Investigation of a MMP-2 Activity-Dependent Anchoring Probe for Nuclear Imaging of Cancer

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Abstract

Purpose: Since matrix metalloproteinase-2 (MMP-2) is an important marker of tumor malignancy, we developed an original drug design strategy, MMP-2 activity dependent anchoring probes (MDAP), for use in MMP-2 activity imaging, and evaluated the usefulness of this probe in in vitro and in vivo experiments.

Methods: We designed and synthesized MDAP1000, MDAP3000, and MDAP5000, which consist of 4 independent moieties: RI unit (111In hydrophilic chelate), MMP-2 substrate unit (short peptide), anchoring unit (alkyl chain), and anchoring inhibition unit (polyethylene glycol (PEGn; where n represents the approximate molecular weight, n = 1000, 3000, and 5000). Probe cleavage was evaluated by chromatography after MMP-2 treatment. Cellular uptake of the probes was then measured. Radioactivity accumulation in tumor xenografts was evaluated after intravenous injection of the probes, and probe cleavage was evaluated in tumor homogenates.

Results: MDAP1000, MDAP3000, and MDAP5000 were cleaved by MMP-2 in a concentration-dependent manner. MDAP3000 pretreated with MMP-2 showed higher accumulation in tumor cells, and was completely blocked by additional treatment with an MMP inhibitor. MDAP3000 exhibited rapid blood clearance and a high tumor accumulation after intravenous injection in a rodent model. Furthermore, pharmacokinetic analysis revealed that MDAP3000 exhibited a considerably slow washout rate from tumors to blood. A certain fraction of cleaved MDAP3000 existed in tumor xenografts in vivo.

Conclusions: The results indicate the possible usefulness of our MDAP strategy for tumor imaging.

Introduction

Tumor metastasis occurs when a subset of tumor cells acquires the ability to break through the basement membrane and invade through dense networks of interstitial extracellular matrix (ECM) proteins [1]. Matrix metalloproteinases (MMPs) constitute the largest family of enzymes responsible for degrading these various ECM components. Since MMP-2 is currently recognized as the subtype that has the best-established association with tumor malignancy [2], in vivo imaging of its activity should be useful for tumor diagnosis. Thus, we aimed to develop a novel nuclear imaging probe capable of estimating in vivo MMP-2 activity with Single Photon Emission Computed Tomography (SPECT).

We originally developed a novel probe design strategy that uses a MMP-2 activity dependent anchoring probe (MDAP) (Fig. 1) to detect MMP-2 activity effectively. Following this MDAP strategy, the probe was expected to be cleaved by MMP-2 enzymatic activity in the vicinity of the tumor, and efficiently trapped in proximal tumor cells. Thus, the radioactivity level detected by SPECT could be correlated with MMP-2 activity in tumors. In this study, we specifically designed and synthesized MDAP1000, MDAP3000, and MDAP5000, consisting of a RI unit (111In DTPA), a MMP-2 substrate unit (short peptide) [3], an anchoring unit (alkyl chains) [4], and an anchoring inhibition unit (polyethylene glycol (PEGn; where n indicates the approximate molecular weight, n = 1000, 3000, and 5000) (Table 1). MDAPCV, which lacks the PEG moiety, served as a control. We evaluated the feasibility of this drug design strategy and the usefulness of the probes in vitro and in vivo.

Materials and Methods

Ethics statement

The animal experiments were conducted in accordance with institutional guidelines and approved by the Kyoto University Animal Care Committee (Permit Number: 2012-49, 2013–33). All
surgery was performed under isoflurane anesthesia, and all efforts were made to minimize suffering.

**General**

Amino acid derivatives were purchased from Watanabe Chemical Industries (Hirosima, Japan) and Iris Biotech GmbH (Marktredwitz, Germany). Matrix Assisted Laser Desorption/Ionization-Mass Spectrometry (MALDI-MS) was performed with an AXIMA-CFR Plus apparatus (Shimadzu Corporation, Kyoto, Japan). Reverse Phase High Performance Liquid Chromatography (RP-HPLC) was performed using a Shimadzu-HPLC-gradient system (LC-20AD; Shimadzu Corporation) equipped with a COSMOSIL 5C18-AR-II column (10×250 mm, Nacalai Tesque, Inc., Kyoto, Japan). Peptides were eluted with a linear gradient starting at 50% solvent B that increased to 80% over 15 min (solvent A: 0.1% trifluoroacetic acid in water [v/v]; solvent B: 0.1% trifluoroacetic acid in acetonitrile [v/v]) at a flow rate of 4.0 ml/min. For tumor metabolism, products were eluted with a linear gradient starting at 50% solvent B that increased to 80% over 30 min at a flow rate of 2.0 ml/min.

**Preparation of probes (Table 1)**

Fmoc-Dap(Boc)-10-Adc-Gly-Pro-Leu-Gly-wang-resin (Dap: 2,3-diamino propionic acid, 10-Adc: 10-amino-decanoic acid) was synthesized by a Fmoc-solid-phase peptide synthesis procedure using a peptide synthesizer (PSSM-8; Shimadzu Corporation) with N<sub>α</sub,N<sub>α</sub>-diisopropylcarbodiimide (1.2 eq), N<sub>α</sub>,N<sub>α</sub>-diisopropylpropylamine (1.0 eq), and 1-hydroxybenzotriazole (1.0 eq) as reagents [5]. After Fmoc group removal with 20% piperidine in N<sub>Me</sub>formamide, palmitic acid was reacted in a similar way to yield palmitoyl-Dap(Boc)-10-Adc-Gly-Pro-Leu-Gly-wang-resin. Palmitoyl-Dap(Boc)-10-Adc-Gly-Pro-Leu-Gly-Val-Arg(pbf)-Gly-Lys(ivDde)-PEG<sub>n</sub>-amide resin (n = 1000, 3000, or 5000) was supplied by Scrum Inc. (Tokyo, Japan). Peptide deprotection and cleavage from the resin were simultaneously performed using trifluoroacetic acid/ethanedithiol/water/triisopropylsilane (95/2.5/2.5/1, v/v). The crude peptides were purified by RP-HPLC, followed by lyophilization. The purified compound was reacted with p-SCN-Bn-DTPA (2 eq) in N<sub>α</sub>,N<sub>α</sub>-dimethylformamide (1 ml) and N<sub>α</sub>,N<sub>α</sub>-diisopropylamine (20–100 µl) to adjust the pH of a solution (pH>6), and incubated at room temperature overnight. The radio-labeling precursors were purified by RP-HPLC and characterized by mass spectrometry. ([111In]InCl<sub>3</sub> (5.55 MBq in 100 µl of HCl solution, Nihon Medi-Physics Co., Ltd., Japan) was added to each purified precursor (2 nmol) in 0.1 M acetate buffer (pH 6.0, 50 µl) and the mixture was incubated at room temperature for 30 min. Radiochemical purity was estimated by RP-HPLC equipped with a radioactivity detector.

**Measurement of partition coefficients**

The experimental determination of probe partition coefficients (log P values) was performed in 1-octanol and 0.02 M phosphate buffer pH 7.4 where the two phases were pre-saturated with each other. 1-Octanol (200 µl) and phosphate buffer (200 µl) were pipetted into LoBind Eppendorf tubes containing 0.37 MBq of probes. The tube was vortexed for 10 seconds, and centrifuged (5 min, 4000×g). Aliquots (50 µl) from the 1-octanol and buffer phases were transferred into two test tubes for radioactivity counting with a NaI well-type scintillation counter (1470 WIZARD, Perkin Elmer, Kanagawa, Japan). The partition coefficient was calculated using the equation $P = (\text{counts/µl in 1-octanol})/(\text{counts/µl in buffer})$.

**Cleavage assay**

Human-recombinant MMP-2 protein (902-MP-010, R & D Systems, Inc., Minneapolis, MN USA) was activated with p-aminophenylmercuric acetate (1 mM) in assay buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM CaCl<sub>2</sub>, 5 µM ZnCl<sub>2</sub>, 0.02% Brij 35, pH 7.5). MDAP<sub>1000</sub>, MDAP<sub>3000</sub>, and MDAP<sub>5000</sub> (370 kBq) were incubated at 37°C for 2 hr with activated MMP-2 (1–5 nM) and a MMP inhibitor (0 or 100 µM, GM6001, Merck KGaA, Darmstadt, Germany). The reaction was quenched by addition of methanol, and the percentage of cleaved peptide was estimated by RP-HPLC.

**Cellular uptake study**

HT1080 human fibrosarcoma cells were purchased from American Type Culture Collection (ATCC, Manassas, VA USA) and cultured in DMEM with low glucose (Invitrogen), 10% fetal bovine serum (FBS), and penicillin/streptomycin. MMP-2 cDNA was cloned into the pENTR vector (Invitrogen) and inserted into the pLent6 vector (Invitrogen) as previously described [6,7]. For the autoactivated MMP-2 mutant, the sequence that encodes the furin cleavage site of MT1-MMP (Gly-Ala-Glu-Ile-Lys-Ala-Asn-Val-Arg-Arg-Lys-Arg) was inserted between Asn109 and Tyr110 in wild-type MMP-2 by PCR. Lentiviral vectors were prepared and transduced into HT1080

**Table 1. Probes evaluated in this study.**

| Probe    | Description                                                                 |
|----------|-----------------------------------------------------------------------------|
| MDAP<sub>1000</sub> | Palmitoyl-Dap(p-SCN-Bn-DTPA-[111In])-10-Adc-Gly-Pro-Leu-Gly-Val-Arg-Gly-Lys(ivDde)-PEG<sub>1000</sub> |
| MDAP<sub>3000</sub> | Palmitoyl-Dap(p-SCN-Bn-DTPA-[111In])-10-Adc-Gly-Pro-Leu-Gly-Val-Arg-Gly-Lys(ivDde)-PEG<sub>3000</sub> |
| MDAP<sub>5000</sub> | Palmitoyl-Dap(p-SCN-Bn-DTPA-[111In])-10-Adc-Gly-Pro-Leu-Gly-Val-Arg-Gly-Lys(ivDde)-PEG<sub>5000</sub> |
| MDAP<sub>CV</sub> | Palmitoyl-Dap(p-SCN-Bn-DTPA-[111In])-10-Adc-Gly-Pro-Leu-Gly-Val-Arg-Gly-Lys(ivDde)-PEG<sub>5000</sub> |

Dap: 2,3-diamino propionic acid, 10-Adc: 10-amino-decanoic acid, PEG: polyethylene glycol.

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cells as previously described [6,7]. After overnight preincubation of 5×10^5 HT1080 cells in FBS-free-DMEM in clean LoBind Eppendorf tubes, cells were incubated with MDAP_{CV}, MDAP_{1000}, MDAP_{3000} or MDAP_{5000} (37 kBq). Thirty min after the initiation, the cells were washed twice with phosphate buffered saline (PBS) (-) (Seikagaku Biobusiness Co., Japan), transferred to another tube, lysed with 0.2 M NaOH, and the radioactivity counted with a NaI well-type scintillation counter. Protein quantitation was performed by BCA protein assay (Thermo Fisher Scientific, Inc. Rockford, IL USA). The cellular uptake study of MDAP_{3000} pretreated with activated MMP-2 (63 nM) in the presence or absence of GM6001 (100 μM) was performed as described above.

Preparation of tumor-bearing mice
Female Balb/c nu-nu mice (5 weeks old, Japan SLC, Inc., Shizuoka, Japan) were housed under a 12-h light/12-h dark cycle and given free access to food and water. HT1080 cells (5×10^6 cells/100 μl PBS (-)) were subcutaneously inoculated into the right hind leg of Balb/c nu-nu mice. Animals were used for experiments two weeks after inoculation when the mean tumor size was 5.7±2.2 mm along the major axis.

In vivo study
MDAP_{1000}, MDAP_{3000} and MDAP_{CV} (37 kBq, 100 μl in PBS including 3% bovine serum albumin and 0.1% Tween 80) were injected intravenously into the tail vein of tumor bearing mice. The mice were sacrificed at various post-injection time points (n = 3 for each time point), and the organs of interest including the tumor tissues were collected for determination of the weights. The radioactivity of each sample was measured with a NaI well-type scintillation counter. From fitting to the two phase decay curves for blood radioactivity data analyzed by GraphPad Prism 6 (GraphPad Software, San Diego, CA), whole body pharmacokinetic parameters such as blood half-lives, distribution volume, mean residence time and total clearance were calculated for each of the probes and the values compared. Simple pharmacokinetic analysis using a single tissue compartment model was applied to the biodistribution data to calculate rate constants (K1 and K2) for radioactivity transfer from blood to tumor and clearance from tumor to blood by PMOD version 3.2. In addition, MDAP_{5000} (7.4 MBq in 200 μl) was injected intravenously into the tail vein of tumor bearing mice for metabolite analysis in tumors excised 3, 6, and 24 hr post injection (n = 2 each). Tumor homogenates were prepared on ice and insoluble material was removed by centrifugation after methanol treatment. The resulting supernatant was analyzed by RP-HPLC.

The above data suggested that MDAP_{5000} underwent intratumoral cleavage to some extent after intravenous injection. Thus, for precise analysis of the MDAP_{5000} metabolite generated by MMP activity in tumors, an intratumoral probe administration method was adopted that avoids the possibility that any metabolite made in other tissues would re-distribute to tumors, which is an inevitable issue in intravenous administration methods. MDAP_{3000} (37 kBq, 10 μl in saline) was intratumorally administered to tumor-bearing mice 30 min after intratumoral injection of GM6001 (100 μM, 20 μl in 1% DMSO saline) or 1% DMSO saline (20 μl). Thirty min later, the mice were sacrificed (n = 3 each), the tumors immediately removed and tumor homogenates were prepared on ice. Insoluble material was removed by centrifugation and the resulting supernatant was analyzed by RP-HPLC for cleavage estimation.

Zymography was performed with excised HT1080 tumor homogenates using a Novex Zymogram Gel (Life Technologies

Figure 2. Reverse phase HPLC analysis after probe radiosynthesis. RI charts of (a) MDAP_{1000}, (b) MDAP_{3000}, (c) MDAP_{5000}, and (d) MDAP_{CV} are shown with UV charts of corresponding nonradioactive compounds. doi:10.1371/journal.pone.0102180.g002
Figure 3. *In vitro* experiment. (a) Cellular accumulation of MDAP<sub>1000</sub>, MDAP<sub>3000</sub>, MDAP<sub>5000</sub> and MDAP<sub>Cu</sub> estimated by radioactivity counting. (b) Cleavage (%) of MDAP<sub>1000</sub>, MDAP<sub>3000</sub>, and MDAP<sub>5000</sub> arising from MMP-2 treatment. Values were estimated by reverse phase HPLC. (c) Cellular accumulation of MDAP<sub>3000</sub> with or without MMP-2 protein and MMP inhibitor (GM6001).

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Table 2. Radioactivity biodistribution after intravenous administration of MDAP<sub>1000</sub> in tumor bearing mice.

| Time after administration (hr) | 0.25 | 1   | 3   | 6    | 24   |
|-------------------------------|------|-----|-----|------|------|
| Blood                         | 46.93±5.71 | 24.81±3.60 | 10.58±1.28 | 5.85±0.74 | 5.26±0.94 |
| Heart                         | 9.40±2.03  | 8.43±1.19  | 7.47±0.45  | 5.71±0.61  | 4.11±0.44  |
| Lung                          | 21.23±2.65 | 12.56±1.70 | 6.17±0.88  | 4.38±0.32  | 2.63±0.28  |
| Liver                         | 34.27±5.87 | 42.27±8.44 | 50.70±7.52 | 30.08±5.98 | 8.89±1.11  |
| Kidneys                       | 14.24±1.90 | 11.66±1.17 | 10.56±1.49 | 9.53±1.08  | 7.01±0.45  |
| Stomach<sup>a</sup>           | 1.03±0.15  | 1.13±0.21  | 1.14±0.33  | 0.73±0.06  | 0.49±0.09  |
| Intestine                     | 4.18±0.58  | 8.97±0.28  | 20.39±4.51 | 13.23±3.92 | 4.90±0.90  |
| Pancreas                      | 4.28±0.41  | 5.52±1.05  | 4.50±1.45  | 5.53±1.77  | 6.21±3.76  |
| Spleen                        | 8.06±0.23  | 7.72±1.86  | 10.01±3.77 | 10.00±2.61 | 4.82±1.10  |
| Muscle                        | 2.15±0.57  | 1.94±0.33  | 2.00±0.38  | 1.68±0.46  | 1.55±0.13  |
| Tumor                         | 1.70±0.85  | 1.90±0.38  | 2.65±0.33  | 3.18±0.69  | 3.33±0.44  |
| T/B                           | 0.04±0.01  | 0.08±0.02  | 0.25±0.05  | 0.55±0.15  | 0.65±0.15  |

Data are presented as % injected dose per gram. Each value represents the mean ± s.d. for 3 animals at each interval. T/B means tumor to blood ratio.

<sup>a</sup>Presented as % injected dose per organ.

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Corporation, Carlsbad, CA USA) following the manufacturer’s protocol.

The effective dose of MDAP3000 was estimated using the area under the non-decay-corrected time radioactivity curves generated from the biodistribution data for each organ with the standard quantitation platform included in Organ Level Internal Dose Assessment software (OLINDA, Vanderbilt University) [8].

### Statistical analysis

Data are presented as mean ± SD. Statistical analysis was performed with the Bonferroni’s Multiple Comparison test or unpaired t test. A two-tailed value of P<0.05 was considered statistically significant.

### Results

#### Preparation of probes

MDAP1000, MDAP5000, MDAP3000 and MDAPCV were obtained with greater than 95%, 97%, 95%, and 98% radiochemical purity, respectively (Fig. 2). The specific radioactivity of the probes was estimated to be 2.8 MBq/nmol, which was dependent on the radioactivity of [111In]InCl3 used for the radiolabeling procedure. The log P values of MDAP1000, MDAP3000, MDAP5000 and MDAPCV were −0.56 ± 0.13, −0.95 ± 0.12, −1.04 ± 0.16 and 0.29 ± 0.07, respectively.

#### In vitro study

As shown in Fig. 3a, MDAP1000 showed high radioactivity accumulation in tumor cells and levels that were similar to...

### Table 3. Radioactivity biodistribution after intravenous administration of MDAP3000 in tumor bearing mice.

| Time after administration (hr) | 0.25   | 1      | 3      | 6      | 24     |
|-------------------------------|--------|--------|--------|--------|--------|
| Blood                         | 44.98±2.53 | 30.48±1.67 | 15.33±0.81 | 8.33±0.58 | 1.61±0.27 |
| Heart                         | 9.26±0.86  | 8.20±0.74  | 6.70±0.59  | 5.64±0.79 | 3.78±0.39 |
| Lung                          | 20.44±4.77 | 15.92±0.58 | 9.72±1.02  | 5.56±0.75 | 2.50±0.44 |
| Liver                         | 18.83±1.38 | 18.45±0.81 | 22.83±2.72 | 17.61±2.53 | 10.51±0.89 |
| Kidneys                       | 11.85±0.84 | 10.60±0.38 | 11.89±0.76 | 11.40±1.18 | 10.81±2.35 |
| Stomach*                      | 0.48±0.04  | 0.69±0.21  | 1.17±0.82  | 0.97±0.46 | 0.54±0.07 |
| Intestine                     | 3.59±0.24  | 5.85±0.11  | 13.13±1.73 | 14.08±2.39 | 3.70±1.06 |
| Pancreas                      | 3.91±0.33  | 4.35±0.74  | 5.25±0.57  | 4.97±1.31 | 3.92±0.68 |
| Spleen                        | 6.53±0.34  | 5.65±0.98  | 5.41±0.64  | 4.94±1.51 | 3.92±0.24 |
| Muscle                        | 1.72±0.76  | 1.27±0.20  | 1.49±0.15  | 1.43±0.28 | 1.02±0.16 |
| Tumor                         | 3.60±0.17  | 5.49±0.53  | 5.49±0.36  | 7.31±1.12 | 4.25±0.79 |
| T/B                           | 0.08±0.01  | 0.18±0.03  | 0.36±0.04  | 0.68±0.14 | 2.74±0.89 |

Data are presented as % injected dose per gram. Each value represents the mean ± s.d. for 3 animals at each interval. T/B means tumor to blood ratio.

*Presented as % injected dose per organ.

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### Table 4. Radioactivity biodistribution after intravenous administration of MDAPCV in tumor bearing mice.

| Time after administration (hr) | 0.25   | 1      | 3      | 6      | 24     |
|-------------------------------|--------|--------|--------|--------|--------|
| Blood                         | 51.19±6.00 | 37.43±5.29 | 30.07±2.16 | 30.99±1.77 | 19.94±2.95 |
| Heart                         | 9.08±1.72  | 8.04±0.37  | 7.09±0.12  | 6.61±0.43 | 4.84±1.43 |
| Lung                          | 25.41±7.86 | 18.25±2.62 | 15.52±3.02 | 13.07±3.55 | 9.08±0.75 |
| Liver                         | 13.05±1.68 | 11.57±2.33 | 8.63±0.32  | 8.47±1.31 | 7.13±0.54 |
| Kidneys                       | 10.48±1.00 | 8.84±1.71  | 7.99±0.42  | 7.83±1.55 | 6.91±0.70 |
| Stomach*                      | 0.60±0.02  | 0.53±0.23  | 0.81±0.40  | 0.82±0.37 | 0.43±0.13 |
| Intestine                     | 2.02±0.23  | 3.37±0.48  | 6.13±3.17  | 14.17±10.09 | 25.12±5.34 |
| Pancreas                      | 2.21±0.45  | 2.61±0.42  | 3.17±0.11  | 2.56±0.94 | 2.22±0.45 |
| Spleen                        | 6.20±0.62  | 4.96±0.40  | 4.12±0.57  | 4.45±0.45 | 4.61±0.22 |
| Muscle                        | 1.07±0.17  | 1.17±0.14  | 1.44±0.24  | 1.50±0.48 | 1.53±0.53 |
| Tumor                         | 3.80±0.90  | 5.94±1.39  | 6.76±0.77  | 7.43±2.71 | 6.08±1.19 |
| T/B                           | 0.08±0.02  | 0.16±0.05  | 0.22±0.01  | 0.24±0.10 | 0.30±0.02 |

Data are presented as % injected dose per gram. Each value represents the mean ± s.d. for 3 animals at each interval. T/B means tumor to blood ratio.

*Presented as % injected dose per organ.

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MDAPCV, while MDAP3000 and MDAP5000 showed significantly lower radioactivity. MMP-2 cleaved MDAP1000, MDAP3000 and MDAP5000 in a concentration-dependent manner (Fig. 3b). MDAP3000 pretreated with MMP-2 accumulated in cells to higher levels, while additional treatment with a MMP inhibitor completely blocked this increased uptake (Fig. 3c).

In vivo study

Radioactivity distribution profiles after intravenous administration of MDAP1000, MDAP3000 and MDAPCV are shown in Tables 2, 3 and 4, respectively. To compare probe pharmacokinetics, changes in radioactivity in the blood and tumor are plotted as a function of time after administration (Fig. 4a, b), and as tumor to blood ratios (Fig. 4c). MDAP3000 exhibited rapid blood clearance (Fig. 4a) and high tumor accumulation (Fig. 4b). Thus, among the probes MDAP3000 achieved significantly higher tumor to blood ratios (2.74 ± 0.89 at 24 hr, Table 3 and Fig. 4c).

MDAP1000 also exhibited rapid blood clearance (Fig. 4a) but showed low tumor accumulation (Fig. 4b). Meanwhile, MDAPCV showed a slow blood clearance (Fig. 4a) and the lowest tumor to blood ratios over the experimental period (0.30 ± 0.02 at 24 hr, Table 4 and Fig. 4c). Pharmacokinetic analyses on whole body (Table 5) and tumors (Table 6) revealed the above points indicated by the biodistribution data more clearly. As shown in Table 5, MDAP3000 and MDAPCV displayed similar distribution volumes while MDAPCV showed slower total clearance as well as longer half-lives and mean residence time than MDAP3000. Table 6 shows an approximately seven-fold slower washout rate of MDAP3000 from the tumor (k2 = 1.2 ± 10^{-3} min^{-1}) compared to MDAPCV (k2 = 8.6 ± 10^{-3} min^{-1}). In addition, HPLC analysis of tumors excised 3, 6 and 24 hr after intravenous injection of MDAP3000 showed a certain fraction of MDAPCV existed in the tumor (Fig. 5, white arrow). The intact form of MDAP3000 indicated by the black arrow disappeared with time as shown in the HPLC chart. A certain fraction of MDAPCV was also present inside the tumor after intratumoral injection of MDAP3000, which could be blocked by inhibitor treatment (Fig. 6). Other peaks observed around 5–10 min could represent further metabolites that are produced inside the cells. MMP-2 enzymatic activity of the tumor homogenate was confirmed by zymography (Fig. 7). The effective dose of MDAP3000 estimated from biodistribution data was 5.08 ± 10^{-2} mSv/MBq.

Discussion

In this study we tested the feasibility and usefulness of our novel drug design strategy wherein the MMP-2 activity-dependent anchoring probe MDAP is used for MMP-2 activity imaging in cancer. Both in vitro and in vivo experiments indicated that MDAP3000 displayed cell membrane anchoring properties following substrate cleavage by MMP-2 in tumors, which led to a somewhat slower washout of radioactivity from tumors. As such, we successfully demonstrated the possible application of our MDAP strategy for tumor imaging.

During the initial stages of probe design, we selected a double alkyl chain as the anchoring unit based on a previous report indicating that double alkyl chains were superior to single alkyl chains for cell membrane anchoring. The length of the double

| Table 5. Whole body pharmacokinetic parameters. |
|-----------------------------------------------|
| Blood half-life (min) | Distribution volume (ml) | Mean residence time (min) | Clearance (ml/min) |
|-----------------------|---------------------------|---------------------------|--------------------|
| Fast                  | Slow                      |                           |                    |
| MDAP1000              | 4.0 x 10                  | 2.6 x 10^3               | 1.71               | 2.1 x 10^2         | 8.3 x 10^{-3} |
| MDAP3000              | 5.6 x 10                  | 4.3 x 10^2               | 1.94               | 2.5 x 10^2         | 7.9 x 10^{-3} |
| MDAPCV                | 2.5 x 10^2                | 5.4 x 10^15              | 2.16               | 8.6 x 10^2         | 2.5 x 10^{-3} |

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alkyl chain (palmitoyl group and 10-amino-decanoic acid in the probe structures) was selected based on results from a preliminary cell binding experiment. We then combined other moieties to fulfill the requirements for the MDAP. Specifically, $^{111}$In was selected for radiolabeling due to its simple and rapid radiosynthesis with a hydrophilic chelating agent (DTPA)-conjugated precursor under mild conditions [9]. Among previously reported MMP-2 substrate peptides, Pro-Leu-Gly-Val-Arg-Gly was selected so that the amino terminus residues (Pro-Leu-Gly) would retain their radioactivity after cleavage and not inhibit the anchoring property of the double alkyl chain [10]. PEG was selected because of the hydrophilicity and steric hindrance that would allow it to function as an inhibitor of the MDAP probes and three PEG molecule types (MW: 1000, 3000, and 5000) were tested to determine the optimum molecular weight both in terms of inhibitive capacity on anchoring and resistance to MMP-2 cleavage. As a result, we successfully obtained radiolabelled MDAP probes with a high radiochemical yield and purity that allowed their use in in vitro and in vivo experiments without additional purification.

PEG is often used to modify molecular probes to alter their hydrophilicity and pharmacokinetics [11,12]. Therefore, we expected both the hydrophilicity and steric hindrance of PEG to alter the properties of MDAP. Cellular accumulation results clearly divided the probes into a high accumulation group (MDAP$_{1000}$ and MDAP$_{CV}$) and low accumulation group (MDAP$_{3000}$ and MDAP$_{5000}$) where the probe hydrophilicity gradually increased with the molecular weight of PEG as shown in the obtained log P values. As such, the steric hindrance rather than the hydrophilicity imparted by PEG functioned as an inhibitor of the anchoring probe moiety. While MDAP$_{1000}$, MDAP$_{3000}$, and MDAP$_{5000}$ were cleaved by MMP-2 as expected, the cleavability of MDAP$_{5000}$ was rather low, which indicated that PEG located around the probe inhibited its interactions with other molecules, including the MMP-2 protein. Thus, the in vivo experiments showed that MDAP$_{5000}$ was recognized as a possible suitable probe for further in vivo evaluation.

The in vivo biodistribution study revealed a rapid blood clearance and high tumor uptake of MDAP$_{5000}$ after intravenous injection, while MDAP$_{CV}$ showed high radioactivity in both blood and tumors. Thus, a significantly higher tumor to blood radioactivity ratio (T/B ratio), an imaging index [9], was obtained for the MDAP$_{5000}$ group, indicating the superior characteristics of MDAP$_{5000}$ as an imaging agent in vivo. The effective dose of MDAP$_{5000}$ estimated from the biodistribution data was acceptable for the use of MDAP$_{5000}$ as an imaging agent [13]. On the other hand, MDAP$_{CV}$ showed high radioactivity remaining in the blood, which implied nonspecific binding with serum proteins and/or blood cells. This result suggested that intravenously administered MDAP$_{CV}$ may bind with serum proteins and exist within the interstitial space of tumors as the complex form. This possibility was also indicated by MDAP$_{CV}$ having the lowest T/B ratios (less than 0.3) over the experimental period. The MDAP$_{CV}$ biodis-

### Table 6. Probe pharmacokinetic parameters between blood and tumors.

|            | K1 (ml/g/min) | %SE | k2 (1/min) | %SE |
|------------|---------------|-----|------------|-----|
| MDAP$_{1000}$ | $7.1 \times 10^{-4}$ | 1.14 | $1.1 \times 10^{-3}$ | 2.11 |
| MDAP$_{3000}$ | $1.3 \times 10^{-3}$ | 4.09 | $1.2 \times 10^{-3}$ | 6.98 |
| MDAP$_{CV}$  | $2.2 \times 10^{-3}$ | 8.2  | $8.6 \times 10^{-3}$ | 7.82 |

Data were calculated by PMOD ver. 3.2 (1-tissue compartment model) with biodistribution results. K1 and k2 are rate constants for transfer from blood to tumor and clearance from tumor to blood, respectively.

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Figure 5. Reverse phase HPLC analysis of tumors excised 3, 6, and 24 hr after intravenous injection of MDAP$_{3000}$. Black and white arrows indicate the intact and cleaved peaks, respectively.

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Figure 6. MDAP$_{CV}$ (%) present in tumors after intratumoral injection of MDAP$_{3000}$ with or without MMP inhibitor (GM6001).

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tration results indicated that MDAPCV generated in other tissues after systemic MDAP administration would be less frequently redistributed to tumors. Thus, tumor accumulation of MDAP3000 was not derived from redistribution of cleaved MDAPCV since MDAP3000 showed rapid blood clearance and increase in the T/B ratio with time after intravenous administration. Nevertheless, there was a measurable percentage of MDAPCV existing in tumors after intravenous and intratumoral injections of MDAP3000, and MDAPCV resulting from intratumoral injection of MDAP3000 was somewhat decreased by the MMP inhibitor. MDAPCV could be generated in the tumor as a result of MDAP3000 cleavage by MMP-2. HPLC analyses on excised tumors also indicate accumulation and retention of MDAP3000 followed by gradual degradation to hydrophilic metabolites in tumors. It is quite reasonable from the MDAP strategy that pharmacokinetic analysis provided a considerably slower washout rate ($k_2$ value) for MDAP3000 compared to MDAPCV, because the slower washout rate for MDAP3000 indicates MDAP3000 radioactivity capture in tumors. Regarding the limitations of this study, direct evidence that MDAP3000 was anchored in the cell membrane after MMP-2 activation in tumor tissues was not obtained. To evaluate whether the MDAP probe is anchored in the cell membrane after MMP-2 activation more precisely, further studies such as HPLC analysis of membrane fractions from the tumor cells alone or confocal microscopy approaches using an appropriate fluorophore introduced into MDAP3000 are needed. However, the in vitro and in vivo studies did show MMP-2 dependent probe cleavage, retention of radioactivity in tumor tissues and slow washout from tumor tissues, indicating the possible application of the MDAP strategy.

Although fluorescence activatable probes have been reported for MMP imaging [14–17], nuclear medical imaging has great potential for in vivo applications because it can quantitatively detect signals from tissues deep in the body but also can be applied for use in cancer therapies involving $\alpha$, $\beta$, or auger electron emitters [18,19]. Since MDAP3000 might allow selective readout of the activated MMP-2 subpopulation due to signal amplification as compared to MMP binding probes, MDAP3000 could be a potential lead compound for use in future applications. Further studies to compare MDAP3000 with other radiolabeled MMP imaging probes are thus warranted [20,21].

Author Contributions

Conceived and designed the experiments: TT HH HS. Performed the experiments: TT HH AY NK KS. Analyzed the data: TT HH AY MO HS. Contributed reagents/materials/analysis tools: TT HH TS MS HS. Wrote the paper: TT HH AY HS.

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