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Effect of Ultrasound-Induced Hyperthermia on Cellular Uptake of P-gp Substrate and Non-P-gp Substrate in MDR Cells

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ABSTRACT – A previous report recently demonstrated that ultrasound-induced hyperthermia (USHT: 0.4 watts (W)/cm^2 at 41°C) could increase cellular uptake of P-glycoprotein (P-gp) substrates in P-gp expressing cancer cell lines. Since P-gp plays a major role in limiting drug permeability in the multi-drug resistant (MDR) cells, studies were conducted to elucidate the mechanism of USHT on cellular accumulation of P-gp and non-P-gp substrate in MDR cells. To accomplish this aim, we studied the effects of USHT on the accumulation of P-gp substrate, R123 and non-P-gp substrate, antipyrine in MDR cells. We demonstrated that USHT increased permeability of hydrophobic molecules (R123 and [^{14}C]-antipyrine). The enhanced permeability is reversible and size-dependent as USHT produces a much larger effect on cellular accumulation of [^{14}C]-antipyrine (MW 188) than that of R123 (MW 380.8). These results suggest that USHT could affect MDR cells more sensitive than BBMECs. Also, the present results point to the potential use of USHT to increase cellular uptake of P-gp recognized substrates, mainly anti-cancer agents into cancer cells.

Key words – Ultrasound-induced hyperthermia (USHT), Multi-drug resistance (MDR), Rhodamine 123 (R123)

P-gp, which is a transmembrane energy-dependent pump system encoded by the MDR-1 gene located on chromosome 7, is one of the best-understood mechanisms of multi-drug resistance (MDR) phenomenon in cancer treatment.[1,2] P-gp is known to recognize and transport a variety of large, structurally- and functionally-unrelated, neutral or cationic anticancer drugs, including vinca alkaloids, anthracyclines, epipodophyllotoxins, and taxanes. By pumping substrate drugs out of the cell, P-gp decreases intracellular drug accumulation, leading to diminished therapeutic efficacy. Clinical studies have also determined that the level of P-gp expression in cancer cells is inversely correlated with treatment response and overall survival.[3-5]

In an attempt to circumvent MDR due to P-gp expression, numerous studies have focused on the use of P-gp modulating agents to block the P-gp-induced drug efflux.[6-8] However, most of these agents show poor selectivity, leading to cytotoxicity.[9] Indeed, achieving a chemosensitizing level for these agents without causing severe tissue toxicity is very difficult. Recently, we have reported that mild hyperthermia (41°C) as induced by low intensity (0.4 W/cm^2) continuous wave high frequency (1 MHz) ultrasound was able to increase cellular uptake and cytotoxicity of several P-gp substrates in P-gp-expressing cancer cell lines.[10] Also, we already published that USHT increased permeability of hydrophobic molecules including R123 and [^{14}C]-antipyrine and not hydrophilic molecules ([^{14}C]-sucrose and 2-[^{14}H]-deoxy-D-glucose) in bovine brain microendothelial cells (BBMECs).[11] Based on these results, we examined that cellular accumulation of P-gp substrate or non-P-gp substrate according to the elapsed time after USHT treatment could be affected in P-gp expressing cancer cell lines.

Our results indicate that USHT does not modulate P-gp activity, but rather it increases cellular accumulation of R123 by altering membrane permeability in a manner that was selective and reversible. Together, the present results point to the potential use of USHT to increase cellular uptake of P-gp recognized substrates, mainly anti-cancer agents into cancer cells.

Materials and Methods

Materials

Cell culture medium and fetal calf serum (FCS) were obtained from Gibco (Grand Island, NY). Rhodamine 123 (R123), vinblastine and verapamil were purchased from Sigma Chemical Co. (St. Louis, MO). [^{14}C]-Antipyrine (0.056 Ci/mmol) was purchased from New England Nuclear (Boston, MA). PSC 833 was purchased from Norvartis (Basel, Switzerland). All other reagents, unless specifically stated otherwise, were purchased from Sigma Chemical Co.

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Cell cultures

KB-3-1 (human epidermoid carcinoma) and its MDR variant KB-V-1 cells were provided by Dr. Michael Gottesman (NCI, NIH).[12] 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. In order to maintain the MDR characteristics, cell culture media for MDR sublines KB-V-1 were supplemented with 1 ug/ml of vinblastine.

Intracellular accumulation of R123

MDR variants of KB cells were seeded in 48-well plates at 1×10^5 cells/well in vinblastine-free MEM one day before experiments. Prior to experiments, cells were washed twice with serum-free MEM. Subsequently, cells were exposed to 0.25 ml serum-free MEM alone or 0.25 ml serum-free MEM containing 4 mM of R123 in the presence or absence of various concentrations of verapamil or PSC 833 for 20 minutes at 37°C. After incubation, cellular accumulation studies were terminated by removing the assay solutions and washing the cells three times with 1.0 ml of ice-cold phosphate buffered saline (PBS) solution. The cells were then solubilized by incubation with 1 ml of 0.2 N NaOH overnight. Aliquots (500 μl and 50 μl) of the cell lysate solution were removed for analysis of R123 and protein content, respectively. The amount of protein in each sample was determined by the Pierce BCA method (Pierce Chemical, Rockford, IL). The concentration of R123 in each sample was determined quantitatively by fluorescence spectrophotometry (Shimadzu RF 1501; Λ_{ex}=492 nm, Λ_{em}=535 nm) as described previously[13] and standardized by the protein content of each sample. All experiments were carried out in triplicate.

Ultrasound apparatus and exposure

The system used to expose MDR cells *in vitro* to USHT has been previously described.[10] In all studies, ultrasound exposure (0.4 W/cm^2 at 1 MHz) was for a period of 20 minutes, 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).

Cellular accumulation of hydrophobic molecules according to the elapsed time after USHT treatment

MDR cells were seeded and grown in 10×33 mm cell culture plates to confluence as described above. Prior to experiments, MDR cells were washed twice and replaced with 12.0 ml 37°C serum-free MEM. Subsequently, MDR cells were either subjected to no USHT (37°C, control) or USHT (0.4 W/cm^2 at 41°C) treatment for 20 min. At indicated time intervals (0, 20, 40, 60, 120 min) after USHT treatment, the culture media of both the control and treatment groups were aspirated and the cells were then incubated with 37°C serum-free medium containing either R123 (4 μM) or [^{14}C]-antipyrine (0.5 μM) for 20 min. After the incubation, cellular accumulation studies were terminated by removing the assay buffer solutions and washing the MDR cells three times with ice-cold PBS. The MDR cells were then solubilized and aliquots of cell lysate solutions removed for analysis of R123 or [^{14}C]-antipyrine and protein content, respectively. The level of radioactivity of [^{14}C]-antipyrine taken into MDR cells was determined using a Beckman LS6000 IC liquid scintillation counter. The concentration of R123 and the amount of protein in each sample were determined as described above.

Western blot analysis for identification of P-gp expression

KB cells were harvested with lysis buffer (New England BioLabs) and disrupted by sonication. Total protein samples (20 μg) were separated by electrophoresis through Ready Gels (4-15% Tris-HCl) (Bio-Rad Laboratories). The proteins were then transferred to a PVDF membrane (Millipore Corp.) using a Trans-Blot SD system (Bio-Rad Laboratories). Membrane was incubated with primary antibody (C219, Signet Pathology Systems) and secondary anti-mouse IgG-HRP (sc-2031, Santa Cruz Biotechnology), respectively.

Statistical analysis

All data are presented as mean ± S.D. from at least three experiments. Treatment groups were compared with control for significance by unpaired Student *t* tests with $P<0.05$ was being considered significant.

Results and Discussion

Intracellular accumulation of R123

When the concentration of verapamil or PSC 833 increased from 1 uM to 200 uM, verapamil and PSC 833 caused a dose-dependent increase in R123 accumulation in MDR cells. Previous publications reported the effects of verapamil and PSC 833 on R123 accumulation in the KB-3-1 cells, parent of KB cells.[14] As published, in KB-3-1 cell lines, incubation with verapamil and PSC 833 resulted in a dose-dependent decrease in R123 accumulation. One such chemical is a lipophilic cationic fluorescent dye, R123, which selectively located in mitochondria,[15] has been found to be relatively non-toxic,[15,16] and

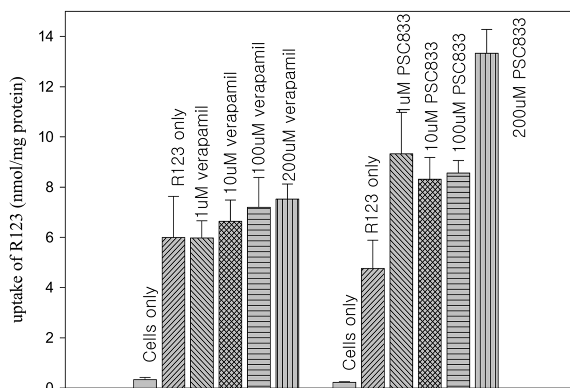

Figure 1−Effect of verapamil or PSC833 on cellular accumulation of R123 by MDR cells.

effluxed more efficiently by MDR cells.[17,18] R123 primarily binds to intact mitochondria and, accordingly, processes that influence cell viability may affect R123 retention by mitochondria and, hence, cellular accumulation of R123. Therefore, R123 has often been used for studying the functional activity of P-gp. In MDR cells, P-gp modulators would be expected to increase R123 accumulation through its P-gp modulating effects. As expected, both verapamil and PSC 833 were effective in increasing R123 accumulation in MDR cells (Figure 1). When R123 used as a molecular probe for study of P-gp activity, it is customary to either follow the accumulation[19-22] or release[21,23,24] of R123 in the presence or absence of P-gp modulators. If the P-gp modulators were to increase R123 accumulation, a conclusion for the presence of P-gp activity is usually drawn.

Simultaneously, to test whether or not P-gp is expressed in the vinblastine-resistant MDR cells, western blot analysis was performed with a C219 monoclonal antibody against the cytoplasmic epitope of P-gp. The vinblastine-resistant MDR cells expressed P-gp as a 170 kDa band while the parent KB-3-1 cells did not (Figure 2). There was a good correlation between the levels of P-gp and cellular accumulation of R123 in the presence of verapamil or PSC 833 in both KB cells. Moreover, by comparing the activity of different P-gp modulators such as verapamil and PSC833 in increasing R123 accumulation, information on the potency of these modulators in reversing P-gp activity can also be obtained that PSC833 is stronger than verapamil.

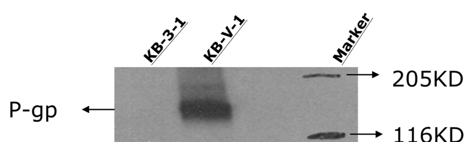

Figure 2−P-gp expression in parent and MDR variant of KB cells.

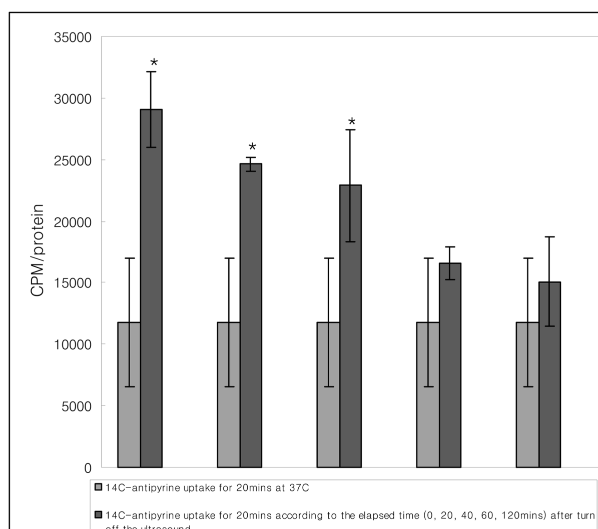

Figure 3−Cellular accumulation of non-P-gp substrate, [^{14}C-antipyrine] according to the elapsed time of USHT treatment. *$p<0.05$, compared with untreated control.

Cellular accumulation of hydrophobic molecules according to the elapsed time after USHT treatment

To determine if USHT mediated its effect on cellular accumulation of R123 by affecting membrane permeability, we compared the cellular accumulation of a non-P-gp hydrophobic substrate ([^{14}C]-antipyrine) with that associated with R123. Further, starting the accumulation studies at various time intervals after the USHT treatment caused a prompt increase of [^{14}C]-antipyrine in MDR cells (Figure 3). Peak accumulation appeared to occur right after USHT treatment and began to decline thereafter. A similar pattern on cellular accumulation

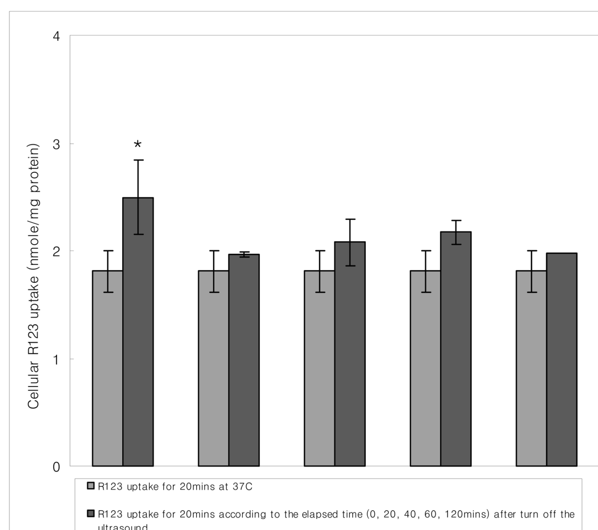

Figure 4−Cellular accumulation of P-gp substrate, R123 according to the elapsed time of USHT treatment. *$p<0.05$, compared with untreated control.

like [^{14}C]-antipyrine was also observed for R123 (Figure 4), although the extent of the increase in cellular accumulation appeared to be smaller than that of [^{14}C]-antipyrine. Further, it also took less time after USHT treatment for R123 accumulation in USHT-treated cells to return to that of the untreated cells (20 min for R123 vs >60 min for antipyrine). Interestingly, different accumulation patterns from the accumulation results of R123 and [^{14}C]-antipyrine observed in BBMECS that USHT substantially increased the accumulation of R123 and [^{14}C]-antipyrine in BBMECS and then accumulation of R123 and [^{14}C]-antipyrine recovered into control status. The accumulation of R123 and [^{14}C]-antipyrine in MDR cells increased right after USHT treatment and then that of R123 and [^{14}C]-antipyrine recovered to control, gradually. These results suggest USHT could affect MDR cells more quickly.

Using doxorubicin as a model drug, several investigators have suggested that the site of action of ultrasound might be the cell membrane,[25-27] where ultrasound-induced changes in the cell membrane could affect the penetration of hydrophobic drugs. We examined this possibility by evaluating cellular accumulation of hydrophobic molecules after USHT treatment. Antipyrine was chosen as hydrophobic permeability markers, respectively, because these molecules are not considered substrates of P-gp or of any known transporter proteins in MDR cells. Cellular accumulation of both R123 (log partition coefficient 0.53) and antipyrine (log partition coefficient 0.4) was increased immediately after USHT treatment. These observations are in agreement with a recent study in which ultrasound increased the uptake of hydrophobic antibiotics into bacterial cells.[28]

Several studies have shown that the activity of several membrane bound proteins is reduced by hyperthermia,[29,30] probably through an irreversible protein transitions found to occur in membranes.[29] Thus, it is possible that the subtle membrane effects produced by USHT may lead to impairment of P-gp activity. In addition, there are also reports, which indicate that hyperthermia might lead to a down-regulation[31] or up-regulation of P-gp.[32] To attempt to discern the possibility that changes in P-gp activity might be partially responsible for the USHT-induced drug accumulation, we focused on functional activity of P-gp rather than on protein expression because P-gp activity has been previously shown to be a more descriptive measure of the importance of this drug efflux system than protein expression.

As such, our findings are in line with others, which indicated that hyperthermia could alter the permeability of the plasma membrane to several compounds.[33] We speculate that this enhanced drug entry rate might transiently overwhelm the P-gp, which in turn leads to more cellular drug uptake.

Conclusion

Interestingly, different accumulation patterns from the accumulation results of R123 and [^{14}C]-antipyrine observed in BBMECS that USHT substantially increased the accumulation of R123 and [^{14}C]-antipyrine in BBMECS and then accumulation of R123 and [^{14}C]-antipyrine recovered into control status. The accumulation of R123 and [^{14}C]-antipyrine in MDR cells increased right after USHT treatment and then that of R123 and [^{14}C]-antipyrine recovered to control, gradually. Nevertheless, the present results coincided that USHT enhanced the accumulation of the hydrophobic molecules of R123 and [^{14}C]-antipyrine in BBMECs.[11] Therefore, these results point to the potential use of USHT to increase cellular uptake of P-gp recognized substrates, mainly anti-cancer agents into cancer cells.

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