Ultrasound in Med. & Biol., Vol. 34, No. 10, pp. 1697–1702, 2008

0301-5629/08/$–see front matter

ELSEVIER

doi:10.1016/j.ultrasmedbio.2008.03.008

● *Original Contribution*

OPTIMIZATION OF ULTRASOUND-MEDIATED *IN VITRO* REVERSAL OF MULTIDRUG RESISTANCE IN HUMAN HEPATOCARCINOMA CELL LINE HEPG2

BAO-JIN ZHAI, ZE-YONG SHAO, CHUN-LIANG ZHAO, KAI HU, DING-MING SHEN, and FENG WU

Clinical Center for Tumor Therapy of 2nd Affiliated Hospital and the Institute of Ultrasonic Engineering in Medicine, Chongqing Medical University, Chongqing, China

(*Received* 3 *September* 2007; *revised* 4 *March* 2008; *in final form* 4 *March* 2008)

Abstract—Previous studies have shown that ultrasound (US) could enhance cellular uptake and cytotoxicity of chemotherapeutic agents in drug-resistant cancer cells. The goal of this study was to investigate the optimization of physical parameters of US exposure for *in vitro* reversal of multidrug resistance (MDR) in human hepatocarcinoma cell line (HepG2). Using a constant total energy density (3.87 J/cm^2) that could maintain cell viability at the 90% level, we exposed parent (HepG2) and MDR variant (HepG2/ADM) tumor cells to US *in vitro* to a variety of US frequency, exposure intensity and duty cycle. After US exposure, flow cytometry was performed to measure retention of rhodamine 123 (Rh123) in both HepG2 and HepG2/MDR cells. The results showed that US frequency and duty cycle (DC) could influence the intracellular retention of Rh123 in HepG2/ADM tumor cells; intensity and exposure duration appeared to be of little importance. At a constant total energy density of 3.87 J/cm^2, the optimal US parameters for *in vitro* reversal of MDR in HepG2/ADM tumor cells appear to be 0.8 MHz, 0.43W/cm^2 and 60% DC, respectively. These findings support our hypothesis that varying the physical parameters would have an effect on efficiency of US-mediated reversal of MDR in cancer cells. (E-mail: mfengwu@yahoo.com) **© 2008 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Therapeutic ultrasound, Multidrug resistance, Neoplasm, High intensity focused ultrasound, Cavitation.**

INTRODUCTION

The phenomenon of multidrug resistance (MDR) in cancer cells is a significant obstacle to successful chemotherapy of human malignancies. By using pharmaceutical agents and small molecules, many strategies to overcome MDR have been explored in the past two decades. But, due to unacceptable side effects, none of them have been proven to be clinically useful in terms of being able to restore the cytotoxicity of anticancer drugs in MDR tumor cells (Fojo and Bates 2003; Ozben 2006). Novel approaches are therefore being investigated for overcoming MDR in cancer cells. Recently, ultrasound (US) has been shown to open the cell membrane temporarily, and allowing delivery of drugs, proteins and genes into viable cells (Mitragotri 2005; Newman and Bettinger 2007; Pitt et al. 2004; ter Haar 2007). US has also been used as a localised approach, in combination with chemotherapeutic agents or MDR modulators, to explore the possibility of enhancing the cytotoxicity of anticancer drugs in MDR tumor cells, and preliminary results were very encouraging (Liu et al. 2001a, 2001b; Rapoport 2004). Our previous study found that US exposure could significantly increase the uptake of rhodamine 123 (Rh123) and adriamycin (ADM) by MDR-resistant tumor cells (HepG2/ADM) and that MDR tumor cells became more sensitive to anticancer agents after US exposure (Shao et al. 2008).

Although US imaging is known to be safe in terms of tissue damage, US bioeffects, including heat and cavitation, for therapeutic purpose are dose-dependent. If US is not properly controlled, unintended bioeffects may be induced, resulting in undesirable tissue damage. Some investigations into US-mediated gene and drug delivery, have shown that inappropriate US exposure caused either cell damage leading to cell death, or inefficient delivery resulting in effects of little clinical significance (Duvs-

Address correspondence to: Dr. Feng Wu, HIFU Unit, The Churchill Hospital, Headington, Oxford, OX3 7LJ, UK. E-mail: mfengwu@yahoo.com

hani-Eshet and Machluf 2005; Larina et al. 2005; Liang et al. 2004; Rahim et al. 2004). In order to properly control low-intensity US for the reversal of MDR in cancer cells, it is important to optimize the US if this technique is to be useful in the clinical setting. The goal of this study, therefore, was to investigate the influence of US parameters, including frequency, duty cycle (DC), intensity and exposure time, on the reversal of MDR in human hepatocarcinoma cell line (HepG2), and to optimize the therapeutic parameters for *in vitro* reversal of MDR in cancer cells.

MATERIALS AND METHODS

Cell preparation

The details of the parent and MDR variant of HepG2 lines have been described previously (Zhai et al. 2006). The HepG2 variant (HepG2/ADM) developed drug resistance after incubation of parent HepG2 cells with increasing concentrations of adriamycin (ADM). In addition to direct resistance to ADM, it was also cross-resistant to vincristine (VCR), etoposide (VP-16), cisplatin (CDDP) and 5-fluorouracil (5-Fu). In order to retain its MDR characteristics, HepG2/ADM variant was maintained in a culture medium containing 1000 μg/L ADM.

Both cell lines were grown as monolayers in RPMI 1640 medium, supplemented with 10% (v/v) heat-inactivated fetal calf serum, 1.0 mmol/L sodium pyruvate at 37°C in a humidified atmosphere containing 5% CO_2. The cells were harvested by trypsin/ethylene diamine tetra-acetic acid (EDTA) digestion, centrifuged at 1500 $\times$g for 5 min, and resuspended in fresh cell media to their final concentration of 1×10^6 cells/ml for US exposure.

Therapeutic device and calibration

As described in detail previously (Shao et al. 2008), a therapeutic US device (Haifu Technology Co. Ltd., Chongqing, China) was used in this study. It principally consisted of therapeutic transducers, a US generator, a water tank and test tubes.

The US beam was produced by planar nonfocusing piezoelectric ceramic transducers, at operating frequencies of 0.4, 0.6, 0.8 or 1.7 MHz, respectively. The radius of the transducers was 2.0 cm and the transducers were driven continuously or intermittently, with duty circles (DC) ranging from 10% to 90%.

The water tank measured $20 \times 20 \times 15$ cm and was filled with degassed water maintained at human body temperature (37°C). The diameter of the polyethylene tube used for cell suspension was 1.0 cm. The bottom of each tube was cut off and a sheet of 25-μm thick polytetrafluoroethylene film was used to make one end of the tube. This film did not measurably attenuate the US beam.

Each tube was filled with 1-ml cell suspension (1×10^6 cells) and the height of the liquid was 1.27 cm. It was then lowered into the water tank and situated 1 cm from the transducer source. The US transducer was placed above the bottom of the water tank pointing upward and its beam aligned axially with the tube. There was the degassed water between the transducer and the tube.

Acoustic calibration measurements were conducted without the polyethylene tube. Calibrations were performed by experts in the national testing center (Wuhan Quality Supervision & Testing Center for Medical Ultrasound Equipment, Wuhan, China], with further technical expertise provided by the Wuhan Institute of Physics and Mathematics of the Chinese Academy of Sciences [Wuhan, China]). Both radiation force balance and hydrophone methods were employed to determine the acoustic pressures and intensities specified in this study. A polyvinylidene difluoride (0.5 mm diameter) needle hydrophone (Jiao Tong University, Shanghai, China) was used to measure the acoustic pressure in water bath. The acoustic pressure delivered to the cells was recorded as a voltage on an oscilloscope and then converted to spatial peak pulse average acoustic intensity *(I_{SPPA})* using the equation as follows: $I_{SPPA} = P^2/\rho c$ where P is the acoustic pressure amplitude, ρ is density of water and c is speed of sound in water.

When the tube was located in the near field, nonlinear distortion of the waveform was minimal and the peak positive and peak negative pressures were the same.

Ultrasound exposure

The total energy density was used as the therapeutic US dose parameter for determining the reversal of MDR in HepG2/ADM cells in this study. The total energy density was calculated as $E = I_{SPPA} \cdot t \cdot DC$, where E is the energy density [J/cm^2], I_{SPPA} is the intensity [W/cm^2], t is the total exposure time [s] and DC is the duty cycle [%].

Our previous study found that the viability of tumor cells varied with US intensity and exposure duration. When an intensity (I_{SPPA}) of 0.43 W/cm^2 was delivered to HepG2/ADM cells for 9 s (0.8 MHz), the cell viability was found to be 90% for a total energy density of 3.87 J/cm^2 (Shao et al. 2008). This total energy density (3.87 J/cm^2) has been taken to be optimal for the viability of HepG2/ADM cells exposed to US and has, therefore, been used for subsequent experiments.

In this study, the total energy density (3.87 J/cm^2) was maintained constant as an optimal parameter for all cell samples exposed to US. However, US frequency, duty cycle and exposure intensity were varied in order to optimise US parameters for reversal of MDR in HepG2/ADM cells. A total energy density of 3.87 J/cm^2 was delivered continuously to cell samples at frequencies of