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Note

Combined cancer therapy by micellar-encapsulated drug and ultrasound

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Abstract

A new modality of drug targeting to tumors that is under development in our lab is based on the drug encapsulation in polymeric micelles followed by a localized release at the tumor site triggered by focused ultrasound. The rationale behind this approach is that drug encapsulation in micelles decreases systemic concentration of drug and provides for a passive drug targeting to tumors via the enhanced penetration and retention (EPR) effect, thus, reducing unwanted drug interactions with healthy tissues. In addition, polymeric micelles sensitize multidrug resistant (MDR) cells to the action of drugs. Upon the accumulation of drug-loaded micelles at the tumor site, ultrasonic irradiation of the tumor is used to provide for the effective intracellular drug uptake. Ultrasound releases drug from micelles and enhances the intracellular uptake of both released and encapsulated drug. An important advantage of ultrasound is that it is noninvasive, can penetrate deep into the interior of the body, can be focused and carefully controlled. The results of the in vitro application of this technique for delivering anthracyclin drugs to ovarian carcinoma A2780 drug-sensitive and MDR cells are described.

Keywords: Ultrasound; Polymeric micelles; Pluronic; Doxorubicin; Ovarian carcinoma

1. Introduction

During the last two decades, a constantly increasing interest has been expressed to polymeric micelles as drug carriers due to a number of advantages that micelles offer over other types of drug carriers. Micelles are self-assembled spherical nanoparticles that have the right size to avoid renal excretion but allow extravasation at the tumor site. This provides for a passive tumor targeting via the enhanced penetration and retention (EPR) effect (Maeda et al., 1992).

When a hydrophobic drug is partitioned into dense micelles, the systemic concentration of free drug is decreased, which diminishes intracellular drug uptake by normal cells and reduces unwanted side effects caused by drug interactions with healthy tissues. However, when drug is encapsulated in micelles, its uptake by cancerous cells is also decreased (Rapoport, 1999; Rapoport et al., 1997, 1999, 2002b; Marin et al., 2001a,b).

To trigger drug release from micelles at the tumor site and enhance drug uptake by tumor cells, we use ultrasonic irradiation of the tumor. Ultrasound triggers controlled release of drugs from micelles at the tumor site and perturbs cell membranes, thus, enhancing the intracellular uptake of both released and micellar-encapsulated drug (Marin et al., 2002; Rapoport et al., 2002a,b, 2003).

This technique promises to overcome two main complications of cancer chemotherapy: severe side effects of toxic drugs and resistance of cancerous cells

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to drug action, which is either inherent or developed in the course of chemotherapy.

The technique we are developing involves four main components: cells, drugs, micelles, and ultrasound. We have studied the individual interactions between the components of the system, which involved interactions between ultrasound and micelles (Husseini et al., 2000a), ultrasound and cells (Rapoport et al., 2002a), and micelles and cells at the presence or absence of ultrasound and drugs (Rapoport, 1999; Rapoport et al., 1999, 2000, 2002a,b, 2003; Husseini et al., 2000a,b; Marin et al., 2001a,b).

Main results of in vitro studies are summarized below. In the majority of the experiments, Pluronic P-105 was used as a micelle-forming polymer. Pluronic is a triblock copolymer poly(ethylene oxide)-co-poly (prolylene oxide)-co-poly(ethylene oxide), with a hydrophobic poly(prolylene oxide) (PPO) central block and two relatively hydrophilic poly(ethylene oxide) flanking blocks. A family of Pluronic copolymers includes a number of members differing in the lengths and ratios of PEO and PPO blocks. Pluronic P-105 comprises 56 propylene oxide units in the central block and 37 ethylene oxide units in each side block. At 37 °C, Pluronic P-105 forms dense micelles with hydrophobic cores at concentrations close to 0.1 wt.%.

2. Materials and methods

2.1. Drug

Doxorubicin (DOX) was obtained from the University of Utah Hospital (SLC, UT) in a 1:5 mixture with lactose; pure DOX was bought from Sigma Chemical Co. (St. Louis, MO). Stock solutions of DOX were kept frozen.

2.2. Cells

Promyelocytic leukemia HL-60 cells grown in suspensions were kindly provided by Dr. B.K. Murrey (Department of Microbiology, Brigham Young University, Provo, UT). They were cultured in RPMI-1640 medium (Sigma) supplemented with 20% fetal calf 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. Minko (Rutgers University, NJ). Cells were cultured in a complete RPMI-1640 medium, which in the case of A2780/ADR cells included 800 ng/ml DOX for maintaining resistance.

2.3. Micelles

A micelle-forming block copolymer, Pluronic P-105 was kindly supplied by the BASF Corporation (Mount Olive, NJ) and used as a 10% solution in either phosphate buffered saline (PBS) (Sigma) (for micellar release measurements) or a complete RPMI-1640 medium (for the intracellular uptake measurements). DOX in desired concentration was introduced into the micellar Pluronic solution from a stock solution; it spontaneously partitioned into the core of Pluronic micelles. Pluronic solutions were sterilized by filtration through a 0.2 μm filter.

2.4. Measuring drug release from micelles

The measurements of the degree of drug release are based on the decrease of DOX fluorescence intensity when DOX is transferred from the hydrophobic environment of micelle cores to the aqueous environment. A custom ultrasonic exposure chamber with real-time fluorescence detection was described previously (Husseini et al., 2000a). Briefly, an argon-ion laser beam of 488 nm was directed to a drug-containing cuvette to excite fluorescence. The emissions were collected using a fiber optic collector and filtered to remove the excitation wavelength. The emissions were quantified using a photodetector, digitized with a 12-bit A/D converter, and stored in a Macintosh computer for further analysis.

Digitized fluorescence intensity data were analyzed to calculate the percent of the drug release from micelles as described previously (Husseini et al., 2000a). Briefly, fluorescence intensity of a 10 μg/ml DOX solution in PBS (I_{pbs}) was measured first; the PBS solution was then carefully sucked out of the cuvette and replaced with a 10 μg/ml DOX solution in 10% Pluronic micelles. Fluorescence of this solution (I_{mic}) was measured, and a difference $I_{mic} - I_{pbs}$ was assumed to correspond to a 100% drug release from mi-

celles. Then ultrasound was switched on, and DOX fluorescence under sonication (I_{us}) was recorded; if sonication induced partial drug release from micelles into the aqueous environment, I_{us} was lower than I_{mic}; the "ultrasound on"/"ultrasound off" cycles were repeated several times to check reproducibility. The length of each ultrasound exposure cycle was 1–2 min. The scatter of the data obtained in various ultrasound cycles did not exceed 20%. The degree of drug release (DDR) was calculated as follows:

$$\mathrm{DDR} = \frac{I_{mic} - I_{us}}{I_{mic} - I_{PBS}} \times 100\% \quad (1)$$

2.5. Sonication

For sonicating HL-60 cells, 3 ml cell suspension in DOX-containing medium was placed in the test tube inserted in the water thermostat maintained at 37 °C; ultrasonic transducer was installed next to the test tube at a distance of 2 mm. Before sonication, cells were equilibrated at 37 °C for 5 min.

A2780, A2780/ADR, and MCF-7 cell were grown in 6-well plates; transducer was placed under a particular well of the plate; acoustic contact between the transducer and the plate was provided by the Aquasonic 100 ultrasound transmission gel (Parker Laboratories, Orange, NJ) placed on the transducer surface. Our experiments showed that there was acoustic contact between the directly sonicated well of the 6-well plate and the wells adjacent to the directly sonicated one. These adjacent wells received about 10% of the acoustic energy supplied to the directly sonicated well. While sonication caused temperature increase in the directly sonicated well, no measurable temperature increase was observed in the wells adjacent to it. These adjacent wells were used for sonicating A2780 and A2780/ADR cells grown in adherent monolayers; regular cell growth medium was replaced by DOX-containing medium heated to 37 °C before the start of the sonication; the plate was then placed into the incubator and cells were equilibrated for 5 min. Sonication lasted for 15–30 s; as mentioned above, no temperature increase was observed in the cell-containing wells during sonication.

Control samples were kept without sonication in the same drug-containing medium at the same temperature and for the same time (in some instances, to produce measurable drug uptake, control samples were incubated for longer time than sonicated samples; this is specified in figure captions).

To generate 1 or 3 MHz ultrasound, a PTI transducer (Omnisound 3000C Accelerated Care Plus, Sparks, NV) was used. Sonication at 67 kHz was performed in the sonication bath (Sonicor Instruments, Copaique, NY).

The acoustic intensity in the tested samples at frequencies of 1 and 3 MHz was measured using a hydrophone (model TNU100A with PFS017A Preamplifier, NTR Systems, Inc., Seattle, WA). The voltage was recorded using Tektronix TDS3012 two-channel color digital phosphor oscilloscope (Tektronix Inc., Beaverton, OR). Ten thousand points were recorded in 0.2 s for each measurement. The signal was averaged using Mean Absolute Value (MAV) method; temporal average power density values are presented in the paper.

A 20-kHz ultrasound was generated by a probe transducer (Sonics and Materials, Newton, CT); at 20 kHz, power density was controlled by varying the amplitude settings of the instrument and was measured with a hydrophone (Bruel & Kjaer, type 8103).

2.6. Measuring the Intracellular Uptake of DOX

The initial concentration of cells ranged from 3×10^6 to 5×10^6 cells/ml as counted using a hemacytometer. After exposure to DOX (10–50 μg/ml in various experiments) and ultrasound (15–30 s), cells were counted again to measure the degree of sonolysis, upon which they were centrifuged, washed by PBS, fixed with a 3% formalin or 2.5% glutaraldehide and analyzed by flow cytometry. Fluorescence histograms were recorded with a FACScan flow cytometer (Beckton Dickinson) and analyzed using CellQuest software supplied by the manufacturer. Minimum of 10,000 events was analyzed to generate each histogram.

The experiments on the effect of ultrasound on the intracellular DOX uptake with and without micelles were always conducted in parallel, at the same day and using the same batch of the cells.

2.7. Fluorescence microscopy

The cells were first fixed with 3% formalin, then washed by 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).

3. Results and discussion

3.1. Ultrasound interaction with polymeric micelles

It was studied using the ultrasonic exposure chamber with real-time fluorescence detection described in detail in (Husseini et al., 2000a), in the frequency range of 20 kHz–3 MHz. The measurements were based on the decrease of the fluorescence intensity when drug was transferred from the micelle core to the aqueous environment. The details of experimental procedure are described in Husseini et al. (2000a). The drop in fluorescence intensity under sonication indicated drug release from the hydrophobic environment of Pluronic micelle cores into the aqueous environment (Fig. 1). This could result either from the ultrasound-induced drug diffusion out of micelles or from micelle perturbation/degradation under sonication. Results of kinetic experiments (Husseini et al., 2002) favored the second hypothesis. It is important to outline that the released drug was quickly re-encapsulated between the pulses of ultrasound, which suggested that upon leaving the sonicated volume, the non-internalized drug would, at least partly, circulate in the encapsulated form.

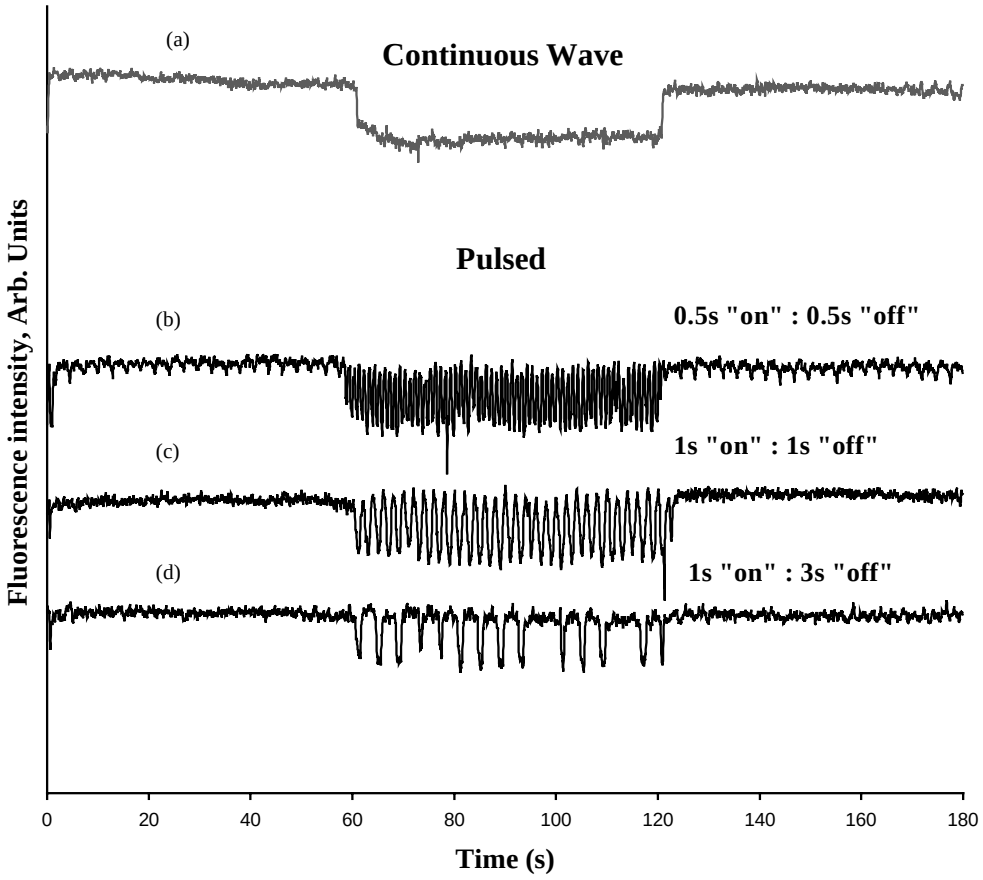

Fig. 1. Doxorubicin (DOX) release profiles from 10% Pluronic micelles under continuous wave (CW) and pulsed 20 kHz ultrasound at a power density of 0.058 W/cm^2 at various durations of ultrasound pulses and inter-pulse intervals indicated in the figure. Measurements are based on the decrease of fluorescence intensity when DOX is transferred from the hydrophobic environment of micelle cores into the aqueous environment. Reprinted with permission from (Husseini et al., 2000a).

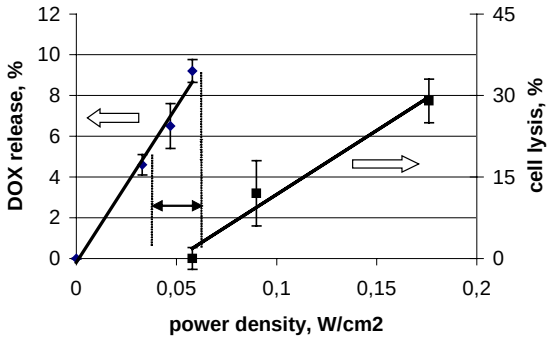

Fig. 2. Dependence of the ultrasound-induced drug release from a 10% Pluronic P-105 micelles and HL-60 cell sonolysis on the ultrasound power density; DOX concentration 10 μg/ml; ultrasound frequency 20 kHz.

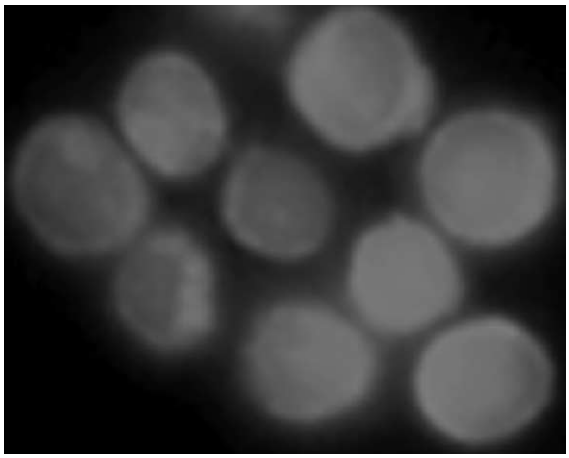

Fig. 3. Fluorescence micrograph illustrating the internalization of Pluronic P-105 by HL-60 cells. Concentration of fluorescently labeled Pluronic P-105 is maintained constant at 0.005%; overall Pluronic concentration is 10%; incubation time is 10 min.

Drug release from micelles proceeded without power density threshold (Fig. 2) indicating that drug release could be induced by mechanical processes other than transient cavitation (Rapoport et al., 2002a).

3.2. Drug/ultrasound interaction

Anticancer drug, DOX was shown to enhance transient cavitation (Rapoport et al., 2002a); encapsulation

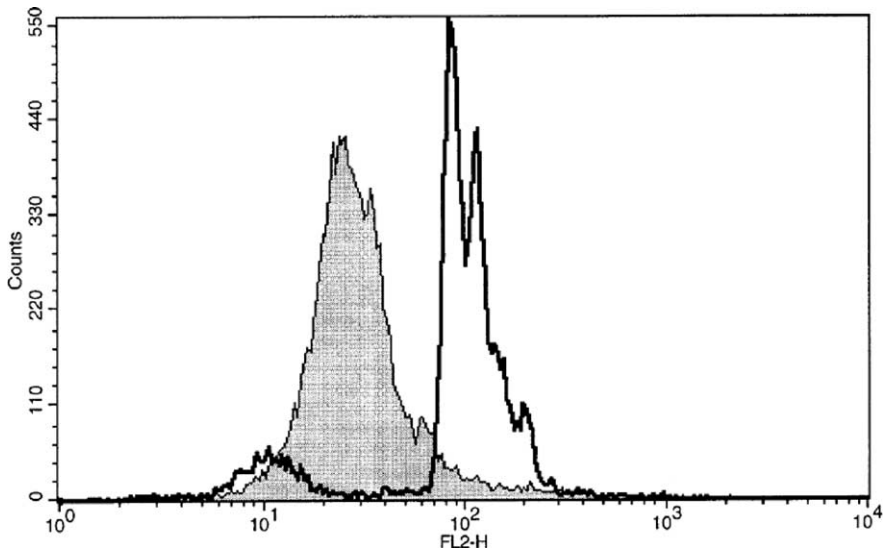

Fig. 4. Flow cytometry histograms illustrating the effect of ultrasound on the DOX uptake from PBS by the drug-sensitive A2780 cells; initial DOX concentration in the incubation medium 20 μg/ml. Unsonicated cells (shaded histogram) were tripsinized, washed by PBS and incubated with DOX for 30 min. Sonicated cells were equilibrated with DOX at 37 °C for 5 min prior to ultrasonic treatment; they were exposed to 1 MHz ultrasound for 15 s, output power density 6 W/cm^2. Upon switching ultrasound on, some fraction of the cells was immediately detached from the substrate; fluorescence histogram presented in figure is for the detached cells. Reprinted with permission from (Marin et al., 2002).

in Pluronic micelles inhibited the cavitation-inducing action of DOX.

3.3. Viable cell/ultrasound interaction

Ultrasound perturbs cell membranes; *above the transient cavitation threshold*, ultrasound severely damages cell membranes and causes cell sonolysis (Fig. 2) (Rapoport et al., 2002b).

3.4. Viable cell/micelle interaction

Both unimers and micelles of amphiphilic polymers are internalized by the cells (an example is shown in Fig. 3); at least partly, unimers and micelles are localized in cell membranes, which results in increased membrane fluidity (Rapoport et al., 2000) and permeability (Rapoport et al., 2002b).

3.5. Viable cell/ultrasound/drug interaction

Below the transient cavitation threshold, ultrasound increased the intracellular uptake of free drug (Fig. 4); above the threshold, severe plasma membrane damage resulted in drug leaking out of the cells (Rapoport et al., 2002b).

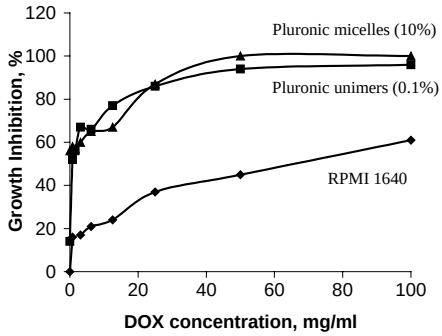

Fig. 6. Growth inhibition of the MDR A2780/ADR cells in the presence of Pluronic unimers and micelles; cell incubation with the drug for 3 h followed by culturing in a drug-free medium for 72 h. Reprinted with permission from (Rapoport et al., 2002a).

3.6. Viable cell/ultrasound/polymeric micelles/drug interaction

Drug intracellular uptake from/with polymeric micelles was enhanced by ultrasound (Fig. 5) (Rapoport, 1999; Rapoport et al., 1999, 2000, 2002a,b, 2003). The data suggested that the enhancement was associated not only with the ultrasound-triggered drug release from micelles but also with the enhanced uptake of micelles containing the encapsulated drug (Marin

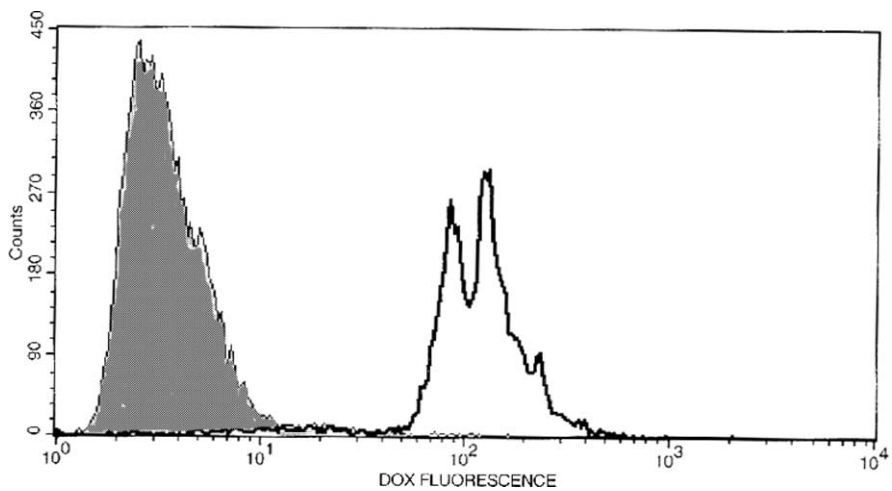

Fig. 5. Fluorescence histograms of the MDR A2780/ADR cells incubated or sonicated in the presence of 10% Pluronic micelles: shaded—unsonicated control (attached cells incubated with DOX for 5.5 min), open—cells exposed to 1 MHz ultrasound for 30 s upon 5 min equilibration with DOX at 37 °C; output power density 6 W/cm^2; initial DOX concentration in the incubation medium 20 μg/ml.

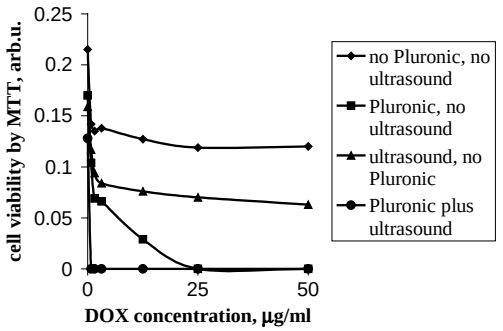

Fig. 7. Effect of Pluronic micelles and ultrasound on the viability of the MDR A2780/ADR cells; cell incubation with Pluronic and DOX for 3 h followed by culturing in a drug-free medium for 72 h. A dramatic drop of IC_{50} of DOX in MDR cells is observed when DOX delivery with Pluronic is combined with the ultrasonic irradiation. Reprinted with permission from (Rapoport et al., 2002a).

et al., 2001a,b). The data also suggested that the permeability of cell membranes (not only plasma membranes but also internal organelle membranes) was enhanced in the presence of Pluronic P-105 (Rapoport et al., 2002b).

3.7. Sensitization of MDR cells under the action of Pluronic micelles and ultrasound

It was reported that Pluronic induced a hypersensitization of MDR cells at concentrations below the CMC, that is, under the action of the individual Pluronic molecules (unimers) (Alakhov et al., 1996; Venne et al., 1996; Batrakova et al., 1999a,b, 2000). In our studies, we observed a comparable sensitization of the MDR ovarian carcinoma A2780/AD cells under the action of Pluronic P-105 unimers and micelles (Fig. 6); the sensitization was additionally enhanced by ultrasound (Fig. 7) (Rapoport et al., 2002a).

At the absence of micelles, the growth of the MDR cells is inhibited by only about 40% at DOX concentrations as high as 50 μg/ml (Fig. 7). At a DOX concentration of 5 μg/ml, only 15% of the MDR cells were killed upon a 3 h incubation with followed by 72 h culturing in a drug-free medium; however, when the MDR cells were incubated with 5 μg/ml DOX in the presence of a unimeric Pluronic solution, cell killing was enhanced to 53%; finally, when the incubation with a drug and a unimeric Pluronic solution was followed by a 10 min sonication by 69 kHz ultrasound at 3.2 W/cm^2, 66% of the cells died upon subsequent cell culturing (data not shown).

Note that *no immediate cell killing by ultrasound was observed in these experiments; cells were killed due to the cytotoxic action of the internalized drug that was enhanced by micelles and ultrasound* (rather than because of the ultrasound-induced mechanical damage).

The results presented above suggested that the drug/micelle/ultrasound technique could effectively deliver drugs to drug-sensitive and MDR tumors in a localized and controlled manner provided that ultrasound was focused on the tumor.

3.8. Ultrasound focusing

Drug targeting requires sharp ultrasound focusing on the tumor. The technique of ultrasound focusing has been developed by the hyperthermia community for tumor ablation. Presently in clinical trials in China and England is JC HIFU system (Kennedy et al., 2003); in the development stage are the instruments based on the time reversed acoustics (Fink, 2003).

The tumor treatment modality suggested here does not require ultra-high ultrasound energies (5–15 kW cm^{-2}) used for tumor ablation. Our technique is based on tumor suppression under the local action of anticancer drugs triggered by focused ultrasound rather than on direct tumor killing by a high-energy ultrasound. However the technology of ultrasound focusing developed for tumor ablation may be adopted for the tumor targeting modality discussed above, which combines micellar-encapsulated drug delivery with triggering a tumor-localized drug uptake by focused ultrasound. An important advantage of this technique over tumor ablation is that is requires orders of magnitude lower ultrasound energies and significantly shorter treatment times. In our first animal studies, we observed the effect of ultrasound on the drug targeting to tumor upon only 30 s sonication at 1.2 W cm^{-2} (compare to 5 kW and 2 h treatment required for ablation).

The results presented above warranted transition to animal experiments that are currently under way in our lab.

Acknowledgements

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References

Alakhov, V., Moskaleva, E., Batrakova, E., Kabanov, A., 1996. Hypersensitization of multidrug resistant human ovarian carcinoma cells by Pluronic P85 block copolymer. Bioconjug. Chem. 7, 209–216.

Batrakova, E., Lee, S., Li, S., Venne, A., Alakhov, V., Kabanov, A., 1999a. Fundamental relationships between the composition of pluronic block copolymers and their hypersensitization effect in MDR cancer cells. Pharm. Res. 16, 1373–1379.

Batrakova, E., Li, S., Miller, D., Kabanov, A., 1999b. Pluronic P85 increases permeability of a broad spectrum of drugs in polarized BBMEC and Caco-2 cell monolayers. Pharm. Res. 16, 1366–1372.

Batrakova, E., Alakhov, V., Kabanov, A., 2000. Selective energy depletion and sensitization of multiple drug resistant cancer cells by Pluronic block copolymer. Polym. Prepr. 41, 1739–1740.

Fink, M., 2003. An overview of time-reversed acoustics. In: Ultrasonics International. Granada, Spain, p. 3.01A.

Husseini, G., Myrup, G., Pitt, W., Christensen, D., Rapoport, N., 2000a. Factors affecting acoustically triggered release of drugs from polymeric micelles. J. Control. Release 69, 43–52.

Husseini, G., El-Fayoumi, R., O'Neill, K., Rapoport, N., Pitt, W.G., 2000b. DNA damage induced by micellar-delivered doxorubicin and ultrasound: comet assay study. Cancer Lett. 154, 211–216.

Husseini, G., Rapoport, N., Christensen, D., Pruitt, J., Pitt, W., 2002. Kinetics of ultrasonic release of doxorubicin from Pluronic P-105 micelles. Colloid Surf. B: Biointerfaces 24, 253–264.

Kennedy, J.E., Wu, F., ter Haar, G.R., Cranston, D., 2003. High-intensity focused ultrasound for treatment of liver and kidney tumors. In: Ultrasonics International. Granada, Spain, p. 3.29B.

Maeda, H., Seymour, L., Miyamoto, Y., 1992. Conjugates of anticancer agents and polymers: advantages of macromolecular therapeutics in vivo. Bioconjug. Chem. 3, 351–362.

Marin, A., Muniruzzaman, M., Rapoport, N., 2001a. Acoustic activation of drug delivery from polymeric micelles: effect of pulsed ultrasound. J. Control. Release 71, 239–249.

Marin, A., Muniruzzaman, M., Rapoport, N., 2001b. Mechanism of the ultrasonic activation of micellar drug delivery. J. Control. Release 75, 69–81.

Marin, A., Sun, H., Husseini, G., Pitt, W., Christensen, D., Rapoport, N., 2002. Drug delivery in Pluronic micelles: effect of high-frequency ultrasound on drug release from micelles and intracellular uptake. J. Control. Release 84, 39–47.

Rapoport, N., Munshi, N., Pitina, L., Pitt, W., 1997. Pluronic micelles as vehicles for tumor-specific delivery of two anticancer drugs to HL-60 cells using acoustic activation. Polymer Preprints 38, 620–621.

Rapoport, N., 1999. Stabilization and activation of Pluronic micelles for tumor-targeted drug delivery. Colloids Surf. B: Biointerfaces 16, 93–111.

Rapoport, N., Herron, J., Pitt, W., Pitina, L., 1999. Micellar delivery of doxorubicin and its paramagnetic analog, ruboxyl, to HL-60: effect of micelle structure and ultrasound on the intracellular drug uptake. J. Control. Release 58, 153–162.

Rapoport, N., Marin, A., Timoshin, A., 2000. Effect of a polymeric surfactant on electron transport in HL60 cells. Arch. Biochem. Biophys. 384, 100–108.

Rapoport, N., Marin, A., Christensen, D., 2002a. Ultrasound-activated micellar drug delivery. Drug Deliv. Systems Sci. 2, 37–46.

Rapoport, N., Marin, A., Luo, Y., Prestwich, G., Muniruzzaman, M., 2002b. Intracellular uptake and trafficking of Pluronic micelles in drug-sensitive and MDR cells: effect on the intracellular drug localization. J. Pharm. Sci. 91, 157–170.

Rapoport, N., Marin, A., Muniruzzaman, M., Christensen, D., 2003. Controlled drug delivery to drug-sensitive and multidrug resistant cells: effects of Pluronic micelles and ultrasound. In: Dinh, S.M., P.L. (Eds.), Advances in Controlled Drug Delivery. Washington, DC, pp. 85–101.

Venne, A., Li, S., Mandeville, R., Kabanov, A., Alakhov, V., 1996. Hypersensitizing effect of Pluronic L61 on cytoxic activity, transport and subcellular distribution of doxorubicin in multiple drug-resistant cells. Cancer Res. 56, 3626–3629.