Journal of Controlled Release 258 (2017) 67–72

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Tumor ablation using low-intensity ultrasound and sound excitable drug

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ARTICLE INFO

Keywords:
Ultrasound
Plasma membrane
Rupture
Sonodynamic therapy
Tumor ablation

ABSTRACT

The cell membrane is a semi-fluid container that defines the boundary of cells, and provides an enclosed environment for vital biological processes. A sound excitable drug (SED) that is non-cytotoxic to cells is developed to disrupt the plasma membrane under gentle ultrasound insonation, 1 MHz, 1 W/cm^2. The frequency and power density of insonation are within the physical therapy and medical imaging windows; thus the applied ultrasound is safe and not harmful to tissues. The insertion of SEDs into the plasma membrane is not toxic to cells; however, the intruding SEDs weaken the membrane's integrity. Under insonation, the ultrasound energy destabilized the SED disrupted membranes, resulting in membrane rupture and eventual cell death. In a xenograft breast tumor model, the SED alone or the ultrasound alone caused little adverse effects to tumor tissue, while the combined treatment triggered necrosis with a brief local insonation of 3 min. The described sono-membrane rupture therapy could be a safe alternative to the currently used high-energy tissue ablation technology, which uses X-rays, gamma rays, electron beams, protons, or high-intensity focused ultrasound.

1. Introduction

Tumor ablation, which uses high-energy particles or waves, is an important and common means of cancer treatment. It is generally effective, but is often associated with severe side effects. The high-energy source causes undesired damage to the tissues along the radiation pathway. A low-energy technology, which could achieve the same ablation effect without damaging normal tissues, would be a preferred choice. In addition, thermal ablation that uses radiofrequency (RF) energy or high intensity focused ultrasound (HIFU) as the heat source are known to have limitations due to the convective cooling of blood flow, which can protect cancer cells near blood vessels from thermal damage. This sometimes results in recurring aggressive tumor growth. A technology based on similar modalities that does not mainly or entirely rely on thermal ablation is therefore highly desirable.

Ultrasound technology has been widely applied in diagnostic imaging, interventional guidance, and physical therapy. In addition to the routine imaging and medical applications, low-intensity ultrasound (< 5.0 W/cm^2) has been introduced in recent years to assist therapies [1,2]. In contrast to the use of HIFU for direct thermal ablation, low-intensity ultrasound works together with chemical cytotoxic agents [3,4]. A few recent studies have reported the use of the combination of chemotherapeutic agents with ultrasound enhances the drug's anti-cancer effects, and sensitizes drug resistant cells. It was believed that ultrasound-induced cavitation weakens the cell membrane and facilitates the intracellular distribution of drugs [5,6]. Direct tumor insonation could also loosen up tight tissue junctions in under-vascularized areas for better intratumoral drug dispersions [7,8]. Most recently, an implantable ultrasound device was used to open up the blood-brain barrier in brain tumor patients to enhance drug delivery [9]. Drugs bound or loaded micrometer sized hollow microbubbles also have been used to deliver drugs [1,10]. Alternatively, microbubbles in conjunction with other carriers, such as liposomes or micelles, could be burst to achieve a local drug release by a locally applied ultrasound energy [11–13].

Sonodynamic therapy (SDT) is similar to the clinically used photodynamic therapy (PDT), but instead of light, ultrasound is used to activate therapeutic sensitizers [14–16]. PDT, which allows for the exclusive eradication of diseased tissue while sparing surrounding healthy cells from damage, suffers from poor tissue penetration and light diffusion. The substitution of ultrasound as the energy source in SDT allows the therapy to overcome these roadblocks. The physics of ultrasound propagation allows for a more favorable and direct deep tissue penetration compared to photons. Most of the reported sonosensitizers are also photosensitizers or derived from photosensitizers; therefore, the accepted mechanism of action of SDT is similar to that

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http://dx.doi.org/10.1016/j.jconrel.2017.05.009
Received 6 September 2016; Received in revised form 11 April 2017; Accepted 9 May 2017
Available online 10 May 2017

of PDT [14,17]. Conversely, a few non-photosensitive sonosensitizers have also been identified [18–20]. These sonosensitizers cannot be excited by light; but under insonation, reactive oxygen species (ROS) were generated, triggering apoptosis. Intrigued by these prior arts, we sought to develop a novel non-toxic non-light-sensitive sound excitable drug (SED) to pair with a low-intensity ultrasound for tumor ablation.

2. Materials and methods

2.1. Synthesis of RB4 (2,3,4,5-tetrachloro-6-(6-hydroxy-2,4,5,7-tetraiodo-3-oxo-3H-xanthen-9-yl)-N-(2-hydroxyethyl)-benzamide)

Rose Bengal (RB), *O*-(Benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), and diisopropoyl ethyl amine (DIEPA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other solvents, including dimethyl formamide (DMF), dichloromethane (DCM), and methanol (MeOH), were purchased from Thermo Fisher (Waltham, MA, USA).

RB4 was synthesized following a published protocol [21]. To a solution of RB (509 mg, 0.5 mmol) in DMF (3 ml) and DIEPA (2 ml) was added HBTU (190 mg, 0.5 mmol) and stirred at room temperature (RT) for 4 h, then 2-aminoethanol (91 μl, 1.5 mmol) was added and reacted overnight at RT. The solvent was removed under reduced pressure. The residue was extracted with DCM and washed with brine, dried over anhydrous sodium sulfate and concentrated, the residue was purified by silica gel column, eluted with DCM, DCM/MeOH = 10/0.5 and 10/1(V/V) to give product as pale yellow solid (161 mg, yield 31.7%). TCL: Rf = 0.3, DCM/MeOH = 10/1. ^{1}H NMR (DMSO-6d, 300 MHz): δ 2.99 (s, 2H), 3.32 (s, 2H), 4.60 (br, 1H), 5.76 (s, 1H), 7.25 (s, 1H), 10.07 (s, 1H). ESI-MS: 1015.57 $(M\text{-}H)^-$. The NMR and Mass spectra of RB4 are included in the Supplementary Materials and Methods.

2.2. Tumor cell culture and animals

MDA-MB-468 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and were maintained in Leibovitz's L-15 medium (Corning, Manassas, VA, USA), and supplemented with 10% fetal bovine serum and antibiotics penicillin (100 μg/ml) and streptomycin (100 μg/ml) without CO_2. HT1080-Luc2 cells obtained from Caliper (Hopkinton, MA, USA) were grown at 37 °C with 5% CO_2 in Eagle's MEM from Corning (Manassas, VA, USA), and supplemented with 10% fetal bovine serum (Seradigm, Radnor, PA, USA) and antibiotics penicillin (100 μg/ml) and streptomycin (100 μg/ml) (Invitrogen, Carlsbad, CA, USA).

All animal studies were performed in compliance with the approved animal protocols and guidelines of the Institutional Animal Care and Use Committee of Weill Cornell Medicine. BALB/c Nu/Nu female nude mice (5–6 weeks) were purchased from Charles River (Wilmington, MA, USA).

2.3. Ultrasound system

A portable bench top ultrasound system (Accusonic plus, Metron, Warrenville, IL) with 1–3 MHz, and 10, 20, 50 and 100% duty cycle capability was used to conduct all studies (Fig. S1). Transducer one (5 cm^2, Model# 901150, Metron) and transducer two (0.75 cm^2, Model# 901175, Metron) were used in the cell culture and animal studies, respectively.

2.4. Sonotoxicity with RB4

MDA-MB-468 cells (5×10^4) in 24 well plates were incubated in complete media with 10 μM RB4 for 1 h prior to the ultrasound treatment. The plates were then placed on a pre-cut gel pad (2 cm × 3 cm, Parker Laboratories, Fairfield, NJ, USA) with multi-purpose ultrasound gel (Parker) over a mounted handheld ultrasound transducer (5 cm^2) at 1 MHz, 1 W/cm^2 for 30 s, 100% duty cycle. Cells were checked under the EVOS® FL Auto Cell Imaging system microscope (Thermo Fisher). Cell viability at 24 h was quantitated using an MTS assay (Promega, Madison, WI, USA). The plate was incubated at 37 °C for 4 h. Absorbance was measured at 490 nm using a microplate reader (Infinite M1000 Pro, Tecan, Männedorf, Switzerland).

2.5. Mechanistic study of death process by flow cytometry

MDA-MB-468 cells (5×10^4) were incubated in complete media with or without 10 μM RB4 for 1 h prior to ultrasound treatment. Then the plates were insonated at 1 MHz, 1 W/cm^2, 30s, 100% duty cycle. Following treatment, cells were re-incubated for 1 day, 4 groups of cells (Control, RB4 alone, Ultrasound alone, and RB4 with Ultrasound) were collected and washed twice with pre-cooled PBS. Cells were stained with FITC-conjugated Annexin-V (Life Technologies) and propidium idodide (PI, Life Technologies) for 15 min as per the manufacture's instructions, and then analyzed by flow cytometry (Gallios, Beckman Coulter). The percentage of dead cells and those undergoing apoptosis were analyzed using Kaluza Software.

2.6. Chemical ROS assay

To study the insonation induced ROS generation in solution, RB4 was tested using a modified 2′, 7′–dichlorofluorescin diacetate (DCFH-DA) assay. DCFH-DA (1 ml, 1 mM in MeOH, Aldrich) was hydrolyzed in NaOH aqueous solution (0.01 N, 4 ml) at RT for 30 min to yield a non-fluorescent DCFH intermediate. The solution was neutralized with 20 ml of NaH_2PO_4 (25 mM) and shielded with aluminum foil. The final solution of DCFH was around 40 μM. RB4 (1.0 mg) was dissolved in DMSO (1 ml), and then diluted with water into a 20 μM solution. The RB4 solution (20 μM, 10 ml) was mixed with a DCFH solution (40 μM, 10 ml) as the test solution. The test solution (0.5 ml) was placed into each well of a 24-well plate. The DCFH solution without RB4 was included as a background control. The plates were treated with ultrasound (0.4–1.2 watt/cm^2) one well by one well for 30 s; the insonated wells were then checked using a fluorescence plate reader, ex 485 nm/em 520 nm.

2.7. Cell based ROS scavenging assay

MDA-MB-468 cells (5×10^4) were treated with free radical scavengers, L-Histidine (10 mM), D-Mannitol (100 mM), *N*-acetyl cysteine (NAC, 0.5 mM), and superoxide dismutase (SOD, 100 μg/ml) for 30 min. The treated cells were then incubated with a fresh media, containing RB4 (10 μM), for an hour. After incubation, the wells were insonated (1 MHz, 1 W/cm^2, 30 s, 100% DC) as described above. One day later, the cell's viability was assessed using the MTS solution.

2.8. In vivo SMRT effect using preloaded tumor cells

MDA-MB-468 cells were suspended in PBS or RB4 (10 μM) in PBS for 1 day. The cells (5×10^6, 0.1 ml) were subcutaneously injected into both flanks. The left tumors, which were only treated with PBS, were the internal control; while the right tumors were treated with RB4 (10 μM) only, ultrasound only (1 MHz, 1 W/cm2, 100% DC, 3 min), or an RB4/ultrasound combination (n = 7). The transducer size is 0.75 cm^2 (Metron). Tumor size was measured with slide calipers on days 7, 10, 14, 17, 21, 24, 28, and 36.

2.9. In vivo SMRT effect with intra-tumoral injected RB4

MDA-MB-468 cells (10^7, 0.1 ml PBS) were subcutaneously inoculated into both flanks of BALB/c Nu/Nu female nude mice (5–6 weeks). The RB4 injections and ultrasound therapies were performed when the tumors had grown to approximately 4–5 mm in diameter, 20–22 days