

In Vitro Study of Ultrasound on Multidrug Resistance in MDR Human Hepatoma HepG₂ Cells

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OBJECTIVE The aim of the study was to examine the reversal effects of ultrasound (US) on the MDR in HepG₂/ADM, a HepG₂ cell line resistant to Adriamycin (ADM), and to study the mechanism of US action.

METHODS Using the MTT assay, the effects of US on MDR in HepG₂/ADR cells were studied. Before and after the treatment with 0.5 W/cm² low intensity ultrasound (LIUS), the expression of the MDR-related genes, *mdr1*, *mrp* and *lrp* was assayed with the reverse transcriptase polymerase chain reaction (RT-PCR) and the levels of their respective protein expression determined by flow cytometry. By using confocal laser scanning microscopy (CLSM), we examined the intracellular daunorubicin (DNR) distribution, and the effects on the cells of treatment with US or DNR.

RESULTS LIUS significantly reversed MDR in HepG₂/ADR cells. After treatment with LIUS at 0.5 W/cm², chemosensitivity to ADM and DNR increased 3.35-fold and 2.81-fold, respectively. The reversal activity by LIUS plus verapamil (VER) was stronger than with either US or VER alone. After treatment with 0.5 W/cm², the expression of both the MDR1 and the MRP mRNA genes began to decline ($P < 0.01$ and $P < 0.05$, respectively); the expression of LRP showed no significant changes. Changes in the expression of the P-glycoprotein (P-gp) and MRP were similar to those of their mRNA expressions. Results of the CLSM showed that administration of US (0.5 W/cm²) or VER (15.7 μM) with DNR to HepG₂/ADM cells showed a significant change in the distribution of DNR in the cells.

CONCLUSION Our results show that LIUS can reverse MDR. The reversal effects are stronger than those of either US or VER alone, when combined with VER administration. As LIUS is non-invasive causing no toxicity, it might have potential for clinical application. The reversal mechanism needs further study.

KEY WORDS: multidrug resistance (MDR), HepG₂/ADM, ultrasound (US), reversal.

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Introduction

The acquisition of resistance to multiple anticancer drugs by human cells results is a serious problem in chemotherapy. One reason for drug resistance is the over-expression of the plasma membrane proteins, such as the *mdr1* gene product P-glycoprotein (P-gp), *mrp* and *lrp*^[1]. These are high molecular weight membrane proteins which act as ATP-dependent pumps to eject anticancer drugs out of cells, and thus decrease their intracellular accumulation and efficacy. Drugs such as verapamil^[2], a calcium channel blocker and cyclosporin-A^[3], an immunosuppressive peptide are two widely studied agents that reverse drug resistance, but they are not totally effective or specific for tumor cells^[4].

The use of ultrasound (US) to enhance cancer therapy has been the subject of numerous biological and clinical investigations. In most studies, US has been used to induce hyperthermia for either direct treatment of small and localized cancerous tumors^[5], or as adjuvant therapy to increase the efficacy of radiotherapy and chemotherapy^[6]. The mechanisms proposed to account for US-enhanced chemotoxicity include both non-thermal and thermal processes. The non-thermal effects of US are those that enhance chemotoxicity at, or below normal physiological temperatures^[7]. Results from experiments conducted at above physiological temperatures ($> 37^{\circ}\text{C}$), indicate that chemotoxicity enhancement is not due to heat alone^[8]. The US non-thermal effects can occur via one or a combination of three mechanisms: cavitation, radiation pressure, and acoustic microstreaming^[9], which have been shown to influence the cell surface^[10] and thereby might promote the cellular drug uptake and enhance chemotoxicity. All three of the non-thermal mechanisms could occur, alone or in a combination to produce chemotoxicity enhancement^[11]. However, the precise mechanism for US-enhanced chemotoxicity and reversal of MDR is still a subject of debate. The purpose of this study was to investigate whether the P-gp or mrp1 or lrp proteins could be induced in vitro by lower intensity ultrasound (LIUS) in HepG₂/ADM cells, and further, to investigate the mechanism of US action.

Methods and Materials

Chemicals

Daunorubicin (DNR) and Verapamil (VER) were obtained from the Shanghai Hualian Pharmaceutical Co. Ltd. (Shanghai, China). The protease inhibitors were from the Sigma Chemical Co. (St. Louis, MO). Polymerase chain reaction (PCR) buffer, avian myeloblastosis virus reverse transcriptase, Taq DNA polymerase, and random hexadeoxynucleotide primers were obtained from Boehringer Mannheim (Mannheim, Germany). Deoxynucleotides and the RNA guard ribonuclease (RNase) inhibitor were from Pharmacia (Uppsala, Sweden), and primers were purchased from DNA Technology (Aarhus, Denmark). All other chemicals were of analytical grade.

Cell lines and cell culture

The cell lines HepG₂/WT (ADM-sensitive) and HepG₂/ADM used in this study were obtained from the Institut of Ultrasound and Engineering in Medicine, Chongqing University of Medical Science, P.R. China. The medium used was RPMI 1640 supplemented with 10% fetal calf serum, L-glutamine (0.29 g/L), penicillin (200,000 IU/L), and streptomycin (50 mg/L). Medium and serum were obtained from Gibco Ltd., and maintained in Dulbecco's modified Eagle's medium incubated at 37°C in 5% CO₂ 95% air under high humidity, and passaged every 2–3 days. The cells were digested with a mixture of 0.025%

trypsin (Gibco BRL) and 0.01% EDTA (Sigma). The medium for HepG₂/ADM cells was further supplemented with ADM (1.0 $\mu\text{g}/\text{ml}$). Before use in experiments the HepG₂/ADR cells were cultured in drug-free medium for 1 week.

The treated sublines HepG₂/WT were derived in vitro by sequential exposure of logarithmically growing HepG₂/WT cells to 4 fractions of 0.5 W/cm² of LIUS. After each exposure, the cells were transferred to 10 ml of culture medium and allowed to repopulate.

The mdr1, mrp and lrp-positive HepG₂/ADM cell line was derived by treating HepG₂ cells with Adriamycin (ADM). To maintain the MDR characteristics, the cell culture media for HepG₂/ADM sublines were supplemented with 1.0 $\mu\text{g}/\text{ml}$ of Adriamycin.

Ultrasonic instrumentation and intensity measurement

The ultrasonic instrument (HIFU CO.LTD, Chongqing, China) used in all the sonication experiments produces LIUS with a resonant frequency of 0.8 MHz. The transducer, with a diameter of 3.0 cm, was horizontally directed to target the sample contained in each well of 6-well culture plates. In these studies, ultrasonic exposure (0.5 W/cm² at 0.8 MHz) was for a period of 5 min, maintaining the treatment temperature at 41°C .

MTT assay of cytotoxic activity

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay for cytotoxic activity was used. Cells were treated with LIUS and other reversal agents, such as VER. MTT assays were performed as follows: HepG₂/WT and HepG₂/ADR cells were each harvested with 0.05% trypsin/EDTA and counted. Cell lines were seeded into 96-well plates at 10^4 viable cells per well, and left to attach to the plate for 24 h. After 24 h, the medium was changed to one containing or lacking test reversal agents or ADM. The final volume was 200 μl per well. The medium was removed after 72 h of incubation. Other medium containing 0.5 mg/ml MTT (Sigma) was added to each well in a volume of 200 μl and incubated for 4 h. The medium was then removed and 180 μl of dimethyl sulphoxide (Sigma) was added to each well for half an hour at room temperature. A 96-well microtitre plate reader (Dynatech, Chantilly, VA, USA) was used to determine A570. The mean concentration in each set of 3 wells was measured. To avoid interference by the red fluorescence of ADM concentrations above 8 μM , a blank well containing the corresponding ADM concentration without MTT was set up and subtracted from the test well absorbance. The absorbance of untreated controls was taken as 100% survival, and the percentage inhibition was calculated as cell survival rate (%) = $100(T - B)/(U - B)$, and growth inhibition (%) = $100 - \text{cell survival rate}(\%)$, where T (treated) was the absorbance of drug-treated cells, U (untreated) was the absorbance of untreated cells, and B (blank) was the absorbance in the absence of both

drug and MTT. IC_{50} values were determined graphically from relative survival curves. The fold reversal was calculated as IC_{50} for ADR/ IC_{50} for ADM US and reversal agents.

The effect of VER on HepG₂/ADM was studied by exposing cells to different concentrations of the cyto-static in the absence or presence of VER (15.7 μ M).

Determination of PGP, MRP and LRP by flow cytometry (FCM)

Detection of expression of the resistance-associated markers (P-gp, MRP and LRP) was conducted by flow cytometry based on the method of Flens et al.^[12] For determination of P-gp expression, suspension of parental and resistant sublines (3×10^6 cells in staining buffer [Dulbecco's PBS supplemented with 5% FCS and 0.2% sodium azide]) were incubated with 10 μ g/ml of either MRK16 or an isotype-matched mouse IgG2a for 1 h at 48°C. The mouse monoclonal antibodies MRK16, MRPr1 and LRP56 were purchased from MAIXIN Reagent Co. Ltd. (FuJian, China).

The cells were washed twice with staining buffer and then incubated with 0.175 mg/ml of FITC-labeled goat antimouse IgG for 1 h at 48°C. After washing with staining buffer, the cells were passed through a 200- μ m nylon mesh and resuspended in staining buffer. Fluorescence intensity (excitation wavelength = 488 nm) was determined by means of an Epics flow cytometer (Elite ESP, Coulter Inc., USA). To estimate mrp or lrp expression, the cells were fixed with formalin by gentle mixing for 10 min at room temperature. The cells were then incubated with 10 μ g/ml of MRPr1, LRP-56 and an isotype-matched rat IgG2a (for MRPr1) or mouse IgG2b (for LRP-56) for 1 h at 48°C. After washing, the cells were incubated with 0.175 mg/ml of FITC-labeled goat anti-rat IgG (for MRPr1) or FITC-labeled goat anti-mouse IgG (for LRP-56) for 1 h at 48°C. Fluorescence intensity was determined by a flow cytometer. The specific binding of primary antibody was estimated by subtracting the fluorescence intensity of samples incubated with isotype-matched IgG from the fluorescence of samples incubated with primary antibody.

The ratio of the specific fluorescence intensity of the resistant sublines to that of parental cells indicated the relative levels of expression. Results were expressed as the ratio of fluorescence intensity values between each experimental sample and control sample of HepG₂/ADM cells.

Measurement of *mdr1*, *mrp* and *lrp* mRNAs by RT-PCR

Primers *mdr1*: 5'-GGC TCC GAT ACA TGG TTT TCC-3'; 3'-TTC AGT GCG ATC TTC CCA GC-5'; *mrp*: 5'-TGA AGG ACT TCG TGT CAG CC-3'; 5'-GTC CAT GAT GGT GTT GAG CC-3'; *lrp*: 5'-CCT CGA GAT CCA TTG TGC TGG-3'; 5'-CAC AGG GTT GGC

CAC TGT GCA-3'; β -actin: 5'-ACC CCC ACT GAA AAA GAT GA-3'; 5'-ATC TTC AAA CCT CCA TGA TG-3'.

β -actin expression was used as control for the amount of RNA used. Total RNA from cells was extracted with TRIzol. *mdr1*, *mrp*, *lrp* and β -actin RNA transcripts were detected by RT-PCR as described by Zhai et al.^[13] An aliquot of each reaction mixture was then analyzed by electrophoresis on a 1.5% agarose gel. Densitometry was performed using a UVP gel image analysis system (BIO-RAD, USA) and the ratio between the target and control PCR products was determined by dividing the densitometric volume of the target band by that of the control band.

Observation of intracellular DNR distribution by CLSM

The experimental procedures were similar to those described by Chen et al.^[14] Cells (1×10^5) were seeded into a 960 mm² petri dish with a slide in it, and incubated in a 5% CO₂ atmosphere at 37°C. After the cells had reached 75% confluence, normal medium was replaced with serum-free RPMI 1640 medium, and cells incubated with DNR at 2 μ g/ml for 1 h. The effect of LIUS was studied after 72 h pre-incubation with ADM or VER. After two washes with PBS and addition of drug-free medium, cells grown on slides were examined with CLSM (MRC-1024ES, BIO-RAD Inc., USA). Coverslips were mounted on slides, supported and sealed on lacquer tiers to prevent compression and drying out. Intracellular drug fluorescence was observed using the 488 nm laser line for excitation and the filter that allowed measurement of emitted light above 515 nm.

Statistical analysis

Levels of statistical significance were evaluated with data from at least three independent experiments by using Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

Enhancement of chemosensitivity in treated cells

Resistant HepG₂ cells (HepG₂/ADM) were derived by treating the cells with stepwise increasing concentrations of doxorubicin, starting at 0.001 μ g/ml and ending up at 1.0 μ g/ml. Multidrug resistance was maintained by culturing the cells at 1.0 μ g/ml doxorubicin. When a cytotoxicity assay was performed, it was found that HepG₂/ADM cells were resistant not only to doxorubicin but also to multiple anticancer drugs. As shown in Table 1, the parental sensitive HepG₂ cells were highly sensitive to DNR and ADM. However, the drug resistant HepG₂/ADM cells were 10¹-fold resistant to DNR and 10⁵-fold to ADM, respectively, as compared with HepG₂ cells. The resistance index (RI) to DNR and ADM in

Table 1. Effect of US on cellular drug sensitivity ($\bar{x} \pm s$).

Cells	IC ₅₀ * (mg/L)		RI**	
	DNR	ADM	DNR	ADM
HepG ₂	0.002 ± 0.0006	0.003 ± 0.0008	1.0	1.0
HepG ₂ /ADM	0.202 ± 0.0076	0.315 ± 0.0254	101	105
HepG ₂ /ADM + US	0.072 ± 0.0002	0.097 ± 0.0009	36.0	31.3
HepG ₂ /ADM + VER	0.084 ± 0.0007	0.094 ± 0.0065	42	31.3
HepG ₂ /ADM + VER + US	0.012 ± 0.0011	0.027 ± 0.0014	6	9

* , mean standard deviation of at least three experiments; **, mean of three experiments; RI, resistance index. All values were calculated as described in MATERIALS AND METHODS. The concentration of verapamil (VER) was 15.7 μ M and the treatment time was 4 h.

HepG₂/ADM cells co-administered with VER (15.7 μ M) or US (0.5 W/cm²) decreased from 10¹ and 10⁵ to 6 and 9, respectively. Treatment with separate VER or US showed a lesser reversal effect on MDR than co-treatment of the cells.

Co-expression of different resistance-associated markers

To examine whether acquisition of Adriamycin resistance was altered at the molecular level, *mdr1*, MRP and LRP mRNA levels in the cells were evaluated by RT-PCR using β -actin as a heterologous internal standard. As demonstrated in Fig.1, *mdr1*, *mrp* and *lrp* mRNAs were detected in all resistant sublines. RNA from untreated cells (HepG₂/ADM) served as a control. In the control HepG₂/ADM cells, *mdr1*, *mrp* and *lrp* gene expression levels were all high. In the control HepG₂/WT cells, the *lrp* genes showed negative results, whereas the *mdr1* and *mrp* genes were expressed positively.

Levels of *mdr1*, *mrp* and *lrp* mRNA expression

As shown by RT-PCR, the amplification products of *mdr1*, *mrp*, *lrp* and β -actin were 181 bp, 242 bp, 377 bp and 620 bp, respectively (Fig.1). Overexpression of *mdr1*, *mrp* and *lrp* mRNAs were detected in HepG₂/

ADM, but a low level mRNA expression in HepG₂/ADM was observed. The β -actin signals in HepG₂, HepG₂/ADM and HepG₂/ADM (US) cells were equally strong, giving a rough estimate that the same amounts of RNA were used in the three compared cell lines. The *mdr1* mRNA, *mrp* mRNA and *lrp* mRNA were determined after exposure to US (0.8 MHz, 0.5 W/cm², 5 min). HepG₂/ADM (US) showed low expression of *mdr1* mRNA and MRP mRNA ($P < 0.05$). The expression of *mdr1* mRNA and *mrp* mRNA decreased approximately 5.5-fold and 3.8-fold, respectively. But the *lrp* mRNA expression level of HepG₂/ADM cells showed no significant change ($P > 0.05$), and reached a level close to that of untreated controls (Fig.2).

Expression of P-gp, MRP and LRP proteins

Flow cytometry analysis was performed to evaluate the involvement of the MDR-associated genes *mdr1*, *mrp* and *lrp* on the protein level in a quantitative manner in the cells. The analysis showed that HepG₂/ADM cells co-express the resistance-associated markers, P-gp, MRP and LRP (Fig.3 A, B and C). HepG₂/WT cells expressed a moderate level of P-gp only. MRP and LRP protein expression was undetectable. After treatment with US, the expression levels of P-gp and MRP in HepG₂/ADM cells

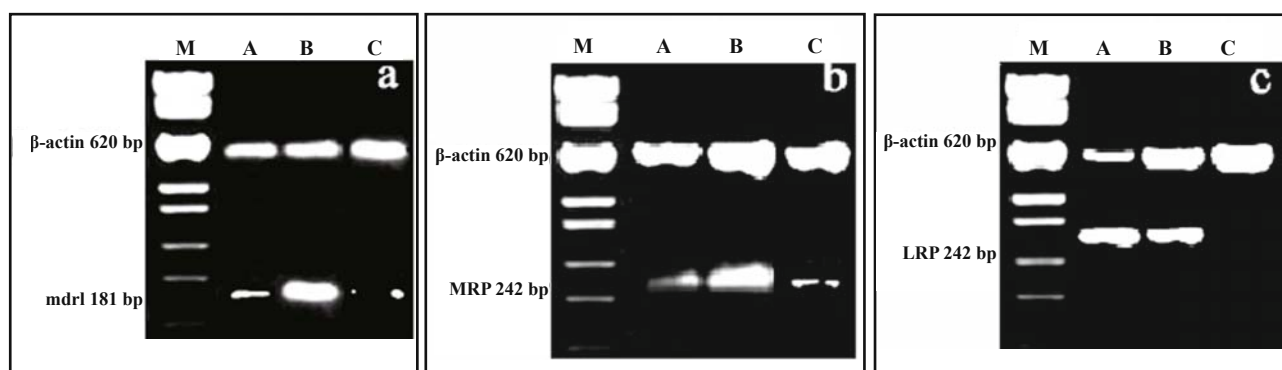


Fig.1. (A, B and C) Target and β -actin RT-PCR mRNA levels of *mdr1*, MRP and LRP. Total RNA (0.20 mg) extracted from parental, resistant sublines and US treated resistant sublines and *mdr1* β -actin (0.8 μ mol), MRP β -actin and LRP β -actin (0.8 μ mol) were used in the PCR reactions. After 32 amplification cycles, the products were resolved on a 1.5% agarose gel and stained with ethidium bromide. Lane M, a 100-bp DNA ladder as a size marker; A, HepG₂/ADM treated by LIPUS; B, HepG₂/ADM; C, parental cell-HepG₂.

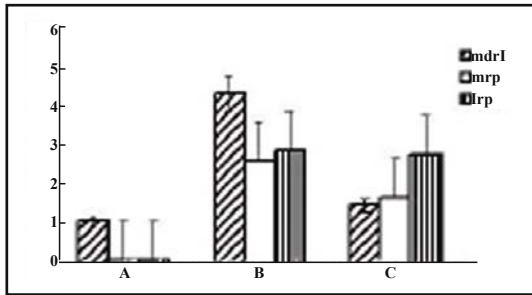


Fig.2. Quantification of PCR. The ratio between the *mdr1* or *lrp* and β -action gene is expressed as described in Materials and Methods. A, parental cell-HepG₂/WT; B, HepG₂/ADM; C, HepG₂/ADM treated with low intensity pulse ultrasound.

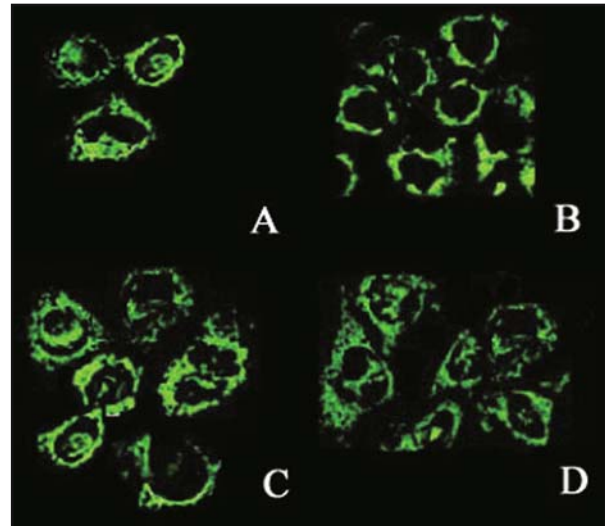


Fig.4. Intracellular DNR concentrations in cells treated with low intensity ultrasound. A, HepG₂/WT treated with DNR; B, HepG₂/ADM treated with DNR; C, HepG₂/ADM treated with US+DNR; D, HepG₂/ADM treated with VER + DNR.

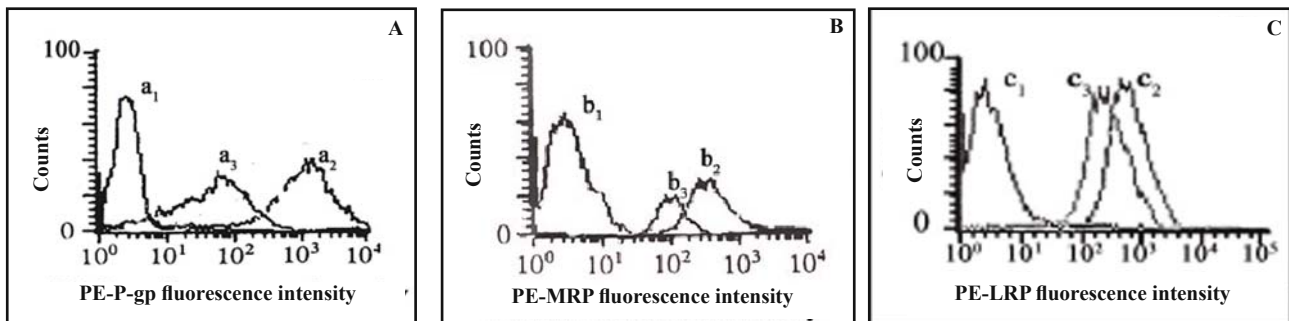


Fig. 3. Expression of resistance-associated markers on parental, resistant sublines and treated resistant sublines. P-gp, MRP or LRP expression was determined by flow cytometry using monoclonal antibodies MRK16, MRPr1 and LRP-56, respectively. The fluorescence of 5,000 cells was measured at an excitation wavelength of 488 nm. The vertical and horizontal axes indicate the relative cell number and fluorescence intensity. Expression of P-gp, MRP or LRP (A, B and C), left-HepG₂ cells (control: a1, b1, c1); Right-HepG₂/ADM cells (a2, b2, c2); middle-HepG₂/ADM cells treated with US (a3, b3, c3).

decreased 68% and 61.2%, respectively. No marked difference was found in LRP protein expression before and after US treatment in HepG₂/ADM cells. In HepG₂ (US) the P-gp protein was almost lost. The effects of US on the expression of drug-resistance protein were similar to those of US on mRNA expression.

Subcellular distribution of DNR

In parental HepG₂/WT cells, DNR fluorescence was mainly located in the nucleus, and was diffusely present in the cytoplasm (Fig.4A). In drug-resistant HepG₂/ADM cells, DNR fluorescence was retained in the perinuclear zone and in peripheral vesicles, with little or no drug in the nuclear zone (Fig.4B). In HepG₂/ADM cells treated separately with US or VER, the subcellular distribution of DNR was similar to parental cells HepG₂/WT, with less intranuclear fluorescence intensity than that in HepG₂/WT cells. Separate treatment of the cells with US or VER did not result in any difference in subcellular DNR distribution (Fig.4 C and D). Co-adminis-

tration of US and VER caused more drug accumulation in the nucleus.

Discussion

Multidrug resistance (MDR) represents a major obstacle to successful chemotherapy of metastatic diseases. This resistance is due to, apart from other causes, the plasma membrane proteins, such as P-glycoprotein (P-gp), transporters of the *mrp* family, and other proteins, which exert an efficient removal of the drugs from the tumor cells. This leads to the requirement of greater amount of therapeutic anti-tumor drugs which can result in greater side effects. One solution to this problem is to inhibit expression of the plasma membrane proteins (P-gp, *mrp*, *lrp*). Ultrasound techniques have been widely applied clinically, and have been studied alone or in combination with other anti-tumor modalities^[15].

It has been reported that parental HepG₂ cells have no or low levels of expression of *mdr1*/P-gp and *mrp*/

P190. However, over-expression of P-gp, MRP and LRP has been observed in drug-resistant HepG₂/ADM cells, which was confirmed in our study. Resistant HepG₂ cells were developed by treating the cells only with doxorubicin, and when resistance developed the mRNA and protein level of P-glycoprotein, MRP and LRP increased significantly, showing that P-glycoprotein, MRP and LRP were not specific for only one drug^[16].

The mechanism by which US induces biological effects is by heat production due to absorption and dissipation of ultrasound energy, free radical formation brought about by inertial cavitation, non-inertial cavitation and other effects which are still poorly characterized. In our study we used a specially designed US set-up that reduces standing wave formation, below threshold values for internal cavitation to occur, and with very minimal heat production^[17]. Therefore, we considered the US in this set-up as thermal and non-internal cavitation ultrasound.

In many studies^[18], US has been as an agent to induce hyperthermia (HT) for either direct treatment of small and localized cancerous tumors, or as adjuvant therapy to increase the efficacy of radiotherapy and chemotherapy. Lower US intensities (0.2 to several W/cm²) produce a mild increase in temperature, and enhance the cytotoxicity of radiation therapy and chemotherapy. US-enhanced delivery into cells has been demonstrated in vitro by uptake of extracellular fluid, drugs, and DNA into cells^[19]. Our study demonstrated that US significantly sensitized the HepG₂ cells to ADM, thus suggesting insights on the mechanism of phonophoresis. The enhancement of chemo-sensitivity to ADM by US and VER was stronger than that by US plus VER alone. The reversal effect of US was similar to that of VER, a classical reversal agent with a high reversal activity. US therefore also has a strong reversal activity.

Semi-quantitative RT-PCR analysis is a sensitive and specific method. A variety of methods using either quantitative or semiquantitative RT-PCR have been used to determine relative initial target mRNA levels in samples. However, in all of these methods undefined variations in amplification complicate the interpretation of results. Most investigators^[20] use internal amplification standards in an attempt to correct variations between tubes. In the present study, as in many other studies, we chose endogenous standards. The reference mRNA and target mRNA are usually processed together throughout the experiments, from RNA extraction until PCR amplification. This tends to minimize differences in RNA yields between samples. The results showed that down-regulation of expression of *mdr1* mRNA/P-gp and *mrp* mRNA/P190 by US accompanied the recovery of drug sensitivity in HepG₂/ADM cells. Drug-resistant HepG₂/ADM cells treated with US and VER showed a 9-fold drug sensitivity to ADM, when compared with drug resistant HepG₂/ADM cells.

Moriyama-Gonda et al.^[21] showed that heating might induce a reduction in P-gp expression, leading to inhi-

bition of drug efflux and enhancement of intracellular drug uptake. These results implied that a mechanism other than modulation of P-gp activity might be responsible for the enhanced drug accumulation by US. Our results suggested that the site of action of US might be the cell membrane, and that US selectively enhanced the permeability of hydrophobic drugs. We also found that US modulation of the *mdr1* and *mrp* genes reduced both mRNA and protein levels of the HepG₂ cells. However, in agreement with our results, Nielsen et al.^[22] has reported irradiation influenced P-gp and *mrp* expression in sensitive Ehrlich ascites tumor cells.

In our study, we also found that US (41°C) at an intensity of 0.5 W/cm² induced apoptosis and the degree transport of cytoskeleton. We observed that in the HepG₂/WT cell line, DNR fluorescence distributed evenly in the nucleus and cytoplasm, while in the HepG₂/ADM cell line, DNR distributed in a punctate pattern in the cytoplasm and was reduced in the nucleus. Treatment by US changed the subcellular DNR distribution pattern in HepG₂/WT cells to that similar to HepG₂/ADM cells. This observation indicated that P-gp or P190 not only pump DNR out of cells, but also transports DNR from the nucleus to the cytoplasm and into some organelles such as the Golgi apparatus. Since P-gp or P190 probably locate in the cell membrane, nuclear membrane, Golgi apparatus, or endoplasmic reticulum, the actions of these two proteins may cause reduction of intracellular and intranuclear drug concentration and drug accumulation in some organelles^[23]. This may account as to why the potent US inhibition of P-gp or P190 expression and intracellular drug concentration was increased and subcellular drug distribution changed, which leads to the reversal of multi-drug resistance in the HepG₂/ADM cell line. Therefore, we believe that over-expression of P-gp and/or P190 is an important mechanism in mediating MDR in the HepG₂/ADM cell line, in spite of the fact that expression of *lrp* was unchanged. When the resistance of tumor cells to anticancer drugs is reduced, smaller amount of drugs can be applied with greater effects. US can therefore reduce not only the side effects of anti-tumor drugs, but also the cost needed for long-term cancer treatment. This application of US has the potential to replace these agents and, thereby, serve as a safer alternative to increase uptake of P-gp or MRP substrates in MDR cells as well as to clinically manage MDR. US is well suited for this application because it can be tailored to the specific treatment requirements for localized concentration of energy in deep tissues by controlling the amount of focusing, frequency, and duration.

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