Passive vs. Active Tumor Targeting using RGD- and NGR-modified Polymeric Nanomedicines

Sijumon Kunjachan[1], Robert Pola[2], Felix Gremse[1], Benjamin Theek[1], Josef Ehling[1], Diana Moeckel[1], Benita Hermanns-Sachweh[3], Michal Pechar[2], Karel Ulbrich[2], Wim E. Hennink[4], Gert Storm[4,5], Wiltrud Lederle[1], Fabian Kiessling[1,*], Twan Lammers[1,4,5,*]

1 Dept. of Experimental Molecular Imaging, University Hospital and Helmholtz Institute for Biomedical Engineering, RWTH Aachen, Pauwelsstrasse 30, 52074 Aachen, Germany

2 Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, Heyrovsky Square 2, 162 06 Prague 6, Czech Republic

3 Electron Microscopy, Institute of Pathology, Medical Faculty, RWTH Aachen, Pauwelstrasse 30, 52074 Aachen, Germany

4 Dept. of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Universiteitsweg 99, 3584 CG Utrecht, The Netherlands

5 Dept. of Controlled Drug Delivery, MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, PO Box 217, 7500 AE, Enschede, The Netherlands

* Corresponding authors: tlammers@ukaachen.de; fkiessling@ukaachen.de

Methods

Synthesis and characterization of the polymeric nanocarriers

N-(2-Hydroxypropyl)methacrylamide (HPMA) was synthesized by reaction of methacryloyl chloride with 1-aminopropan-2-ol (AP) in dichloromethane in the presence of sodium carbonate as described in the literature [1]. N-Methacryloylglycylglycine (Ma-GG-OH) was prepared by the Schotten−Baumann acylation of glycylglycine with methacryloyl chloride in an aqueous alkaline medium [2]. 3-(N-Methacryloylglycylglycyl) thiazolidine-2-thione (Ma-GG-TT) was prepared by the reaction of Ma-GG-OH with 4,5-dihydrothiazole-2-thiol in DMF with N,N′-dicyclo-

hexylcarbodiimide [3]. The multivalent random copolymer precursor poly(HPMA-co-Ma-GG-TT) was prepared by solution radical copolymerization of HPMA (85 mol%) and Ma-GG-TT (15 mol%) performed in DMSO at 60°C for 6 h using AIBN as initiator. The concentration of monomers in the copolymerization mixture was 13% w/w, and that of AIBN initiator was 2% w/w. The molecular weight parameters of polymer precursor are Mw = 24,000 and Mw/ Mn = 1.7 (Figure 2B). The polymer precursor poly(HPMA-co-Ma-GG-TT) (39 mg, 0.78 mmol TT group per gram of polymer) was dissolved in methanol (0.6 mL). Next, DY-750-NH2 (1 mg; 1.26 μmol) and DIPEA (0.45 mL, 2.52 mmol) were added. After a 0.5 h reaction with the fluorescent dye, the polymer was aminolyzed with 1-aminopropan-2-ol (5 μL). Fifteen min later, the aminolyzed copolymer was isolated by precipitation with diethyl ether, followed by centrifugation and lyophilization. The copolymer was dissolved in water, and purified by gel filtration on Sephadex G-25 in water (PD 10 column; Pharmacia) and freeze-dried, yielding 31 mg (80%) of p(HPMA-co-Ma-GG-DY-750-co-Ma-GG-AP): P-CON [1,4,5].

The cyclic peptide cyclo(RGDfK) and its scrambled control cyclo(DRfGK) were prepared using solid phase peptide synthesis [6]. The linear CNGRC peptide was assembled using an AVSP-2 multiple automatic peptide synthesizer, starting from the C-terminus using standard Fmoc procedures, by consecutive addition of N-Fmoc-protected amino acid (2.5 eq), (benzotriazol-1-yloxy)-trispyrrolidinophosphonium hexafluorophosphate (2.5 eq), 1-hydroxybenzotriazole (2.5 eq) and ethyldiisopropylamine (5.0 eq) in DMF. The peptide was cleaved from the resin using trifluoroacetic acid : ethane-1,2-dithiol : thioanisole : phenol : H_2O : triisopropylsilane, in a molar ratio of 68.5 : 10 : 10 : 5.5 : 5 : 1. The cyclization of the peptide was performed in 5% NH3 solution using charcoal-air-mediated intramolecular disulfide bond formation. After 4 h, the solution was lyophilized, with a yield of >90%. Cyclo(CNGRC) was characterized by MALDI-TOF MS and reverse-phase HPLC, showing a single peak with a retention time of 8.2 min (Chromolith C18 column, linear gradient water-acetonitrile, 0-100% acetonitrile, 0.1% TFA and UV detection at 220 nm).

The polymer precursor poly(HPMA-co-Ma-GG-TT) (18.8 mg, 0.84 mmol TT per g polymer) was dissolved in 0.15 M phosphate buffer. After a 0.5 h reaction with DY-676-NH_2 (0.25 mg, 0.6 μmol), the peptide c(RGDfK) (7.9 mg, 6.6 μmol) was added. The progress of the reaction was monitored by HPLC and all peptide was bound to the polymer within 24 h. Remaining TT groups in the copolymer were aminolyzed using a solution of 1-aminopropan-2-ol (2 % aqueous solution, pH 7.8 adjusted by HCl). After 15 min, the aminolyzed copolymer was purified by gel filtration on a Sephadex G-25 using water as eluent (PD 10 column; Pharmacia), and the blue fraction was lyophilized and purified on a semi-preparative Chromolith C18 column using a linear gradient water-acetonitrile, 20-80% acetonitrile with 0.1% TFA. The same procedure was used for the synthesis of the conjugates with scrambled sequence cyclo(DRGfK) and cyclo(CNGRC).

Animal experiments and tumor models

All animal experiments were approved by the local and national regulatory authorities and by ethical animal welfare committee. Eight-week old CD1-nu/nu mice (Charles River Laboratories, Wilmington, USA) weighing ~25 g were fed with chlorophyll-free food pellets and water ad libitum. Mice were housed in ventilated cages and placed in clinically controlled room with customized conditions. CT26 murine colon carcinoma tumor cells (ATCC, USA) were cultured in Dulbecco's modified eagle's medium (DMEM; Gibco, Invitrogen, Germany), supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Germany) and 1% Pen/strep (10,000 U/mL penicillin; 10,000 µg/mL streptomycin, Invitrogen, Germany) at 37°C and 5% CO2, in a humidified atmosphere. Similarly, BxPC3 human pancreatic adenocarcinoma cells (ATCC, USA) were cultured using RPMI 1640 medium (Gibco, Invitrogen, Germany) containing 10% FBS. Mice were inoculated with CT26 (1x106 cells / 100 µL) and BxCP3 tumor cells (5x106 cells / 100 µL) by subcutaneous inoculation into the dorsolateral right flank. A tumor size of ~8 mm was obtained within 10-14 days for CT26 cells and within 120–150 days for BxPC3. The animals were conditionally inhalation-anaesthetized during all experimental operations.

Probe calibration and administration

The polymer-peptide constructs P-RGD, P-NGR, P-DRG (coupled to DY676) and P-CON (coupled to DY750) were solubilized in PBS and calibrated in the FMT device using standard calibration protocols. Based on the calibration, a scaling factor, corresponding to the probe's fluorescence intensity, was generated. Upon internal validation, to confirm its compliance with the theoretical yield of fluorescence, the dose was adjusted for injection purposes. Each polymer-peptide construct was intravenously co-injected into the same mouse with the peptide-free control copolymer P-CON via the tail vein, at a dose of 2.5 nmol (DY-676 and DY-750 eq). Subsequently, their biodistribution and tumor accumulation was monitored, according to the imaging protocols described below.

Imaging protocols

CD-1 nude mice bearing CT26 and BxPC3 tumors of size of ~8 mm in diameter were anaesthetized (2.5 % mixture of isoflurane / O_2) and placed on a multi-modal imaging cassette (CT-imaging, Erlangen, Germany) which is manually adjusted to hold the mice with the tumors projecting at the center. High-resolution dual-energy micro-CT imaging (Tomoscope DUO; µCT Imaging, Erlangen, Germany) was performed using the following scanning protocols: Both tubes were run at 65 kV and a current of 0.5 mA; each flat panel detector acquired 720 projections containing 1032 x 1012 pixels in a full gantry rotation with duration of 90 s. All images were reconstructed using an isotropic voxel size of 35 µm, using a modified Feldkamp algorithm with

a smooth kernel. After the μCT scan, without changing the position and orientation of mouse, it was transferred to the FMT (FMT2500; PerkinElmer). Process parameters such as excitation wavelength and calibration agent were preset by the FMT based on earlier defined calibration details. Laser scanning depth of the imaging cassette was automatically detected and set by the FMT machine. To start with, whole body images of the mice were captured using FRI. Based on the 2D FRI images, the region of interest was defined and 3D FMT scans were subsequently performed. The spatial density of laser scanning was manually set to 'medium' (i.e. the 3 x 3 mm-default setting). Animals were pre-scanned to detect and rule out the chances of auto-fluorescence. 3D FMT imaging was then carried out for respective CT-imaged mice at 0.25, 0.5, 1, 4, 24, 48, and 72 h. After the final scanning, FITC-lectin (1mg/ml; 60μL) was injected into the tail vein, and mice were sacrificed for ex vivo tumor validation. The μCT and FMT image data sets were fused by rigid transformation using fiducial markers in imaging casette. Based on μCT data, tumors were interactively segmented using Imalytics Research Workstation (Philips Technologie GmbH Innovative Technologies, Aachen, Germany). Fluorescence signals from FMT were overlaid with respective organ-segmented μCT images, and the presence of P-NGR, P-RGD, P-DRG, and P-CON were qualitatively and quantitatively estimated. The percentage injected dose (%ID) for each segmented tumor was normalized to its respective average volume.

Immunohistochemical analysis

Histological stainings were performed to analyze the tumor micromorphology and the tumor accumulation of different polymeric nanocarriers. To this end, snap-frozen 8 μm-thick sections were prepared and stained using Hoechst 33258 (Sigma Aldrich, Germany) for nuclei, rat anti-mouse CD31 (BD Biosciences, San José, CA, USA) for blood vessels, αSMA-biotin (Progen, Heidelberg, Germany), rabbit anti-mouse CD13 (Abcam, Germany) for aminopeptidase-N and CD51 (eBioscience, Germany) for assessing αv-integrins (CD51). The respective labeled secondary antibodies were obtained (Dianova, Hamburg, Germany). Sections were mounted using Mowiol and fluorescence microscopy imaging was performed using an Axio Imager M2 microscope and a high-resolution AxioCam MRm Rev.3 camera, at different magnifications. The images were further post-processed using AxioVision Rel 4.8 software (Carl Zeiss Microimaging GmbH, Göttingen, Germany) and analyzed.

Tumor vascular permeability studies

Tumor vascular permeability of CT26 and BxPC3 tumors was assessed using Evans blue 53,54. To this end, Evans blue dye (1 mg/mL) dissolved in PBS was injected at a dose of 10 mg/kg to tumor-bearing mice. Tumors were dissected and normal white light photographs of Evans blue- and PBS- treated tumors were captured at 48 h p.i. Evans blue was extracted using formamide (0.01 mL/mg of tumor tissue) ~0.5 g weighed tumor portions, and its absorbance was measured using spectrophotometer (Cary UV-Vis spectrophotometer, Germany). Furthermore, the tumors were snap-frozen and sliced into 1 μm sections to be analyzed in the bright-light microscopy at 400-fold magnification.

High-resolution transmission electron microscopy and scanning electron microscopy imaging (TEM EM 400T; Philips, Eindhoven, The Netherlands) was carried out for CT26 and BxPC3 tumor sections. Tumor tissue (and kidney as control) pieces of 1 mm3 was obtained, and the tissue was fixed using a mixture of 2.5% glutaraldehyde in 0.2 M Sorensen buffer at pH 7.0. Thin tissue sections were sliced from the pieces using an ultracryotome. Followed by washing, staining with 2.0% aqueous uranylacetate was performed for ~2 h and the tumor tissue was placed at 4°C in dark. After dehydrating with 100 % ethanol and propylene oxide, the tissue was embedded onto an Epon™ (liquid epoxy) resin. High-resolution electron microscopy was carried out at different magnifications (4000-13000x), and the electron micrographs of tumor vessels and capillaries of CT26 and BxPC3 tumors were visualized.

Statistical Analysis

Results are presented as average ± standard deviation. Statistical analyses were performed using GraphPad Prism 5.01, employing the standard student's t test. $P<0.05$ was considered to represent statistical significance.

Supplementary Figure S1

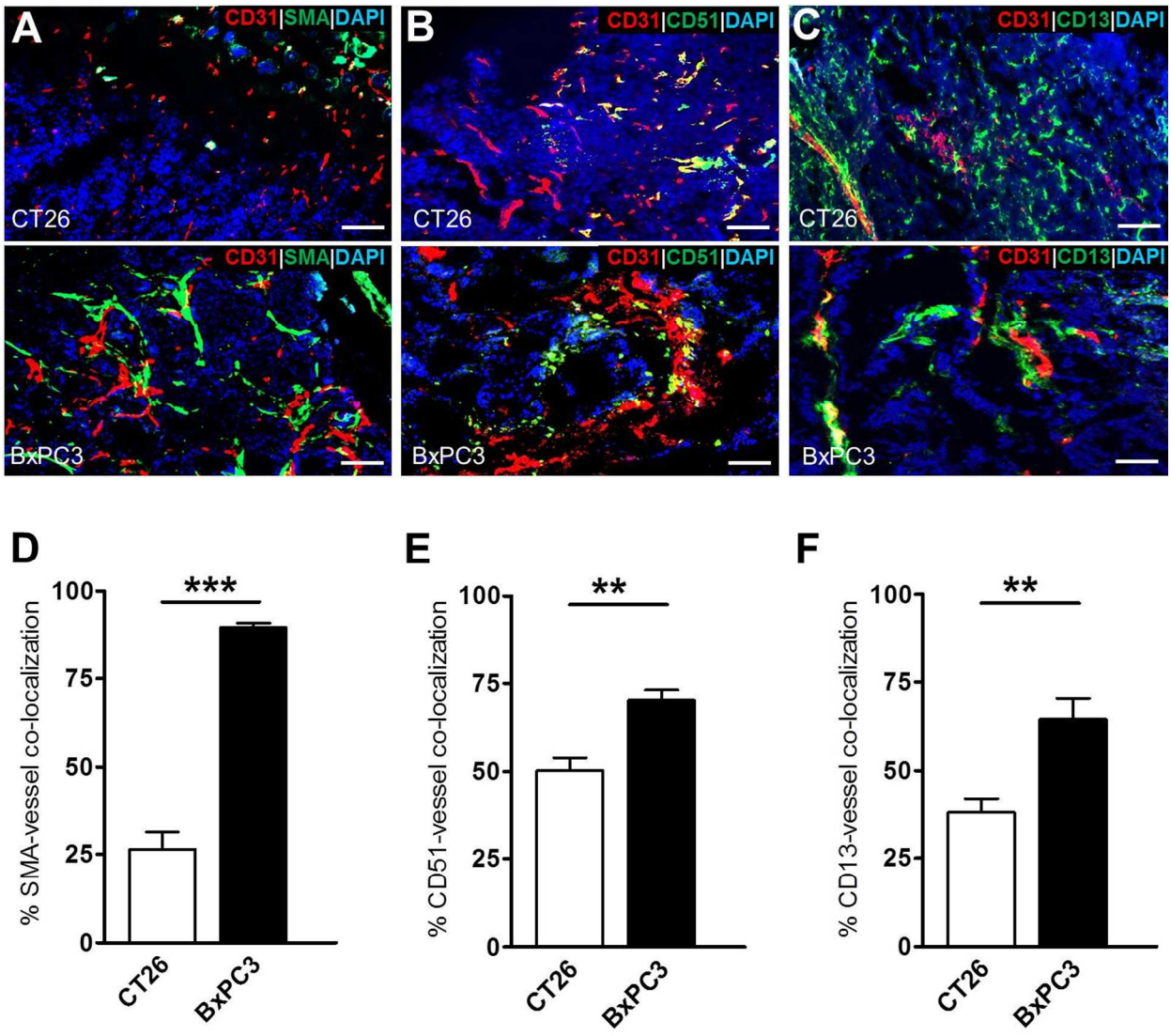

Figure S1: Immunohistochemical analysis of vessel maturation and receptor expression. A. α-Smooth muscle actin (α-SMA) staining of CT26 and BxPC3 tumors, illustrating that blood vessels in BxPC3 tumors were much more mature than those in CT26 tumors. B-C. Integrin (CD51) and aminopeptidase N (CD13) receptor expression in CT26 and BxPC3 tumors. Scale bar: 10 μm. D-F. Quantification of the percentage of α-SMA-, CD51- and CD13-positive vessels. Thirty-six images were analyzed per condition. Values represent average ± SD. ** denotes $P<0.01$, *** $P<0.005$.

References

1. Ulbrich, K.; Subr, V.; Strohalm, J.; Plocova, D.; JelinKova, M.; Rihova, B. *J Control Release* **2000,** *64*, 63-79.

2. Rejmanova, P.; Labski, J.; Kopeck, J. *Makromolekulare Chemie* **1977,** *178*, 2159-2168.

3. Subr, V.; Ulbrich, K. *Reactive and Functional Polymers* **2006,** *66*, 1525-1538.

4. Chytil, P.; Etrych, T.; Konak, C.; Sirova, M.; Mrkvan, T.; Boucek, J.; Rihova; Ulbrich . *J. Control. Release* **2008,** *127*, 121-130.

5. Etrych, T.; Mrkvan, T.; Rihova, B.; Ulbrich, K. *J. Control. Release* **2007,** *122*, 31-38.

6. Pola, R.; Studenovsky, M.; Pechar, M.; Ulbrich, K.; Hovorka, O.; Vetvicka, D.; Rihova, B. *J Drug Target* **2009,** *17*, 763-766.