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● *Original Contribution*

BLOCKING THE GLYCOLYTIC PATHWAY SENSITIZES BREAST CANCER TO SONODYNAMIC THERAPY

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Abstract—Inhibition of the increased aerobic glycolysis in cancer cells is a promising methodology for various malignant tumor therapies but is limited by systemic toxicity, at least in part. Recent studies suggest that dual restriction of glycolysis and mitochondrial function may overcome this issue. Sonodynamic therapy (SDT), a prospective therapeutic modality for cancers, has been reported to induce mitochondria-dependent cell damage. Here, we investigated the combined effect of SDT and 2-deoxyglucose (2DG), an anti-glycolytic agent, on breast cancer both *in vitro* and *in vivo*. *In vitro*, we found that, compared with a single treatment, SDT + 2DG co-treatment significantly decreased cell viability and increased cell apoptosis. Moreover, the generation of reactive oxygen species was enhanced and mitochondrial membrane potential (MMP) was reduced after SDT + 2DG co-treatment. Furthermore, the oxidative phosphorylation was also restrained after SDT + 2DG co-treatment, further to cause the blockage of ATP provision. *In vivo*, SDT + 2DG markedly reduced tumor volume and weight, consistent with the *in vitro* findings. Furthermore, toxicology tests concurrently indicated that the dosages of sinoporphyrin sodium and 2DG were comparatively tolerable. Generally, these results indicated that SDT + 2DG combination therapy may be an available, promising therapy for highly metastatic breast cancer. (E-mail: zkxian@snnu.edu.cn)

Key Words: **2-deoxyglucose, Glycolysis, Sonodynamic therapy, Energy metabolism, Breast cancer.**

INTRODUCTION

Cancer remains a rigorous challenge for governments and researchers worldwide despite the increasing development of diagnostic methods and therapies in past decades (Siegel et al. 2016). As the most common malignant cancer in females, breast cancer has a high incidence, high rate of malignancy and high frequency of recurrence and metastasis (Siegel et al. 2016). Surgical resection, chemotherapy and radiotherapy remain the main therapeutic methods for breast cancer (Santa-Maria et al. 2015). Drug resistance and tumor recurrence, as well as severe toxicity, are also inevitable risks (Yano et al. 2011). New therapeutic strategies and medicines for breast cancer are urgently needed.

Cancer cells differ from most normal differentiated cells with respect to their metabolic requirement for increased glycolysis, also known as the Warburg effect, which generally helps facilitate metastasis and inhibit apoptosis (Cairns et al. 2011; Hanahan and Weinberg 2011; Vander Heiden et al. 2009; Warburg 1956). Thus, elevated glycolysis in tumor cells makes it possible to target tumor metabolism to treat cancer. The most frequently used anti-glycolytic agent is 2-deoxyglucose (2DG), which is phosphorylated by hexokinase II, which is highly expressed in tumor cells, and subsequently inhibits ATP generation to damage malignant cells (Feng et al. 2015; Giammarioli et al. 2012; Golding et al. 2013). Nonetheless, 2DG has proved unsatisfactory in clinical trials for some cancer treatments because of its systemic toxicity (Maschek et al. 2004). On the other hand, multiple researchers have reported that 2DG manifests synergetic effects in inhibiting cancer cell proliferation by cooperating with various drugs such as cisplatin, adriamycin and paclitaxel (Cao et al. 2013; Maschek et al. 2004; Simons et al. 2007). Therefore, the combination of 2DG with other

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therapies may prove to be a promising therapeutic strategy for various cancers (Coleman et al. 2008; Feng et al. 2015; Xue et al. 2016).

Sonodynamic therapy (SDT) also has a synergistic effect, in which ultrasound of the appropriate wavelength facilitates the preferential uptake of sonosensitizers by tumor cells, followed by their activation (Yumita and Umemura 2004; Yumita et al. 2010). Because it is non-invasive, penetrates deeply, involves painless manipulation of patients and has a maneuverable frame, SDT has been extensively studied for the treatment of multiple cancers and other diseases in recent years (Milowska and Gabryelak 2005). The data have illustrated the anti-cancer effect of SDT both *in vitro* an *in vivo* (Li et al. 2014a; Tsuru et al. 2012; Zheng et al. 2014). Furthermore, along with other therapies, SDT is also reported to have a significant therapeutic effect, pointing to its potential as an anti-cancer strategy (Inui et al. 2014). Sonosensitizer is a critical component in SDT (Bellnier et al. 1989). Sinoporphyrin sodium (DVDMS), a derivative of photofrin, but with excellent sensitization activity compared with photofrin, has been granted independent intellectual property rights in China (Suslick 1990). In previous studies, we found that DVDMS could be effectively activated by ultrasound to exert obvious anti-cancer activity *in vivo* and *in vitro* (Li et al. 2014b; Wang et al. 2015; Xiong et al. 2015). Furthermore, we also found that the activated DVDMS induces mitochondria-dependent cancer cell damage because it locates mainly in mitochondria (Wang et al. 2015).

We hypothesized that targeting both mitochondria and aerobic glycolysis using SDT and 2DG would be more efficient than using either alone agent against breast cancer *in vitro* and *in vivo*. The underlying molecular mechanism was also explored, and it was found that adenosine triphosphate (ATP) depletion and mitochondrial disruption were drastically promoted in combination therapy.

METHODS

Reagents

Sinoporphyrin sodium (DVDMS, molecular weight: 1230.265) was provided by Qinglong Hi-Tech (Jiangxi, China) and dissolved in saline in dark environment to a 1 mM stock concentration; 2-deoxyglucose (2DG) was purchased from Sigma Aldrich (Poole, UK), and stored in sterilized phosphate-buffered saline solution (PBS, 0.1 M, pH 7.4) to a 1 M stock concentration.

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltertrazolium bromide tetrazolium (MTT) and 3, 6-diamino-9-[2-(methoxycarbonyl) phenyl] xanthylium chloride (Rho123) were available from Sigma Aldrich. 2', 7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) was purchased from Invitrogen (Paisley, UK). The Annexin V–FITC Apoptosis Detection Kit was obtained from Keygen Technology (Nanjing, China). The Calcein-AM/PI Double Stain Kit was purchased from Yeasen Biotechnology (Shanghai, China). Primary antibody against proliferating cell nuclear antigen (PCNA) was provided by Abcam (Cambridge, UK). Secondary antibody was supplied by Zhong Shan Golden Bridge Biotechnology (Beijing, China). Glutamic-pyruvic transaminase (GPT), glutamic-oxaloacetic transaminase (GOT) and blood urea nitrogen (BUN) assay kits came from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Cells and animals

Human breast cancer MDA-MB-231 and MCF-7 cell lines and human umbilical vein endothelial cells (HUVEC) were obtained from the cell bank of the Chinese Academy of Science (Shanghai, China). The murine breast cancer 4T1 cell line was purchased from the Department of Basic Medicine, Union Medical College (Beijing, China). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco Life Technologies, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Life Technologies, Carlsbad, CA, USA), 1% (v/v) 100 U/mL penicillin-streptomycin solution (Hyclone, Logan, UT, USA) and 1 mmol/L glutamine (Sigma Aldrich, Poole, UK) in a 37 °C humid incubator with 5% CO_2.

BALB/c mice (female, 18–23 g weight) were supplied by the Experimental Animal Center of the Fourth Military Medical University (Xi'an, China), and kept in an air-conditioned room with free access to food or water at 23 ± 2 °C. In experiments, 5×10^6 4T1 cells in 0.1 mL serum-free medium were subcutaneously injected into the left armpit region of BALB/c mice. The mice were grouped when tumor diameters reached the range 3 to 4 mm. All animal experiments were approved by the University's Institutional Animal Care and Use Committee and executed in accordance with the National Institute of Health's *Guide for the Care and Use of Laboratory Animals.*

Experimental protocol

In vitro, a therapeutic pulsed ultrasonic apparatus (Model 838 A-H-L-S) manufactured by Sheng Xiang High Technology (Shenzhen, China) was used in this study. The application parameters were as follows: frequency of 840 kHz, intensity of 0.2 W/cm^2, duration of 60 s, planar transducer diameter of 35 mm. The transducer works at an intermittent mode of 5 s on and 2 s off. The area between the transducer and cell culture plate was filled with acoustic couplant to mediate the transmission of hyperacoustic waves. There was no obvious change in the temperature, as assessed with a digital thermometer, in this process. Thus, ultrasound was responsible for the biological reaction in the experiment.

Cells in the exponential phase were collected, suspended at 2×10^5 cells/mL and cultured in a 35-mm cell