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ORIGINAL PAPER

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Micelle delivery of doxorubicin increases cytotoxicity to prostate carcinoma cells

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Abstract The use of doxorubicin as a chemotherapeutic agent is hindered by its toxic side effects on the normal cells of the body. The objective of this study was to determine if micelle-delivered doxorubicin could increase the effectiveness of doxorubicin against prostate carcinoma cells. Rat prostate carcinoma cells (MatLu) were cultured under standard conditions. Phosphate-buffered saline (PBS), doxorubicin and/or micelle solution (Pluronic 10500 solution) was added to the cell suspensions and incubated for 3 h. After incubation, cells were washed twice. Analysis consisted of: 1) immediate cell count and 2) proliferation assay at 24 and 144 h. After 24 h, samples with micelle-incorporated doxorubicin had 75% (10% pluronic with 10 μg/ml doxorubicin) and 80% (1% pluronic with 10 μg/ml doxorubicin) cell proliferation results compared with the control group. After 144-h incubation, these same two groups demonstrated cell proliferation results of only 30 and 43% of the control group. The in vitro cytotoxicity of doxorubicin against prostate carcinoma cells was dramatically increased by incorporating the molecule with polymeric micelles.

Keywords Prostate carcinoma · Micelle · Doxorubicin · Drug therapy

Introduction

The ideal method for anti-tumor therapy would be to deliver chemotherapeutic agents possessing highly effective cytotoxic activity specifically to tumor cells while normal cells remain unaffected. Conventional chemotherapeutic treatment such as that with the agent doxorubicin is limited because of the toxic side effects that arise. Doxorubicin is a highly potent chemotherapeutic agent. It acts effectively against various types of cancers including breast cancer [7] and urothelial cancer [8, 6] and can be used as a single agent or in a combination as a therapeutic regime. Doxorubicine has also been introduced as chemotherapeutic agent for the treatment of human prostate cancer [1] and has shown in vitro cytotoxic effects on prostate cancer cells [5].

Unfortunately, it also exhibits severe cardiotoxicity. This cardiotoxicity limits the maximum effective dose. Several options have been investigated to create targeted methods of drug delivery to tumor cells. One highly investigated field is the use of conjugates, either as monoclonal antibodies directed at tumor antigens [18, 23], or proteins which render the drug inactive until activated by an enzymatic mechanism of the cancer cells [9, 24]. However, the disadvantage of these systems is that they can provoke an immune system reaction, due to the non-human components, which decreases effectiveness and may result in a suppression of the immune system [18]. Additionally, they are subject to accelerated clearance from the body [19].

Another possibility is to take advantage of the hyperpermeability of vascular endothelia at tumor sites by using passive drug carriers. Three main areas of research have developed from this line of inquiry: the use of polymers [17, 10, 21], liposomes [10, 15] and polymeric micelles [11, 13, 20, 25]. Matsumura and Maeda [17] describe an increased accumulation of polymeric drugs and macromolecules within solid tumors based on the enhanced permeability and retention (EPR) principle. EPR is based on characteristics of solid tumors such as high vascular density, reduced lymphatic drainage, extensive production of vascular mediators and defects in the vascular structure [16]. Polymeric micelles offer several advantages as a passive drug delivery option. In particular Pluronicpolymers, which form micelles in aqueous solutions, have been the subject of drug delivery

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investigations and, more recently, also as inhibitors of drug efflux systems [4, 22]. Batrakova et al. [3] reported that the Pluronic polymer P85 inhibits the P-glycoprotein efflux mechanism of cells through energy depletion and membrance fluidization. Commercially produced Pluronic copolymers, consisting of a triblock of polyethylene oxide-polypropylene oxide-polyethylene oxide, form micelles when dissolved in water at correct temperatures. The hydrophobic nature of many drugs then facilitates the process of incorporation within the micelle [2]. The advantage of this system is that certain drugs can be easily, non-covalently incorporated into micelles. Additionally, the small size of the micelles, 10–80 nm, allows extravasation into tumor sites and evasion from the RES and renal secretion [14], providing a longer circulation time.

This study was designed to evaluate the potential of micelle-delivered doxorubicin to prostate carcinoma cells. Previous in vitro studies have demonstrated the effectiveness of this method against colon adenocarcinoma tumors and HL-60 human leukemia cells. Solid tumors such as found in prostate cancer provide the ideal micro-environment in which micelles should optimally function. In the treatment of prostate cancer cells, standard doxorubicin demonstrates only a low cytotoxic effect due to the low mitotic rate of this type of cancer and is therefore not routinely used clinically. The incorporation of doxorubicin and micelles provides the drug with a vehicle to the tumor site and could prevent efflux of the drug and promote its accumulation within the cell, resulting in a higher cytotoxic effect. The goal of this study is to determine whether the cytotoxic activity of doxorubicin on prostate carcinoma cells can be increased by the use of a micelle delivery system.

Materials and method

Cell cultures

The rat prostate carcinoma cell line, Dunning R 3327 (MatLu), was obtained from the ECACC (Ref No. 94102735, Salisbury, England) and cultured in RPMI 1640 (Life Technologies, Karlsruhe, Germany) media supplemented with 10% FCS (Life Technologies, Karlsruhe, Germany), 4 mM glutamine (Life Technologies, Karlsruhe, Germany) and 250 nM dexamethasone (Sigma, Munich, Germany). Cultures were grown as monolayer cultures at 37°C in 5% CO_2. Under these conditions cells proliferated at a doubling time of 24–30 h. Cells were harvested with 2.5 ml 0.25% trypsin/EDTA before reaching confluency. Trypsin was neutralized with twice the amount of media, and cells were then centrifuged and resuspended in fresh media. Cell concentration and viability was determined by trypan blue staining analysis. Samples were next aliquoted at 3.5×10^6 cells/ml into NUNC cryotubes (Fisher Scientific, Heidelberg, Germany). Lastly, samples were centrifuged, media removed and the appropriate treatment option was added to the pelleted cells.

Preparation of micelles

A 20% stock solution of Pluronic 10500, molecular weight 6,500 (kindly supplied by the BASF, Ludwigshafen, Germany), consisting of 50% ethylene oxide, was made. Pluronic solutions of 10 and 1% w/v were formulated from this stock solution. Micelle solutions were made by adding 10 or 1 μg/ml doxorubicin to each Pluronic solution. All treatment solutions were sonicated for 3 min at 35 kHz using a Sonorex RK255H (Bandelin, Berlin, Germany) and then incubated at 37°C with medium shaking for 60 min.

Cell treatments

The treatment groups included: 1) a control group—suspended in phosphate-buffered saline (PBS); 2) 10 and 1 μg/ml doxorubicin in PBS; 3) 10% and 1% Pluronic polymer solution; 4) 10% Pluronic polymer solution with 10 or 1 μg/ml doxorubicin; and 5) 1% Pluronic polymer solution with either 10 or 1 μg/ml doxorubicin. Treatment solutions were added to the cells, briefly vortexed, and placed in a 37°C water bath for 3 h. Samples were again vortexed approximately halfway through the incubation period. After incubation, samples were washed twice with fresh media using twice the volume of the treatment solution.

Analysis

Three levels of analysis were performed after treatment. First, cell counts were performed immediately after the washing step. The number and viability of cells were calculated for each sample by trypan blue staining analysis and the Beckman Coulter AcT diff Analysation cell counter (Coulter Corporation, Miami, Florida). The number of cells in each of the treated groups was compared with the control, resulting in a percentage comparison. Second, cells were diluted and aliquoted in a Falcon 96 well plate (Becton Dickenson, Heidelberg, Germany), at 1×10^5 cells/well in 200-μl media, for a proliferation assay. After 24 h, 20 μl of Promega Cell Titer 96 Aqueous One Solution Cell Proliferation Assay solution (Promega, Mannheim, Germany) was added to each well. Samples were incubated for a further 2 h and the absorbance was measured at 490 nm. The absorbance of the sample is proportional to the number of living cells, thus the absorption of cells in the treatment group can be compared with the absorption of the control groups. A second proliferation assay was performed with an incubation of 144 h. The same procedure as for the 24-h assay was used, except the initial cell number was 1×10^3 cells/well. An incubation period of 144 h was chosen to allow a minimum of five doubling periods. Again, absorbencies from the treated groups were compared with that of the control to determine proliferation ability. Cells were also plated in six well plates (Becton Dickenson, Heidelberg, Germany) at 1×10^4 cells/well, incubated for 144 h and stained with 0.5% methylene blue in 50% ethanol for colony observance.

Statistics

Results are expressed as means ± SE. Statistical significance of results was determined by comparison of results using a one-tailed analysis of variance (ANOVA) and the Tukey multiple comparison test. P values of less than 0.05 were considered significant.

Results

Micelle-delivered doxorubicin greatly increased the cytotoxic effects of doxorubicin against prostate carcinoma cells in vitro. Cell counts conducted within the first hour after treatment revealed the expected decrease in cell number in samples treated with pure doxorubicin under both concentrations tested. Samples

treated with 10 μg/ml doxorubicin showed an average of 14% fewer cells than the control group and samples treated with 1 μg/ml showed an average of 9% fewer cells than the control group. All other samples had, if any, insignificant differences to the control group.

After 24 and 144 h, proliferation assays were used to compare the proliferation ability of treated groups with the untreated control. Average absorbencies of cells in the treated groups were calculated as a percentage of the control group. After 24 h, significant differences appeared in groups treated with 10 μg/ml doxorubicin which had been incorporated with either 10 or 1% Pluronic 10500. Both groups had approximately 25% fewer cells than the untreated control group. Groups treated with only doxorubicin or only Pluronic solution demonstrated no difference in proliferation ability in comparison to the control.

The second proliferation assay was analyzed after a 144-h incubation period at 37°C with 5% CO_2. This length of time allows at least five doublings for MatLu cells to ensure reasonable estimation of proliferation ability. Subsequently, a highly significant difference was observed in the samples treated with 10 μg/ml doxorubicin incorporated with 10 and 1% Pluronic 10500. The absorbance in groups treated with 10 μg/ml doxorubicin/10% Pluronic 10500 was 70% less than that of the control group. In the group treated with 10μg/ml doxorubicin/1% Pluronic 10500, absorbance was 58% less than that of the control group. Smaller differences were observed in groups treated with 10 μg/ml doxorubicin alone and in the groups treated with 1 μg/ml doxorubicin incorporated with 10 or 1% Pluronic 10500 (Fig. 1). The colony assay and microscopic photos demonstrate optically the reduced proliferation and different appearance of cells treated with the Pluronic/doxorubicin combination (Fig. 2).

Interesting trends within the treatment modalities can be seen when plotted in comparison to the control. Control groups compared only with groups treated with pure doxorubicin demonstrate similar proliferation ability 24 h after treatment, but begin to decline slightly in number after 144 h (Fig. 3a). Control groups in comparison to groups treated with Pluronic solutions (without drug incorporation) demonstrate some loss of proliferation in the first 24 h, but later regain normal proliferation ability (Fig. 3b). However, cells treated with Pluronic solutions incorporating doxorubicin demonstrate very significant losses of cell proliferation ability. Most notable within these groups are those treated with 10% Pluronic with 10 μg/ml and 1% Pluronic with 10 μg/ml (Fig. 3c).

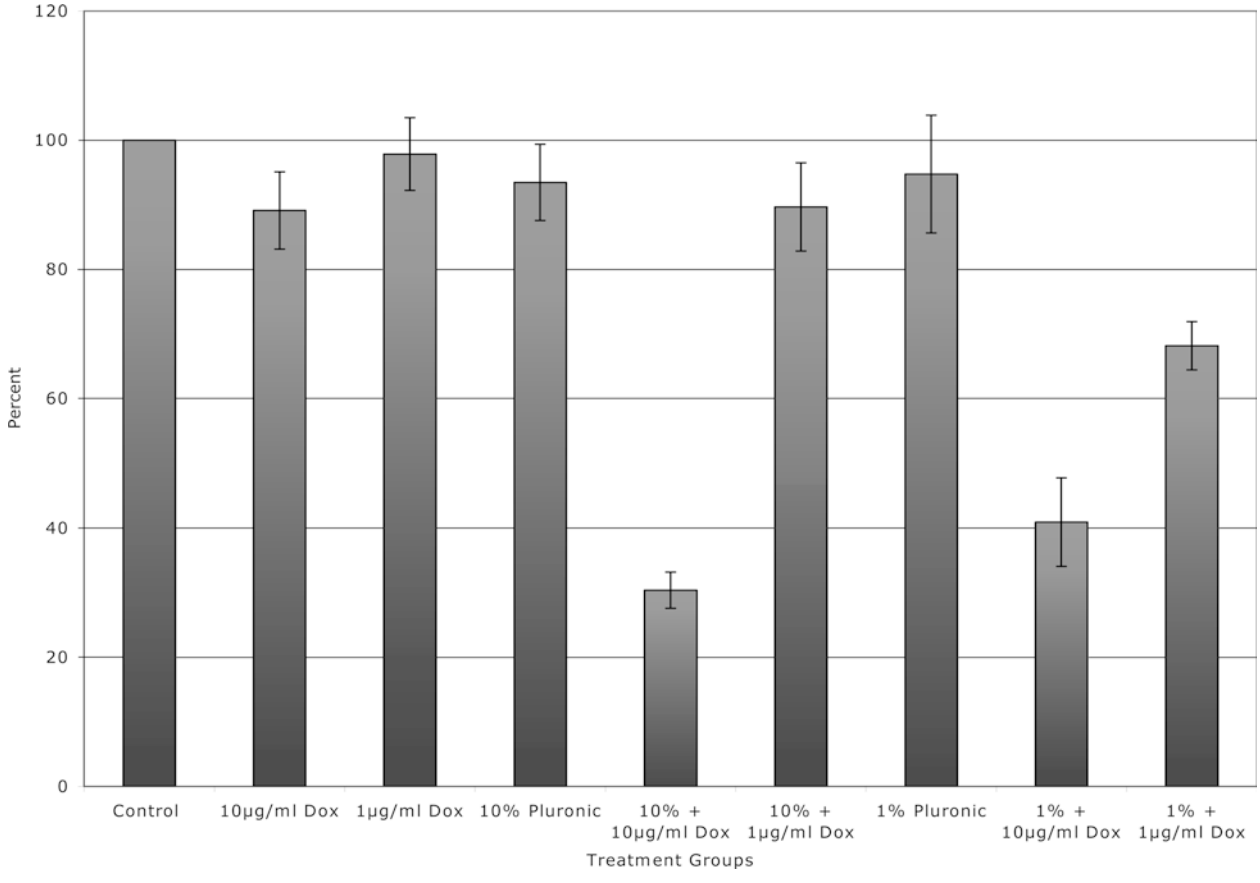

Fig. 1 Comparison of cell proliferation ability after a 144-h incubation period. Percents diagrammed are based on absorbance of samples at 490 nm in comparison to a control group after treatment with Promega One Step Solution. Results are average percentages ± SE

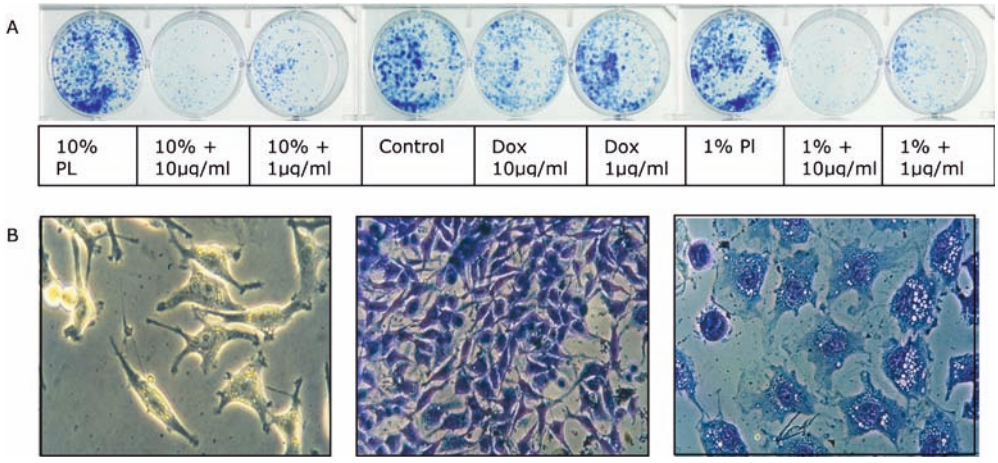

Fig. 2 **a** Colony appearance of control and treated groups 144 h after treatment. Groups treated with a combination of Pluronic polymer solution and doxorubicin demonstrate a distinctly inhibited proliferation ability. **b** The first photo demonstrates normal MatLu cells in culture. The middle photo shows control group cells stained with methylene blue and the last photo shows cells stained with methylene blue after treatment with 10 μg/ml doxorubicin in 10% Pluronic solution, after the 144-h incubation period. The cells in the last photo demonstrate characteristics of cellular senesence

Statistical analysis using ANOVA and the Tukey multiple comparison test disclosed significant differences (ANOVA, $p < 0.05$) between the groups treated with 10 μg/ml doxorubicin in combination with either 10 or 1% Pluronic solution and the rest of the groups in the experiment. There were additional significant differences between these two groups.

Discussion

In recent years, the aim of investigators has been to search for optimal methods of targeting and delivering therapeutic agents to cancer cells while simultaneously protecting normal cells against toxicity. As, in contrast to bacteria and viruses, cancer cells are not foreign to the body, treatment specifically aimed at tumors has been a difficult process. Most cytotoxic treatment modalities focus on drugs that act on the proliferative ability of the cell. However, these drugs also affect the proliferative ability of normal cells, hence the search for a reliable procedure that targets the drugs only at cancer cells. Options for targeting drugs to cancer cells include antigen targeting therapy [18], passive carriers [10] and pro-drug directed therapy [9, 24]. The idea behind these therapies is to maximize the use of properties of tumors that differ from normal cells.

One main feature of tumors different from normal tissue is the character of the blood vessels. In rapidly growing, solid tumors, vasculature may be defective in numerous ways. Additionally, cellular adhesion can be poor, as well as the lymphatic drainage of the tumor site. These characteristics of solid tumors can be and have been utilized in the concept of passive targeting [16]. Passive targeting research has investigated the use of carrier molecules such as polymers [17], liposomes [10], and protein conjugates [9, 24].

The major result of this study has demonstrated increased cytotoxicity of doxorubicin incorporated with micelles when delivered to prostate carcinoma cells in contrast to standard doxorubicin. By incorporating doxorubicin into Pluronic micelles, the cytotoxic activity increased over threefold. Although several studies have examined the potential of Pluronic micelles for use as drug carriers, this study is the first to examine their effect on prostate cancer—a cancer with a recognized low mitotic rate. A key to the results presented here is the time of incubation after treatment. No obvious effect from micelle incorporation with doxorubicin was seen until 24 h after treatment and significant effects were not observed until 144 h after treatment. This trend implies that the micelle/doxorubicin is taken up by the cell, most likely through endocytosis, and then processed by cellular mechanisms, either in the cytosol or the nucleus. This process then allows a greater accumulation of doxorubicin in the cell, which is key for producing cytotoxicity in cells with a low mitotic activity. Not only accumulation but also maintenance of doxorubicin levels inside the cell or nucleus for a longer time could enable better intercalation of the drug within the DNA. However, doxorubicin is also thought to function by numerous other methods that could be aided by a higher concentration and longer presence within the cell. For example, the enzymatic reduction of large quantities of doxorubicin that results in the production of free radicals may contribute to the high inhibition of cellular proliferation ability seen in cells treated with micelles incorporated with 10 μg/ml doxorubicin.

Determination of the process of proliferation inhibition by micelles incorporated with doxorubicin will assist optimization of this treatment modality. Future in vivo animal trials will contribute to the theory that solid tumors exhibit characteristics that allow a greater accumulation of micelles.

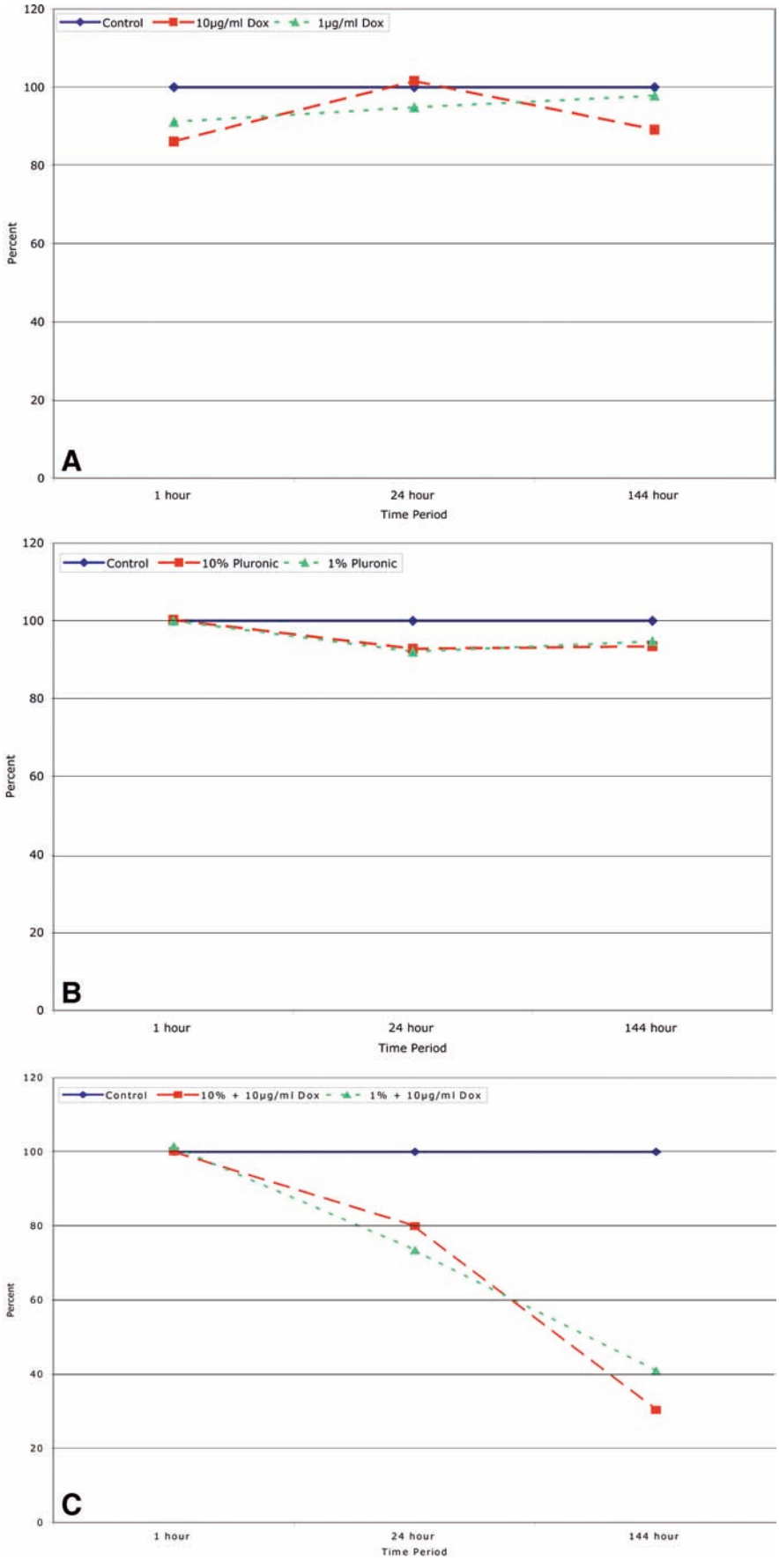

Fig. 3a–c Trends in cell proliferation ability over a 144-h period. **a** Control vs. treatment with doxorubicin alone. **b** Control vs. treatment with Pluronic solution alone. **c** Control vs. treatment with Pluronic micelles incorporated with doxorubicin

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