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● *Original Contribution*

ULTRASOUND AND MICROBUBBLE-INDUCED LOCAL DELIVERY OF MICRORNA-BASED THERAPEUTICS

Rick F. J. Kwekkeboom,* Zhiyong Lei,† Sylvia J. P. Bogaards,* Eric Aiazian,‡§ Otto Kamp,§ Walter J. Paulus,* Joost P. G. Sluijter,† and René J. P. Musters*

*Department of Physiology, VU University Medical Center, Amsterdam, The Netherlands; †Department of Experimental Cardiology, University Medical Center Utrecht, Utrecht, The Netherlands; ‡Axle International, The Hague, The Netherlands; and §Department of Cardiology, VU University Medical Center, Amsterdam, The Netherlands

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Abstract—MicroRNAs are involved in many pathologic processes and are a promising target for therapeutic intervention. However, successful, localized delivery of microRNA-based therapeutics is lacking. In this study, cationic ultrasound-responsive microbubbles (MBs) were used to deliver microRNA blockers and mimics *in vitro* and *in vivo*. Cationic MBs successfully delivered microRNA blockers to human endothelial cells on ultrasound (US) exposure *in vitro*. This *in vitro* US protocol did not successfully deliver microRNA mimics to skeletal muscle of mice, whereas an US protocol that is routinely used for contrast imaging did. Additionally, we used cationic MBs and US to locally deliver antimiR and antagomiR molecules with US causing inertial cavitation. Delivery of antimiR to the extracellular compartments of the muscle was only slightly increased, whereas delivery of antagomiR to the capillaries, myocytes and extracellular space was significantly increased. AntagomiR seems to be a more suitable microRNA blocker than antimiR for use in combination with MBs and US for local delivery. (E-mail: R.kwekkeboom@vumc.nl) © **2015 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Ultrasound, Microbubbles, MicroRNA, AntimiR, AntagomiR, Drug delivery, Endothelial cells.**

INTRODUCTION

Soon after their discovery in humans (Pasquinelli et al. 2000), microRNAs (miRNAs) were recognized as a potential target for pharmaceutical intervention (Van Mil et al. 2009). Therapy can focus on either gain of function (*e.g.*, with miRNA mimics) or inhibition (*e.g.*, with antimiRs or antagomiRs) of miRNA. AntimiRs are single stranded, RNase-resistant RNA molecules that can block miRNA function (Calin et al. 2002). AntimiRs are hydrophilic and are considered not to be taken up efficiently by cells. An antimiR can be modified to create an antagomiR by adding a cholesterol group to the 3′ end of the antimiR, resulting in its cellular uptake on systemic injection (Krutzfeldt et al. 2005). MicroRNA activity can also be boosted using miRNA mimics. Developments in miR mimic chemistry have not led to substantial success in *in vivo* miR mimic delivery (Van Rooij and Olson 2012). Ideally, antimiR, antagomiR and miRNA mimics would be injected in the blood and taken up only by specific tissues or cells. Development of drug delivery vehicles for antimiR and miRNA mimics is still in its infancy (Akinc et al. 2008; Kota et al. 2009; Thum 2012), and localized delivery of antimiR, antagomiR and miRNA mimics remains a challenge.

One type of drug delivery vehicle for localized delivery is the microbubble (Hernot and Klibanov 2008). Microbubbles (MBs) are gas-filled spheres with a micrometer diameter between 1 and 10 μm and are used as a contrast agent in ultrasonography. These gas-filled spheres can be given a positive net charge and loaded with molecules such as oligodeoxynucleotides (Haag et al. 2006) and siRNA (Carson et al. 2012). After intravenous injection of these "loaded" MBs, ultrasound (US) can locally cause MBs in the ultrasonic field to cavitate. Cavitation of MBs causes loss of their payload and permeabilizes the vasculature, leading to an increased localized release and uptake of therapeutics at the site of US treatment (Carson et al. 2012; Chappell et al. 2008; Christiansen et al. 2003; Haag et al. 2006; Leong-Poi et al. 2007). *In vitro* studies have revealed that

Address correspondence to: Rick F.J. Kwekkeboom, Department of Physiology, VU University Medical Center, Van der Boechorststraat 7, 1081 BT, Amsterdam, The Netherlands. E-mail: R.kwekkeboom@vumc.nl

MB + US-induced drug delivery to cells can be achieved at low-mechanical-index (MI, ranging from 0.1 to 0.4) US, causing stable oscillation of MBs (Karshafian et al. 2009). On the other hand, *in vivo* studies report the use of high-MI (>1) US, leading to destruction of MBs (inertial cavitation). These high-MI *in vivo* approaches have been successful in delivering drugs locally without causing major damage (Carson et al. 2012; Chappell et al. 2008; Christiansen et al. 2003; Haag et al. 2006; Leong-Poi et al. 2007). This discrepancy in US parameters between *in vitro* and *in vivo* raises the question of whether the mechanism we are looking at *in vitro* is the same as the *in vivo* mechanism of drug delivery.

The first goal of this study was to test whether antimiR and antagomiR molecules can be loaded on cationic MBs and subsequently delivered intracellularly to cultured human umbilical vein endothelial cells (HUVECs). The second goal of this study was to test whether US settings that successfully deliver antimiR and antagomiR *in vitro* can be used for *in vivo* delivery of miRNA mimics (as a model small RNA) to skeletal muscles of mice. The third goal was to compare antimiR and antagomiR with respect to tissue distribution in skeletal muscles of mice after MB + US-induced local delivery at 7 and 2 MHz. The final aim of this study was to establish what kind of miR blocking agent is most suitable for local delivery using MBs and US and to re-evaluate the relevance of *in vitro* data for *in vivo* small RNA delivery.

METHODS

AntimiR, antagomiR and miRNA mimics

For *in vitro* transfection experiments and *in vivo* delivery experiments, antimiRs and antagomiRs targeting miR-214 were designed and synthesized (VBC-Biotech, Vienna, Austria), as were mismatch control antimiRs and antagomiRs (see Supplementary Fig. A in the online version at http://dx.doi.org/10.1016/j.ultrasmedbio.2014.08.012). For *in vivo* miR mimic delivery, a commercially available double-stranded Ambion miR-159a mimic (Life Technologies, Bleiswijk, Netherlands) was used; miR-159a is not endogenously expressed by mice.

Fluorescence imaging

Microscopic imaging was performed on a ZEISS Axiovert Marianas 200M inverted fluorescence microscope (Intelligent Imaging Innovations, Denver, CO, USA) in combination with Slidebook 5.0 software (Intelligent Imaging Innovations) for qualitative and quantitative analysis.

Cationic MB production and antimiR complex formation

Cationic MB design was based on work by Christiansen et al. (2003), but altered to improve MB–antimiR/antagomiR binding. Distearoylphosphatidylcholine (DSPC, Avanti Polar Lipids, Alabaster, AL, USA), distearoyl-tri-ammoniumpropane (DSTAP, Avanti Polar Lipids) and polyethylene glycol-40 (PEG40) stearate (Life Technologies) were dissolved in glycerol (Invitrogen, Life Technologies) at 10 mg/mL at 70°C. Phospholipids and PEG40 stearate were transferred to a 2-mL reaction tube in a DSPC:DSTAP:-PEG40-stearate weight ratio of 8:4:1 to an H_2O:glycerol:-propylene glycol mixture with a 24:14:3 volume ratio. Perfluorobutane gas (C_4 $F_{10,}$ F2 Chemicals, Lancashire, UK) was added to the capspace of the reaction tube, after which the phospholipid mixture was placed in a Decon FS200 ultrasonic bath (Decon Ultrasonics, East Sussex, UK) for 10 min. MBs were created by high-speed shaking (4500 rpm) using a Vialmix device (Lantheus Medical Imaging, North Billerica, MA, USA). MBs were washed three times by centrifugal flotation. MB size distribution and amount were determined using a Multisizer 3 (Beckman Coulter Nederland, Woerden, Netherlands). Next, MBs were tested for their ability to load fluorescein isothiocyanate-labeled antimiR, antagomiR and Ambion miRNA mimic (Life Technologies) molecules. MBs were diluted to 500×10^6 MBs/mL, and antimiR, antagomiR or miR mimic was added to a concentration of 1.4, 2.9 or 3.3 nmol/mL, respectively. After 5 min of incubation, complexes were analyzed microscopically using differential inference contrast imaging and fluorescein isothiocyanate fluorescence imaging. Additionally, the cationic MB payload was determined for antimiR molecules. Two nanomoles of fluorescein isothiocyanate-labeled antimiR was added to 200×10^6 cationic MBs in a volume of 300 μL, and the mixture was allowed to form complexes for 5 min. Degassed H_2O was subsequently added up to a volume of 3 mL, and unbound antimiR was separated from the cationic MBs using centrifugal flotation at 300*g* for 10 min. Unbound antimiR was collected, and its concentration was determined using a FLUOstar Galaxy microplate reader (MTX Lab Systems, Vienna, VA, USA). The percentage of antimiR bound to the MBs was determined by subtracting the unbound amount of antimiR from the added amount of antimiR.

MB-induced transfection of HUVECs with antimiR at an angle of 90°

A custom-designed *in vitro* US treatment chamber was manufactured for treatment of HUVEC monolayers at an angle of 90°, that is, pushing MBs to the cells (see Supplementary Fig. B, *In vitro* system 1, in the online version at http://dx.doi.org/10.1016/j.ultrasmedbio.