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Overcoming MDR by Ultrasound-Induced Hyperthermia and P-Glycoprotein Modulation

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We assessed the effects of combining ultrasound-induced hyperthermia (USHT) with the P-glycoprotein modulator PSC 833 on cellular retention and cytotoxicity of rhodamine 123 (R123) and doxorubicin in the parent and multidrug resistance (MDR) variants of two human cancer lines. USHT significantly increased cellular uptake of R123 and doxorubicin. Without PSC 833, release of R123 and doxorubicin from both USHT-treated and untreated cells was rapid. As expected, PSC 833 (0.5 μM) only slowed their release into the MDR lines. Interestingly, despite the differences in their starting amounts, PSC 833 was effective in prolonging R123 and doxorubicin release from both USHT-treated and untreated MDR cells. PSC 833 did not augment the cytotoxicity of doxorubicin in parent lines but did cause a significant increase in cytotoxicity of doxorubicin in the MDR lines. However, the combined effect of USHT and PSC 833 on cytotoxicity of doxorubicin far exceeded that produced by USHT or PSC 833 alone.

Key Words: **P-glycoprotein; rhodamine 123; multidrug resistance; MDR; efflux; uptake; cancer; doxorubicin; ultrasound; hyperthermia.**

Multidrug resistance (MDR) represents a major obstacle to successful chemotherapy of metastatic diseases (1). One of the best-understood mechanisms of MDR is that of overexpression of P-glycoprotein (P-gp) (2). This 170-kDa plasma membrane protein belongs to a larger family of ATP-binding cassette proteins and confers resistance to tumor cells by extruding many structurally and functionally unrelated hydrophobic anticancer drugs using the energy of ATP hydrolysis (3). In an effort to restore cytotoxicity to many of these anticancer drugs, many agents have been studied for their roles in reversing or modulating P-gp activity (4). In particular, a relatively nontoxic cyclosporin D analog, PSC 833, has been shown to be a potent P-gp modulating agent. Indeed, human clinical trials evaluating continuous infusion of PSC 833 given in combination with anticancer drugs have shown that PSC 833 can reverse MDR. However, the effect of PSC 833 in these studies has been modest and its value in reversing resistance mediated by P-gp remains to be determined (5–8). Thus, parallel search for more effective methods in overcoming MDR due to P-gp are needed.

Since the focus of ultrasonic waves can be localized and 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 these studies, ultrasound has either been investigated for its ability to heat tumor tissues for direct treatment of small and localized cancerous tumors (9, 10) or as adjuvant therapy to increase the efficacy of radiotherapy (11) and chemotherapy (12). In earlier studies, we reported that mild hyperthermia (41°C) as induced by ultrasound (USHT) could be used to increase cellular uptake and cytotoxicity of P-gp substrate doxorubicin in P-gp overexpressing cells (13). However, our recent data did not indicate that USHT could modulate P-gp activity, but rather it enhances cellular uptake and cytotoxicity of doxorubicin by mostly a thermal effect that increases membrane permeation to P-gp substrates (14). To that effect, it is reckoned if the enhanced drug uptake could be maintained in the P-gp expressing cells longer, an augmented binding of the anticancer drug to its target and, therefore, increased cytotoxicity might be obtained. Here, utilizing the parent and P-gp over-expressing MDR human metastatic lung carcinoma (MV522) (15) and epidermoid carcinoma (KB) cell lines (16), studies were carried out to examine whether combination of USHT, which in-

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creases cellular uptake of P-gp substrate, with P-gp modulating agent PSC 833, which inhibits P-gp mediated drug extrusion, would further augment the effect of anticancer drug and P-gp substrate doxorubicin.

MATERIALS AND METHODS

Materials. Cell culture medium and fetal calf serum (FCS) were obtained from GIBCO (Grand Island, NY). Rhodamine 123 (R123) and doxorubicin were purchased from Sigma Chemical Co. (St. Louis, MO). PSC 833 was purchased from Norvartis (Basel, Switzerland). All other reagents, unless specifically stated otherwise, were purchased from Sigma Chemical Co.

Cell cultures. The parent and MDR variant of MV522 and KB cell lines were used as previously described (13). 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% FCS and 2 mM glutamine. To maintain the MDR characteristics; cell culture media for MDR sublines MV522/Q6 and KB-V-1 were supplemented with 40 ng/ml and 1 μg/ml of vinblastine, respectively.

Ultrasound apparatus and exposure. The system used to expose the cells *in vitro* to USHT has been previously described (13). In all studies, ultrasound exposure (0.4 W/cm^2 at 1 MHz) was for a period of 20 min, and treatment temperature was maintained at 41°C. The accuracy of the power output (W/cm^2) from the ultrasound unit was confirmed by the radiation balance technique using a commercially available radiation balance (UPM DT-10 Ultrasound Powermeter, Ohmic Instruments, Easton, MD).

Evaluation of cytotoxic effect of PSC 833. The parent and MDR variant of MV522 or KB cell lines were seeded in six-well plates at 2.5×10^5 cells/well in 10% FCS MEM without vinblastine 1 day before experiments. Prior to experiments, cells were washed twice with 37°C serum-free MEM. Subsequently, cells were exposed to 4 ml MEM containing 10% FCS or 4 ml MEM containing 10% FCS and various concentrations of PSC 833 for 72 h at 37°C. After drug exposure, the cells were collected for determination of cell growth and viability using our previously described procedure (13).

Cellular efflux of R123. The parent and MDR variants of MV522 and KB cell lines were seeded in six-well plates at 1×10^6 cells/well in 10% FCS MEM without vinblastine 1 day before experiments. Prior to the experiments, cells were washed twice with 37°C serum-free MEM. Subsequently, the cells were incubated with 1 ml serum-free MEM containing 4 μM R123 for 60 min at 37°C. After the incubation was complete, the culture medium was aspirated gently, and cells were washed three times with 1 ml of 37°C serum-free MEM to remove extracellular R123. After the washing was complete, cells were incubated at 37°C with 1 ml of either MEM containing 10% FCS or MEM containing 10% FSC and 0.5 μM PSC 833. At designated time intervals, culture medium was removed and cells were washed three times with 1.0 ml of ice-cold PBS. The cells were then solubilized and assayed for R123 and protein contents as previously described (13).

Combinative Use of USHT and PSC 833 on cellular retention of R123 and doxorubicin. Parent or MDR variant of MV522 and KB cells were seeded in 10 × 33-mm tissue culture dishes at 1×10^6 cells/dish in 10% FCS MEM without vinblastine 1 day before the experiments. Prior to the experiments, cells were washed twice with 37°C serum-free MEM and replaced with fresh serum-free MEM containing either 4 μM R123 or 25 μM doxorubicin. Subsequently, the cells either received no treatment (37°C) or treatment with USHT at 41°C for 20 min. Afterward, the media for both treated and untreated cells was removed and the cells were rinsed three times with 37°C serum-free MEM. The cells were then incubated at 37°C with 1 ml serum-free MEM containing the presence or absence of 0.5 μM PSC 833 for 60 min. After the incubation was complete, the culture media was removed and cells were washed three times with 1 ml of ice-cold serum-free MEM to remove any extracellular R123 or doxorubicin. The cells were then solubilized and aliquots of cell lysate solutions collected for protein measurements and fluorescence detection of R123 or doxorubicin as previously described (13).

Combinative use of USHT and PSC 833 on cytotoxicity of doxorubicin. Parent or MDR variant of MV522 and KB cells were seeded in 10 × 33-mm tissue culture dishes at 2.5×10^5 cells/dish in 10% FCS MEM without vinblastine 1 day before the experiments. Prior to the experiments, the cells were washed twice with 37°C serum-free MEM and replaced with serum-free MEM containing either no drug or 25 μM doxorubicin. Subsequently, the cells either received no treatment (37°C) or treatment with USHT at 41°C for 20 min. Afterward, the medium for both the treated and untreated cells was removed and the cells were rinsed three times with 37°C serum-free MEM. The cells were then incubated at 37°C with 1 ml serum-supplemented MEM (10% FCS) containing the presence or absence of 0.5 μM PSC 833 for 24 h, after which the concentration of PSC 833 in the tissue culture dishes was reduced to 0.125 μM by addition of 3 ml serum-supplemented MEM (10% FCS). The cells were allowed to grow for an additional 48 h, after which they were collected for determination of cell growth and viability as described above.

Statistical analysis. All data are presented as means ± SD. Mean values were calculated from at least three experiments. The effects of USHT and/or PSC 833 were compared using analysis of variance in combination with Duncan grouping or Scheffé's test as post hoc analyses. Time-dependent data within each treatment group were analyzed using repeated-measures analysis of variance (longitudinal analysis); effects between groups at each time point were compared using Student's unpaired *t* test or analysis of variance as appropriate (vertical analysis). All statistical comparisons and distribution statistics were calculated using the SPSS software package (version 10.0, SPSS Inc., Chicago, IL). $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Cytotoxic and P-Glycoprotein Modulating Effect of PSC 833

PSC 833, which is a high-affinity and noncompetitive inhibitor of P-gp, has been widely used for its ability to reverse P-gp activity and enhance cytotoxicity of chemotherapeutic agents both *in vitro* and *in vivo* (4). However, recent reports have indicated that PSC 833 can also act as a cytotoxic agent (17–19). In that regard, preliminary experiments were carried out to identify a concentration of PSC 833 that is both nontoxic and effective in modulating P-gp activity. Figure 1 shows that incubation of the parent and MDR variant of MV522 and KB lines with PSC 833 both resulted in dose-dependent cytotoxicity. The data indicated that the presence of P-gp renders no protective effect against the cytotoxicity of PSC 833 in MDR cells. These results are rather expected, as PSC 833 is a poor substrate for P-gp-mediated transport (20). In both the parent and MDR variant of KB and MV522 lines, 0.5 μM PSC 833 produced negligible or no effect on cell viability. However, PSC 833-mediated cytotoxicity began as low as 1 μM, with MV522 line displaying more sensitivity to PSC 833 than the KB lines. Based on

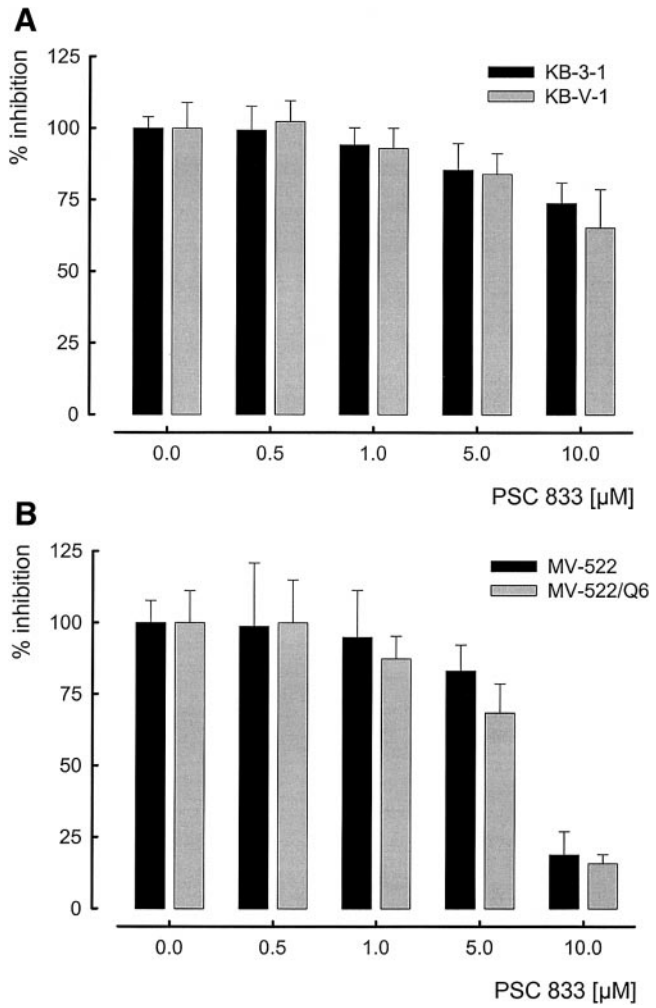

FIG. 1. Effects of different concentrations of PSC 833 on proliferation of the parent and MDR variant of MV522 and KB lines. Data were expressed as percentage inhibition calculated by the formula % inhibition = [1 − (counts of viable drug-exposed cells/counts of viable non-drug-exposed cells)] × 100 and are presented as means ± standard deviations. Cell viability was determined by hemocytometry technique after trypan blue staining. All experiments were carried out in triplicate (means ± standard deviation).

these results, we next examined the effect of 0.5 μM PSC 833 on cellular efflux of P-gp substrate R123 from the parent and MDR variant of KB and MV522 cells preloaded with R123 (Fig. 2). In both the MV522 and KB lines, efflux of R123 was both greater and faster in the MDR than the parent lines. Addition of PSC 833 did not affect R123 efflux in the parent KB and MV522 lines. In contrast, efflux of R123 was significantly reduced by PSC 833 in both MDR variants of the KB and MV522 lines. PSC 833 also significantly extended the cellular retention time of R123 in the MDR cells when compared to controls (no PSC 833). For instance, without PSC 833, approximately 20% of R123 was found to remain inside the KB-V-1 cells after a 2-h release study. The retention of similar amount of R123 by KB-V1 cells, however, was extended by approximately 8 h with the addition of 0.5 μM PSC 833. Taken together, the results indicate that at 0.5 μM, PSC 833 is both nontoxic and effective in modulating P-gp activity. This concentration, however, is slightly lower than the concentration considered being necessary to inhibit P-gp both *in vitro* and *in vivo* (4).

Effect of Combined Use of USHT and PSC 833 on Cellular Retention of R123 and Doxorubicin

We showed that 0.5 μM PSC 833 reduces efflux as well as increases cellular retention time of P-gp substrates in P-gp-expressing cells. As a next step, we determined if this same concentration of PSC 833 could

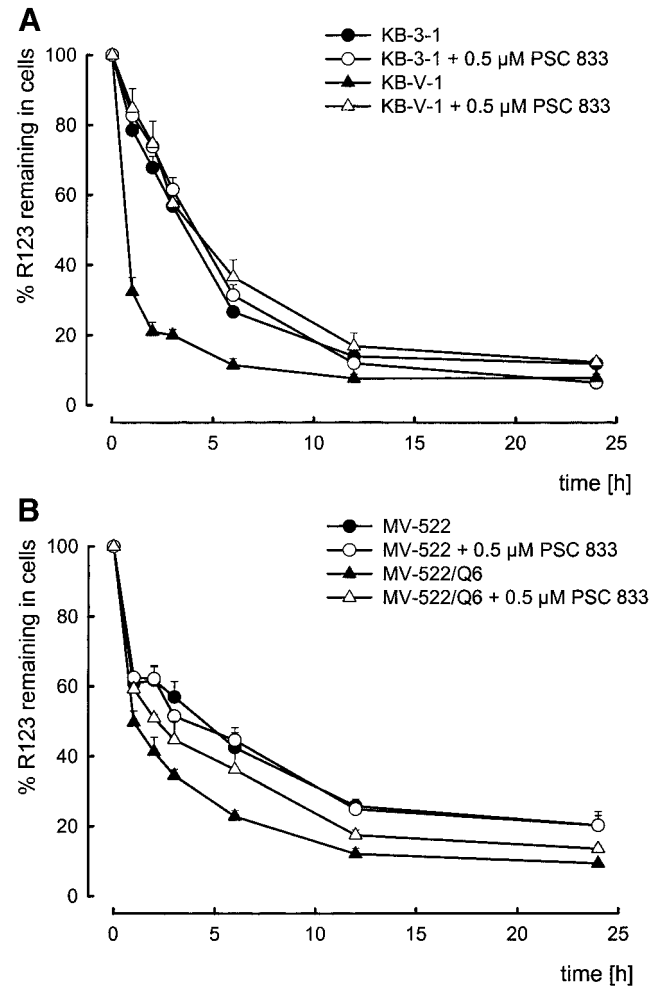

FIG. 2. Effects of absence or presence of 0.5 μM PSC 833 on cellular retention of R123 in the parent and MDR variant of MV522 and KB lines. R123 retention normalized to protein content was expressed as percentage R123 remaining inside the cells. Retention at the beginning of the release study (i.e., at time = 0 h) was defined as equal to 100%. All experiments were carried out in triplicate (means ± standard deviation).

TABLE 1

Effects of PSC 833 on Release of R123 and Doxorubicin from Parent Tumor Cells (KB-3-1 and MV522) and Their MDR Counterparts (KB-V-1 and MV522/Q6) after Loading at 37°C and USHT (41°C)

A

Cells	Substrate	Temperature (°C)	Loading (fluorescence/mg protein)	Release in 1 h without PSC (%)	Release in 1 h with PSC (%)
KB-3-1	R123	37	5.9 ± 0.1	−35.9 ± 7.8	−42.0 ± 1.6
		41 (USHT)	14.9 ± 4.0	−25.5 ± 23.6	−17.2 ± 35.3
	Doxorubicin	37	128.8 ± 13.8	−20.6 ± 11.4	−10.5 ± 10.6
		41 (USHT)	316.8 ± 14.7	−9.8 ± 3.1	−11.0 ± 23.8
KB-V-1	R123	37	5.1 ± 0.6	−78.2 ± 3.0	−63.8 ± 7.9
		41 (USHT)	17.2 ± 3.4	−83.4 ± 1.1	−64.7 ± 5.3
	Doxorubicin	37	117.4 ± 5.8	−58.3 ± 9.8	−22.7 ± 10.5
		41 (USHT)	307.9 ± 47.2	−52.7 ± 5.0	−34.1 ± 13.0
MV522	R123	37	6.2 ± 0.7	−28.8 ± 8.4	−16.7 ± 14.2
		41 (USHT)	13.5 ± 1.4	−27.2 ± 10.5	−26.1 ± 6.7
	Doxorubicin	37	125.8 ± 30.3	−22.6 ± 11.5	−23.9 ± 15.5
		41 (USHT)	278.1 ± 75.8	−13.6 ± 19.7	−28.4
MV522/Q6	R123	37	4.0 ± 0.0	−42.9	−18.5 ± 1.8
		41 (USHT)	11.1 ± 0.8	−43.9 ± 7.1	−2.0 ± 9.7
	Doxorubicin	37	127.0 ± 6.0	−57.2 ± 6.2	−28.3 ± 5.4
		41 (USHT)	207.6 ± 21.4	−29.8 ± 11.8	+7.0 ± 27.5

B

	$P <$			
	KB-3-1	KB-V-1	MV522	MV522/Q6
Doxorubicin				
37°C versus USHT (41°C)				
Loading	0.001	0.001	0.01	0.003
−PSC 833	0.001	0.015	0.008	0.001
+PSC 833	0.003	0.002	0.024	0.001
With versus without PSC 833				
37°C	1.00	0.41	1.00	0.30
41°C (USHT)	1.00	0.06	1.00	0.06
R123				
37°C versus USHT (41°C)				
Loading	0.002	0.001	0.001	0.001
−PSC 833	0.017	0.145	0.005	0.001
+PSC 833	0.005	0.977	0.012	0.001
With versus without PSC 833				
37°C	1.00	0.99	1.00	0.55
41°C (USHT)	0.99	0.42	0.99	0.001

Note. Table 1A shows the effect of USHT on loading of tumor cells and their P-gp-overexpressing versions with R123 and doxorubicin and the effect of USHT and the P-gp modulator PSC 833 on release of the drugs from the cells after 1 h (in percentage of intracellular concentration change of R123 and doxorubicin after loading). Data are presented as means ± standard deviation ($n = 3$). Table 1B shows the statistical comparison of the results presented in Table 1A. An analysis of variance in combination with Scheffé's test as post hoc analysis was used. The analyses of variance showed differences with $P < 0.001$. The results of Scheffé's test are presented in Table 1B. Abbreviations used: R123, rhodamine 123; USHT, ultrasound-induced hyperthermia.

prolong cellular retention of P-gp substrates previously loaded into P-gp expressing cells by USHT. The results from such studies are shown in Table 1. As previously shown before (13), brief USHT treatment (20 min) resulted in significant increase in cellular loading of R123 and doxorubicin, when compared to controls (no USHT treatment) (see Table 1B). Without PSC 833, both USHT-treated and untreated cells rapidly lost their R123 and doxorubicin contents, with faster efflux occurring in the MDR lines than the parent lines. As expected, inclusion of PSC 833 in the release study produced little effect on efflux of R123 and doxorubicin from the parent lines, but it significantly reduced R123 or doxorubicin efflux from the MDR lines (see Table

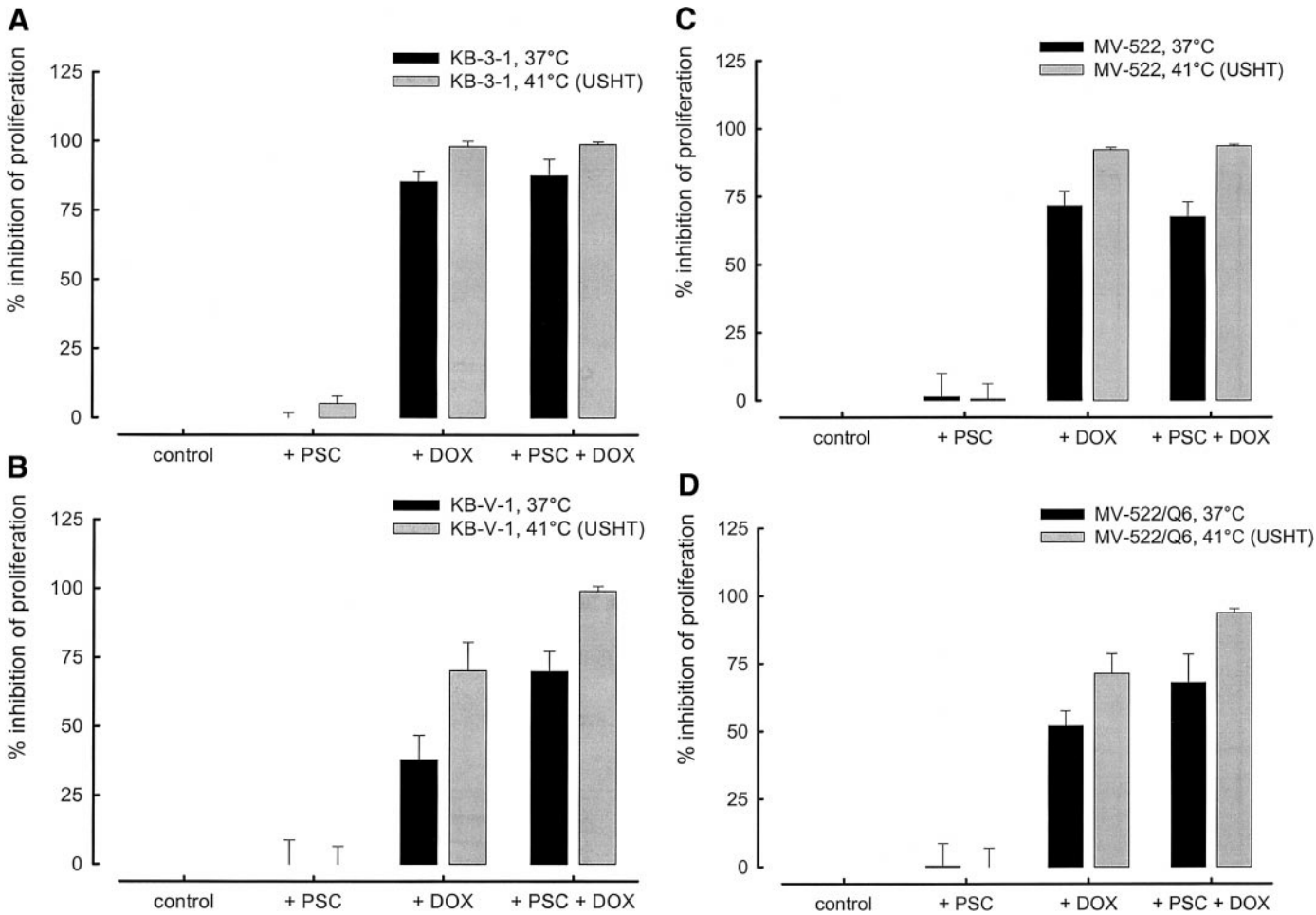

FIG. 3. Effect of combined use of USHT and PSC 833 on cytotoxicity of doxorubicin in the parent and MDR variant of MV522 and KB lines. Data were expressed as percentage inhibition calculated by the formula % inhibition = [1 − (counts of viable drug-exposed cells/counts of viable non-drug-exposed cells)] × 100. Cell viability was determined by hemocytometry technique after trypan blue staining. All experiments were carried out in triplicate (means ± standard deviation). The statistical analysis of the results is presented in Table 2.

1B). It is interesting to note that despite the disproportionate starting amounts of R123 and doxorubicin in the USHT-treated and untreated MDR cells (Table 1), 0.5 μM PSC 833 was effective in prolonging R123 and doxorubicin release in both cells (see Table 1). These results imply that as long as the amounts of P-gp substrate inside a P-gp-expressing cell are not too overwhelming for P-gp from where passive diffusion becomes a major pathway for efflux, it is highly probable to increase cellular retention of P-gp substrates by means of P-gp modulation. However, the effect of PSC 833 on intracellular doxorubicin concentrations after 1 h reached statistical significance only after USHT treatment of the MDR cell lines included in our study (Table 1B). Based on these results, we next investigated the combinative use of USHT and PSC 833 to increase the cytotoxicity of P-gp substrate doxorubicin in the parent and MDR variant of KB and MV522 cells.

Effect of Combined Use of USHT and PSC 833 on Cytotoxicity of Doxorubicin

It is reckoned if the concentration of a P-gp recognized chemotherapeutic agents could be increased and maintained in a P-gp expressing cancer cell longer, it should lead to a much-enhanced chemotherapy. To that end, we tested the effects of combining USHT, which enhances cellular drug uptake, with PSC 833, which modulates P-gp activity and hence promotes cellular drug retention, on cytotoxicity of doxorubicin in the parent and MDR cells. Consistent with our previous findings (13), brief USHT treatment increased cytotoxicity of doxorubicin in both the parent and MDR cells (Fig. 3, Table 2). PSC 833 (0.5 μM) did not affect cytotoxicity of doxorubicin in either the USHT-treated or untreated parent cells (Fig. 3). In contrast, PSC 833 increased the cytotoxicity of doxorubicin in the MDR cells (Fig. 3, Table 2). The results also indicated that

TABLE 2

Comparison of the Inhibitory Effect of Doxorubicin on the Proliferation of Parent Tumor Cells (KB-3-1 and MV522) and Their MDR Versions (KB-V-1) and (MV522/Q6) in Combination with USHT and PSC 833

	KB-3-1	KB-V-1	MV522	MV522/Q6
37°C versus USHT (41°C)				
+DOX	0.72	0.02	0.24	0.17
+DOX + PSC	0.84	0.06	0.07	0.05
DOX with versus without PSC 833				
37°C	1.00	0.02	1.00	0.39
41°C (USHT)	1.00	0.07	1.00	0.14

Note. The data are presented in Fig. 3. An analysis of variance in combination with Scheffé's test as post hoc analysis was used. The analysis of variance indicated highly significant differences ($P < 0.001$). The results of the post hoc analysis are shown. Abbreviations used: DOX, doxorubicin; USHT, ultrasound-induced hyperthermia.

the combinative approach (i.e., USHT + PSC 833) produced cytotoxic effects in the MDR cells that exceeded those generated by either USHT or PSC 833 alone (Fig. 3, Table 2). For instance, treatment of MDR variants with USHT or PSC 833 increased the anti-proliferative effect of doxorubicin from 52.1% (controls at 37°C) to 71.4% (USHT) or 68.1% (PSC 833) in MV522/Q6, and from 37.7 to 70.1% (USHT) or 69.9% (PSC 833) in KB-V-1 cells, respectively (Fig. 3). Combination of USHT and PSC 833, however, resulted in almost complete inhibition of proliferation in both the MV522/Q6 (93.7%) and KB-V-1 (98.9%) lines (Fig. 3).

Our results show for the first time that cytotoxicity of anticancer drugs in P-gp-expressing cells can significantly be enhanced by the combined use of USHT and P-gp-modulating agent, where USHT increases uptake and the P-gp inhibitor reduces efflux of the cytotoxic agent from the cell resulting in significantly increased exposure and efficacy. These findings have significant implications for combined therapy using PSC 833 with cytotoxic anticancer drugs. For instance, PSC 833 has been found to alter the pharmacokinetic profile of the concomitant anticancer drugs, which leads to unexpected toxicity (4). As a result, dosage reductions for anticancer drugs are often needed to avert unexpected toxicity. In the context of the present study, this reduction in dosage may be compensated by the USHT, which specifically increases cellular uptake of cytotoxic agent into the tumor. Thus, our findings may open new avenues for therapeutic intervention of MDR and treatment of MDR cancers.

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