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In vivo biodistribution of radiolabeled MMP-2/9 activatable cell-penetrating peptide probes in tumor-bearing mice

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Matrix metalloproteinases (MMPs) play a pivotal role in cancer progression and present therefore an interesting biomarker for early diagnosis, staging and therapy evaluation. Consequently, MMP-specific molecular imaging probes have been proposed for noninvasive visualization and quantification of MMP activity. An interesting approach is MMP-2/9 activatable cell-penetrating peptides (ACPP) that accumulate in the tumor tissue after activation. However, a recent study revealed that probe activation occurred already in the vasculature followed by nonspecific tumor targeting. In the latter study, biodistribution was determined 6 and 24 h post-ACPP injection. An alternative explanation could still be that the kinetics of tumor-specific activation is faster than that of blood activation plus subsequent nonspecific uptake in tumor. The aim of this study was to assess if tumor-specific ACPP activation occurs in mice with MMP-2/9 positive subcutaneous HT-1080 tumors at 3 h post-injection. As control, we studied the MMP-2/9 sensitive ACPP in mice bearing subcutaneous BT-20 tumors with low MMP-2/9 expression to test if probe cleavage correlates with tumoral MMP expression. *Ex vivo* biodistribution showed no improved tumoral ACPP activation in HT-1080 tumor-bearing mice at 3 h post-injection compared with previous reported data collected at 24 h post-injection. Furthermore, tumoral uptake and relative tumoral activation for ACPP were similar in both BT-20 and HT-1080 tumor-bearing mice. In conclusion, this study suggests that tumoral ACPP uptake in these tumor models originates from probe activation in the vasculature instead of tumor-specific MMP activation. Novel ACPPs that target tissue-specific proteases without nonspecific activation may unleash the full potential of the elegant ACPP concept. Copyright © 2014 John Wiley & Sons, Ltd.

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Keywords: molecular imaging; dual-isotope; activatable probe; matrix metalloproteinase; cancer

1. INTRODUCTION

The family of matrix metalloproteinases (MMPs) plays a pivotal role in the remodeling of the extracellular matrix (ECM) (1). Since extensive remodeling is a hallmark of many diseases, including cancer (2), development of imaging strategies to detect MMP activity *in vivo*, aiming for improved diagnosis or therapy evaluation, is of great interest. The current toolbox for *in vivo* monitoring of MMP levels is mostly based on probes showing a 1:1 probe-target binding fashion, which lacks amplification of the imaging signal (3,4). The assessment of MMP specificity for these probes is seldom available (5). Two recent studies highlighted a rather low specificity of 18–30% (6,7), indicating that MMP specificity can be difficult to reach. Novel imaging strategies focus on MMP responsive probes in which enzymatic probe activation drives signal enhancement and/or probe accumulation at the target site (8–14). The group of Roger Tsien employed a strategy that centered on an activatable cell penetrating peptide (ACPP) consisting of an MMP-2/9 substrate inserted in between a polyanionic inhibitory peptide and a polycationic cell penetrating peptide (CPP) (15–17). Cleavage of the substrate by tumoral MMP-2/9 released the polycationic from the polyanionic domain, thereby triggering cellular adhesion and subsequent uptake of the imaging label-functionalized polycationic peptide domain in tumors. Although this is a very elegant concept, we showed

that uptake of the activated ACPP probe in a subcutaneous HT-1080 tumor model in mice at least partly originated from probe activation in the vasculature instead of MMP-related activation in the tumor compartment (18). In our approach, a single-isotope ACPP approach, potentially suitable for imaging/therapy, was extended to a dual-isotope ACPP research strategy. The dual-isotope ACPP strategy has been shown to be a powerful preclinical research tool to study ACPP behaviour and activation in more detail. Specifically, this strategy enabled discrimination between activated ACPP and uncleaved ACPP *in vivo*, which is not possible for monolabeled probes (Fig. 1). Inspired by previous work on the simultaneous use of the orthogonal radioisotopes

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Figure 1. Rationale of the use of a dual-isotope labeled MMP-2/9 activatable dual-isotope radiolabeled ACPP probe (dACPP). (A) The cell-penetrating property of a polycationic peptide is masked by a polyanionic peptide. Cleavage of the linker by MMP-2/9 releases the polycationic cell-penetrating peptide and its radionuclide cargo (in blue), which will be retained by the target tissue. The polyanionic peptide and its radionuclide cargo (in red) will be cleared from the target tissue. The ratio of the radionuclide cargoes (blue-over-red) is a measure for local dACPP activation. (B) Peptide structure of MMP-2/9 sensitive dACPP. MMP-2/9 will cleave the linker of dACPP between glycine and leucine residue. X denotes amino-hexanoic acid. Lower case letters indicate D-amino acids. Molecular structure of dACPP is shown in Fig. S1 in the Supporting Information.

^{177}Lu (energy of γ -ray emitted by isotope [E_γ] = 208 keV) and ^{125}I (E_γ = 35 keV) in biodistribution studies (19), we labeled the polycationic CPP domain and polyanionic inhibitory peptide domain with ^{177}Lu and ^{125}I , respectively. A large ratio of ^{177}Lu over ^{125}I indicated cleavage of the probe and subsequent uptake of the ^{177}Lu -radiolabeled CPP domain. We showed that the dual-isotope radiolabeled ACPP probe (dACPP), which demonstrated specific sensitivity towards MMP-2/9 without cross-reactions with other members of the MMP-family, was taken up and retained in all tissues in its activated form (18). Furthermore, the *in vivo* biodistribution of ^{177}Lu -CPP, the activated CPP domain of ACPP serving as a positive control revealed similar tumor-to-tissue ratios compared with monolabeled ^{177}Lu -ACPP, indicating that ACPP was most likely activated in the vascular compartment rather than in the tumor (18). Notably, the biodistribution studies were performed 6 and 24 h after injection of the radiolabeled ACPP probes. We and others (20) recognized that an alternative explanation could still be that kinetics of tumor-specific activation is faster than that of blood activation plus subsequent nonspecific uptake in tumor. If so, tumor-specific activation might be detectable at early time points post-dACPP injection. Therefore, we here explored the biodistribution of the MMP-2/9 sensitive dual-isotope radiolabeled dACPPs in MMP-expressing HT-1080 tumor-bearing mice at 3 h post-injection. Furthermore, if tumor-specific MMP activation plays a dominant role in probe cleavage and subsequent tumor uptake, the latter should correlate with tumoral MMP expression. Therefore, dACPP biodistribution was in this study also determined in BT-20 tumor-bearing mice with low tumoral MMP-2/9 expression (4,8) for comparison with data obtained with the high MMP-2/9-expressing HT-1080 tumor. We furthermore included a negative control and positive control probe in this study. As dACPP exists either in an intact or activated entity upon *in vivo* administration, a noncleavable dACPP (non-dACPP) was used as

negative control to represent the intact dACPP and a CPP domain was used as a positive control to represent the activated dACPP.

2. MATERIALS AND METHODS

2.1. Probe Synthesis

Peptides Ac-y-e₉-x-PLGLAG-r₉-x-k(DOTA)-NH₂ (dACPP), Ac-y-e₉-x-LALGPG-r₉-x-k(DOTA)-NH₂ (non-dACPP) and Ac-LAG-r₉-x-k(DOTA)-NH₂ (CPP) were synthesized as previously reported (18). D-Amino acids are denoted in lower case and x represents 6-amino-hexanoic acid. Additionally, fluorescence resonance energy transfer (FRET) ACPP and non-ACPP analogs were synthesized. The synthetic procedure for these peptides, the serum stability experimental procedure and the method for determination of FRET-ACPP enzyme kinetics with MMP-2 and MMP-9 are described in the Supporting Information.

2.2. Radiolabeling

Radiolabeling of non-dACPP, dACPP and CPP with ^{177}Lu and/or ^{125}I was performed following a procedure reported earlier (18). $^{177}\text{LuCl}_3$ (PerkinElmer) in 0.05 M HCl (5.0 μL , 5.0–10.0 MBq) was mixed with dACPP, non-dACPP (38.8 μL , 300 nmol) or CPP (31.5 μL , 300 nmol) in MilliQ water and metal-free 0.9% NaCl (450 μL) for 20 min at 300 rpm and 90 °C. ^{125}I (PerkinElmer) in 1 mM NaOH (5.0 μL , 2.5 MBq) was mixed with dACPP or non-dACPP in MilliQ water (38.8 μL , 300 nmol) and phosphate-buffered saline (PBS; 225 μL) in an IODOGEN iodination tube for 7 min at 300 rpm and 25 °C and transferred to a siliconized 1.5 mL tube together with 250 μL of 0.9% NaCl. The ^{177}Lu and ^{125}I labeling yields were determined by radio thin-layer chromatography, using instant thin-layer chromatography silica gel strips (Pall) eluted with 200 mM

ethylenediaminetetraacetic acid in 0.9% NaCl and 20 mM citric acid at pH 5.2, respectively. The yields were imaged on a phosphor imager (FLA-7000; Fujifilm) and quantified with AIDA Image Analyzer software. Analytical radio-HPLC was performed on an Agilent 1100 system equipped with a C₁₈ Eclipse XBD column (150 × 4.6 mm; particle size, 5 μm) and a Gabi radioactive detector (Raytest). The radiochemical purities were 95% or higher. ¹⁷⁷Lu-dACPP was mixed with ¹²⁵I-dACPP in a 1:1 molar ratio.

2.3. Animal Studies

All animal procedures were approved by the ethical review committee of the Maastricht University Hospital (The Netherlands), and were performed according to the principles of laboratory animal care (NIH publication 85-23, revised 1985) (21), and the Dutch national law 'Wet op Dierproeven' (Stb 1985, 336). Six-week old athymic female mice (CD-1 nu/nu, body weight 22–30 g, Charles River Laboratories) were housed in an enriched environment under standard conditions: 23–25 °C, 50–60% humidity and 12 h light/dark cycles for >1 week. Food and water were freely available.

2.4. HT-1080 Fibrosarcoma/BT-20 Breast Carcinoma Model

HT-1080 fibrosarcoma and BT-20 breast carcinoma cells acquired from the American Type Culture Collection were maintained under standard culture conditions in Eagle's minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), penicillin (100 U/ml), streptomycin (100 μg/ml) and 2 mM Glutamax (Gibco). HT-1080 or BT-20 cells at 80–90% confluency were harvested by trypsination. The trypsin activity was inhibited by addition of MEM, after which cells were isolated by centrifugation. The cells were washed 1× with sterile Dulbecco's PBS and concentrated to 3.0×10^7 HT-1080 or 6.0×10^7 BT-20 cells/ml in sterile Dulbecco's PBS. BT-20 cells were diluted once to 3.0×10^7 BT-20 cells/ml by addition of ice-cold matrigel. Approximately 3.0×10^6 HT-1080 or BT-20 cells were subcutaneously injected on the left flank of a mouse. The tumor sizes were determined with a caliper every 2 days and their volumes were calculated using the formula: $\frac{1}{2} \times \text{length} \times \text{width} \times \text{height}$. When tumors reached a size of 8–50 mm³ (typically 1–2 and 3–5 weeks after s.c. injection of HT-1080 and BT-20 cells, respectively), the mice were used for *in vivo* studies.

2.5. Biodistribution Experiments

Biodistribution experiments were performed on HT-1080 tumor-bearing mice and BT-20 tumor-bearing mice with group sizes of $n = 3$ or $n = 4$ (group sizes are specified in the respective tables and figure legends). The mice were injected i.v. with ¹⁷⁷Lu/¹²⁵I-dACPP (60 nmol/100 μl, ca. 1.0 MBq ¹⁷⁷Lu, ca. 0.25 MBq ¹²⁵I), ¹⁷⁷Lu/¹²⁵I-non-dACPP (60 nmol/100 μl, ca. 1.0 MBq ¹⁷⁷Lu, ca. 0.25 MBq ¹²⁵I) or ¹⁷⁷Lu-CPP (60 nmol/100 μl, ca. 1.0 MBq). The mice were anesthetized with isoflurane 3 or 24 h after i.v. injection of the above probes, subjected to cardiac puncture and sacrificed by cervical dislocation. Organs and tissues of interest were harvested and weighed, and sample radioactivity was measured in a γ-counter (Wizard 1480, PerkinElmer) along with standards to determine the percentage injected dose per gram (%ID/g). Specifically, a dual-isotope protocol (10–80 and 155–380 keV energy windows for ¹²⁵I and ¹⁷⁷Lu, respectively) with cross-contamination correction was used.

2.6. Sample Preparation for Gelatin Zymography

After γ-counting, tissue samples were homogenized in 20 vols of 50 mM Tris, 200 mM NaCl, 10 mM CaCl₂ and 10 μM ZnCl₂ at pH 7.5, 4 °C and 30 Hz for 5 min using a tissue lyser (Qiagen), mixed for 4 h at 4 °C and centrifuged at 10,000 rpm for 5 min. The supernatants were aliquoted and stored at –80 °C until zymography analysis. Typically, 11.2 μl tissue supernatant (from 0.53 mg tissue) was used per analysis.

2.7. Gelatin Zymography

Samples were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels containing 0.1% (w/v) gelatin (Biorad). Active MMP-2 (0.21 ng) was loaded as an internal standard used to normalize activities between gels. After running the gel at 150 V, it was washed with MilliQ water, incubated for 3 × 20 min in 2.5% Triton-X (60 rpm) to remove SDS, washed with MilliQ water, and incubated in 50 mM Tris, 200 mM NaCl, 5 mM CaCl₂, 0.1% (w/v) NaN₃, and 0.02% (w/v) Brij-35 at pH 7.6 at 37 °C for 2 days. Gels were stained for 2 h with 0.25% Coomassie Blue in 60% (v/v) MilliQ water, 30% (v/v) methanol and 10% (v/v) acetic acid, and destained for >24 h with 67.5% (v/v) MilliQ water, 25% (v/v) methanol and 7.5% (v/v) acetic acid. Gelatin activity showed up as clear bands against a dark background. Zymograms were imaged (Epson Perfection V700 Photo scanner) and band intensities were quantified using ImageJ.

2.8. Statistical Methods

Quantitative data were expressed as means ± SD. Comparisons between the means of the groups were performed by the (paired) *t*-test. All tests were two-tailed, and *p*-values of <0.05 indicate significant differences. GraphPad Prism was used for all statistical calculations.

3. RESULTS

Following successful synthesis (18) and radiolabeling (Supporting Information, Figs S2–S4), ¹⁷⁷Lu/¹²⁵I-dACPP, the noncleavable control peptide ¹⁷⁷Lu/¹²⁵I-non-dACPP, and the positive control peptide ¹⁷⁷Lu-CPP were studied in HT-1080 tumor-bearing mice and BT-20 tumor-bearing mice. Tables 1 and 2 and Supporting Information, Tables S1 and S2 show respectively the biodistribution data for dACPP in HT-1080 and BT-20 tumor-bearing mice at 3 h post-injection (Table 1) and at 20 h post-injection (Table 2), non-dACPP in HT-1080 tumor-bearing mice at 3 and 20 h post-injection (Supporting Information, Table S1), and CPP in HT-1080 and BT-20 tumor-bearing mice at 3 and 20 h post-injection (Supporting Information, Table S2). The data for ¹⁷⁷Lu biodistribution of dACPP in HT-1080 tumor-bearing mice at 3 h post-probe injection were in agreement with the data collected at 6 and 24 h post-injection, as reported previously (18). First, for dACPP, the tumor uptake of ¹⁷⁷Lu was significantly higher compared with ¹⁷⁷Lu uptake in muscle ($p < 0.05$, Table 1). Furthermore, the tumor uptake of ¹⁷⁷Lu for dACPP was significantly higher compared with tumor uptake of ¹⁷⁷Lu for the negative control non-dACPP at 3 h post-injection ($p < 0.05$, Table 1 and Supporting Information, Table S1). Next, for non-dACPP, the ¹⁷⁷Lu uptake was significantly higher in tumor compared with muscle and heart ($p < 0.001$, Supporting Information, Table S1). Finally, the relative difference between ¹⁷⁷Lu uptake for dACPP and for non-dACPP in tumor (0.904 ± 0.135) was in a similar range ($p > 0.05$) compared

Table 1. Biodistribution results of 60 nmol $^{177}\text{Lu}/^{125}\text{I}$ -dACPP (dual-isotope radiolabeled ACPP probe) in HT-1080 tumor-bearing mice and BT-20 tumor-bearing mice at 3 h post-injection ($n=3$). The data are presented as means $\pm$ SD

3 h, dACPP	HT-1080 tumor-bearing mice			BT-20 tumor-bearing mice		
	^{177}Lu (%ID/g)	^{125}I (%ID/g)	Ratio $^{177}\text{Lu}/^{125}\text{I}$	^{177}Lu (%ID/g)	^{125}I (%ID/g)	Ratio $^{177}\text{Lu}/^{125}\text{I}$
Blood	0.64 $\pm$ 0.23	0.52 $\pm$ 0.10	1.3 $\pm$ 0.8	0.48 $\pm$ 0.22	0.16 $\pm$ 0.08	3.2 $\pm$ 1.4
HT-1080 tumor	3.36 $\pm$ 0.18	0.65 $\pm$ 0.28	6.0 $\pm$ 2.8	—	—	—
BT-20 tumor	—	—	—	5.26 $\pm$ 2.59	0.25 $\pm$ 0.20	25.5 $\pm$ 14.1
Muscle	0.59 $\pm$ 0.09	0.16 $\pm$ 0.08	4.2 $\pm$ 1.8	0.41 $\pm$ 0.14	0.04 $\pm$ 0.02	12.5 $\pm$ 3.4
Heart	0.69 $\pm$ 0.11	0.21 $\pm$ 0.04	3.3 $\pm$ 1.2	0.56 $\pm$ 0.11	0.07 $\pm$ 0.03	8.6 $\pm$ 3.5
Lung	1.90 $\pm$ 0.26	0.41 $\pm$ 0.08	4.8 $\pm$ 1.5	1.93 $\pm$ 0.38	0.28 $\pm$ 0.07	6.9 $\pm$ 0.9
Spleen	3.18 $\pm$ 0.36	0.38 $\pm$ 0.05	8.4 $\pm$ 0.8	3.68 $\pm$ 0.48	0.18 $\pm$ 0.08	22.1 $\pm$ 6.2
Liver	12.5 $\pm$ 0.31	0.51 $\pm$ 0.01	24.3 $\pm$ 0.8	9.71 $\pm$ 1.39	0.32 $\pm$ 0.07	30.6 $\pm$ 4.2
Kidney	59.9 $\pm$ 16.7	24.4 $\pm$ 6.17	2.5 $\pm$ 0.2	62.4 $\pm$ 7.82	30.9 $\pm$ 4.19	2.0 $\pm$ 0.2
Fat	0.29 $\pm$ 0.04	0.09 $\pm$ 0.02	3.5 $\pm$ 1.4	0.26 $\pm$ 0.10	0.05 $\pm$ 0.02	5.7 $\pm$ 0.3
Thigh bone	3.12 $\pm$ 0.19	0.94 $\pm$ 0.19	3.4 $\pm$ 0.5	2.75 $\pm$ 0.54	0.56 $\pm$ 0.16	5.1 $\pm$ 1.1
Brain	0.04 $\pm$ 0.01	0.03 $\pm$ 0.01	1.2 $\pm$ 0.5	0.03 $\pm$ 0.01	0.02 $\pm$ 0.01	ND

ND, Not determined; %ID/g, percentage injected dose per gram.

Table 2. Biodistribution results of 60 nmol $^{177}\text{Lu}/^{125}\text{I}$ -dACPP in HT-1080 tumor-bearing mice [$n=6$, reprinted with permission from (18)] and BT-20 tumor-bearing mice ($n=4$) at 24 h post-injection. The data are presented as means $\pm$ SD

24 h, dACPP	HT-1080 tumor-bearing mice			BT-20 tumor-bearing mice		
	^{177}Lu (%ID/g)	^{125}I (%ID/g)	Ratio $^{177}\text{Lu}/^{125}\text{I}$	^{177}Lu (%ID/g)	^{125}I (%ID/g)	Ratio $^{177}\text{Lu}/^{125}\text{I}$
Blood	0.01 $\pm$ 0.02	0.01 $\pm$ 0.01	ND	0.00 $\pm$ 0.01	0.01 $\pm$ 0.00	ND
HT-1080 tumor	1.65 $\pm$ 0.66	0.13 $\pm$ 0.03	13.4 $\pm$ 6.2	—	—	—
BT-20 tumor	—	—	—	1.57 $\pm$ 0.72	0.06 $\pm$ 0.02	27.2 $\pm$ 5.6
Muscle	0.47 $\pm$ 0.18	0.02 $\pm$ 0.01	22.1 $\pm$ 3.8	0.24 $\pm$ 0.06	0.01 $\pm$ 0.01	18.3 $\pm$ 8.0
Heart	0.23 $\pm$ 0.07	0.02 $\pm$ 0.01	10.6 $\pm$ 4.4	0.23 $\pm$ 0.06	0.02 $\pm$ 0.01	13.2 $\pm$ 4.5
Lung	0.61 $\pm$ 0.25	0.05 $\pm$ 0.01	11.4 $\pm$ 3.3	0.57 $\pm$ 0.18	0.10 $\pm$ 0.09	8.8 $\pm$ 5.5
Spleen	3.23 $\pm$ 1.63	0.07 $\pm$ 0.02	46.2 $\pm$ 18.2	3.69 $\pm$ 1.08	0.05 $\pm$ 0.01	69.8 $\pm$ 22.1
Liver	18.4 $\pm$ 3.67	0.25 $\pm$ 0.05	77.5 $\pm$ 25.8	17.4 $\pm$ 3.77	0.19 $\pm$ 0.10	116 $\pm$ 59.4
Kidney	47.5 $\pm$ 7.70	17.0 $\pm$ 2.97	2.8 $\pm$ 0.4	51.8 $\pm$ 8.14	19.8 $\pm$ 5.40	2.7 $\pm$ 0.5
Thigh bone	2.57 $\pm$ 0.38	0.55 $\pm$ 0.11	4.8 $\pm$ 1.3	2.03 $\pm$ 0.46	0.26 $\pm$ 0.20	11.8 $\pm$ 7.3
Fat	0.26 $\pm$ 0.15	0.02 $\pm$ 0.01	ND	0.18 $\pm$ 0.07	0.02 $\pm$ 0.01	ND
Brain	0.01 $\pm$ 0.01	0.00 $\pm$ 0.01	ND	0.01 $\pm$ 0.01	0.00 $\pm$ 0.01	ND

Figure 2. Comparison of the *in vivo* biodistribution of 60 nmol $^{177}\text{Lu}/^{125}\text{I}$ -dACPP ($n=3$) in HT-1080 tumor-bearing mice and in BT-20 tumor-bearing mice at 3 h post-injection. The data are presented as means $\pm$ SD.

with the relative difference observed for muscle and heart (0.932 ± 0.135 and 0.957 ± 0.327 , respectively) at 3 h post-injection.

A comparison of dACPP in HT-1080 and BT-20 tumor-bearing mice revealed no significant tumoral differences for both uptake

and the ^{177}Lu -to- ^{125}I ratio at 3 h post-injection ($p > 0.05$, Fig. 2, Table 1). Furthermore, dACPP biodistribution in BT-20 tumor-bearing mice at 24 h post-injection was consistent with earlier findings in HT-1080 tumor-bearing mice at 24 h post-injection

(Table 2) and no differences between the ^{177}Lu -to- ^{125}I ratios in HT-1080 tumor and BT-20 tumor were found ($p > 0.05$). Gelatin zymography showed that most MMPs were present as inactive zymogens for BT-20 tumors, while for HT-1080 tumors MMPs were predominantly present in their activated form (Fig. 3). Surprisingly, the ^{177}Lu -to- ^{125}I ratio in BT-20 tumor tends to be higher than for HT-1080-tumor at 3 h post-injection ($p = 0.07$). Comparing the ^{177}Lu -to- ^{125}I ratios for dACPP between 3 and 24 h post-injection, lower ratios are observed in most tissues at 3 h post-injection compared with 24 h post-injection in both tumor-types (Tables 1 and 2). This is most likely caused by a contribution of background signal from the blood in these tissues owing to the incomplete clearance of the $^{177}\text{Lu}/^{125}\text{I}$ labeled probe and cleaved ^{125}I -labeled inhibitory domain from the circulation/target tissue at this early time point. Furthermore, ^{177}Lu -to- ^{125}I ratios for dACPP were significantly higher compared with the negative control in HT-1080 tumors with high gelatinase expression at both 3 and 24 h post-injection, but also in muscle and heart tissue and BT-20 tumors (24 h post-injection) with low gelatinase expression ($p < 0.05$).

The *in vivo* biodistribution of ^{177}Lu -CPP (Supporting Information, Table S2), with the activated cell-penetrating peptide domain of ACPP serving as positive control, revealed significantly higher tumor uptake levels compared with muscle and heart in both HT-1080 and BT-20 tumor-bearing mice ($p < 0.05$). Owing to differences in pharmacokinetics and bioavailability for dACPP and CPP (i.e. CPP is directly available for tissue uptake, while ACPP has first to be activated), we compared ^{177}Lu tumor-to-tissue ratios for dACPP and CPP instead of absolute uptake values. The tumor-to-tissue ratios showed no significant differences for both tumor types at 3 and 24 h post-injection ($p > 0.05$, Fig. 4, Supporting Information, Table S3).

4. DISCUSSION

The use of ACPPs has been advocated as a promising strategy for the *in vivo* imaging of tumoral proteolytic activity (15). The systemic delivery of MMP responsive ACPP probes showed at least a 3-fold increase in tumoral uptake compared with a noncleavable negative control (non-ACPP) (15–17). However, we recently showed that ACPP probe uptake was also several fold higher in tissues with low MMP expression compared with the negative control (18). To better understand the ACPP behaviour *in vivo* and to be able to discriminate between activated ACPP and uncleaved ACPP *in vivo*, we developed a dual-isotope

Figure 4. Comparison of ^{177}Lu tumor-to-tissue ratios for 60 nmol dACPP and 60 nmol cell penetrating peptide (CPP) in HT-1080 tumor-bearing mice [$n = 6$, reprinted with permission from (18)] and 60 nmol dACPP ($n = 4$) and 60 nmol CPP ($n = 3$) in BT-20 tumor-bearing mice at 24 h post-injection. The data are presented as mean $\pm$ SD.

preclinical research tool by labeling the MMP responsive ACPP construct with ^{177}Lu and ^{125}I (18). The added value of the simultaneous use of orthogonal radioisotopes ^{177}Lu and ^{125}I in preclinical biodistribution studies has recently been demonstrated in a study on tumor pretargeting (19). In the latter study, the application of ^{177}Lu and ^{125}I radiolabeled pretargeting components made it possible to assess the *in vivo* reaction between the two components in mice. In this study, the cationic polyarginine domain of ACPP was labeled with ^{177}Lu , while ^{125}I was conjugated to the inhibitory polyanionic domain. By conjugating ^{125}I to the noncell-penetrating inhibitory domain, we expected for ^{125}I minimal intracellular-associated dehalogenation and subsequent cellular release of ^{125}I . Previously, we indeed showed that radioiodinated ACPP was stable and not subject to significant dehalogenation, as evidenced by the small amount of ^{125}I measured in thyroids and the stomach (18). In that study, *in vivo* biodistribution experiments with $^{177}\text{Lu}/^{125}\text{I}$ -dACPP revealed high ^{177}Lu -to- ^{125}I ratios in tumor as well as in muscle and heart tissue. This indicated that ACPP activation and accumulation were not specific to the tumor tissue. Furthermore, an *in vivo* assessment comparing ^{177}Lu -ACPP and the positive control ^{177}Lu -CPP revealed similar biodistribution profiles and suggests a systemic ACPP activation in the circulation prior to tumor uptake (18). If our hypothesis of activation in the vascular system with subsequent uptake in the tumor holds, it should be independent from MMP expression levels in the tumor

Figure 3. (A) Representative gelatin zymography of tissue homogenates on 10% SDS-PAGE gel containing 0.1% w/v gelatin A. Lane 1 = HT-1080 tumor; lane 2 = muscle from HT-1080 tumor-bearing mice; lane 3, BT-20 tumor; lane 4, muscle from BT-20 tumor-bearing mice; and lane 5, active MMP-2 (0.21 ng). (B) Gelatinase expression levels of HT-1080 tumors ($n = 3$) and BT-20 tumors ($n = 5$). The data are presented as means $\pm$ SD.

and consequently occur as well in low MMP-expressing tumors. As the ACPP-based approach has so far only been tested in a variety of MMP-2 overexpressing tumors (17,18), we therefore performed an *in vivo* comparison of the MMP responsive dual-isotope labeled ACPP concept in a tumor-bearing mouse model with low MMP expression (BT-20 breast carcinoma) (4,8) to a tumor-bearing mouse model showing high MMP expression (HT-1080 fibrosarcoma).

Furthermore, we and others (20) realized that tumor-specific activation could be faster than nonspecific cleavage and uptake in tumor and that tumor-specific activation may be detectable at early time points post-dACPP injection. Therefore, we assessed here the biodistribution of dACPP at 3 h post-injection in both HT-1080 and BT-20 tumor-bearing mice complementing our earlier time points of 6 and 24 h (18). Overall, we have with this and our earlier study a comprehensive dataset that shows similar dACPP biodistribution pattern in HT-1080 tumor-bearing mice at both early (3 h) and late (24 h) time points after probe injection. This finding does not suggest that tumor-specific activation has occurred at early time points but escaped detection at the 6 and 24 h time point investigated in our previous paper.

Moreover, tumor-to-tissue ratios for ACPP and the positive control CPP were similar for HT-1080 and BT-20 tumors, suggesting that tumoral ACPP uptake originates from probe activation in the vasculature prior to nonspecific tumor targeting of the activated CPP domain. The increased ^{177}Lu blood levels of dACPP (0.48–0.64 %ID/g) and CPP (0.75–0.91 %ID/g) compared with non-dACPP (0.01 %ID/g) in both HT-1080 and BT-20 tumor-bearing mice also point to the presence of the activated CPP domain in blood for dACPP at 3 h-post-injection. We earlier reported on the blood kinetic profiles and volume-of-distribution of these probes (18). Initially dACPP and non-dACPP behaved similarly (same volume-of-distribution). In accordance with this study, dACPP behaved more like the highly positively charged CPP (2-fold difference in %ID/g for ^{177}Lu) at 3 h post-injection, while a roughly 10-to-20 fold difference between dACPP and non-dACPP was found (18). The higher blood levels at 3 h-post-injection for CPP and dACPP compared with non-dACPP might be caused by (activated) CPP binding to serum proteins (22) or circulating cells and subsequent reduced clearance.

Next, HT-1080 tumor-bearing mice and BT-20 tumor-bearing mice showed similar $^{177}\text{Lu}/^{125}\text{I}$ -dACPP biodistribution. Surprisingly, dACPP tended to show a higher ^{177}Lu -to- ^{125}I ratio in BT-20 tumor compared with HT-1080-tumor ($p = 0.07$), while, in agreement with the literature, MMP expression levels were significantly lower in BT-20 tumors compared with HT-1080 tumors (8,4). For BT-20 tumors the MMPs were mostly present as inactive zymogens, while for HT-1080 tumors most MMPs were present in its activated form, although possible inhibition of these active MMPs by tissue inhibitors of metalloproteinases cannot be excluded by gelatin zymography. These observations do not support a hypothesis taking tumor-associated MMP-2/9 cleavage of ACPP as the main mechanism for ACPP activation in our investigated tumor models. On the contrary, differences in tissue architecture between tumor tissue and healthy tissue types, specifically enhanced permeability of tumor tissue, is likely to be the main reason for elevated ACPP uptake after activation of the probe in the vascular system. During the preparation of this manuscript, Savariar *et al.* reported on the *in vivo* biodistribution of a dual-fluorophore Cy5/Cy7 labeled MMP-2/9 sensitive ACPP (23). In contrast to our data showing systemic activation, the latter study showed both systemic and specific activation of ACPP in HT-1080 tumors at 2 h post-injection upon intravenous administration of ACPP. A comparison of both

studies revealed an important difference in the study design that may explain the different results. Specifically, the HT-1080 tumor micro-environment was different for both studies associated with different tumor locations. In our studies, the HT-1080 tumors were grown subcutaneously on the left flank, while Savariar *et al.* injected the HT-1080 tumor cells in the mammary fat pad (24). Recently, a greater tumor vascular density as well as an increased vascular permeability has been observed for tumors grown in the mammary fat pad compared with tumors grown subcutaneously (24). Although the latter study used a different tumor cell line, these results may imply a better tumor penetration and tumor availability of ACPP in the mammary fat pad HT-1080 model compared with a subcutaneous HT-1080 model and, therefore, will increase the chances for local ACPP activation. Furthermore, substantial MMP inhibition has been reported in the subcutaneous HT-1080 model (25), while to our knowledge no data exist for the mammary fat pad HT-1080 model. If a higher absolute MMP activity is present in the mammary fat pad HT-1080 model, this could explain the differences in ACPP activation in both tumor models.

We realized that an experiment demonstrating ACPP cleavage *in vitro* in serum would further support our conclusion of *in vivo* ACPP activation in the vascular system. We however were unable to quantify the kinetics of ACPP in serum. For γ -HPLC, the radiolabeled ACPP probes and/or ACPP fragments had to be isolated from serum proteins to avoid column contamination. However, a serum protein precipitation approach resulted in the significant presence of ACPP/CPP probes in the protein pellet, most likely owing to binding of the charged probes to serum proteins as reported by others (22,26). For γ -SEC, the partial overlap of the elution profiles for radiolabeled ACPP and CPP prevented quantification of ACPP cleavage in serum samples. As alternative, the serum stability of a fluorescence resonance energy transfer (FRET) ACPP analog was studied by fluorescence spectroscopy. This probe contains a Cy-5 fluorophore conjugated to the CPP domain and a QSY21 quencher conjugated to the inhibitory domain. FRET-ACPP was incubated with commercial mouse serum in the presence/absence of GM6001, a broad spectrum MMP inhibitor. We detected reduced Cy-5 release for FRET-ACPP in the presence of GM6001 (Supporting Information, Fig. S5), suggesting a certain degree of MMP-mediated activation in serum. Surprisingly, the negative control probe FRET-non-ACPP also showed significant activation, which was not observed for the radiolabeled negative control non-dACPP during the *in vivo* studies. There, the ratios of ^{177}Lu to ^{125}I were close to unity in most tissues, indicating no activation. Therefore, the results for FRET-ACPP activation in serum *in vitro* do not appear to be representative for the *in vivo* experiments.

Recently, Linder *et al.* reported that another MMP sensitive probe, IRDye800CW-PLG-LK(BHQ-3)AR-NH₂, was degraded rapidly at the MMP cleavage site in normal mice, resulting in the presence of the metabolized probe in plasma (27). Although the molecular source and location of activation were not revealed, it was suggested that MMPs were responsible for probe degradation. In blood, proteases such as MMPs are generally bound by the proteinase inhibitor α_2 -macroglobulin ($\alpha_2\text{M}$) (28). Importantly, these protease/ $\alpha_2\text{M}$ complexes are not active towards endogenous macromolecular substrates, but remain active and accessible for low-molecular-weight (synthetic) substrates (29,30). For example, the MMP/ $\alpha_2\text{M}$ complex efficiently cleaved a small fluorogenic substrate, while a high-molecular weight substrate could not be degraded (31). In this view, the low molecular weight MMP-2/9 sensitive ACPP and the probe of Linder *et al.* may have

been activated in the circulation by baseline levels of the MMP/ α_2 M complex. Furthermore, we earlier showed that the PLGLAG linker in ACPD can be cleaved by trypsin, while the linker LALGPG in non-ACPD could not be cleaved by trypsin (18). In the circulation, trypsin directly forms a trypsin/ α_2 M complex that, importantly, still may have displayed proteolytic activity towards ACPD.

We and others showed that the PLGLAG substrate incorporated in the ACPD probe was predominantly sensitive for MMP-2 and -9, and to some extent for MMP-14 (16,18). Other proteases (MMP-1, -3, and -7, urokinase, plasmin, thrombin, cathepsin B, tissue plasminogen activator, prostate-specific antigen, neprilysin) showed minimal to no detectable ACPD degradation. However, it cannot be excluded that other proteases might have facilitated ACPD degradation in the circulation, for example, trypsin as discussed before. Furthermore, the substrate PLGLAG forms a hairpin-like structure in the ACPD probes (15), which might be more prone to systemic peptidase degradation compared with linear PLGLAG substrates. Future research should reveal the exact molecular source of systemic ACPD activation.

The nonspecific activation of the studied MMP-2/9 ACPD prevents the clinical translation of this specific ACPD probe. However, novel ACPDs that are specifically activated in the tumor tissue with no or very limited nonspecific activation may find application in the clinic. In this respect, a recent interesting approach was focused on phage display maturation screens to identify novel ACPDs sequences with improved tumor targeting properties (32). The latter study revealed an ACPD sequence that was highly sensitive to tumor expressed plasmin and elastase and highlighted the value of unbiased selection schemes to improve the effectiveness of ACPD probes.

While we here employed a dual-labeling tool to study probe biodistribution in a preclinical research setting, development of single labeled novel ACPD probes with ^{111}In or $^{99\text{m}}\text{Tc}$, or ^{68}Ga or ^{18}F radiolabels may allow (pre-)clinical translation towards single photon emission computed tomography (SPECT) or positron emission tomography (PET), respectively.

5. CONCLUSION

We showed that probe activation for a MMP responsive radiolabeled ACPD probe occurred already in the vasculature early after systemic administration, followed by nonspecific uptake in subcutaneous tumors that is comparable for high and low MMP-expressing tumors. Future clinical translation of the elegant ACPD concept may be achieved by novel ACPD sequences that are not subject to nonspecific activation in the blood pool and target more tissue-specific proteases.

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