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Increased local delivery of antagomir therapeutics to the rodent myocardium using ultrasound and microbubbles

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ABSTRACT

Recent developments in microRNA (miRNA) research have identified these as important mediators in the pathophysiological response upon myocardial infarction (MI). Specific miRNAs can inhibit the translation of entire groups of mRNAs, which are involved in specific processes in the pathophysiology after MI, e.g. the fibrotic, apoptotic or angiogenic response. By modulating miRNAs in the heart, these processes can be tuned to improve cardiac function. Antagomirs are effective miRNA-inhibitors, but have a low myocardial specificity and cardiac antagomir treatment therefore requires high doses, which causes side effects. In the present study, ultrasound-triggered microbubble destruction (UTMD) was studied to increase specific delivery of antagomir to the myocardium. Healthy control mice were treated with UTMD and sacrificed at 30 min, 24 h and 48 h, after which antagomir delivery in the heart was analyzed, both qualitatively and quantitatively. Additionally, potential harmful effects of treatment were analyzed by monitoring ECG, analyzing neutrophil invasion and cell death in the heart, and measuring troponin I after treatment. Finally, UTMD was tested for delivery of antagomir in a model of ischemia–reperfusion (I/R) injury. We found that UTMD can significantly increase local antagomir delivery to the non-ischemic heart with modest side-effects like neutrophil invasion without causing apoptosis. Delivered antagomirs enter cardiomyocytes within 30 min after treatment and remains there for at least 48 h. Interestingly, after I/R injury antagomir already readily enters the infarcted zone and we observed no additional benefit of UTMD for antagomir delivery. This study is the first to explore cardiac antagomir delivery using UTMD. In addition, it is the first to study tissue distribution of short RNA based therapeutics (~22 base pairs) at both the cellular and organ levels after UTMD to the heart in general. In summary, UTMD provides a myocardial delivery strategy for non-vascular permeable cardiac conditions later in the I/R response or chronic conditions like cardiac hypertrophy.

1. Introduction

Since the discovery of microRNAs (miRNAs) in humans [1], many of these small non-coding RNA molecules have been found to be involved in progressive pathological conditions, including several cardiac diseases [2]. miRNAs are small RNA molecules that bind to the 3′ untranslated region of mRNA molecules and thereby block their translation. One miRNA can potentially bind to whole groups of mRNA targets that are directly involved in specific processes like apoptosis, angiogenesis or fibrosis formation [3]. Blocking the functionality of specific miRNAs has been feasible for several years [4] and by this approach cardiac function was improved in several models of disease [5,6]. However, current approaches that use miRNA inhibitors have low organ specificity [7] and therefore employ high doses, making side effects and cost an issue and creating the need for better delivery strategies. One such delivery strategy is the use of gas-filled microbubbles (MB) in combination with ultrasound (US) [8]. Intravascular MB can, upon destruction by US Triggered MB Destruction (UTMD), locally increase vascular permeability and cellular membrane permeability [9,10]. Several studies have successfully delivered plasmid DNA specifically to the heart to improve cardiac function [11–13]. As this strategy has also been used to locally deliver siRNA as a proof of concept [14], UTMD is an interesting option to increase cardiac local delivery of miRNA inhibitors due to similarities in chemical structure with siRNAs.

In this study, we hypothesized that UTMD can be used to increase antagomir inhibitor delivery to the healthy and diseased hearts. Antagomirs are single stranded, 2′-*O*-methyl and phosphorothioate linked oligoribonucleotides with a sequence that is

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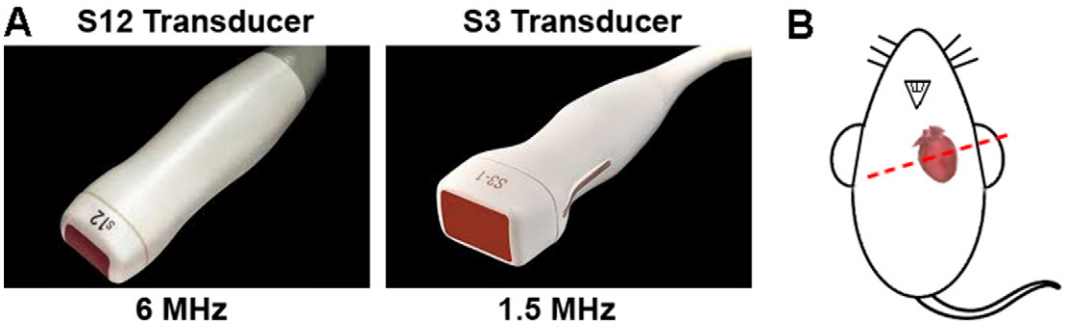

Fig. 1. Used US-transducers for UTMD. A) Transducers operate at different frequencies as displayed below the images. The S3 transducer has an imaging surface which is 4× as large as the S12 transducer, making it more difficult to precisely place the transducer over a mouse heart. B) The dotted red line represents the imaging/treatment plane that was used for UTMD.

exactly complementary to its miRNA-target and a cholesterol attached to the 3′ end. As a highly relevant cardiac disease we choose a model of ischemia–reperfusion (I/R) to test UTMD effectivity. We investigated the localization of these delivered antagomirs at both the whole organ as well as at the cellular level to determine feasible cellular targets for miRNA modulation, specific for UTMD. In addition, we determined the localization of delivered antagomir over time (up to 48 h) and investigated the acute functional response of the heart to UTMD and the later (patho) physiological response of the heart to UTMD at a cellular level.

2. Material and methods

2.1. Antagomir–microbubble preparation

Cationic microbubbles (cMB) were produced as described previously [15]. Briefly, a combination of 1,2-distearoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids, Alabaster, LA, USA), 1,2-stearoyl-3-trimethylammonnium-propane (Avanti Polar Lipids) and polyoxyethylene-40-stearate (Life Technologies, Bleiswijk, The Netherlands), in a molecular ratio of 2:1:0.6, was solved in a $H_2O_{(l)}$, glycerol (Life technologies), propylene glycol (Sigma-Aldrich, Zwijndrecht, The Netherlands) mixture (volume 24:13:3) in a 2 ml tube with perfluorobutane gas ($C_4F_{10(g)}$, F2 Chemicals, Lancashire, UK) in the capspace. cMB were produced by means of mechanical agitation using a Vialmix™ (Lantheus Medical Imaging, North Billerica, MA, USA) high-speed shaker. Subsequently, cMB were washed 3 times using centrifugal flotation. Size distribution and amount of washed cMB were determined using a Multisizer 3 (Beckman Coulter Nederland, Woerden, Netherlands) and concentrated to $2 * 10^9$ cMB/ml in H_2O for in vivo experiments. Antagomir formulation for analysis time points 30 min and 24 h was Cy3-5′-a*c*ucccugccuuucccuua*u*g*u*-3′-cholesterol. For the 48 h timepoint it was Cy3-5′-a*c*ugccugucugugccugc*u*g*u*-3′-cholesterol. Antagomirs are 3′ cholesterol modificated, 2′-*O*-methylated, and contain PTO-linkages (*) at the first two and last four nucleotides. Antagomirs were custom-ordered from VBC-Biotech, Vienna, Austria as reported before [16]. Prior to UTMD treatment, 2 nmol of antagomir was added to $200 * 10^6$ cMB in 100 μl H_2O at room temperature and incubated for 5 min to bind the negatively charged antagomir

Table 1
Ultrasound application protocols.

Treatment protocol:	I.v. injection:	Transducer:	US mode:	MI:	Frequency:	Focus/depth:	Time points:
Control	Antagomir only	S3	Power Doppler	1.4	1.5 MHz	4 cm/9 cm	30 min, 24 h, 48 h
S12 B-mode – 1 (Fusion 1)	Antagomir + cMB	S12	B-mode	1.8	7 MHz	1.5 cm/9 cm	30 min
S12 B-mode – 2 (Fusion 5)	Antagomir + cMB	S12	B-mode	1.8	7 MHz	1.5 cm/9 cm	30 min
S3 Power Doppler	Antagomir + cMB	S3	Power Doppler	1.4	1.5 MHz	4 cm/9 cm	30 min, 24 h
S12 Power Doppler 1	Antagomir + cMB	S12	Power Doppler	1.5	7 MHz	1.5 cm/9 cm	24 h, 48 h
S12 Power Doppler 2	Antagomir + cMB	S12	Power Doppler	1.5	6 MHz	3 cm/16 cm	48 h

Table of treatment parameters. I.v. = intravenous, MI = mechanical index.

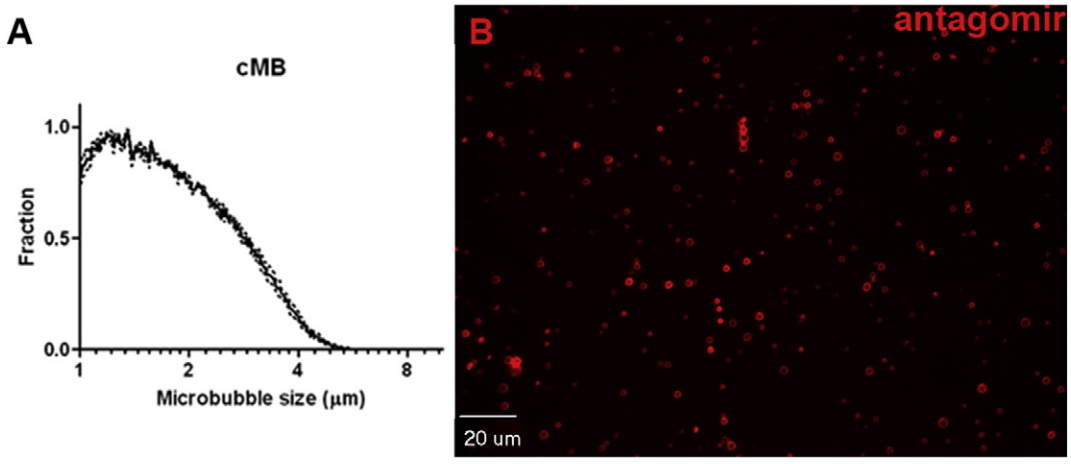

Fig. 2. Cationic microbubble size distribution and antagomir attachment. A) Graph of microbubble size distribution. On the x-axis the microbubble diameter is depicted. On the y-axis, the amount of microbubbles of a particular size is displayed as a fraction of the most-abundant size. B) Fluorescence microscope image of antagomir bound to cationic microbubbles.