained with 10 µl of Annexin V solution (#1828681, Roche Diagnostics, Basel, Switzerland), and 10 µl propidium iodide (50 µg/ml solution in PBS) for 15 min. Immediately after that, the cells were supplemented with 0.4 ml of assay buffer (HEPES 10 mM, NaCl 140 mM, CaCl_2 5 mM, pH 7.4) and analyzed by a flow cytometry. Fluorescent histograms were recorded with a FAC-StarPlus flow cytometer (Becton Dickinson) operating under Lysis II.

Later stages of apoptosis involving DNA fragmentation were determined using terminal deoxynucleotidyl transferase mediated fluorescein nick end labeling method ("TUNEL") [19]. Following treatment with Dox and P85 (as described above) and incubation for 24 h in P85-free and drug-free media, the cells ($1 \times 10^6/100 \mu\text{l}$) were washed with ice-cold PBS, fixed in paraformaldehyde solution (4% in PBS, pH 7.4), and permeabilized with 100 µl of solution containing 0.1% Triton X-100 and 0.1% sodium citrate for 2 min on ice. After that, the cells were stained with TUNEL reaction mixture (#1684795, Roche Diagnostics, Basel, Switzerland) for 1 h at 37 °C according to manufacturer's protocol and analyzed by the flow cytometry [19]. The cells stained with the labeling solution in the absence of deoxynucleotidyl transferase were used as a negative control.

2.4. Expression of genes related to apoptosis

MCF7/ADR cells were grown in flasks and incubated with the media, 1% P85, 1 µg/ml Dox, and 1 µg/ml Dox in 1% P85 for 2 h. After the treatment, the cells were incubated in P85-free and drug-free media for another 24, 48, and 72 h. Then, the Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) was used for the analysis of gene expression in the cells. For this purpose, the total cellular RNA was isolated using an RNeasy kit (Qiagen, Valencia, CA, USA). First-strand cDNA was synthesized by Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences, Piscataway, NJ, USA) with 1 µg of the total cellular RNA (from 1×10^7 cells) and 100 ng of random hexadeoxynucleotide primer (Amersham Biosciences, Piscataway, NJ, USA). After synthesis, the reaction mixture was immediately subjected to PCR, which was carried out using GenAmp PCR System 2400 (Perkin Elmer, Shelton, CT, USA). The pairs of primers (shown in Table 1) used to amplify each type of cDNA and the PCR regimes have been previously described [20,21]. PCR regimen was: 94 °C/4 min, 55 °C/1 min, 72 °C/1 min for 1 cycle; 94 °C/1 min, 55 °C/50 s, 72 °C/1 min for 28 cycles, 60 °C for 10 min. PCR products were separated in 4% NuSieve 3:1 Reliant® agarose gels (BMA, Rockland, ME, USA) in $1 \times$ TBE buffer (0.089 M Tris/Borate, 0.002 M EDTA, pH 8.3; Research Organics Inc., Cleveland, OH, USA) by the submarine electrophoresis. The gels were stained with ethidium bromide, digitally photographed, and scanned using Gel Documentation System 920 (NucleoTech, San Mateo, CA, USA). To correct for loading differences, the gene expression was calculated as a ratio of mean band density of the analyzed RT-PCR product to

that of the internal standard (β_2 -microglobulin, β_2 -m). The expression of β_2 -microglobulin did not change in different experimental series.

2.5. Localization of P85 by fluorescent microscopy

For this study, P85 was labeled with fluorescein-5-isothiocyanate (FITC) as described previously [22]. MCF7/ADR cells grown on chamber slides (Fisher, St. Louis, MO, USA) were incubated with 0.1% P85-FITC in assay buffer for 2 h at 37 °C. After this period, cells were treated for 10 min with a staining solution containing 100 nM of the mitochondrial dye, MitoTracker-Red (Molecular Probes, Inc., Eugene, OR, USA). Then, the loading solution was removed, the cell monolayers were washed three times with ice-cold PBS containing 1% bovine serum albumin, and examined by confocal laser microscope ACAS-570 Meridian Instruments (Okimos, MI, USA).

2.6. Statistical analysis

Data obtained were analyzed using descriptive statistics, single factor analysis of variance (ANOVA) and presented as mean value $\pm$ SD from the four independent measurements.

3. Results

3.1. Effects of P85 on drug-induced apoptosis by annexin V and tunel methods

The earlier stages of apoptosis in KBv cells treated with Dox alone and Dox in combination with P85 were detected by Annexin V assay (Fig. 1A). The induction of apoptosis in the cells treated with Dox/P85 formulation was significantly more pronounced than in those treated with free Dox, almost two times ($40.7 \pm 3\%$ vs. $21.6 \pm 1.7\%$, $p < 0.05$). The similar result was obtained by TUNEL method that detects later stages of apoptosis (Fig. 1B); a considerable increase in the number of apoptotic and decrease in living cells followed by the treatment with Dox formulated with P85 was found compared to the cells treated with Dox alone.

3.2. Effect of P85 on drug-induced expression of antiapoptotic genes

Expression of two genes related to antiapoptotic defense, *BCL2* and *BCLXL*, was examined in MCF7/ADR cells incubated with the medium (control), P85 alone, Dox alone, and Dox formulated with P85 (Fig. 2). Three separate groups represent the cells incubated with P85-free and Dox-free medium for 24, 48, and 72 h followed by the treatment with solutions described above. As is seen in the figure, incubation of the cells with P85 alone did not affect the expression levels of these genes (bars # 2, 6, and 10) for all times of post-incubation in P85-free and Dox-free medium. The treatment with Dox alone caused statistically significant activation of an antiapoptotic cellular defense by the overexpressing *BCL2* and *BCLXL* (bars # 3, 7, and 11). Contrary to the above, treatment of the cells with Dox formulated with P85 (bars # 4, 8, and 12) considerably decreased the expression levels of the *BCL2* and *BCLXL* genes (bars # 4, 8, and 12) at all examined times of post-incubation.

3.3. Effect of P85 on drug-induced expression of proapoptotic genes

The effect of P85 on the Dox-induced expression of genes involved into the cellular proapoptotic signal transduction, *BAX*, *P53*, *APAF1*, *Caspase 9* and *Caspase 3* (Fig. 3–Fig. 5A) was studied in MCF7/ADR cells. The obtained data indicate that P85 alone (bars #2 in Fig. 3–Fig. 5A) does not affect the expression of proapoptotic genes following 2-h treatment and 24-h incubation in P85-free media (except significant increases in Caspase 3 levels). However, post-treatment incubation for longer time periods (48 and 72 h) resulted in the increased expression levels for almost the all studied proapoptotic genes (bars # 6 and 10 in Fig. 3–Fig. 5A) compared with the control non-treated cells. Further, the incubation of the cells with Dox alone led to the profound up-regulation of all proapoptotic genes (bars # 3, 7, and 11

in Fig. 3–Fig. 5A) for the all post-incubation periods in copolymer-free and drug-free media. Remarkable, that incubation of the cells with Dox/P85 formulation caused further increases in the expression of proapoptotic genes (bars # 4, 8, and 12 in Fig. 3–Fig. 5A). Overall, all the examined proapoptotic genes were considerably up-regulated following the treatment with Dox/P85 formulation signifying the greater induction of apoptosis compared with the cells treated with Dox alone.

3.4. Intracellular localization of P85

The distribution of P85 in MCF7/ADR cells was examined using confocal microscopy. Fig. 6 presents the fluorescence microphotographs of MCF7/ADR, treated with FITC-P85 (0.1%) for 2 h. In the same experiment the cells were stained with a mitochondrial dye, MitoTracker-Red to visualize localization of mitochondria. Fig. 6A shows the fluorescence of FITC-P85. This data suggest that the block copolymer is internalized within MCF7/ADR cells. Fig. 6B shows the fluorescence of MitoTracker Red dye. Co-localization of the two staining in yellow color is shown on Fig. 6C, indicating practically identical intracellular localization of FITC-P85 and Mito-Tracker Red. Therefore, the block copolymer spreads throughout the cell, where it may interact with various intracellular targets, including the same organelles where the MitoTracker Red is accumulated.

4. Discussion

Pluronic block copolymer P85 significantly enhances therapeutic activity of anticancer agents in MDR cancer cells affecting various mechanisms of resistance. First, P85 causes the inhibition of the drug efflux transporter, Pgp, and the increase in intracellular accumulation levels of drugs, Pgp substrates [23]. Second, P85 significantly inhibits GSH/GST detoxification system [7] that plays an important role in drug resistance, especially in the human breast cancer cells [24]. Finally, P85 abolishes the drug sequestration within cytoplasmic vesicles facilitating the drug release to the cytoplasm and its accumulation in the nucleus [1]. Nevertheless, this list of P85 effects displayed in MDR cells is not complete. Data obtained in the present work indicate that, at least, one more effect, specifically, alterations in drug-induced apoptosis is responsible for P85 sensitization effects in MDR cells. Thus, formulation of Dox with P85 greatly enhanced drug-induced apoptosis in MDR cells compared with Dox alone, as was shown by Annexin V and TUNEL methods.

More detailed information obtained by RT-PCR indicated that addition of P85 to Dox altered the expression of genes related to apoptosis in resistant cancer cells. In particular, a significant *downregulation* of the intracellular levels of antiapoptotic genes, *BCL2* and *BCLXL*, which are known to prevent programmed cell death, was found in the cells treated with Dox/P85 formulation. In contrast, treatment of the cells with Dox alone increased intracellular levels of *BCL2* and *BCLXL* inhibiting the antiapoptotic defense in the resistant cells, which is in agreement with the earlier report [25]. This effect is of great importance due to the fact that down-regulation of these genes is associated with better therapeutic outcome, specifically, in breast cancer patients [26,27]. Moreover, overexpression of BCL2 protein in cancer cells confers resistance to a broad spectrum of anticancer drugs and associated with a poor prognosis and low response to chemotherapy in many human cancers [11,28–30]. Therefore, the inhibition effect of Dox/P85 formulation on the expression of antiapoptotic genes in human breast cancer cells may have a potential therapeutic significance.

Formulation of Dox with P85 altered the expression levels of the proapoptotic genes (*BAX*, *P53*, *APAF1*, *Caspase 9* and *Caspase 3*) in MDR cells. Although the treatment of the cells with Dox alone elevated the expression levels of these genes, addition of P85 to the drug amplified further the proapoptotic activation. Thus, incubation of MCF7/ADR cells with Dox/P85 increased central cell death signal by overexpressing of *P53* mRNA, as well as an apoptotic

protease activating factor APAF1 in a time-dependent manner. The latter activates a caspase-dependent pathway of apoptosis by overexpressing an apoptosis initiator, Caspase 9, and an apoptosis executor, Caspase 3. Remarkably, P85 alone also showed proapoptotic effect, although, at lesser extent compared with the Dox/P85 formulation.

The overall effect of P85 on apoptosis in MDR cancer cells can be summarized in a *BAX/BCL2* ratio of the proapoptotic activity gene (*BAX*) and the antiapoptotic cellular defense (*BCL2*), which reflects the activation of a proapoptotic mitochondria-derived signal. As is seen in Fig. 3B treatment of the cells with Dox alone simultaneously activates the proapoptotic signal and antiapoptotic cellular defense. As a result, the apoptosis induction by Dox (reflected in *BAX/BCL2* ratio) is substantially limited. In contrast, the *BAX/BCL2* ratio was considerably increased in the cells treated with the Dox/P85 formulations (Fig. 3B, bars # 4, 8, 12), which is likely to be related to the strong cytotoxic response of the resistant cells to the formulation. Noteworthy, the treatment with P85 alone also enhanced the *BAX/BCL2* ratio, although to a lesser extent. Overall, this study suggested that incubation of the resistant cells with Dox/P85 formulation enhanced the proapoptotic activity of the drug and prevents the activation of the anti-apoptotic cellular defense.

It has been demonstrated earlier that synthetic polymers conjugated to anticancer drugs can considerably affect the apoptosis signaling pathways in sensitive and resistant human ovarian carcinoma cell lines, A2780 and A2780/AD [9,10]. Specifically, conjugation of Dox with poly (2-hydroxypropylmethacrylate) (PHPMA) induced apoptosis by up-regulating *Caspases* 3,6,7,8, and 9, while the free drug caused induction of only three *Caspases* 9,3, and 7 in drug resistant A2780/AD cells [9]. More-over, the *BCL2* gene responsible for the cellular defense mechanism was down-regulated following treatment of the cells with PHPMA–Dox conjugate, resulting in the further stimulation of the proapoptotic route. Conversely, free Dox increased expression of *BCL2* gene, which partially mitigated its cytotoxic activity. Remarkably, the overall pattern of the effects demonstrated for the copolymer–drug conjugate [9] resembles those for the copolymer that are not covalently bound to the drug (reported in this work); both polymer drug formulations cause further activation of proapoptotic signals and down-regulation of antiapoptotic defense compared with the free drug.

It is well documented that mitochondria play a crucial role in regulating apoptosis in vertebrates by releasing cytochrome *c* [31–33]. This release is partly regulated by several pro- and antiapoptotic proteins, positioned in the outer mitochondrial membrane [34]. Majority of proapoptotic stimuli, including anticancer drugs, require a mitochondrion-dependent step that involves permeabilization of the outer mitochondrial membrane and the release of mitochondrial proteins normally located in the intermembrane space [35–38]. In this respect, indications that P85 can reach and affect mitochondria in the cancer cells become crucial. Co-localization of FITC-labeled P85 and Mitotracker Red staining mitochondria in MCF7/ADR cells demonstrated in this study supports this statement. Direct indication of P85 effect on mitochondria activity was shown in our earlier papers [2,39], where a significant ATP depletion caused by P85 in the drug resistant cancer cells was reported. The unpublished data related to the inhibition of respiration in these cells by P85 reinforce this statement (in collaboration with N. Rapoport, University of Utah). It suggests that P85 molecules incorporate into mitochondria membranes and interrupt the essential processes, including respiration, ATP synthesis, and release of antiapoptotic and proapoptotic proteins.

Based on the in vivo efficacy evaluation the formulation of doxorubicin with Pluronic (“SP1049C”) was selected for clinical development. Open labeled two-site Phase I clinical trial of SP1049C has been completed [5]. Based on this trial results the dose-limiting toxicity of SP1049C was myelosuppression reached at 90 mg/m² (maximum tolerated dose was 70 mg/m²). Evidence of antitumour activity was seen in some patients with advanced resistant solid

tumours. The phase II clinical trial of this formulation to treat advanced oesophageal adenocarcinoma is currently being conducted [40]. The reported data suggest that SP1049C appears to be active based on the preliminary results of the first 10 patients and the study will continue accrual to a total of 24 available patients. Overall, Pluronic block copolymers represent a potent chemosensitizer of MDR cancer cells displaying complex effects in drug resistant cells that are currently under investigation.

Acknowledgments

This study was supported by National Institutes of Health grants CA89225. The authors thank Charles Kuszynski for the assistance with flow cytometry studies, and Janice Taylor of the Confocal Laser Scanning Microscope Core Facility at the UNMC for the assistance with confocal microscopy.

References

1. Venne A, Li S, Mandeville R, Kabanov A, Alakhov V. Hypersensitizing effect of pluronic L61 on cytotoxic activity, transport, and subcellular distribution of doxorubicin in multiple drug-resistant cells. *Cancer Res* 1996;56:3626–3629. [PubMed: 8705995]
2. Batrakova EV, Li S, Elmquist WF, Miller DW, Alakhov VY, Kabanov AV. Mechanism of sensitization of MDR cancer cells by Pluronic block copolymers: selective energy depletion. *Br. J. Cancer* 2001;85:1987–1997. [PubMed: 11747344]
3. Kabanov A, Batrakova E, Alakhov V. Pluronic block copolymers for overcoming drug resistance in cancer. *Adv. Drug Deliv. Rev* 2002;54:759–779. [PubMed: 12204601]
4. Batrakova E, Li S, Alakhov V, Miller D, Kabanov A. Optimal structure requirements for pluronic block copolymers in modifying P-glycoprotein drug efflux transporter activity in bovine brain microvessel endothelial cells. *J. Pharmacol. Exp. Ther* 2003;304:845–854. [PubMed: 12538842]
5. Danson S, Ferry D, Alakhov V, Margison J, Kerr D, Jowle D, Brampton M, Halbert G, Ranson M. Phase I dose escalation and pharmacokinetic study of pluronic polymer-bound doxorubicin (SP1049C) in patients with advanced cancer. *Br. J. Cancer* 2004;90:2085–2091. [PubMed: 15150584]
6. Batrakova E, Li S, Vinogradov S, Alakhov V, Miller D, Kabanov A. Mechanism of pluronic effect on P-glycoprotein efflux system in blood–brain barrier: contributions of energy depletion and membrane fluidization. *J. Pharmacol. Exp. Ther* 2001;299:483–493. [PubMed: 11602658]
7. Batrakova EV, Li S, Alakhov VY, Elmquist WF, Miller DW, Kabanov AV. Sensitization of cells overexpressing multidrug-resistant proteins by pluronic P85. *Pharm. Res* 2003;20:1581–1590. [PubMed: 14620511]
8. Kabanov AV, Batrakova EV, Sriadibhatla S, Yang Z, Kelly DL, Alakov VY. Polymer genomics: shifting the gene and drug delivery paradigms. *J. Control. Release* 2005;101:259–271. [PubMed: 15588910]
9. Minko T, Kopeckova P, Kopecek J. Preliminary evaluation of caspases-dependent apoptosis signaling pathways of free and HPMa copolymer-bound doxorubicin in human ovarian carcinoma cells. *J. Control Release* 2001;71:227–237. [PubMed: 11295216]
10. Dharap SS, Qiu B, Williams GC, Sinko P, Stein S, Minko T. Molecular targeting of drug delivery systems to ovarian cancer by BH3 and LHRH peptides. *J. Control Release* 2003;91:61–73. [PubMed: 12932638]
11. Reed JC. Bcl-2: prevention of apoptosis as a mechanism of drug resistance. *Hematol./Oncol. Clin. North Am* 1995;9:451–473.
12. Tsujimoto Y, Cossman J, Jaffe E, Croce CM. Involvement of the bcl-2 gene in human follicular lymphoma. *Science* 1985;228:1440–1443. [PubMed: 3874430]
13. De Benedetti V, Bennett WP, Greenblatt MS, Harris CC. p53 tumor suppressor gene: implications for iatrogenic cancer and cancer therapy. *Med. Pediatr. Oncol. Suppl* 1996;1:2–11. [PubMed: 8643042]
14. Linn SC, Honkoop AH, Hoekman K, van der Valk P, Pinedo HM, Giaccone G. p53 and P-glycoprotein are often co-expressed and are associated with poor prognosis in breast cancer. *Br. J. Cancer* 1996;74:63–68. [PubMed: 8679460]

15. Smyth MJ, Krasovskis E, Sutton VR, Johnstone RW. The drug efflux protein, P-glycoprotein, additionally protects drug-resistant tumor cells from multiple forms of caspase-dependent apoptosis. *Proc. Natl. Acad. Sci. U. S. A* 1998;95:7024–7029. [PubMed: 9618532]
16. Johnstone RW, Cretney E, Smyth MJ. P-glycoprotein protects leukemia cells against caspase-dependent, but not caspase-independent, cell death. *Blood* 1999;93:1075–1085. [PubMed: 9920858]
17. Robinson LJ, Roberts WK, Ling TT, Lamming D, Sternberg SS, Roepe PD. Human MDR 1 protein overexpression delays the apoptotic cascade in Chinese hamster ovary fibroblasts. *Biochemistry* 1997;36:11169–11178. [PubMed: 9287159]
18. Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin V. *J. Immunol. Methods* 1995;184:39–51. [PubMed: 7622868]
19. Gorczyca W, Bigman K, Mittelman A, Ahmed T, Gong J, Melamed MR, Darzynkiewicz Z. Induction of DNA strand breaks associated with apoptosis during treatment of leukemia. *Leukemia* 1993;7:659–670. [PubMed: 8483318]
20. Minko T, Kopeckova P, Kopecek J. Efficacy of the chemo-therapeutic action of HPMA copolymer-bound doxorubicin in a solid tumor model of ovarian carcinoma. *Int. J. Cancer* 2000;86:108–117. [PubMed: 10728603]
21. Minko T, Dharap SS, Fabbriatore AT. Enhancing the efficacy of chemotherapeutic drugs by the suppression of antiapoptotic cellular defense. *Cancer Detec. Prev* 2003;27:193–202. [PubMed: 12787726]
22. Kabanov A, Batrakova E, Melik-Nubarov N, Fedoseev N, Dorodnich T, Alakhov V, Chekhonin V, Nazarova I, Kabanov V. A new class of drug carriers: micelles of poly (oxyethylene)-poly (oxupropylene) block copolymers as micro-containers for drug targeting from blood in brain. *J. Control. Release* 1992;22:141–158.
23. Alakhov V, Moskaleva E, Batrakova E, Kabanov A. Hypersensitization of multidrug resistant human ovarian carcinoma cells by pluronic P85 block copolymer. *Bioconjug. Chem* 1996;7:209–216. [PubMed: 8983343]
24. Su F, Hu X, Jia W, Gong C, Song E, Hamar P. Glutathione S transferase pi indicates chemotherapy resistance in breast cancer. *J. Surg. Res* 2003;113:102–108. [PubMed: 12943817]
25. Knowlton K, Mancini M, Creason S, Morales C, Hockenbery D, Anderson BO. Bcl-2 slows in vitro breast cancer growth despite its antiapoptotic effect. *J. Surg. Res* 1998;76:22–26. [PubMed: 9695733]
26. Joensuu H, Pylkkanen L, Toikkanen S. Bcl-2 protein expression and long-term survival in breast cancer. *Am. J. Pathol* 1994;145:1191–1198. [PubMed: 7977649]
27. Gasparini G, Barbareschi M, Doglioni C, Palma PD, Mauri FA, Boracchi P, Bevilacqua P, Caffo O, Morelli L, Verderio P, et al. Expression of bcl-2 protein predicts efficacy of adjuvant treatments in operable node-positive breast cancer. *Clin. Cancer Res* 1995;1:189–198. [PubMed: 9815973]
28. Buchholz TA, Davis DW, McConkey DJ, Symmans WF, Valero V, Jhingran A, Tucker SL, Pusztai L, Cristofanilli M, Esteva FJ, Hortobagyi GN, Sahin AA. Chemotherapy-induced apoptosis and Bcl-2 levels correlate with breast cancer response to chemotherapy. *Cancer J* 2003;9:33–41. [PubMed: 12602766]
29. Campos L, Rouault JP, Sabido O, Oriol P, Roubi N, Vasselon C, Archimbaud E, Magaud JP, Guyotat D. High expression of bcl-2 protein in acute myeloid leukemia cells is associated with poor response to chemotherapy. *Blood* 1993;81:3091–3096. [PubMed: 7684624]
30. Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. *Genes Dev* 1999;13:1899–1911. [PubMed: 10444588]
31. Duke RC, Ojcius DM, Young JD. Cell suicide in health and disease. *Sci. Am* 1996;275:80–87. [PubMed: 8923763]
32. Green DR, Reed JC. Mitochondria and apoptosis. *Science* 1998;281:1309–1312. [PubMed: 9721092]
33. Lee HC, Wei YH. Mitochondrial role in life and death of the cell. *J. Biomed. Sci* 2000;7:2–15. [PubMed: 10644884]
34. Susin SA, Zamzami N, Kroemer G. Mitochondria as regulators of apoptosis: doubt no more. *Biochim. Biophys. Acta* 1998;1366:151–165. [PubMed: 9714783]

35. Petit PX, Lecoœur H, Zorn E, Dauguet C, Mignotte B, Gougeon ML. Alterations in mitochondrial structure and function are early events of dexamethasone-induced thymocyte apoptosis. *J. Cell Biol* 1995;130:157–167. [PubMed: 7790370]
36. Petit PX, Zamzami N, Vayssière JL, Mignotte B, Kroemer G, Castedo M. Implication of mitochondria in apoptosis. *Mol. Cell. Biochem* 1997;174:185–188. [PubMed: 9309685]
37. Zamzami N, Marchetti P, Castedo M, Zanin C, Vayssière JL, Petit PX, Kroemer G. Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo. *J. Exp. Med* 1995;181:1661–1672. [PubMed: 7722446]
38. Krajewski S, Tanaka S, Takayama S, Schibler MJ, Fenton W, Reed JC. Investigation of the subcellular distribution of the bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. *Cancer Res* 1993;53:4701–4714. [PubMed: 8402648]
39. Batrakova E, Li S, Alakhov V, Kabanov A. Selective energy depletion and sensitization of multiple drug resistant cancer cells by pluronic block copolymers. *Polym. Prepr. (Am. Chem. Soc., Div. Polym. Chem.)* 2000;41:1639–1640.
40. Valle JW, Lawrance J, Brewer J, Clayton A, Corrie P, Alakhov V, Ranson M. A phase II, window study of SP1049C as first-line therapy in inoperable metastatic adenocarcinoma of the oesophagus. *J. Clin. Oncol* 2004;22:4195.(ASCO Annual Meeting Proceedings (Post-Meeting Edition))

Fig. 1.

The effect of Dox formulated with Pluronic on apoptosis in KBv cells. The cells were treated with 1 $\mu\text{g/ml}$ Dox with or without 1% P85 for 2 h, and then incubated in the copolymer-free and drug-free media for 24 h. Percentages of living cells (grey bars), apoptotic cells (black bars), and dead cells (white bars) were recorded on the early and late stages of apoptosis in the cells analyzed with Annexin V (A) and TUNEL (B) methods based on the flow cytometry analysis. Statistical significance of the Dox/P85 effect compared to the free Dox is shown in (*), $p < 0.05$; and (**), $p < 0.005$.

Fig. 2.

The typical images of the gel electrophoresis of RT-PCR products and expression of genes (% of internal standard, β_2 -microglobulin) encoding BCL2 and BCLXL proteins in multidrug resistant human breast (MCF-7/ADR) cancer cells treated with: the media (1, 5, 9); 1% P85 (2, 6, 10); 1 μ g/ml Dox (3, 7, 11); and 1 μ g/ml Dox in 1% P85 (4, 8, 12) for 2 h. Following the treatment the cells were incubated in the copolymer-free and drug-free media for 24, 48 or 72 h. Means $\pm$ SD from four independent measurements are shown. Statistical significance of the Dox effect compared to the free-Dox media is shown in (*), $p < 0.005$; and the Dox/P85 effect compared to the Dox is shown in (†), $p < 0.005$.

Fig. 3.

The typical images of gel electrophoresis of RT-PCR products and expression of gene (% of internal standard, β_2 -microglobulin) encoding BAX protein (A), and the ratio of BAX/BCL2 expression (B) in multidrug resistant human breast (MCF-7/ADR) cancer cells treated with: the media (1, 5, 9); 1% P85 (2, 6, 10); 1 μ g/ml Dox (3, 7, 11); and 1 μ g/ml Dox in 1% P85 (4, 8, 12) for 2 h. Following treatment the cells were incubated in the copolymer-free and drug-free media for 24, 48 or 72 h. Means $\pm$ SD from four independent measurements are shown. Statistical significance of the Dox effect compared to the free-Dox media is shown in (*), $p < 0.005$; and the Dox/P85 effect compared to the Dox is shown in (†), $p < 0.005$.

Fig. 4.

The typical images of gel electrophoresis of RT-PCR products and expression of genes (percentage of internal standard, β_2 -microglobulin) encoding P53 and APAF1 proteins in multidrug resistant human breast (MCF-7/ADR) cancer cells treated with: the media (1, 5, 9); 1% P85 (2, 6, 10); 1 μ g/ml Dox (3, 7, 11); and 1 μ g/ml Dox in 1% P85 (4, 8, 12) for 2 h. Following treatment the cells were incubated in the copolymer-free and drug-free media for 24, 48 or 72 h. Means $\pm$ SD from four independent measurements are shown. Statistical significance of the Doxalone or P85 alone effect compared to the free-Dox media is shown in (*), $p < 0.005$; and the Dox/P85 effect compared to the Dox is shown in (†), $p < 0.005$.

Fig. 5.

The typical images of gel electrophoresis of RT-PCR products and expression of genes (percentage of internal standard, β_2 -microglobulin) encoding Caspases 9 and 3 in multidrug resistant human breast (MCF-7/ADR) cancer cells treated with: the media (1, 5, 9); 1% P85 (2, 6, 10); 1 μ g/ml Dox (3, 7, 11); and 1 μ g/ml Dox in 1% P85 (4, 8, 12) for 2 h. Following treatment the cells were incubated in the copolymer-free and drug-free media for 24, 48 or 72 h. Means $\pm$ SD from four independent measurements are shown. Statistical significance of the Dox effect compared to the free-Dox media is shown in (*), $p < 0.005$; and the Dox/P85 effect compared to the Dox is shown in (†), $p < 0.005$.

Fig. 6. The confocal microscopy images showing the intracellular localization of (A) FITC-P85, and (B) Mitotracker-Red in MCF7/ADR cells. The cells were exposed to 0.1% of FITC-P85 for 2 h, washed with PBS, and stained with 100 nM MitoTracker-Red. A co-localization of FITC-P85 and mitochondria is shown on (C).

Table 1

The list of primers used in RT-PCR

Genes	Primers (5' to 3')	
	Sense primer	Antisense primer
<i>BCL2</i>	ggattgtggccttcttgag	ccaaactgagcagagtcttc
<i>BCLXL</i>	atgaactcttcgggatgg	tggatccaaggctctagggtg
<i>BAX</i>	tttcttcagggtttcatcc	gccactcggaaaaagacctc
<i>P53</i>	gaagaccagggtccagatga	ggtaggttttctgggaaggg
<i>APAF1</i>	gggtttcagttgggaacaa	caccaagagtcccaaacat
<i>Caspase 3</i>	tggaattgatcgtgatgtt	ggcaggcctgaataatgaaa
<i>Caspase 9</i>	tgactgccaagaaatgggtg	cagctggtcccattgaagat
β_2 -m	acccccactgaaaaagatga	atcttcaaacctccatgatg
(Internal standard)		