Intracellular Uptake and Trafficking of Pluronic Micelles in Drug-Sensitive and MDR Cells: Effect on the Intracellular Drug Localization

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ABSTRACT: The intracellular uptake and localization of a fluorescently labeled Pluronic P-105 in HL-60 leukemia cells and in A2780 drug-sensitive and A2780/ADR MDR ovarian carcinoma cells were characterized by flow cytometry and fluorescence microscopy. Pluronic P-105 molecules were labeled with a pH-sensitive fluorescent label, 5-(and 6-)carboxy-2′7′-dichlorofluorescein. The fluorescence intensity of labeled Pluronic was about twofold higher at pH 7.4 than at pH 5.5. At Pluronic concentrations exceeding the critical micelle concentration (cmc), flow cytometry histograms manifested bimodal distribution of cell fluorescence for all types of cells. Cell population characterized by higher fluorescence intensity presumably resulted from Pluronic transfer from the acidic environment of cytoplasmic vesicles (endosomes or lysosomes) into the neutral environment of the cytoplasm and cell nuclei, which suggested the permeabilization of the membranes of acidic vesicle by Pluronic molecules. For the MDR cells, the bimodal distribution of cell fluorescence was already observed at very low Pluronic concentrations in the incubation medium (i.e., below the cmc). The data suggest that the membranes of acidic vesicles of MDR cells are more susceptible to the action of polymeric surfactants than those of drug-sensitive cells. Permeabilization of acidic vesicles had a dramatic effect on the intracellular trafficking of drugs: when delivered in PBS, the anthracyclin drug ruboxyl (Rb) sequestered in cytoplasmic vesicles and was excluded from cell nuclei; however, when delivered in Pluronic micelles, drug accumulated in cell nuclei. Drug uptake from/with Pluronic micelles was substantially enhanced by ultrasound. These findings suggest that the nuclear accumulation of drugs internalized via fluid-phase endocytosis can be enhanced by the application of Pluronic micelles and can be further augmented by ultrasonic irradiation. J Pharm Sci 91:157–170, 2002

Keywords: endosomal release; intracellular trafficking; Pluronic micelles; MDR cells; drug-sensitive cells; HL-60 cells; A2780 cells; A2780/ADR cells

INTRODUCTION

Polymeric micelles have been suggested as drug carriers because of a number of advantageous properties that include (1) an appropriate size to escape renal excretion, allowing however for the extravasation at the tumor site; (2) an enhanced penetration and retention (EPR) effect that provides for a selective accumulation of micellar-encapsulated drugs in solid tumors;[1–3] (3) a shielding effect that diminishes drug interaction with healthy tissues;[4–12] (4) the opportunity to release drug from micelles in a spatially and

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temporally controlled manner by ultrasonic irradiation;[4,5,7–13] (5) enhanced intracellular uptake of a micellar-encapsulated drug under the action of ultrasound;[11–13] and (6) the uptake of micellar-encapsulated drugs via a fluid-phase endocytosis rather than a passive diffusion. The latter factor is important for overcoming drug resistance, which is a significant problem in cancer chemotherapy. Drug resistance and multidrug resistance (MDR) of the cells are attributed to the work of the ATP-dependent efflux pumps localized in the cytoplasmic membranes. Drugs internalized by endocytosis bypass efflux pumps;[14,15] however, endocytosed drugs are sequestered in the cytoplasmic acidic vesicles, which create a second barrier on the way of the drug toward its target. For anticancer drugs, the targets are commonly located outside of the cytoplasmic acidic vesicles; in particular, for anthracyclin drugs, the DNA target is localized in cell nuclei.

Positively charged drugs usually end up in acidic vesicles; this phenomenon is especially pronounced in MDR cells because of the high pH gradient between these vesicles and the cytosol.[16,17] It was reported that ionoforic compounds that reduced the pH gradient between acidic vesicles and cytosol reversed the multidrug resistance of MDR cells.[16] Enhancing drug release from the acidic vesicles is an important task pertinent not only to drug delivery but also to efficient gene delivery.[18–20]

It was recently reported by Kabanov and Alakhov's group that doxorubicin (DOX) formulated with unimeric concentrations of Pluronic L61 copolymer not only effectively penetrated plasma membranes of drug-resistant cells but also avoided sequestration in acidic vesicles.[14,21] By using acridine orange as a fluorescent probe, these authors showed that Pluronic L61 decreased the number of the cytoplasmic vesicles characterized by a high pH gradient with cytosol,[21] which resulted in the hypersensitization of drug-resistant cells.[22–25] Pluronic (or Poloxamer) copolymers are triblock copolymers of poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO), often denoted PEO-PPO-PEO. The fundamental relationship between the composition of Pluronic block copolymers and their hypersensitization effect in MDR cancer cells was recently established by Kabanov et al.[25] Importantly, Pluronic copolymers have a relatively low *in vivo* toxicity (see, e.g., ref. 21).

The aggregation state of Pluronic copolymers can be controlled by choosing the copolymer with the appropriate molecular weight and PPO/PEO block-length ratio, and by adjusting the concentration.[25–27] There is extensive information in the literature on the structure and properties of Pluronic micelles, including a comprehensive review by Alexandridis et al.[27]

In our work, we use Pluronic copolymers at concentrations that are above the critical micelle concentration (cmc),[4–13] which results in self-assembly of Pluronic molecules into dense micelles; the core-forming block in aqueous solutions is PPO, whereas PEO blocks form micelle coronas. The hydrodynamic radii of Pluronic micelles at physiological temperatures range between 10 and 20 nm.

The application of Pluronic micelles rather than unimers (i.e., individual molecules) has a number of advantages, the most important of which are (i) the possibility of drug targeting to tumors while avoiding damage to healthy tissues, and (ii) the chances of overcoming drug resistance by switching drug transport from passive diffusion to endocytosis. We use ultrasound to enhance the intracellular uptake of micellar-encapsulated drugs by tumor cells.

Important information on the Pluronic uptake and distribution in drug-sensitive and MDR cells is absent in the literature. In the present paper we report on the effect of the extracellular Pluronic concentration, which controls the aggregation state of Pluronics, on the intracellular uptake and distribution of Pluronic P-105 in drug-sensitive and MDR cells. For this study, Pluronic P-105 was fluorescently labeled with a pH-sensitive fluorescent label, and its uptake and distribution were studied by flow cytometry.

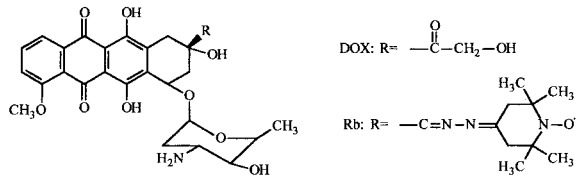
MATERIALS AND METHODS

Materials

Pluronic P-105, with an average molecular weight of 6500 and the number of monomeric units in PEO and PPO blocks being 37 and 56, respectively, was kindly supplied by the BASF Corporation (Mount Olive, NJ. Pluronic solution in phosphate-buffered saline (PBS) was used for the experimennts. Dulbecco's PBS, pH 7.4, was purchased from Sigma and used as received.

Anthracycline Drug

Two anticancer anthracycline drugs, doxorubicin (DOX) and ruboxyl (Rb), were used in this study.



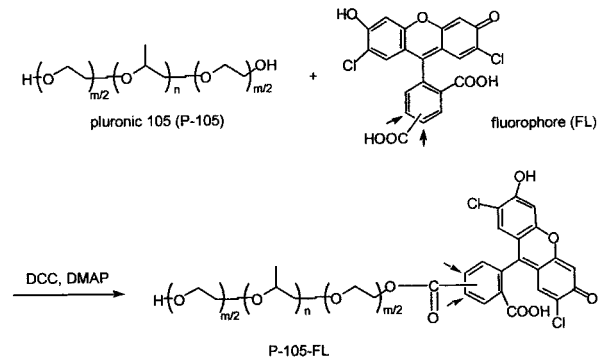
Rb, a paramagnetic analog of DOX, was synthesized by conjugating a paramagnetic Tempo-type nitroxyl radical (1-oxo-2,2,6,6-piperidone-4-hydrazone) to DOX (US Patent 4,332,934, 1982; see Scheme 1). This drug is presently in phase II of clinical trials in Russia. Rb was found to be effective against breast and colon carcinoma and bone sarcoma. Rb is an intercalating drug that stacks between paired bases in DNA. In this study, Rb was chosen because it is more lipophilic than DOX; within the cells, Rb partitions between DNA and phospholipid membranes.[7,11]. We were interested in the effect of Pluronic micelles on the intracellular distribution of the drug that not only intercalates DNA but is also partially localized in cell membranes.

Synthesis of a Fluorescently-Labeled Pluronic P105

Pluronic-105 (MW = 6500; 28.7 mg, which corresponds to 8.8 μmol of free OH group) was dissolved in 10 mL of dimethylformamide (DMF), and 3.94 mg (8.8 μmol) of 5-(and 6-)carboxy-2′7′-dichlorofluorescein mixed isomers (Molecular Probes, Inc., Eugene, OR) in 5 mL of DMF was added. Next, 18.3 mg of dicycohexylcarbodimide (8.8 μmol) and 2.2 mg of dimethylaminopyridine (1.7 μmol) were added in solid form. The reaction was stirred at room temperature for 3 days, then diluted with 100 mL of ice-cold water. The mixture was dialyzed against water for 7 days (3 changes/day), and the purity of fluorescently labeled Pluronic 105 was monitored by gel permeation chromatography (GPC). The product was lyophilized to obtain the dry powder of a fluorescently labeled Pluronic 105. The reaction is schematically shown in Scheme 2.

Cell Lines

HL-60 promyelocytic cells were kindly provided by Dr. B.K. Murrey (Department of Microbiology, Brigham Young University, Provo, UH). They were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.2% sodium bicarbonate, and



50 μg/mL gentamicin at 37 °C in humidified air containing 5% CO_2.

Drug-sensitive A2780 and multidrug resistant (MDR) ovarian carcinoma A2780/ADR cells growing in adherent monolayers were kindly provided by Dr. T.C. Hamilton (Fox Chase Cancer Center, PA). Cells were cultured in a complete RPMI 1640 medium, supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 μg/mL insulin, which in the case of A2780/ADR cells included 100 ng/mL DOX for maintaining resistance.

Cell Incubation With Fluorescently Labeled Pluronic

Cell were harvested, washed with PBS, and resuspended in a solution of a fluorescently labeled Pluronic; overall Pluronic concentration in a solution ranged from 0.0002 to 5 wt %.

Two sets of experiments were made. In the first set, HL-60 cells were incubated with progressively increasing concentrations of fluorescently labeled Pluronic. In the second set, the concentration of a fluorescently labeled Pluronic in the incubation medium remained constant at 10, 20 or 50 μg/mL, and nonlabeled Pluronic was added to the incubation medium at concentrations below and above the cmc of Pluronic P-105 (which is 0.004 wt % at 37°C). Following incubation with a Pluronic solution, cells were analyzed by flow cytometry and fluorescence microscopy. Pluronic uptake by the cells was quantitatively measured by fluorescence depletion from the incubation medium.

Sonication

Polystyrene test tubes (diameter, 10 mm) containing cell suspensions were positioned along the

circumference of a circular holder; they were placed in water bath thermostated at 37°C. Ultrasound at 20 kHz was generated by a microprobe transducer (tip diameter, 13 mm; Sonics and Materials, Newton, CT) positioned in the center of the sample holder at a distance of 3 cm from the samples. Power density was controlled by varying the amplitude settings of the instrument and was measured with a hydrophone (Bruel & Kjaer, type 8103). Sonication of adherent monolayers of A2780 and A2780/ADR cells at 67 kHz was performed in Sonicor SC 100 sonication bath (Sonicor Instruments, Copaique, NY) in 6-well plates. We could not measure power density received by the monolayer, but indirect evidence indicates that cells experienced mild insonation because no detachment of cells from monolayers was observed in our experiments.

Flow Cytometry

Fluorescence histograms were recorded with a Facscan (Beckton Dickinson) flow cytometer and analyzed using CellQuest software supplied by the manufacturer. Minimums of 10,000 events were analyzed to generate each histogram. To prevent Pluronic efflux from the cells at washing, the cells were injected into the flow cytometer in the initial incubation medium.

Fluorescence Microscopy

The cells were first fixed with 3% formalin, then washed with PBS containing 3% formalin, sealed on glass slides, and visualized at 100× magnification by fluorescence microscopy with 527–552 nm excitation and 577–632 nm emission wavelengths (Eclipse E800, Nikon).

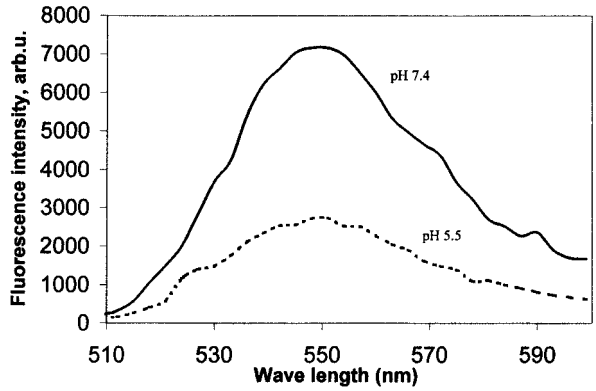


Figure 1. Fluorescence spectra of aqueous solutions of fluorescently labeled Pluronic P-105 at pH 5.5 and 7.4.

RESULTS AND DISCUSSION

Fluorescence Spectra

The fluorescence spectra of fluorescently labeled Pluronic at neutral (pH 7.4) and acidic (pH 5.5) pHs are presented in Figure 1. The fluorescence intensity of labeled Pluronic at neutral pH more than doubles compared with the acidic environment. This feature was used to study the intracellular distribution of fluorescently labeled Pluronic.

Intracellular Pluronic Distribution in HL-60 Cells

Fluorescence histograms of drug-sensitive promyelocytic HL-60 cells incubated with various concentrations of fluorescently labeled Pluronic P-105 are presented in Figure 2. A clearly bimodal character of fluorescence histograms is observed at Pluronic concentrations $\geq 0.2\%$; these concentrations are above the cmc of Pluronic P-105 and correspond to micellar solutions.

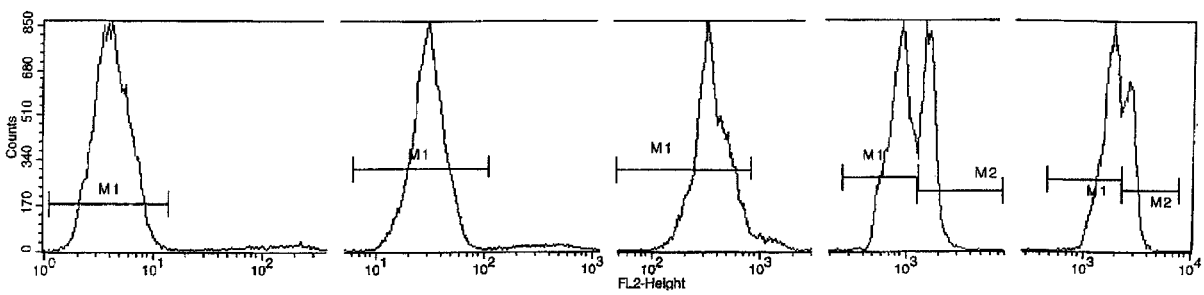


Figure 2. Fluorescence histograms of HL-60 cells incubated with fluorescently labeled Pluronic P-105; initial concentration of a labeled Pluronic P-105 in the incubation medium (left to right) 2×10^{-4} %wt, 2×10^{-3} %wt, 0.02%, 0.2%, and 0.2 %wt; incubation time: 60 min.

We hypothesize that the population of the cells with a higher fluorescence intensity resulted from the disruption of the pH gradient on acidic vesicle membranes followed by the release of Pluronic molecules from the acidic environment of the cytoplasmic vesicles into the neutral environment of the cytosol. This result may, in turn, be caused by the fluidization of phospholipid membranes under the action of the internalization Pluronic micelles. The electron paramagnetic resonance (EPR) spectra of a membrane-localized spin probe 16-doxyl stearic acid methyl ester (16-DSME) in HL-60 cells indicated that Pluronic intracellular uptake from micellar solutions resulted in a decrease of a solid–fluid transition temperature and intensification of probe motion in phospholipid membranes.[10]

The hypothesis of the self-release of Pluronic from acidic vesicles was confirmed by the results of the experiment presented in Figure 3 and by a direct observation of the cells by fluorescence microscopy (Figure 4). In the experiment presented in Figure 3, the cells were incubated with a mixture of fluorescently labeled and unlabeled Pluronic P-105. The initial concentration of labeled Pluronic in the incubation medium was

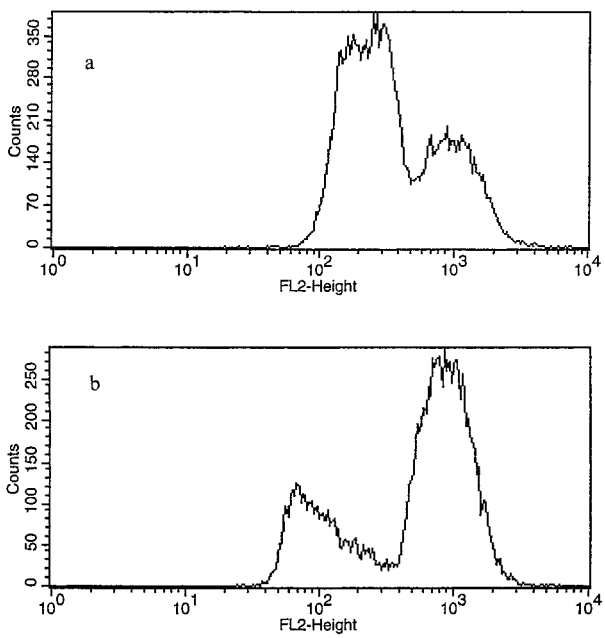


Figure 3. Fluorescence histograms of HL-60 cells incubated with a mixture of fluorescently labeled and unlabeled Pluronic P-105. The concentration of labeled Pluronic in the incubation medium is maintained constant at 0.002%; the concentration of unlabeled Pluronic is 0.02% (a) and 0.2% (b); the incubation time is 30 min.

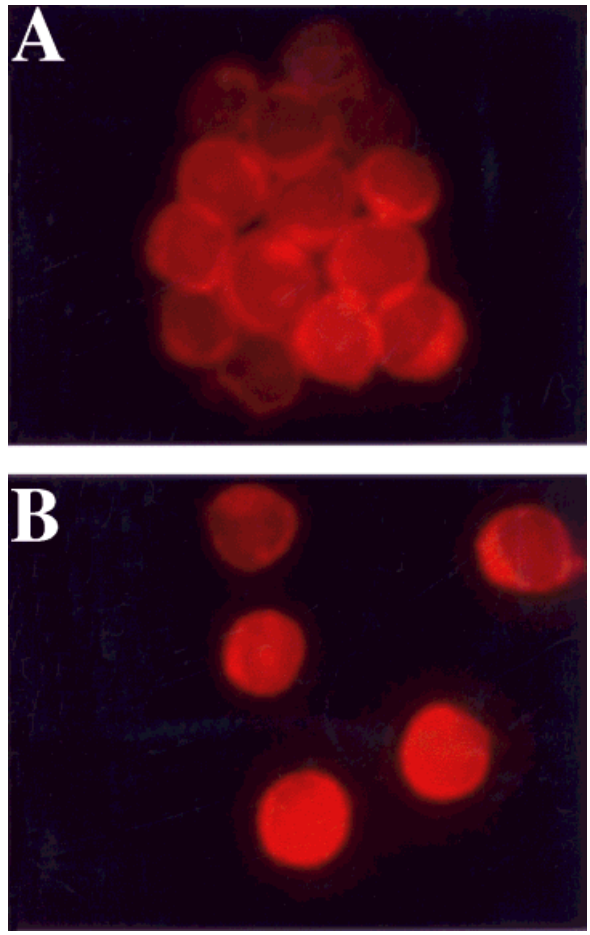


Figure 4. Effect of Pluronic P-105 concentration in the incubation medium on its intracellular distribution. The concentration of fluorescently labeled Pluronic P-105 is maintained constant at 0.005%. The overall Pluronic concentration is either (A) 0.1% or (B); 10%. The incubation time is 10 min. The cells of the same sample are characterized by a different intracellular distribution of a fluorescently labeled Pluronic P-105. With increasing Pluronic concentration, release of Pluronic from sequestering compartments is observed.

the same for both A and B samples, but the concentration of unlabeled Pluronic was an order of magnitude higher in sample B than in sample A (both concentrations being above the cmc). As seen from Figure 3, the population of the cells with a stronger fluorescence was much higher in sample B than in sample A, presumably because of the Pluronic-induced permeabilization of the endosomal (and/or lysosomal) membranes, which resulted in Pluronic transfer to the environment with a higher pH.

For the samples characterized by bimodal fluorescence histograms, fluorescence micrographs

revealed distinctly different distribution of fluorescence for the cells belonging to the same sample (Figure 4). Figure 4B shows that fluorescence of some cells is relatively low and concentrated around the nucleus, suggesting that Pluronic is sequestered in cytoplasmic vesicles. In contrast, fluorescence of other cells is higher and more evenly distributed, suggesting that Pluronic is released from cytoplasmic vesicles. The observed differences may be due to the differences in the intracellular Pluronic concentration within the cell ensemble; alternatively, for the cells that are at different stages of the cell cycle, the membranes of acidic vesicles may have different susceptibility to the action of a polymeric surfactant. The fraction of the cells with stronger and more evenly distributed fluorescence grew with Pluronic concentration, as indicated by flow cytometry (Figure 2) and confirmed by fluorescence microscopy.

The duration of cell incubation with Pluronic substantially affected the intracellular distribution of Pluronic. For all Pluronic concentrations, Pluronic release from acidic vesicles increased with the incubation time.

Ultrasound was another important factor that modulated the intracellular uptake and distribution of Pluronic molecules.[12] Figure 12 of ref. 12 suggests that ultrasound increased Pluronic transfer from acidic vesicles, presumably due to the perturbation of their membranes resulting in their enhanced permeabilization; the latter was observed even for a short-term (10 min) cell sonication with relatively low Pluronic concentrations (0.005%). The application of ultrasound allowed decreasing Pluronic concentration and shortening of the incubation time needed for the permeabilization of the cytoplasmic vesicles.

Pluronic Uptake and Distribution in Ovarian Carcinoma Cells

These experiments were performed with drug-sensitive A2780 and MDR A2780/ADR ovarian carcinoma cells. Cells were incubated with a mixture of a fluorescently labeled and unlabeled Pluronic P-105; the concentration of the labeled Pluronic in the incubation medium was maintained constant at 0.001% whereas the concentration of the unlabeled Pluronic progressively increased, as indicated in Figure 5.

For drug-resistant cells, the overall Pluronic uptake measured by fluorescence depletion from the incubation medium was slightly lower than for drug-sensitive cells (Table 1). For both types of cells, the uptake of a fluorescently labeled Pluronic dropped with increasing concentration of the unlabeled Pluronic (Table 1).

Fluorescence histograms are presented in Figure 5; intensities and fractions of the respective fluorescence peaks are presented in Table 2. Fluorescence histograms revealed differences in Pluronic distribution in drug-sensitive and MDR cells. There is an obvious discrepancy between the data of Tables 1 and 2: whereas the uptake of fluorescently labeled Pluronic dropped with increasing concentration of the unlabeled Pluronic (Table 1), the mean intracellular fluorescence of the cells went through a flat maximum in drug-sensitive cells and even increased in drug-resistant cells (Table 2). In other words, the uptake of the unlabeled Pluronic resulted in a higher fluorescence of the cells that comprised a lower intracellular concentration of a fluorescently labeled Pluronic. This result presumably occurred because of the increase of pH of the probe environment, which, as shown in Figure 1, resulted in an increase of probe fluorescence.

The data just presented confirm the permeabilization of the membranes of acidic vesicles under the action of Pluronic copolymer, which may allow the release of Pluronic molecules from acidic vesicles into cytosol.

Some indications of the transfer of Pluronic from the cytoplasmic acidic vesicles into the cytosol were obtained in ref. 28; the author conjugated peroxidase to Pluronic P-85 and introduced the conjugate encapsulated in Pluronic P-85 micelles into Jurkat cells.[28] The conjugate was taken up by the cells via endocytosis and was localized in the acidic vesicles. However, the insoluble product of the peroxidase reaction with its substrate was observed not only in the vesicles but also in the cytosol, indicating that the conjugate was partly released from acidic vesicles. This result is consistent with our results already presented.

For the MDR cells, a bimodal distribution of cell fluorescence was already observed at a very low Pluronic concentration in the incubation medium that corresponded to Pluronic unimers (Figure 5). In contrast, for the sensitive cells, the distinctly bimodal distribution of cell fluorescence was observed only at Pluronic concentrations corresponding to micellar solutions. These data suggest that MDR cells are more susceptible to the action of a Pluronic copolymer than drug-sensitive cells, which may point to the differences

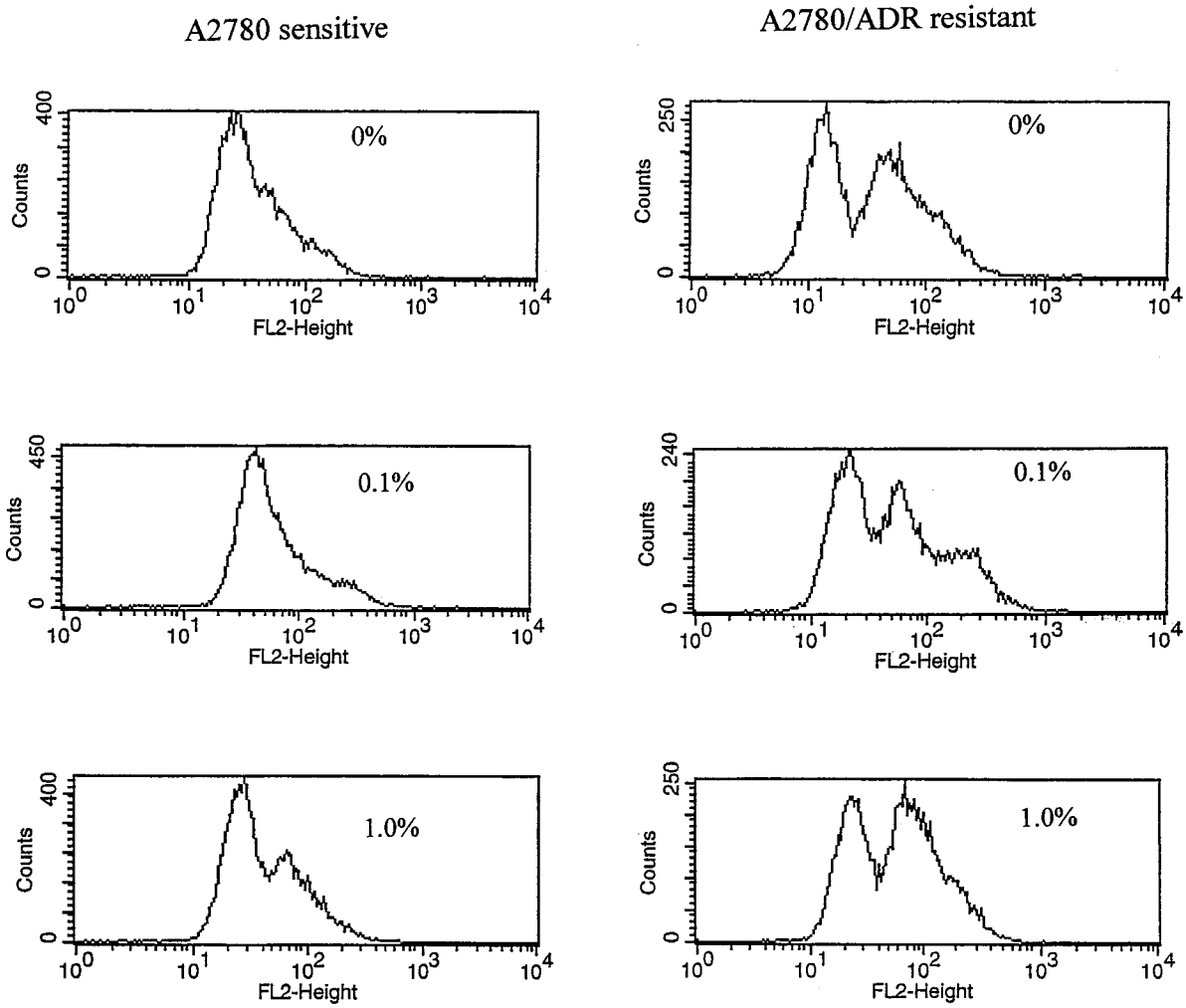


Figure 5. Fluorescence histograms of drug-sensitive A2780 cells (left column) and MDR A2780/ADR cells (right column) incubated with a mixture of a fluorescently labeled and unlabeled Pluronic P-105. The concentration of labeled Pluronic is maintained constant at 0.001%, and the concentration of unlabeled Pluronic P-105 is indicated in the Figure.

Table 1. Pluronic P-105 Uptake by Drug-Sensitive and MDR A2780 Cells Measured by Depletion From the Incubation Medium[a]

Cell Type	Total Pluronic P-105 Concentration, % wt/v	Uptake of a Fluorescently Labeled Pluronic, μg/10^6 cells
Drug-sensitive A2780 cells	0.001	0.53 ± 0.02
	0.011	0.19 ± 0.02
	0.1	0.11 ± 0.01
	2.0	0.08 ± 0.01
Drug-resistant A2780/ADR cells	0.001	0.44 ± 0.02
	0.011	0.18 ± 0.02
	0.1	0.08 ± 0.01
	2.0	0.04 ± 0.01

[a]Concentration of fluorescently labeled Pluronic P-105 is 0.001% for all samples; incubation time 30 min.

Table 2. Flow Cytometry Data for A2780 and A2780/ADR Cells Incubated With a Mixture of a Fluorescently Labeled and Unlabeled Pluronic P-105[a]

	Drug-Sensitive A2780 Cells					Drug-Resistant A2780/ADR Cells				
Pluronic Concentration, %	M_1[b]	Fraction M_1,%	M_2	Fraction M_{25},%	Mean-Fluorescence	M_1	Fraction M_1,%	M_2	Fraction M_{25},%	Mean-Fluorescence
0.001	—	—	—	—	35	13	47	55	53	28
0.01	—	—	—	—	46	20	51	82	49	40
0.1	—	—	—	—	57	20	49	87	51	43
1.0	24	57	79	43	40	23	43	96	57	53
2.0	26	56	80	44	43	24	47	96	53	50

[a]Concentration of fluorescently labeled Pluronic P-105—0.001% for all samples; incubation time 30 min.
[b]M_1 and M_2 are mean fluorescence intensities of the first and the second pick, respectively.

in the structure of the acidic vesicle membranes in drug-sensitive and drug-resistant cells. This suggestion is in agreement with Kabanov and Alakhov's results.[21] These authors observed a reduction of high pH gradients in MDR cells under the action of unimeric concentrations of Pluronic L61; the reduction of pH gradients indicated that Pluronic had ionoforic properties, which resulted in the increased release of positively charged compounds (e.g., DOX) from acidic vesicles.[21] Our data suggest that not only ions but also neutral polymeric molecules (i.e., Pluronic P-105 molecules; MW 6500 Da) can experience Pluronic-induced enhanced release from acidic vesicles. The data just presented support Kabanov and Alakhov's hypothesis that the permeabilization of the cytoplasmic vesicles of MDR cells by Pluronic molecules may play an important role in the hypersensitization of drug-resistant cells by Pluronic unimers.[21]

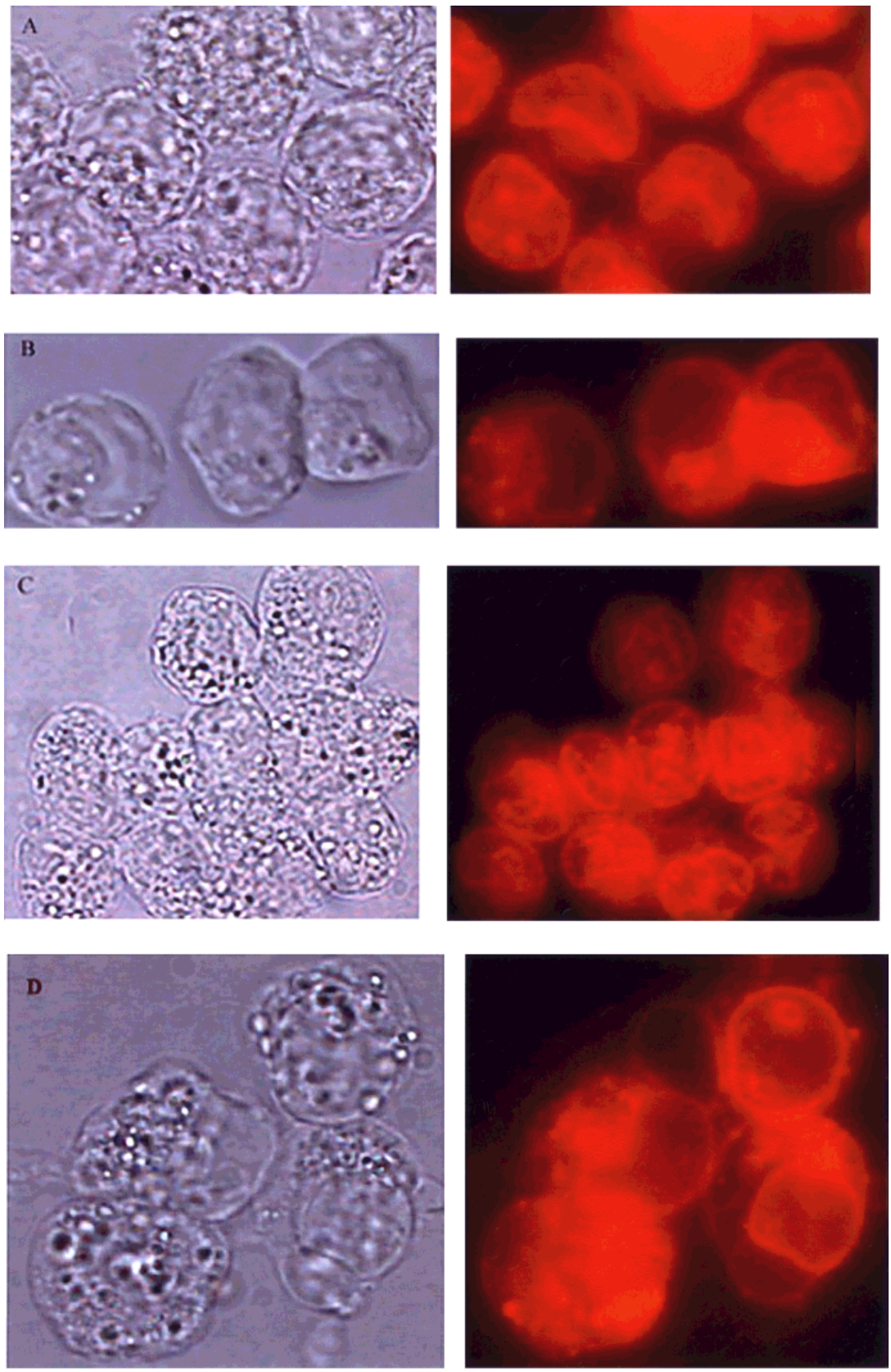
Effect of Pluronic on Drug Trafficking in Drug-Sensitive and MDR Cells

Anthracyclin drugs DOX and Rb were used in these experiments; conventional and confocal fluorescence microscopy was exploited. DOX and Rb are the DNA-intercalating drugs; DOX has significantly higher affinity to DNA than Rb [6,10]; in HL-60 cells, DOX preferentially accumulates in cell nuclei while Rb partitions between nuclei and cell membranes.[10]

Drug intracellular drug distribution in ovarian carcinoma cells depended on cell type (sensitive, resistant), drug type (Rb, DOX), and delivery system (PBS, Pluronic micelles). In drug-sensitive A2780 cells, both DOX and Rb preferentially accumulated in cell nuclei independently on the delivery system; location of the nuclei was verified by the DAPI uptake. For Rb, some fraction of the drug also partitioned into the membranes and perinuclear compartments. These data suggest that in drug-sensitive cells, drug accumulation in acidic vesicles was not significant, presumably due to the low pH gradient between acidic vesicles and cytosol.[17] This gradient provided for drug trafficking into cell nuclei.

For drug-resistant A2780/ADR cells, drug distribution was distinctly different for DOX and Rb. Whereas DOX accumulated in cell nuclei (Fig. 6A), Rb was completely excluded from the nuclei (Fig. 6B). Confocal microscopy revealed Rb partitioning into plasma membranes, intracellular membranes, and intracellular vesicles (data not

Figure 6. Phase contrast and fluorescence micrographs of A2780/ADR cells incubated for 30 min with (A) DOX (10 μg/mL) dissolved in PBS or Rb (10 μg/mL) dissolved in (B) PBS, (C) 1.0% Pluronic P-105, or (D); 0.1% Pluronic P-105. When delivered in PBS, DOX accumulates in cells nuclei whereas Rb is excluded from cell nuclei [compare (A) and (B)]; when delivered in 1.0% Pluronic micelles, Rb penetrates into cell nuclei [compare (B) and (C)]; at 0.1% Pluronic solution, Rb does not penetrate into nuclei but accumulates in plasma membranes, cytoplasmic vesicles, and perinuclear compartments (D).


A
B
C
D

shown). The Rb molecule is bulkier and more hydrophobic than that of DOX and much less DNA-tropic.[6] Our previous experiments using HL-60 cells showed that ~40% of the intracellular Rb delivered in PBS was retained in cell membranes.[10]

Introduction of Rb in Pluronic micelles dramatically changed the intracellular trafficking of Rb in MDR cells. In this case, Rb preferentially accumulated in the cell nuclei (Fig. 6C). No drug was observed in cell membranes, vesicles, or cytosol, suggesting that Pluronic induced Rb release from acidic vesicles and extraction from membranes. Interestingly, substantial nuclear accumulation of Rb in MDR cells required much higher Pluronic concentrations than those that caused disruption of the pH gradient of acidic vesicles of MDR cells. At Pluronic concentration of 0.1%, Rb accumulated in the perinuclear compartments of the MDR cells and did not penetrate into the nuclei (Fig. 6D); nuclear accumulation was observed at Pluronic concentrations of 1% and higher (Fig. 6C). It seems that for lipophilic drugs like Rb, substantial nuclear accumulation involves not only drug release from acidic vesicles but also drug extraction from cell membranes, which in turn requires rather high concentrations of polymeric surfactants.

It should be noted that Rb delivery in a complete RPMI-1640 medium rather than in PBS increased Rb accumulation in cell nuclei. Moreover, if the cells were first drug-loaded in PBS and then incubated in a complete RPMI-1640 medium, Rb gradually accumulated in the nuclei. This result indicates that some component (or components) of the complete RPMI-1640 medium exerted the same effect as Pluronic micelles on Rb release from acidic vesicles and cell membranes.

Effect of Pluronic on the Intracellular Drug Uptake by Drug-Sensitive and MDR Cells

Drug-sensitive and MDR cells differed dramatically in their response to drug introduction in Pluronic micelles. When DOX was delivered in Pluronic micelles, its uptake by drug-sensitive cells dropped, whereas the uptake by MDR cells increased. Typical flow cytometry results are presented in Tables 3 and 4 (the data of Tables 3 and 4 were acquired with different cell batches; only minor quantitative differences were observed for non-sonicated samples, the general pattern being the same). It should be noted that for DOX, flow cytometry results agree with the direct measurements of DOX uptake based on drug depletion from the incubation medium. This agreement allows using flow cytometry for comparing DOX uptake by various samples.

The micellar-induced decrease of DOX uptake by drug-sensitive cells was previously reported by us,[5–13] and the mechanism of this effect is discussed in details in a recent paper.[12] The increase of DOX uptake by MDR cells in the presence of Pluronic micelles is observed for the first time; the effect was presumably caused by a Pluronic-induced deactivation of drug efflux pumps in MDR cells, as was previously reported for Pluronic unimers.[14,21–26] Note that the increased DOX uptake was more pronounced at Pluronic concentration of 0.1% than at 10% (Tables 3 and 4). At a concentration of 0.1%, Pluronic P-105 does not exert a shielding effect on drug uptake and therefore the main effect of

Table 3. Effect of Pluronic Micelles and Ultrasound on DOX Uptake by Adherent A2780 and A2780/ADR Cells[a]

Cell Line	Pluronic P-105 Concentration in RPMI-1640, wt %	Mean Fluorescence, arb.u.	
		Non-sonicated	Sonicated
Drug-Sensitive A2780	0	233.1 ± 17.7	230.8 ± 21.8
	0.1	233.6 ± 18.7	238.0 ± 26.0
	10	111.6 ± 11.2	132.2 ± 6.1
Drug-resistant A2780/ADR	0	31.8 ± 0.1	35.5 ± 0.1
	0.1	125.5 ± 1.5	137.9 ± 12.4
	10	66.5 ± 4.0	90.0 ± 0.8

[a]Initial DOX concentration, 50 μg/mL; continuous wave sonication for 10 min; ultrasound frequency, 69 kHz; ultrasound power density in the incubation medium, 3.2 W/cm^2. The experiments were made in duplicate; average values ± standard deviations are presented.

Table 4. Effect of Pluronic Micelles and Ultrasound on DOX Uptake by Adherent A2780 and A2780/ADR Cells[a]

Cell Line	Pluronic Concentration in RPMI-1640, wt %	Mean fluorescence, arb.u.[b]	
		Non-sonicated	Sonicated
A2780	0	229.2 ± 15.4	294.1 ± 21.8
	0.1	237.4 ± 16.0	274.7 ± 24.0
	10	124.4 ± 11.2	160.0 ± 6.1
A2780/ADR	0	52.3 ± 5.0	58.2 ± 5.3
	0.1	143.6 ± 15.4	191.8 ± 15.6
	10	72.8 ± 4.1	104.7 ± 5.2

[a]Initial DOX concentration, 50 μg/mL; continuous wave sonication for 10 min; ultrasound frequency, 69 kHz; ultrasound power density in the incubation medium, 3.2 W/cm^2. The experiments were made in triplicate; average values ± standard deviations are presented.

[b]Statistically significant differences ($p < 0.05$) were observed between drug-sensitive and drug-resistant cells in all cases. Across various Pluronic concentrations, for the drug-sensitive cells, statistically significant differences were observed between a 10% Pluronic solution and a RPMI-1640 or 0.1% Pluronic solution. For the MDR cells, across various Pluronic concentrations, statistically significant differences were observed in all cases. The sonication effect was statistically significant in all cases with the exception of the MDR cells incubated in RPMI-1640 medium; in the latter case, substantial cell sonolysis was observed, indicating considerable damage of plasma membranes that could cause drug leaking from the cells.

Pluronic is that of deactivating efflux pumps. In contrast, at a concentration of 10%, Pluronic micelles exert a shielding effect on the drug uptake; this effect is opposite in direction to the deactivation of efflux pumps. The final result depends on the competition of two processes; as follows from the data of Tables 3 and 4, for DOX in the MDR cells, the deactivation of efflux pumps prevailed over the shielding effect of Pluronic micelles.

For Rb, the situation was more complicated. As already mentioned, Rb partitions between various cellular compartments (i.e., nuclear DNA, membranes, cytoplasmic vesicles). Rb fluorescence depends strongly on its localization. Compared with an aqueous environment, fluorescence is enhanced in phospholipid membranes and quenched with Rb intercalation into DNA.[6] The resulting fluorescence of Rb-loaded cells depends on Rb partitioning between various cellular compartments. Therefore, for Rb, in contrast to DOX, fluorescence intensity is not a direct function of the intracellular concentration. This difference complicates quantitative measurements but provides additional information on Rb partitioning.

At a low Rb concentration in the incubation medium (10 μg/mL), the effect of Pluronic micelles on Rb uptake by drug-sensitive and MDR cells was similar to that observed for DOX. Based on the direct measurements of drug depletion from the incubation medium, the uptake of Rb by the drug-sensitive A2780 cells dropped whereas that by the MDR cells increased when Rb was delivered in Pluronic micelles (Table 5). In the drug-sensitive cells, Rb predominantly accumulated in cell nuclei independently of the delivery system; in this case, a decrease in drug uptake was in agreement with a decrease in cell

Table 5. Effect of Pluronic Micelles on Rb Uptake and Intracellular Fluorescence for Drug-sensitive and MDR Cells[a]

Cell Line	Pluronic Concentration in RPMI-1640, %	Rb Uptake, μg/10^6 Cells	Mean Fluorescence, arb.u. (flow cytometry)	Fluorescence/Uptake Ratio, arb.u.
Drug-Sensitive A2780	0	1.30 ± 0.13	79.8	62
	10	0.38 ± 0.04	22.9	61
MDR A2780/ADR	0	0.17 ± 0.02	31.2	185
	10	0.30 ± 0.03	24.7	82

[a]Rb concentration in the incubation medium, 10 μg/mL.

fluorescence (Table 5). In contrast, for the MDR cells, the increased drug uptake in the presence of Pluronic micelles was accompanied by a slight drop of cell fluorescence (Table 5), indicating the enhanced nuclear localization. This result is in agreement with the data provided by fluorescence microscopy (see Figure 6).

An important difference between DOX and Rb is that the uptake of DOX gradually levels off with increasing drug concentration in the incubation medium, whereas the uptake of Rb significantly increases in the same concentration range.[12] For DOX, the upper limit of drug uptake is controlled by drug intercalation into DNA. For Rb, uptake proceeds above this limit[12] because of the drug accumulation in the membranes and cytoplasmic vesicles. Rb localization in mitochondrial membranes was confirmed by the intracellular bioreduction of the nitroxide moiety that proceeded in mitochondrial membranes.[10] For Rb, the difference between the fluorescence of drug-sensitive and MDR cells significantly dropped with increasing drug concentration in the incubation medium (Table 6), presumably because of Rb accumulation in the membranes and vesicles of the MDR cells. These data suggest that efflux pumps do not affect a fraction of drug localized in vesicles and membranes.

Effect of Ultrasound on the Intracellular Uptake of DOX and Rb by Drug-Sensitive and MDR Cells

As was previously observed for drug-sensitive cells, ultrasound substantially enhanced the intracellular DOX and Rb uptake by the MDR cells in the presence of 10% Pluronic micelles. Typical results for DOX are presented in Tables 3 and 4. It is noteworthy that the effect of ultrasound on the drug uptake by both drug-sensitive and MDR cells was much more pronounced in the presence of 10% Pluronic micelles than in PBS or 0.1% Pluronic P-105 solution suggesting that Pluronic micelles and ultrasound acted synergistically. The mechanism of the ultrasonic enhancement of drug uptake from micellar solutions was previously discussed in detail.[12] Briefly, a new equilibrium between the external and internalized drug is established under ultrasound, being shifted towards the internalized drug. Upon turning ultrasound off, the initial external–internal drug equilibrium is not restored if ultrasound-induced plasma membrane defects are sealed, which results in the "supersaturation" of cells with drug. We have reported that 10% Pluronic micelles exerted a protective effect on the cells during the sonication process, presumably because of the membrane fluidization that resulted in the accelerated sealing of the ultrasound-induced membrane defects.[11,12] No cell protection was exerted by PBS, RPMI-1640, or a 0.1% Pluronic solution; therefore, the internalized drug could partly leak out of the cells through the ultrasound-induced membrane defects, thus decreasing the resulting drug uptake. These data show that drug delivery in polymeric micelles combined with the application of ultrasound might be an effective way of drug targeting to drug-sensitive and MDR tumors.

CONCLUSIONS

The intracellular uptake of Pluronic P-105 resulted in the permeabilization of acidic vesicles and disruption of a pH gradient at their membranes. In MDR cells, the permeabilization occurred at a significantly lower Pluronic concentrations than

Table 6. Effect of Rb Concentration in the Incubation Medium on the Intracellular Fluorescence of Rb in A2780 Drug-Sensitive and MDR Cells

		Mean Fluorescence, arb.u.		Fluorescence Ratio, Drug-Sensitive/MDR	
		Rb Concentration in the Incubation Medium, μg/mL		Rb Concentration in the Incubation Medium, μg/mL	
Cell Type	Pluronic P-105 Concentration, wt%	10	50	10	50
Drug-Sensitive	0	79.8	367	2.6	1.3
	10	22.9	95	0.9	1.5
MDR	0	31.2	284	—	—
	10	24.7	60	—	—

in drug-sensitive cells, suggesting a higher sensitivity of drug-resistant cells to the action of polymeric surfactants. The permeabilization of acidic vesicles caused dramatic changes of the intracellular localization of the anthracyclin drug Rb. When delivered in PBS, the drug was excluded from the nuclei of MDR cells, whereas delivery in Pluronic micelles resulted in drug release from the cytoplasmic vesicles and cell membranes and accumulation in cell nuclei. Drug delivery in Pluronic micelles combined with the application of ultrasound substantially increased drug uptake by the MDR cells. These findings suggest that the nuclear accumulation of drugs internalized via fluid-phase endocytosis can be enhanced by the application of Pluronic micelles and can be further augmented by ultrasonic irradiation.

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