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Multivalent nanoparticles bind the retinal and choroidal vasculature☆

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ABSTRACT

The angiotensin II receptor type 1 (AT_1R), which is expressed in blood vessels of the posterior eye, is of paramount significance in the pathogenesis of severe ocular diseases such as diabetic retinopathy and age-related macular degeneration. However, small molecule angiotensin receptor blockers (ARBs) have not proven to be a significant therapeutic success. We report here on a nanoparticle system consisting of ARB molecules presented in a multivalent fashion on the surface of quantum dots (Qdots). As a result of the multivalent receptor binding, nanoparticles targeted cells with high AT_1R expression and inhibited their angiotensin receptor signaling with an IC_{50} of 3.8 nM while showing only minor association to cells with low AT_1R expression. After intravenous injection into the tail vein of mice, multivalent nanoparticles accumulated in retinal and choroidal blood vessels of the posterior eye. At the same time, multivalent ligand display doubled the Qdot concentration in the blood vessels compared to non-targeted Qdots. Remarkably, ARB-targeted Qdots showed no pronounced accumulation in AT_1R-expressing off-target tissues such as the kidney. Following systemic application, this multivalent targeting approach has the potential to amplify AT_1R blockade in the eye and concomitantly deliver a therapeutic payload into ocular lesions.

1. Introduction

The angiotensin II receptor type 1 (AT_1R) is a G protein-coupled receptor (GPCR) that is known for its role in regulating physiologic fluid and salt homeostasis and blood pressure [1]. However, it also serves as a key mediator of inflammation [2] and potent growth factor [3]. As such, AT_1R and its ligand angiotensin II are critically important in pathological angiogenesis [4]. Two prominent examples of pathologies in which the receptor plays a pivotal role are exudative age-related macular degeneration (wet AMD) and proliferative diabetic retinopathy, diseases of the posterior eye that lead to blindness and severe visual impairment in a large number of patients each year [5,6]. In both of these neovascular diseases, inflammation mediated by AT_1R appears to be crucial for pathogenesis and disease progression [7,8]. This is not surprising, since angiotensin II stimulates formation of retinal blood vessels and concomitantly increases their leakiness [9]. Despite this clear link between AT_1R and ocular disease, the effect of angiotensin receptor blockers (ARBs) on therapeutic outcomes has only been modest [10–12]. Since ARBs display enormous plasma protein binding of more than 95% [13,14] and may be taken up by several other tissues such as kidneys, adrenal glands, vascular smooth muscle cells and heart, all of which abundantly express the receptor [15], it is possible that therapeutic ARB levels cannot be maintained in the eye.

To improve AT_1R antagonism in the ocular vasculature we developed a nanoparticle system that displays multiple receptor antagonists at the surface of a colloid, thus allowing the formation of multivalent ligand-receptor interactions towards AT_1 receptors [16]. The nanoparticles retain overall binding affinity while each individual ligand receptor-interaction is reduced. This gives these colloids the capability to differentially target tissues with higher AT_1R expression. We envision a scenario in which the enhanced receptor binding – resulting in prolonged contact times on the cell surface – and the nanoparticles' diminished off-target tissue accumulation could enhance the ocular bioavailability of ARBs and thus amplify their therapeutic benefit.

Although *in vitro* data have convincingly proven that multivalent nanoparticles bind AT_1R-expressing cells [16], the capability of these nanomaterials to interact with the choroidal and retinal blood vessels has not been assessed so far. Since the choroidal blood vessels, the major underlying blood vessels of the retina, have the highest perfusion of all vascular beds within the human body [17], only a limited timeframe is available for the colloids to establish strong nanoparticle-cell interaction and thus bind endothelial cells. Therefore, this study aims to explore the *in vivo* potential of nanomaterials that use ARBs as

☆ This article is dedicated to Manfred Scheer on the occasion of his 60th birthday.

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multivalent targeting ligands and not as the nanoparticles themselves [18] or as a nanoparticle payload [19,20]. EXP3174, the active metabolite of the prominent ARB losartan, was covalently immobilized on the surface of highly fluorescent PEGylated quantum dots (Qdots), which were used as model colloids due to their intense fluorescence and highly monodisperse size [21]. *In vitro* binding and receptor affinity of nanoparticles to cell lines with different levels of AT_1R expression was investigated with confocal microscopy and intracellular calcium measurements. Multi-spectral imaging, immunohistochemistry and inductively coupled plasma mass spectrometry (ICP-MS) were utilized to follow the nanoparticles' fate *in vivo* and gain insight into their biodistribution and tissue accumulation.

2. Materials and methods

2.1. Materials

EXP3174, also known as losartan carboxylic acid, was purchased from Santa Cruz (Heidelberg, Germany). Qdots® 655 ITK™ amino PEG (Life Technologies, Carlsbad, CA, USA) were used as nanoparticle starting material. All chemicals were obtained from Sigma Aldrich (Taufkirchen, Germany) in analytical grade unless stated otherwise. All buffers and solutions were prepared using ultrapure water, which was obtained from a Milli-Q water purification system (Millipore, Billerica, MA, USA). Buffers used for nanoparticle labeling and purification were 10 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer pH 4.8, 50 mM borate buffer pH 8.5 and Dulbecco's phosphate buffered saline (DPBS) pH 7.4 consisting of 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.7 mM KCl and 138 mM NaCl.

Mowiol mounting medium was prepared by mixing 6.9 g glycerol 87% (AppliChem GmbH, Darmstadt, Germany) with 2.4 g Mowiol 4-88, 5.1 mL H_2O and 12.0 mL Tris buffered solution (0.2 M, pH 8.50). Mowiol mounting medium was prepared without the antifading agent 1,4-diazabicyclo[2.2.2]octane (DABCO) since it potentially quenches the Qdot fluorescence [22].

2.2. Cell culture

Human adrenal gland carcinoma cells NCI-H295R (ATCC No. CRL-2128) were cultured in RPMI1640 medium containing 10% fetal bovine serum (Sigma Aldrich, Taufkirchen, Germany) and supplemented with insulin-transferrin-selenium (Life Technologies, Carlsbad, CA, USA), penicillin-streptomycin (Life Technologies, Carlsbad, CA, USA) and 100 nM hydrocortisone. HeLa cells (ATCC No. CCL-2) were maintained in Eagle's Minimum Essential Medium (EMEM) containing 10% fetal bovine serum and 1 mM sodium pyruvate. All cells were cultured in T-75 cell culture flasks (Corning, Corning, NY, USA).

2.3. Real-time PCR

To quantify the AT_1 receptor expression on NCI-H295R and HeLa cells, real-time reverse-transcriptase polymerase chain reaction (qRT-PCR) was conducted. For that reason, cells were cultured in T-25 cell culture flasks (Corning, Corning, NY, USA). After the cells had reached subconfluency, RNA was isolated using the peqGOLD total RNA kit (Peqlab, Erlangen, Germany) including an on-column DNAse digestion step (peqGOLD DNase I Digest Kit, Peqlab, Erlangen, Germany). The purity and concentration of RNA was determined with the help of a Nanodrop ND-1000 spectrophotometer (Peqlab, Erlangen, Germany). To generate single-stranded cDNA, 3 μg of the isolated RNA were reversely transcribed using the cDNA-Synthesis Kit H Minus (Peqlab, Erlangen, Germany) according to the manufacturer's instructions. For quantification of human AT_1R expression (sense: 5′-GGC CAG TGT TTT TCT TTT GAA TTT AGC AC-3′, antisense: 5′-TGA ACA ATA GCC AGG TAT CGA TCA ATG C-3′), real-time RT-PCR was performed using a LightCycler® Instrument (Roche Diagnostics, Rotkreuz, Switzerland) and a LightCycler® 480 SYBR Green I Master (Roche Diagnostics, Rotkreuz, Switzerland). Due to the different tissue origins of both carcinoma cell lines we related the AT_1R expression to the expression of three different housekeeping genes: glyceraldehyde 3-phosphate dehydrogenase GAPDH (sense: 5′-GAA GGT GAA GGT CGG AGT C-3′, antisense: 5′-GAA GAT GGT GAT GGG ATT TC-3′), β-actin (sense: 5′-AGC CTC GCC TTT GCC GA-3′, antisense: 5′-CTG GTG CCT GGG GCG-3′) and RNA polymerase II (sense: 5′-GCA CCA CGT CCA ATG ACA T-3′, antisense: 5′-GTG CGG CTG CTT CCA TAA-3′). Especially RPII has been shown to have a constant expression in different tissues [23], which makes it an ideal reference gene when comparing expression levels between different tissues. Primers were purchased from Eurofins Genomics (Ebersberg, Germany). Real-time PCR was run for 50 cycles with 15 s denaturation at 95 °C, 20 s annealing at 60 °C, and 20 s elongation at 72 °C. The accuracy of the amplicon was verified by performing a melting-curve analysis after amplification. Data is expressed as mean ± standard deviation ($n = 3$).

2.4. Nanoparticle labeling

EXP3174 (16 nmol) was activated using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC, 10 mM) and N-hydroxysulfosuccinimide (sulfo-NHS, 5 mM) in MES-buffered solution. After activation of the carboxylic group of EXP3174 for 1 h, the pH was raised by addition of 50 mM borate buffer at pH 8.5. Qdots® 655 ITK™ amino PEG (160 pmol) were immediately added to the reaction mixture for the labeling reaction. After 16 h of gentle shaking, EXP3174-modified Qdots were purified by size exclusion chromatography using Sephadex G-25 resin in a PD-10 column (GE Healthcare, Munich, Germany) with DPBS as eluent. Fractions containing EXP3174-coupled Qdots were pooled and further purified by ultrafiltration using a 100 kDa molecular weight cut-off Amicon Ultra-4 filter unit (Millipore, Billerica, MA, USA). Finally, Qdot concentration was determined by fluorescence measurement in a range of 0 to 10 nM using a FluoStar Omega fluorescence microplate reader (BMG Labtech, Ortenberg, Germany) with an excitation and emission wavelength of 450 nm and 650 nm, respectively.

2.5. Confocal laser scanning microscopy

Adrenal gland carcinoma NCI-H295R and HeLa cells were seeded into 8-well μ-slides (Ibidi, Martinsried, Germany) at a density of 10,000 cells per well. After 96 h (NCI-H295R) or 24 h (HeLa) of adhering and growing, nanoparticle binding to the cells was analyzed. In a first step, cells were washed with warm DPBS. Pre-warmed nanoparticle test solution in Leibovitz's medium supplemented with 0.5% bovine serum albumin (BSA) was pipetted onto the cells and incubated for 1 h at 37 °C. BSA was added to the nanoparticle solutions to block non-specific nanoparticle binding to the cells. After the incubation period, cells were washed with DPBS, fixed with 2% paraformaldehyde in DPBS for 10 min at room temperature and washed extensively with DPBS. Analysis of nanoparticle binding to the cells was conducted using a Zeiss Axiovert 200 microscope combined with a LSM 510 laser-scanning device using a 63× Plan-Apochromat (NA 1.4) objective (Zeiss, Jena, Germany). Qdot fluorescence was excited with an argon laser at 488 nm. Qdot emission was recorded after a 650 nm longpass filter. The focal plane was adjusted to 1.1 μm. For image acquisition and processing AIM 4.2 (Zeiss, Jena, Germany) was used. Qdot fluorescence was displayed in false color (green) for better visibility.

2.6. Intracellular calcium mobilization assay

The affinity of the EXP3174-modified Qdots for AT_1R was investigated by the fluorescent fura-2 Ca^{2+} chelator method as originally established by Grynkiewicz et al. [24] and as recently described [16]. Confluent HeLa and NCI-H295R cells grown in cell culture flasks were harvested with trypsin, centrifuged (5 min, 200 g, RT) and loaded in