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Research Article

Synthesis, quality control and *in vivo* evaluation of [^{123}I] rhTIMP-2, a potential tumour-imaging agent

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Summary

Matrix metalloproteinases (MMPs) are enzymes involved in the turnover of the extracellular matrix. Their overexpression in tumours may provide a target for diagnostic imaging by using labelled MMP inhibitors. MMPs are inhibited by endogenous tissue inhibitors of metalloproteinases (TIMPs). The enhanced production of MT1-MMP, located on the surface of cells within or in the direct vicinity of the tumour, and the high affinity interaction between TIMP-2 and MT1-MMP suggested that TIMP-2 could be a potential agent for non-invasive monitoring of cancer MMP levels, diagnosis of primary and secondary tumours and tumour response to MMP inhibitor therapy. There is also evidence that ^{125}I-rhTIMP-2 internalizes, which is an important feature for its possible use as a radiotherapeuticum if labelled with ^{131}I. Labelling of rhTIMP-2 was performed using the iodogen method resulting in a radiochemical yield of 51.1 $\pm$ 11.8% ($n = 5$) and a radiochemical purity of $>98\%$. The trichloroacetic acid (TCA) precipitability of ^{123}I rhTIMP-2 was 95.2%. SDS-PAGE confirmed the correct size (21 kDa) of the purified ^{123}I rhTIMP-2 without degradation. HPLC showed one radioactive peak with a retention time corresponding to the non-labelled rhTIMP-2. *In vivo* biodistribution showed no long-term accumulation in organs and the possibility to accumulate in the tumour. These results show the potential of ^{123}I rhTIMP-2 as tumour-imaging agent. Copyright © 2005 John Wiley & Sons, Ltd.

Key Words: radiolabelled rhTIMP-2; iodine-123; *in vivo* biodistribution; SPECT; tumour imaging

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Introduction

Matrix metalloproteinases (MMPs) form a family of neutral zinc endopeptidases which play pivotal roles in several normal and pathological processes, including embryogenesis, tumour invasion, metastatic dissemination and angiogenesis.[1–3] MMPs are subgrouped into soluble and membrane-anchored MMPs, called membrane-type MMPs (MT-MMPs).[4] Membrane-type 1 matrix metalloproteinase (MT1-MMP) has been shown to be a key enzyme in tumour angiogenesis and metastasis.[5] Research results have suggested that the cell–cell contact between carcinoma cells and normal fibroblasts enhances the production of MT1-MMP followed by sequential activation of progelatinase A (pro-MMP-2) on the tumour cell surface, which may be closely involved in tumour invasion *in vivo*.[6] Soluble constructs of MT1-MMP were used to demonstrate that binding with TIMP-2 occurs primarily through N-terminal domain interactions. Subsequently the C-terminal domain is free for interactions with progelatinase A[7] forming a ternary complex, which permits pro-MMP-2 activation by a TIMP-2-free neighbouring MT1-MMP.[8] Studies to displace MT1-MMP-bound TIMP-2 in a purified system with active MMP-2 show minimal displacement of inhibitor, under the experimental conditions, due to the high affinity interaction between TIMP-2 and MT1-MMP.[8] The enhanced production of MT1-MMP, located on the tumour cell surface, and the high affinity interaction between TIMP-2 and MT1-MMP may suggest that TIMP-2 could be a potential agent for non-invasive monitoring of cancer MMP levels, diagnosis of primary and secondary tumours and tumour response to MMP inhibitor therapy. There is also evidence that ^{125}I rhTIMP-2 internalizes and that this reflects the internalization of MT1-MMP.[9] The internalization of radioiodinated rhTIMP-2 is an important feature for the possible use as a radiotherapeuticum. A rapid clearance by the kidneys is expected owing to the modest molecular weight of 21 kDa. Compared to antibodies, imaging time points should be shorter because of a faster blood clearance and good tumour to blood ratios should be reached sooner resulting in a lower radiation dose. This study reports the labelling, quality control and *in vivo* evaluation of ^{123}I rhTIMP-2. These results will be used for further *in vivo* evaluation in tumour-bearing athymic mice and SPECT imaging.

Experimental

Production and purification of rhTIMP-2

Production and purification of rhTIMP-2 was conducted at the Laboratory of Tumour and Developmental Biology, University of Liège, Sart-Tilman, as described previously.[10] Briefly, human recombinant TIMP-2 (rhTIMP-2) was purified from Chinese Hamster Ovary (CHO) cells transfected with the

pDSRa2 vector containing human TIMP-2 cDNA. Recombinant rhTIMP-2 was purified as described before.[11]

Synthesis of ^{123}I rhTIMP-2

Radiosynthesis was performed using the Iodogen technique as described before by Haisma *et al.*[12] Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycouracil, Pierce, Aalst, Belgium) was coated to polypropylene vials using 70 µg of Iodogen in 200 µl of chloroform for each vial (Alldrich, Bornem, Belgium). After evaporation of the solvent under N_2-atmosphere at room temperature, the vials were stored at 4°C. Eight micrograms of rhTIMP-2 (University of Liège, Sart-Tilman) in 10 µl Trisbuffer and n.c.a. (1–20 mCi dependent from the experiment) carrier-free Na^{123}I (Bristol-Meyers Squibb, Brussels, Belgium) were added to a Iodogen-coated vial. Total reaction volume was adjusted to 200 µl, using a 0.5 M sodiumphosphate buffer at pH 8.5. After 10 min the mixture was removed from the Iodogen vial to stop the reaction. Bovine serum albumin solution was added (BSA, Sigma, Bornem, Belgium; 250 mg BSA/100 ml 0.1 M phosphate buffered saline (PBS) at pH 7.5, referred to as PBS/BSA) to a volume of 1 ml. ^{123}I rhTIMP-2 and remaining radio-iodide were separated using a standard prepacked PD-10 SEC column (Size Exclusion Chromatography, Amersham Pharmacia Biotech, Uppsala, Sweden) using PBS/BSA as the eluting agent. The adsorption of the tracer to the preparative SEC column was checked using a purified ^{123}I rhTIMP-2 fraction in PBS/BSA. From this adsorption value and the initial mass of protein used, the molar fraction eluted from the column was calculated. Specific activity was calculated as the amount of radioactivity per µg rhTIMP-2 recovered at the end of purification.

Quality control of ^{123}I rhTIMP-2

TCA precipitation. TCA (Trichloroacetic acid, Alldrich, Bornem, Belgium) precipitation is conducted as control for radiochemical purity and the calculation of specific activity as well as for concentration of the samples for analysis on SDS-PAGE. To 900 µl reaction mixture was added 100 µl 100% TCA solution and 20 µl 3% deoxycholaat (DOC, Alldrich, Bornem, Belgium) solution. After 1 h of incubation at −20°C the tube was spinned in the microcentrifuge (Biofuge*pico*, Heraeus, Germany) for 40 min at 14 000 rpm. The supernatant was removed and the pellet counted for radioactivity. The percentage of radioactivity in the pellet to the total activity before precipitation divided by the used amount of protein results in the specific activity.

SDS-PAGE. The pellet (2–5 µg protein) resulting from TCA-precipitation is washed twice with aceton and once with 70% Ethanol. Every washing step is

followed by vortexing (REAXtop, Heidolph, Germany) and centrifugation for 20 min at 14 000 rpm. The pellet is then dried in an exciccator to remove all aceton and dissolved in 25 µl water. To each sample (^{123}I rhTIMP-2 and rhTIMP-2) was added an equal volume of Laemmli buffer (Biorad laboratories, Eke, Belgium) to a maximum of 50 µl. Samples were placed in a 95°C heat block for 5 min and centrifuged shortly. A Kaleidoscope prestained standard (Biorad laboratories, Eke, Belgium) and a precision protein standard were included. Gel was loaded and run at 200 V for approximately 40 min. Gel was coloured overnight with Coumassie (Biorad laboratories, Eke, Belgium) and destained (Methanol:Aceticacid:water, 40:10:50).

HPLC. HPLC analysis was used for identification and purity determination of ^{123}I rhTIMP-2 and was performed using a Shodex column (SEC, 8×300 mm) and a Waters 510 dual head pump (Figure 1). The effluent was monitored with an UV-VIS detector at $\lambda = 280$ nm (PU 4110 UV/VIS, Philips) and a NaI detector (Bicron Frisktech^{TM}, probe 1×1 in). Non-labelled rhTIMP-2 and PD-10 SEC column purified ^{123}I rhTIMP-2 were analysed, 0.1 M potassiumphosphate buffer pH 7 was used as mobile phase at a flow rate of 0.8 ml/min.

Stability. Stability of the tracer was assessed in both PBS and cell growth medium (Dulbecco's Modified Eagle's Medium) after various times points and temperatures of incubation, using a PD-10 SEC column (Tables 1 and 2).

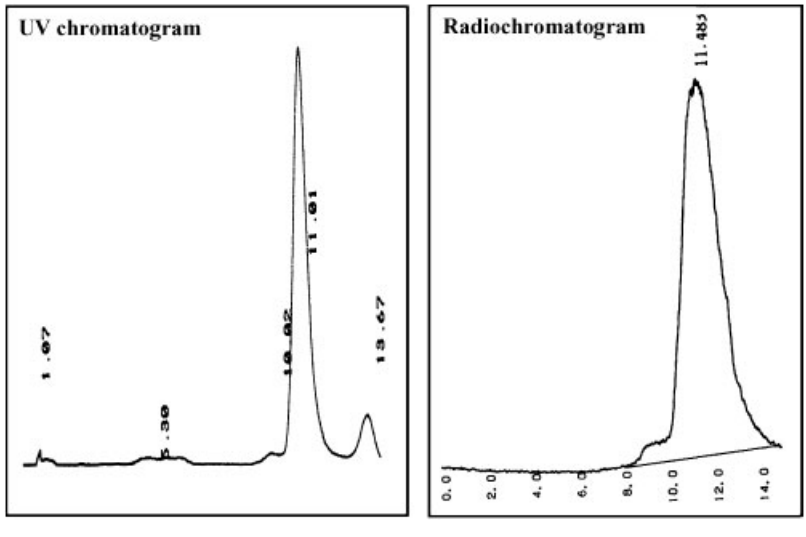

Figure 1. HPLC evaluation of rhTIMP-2 and ^{123}I rhTIMP-2. Shodex column; 0.1 M KH_2PO_4, pH 7; 0.8 ml/min; $\lambda = 280$

Table 1. Stability at 4 h

	PBS (1 ×) (%)	Medium (%)
4°C	98.8 ± 0.3	98.9 ± 0.5
37°C	93.8 ± 2.0	93.17 ± 1.5

Stability was ⩾93.17 ± 1.5% at 4 h in all conditions.

Table 2. Stability at 24 h

	PBS (1 ×) (%)	Medium (%)
4°C	92.1 ± 3.2	96.7 ± 0.5
37°C	89.9 ± 3.0	92.9 ± 1.0

Stability was ⩾89.9 ± 3.0% at 24 h in all conditions.

Biodistribution of ^{123}I rhTIMP-2 in NMRI mice

All animals were treated according to the regulations of the Belgian law and the local Ethical Committee.

Approximately 37 kBq (1 μCi) of ^{123}I rhTIMP-2 dissolved in ethanol/water (200 μl, 5/95), was injected in the tail vein of white mice (NMRI, 20–25 g) of either sex. At 20 and 40 s, 1, 1.5, 2, 3, 5, 10, 20 and 40 min and 1, 2, 3, 6, 9, 15, 24 and 48 h post injection (p.i.), animals ($n = 3$) were sacrificed by decapitation after halothane anaesthesia. Blood was taken, organs were excised, washed and dried and excretion was collected. All tissues were weighed and counted for radioactivity with a Cobra automated gamma counter (Cobra II Series, Canberra Packard, Meriden, CT, USA Cobra). The concentration of radioactivity was expressed as a percentage of the injected dose/g (% ID/g) of tissue and decay corrected. The excretion results were expressed as a % ID (Table 3).

PCR evaluation of MT1-MMP in cancer cell lines

The expression profile of gelatinase A, gelatinase B, TIMP-1, TIMP-2 and MT1-MMP has been determined in several human breast carcinoma cell lines (SKBR3, MDA-MB231) and a mouse mammary carcinoma EF43.fgf-4,[13] pancreas carcinoma (Aspc-1), lung carcinoma (A549), fibrosarcoma (HT1080) and A2058 human melanoma cells transfected with MT1-MMP cDNA (S.I.5 clone) or control vector (C.IV.3 clone). Analysis was performed as described before[14] using RNeasy Protect Kit and QIAshredder Kit (QIAGEN Benelux, Venlo, Netherlands). These results were used to select a suitable cell line to inoculate in athymic mice.

Table 3. Tissue concentrations of radioactivity at various time points post-i.v. administration of [^{123}I] rhTIMP-2[a]

	Time (min)										Time (h)							
	0.33	0.66	1	1.5	2	3	5	10	20	40	1	2	3	6	9	15	24	48
Blood	45.7 ± 2.5	36.1 ± 3.9	28.9 ± 1.0	30.0 ± 1.2	27.5 ± 2.6	24.8 ± 2.9	24.0 ± 3.0	19.0 ± 1.0	14.9 ± 2.1	9.7 ± 0.3	8.2 ± 1.3	4.0 ± 0.8	3.3 ± 0.5	1.6 ± 0.6	1.1 ± 0.7	0.2 ± 0.1	0.1 ± 0.02	0.2 ± 0.2
Brain	1.0 ± 0.2	1.1 ± 0.2	0.7 ± 0.2	0.8 ± 0.1	0.9 ± 0.2	0.6 ± 0.1	0.7 ± 0.05	0.5 ± 0.01	0.5 ± 0.3	0.4 ± 0.1	0.3 ± 0.1	0.2 ± 0.03	0.1 ± 0.03	0.1 ± 0.02	0.04 ± 0.02	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.00
Heart	10.3 ± 0.6	7.4 ± 0.5	8.5 ± 3.0	7.4 ± 2.3	7.8 ± 1.6	6.0 ± 1.6	6.8 ± 1.9	5.9 ± 0.9	5.0 ± 0.4	4.5 ± 0.7	3.4 ± 0.5	2.1 ± 0.4	1.7 ± 0.1	1.0 ± 0.5	0.5 ± 0.3	0.1 ± 0.04	0.1 ± 0.01	0.1 ± 0.03
Lungs	13.9 ± 8.3	10.8 ± 4.6	10.4 ± 3.9	9.1 ± 1.6	9.6 ± 1.7	8.5 ± 1.7	8.1 ± 0.5	4.2 ± 3.7	7.4 ± 0.9	5.6 ± 0.9	5.0 ± 1.3	2.6 ± 0.9	2.3 ± 0.1	1.2 ± 0.6	0.7 ± 0.4	0.2 ± 0.2	0.1 ± 0.03	0.1 ± 0.1
Stomach	0.3 ± 0.1	0.6 ± 0.1	0.8 ± 0.4	0.9 ± 0.4	1.2 ± 0.6	0.5 ± 0.4	1.3 ± 0.9	2.1 ± 1.6	7.1 ± 2.1	16.9 ± 5.1	24.5 ± 11.9	22.5 ± 2.0	20.8 ± 1.7	13.1 ± 9.5	7.6 ± 6.6	0.6 ± 0.6	0.2 ± 0.1	0.5 ± 0.4
Spleen	0.5 ± 0.7	2.2 ± 0.3	3.8 ± 1.0	33.3 ± 48.2	4.4 ± 3.6	4.8 ± 3.1	9.2 ± 0.6	8.2 ± 1.3	8.0 ± 2.1	6.6 ± 0.6	5.3 ± 1.1	6.6 ± 7.4	1.8 ± 0.2	0.9 ± 0.4	0.5 ± 0.4	0.1 ± 0.07	0.1 ± 0.01	0.1 ± 0.02
Liver	3.2 ± 1.1	7.7 ± 1.1	6.9 ± 0.4	5.6 ± 4.6	7.7 ± 1.4	8.4 ± 0.8	9.6 ± 1.9	8.3 ± 2.2	6.9 ± 1.6	4.5 ± 0.3	3.4 ± 0.2	1.1 ± 0.9	1.4 ± 0.1	0.7 ± 0.3	0.6 ± 0.3	0.2 ± 0.07	0.1 ± 0.02	0.1 ± 0.1
Kidneys	10.7 ± 1.3	17.8 ± 1.8	23.7 ± 3.0	36.6 ± 5.4	35.6 ± 3.1	46.7 ± 8.0	60.4 ± 5.7	25.4 ± 22.4	21.2 ± 4.9	16.1 ± 1.4	11.7 ± 2.7	6.1 ± 1.3	3.7 ± 0.4	2.3 ± 0.8	2.9 ± 2.1	0.4 ± 0.3	0.4 ± 0.2	0.2 ± 0.04
Small intestine	0.6 ± 0.3	1.0 ± 0.3	1.2 ± 0.2	1.5 ± 0.5	1.6 ± 0.5	1.6 ± 0.3	1.8 ± 0.2	1.9 ± 0.8	3.1 ± 0.8	3.5 ± 0.6	3.3 ± 0.8	2.1 ± 0.3	1.8 ± 0.2	0.9 ± 0.3	0.7 ± 0.5	0.1 ± 0.07	0.1 ± 0.02	0.1 ± 0.04
Large intestine	0.3 ± 0.2	0.7 ± 0.1	0.7 ± 0.1	0.8 ± 0.2	0.7 ± 0.2	0.5 ± 0.4	0.9 ± 0.2	0.9 ± 0.2	0.9 ± 0.5	1.5 ± 0.2	1.5 ± 0.5	1.2 ± 0.3	1.3 ± 0.3	1.3 ± 0.5	0.8 ± 0.4	0.1 ± 0.06	0.1 ± 0.02	0.1 ± 0.1
Bladder	1.4 ± 0.2	1.5 ± 0.2	1.3 ± 0.4	1.2 ± 0.4	1.9 ± 0.3	1.6 ± 0.6	2.2 ± 0.1	2.0 ± 0.7	4.1 ± 1.6	3.7 ± 1.6	3.5 ± 0.5	19.1 ± 22.1	9.1 ± 6.1	3.0 ± 0.8	2.9 ± 1.2	0.1 ± 0.09	0.04 ± 0.06	0.1 ± 0.08
Fat	1.4 ± 0.4	2.1 ± 0.7	1.8 ± 0.1	2.4 ± 0.3	2.3 ± 0.7	1.7 ± 1.5	2.5 ± 0.6	2.1 ± 2.0	3.0 ± 0.7	2.8 ± 0.7	2.6 ± 0.7	1.8 ± 0.4	1.5 ± 0.4	0.5 ± 0.3	0.5 ± 0.1	0.04 ± 0.04	0.1 ± 0.02	0.1 ± 0.07
Excretion[b]	ND ND	ND ND	ND ND	ND ND	ND ND	ND ND	ND ND	1.2 ± 1.0	0.9 ± 0.9	6.6 ± 2.2	8.7 ± 5.7	12.8 ± 1.0	16.4 ± 1.7	22.3 ± 5.2	35.0 ± 2.1	45.9 ± 4.8	51.7 ± 3.5	66.5 ± 5.4

[a]Animals were injected intravenously with 37 kBq [^{123}I] rhTIMP-2 and sacrificed at designated times. Units are expressed as % ID/g of tissue ($n = 3$) corrected for background radiation and averaged. Below are reported the standard deviations.
[b]The results of the excretion are expressed as % ID. ND: not determined.

Results and discussion

The overall radiochemical yield using freshly prepared Iodogen vials is $51.1 \pm 11.8\%$ ($n = 5$) for [^{123}I] rhTIMP-2. An elution of purified [^{123}I] rhTIMP-2 with PBS/BSA on PD-10 gives rise to an eluted fraction of 96.6%. Starting from an 8 μg solution rhTIMP-2, 7.7 μg is recovered after purification. Calculation of specific activity results in a range from 1.35×10^2 Ci/mmol to 2.7×10^4 Ci/mmol for a peak fraction of 50 μCi–10 mCi dependent on the experiment. Radiochemical purity of the ^{123}I tracer recovered at end of synthesis was >98%. The TCA precipitability of ^{123}I rhTIMP-2 was 95.2%. SDS-PAGE confirmed the correct size (21 kDa) of the purified ^{123}I rhTIMP-2 without degradation (Figure 2). HPLC showed one radioactive peak with a retention time corresponding to the non-labelled rhTIMP-2 (retention time 11.4 min). Stability was $\geqslant 89.9 \pm 3.0\%$ at 24 h in all conditions.

Biodistribution in NMRI mice showed no long-term accumulation in heart, lungs, liver and kidneys (9 h: <1.6% ID/g). A rapid clearance by the kidneys is seen related to its modest molecular weight of 21 kDa. Up to 1.1% ID/g was accumulated in blood until 9 h p.i., indicating the potential to accumulate in the tumour. Dehalogenation was indicated by an uptake of 24.5% ID/g in the stomach. Although stomach uptake is still significantly high, it is still 2–3 times lower as for other mentioned radioiodinated peptides such as [^{123}I] I-Annexin V.[15] Also an uptake in the fatty tissue (20 min: 3.0% ID/g) was observed. The radioactivity was cleared from the body after 48 h estimating a low radiation burden.

The expression profile of gelatinase A, gelatinase B, TIMP-1, TIMP-2 and MT1-MMP in different cell lines is shown in Figure 3. These results were used to select a common cell line to inoculate in athymic mice. Taking into account the expression of MT1-MMP and the ease of tumour growth after inoculation,

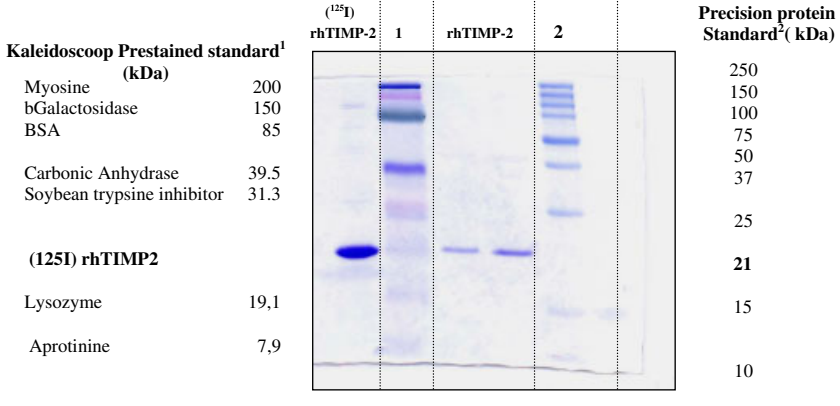

Figure 2. SDS-PAGE evaluation of labelled ^{123}I rhTIMP-2

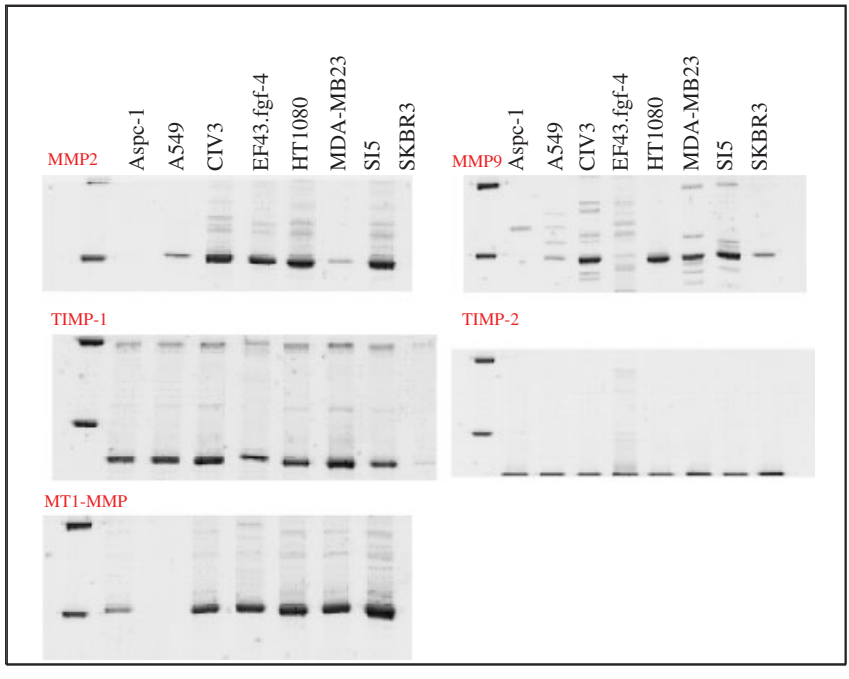

Figure 3. The expression profile of gelatinase A, gelatinase B, TIMP-1, TIMP-2 and MT1-MMP in several breast carcinoma cell lines (SKBR3, MDA-MB231, EF43fgf4), pancreas carcinoma (Aspc-1), lung carcinoma (A549), fibrosarcoma (HT1080) and A2058 human melanoma cells transfected with MT1-MMP cDNA (S.I.5 clone) or control vector (C.IV.3 clone)

EF43.fgf-4 cells,[13] established by stably infecting the normal mouse mammary EF43 cells with a retroviral expression vector for the fgf-4 oncogene, will be inoculated to evaluate tumour uptake and tumour to blood, tumour to muscle and tumour to fat ratio's [16]. Starting from these results SPECT images will be acquired. In comparison to antibodies, imaging time points should be shorter because of a faster blood clearance and good tumour to blood ratios should be reached sooner resulting in a lower radiation dose. Also a fragment of rhTIMP-2 namely rhTIMP-2ΔC will be investigated in this context because it is known that smaller proteins have a faster blood clearance and consequently imaging time points and tumour to blood ratios are expected to be better. These features would probably also result in lower radiation burden.

Conclusion

rhTIMP-2 was radioiodinated with radiochemical yield of $51.1 \pm 11.8\%$ ($n = 5$), a radiochemical purity $> 98\%$ and a specific activity of 1.74×10^3 Ci/

mmol. The trichloroacetic acid (TCA) precipitability of ^{123}I rhTIMP-2 was 95.2%. SDS-PAGE confirmed the correct size (21 kDa) and no degradation of the purified ^{123}I rhTIMP-2. HPLC showed one radioactive peak with a retention time corresponding to the non-labelled rhTIMP-2. Stability was $\geqslant 89.9 \pm 3.0\%$ at 24 h in all conditions. *In vivo* biodistribution showed no long-term accumulation in organs and the possibility to accumulate in the tumour. These data suggest that rhTIMP-2 may be a potential useful agent for non-invasive monitoring of cancer MMP levels *in vivo*, diagnosis of primary and secondary tumours and tumour response to MMP inhibitor therapy using SPECT. These results also warrant further evaluation of tumour uptake in tumour-bearing athymic mice, based on the PCR results for expression of MT1-MMP, metabolite studies of the radioiodinated rhTIMP-2 and SPECT evaluation.

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