Ultrasound-Induced Hyperthermia Increases Cellular Uptake and Cytotoxicity of P-Glycoprotein Substrates in Multi-Drug Resistant Cells

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Purpose. Localized hyperthermia has been shown previously to augment the cytotoxicity of some lipophilic anticancer drugs. Because many of the substrates for the multi-drug resistance (MDR) transporter P-glycoprotein (P-gp) are lipophilic in nature, studies were conducted to test the hypothesis that hyperthermia induced by ultrasound could also increase cellular uptake and cytotoxicity of P-gp substrates by P-gp-expressing cells.

Methods. To test this hypothesis, we studied the effects of hyperthermia and ultrasound on cellular accumulation of putative P-gp substrates, rhodamine 123 (R123) and doxorubicin (DOX), and cytotoxicity of DOX in the parent and MDR variants of two human cancer cell lines.

Results. Treatment of cells with hyperthermia or ultrasound (20 min at 41°C) both caused a significant increase over controls (no ultrasound treatment) in R123 and DOX accumulation in the parent and MDR lines of MV522 and KB cells. Ultrasound also substantially increased the antiproliferative effects of DOX in both the parent and MDR variants of MV522 and KB cell lines when compared with controls. Our results also indicated that ultrasound exerted a much greater effect on cellular accumulation of R123 and DOX and cytotoxicity enhancement of DOX in the MDR variants than putative P-gp antagonist such as verapamil.

Conclusion. The present results point to the potential use of ultrasound-induced hyperthermia as a much safer alternative to P-gp antagonist for reversal of MDR.

KEY WORDS: ultrasound; hyperthermia; multi-drug resistance; MDR; P-glycoprotein; P-glycoprotein modulating agents; verapamil; anticancer drugs.

INTRODUCTION

Hyperthermia (HT) has been used in the clinical management of various cancers (1). Because the focus of ultrasonic waves can be localized and because its intensity level is relatively easy to control, the use of ultrasound for enhanced cancer therapy has been the subject of much cancer research. In most of these studies, ultrasound has either been investigated for direct treatment of small and localized cancerous tumors (2,3) or as adjuvant therapy to increase the efficacy of radiotherapy (4) and chemotherapy (5). The difference between these two treatment modalities lies in the levels of energy, as well as the final treatment temperature used. For instance, acoustic intensities and final treatment temperatures in the range of thousands of watts/cm^2 and 98°C are often needed for direct treatment of cancerous tumors (6). Because of this extreme temperature and energy, this form of HT is often referred to as thermal therapy (7). In contrast, ultrasound ranging from 0.2 W/cm^2 to several watts produces a mild increase in temperature (41 to 45°C) and enhances the cytotoxicity of a large number of anticancer agents (8–11). Although the mechanisms for such enhanced cytotoxicity are still under debate, one possible explanation is that HT increases cellular uptake of these anticancer drugs (9,11–14). Interestingly, close examination of these anticancer drugs has revealed that they are essentially low-molecular-weight hydrophobic drugs.

Because P-glycoprotein (P-gp), which is one of the best-understood mechanisms of multi-drug resistance (MDR) (15), recognizes and extrudes a diverse class of low-molecular-weight hydrophobic drugs (16), it will be interesting to ascertain whether HT produced by ultrasound could increase cellular uptake of P-gp substrates and thereby reduce the clinical effects of MDR. To examine this possibility, we first studied how HT or ultrasound at 41°C effects the cellular uptake of P-gp substrates rhodamine 123 (R123) and DOX in the parent and MDR variants of two human cancer cell lines, MV522 and KB. Using these same cell lines, we then studied the effects of HT or ultrasound on the cytotoxicity of DOX. Our results indicated that both methods were able to increase cellular uptake of R123 and DOX in the parent and MDR variants of the two cell lines. Furthermore, the cytotoxicity of DOX was enhanced significantly by treatment with HT or ultrasound. Most importantly, the enhancement in uptake of R123 and DOX, as well as in the cytotoxicity of DOX, produced by HT or ultrasound is far better than that produced by the P-gp antagonist, verapamil. Together, these data suggest a novel way to increase uptake of P-gp substrates in MDR cells, as well as to clinically manage MDR.

MATERIALS AND METHODS

MV-522 and KB-3-1 Cell Line Cultures

MV522 (human metastatic lung carcinoma) and its MDR variant MV522/Q6 cells (a transfectoma that expresses high levels of the MDR1 gene-encoded 170-kd P-glycoprotein) were provided by Dr. Michael J. Kelner (University of California, San Diego, CA) (17). Dr. Michael Gottesman (National Cancer Institute, National Institute of Health) provided KB-3-1 (human epidermoid carcinoma) and its MDR variant KB-V1 cells (18). All cell lines were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air in minimal essential medium (MEM) supplemented with 10% fetal calf serum and 2 mM glutamine. To maintain the MDR characteristics, cell culture media for MDR sublines MV522/Q6 and KB-V1 were supplemented with 40 ng/mL and 1 μg/mL of vinblastine (VIN), respectively.

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Ultrasound Dosimetry and Apparatus

A commercial therapeutic ultrasound unit (Rich-Mar Model 25, Inola, OK) was used for generating high-frequency (1 MHz) and low-intensity ultrasound. To verify the uniformity of the sound field of the transducer head, a two-dimensional intensity map was generated. This was obtained by positioning a pizoelectric hydrophone at a distance of 0.5 cm in front of the transducer. The hydrophone was then moved in a x–y pattern across the face of the transducer, covering a 3 × 3 cm area. The data indicated that the sound field in the central area of the ultrasonic beam (1.2 cm diameter) was uniform. The accuracy of the power output from the ultrasound unit was measured by the radiation balance technique using a commercially available radiation balance (UPM DT-10 Ultrasound Powermeter, Ohmic Instruments, Easton, MD). The measured power (watts/cm^2) corresponded to the instrument settings.

Ultrasound Exposure

Figure 1 shows a schematic diagram of the set-up used to expose MV522, KB-3-1, and their MDR variants *in vitro* to ultrasound. Essentially, a 37°C circulating water bath was placed directly into a 10 × 33 mm tissue culture dish containing 2.5 × 10^5 to 1 × 10^6 cells in serum-free media. The cells were exposed to continuous-wave ultrasound by immersing the head of the transducer into the water bath. The water bath couples the ultrasonic waves, eliminating any possibility of heat contribution from nonultrasound sources, such as inefficient electrical conversion to ultrasound by the transducer, and assuring that HT in the current study was derived mainly from ultrasound. To optimize ultrasound exposure, the cells were positioned in the near field of the ultrasound beam, which was measured at 0.5 cm from the plane of the transducer head. In all studies, ultrasound exposure (0.4 watts/cm^2 at MHz) was for a period of 20 min, and the duty cycle was varied from 50% to 100% to maintain the treatment temperature at 41°C throughout the entire study.

Ultrasound-Induced Hyperthermia on Cellular Accumulation of R123 and Doxorubicin

Parent or MDR variants of MV522 and KB cells were seeded in 10 × 33 mm tissue culture dishes at 1 × 10^6 cells/dish in VIN-free MEM 1 day before the experiments. Before the experiments, cells were washed twice with 37°C serum-free MEM and replaced with fresh serum-free MEM containing no drug, 4 μM R123, or 25 μM DOX. Subsequently, the cells either received no treatment (37°C) or treatment with ultrasound or 41°C for 20 min. As control, another group of cells was treated with serum-free media containing 4 μM R123 plus P-gp antagonist (10 μM verapamil) or 25 μM DOX plus 10 μM verapamil for 20 min at 37°C. Cellular accumulation studies were terminated by aspiration of media and washed three times with 1.0 mL of ice-cold phosphate-buffered saline (PBS). The cells were then solubilized and aliquots of cell lysate solutions collected for protein measurements and fluorescence detection of R123 (Shimadzu RF 1501; λ_{ex} = 492 nn, λ_{em} = 535 nm) (19) or DOX (Shimadzu RF 1501; λ_{ex} = 465 nm, λ_{em} = 580 nm) (20). The amount of protein in each sample was determined by the Pierce BCA method (Pierce Chemical, Rockford, IL). The amount of R123 or DOX was standardized by the protein content of each sample. All experiments were performed in triplicate.

Short-Term Drug Exposure on Growth Inhibition Properties of Doxorubicin

Parent or MDR variants of MV522 and KB cells were seeded in 10 × 33 mm tissue culture dishes at 5 × 10^5 cells/dish in VIN-free MEM 1 day before the experiments. On the day of the experiments, cells were washed twice with 37°C serum-free MEM and treated with serum-free MEM containing various concentrations of DOX (0, 12.5, 25, 50, 100, and 200 μM)

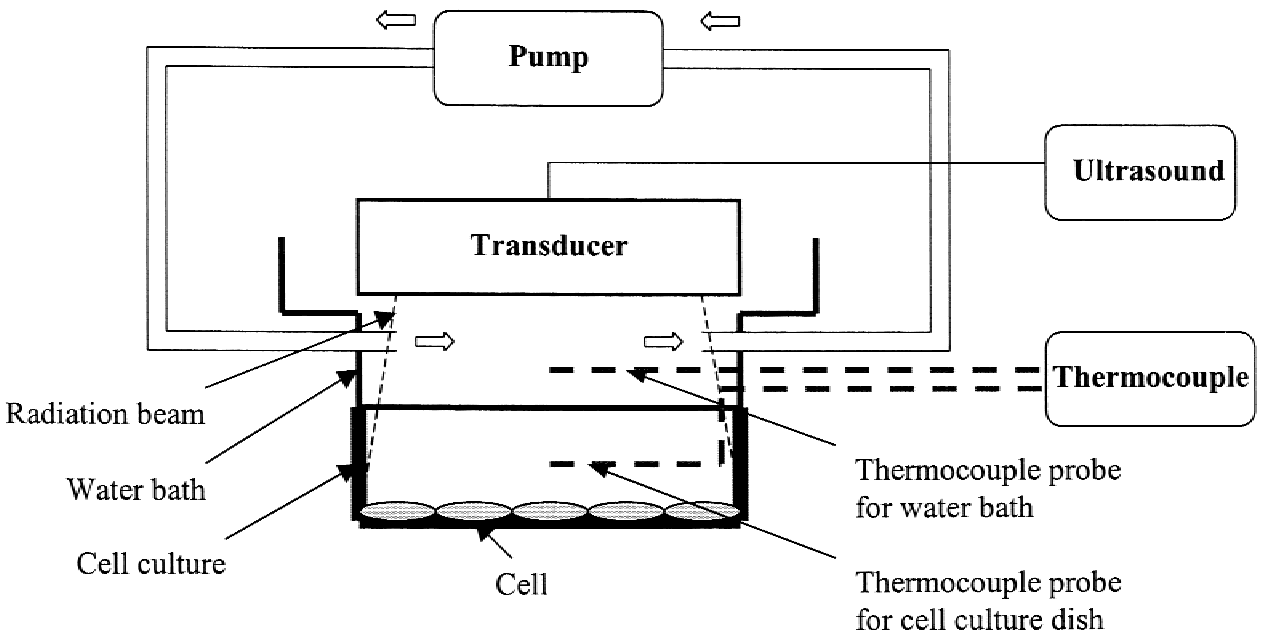

Fig. 1. A schematic diagram of the set up used to expose the cells *in vitro* to ultrasound.

for 20 min at 37°C. After drug exposure, cells were washed three times with 5 mL of room temperature MEM and replaced with 4 mL of pre-warmed (37°C) MEM containing 10% FBS. Cells were allowed to grow for 72 h, after which they were collected to determine cell growth and viability (through trypan blue exclusion). Briefly, both suspended cells in culture media (due to detachment from tissue culture dish) and adhered cells collected after trypsinization with 300 μL of 0.25% trypsin/0.1% EDTA solution were combined and centrifuged. Cell pellets were later resuspended in 5 mL of MEM and 100 μL of 2% trypan blue were then added. After 1 min of incubation and gentle mixing, 10 μL of cell suspension were collected and viable cells were counted by the hemocytometry technique. All experiments were performed in triplicate. The percent of inhibition was determined according to the equation:

$$\% \text{ Inhibition} = \left[1 - \frac{\text{Counts of viable drug exposed cells}}{\text{Counts of viable non–drug exposed cells}} \right] \times 100$$

Ultrasound-Induced Hyperthermia on the Cytotoxicity of Doxorubicin

Parent or MDR variants of MV522 and KB cells were seeded in 10 × 33 mm tissue culture dishes at 2.5×10^5 cells/dish in VIN-free MEM 1 day before the experiments. Prior to the experiments, the cells were washed twice with 37°C serum- free MEM and replaced with DOX-loaded serum-free MEM using the following combinations: 12.5 μM DOX for MV522 and KB-3-1 cells, 25 μM DOX for MV522/Q6, and KB-V-1 cells. Subsequently, the cells either received no treatment (37°C) or treatment with ultrasound or HT at 41°C for 20 min. To compare the effects of ultrasound with P-gp antagonist, another group of cells was treated with serum-free media containing DOX plus verapamil for 20 min at 37°C using the following combinations: 12.5 μM DOX plus 10 μM verapamil for MV522 and KB-3-1 cells and 25 μM DOX plus 10 μM verapamil for MV522/Q6 and KB-V-1 cells. As controls, a group of cells was treated with 10 μM verapamil for 20 min at 37°C or ultrasound at 41°C for 20 min. After drug exposure, cells were washed three times with 5 mL of room temperature MEM and replaced with 4 mL of pre-warmed (37°C) MEM containing 10% FBS. Cells were allowed to grow for 72 h and then collected to determine cell growth and viability (through trypan blue exclusion) using the procedure as described above. All experiments were performed in triplicate.

Statistical Analysis

All data are presented as mean ± SD. Mean values were calculated using the triplicates of at least two experiments. Treatment groups were compared with controls using Student's unpaired *t* tests. $P < 0.05$ was considered significant.

RESULTS

Effect of Ultrasound-Induced Hyperthermia on Cellular Accumulation of R123 and Doxorubicin

Figure 2 shows that HT and ultrasound produced a similar increase in R123 and DOX uptake in both the parent and MDR variants of MV522 and KB cell lines when compared with controls (37°C). Furthermore, treatment with HT or ultrasound resulted in a much larger increase in uptake of R123 and DOX in the parent cells (2- to 6-fold) than in the MDR variants (1- to 2-fold). Most interestingly, both methods appeared to exert a much greater effect on cellular uptake of R123 and DOX than putative P-gp antagonist verapamil does in the MDR variants. As opposed to the 1- to 2-fold increase seen with HT, incubating MDR cells with 10 μM verapamil resulted in either no or less than 10% increase in R123 and DOX uptake.

Effect of Short-Term Drug Exposure on Growth Inhibition Properties of Doxorubicin

The effect of short-term drug exposure on growth inhibition properties of DOX in the parent and MDR variants of MV522 was studied. The goal was to identify optimal drug concentrations to use in a subsequent ultrasound cytotoxicity study, which requires a brief ultrasound exposure time. An optimal concentration in that context refers to a concentration of drug that is neither too low to induce any inhibition nor too high to induce complete growth inhibition. Performing studies at both extreme concentrations might inadvertently mask any potential ultrasound effects on growth inhibition. In the case of the present study, optimal concentrations are chosen as those that fall in the steepest part of the growth inhibition curves (hence higher cellular drug response should be expected) or those that induce 35–50% growth inhibition, depending on which specific cell type. Figure 3 shows the effects of 20-min exposure to various concentrations of DOX on growth inhibition in the parent and MDR variants of MV522 and KB cell lines. For the most part, substantial growth inhibition was observed when both the parent cells and MDR variants were treated with DOX. In both cell types, a higher drug concentration was required to induce a similar level of growth inhibition in the MDR variants versus the parent cells. In the case of the KB cell line, the MDR variants were 5-fold more resistant to DOX with respect to the parent cells, based on the drug concentration required to induce a 50% growth inhibition. This is in contrast to the MV522 cell line, where the MDR variants were only 1-fold more resistant to DOX than the parent cells. It is interesting to point out that among the four cell types, KB-V1 cells appear to be the most resistant to the cytotoxic effect of DOX, as treatment of KB-V1 cells with up to 200 μM of DOX could only lead to about a 70% growth inhibition. In contrast, DOX in concentration as low as 50 μM caused a maximum growth inhibitory effect in KB-3-1 cells (~100% inhibition) and MV522 and MV522/Q6 cells (~80% inhibition). Based on this data, the following combinations were chosen for the subsequent ultrasound cytotoxicity studies: 12.5 μM DOX for MV522 and KB-3-1 cells and 25 μM DOX for MV522/Q6 and KB-V-1 cells. Incidentally, these concentrations happened to be within the clinically relevant concentrations of DOX (21).

Effect of Ultrasound-Induced Hyperthermia on Cytotoxicity of Doxorubicin

Figure 4 shows the effects of HT, ultrasound, and verapamil on the cytotoxicity of DOX in the parent and MDR variants of MV522 and KB cell lines. In all cases and regardless of cell types, treatment with HT or ultrasound enhanced the cytotoxicity of DOX and resulted in higher growth inhibition than treatment with drugs alone (37°C). When the cytotoxicity enhancement of HT or ultrasound was compared,

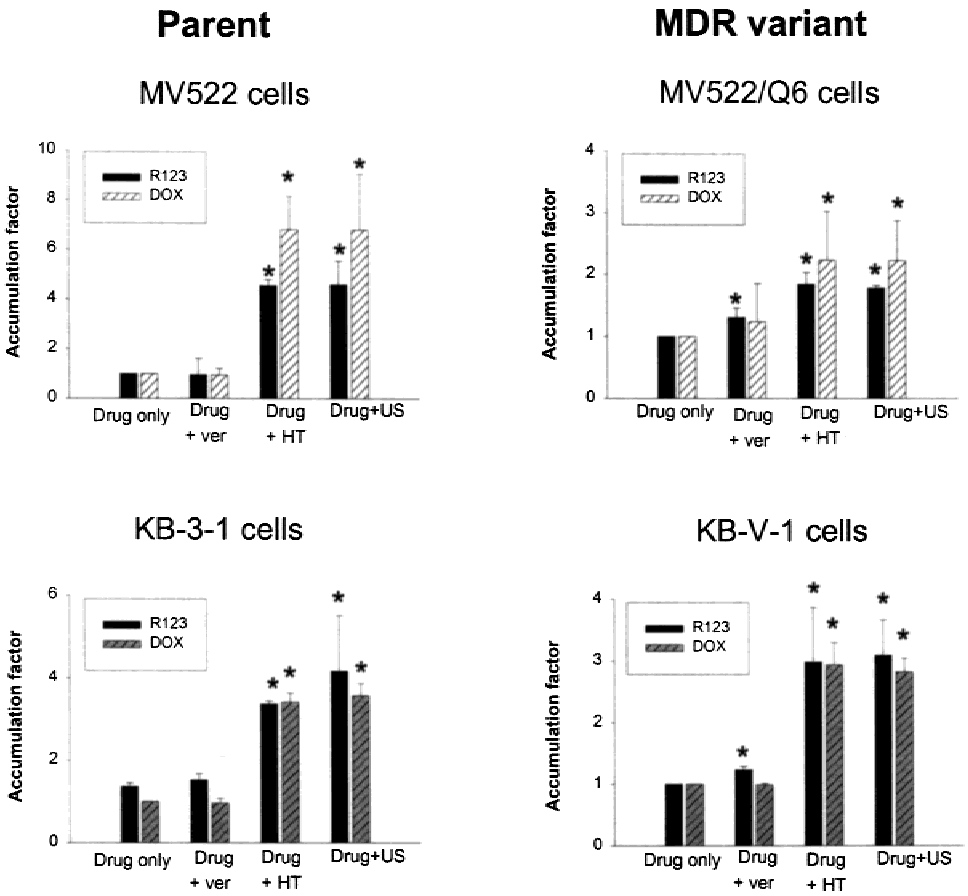

Fig. 2. Effect of HT (41°C), ultrasound (US), or verapamil (ver) on cellular uptake of R123 and doxorubicin (DOX) by the parent or multi-drug resistance variants of MV522 and KB cell lines. Cellular uptake data are presented as accumulation factor. Accumulation factor for control cells is defined as equal to 1. Accumulation factor for treated cells is defined as ratio of cellular R123 or DOX accumulation in the presence of HT, ultrasound, or verapamil to cellular R123 or DOX accumulation in the absence of HT, ultrasound, or verapamil. All experiments were performed in triplicate. $*P < 0.05$ was considered significant.

no significant difference was observed. Further, incubating the cells with verapamil did not result in any cytotoxic enhancement of DOX in the parent cells, but it did slightly increase the cytotoxicity of DOX in the MDR variants. This enhancement of cytotoxicity, however, is far less than that produced by the HT or ultrasound. The data also demonstrated the relatively harmless effect of short ultrasound exposure, because it did not result in any significant growth inhibition in either the parent or MDR variants of the MV522 and KB cell lines on its own, rather it enhanced the efficiency of the cytotoxic agents.

DISCUSSION

MDR represents a major obstacle to successful chemotherapy of metastatic diseases (22). One of the best-understood mechanisms of MDR is the expression of P-gp encoded by the MDR-1 gene located on chromosome 7. P-gp is a transmembrane, energy-dependent, pump system that extrudes anticancer drugs from cells. P-gp is known to recognize and transport many structurally and functionally unrelated anticancer drugs (16). The role of P-gp in MDR has been implicated both *in vitro* (23) and *in vivo* (2425). Given the clinical impact of MDR on the success of chemotherapy, it is no wonder a great deal of research effort has been directed towards manipulating P-gp expression and function, thereby reducing the clinical effects of MDR.

This study examined the use of HT (41°C) as induced by ultrasound to increase cellular uptake and cytotoxicity of P-gp substrates. Ultrasound was chosen in the present study to induce HT because ultrasound can be easily focused to reach various heating depths with frequency variations (26). This property makes ultrasound a very attractive energy source for future interstitial HT therapy. In addition, the present work is the result of the combination of two separate observations. For instance, previous work by others (9,11–14) and within our laboratories (unpublished results) has indicated that the treatment of cells with HT can increase cellular uptake and cytotoxicity of low molecular weight hydrophobic drugs. When this information was combined with the knowledge that the majority of the P-gp substrates are low molecular weight hydrophobic molecules (16), we surmised the possibility that HT might enhance cellular uptake of P-gp substrates and, therefore, be used to reduce the clinical effect of MDR. Surprisingly, not many studies today have investigated the use of HT to increase cellular uptake and cytotoxicity of P-gp

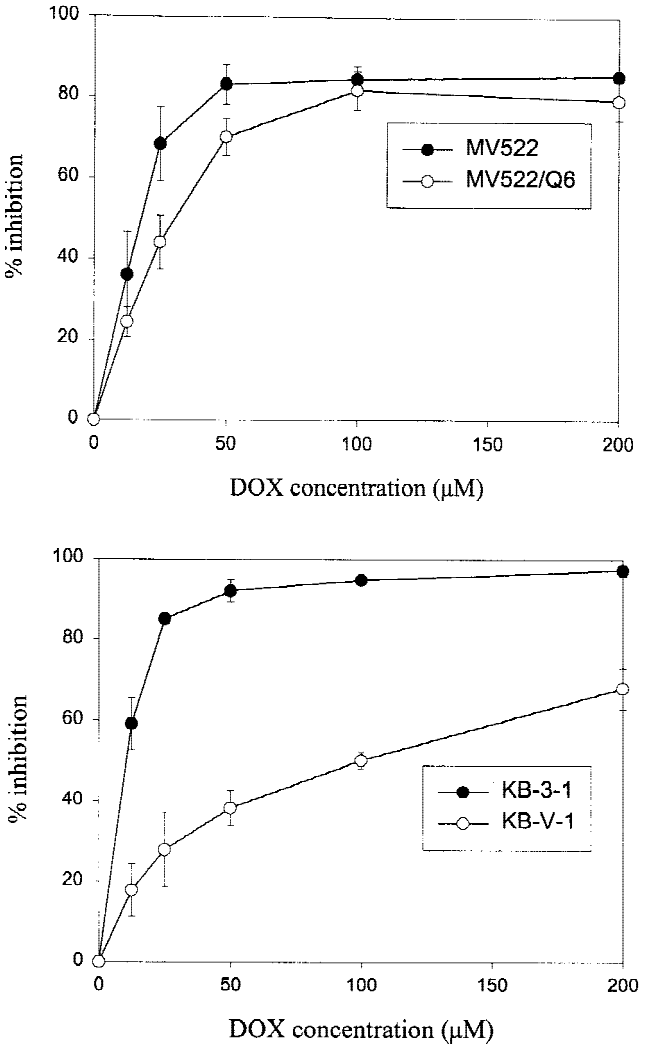

Fig. 3. Effect of short-term doxorubicin exposure on cell growth of parent and multi-drug resistance variants of MV522 and KB cell lines. Data were expressed as percent inhibition calculated by the following formula: % inhibition = [1 – (counts of viable drug-exposed cells/ counts of viable non-drug exposed cells)] × 100. Cell viability was determined by hemocytometry technique after trypan blue staining. All experiments were performed in triplicate.

substrates. Bates and Mackillup, however, did explore the use of HT (40 to 50°C) to overcome drug resistance associated with increased drug efflux (27,28). But their results were negative in that at temperature normally used for HT (41–45°C) (5), significant increase in drug uptake and cytotoxicity was only observed in resistant cells for melphalan (28), which is not a P-gp-recognized substrate (29), and not for DOX. HT at much higher temperature (50°C), however, did induce a smaller change in the intracellular level of DOX in resistant cells in the same studies. But this temperature is not seen as attractive in future thermochemotherapy. This is because HT at such a high temperature is known to induce cell death in most cells. Thus, inadvertent killing of innocent bystander cells can be a major possible side effect. Although it is currently unclear why our results differ from that of Bates *et al.* when temperatures in the range of 41–43°C were used, we speculate that there are two possible explanations (27,28). The first is that the drug concentrations used in the two studies are different (25 μM vs. the 1.8 μM used in the studies of Bates and Mackillop). The second is that the resistant cells used by Bates *et al.* might have a higher P-gp expression. Taken together, this would imply a more efficient efflux of DOX by resistant cells in the studies performed by Bates *et al.,* thus resulting in insignificant uptake of DOX under the influence of HT.

Although HT and ultrasound were shown to increase R123 and DOX uptake in both the parent and MDR variants of MV522 and KB cell lines (Fig. 2), it also increased the efflux of R123 and DOX from the parent and MDR cells (data not shown). These results are in agreement with the studies performed by Bates and Mackillop (27,28). Despite the increase in efflux at higher temperature, the intracellular level of drug in both the parent and MDR cells increased with temperature when extracellular drug was present. When the extent of increase of drugs for the parent and MDR variants in both cell types was compared, a notable difference was observed (Fig. 2). For instance, both treatments caused a 2- to 6-fold increase in R123 and DOX in the parent MV522 or KB cell lines, but it only caused a 1- to 2-fold increase in their MDR counterparts. These results make sense, as the presence of P-gp should reduce the overall cellular uptake of drugs in the MDR cells. Although there is no direct evidence, our findings that uptake of drugs in the MDR variants is much lower than that of the parent cells imply the following two things: 1) brief exposure of cells to ultrasound did not seem to alter P-gp activity extensively and 2) the mechanism for HT-induced increase in drug uptake is mostly a result of enhanced membrane permeability rather than a modulation of P-gp activity.

Treatment with HT or ultrasound also enhanced the cytotoxicity of DOX in both the parent and MDR variants (Fig. 4). Because DOX is a putative P-gp substrate (29), these findings imply that ultrasound could potentially increase the cytotoxicity of P-gp recognized anticancer drugs and, therefore, improve the clinical outcomes of chemotherapy in tumors where P-gp is the predominant mechanism of MDR. Because of these exciting findings and implications, it is both interesting and important to comment on this enhancement effect. In the presence of HT or ultrasound, DOX was able to induce about 80% of growth inhibition in the MV522, MV522/Q6 and KB-3-1 cells and approximately 50% in KB-V1 cells (Fig. 4). Thus, when compared to the effects of treating the cells with DOX alone, HT or ultrasound improves the effect of DOX by approximately 40–70% in MV522, MV522/Q6, and KB-3-1 cells, and about 140% in KB-V1 cells. Because HT or ultrasound almost doubled the increase in DOX uptake in KB-V-1 cells, one could at least attribute the enhanced cytotoxicity seen in this cell line to increased cellular uptake of drugs at elevated temperature. However, explaining the enhanced cytotoxicity in terms of increased drug uptake might prove quite tricky for MV522, MV522/Q6 and KB-3-1 cells. For instance, HT increased DOX uptake in these cell lines by more than 2- to 6-fold (Fig. 2), but it only caused a 1- to 2-fold increase in cytotoxicity (Fig. 4). A possible explanation for these paradoxical observations can be obtained in the growth inhibition properties of DOX under short-term drug exposure conditions (Fig. 3). Under these conditions, DOX induced a maximum growth inhibition effect on MV522, MV522/Q6 and KB-3-1 cells and not on KB-V-1 cells (Fig. 3). Because intracellular concentration of DOX increases in a dose-dependent

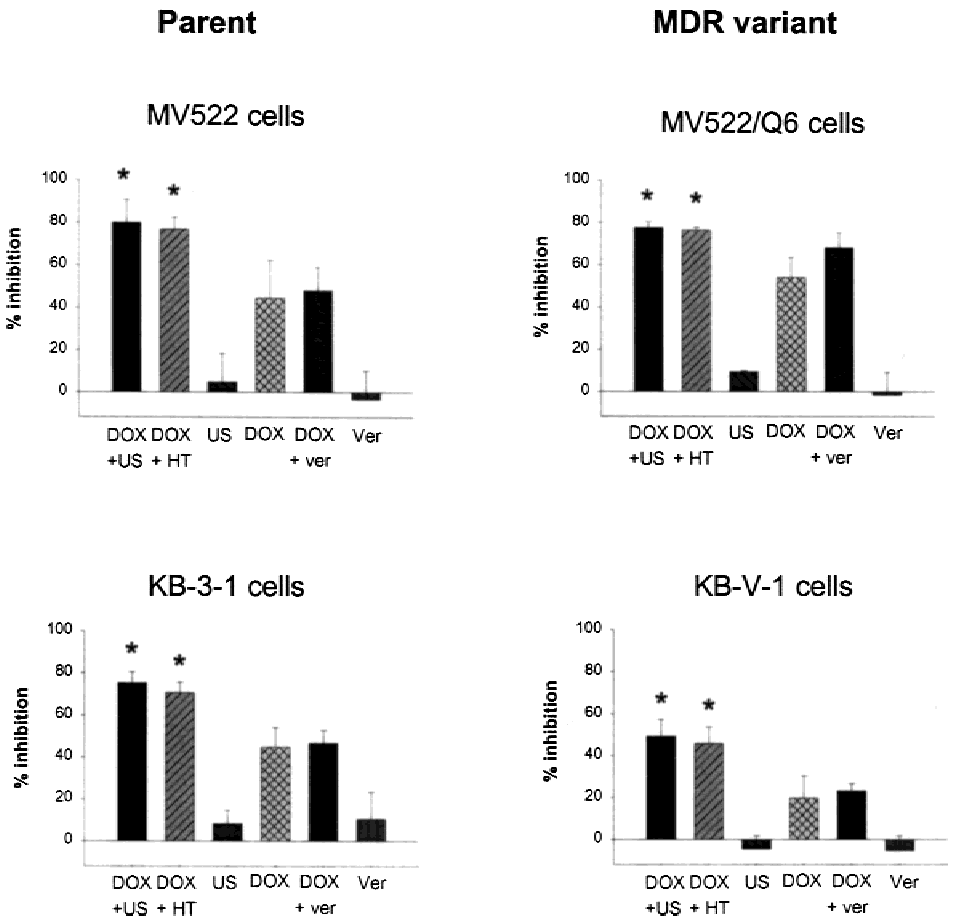

Fig. 4. Effects of HT (41°C), ultrasound (US), or verapamil (Ver) on cytotoxicity enhancement of doxorubicin in the parent and multi-drug resistant variants of MV522 and KB cell lines. Data were expressed as % inhibition calculated by the following formula: % inhibition = [1 – (counts of viable drug-exposed cells/counts of viable non-drug exposed cells)] × 100. Cell viability was determined by hemocytometry technique after trypan blue staining. All experiments were performed in triplicate. **P* < 0.05 was considered significant.

manner with increasing extracellular drug concentration among all cell types (data not shown), these results would imply a cause-and-effect relationship between drug uptake and cytotoxicity for only the KB-V-1 cells, and not MV522, MV522/Q6, and KB-3-1 cells. Thus, it is apparent why the many fold increases in drug uptake in the ultrasound study did not match the increase in growth inhibition in MV522, MV522/Q6, and KB-3-1 cells.

What is most surprising in this study is the finding that treatment of MDR cells (P-gp expression and function confirmed by western blot analysis and R123 efflux assay in the presence or absence of P-gp modulating agents such as 10 μM verapamil) with HT or ultrasound produced a much greater effect on the cellular uptake and cytotoxicity of DOX than that produced by putative P-gp antagonist, verapamil. It has been shown previously that heating might induce reduction in P-gp expression, hence leading to inhibition of drug efflux and enhancement of intracellular drug uptake (30). But this mechanism might prove insufficient to explain our observations for the following two reasons. The first is that reduction in P-gp expression in this study was only observed after cells were preheated at 44°C for 1 h, a temperature and duration that are much higher than the one reported in the present study. The second is that P-gp expression in cells preheated at 41°C and control cells (37°C) was found to be not statistically different. Although it is currently unclear why HT or ultrasound should induce a much greater effect than P-gp modulating agent on cellular uptake of P-gp substrates, we speculate that this difference could lie in how fast the P-gp could handle P-gp substrates as they enter into the cells. In the case of HT-treated cells, drug entry rate is expected to be much faster because of the enhancement effects of HT on cellular permeability. This enhanced drug entry rate might transiently overwhelm the P-gp, which in turn leads to more cellular drug uptake. Because most of the P-gp antagonists currently in use today are cytotoxic because of their lack of specificity (29) and abundance of P-gp in other bodily sites (31), our findings thus point to a much safer means of overcoming the effects of MDR. This is because ultrasound can easily be focused and directed in a non-invasive manner to reach any potential tissue sites, to achieve targeted, drug-to-tumor delivery. Further, since multi-drug resistance associated protein (MRP), which is another MDR drug efflux protein, and P-gp share many of the substrates together (22), ultrasound may also prove useful increasing cellular uptake of substrates of MRP and possibly other putative MDR proteins.

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