

Selective *In Vivo* Imaging of Syngeneic, Spontaneous, and Xenograft Tumors Using a Novel Tumor Cell-Specific Hsp70 Peptide-Based Probe

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Abstract

Although *in vivo* targeting of tumors using fluorescently labeled probes has greatly gained in importance over the last few years, most of the clinically applied reagents lack tumor cell specificity. Our novel tumor cell-penetrating peptide-based probe (TPP) recognizes an epitope of Hsp70 that is exclusively present on the cell surface of a broad variety of human and mouse tumors and metastases, but not on normal tissues. Because of the rapid turnover rate of membrane Hsp70, fluorescently labeled TPP is continuously internalized into syngeneic, spontaneous, chemically/genetically induced and xenograft tumors following intravenous administration, thereby enabling site-specific labeling of primary tumors and metastases. In contrast with the commercially available nonpeptide small molecule $\alpha_v\beta_3$ -integrin antagonist IntegriSense, TPP exhibits a significantly higher tumor-to-background contrast and stronger tumor-specific signal intensity in all tested tumor models. Moreover, in contrast with IntegriSense, TPP reliably differentiates between tumor cells and cells of the tumor microenvironment, such as tumor-associated macrophages and fibroblasts, which were found to be membrane-Hsp70 negative. Therefore, TPP provides a useful tool for multimodal imaging of tumors and metastases that might help to improve our understanding of tumorigenesis and allow the establishment of improved diagnostic procedures and more accurate therapeutic monitoring. TPP might also be a promising platform for tumor-specific drug delivery and other Hsp70-based targeted therapies. *Cancer Res*; 74(23); 6903–12. ©2014 AACR.

Introduction

Tumor targeting peptides represent a promising new class of prognostic tools due to their advantageous biodistribution with fast body clearance and their capacity to effectively penetrate viable cells (1–3). However, it is essential that such probes exhibit specific binding to tumor cells and exclude normal tissue. The identification of tumor-selective probes is therefore essential.

In addition to its physiologic, stress-induced appearance in the cytoplasm, HSP70 (Hsp70-1, HspA1A, #3303) has been found to be selectively localized on the plasma

membrane of a broad variety of different murine and human tumors. Because of its absence in the membranes of normal cells, targeting membrane Hsp70 on tumors offers unique opportunities for the detection, screening, and staging of diseases (4–7), as well as for drug development and treatment evaluation of a wide variety of neoplasms in future theranostic approaches (8–10).

Screening of tumor biopsies and their corresponding normal tissues of well over 1,000 patients has shown that the majority of the primarily diagnosed carcinoma samples, but none of the tested corresponding normal tissues, exhibited an Hsp70-membrane-positive phenotype (5). Furthermore, we have previously shown that Hsp70-membrane positivity is increased in stressed tumor cells that also exhibit elevated cytosolic Hsp70 levels. In the cytosol, Hsp70 acts in an antiapoptotic manner and enhances survival of the tumor cells following therapies such as chemo- and radiotherapy (11). Most importantly, an Hsp70-membrane-positive tumor phenotype has been shown to be associated with a significantly decreased overall survival in cancerous diseases such as leukemia (12), lung, breast (13), lower rectal carcinomas (14), prostate (8), and liver cancer (15). These findings demonstrate that Hsp70 positivity might serve as a useful marker for prediction of clinical outcome. The potential therapeutic value of membrane Hsp70 has been demonstrated in a

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previous study that reported on the generation of an Hsp70-specific antibody termed cmHsp70.1 to specifically induce antibody-dependent cellular cytotoxicity of tumor cells *in vitro* and *in vivo* (16, 17).

Herein, we describe a novel peptide-based probe that binds to membrane Hsp70. Because of its remarkable tumor specificity and capacity for internalization (18), this probe has been termed Hsp70 tumor cell-penetrating peptide (TPP). TPP, which comprises the amino acid sequence aa_{450–463} (TKDNNLLGRFELSG) of the C-terminal oligomerization domain of Hsp70, binds to tumor cells with high affinity *in vivo* as well as *in vitro*. The specificity of TPP toward Hsp70 was shown by ELISA (Supplementary Fig. S1). As recently shown by Mahalka and colleagues (19), the C-terminal and N-terminal domain of Hsp70 are exposed to the extracellular milieu of tumor cells if presented on the plasma membrane. Although *in vitro* TPP exhibits similar binding characteristics toward Hsp70 than the cmHsp70.1 monoclonal antibody (16, 17), TPP exerts a better bio-distribution, *in vivo*.

Materials and Methods

Animals

BALB/c, FvB, and SHO-*Prkdc^{scid}H^{tr}* (SHO) mice were obtained from an animal breeding colony (Charles River and Harlan Winkelmann) and were maintained in pathogen-free, individually ventilated cages (Tecniplast). All animal experiments were approved by the District Government of Upper Bavaria and performed in accordance with the German Animal Welfare and Ethical Guidelines of the Klinikum rechts der Isar, TUM, Munich, Germany. Tumors were examined after the animals were sacrificed by cervical dislocation.

Syngeneic tumor models

CT26 mouse colon adenocarcinoma cells (5×10^5) or 4T1 mammary carcinoma cells (2.5×10^5 ; ATCC, authentication not applicable for mouse cell lines) were harvested in the exponential growth phase and injected subcutaneously in the neck or orthotopically into the mammary fat pad of 8-week-old, female BALB/c mice, respectively. Primary tumor growth was followed by ultrasonic measurements (Logiq-5, GE Healthcare). Intraoperative *in vivo* imaging was performed post mortem on exposed tumors when the CT26 tumors reached a size of approximately 0.4 cm^3 . 4T1-derived tumors and lung metastases were investigated after 25 to 30 days of tumor growth.

Xenograft tumor models

To test the capacity of TPP to recognize human tumors, 5×10^6 cells from the following human carcinoma cell lines were implanted subcutaneously into the neck area or orthotopically into the mammary fat pad of 8- to 10-week-old, female SHO mice: colon carcinoma cell lines HCT-116 (ATCC) and CX-2 (DKFZ), mammary carcinoma cell lines MCF-7, MDA-MB231 and T-47D (ATCC), pancreas carcinoma cell lines Panc-1, MIA PaCa-2 (provided by J.T. Siveke) and COLO357 (Leibniz-Institute DSMZ, Braunschweig,

Germany), small-cell lung cancer H1339, non-small cell lung cancer (NSCLC) A549 (n.t.), head and neck carcinoma FaDu (all ATCC) and Cal-33 (Leibniz-Institute DSMZ), as well as the human cervix carcinoma HeLa (ATCC). Mice were sacrificed and intraoperative *in vivo* imaging was performed on the exposed tumors when tumors had reached a volume of 0.2 to 0.4 cm^3 . If not stated otherwise, cell line authentication was performed by short tandem repeat profiles that are generated by simultaneous amplification of multiple short tandem repeat loci by ATCC and DSMZ. Cells were not cultured for longer than 5 months after testing.

Colitis-associated spontaneous tumor model

Spontaneous colonic tumors were induced in 8-week-old female FvB mice (Charles River) using an established approach. Briefly, mice were injected intraperitoneally with a single dose of azoxymethane (AOM; Sigma-Aldrich) at 10 mg/kg body weight. Following AOM treatment, the mice were subjected to three cycles of dextran sulfate sodium salt (MP Biomedicals) administration in their drinking water (20). After 15 to 18 weeks of tumor development, the mice were sacrificed and the colon was resected for experiments. The presence of colorectal tumors was confirmed using standard histologic techniques.

Endogenous pancreatic ductal adenocarcinoma mouse model

The spontaneous pancreatic ductal adenocarcinoma (PDAC) mouse model (*Ptf1a^{+/Cre};Kras^{+/LSL-G12D};p53^{LoxP/LoxP}*, CKP) has been described previously (21). After sacrificing, the abdominal cavity of tumor-bearing mice, including the pancreas, was exposed for *in vivo* imaging of the tumor area and surrounding normal tissue.

Toxicity testing

Potential toxic effects of TPP were investigated by injecting healthy BALB/c mice intravenously with 500 and $1050 \mu\text{g}$ of TPP. Concentrations up to $500 \mu\text{g}$ of TPP per mouse were tested in tumor-bearing mice (s.c. tumors, size 0.4 cm^3). The health status and the general behavior of mice were inspected. Animals were sacrificed on day 5 and the heart, liver, spleen, lung, and kidneys examined for pathologic changes using standard histologic techniques.

Flow cytometry

The membrane-Hsp70 phenotype of tumors grown *in vivo* was determined by flow-cytometric analysis of single cell suspensions that were generated following 30 minutes collagenase/dispase digestion using FITC-conjugated cmHsp70.1 mAb (IgG1, multimmune GmbH) or carboxyfluorescein-conjugated TPP (TPP[CF]). Cell suspensions were incubated with the appropriate reagent for 30 minutes at 4°C , after which they were washed and viable (7-AAD negative) cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson). Gating was applied to distinguish the $\text{CD45}^-/\text{CD140b}^-$ tumor cell population from $\text{CD45}^-/\text{CD140b}^+$ fibroblasts and $\text{CD45}^+/\text{F4}/80^+$ macrophages.

TPP, control peptide, and IntegriSense 750 in imaging procedures

In *in vivo* imaging experiments, Cy5.5 or DyLight750-conjugated Hsp70-specific 14-mer TPP (OEM manufactured by Thermo Fischer) was compared with the unspecific scrambled control peptide (CP, OEM manufactured by Thermo Fischer) and with IntegriSense 750 (IS750, PerkinElmer; Supplementary Fig. S2). The intraoperative imaging procedure, which captured color and fluorescence images of the exposed tumors and its surrounding normal tissue, was configured to simulate an intraoperative imaging situation. TPP[Cy5.5] or CP[DL750] (100 μ g, equal to 45 nmol per animal) or IS750, at the recommended dose of 2 nmol per animal, was injected into the tail vein of tumor-bearing mice. Concentrations above 2 nmol resulted in a higher staining of healthy tissues and thus negatively affected the tumor-to-background ratio (TBR; data not shown). Signal specificity was determined by calculating the ratio of the mean signal intensity in the exposed tumor tissue and that of the surrounding normal tissue (TBR; ref. 22). Kinetic studies were performed 12, 24, 48, and 72 hours after tail vein bolus injection of TPP[Cy5.5] and CP[DL750]. All other experiments were performed 24 hours after intravenous injection of the respective probes.

Fluorescent images were acquired by illuminating the specimens using 670 and 740 nm diode lasers and guiding the emitted fluorescence through appropriate emission filters before capturing it using a back illuminated EM-CCD camera (iXon DU888, Andor), as described previously (16). A more detailed description of the imaging procedures is shown in the Supplementary Section.

Biodistribution studies

For biodistribution, tumor-bearing mice were sacrificed 12, 24, or 48 hours after intravenous injection of TPP[Cy5.5]. Fluorescent signal intensities of 0.25 cm³ tissue cubes, taken from the tumor, spleen, pancreas, liver, lung, duodenum, kidney, heart, and 0.25 mL of peripheral blood were measured as described above. Agarose-gel cubes with standardized concentrations were used to calculate the probe concentrations in the investigated tissue samples. Detailed information concerning the quantification of TPP[Cy5.5] is provided in Supplementary Fig. S6.

Histology, IHC, and fluorescence microscopy

Paraformaldehyde-fixed and paraffin-embedded tissue sections (2 μ m) were stained with hematoxylin and eosin using standard procedures. For IHC on cryosections, unspecific mouse-on-mouse reactions were blocked using a M.O.M. kit (Vector Labs) and stained with cmHsp70.1 mAb following antigen retrieval in citrate buffer. The quantification and volumetric analysis of lung metastases were performed using consecutive tissue sections (100 μ m) using Aperio ImageScope software (Leica).

For microscopic *in situ* fluorescence analysis, TPP[CF] (100 μ g) and the recommended dose of IntegriSense 750 (IS[750]) were injected intravenously into tumor-bearing mice and allowed to circulate for 24 hours. Tissue was collected and 8 μ m cryosections counterstained with DAPI (DAKO) and

examined microscopically using a Zeiss Observer Z1 (Carl Zeiss) equipped with standard filters for GFP and Cy7 after 4 minutes of fixation with 1.8% w/v PFA.

Statistical analysis

Statistical analysis was performed using the Student *t* test. Data are mean $\pm$ SD. *P* values $\leq$ 0.05 were considered as being significant different between the compared groups.

Results

The membrane-bound form of Hsp70, but not β 3-integrin, is present on a wide variety of human and murine tumors

To examine the presence of the investigated reporter epitopes on cells *in vivo*, a broad variety of murine and human tumors were screened for the presence of membrane Hsp70 and β 3-integrin (CD61), the target epitopes for TPP and IntegriSense, respectively. This screening included colon, mammary, pancreatic, lung, head and neck, and cervical carcinomas derived from syngeneic, chemically induced, spontaneous and xenograft mouse models. Flow-cytometric analysis of viable cells derived from the solid tumors demonstrated that all of the cells examined exhibited a comparable membrane Hsp70 staining pattern using the cmHsp70.1 monoclonal antibody or the TPP probe (Fig. 1; Table 1). This finding further emphasizes the fact that targeting membrane Hsp70 could serve as a universal approach for diagnosing a wide variety of tumor entities.

For flow-cytometric analysis, a cell population was defined to be membrane positive if as more than 20% of the cells were stained. The threshold of 20% was defined upon screening of a large panel of healthy human tissues, including human peripheral blood lymphocytes, fibroblast, and healthy tissues surrounding tumor (*n* = 86). CD61 staining revealed $\alpha_v\beta_3$ -integrin positivity on mouse colon (CT26) and mammary (4T1) carcinoma, on human mammary (MDA-MB231, T-47D) and head and neck (CAL-33) carcinoma, as well as on human and mouse pancreatic carcinoma (PDAC, Panc-1, MIA PaCa-2, COLO357). However, no β_3 -integrin expression could be detected on human colon (HCT-116, CX-2), mammary (MCF-7), lung (H-1339), or cervical (HeLa) carcinoma cells. Cells derived from a murine colitis-associated carcinoma (CAC) model showed a heterogeneous CD61 expression. These data indicate that, in comparison with β_3 -integrin, Hsp70 is more frequently expressed on human and murine tumors. However, tumor cell specificity is critical for targeting strategies. Therefore, the presence of membrane Hsp70 and β 3-integrin on tumor-residing CD45⁺/F4/80⁺ macrophages and CD45⁺/CD140b⁺ fibroblasts was investigated. TPP only weakly stains (below 10%) tumor-associated fibroblasts and tumor-infiltrating macrophages; in contrast, β 3-integrin positivity was found on more than 60% of tumor-associated fibroblasts and tumor-infiltrating macrophages (Fig. 1B). To test Hsp70 positivity on tumors, cryosections were stained with the Hsp70-specific antibody cmHsp70.1. Although tumor cells of the various tumor models were positive, cells of the tumor microenvironment remained negative for Hsp70. A representative staining of a PDAC tumor is shown in Supplementary Fig. S3.

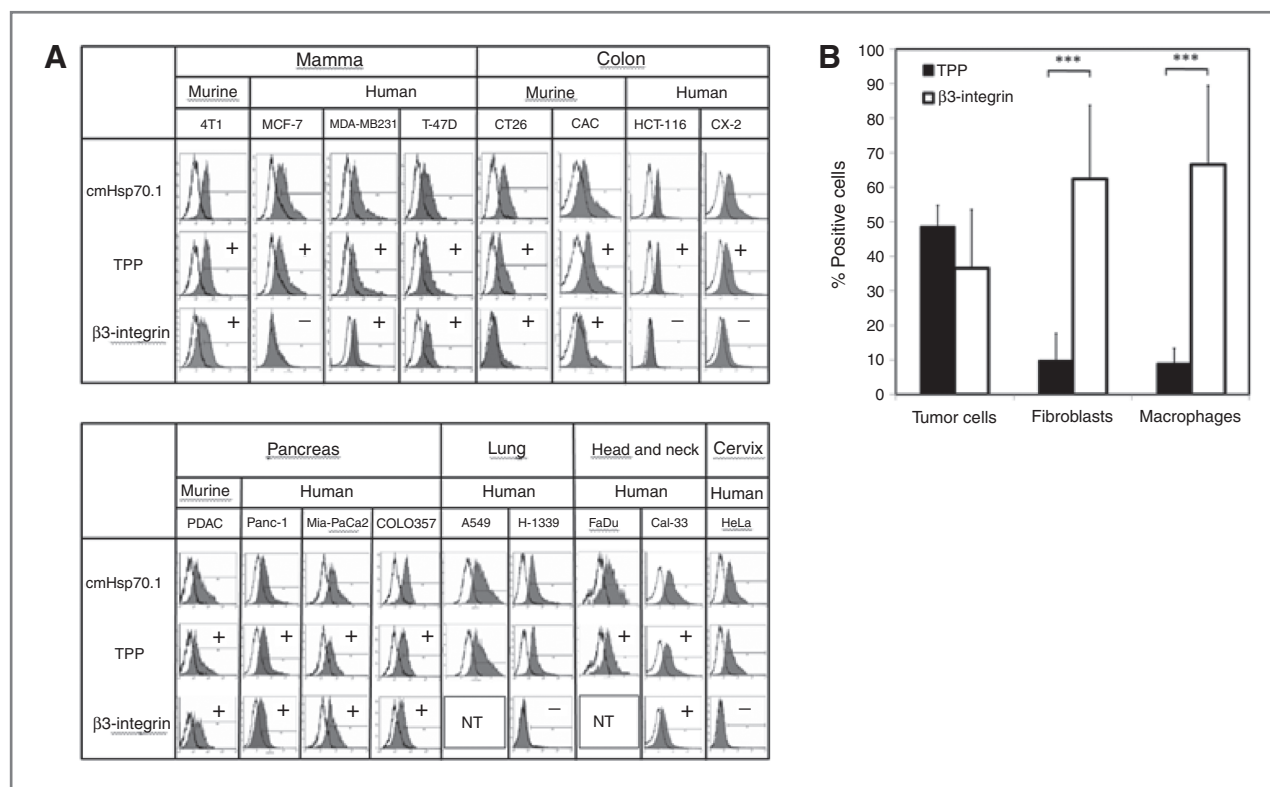


Figure 1. Membrane-Hsp70 and $\beta 3$ -integrin expression on single cell suspensions of human (h) and murine (m) tumors that were grown in mice. A, comparison of the binding capacity of cmHsp70.1 full-length antibody (top), TPP (middle), and $\beta 3$ -integrin antibody (bottom) to mammary (m4T1, hMCF-7, hMDA-MB231, hT-47D), colon (mCT26, mCAC, hHCT-116, hCX-2), pancreas (mPDAC, hPanc-1, hMiaPaCa2, hCOLO357), lung (hA549, hH-1339), head and neck (hFaDu, hCal-33), and cervix (hHeLa) carcinomas. Results are expressed as log fluorescence intensity versus relative cell numbers. The IgG1 isotype-matched control is indicated in white and membrane staining in gray histograms. B, membrane positivity of TPP (black bars) and $\beta 3$ -integrin (white bars) on cells of the tumor microenvironment in comparison with $\beta 3$ -integrin-positive tumors. Membrane staining of TPP remained significantly lower in macrophages ($P = 1.54 \times 10^{-7}$) and fibroblasts ($P = 4.67 \times 10^{-8}$), compared with $\beta 3$ -integrin. Averaged tumor cell positivity revealed no significant difference between TPP and $\beta 3$ -integrin staining.

TPP is not toxic *in vivo*

To examine potential toxic side effects of TPP, healthy and tumor-bearing BALB/c mice were injected intravenously with 500 to 1,050 μg of TPP, which is equivalent to 25 to 50 $\mu\text{g/g}$ of their body weight. Gross monitoring of the mice did not show any toxic reactions such as skin ulcerations or toxic death at any of the tested doses. Nor did any animal exhibit any loss of weight or change in mobility. Histologic examination of the heart, liver, spleen, lung, and kidney on day 5 after injection by a veterinarian, revealed no pathologic changes. An examination of the effects of TPP concentrations up to 500 μg in tumor-bearing mice also did not exert any side effects.

TPP accumulates within the tumor with maximal specificity 24 hours postintravenous injection

To investigate the *in vivo* circulation and tumor-homing properties of TPP, we analyzed its biodistribution in CT26 tumor-bearing mice. The accumulation of TPP in different organs was determined 12, 24, and 48 hours after intravenous injection of 100 μg TPP[$\text{Cy}5.5$] by measuring fluorescent signal intensities in the tumor, spleen, pancreas, liver, lung, duodenum, kidney, heart, and in the peripheral blood (Fig. 2).

A strong TPP[$\text{Cy}5.5$] signal could be detected within the tumor ($7.6 \pm 0.7 \mu\text{g}/\text{cm}^3$) 12 hours after intravenous injection. This reached a maximum after 24 hours ($10.4 \pm 2.2 \mu\text{g}/\text{cm}^3$) and dropped after 48 hours. TPP accumulation was significantly lower at all investigated time points in all other tested organs, except the kidney. The concentration of TPP[$\text{Cy}5.5$] reached its maximum in the kidney after 12 hours at $13.7 \pm 0.6 \mu\text{g}/\text{cm}^3$, and this accumulation was followed by a sharp drop, 24 and 48 hours postinjection. These data further confirm the tumor specificity of TPP and indicate that TPP is cleared from the body by renal excretion.

In further experiments, we investigated the optimal time point for *in vivo* imaging in a CT26 tumor model (subcutaneously) 2 weeks after tumor injection (Supplementary Fig. S5A) and a metastasizing 4T1 mammary carcinoma model (orthotopically) 3 to 4 weeks after tumor injection (Supplementary Figs. S5B and S5C and S7). TPP[$\text{Cy}5.5$] yielded maximal TBR in primary tumors 24 hours after injection of the probe, whereas the peak TBR in metastasized lungs occurred after 48 hours. The scrambled control peptide CP[DL750] did not show any specific accumulation in tumor tissue (Supplementary Fig. S5A–S5C).

Table 1. Binding of TPP to different membrane-Hsp70–positive colon, mammary, pancreas, lung, head and neck, and cervix mouse and human tumors

%	Colon				Mammary			
	Murine		Human		Murine		Human	
	CT26	CAC	HCT-116	CX-2	4T1	MCF-7	MDA-MB231	T-47D
Hsp700	55.5 ± 5.5	51.2 ± 0.8	63.3 ± 9.3	56.6 ± 8.3	56.3 ± 3.5	58.0 ± 3.6	63.3 ± 13.3	48.0 ± 4.2
TPP	42.0 ± 4.3	52.8 ± 7.5	59.0 ± 7.9	45.2 ± 5.2	47.5 ± 2.1	46.0 ± 5.6	62.5 ± 15.6	43.5 ± 2.1
CD61	26.5 ± 7.8	23.0 ± 7.1	15.3 ± 1.5	14.0 ± 5.0	38.3 ± 3.2	13.0 ± 1.7	20.0 ± 5.6	55.5 ± 2.1

%	Pancreas				Lung		Head and neck		Cervix
	Murine		Human		Human		Human		Human
	PDAC	Panc-1	MIAPaCa-2	COLO357	A549	H-1339	FaDu	Cal-33	HeLa
Hsp70	50.0 ± 8.6	46.0 ± 4.2	58.5 ± 7.6	57.8 ± 9.3	76.3 ± 1.5	60.0 ± 7.5	59.0 ± 8.5	68.1 ± 8.5	55.0 ± 18.4
TPP	45.0 ± 1.4	47.0 ± 7.1	42.0 ± 6.6	49.5 ± 4.9	58.3 ± 7.6	67.0 ± 5.7	53.5 ± 0.7	52.0 ± 4.2	63.0 ± 4.2
CD61	50.0 ± 5.7	36.0 ± 2.8	70.0 ± 11.3	20.0 ± 9.9	n.t.	4.0 ± 1.8	n.t.	23.5 ± 3.5	5.0 ± 5.6

NOTE: The proportion of membrane-Hsp70–positive cells was measured with cmHsp70.1 antibody and TPP peptide on single cell suspensions of *in vivo*-grown human and mouse tumors of different entities. The expression of CD61 (β3-integrin) was also determined on these tumor cells. A sample was determined as membrane positive if more than 20% of the cells showed a positive staining. Abbreviation: n.t., not tested.

The specific accumulation of TPP in syngeneic primary tumors and metastases is superior to that of IntegriSense

To confirm the specificity of TPP[Cy5.5] for primary tumors and metastases *in vivo*, we simultaneously injected 100 μg of TPP[Cy5.5] and the recommended dose of IntegriSense 750 (IS[750]) into CT26 and 4T1 tumor-bearing mice,

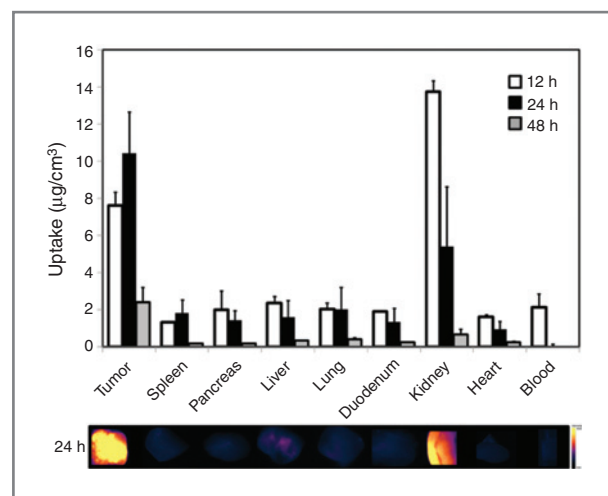


Figure 2. Biodistribution of TPP[Cy5.5] in CT26 tumor-bearing mice, assessed by NIRF quantification. Tumors and organs of CT26 tumor-bearing mice were collected 12, 24, and 48 hours (white, black, and gray bars, respectively) after intravenous injection of TPP[Cy5.5] into the tail vein. The fluorescence signals of the probe were analyzed, *ex vivo*. Two to three mice per time point were assessed; graph bars show mean values ± SD.

24 hours before *in vivo* imaging. Although TPP[Cy5.5] and IS[750] both clearly delineate the tumor, IS[750] signals can also be found in normal tissue surrounding the tumor (Fig. 3A and B, true color images of the region of interest is depicted on the left). TPP[Cy5.5] exhibited a significantly higher TBR than IS[750] ($P = 0.0004$ and 0.003 , respectively). To further investigate the specificity of the probes, *ex vivo* imaging of dissected lungs containing metastases was performed. Quantification of metastases can be found in Supplementary Fig. S4. The TBR for IS[750] imaging was also lower than that for TPP[Cy5.5] ($P = 0.04$; Fig. 3C, bottom) in distant lesions. Although the delineation of metastases by TPP[Cy5.5] and IS[750] was comparable, the enrichment of IS[750] in normal lung tissue is marginally higher (Fig. 3C, top). The specificity and colocalization of TPP and IS was further confirmed on cryo-sections of the lesions, which were obtained 24 hours after intravenous injection of TPP[CF] and IS[750] (Fig. 3D). Both probes specifically enriched in the primary tumor and lung metastases, however, a background staining of IS[750] remained in healthy lung tissue. These data demonstrate that, although both compounds specifically stain tumors and metastases, TPP is more effectively washed out of normal tissue and therefore provides a better signal-to-background contrast than IntegriSense.

The specific tumor-homing capacity of TPP is higher than that of IntegriSense in endogenous tumor models

To more realistically mimic the histologic and molecular features of human tumors in *in vivo* imaging, we took advantage of chemically induced and endogenous mouse models of colorectal (CAC) and pancreatic (PDAC) cancer. Flow-

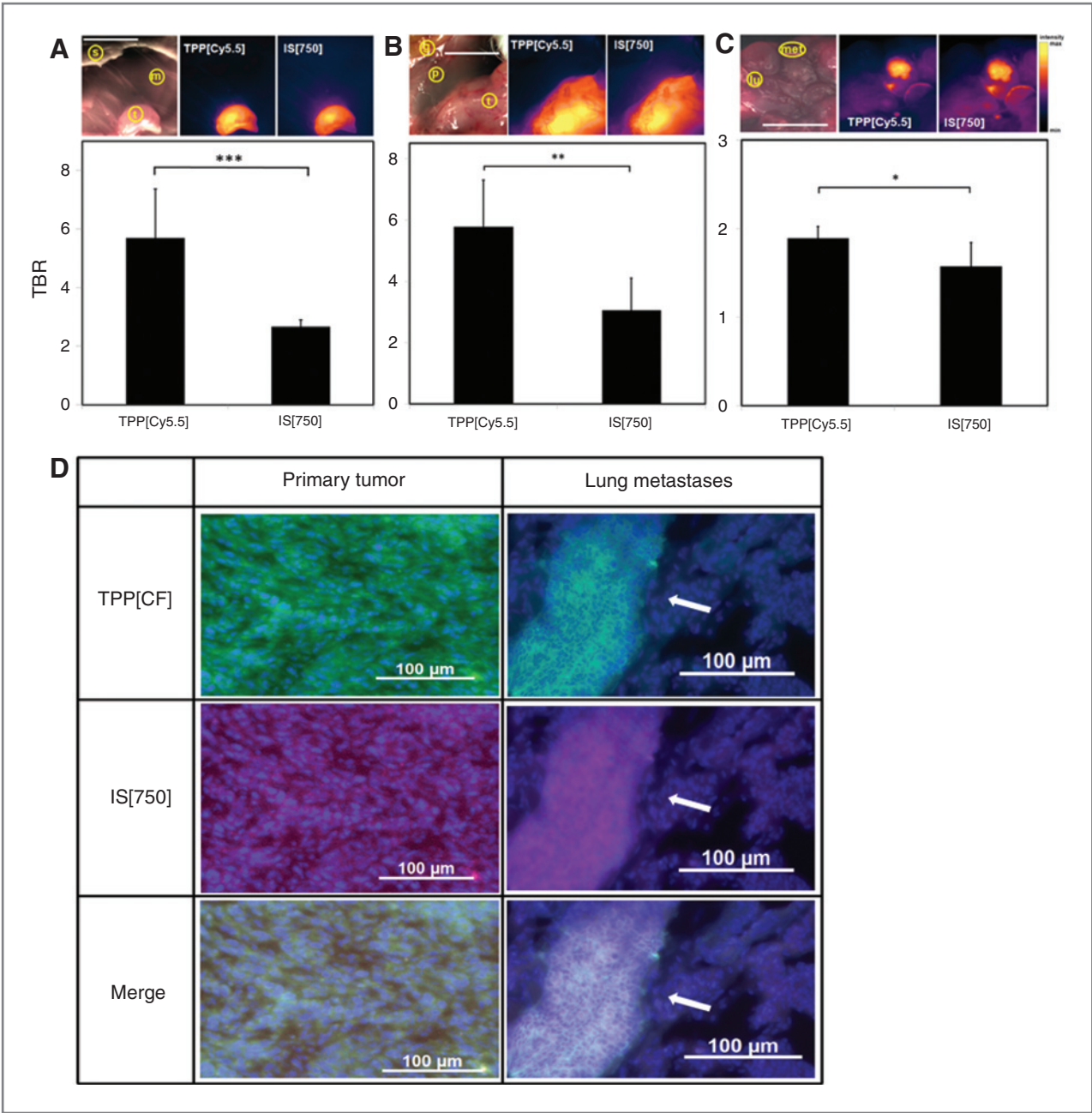


Figure 3. Intraoperative *in vivo* imaging and fluorescence microscopy of syngeneic tumor models after the injection of TPP[Cy5.5] and IS[750]. **A**, NIRF imaging of CT26 tumors that were injected subcutaneously into the neck area, **B** and **C**, 4T1 tumors injected orthotopically (**B**) and lung metastases (**C**) derived thereof. Top, true color images of tumor (t) and metastases (met), as well as surrounding normal tissue, including muscle (m), skin (s), gut (g), peritoneum (p), and lung (lu; left, scale bars, 5 mm). Corresponding pseudocolor fluorescence images were taken 24 hours after intravenous injection of TPP[Cy5.5] (middle) and IS[750] (right) into the same animal. Bottom, TBR, 24 hours after intravenous injection of TPP[Cy5.5] and IS[750]. The data are shown as mean $\pm$ SD [$n = 4$ (CT26) and 6 (4T1)]; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. **D**, fluorescence microscopy of primary tumor (left) and lung tissue, including metastases, indicated by arrows (right), 24 hours after intravenous injection of TPP[CF] (top) and IS[750] (middle) in the same animal. Bottom, an overlay of both compounds. Scale bars, 100 μ m.

cytometric analysis of isolated tumor cells revealed Hsp70 and β 3-integrin positivity in both models (Table 1). To investigate the *in vivo* tumor targeting properties in these models, NIR imaging of tumors was performed 24 hours after intravenous injection of TPP[Cy5.5], CP[DL750], and IS[750] (Fig. 4).

Although the fluorescence signals of both TPP[Cy5.5] and IS[750] clearly delineated the exposed colon and pancreatic tumors, a clear background staining in the tumor-surrounding normal colon tissue was observed following administration of IS[750]. In the colitis-induced tumors, the TBR of TPP[Cy5.5]

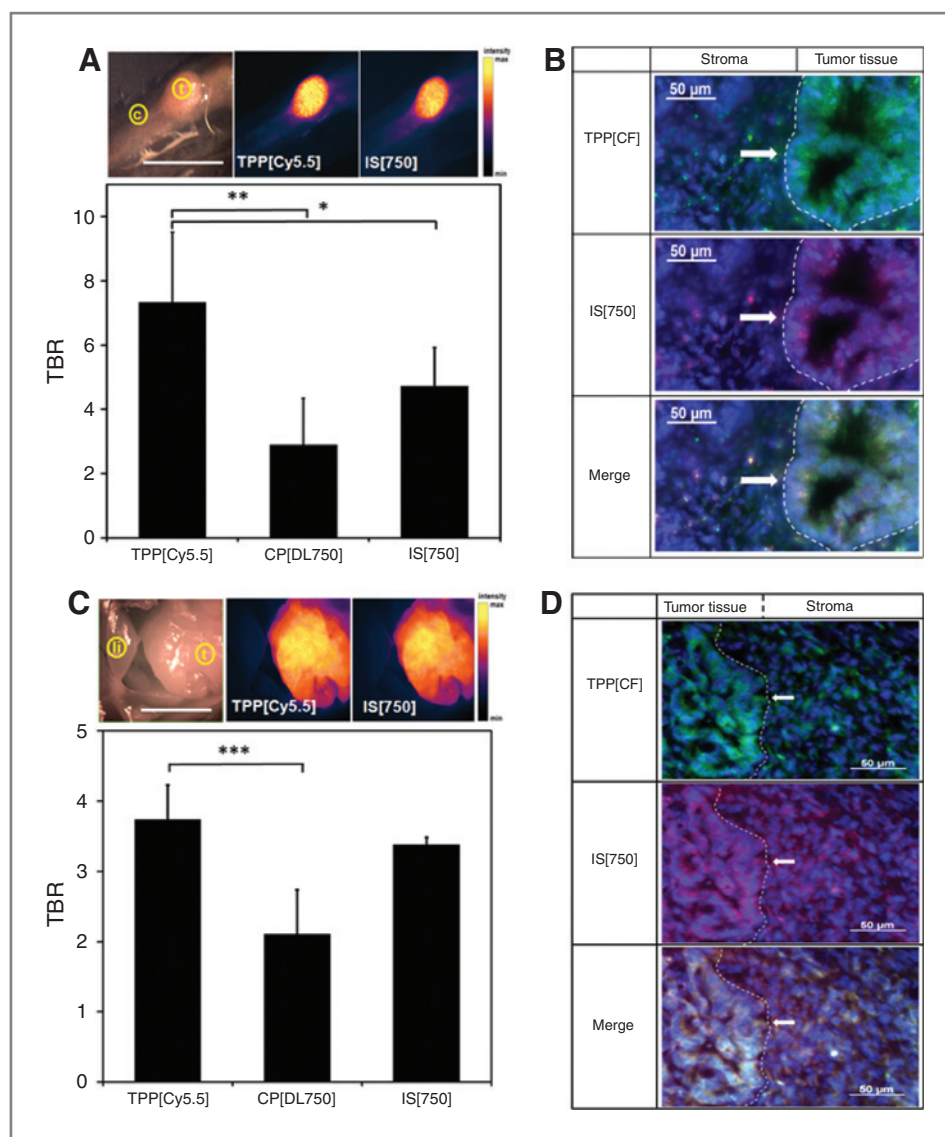


Figure 4. Intraoperative *in vivo* imaging and fluorescence microscopy in spontaneous tumor mouse models 24 hours after injection of TPP[Cy5.5], CP[DL750], and IS[750]. **A**, NIRF imaging of tumor (t) and surrounding colon tissue (c; scale bar, 5 mm), with corresponding pseudocolor fluorescence images 24 hours after intravenous injection of TPP[Cy5.5] (middle) and IS[750] (right). Bottom, TBR, 24 hours after intravenous injection of TPP[Cy5.5], CP[DL750], and IS[750]. The data are shown as mean $\pm$ SD, $n = 5$ (TPP[Cy5.5]), 4 (CP[DL750]), and 3 (IS[750]). *, $P < 0.05$; **, $P < 0.01$. **B**, fluorescence microscopy of identical cryosections of a representative primary tumor (left) and normal tissue (right) 24 hours after intravenous injection of TPP[CF] (top) and IS[750] (middle) into the same animal. Bottom, an overlay of both compounds with enhanced enrichment of TPP[CF] in crypt-like neoplastic tissue (arrows), compared with IS[750]. Normal tissue remained negative for both compounds. Scale bars, 50 μ m. **C**, NIRF imaging of PDAC. Top, true color image of tumor (t) and surrounding normal tissue, including liver (li; left, scale bar, 5 mm), and corresponding pseudocolor fluorescence images, 24 hours after intravenous injection of TPP[Cy5.5] (middle) and IS[750] (right). Bottom, TBR 24 hours after intravenous injection of the compounds. The data are shown as mean $\pm$ SD of $n = 7$ (TPP[Cy5.5]), 6 (CP[DL750]), and 2 (IS[750]) animals. ***, $P < 0.001$. **D**, fluorescence microscopy of identical cryosections of primary tumor tissue (left) including fibrosis of the tumor microenvironment (right) 24 hours after intravenous injection of TPP[CF] (top) and IS[750] (middle). Bottom, an overlay with a comparable enrichment of both compounds in carcinoma tissue and additional IS[750] staining of cells of the tumor microenvironment. Scale bar, 50 μ m.

was significantly higher than that of CP[DL750] ($P = 0.005$) and IS[750] ($P = 0.016$; Fig. 4A). The cellular distribution of the compounds at a microscopic level was analyzed using cryosections of the resected tumors that were obtained 24 hours after intravenous injection of TPP[CF] and IS[750]. Although a strong cytosolic signal of TPP[CF] in predominantly crypt-like

tumor structures (white arrows) was observed, IS[750] treatment resulted in a weaker and more heterogeneous staining pattern (Fig. 4B). Our recent demonstration that membrane Hsp70 is rapidly endocytosed in tumor cells under physiologic conditions (18) provides an explanation for the cytosolic staining pattern of tumors.

The uptake capacities of TPP and IntegriSense were also investigated using an endogenous pancreatic PDAC model, which has an activating mutation in the *Kras* oncogene concomitant with a deletion of the tumor suppressor *Tp53* (21). Tumor cells of this model exhibited strong membrane positivity of both Hsp70 and β_3 -integrin (Table 1). Imaging of the pancreatic tumors was performed after opening of the peritoneal cavity; the stomach, liver, and gut were used to calculate the background fluorescence. The TBR for TPP[Cy5.5] was significantly higher than that of the control peptide ($P = 0.0001$) and comparable with that of the integrin $\alpha_v\beta_3$ -targeting compound IS[750] (Fig. 4C). Figure 4D shows fluorescence images of a cryo-sliced pancreatic tumors 24 hours after simultaneous injection of TPP[CF] and IS[750]. Although both compounds stained tumor cells equally (left, white arrows), cells of the tumor microenvironment showed a weaker staining with TPP[CF] (right).

TPP has a higher capacity to specifically target human xenograft tumors than IntegriSense

To confirm the tumor homing capacities of TPP[Cy5.5] in human tumors, imaging experiments were performed in 13 different human tumor cell lines derived from six entities (colon, mammary, pancreas, head and neck, cervix, and lung; Fig. 5). The TBRs for TPP[Cy5.5] in the xenograft tumors ranged from 5.6 ± 1.6 (mammary carcinoma cell line, MDA-MB231) to 7.8 ± 1.4 (squamous cell carcinoma cell line of the head and neck, Cal-33), all of which were significantly higher than those of CP[DL750]. This indicates a specific enrichment of TPP[Cy5.5] in all of the investigated xenograft tumors. Because it is known that the majority of human pancreatic carcinomas are positive for β_3 -integrin (22), we included three pancreas tumor xenograft models

(Panc-1, MiaPaCa-2, COLO357) in our experiment. These models revealed substantial Hsp70 and β_3 -integrin positivity (Table 1; Fig. 1). Although IS[750] produced higher TBRs in the pancreatic tumors compared with the other xenograft models that were examined, TPP[Cy5.5] yielded significantly higher ratios in two of these three models [$P = 0.04$ (Panc-1) and $P = 0.03$ (COLO357)].

Other human carcinoma cell lines that were determined as being β_3 -integrin positive were T-47D, MDA-MB-231 (mammary carcinomas), and Cal-33 (squamous cell carcinoma of the head and neck; see Table 1). The TBRs of IS[750] fluorescence of T-47D and Cal-33 also were significantly lower than the TBRs for TPP[Cy5.5] ($P = 0.04$ and 0.02 , respectively). The TBR for IS[750] in all β_3 -integrin negative tumors remained clearly below the TBRs for TPP[750]. These data indicate that TPP might be a powerful tumor targeting agent for the detection, diagnosis, and targeting of several entities in humans.

Discussion

Interest in the use of peptides as innovative cancer diagnostic and tumor-specific drug delivery tools has increased (1–3). However, one of the most critical issues in cancer detection using tumor-targeting probes is specificity, as most of the recently used markers also bind to tumor-associated stroma and other cells. Despite many reports on tumor markers in oncology, the number of clinically useful, tumor-specific markers with prognostic relevance remains low (23–26). It was shown that membrane Hsp70 is present on nearly all aggressive tumor cells from a wide variety of murine and human tumor entities (4–8, 11–15). Therefore, membrane Hsp70 is of prognostic value and fulfills the criteria for an effective tumor targeting molecule.

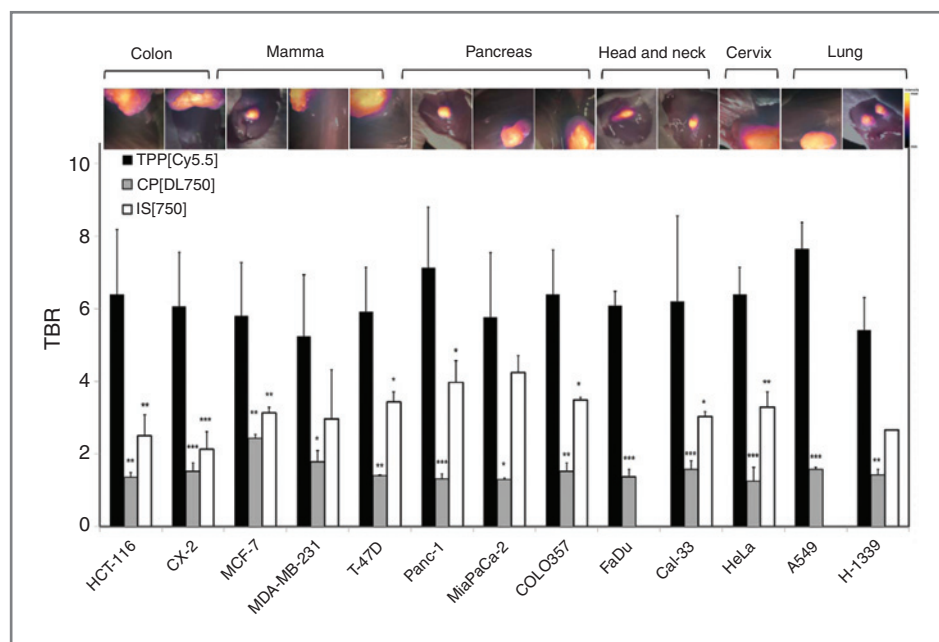


Figure 5. Intraoperative *in vivo* imaging of xenograft tumors 24 hours after intravenous injection of TPP[Cy5.5], CP[DL750], and IS[750]. Top, overlay of intraoperative true color and fluorescence pseudocolor images of subcutaneous tumors and the surrounding normal tissue 24 hours after intravenous injection of TPP[Cy5.5]. Bottom, TBR, 24 hours after intravenous injection of TPP[Cy5.5] (black bars), CP[DL750] (gray bars), and IS[750] (white bars). The data are shown as mean $\pm$ SD of two to five mice (H-1339, IS[750]); significance was calculated in comparison with TPP[Cy5.5]; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Given the widespread expression of membrane Hsp70 in different tumor entities and its prognostic relevance, this study evaluated TPP as a tumor homing peptide probe in a wide range of murine and human tumor models. TPP was shown to be highly specific (18) and is therefore an extremely useful tool for *in vivo* tumor detection and diagnosis. Targeting membrane Hsp70 with TPP is advantageous due to its rapid intracellular uptake, biodistribution, and tumor cell-selective accumulation. Similar to Hsp70, an ectopic and tumor-selective expression of Hsp90 has been utilized for the internalization of optical and radioiodinated tethered Hsp90 inhibitors (27).

The use of a colitis-associated colon carcinoma (20) and an endogenous pancreatic tumor model (21) allowed ample mimicking of the human situation in terms of tumor morphology and microenvironment, whereas the xenograft models of colon, mammary, pancreas, lung, head and neck, and cervix carcinoma were used to screen the performance of TPP in numerous human-derived malignant tumors *in vivo*. TPP was able to specifically detect and penetrate each of the tested tumors at high yields, *in vivo* (Figs. 3–5; Supplementary Fig. S7A and S7B). Furthermore, TPP specifically stains single cell suspensions of solid tumors, *in vitro* (Fig. 1A; Table 1). We also compared the *in vitro* and *in vivo* binding specificity of TPP with the neoplastic cells of various tumor models and nontransformed cells of the tumor stroma with that of a scrambled control peptide and the commercially available marker IntegriSense, which has been shown to stain different tumor entities, including pancreatic cancers, *in vivo* (28–31). The TBRs for TPP were higher than that of a control peptide and IntegriSense for all of the investigated tumors.

Flow-cytometric analysis revealed that the ligand of IntegriSense, β_3 -integrin, was absent in five of the 11 human tumor xenografts, whereas all tested human and murine tumors were found to be highly membrane-Hsp70 positive (Fig. 1A; Table 1).

To reflect the more complex *in vivo* situation, we broadened our investigations to non-neoplastic cells of the tumor stroma. We used flow cytometry to analyze the presence of membrane Hsp70 and β_3 -integrin on macrophages and fibroblasts in single cell suspensions derived from solid tumors. Both cell types show a low background fluorescence of membrane-Hsp70 but high β_3 -integrin positivity (Fig. 1B). Although macrophages internalize IntegriSense and TPP *in vivo*, only a minimal level of TPP staining was detected in tumor-associated fibroblasts. Other studies demonstrated the presence of integrins, including β_3 -integrin, on tumor endothelial cells (32, 33), which were negative for membrane Hsp70 (34).

Another recently discovered tumor targeting strategy involves the chemokine receptor CXCR4. Similar to Hsp70, CXCR4 offers the possibility to target different tumor entities.

However, some carcinomas, like the NSCLC, do not or only weakly express CXCR4 on their plasma membranes (35, 36). In contrast, Hsp70 is present on all tested NSCLC tumors (xenograft, primary human tumors; ref. 5). Furthermore, potential adverse effects of targeting CXCR4, such as the mobilization of hematopoietic progenitor cells by CXCR4 antagonists (37), remain to be elucidated.

The current study found no evidence that TPP has any toxic side effects, even if it was administered at 10-fold higher doses than those that were used for *in vivo* imaging. *Ex vivo* biodistribution analysis revealed the beneficial tumor-homing capacities of the TPP peptide. Although TPP was effectively washed out of the normal tissue 24 hours after its administration, pronounced levels remained within the tumor tissue at this time point (Fig. 2). The small-molecular weight of TPP ensures that it is cleared via the kidneys and does not accumulate in the liver like high-molecular weight targeting probes such as antibodies. As a consequence, TPP did not result in nonspecific background signals in the liver. Therefore, TPP could be used as an imaging probe for hepatocellular carcinoma and liver metastases (15).

In conclusion, TPP might be a useful peptide-based marker for diagnostic tumor targeting, as well as an efficient tool for drug delivery for a broad variety of human tumors.

Disclosure of Potential Conflicts of Interest

S. Stangl and G. Multhoff have ownership interest in a patent. V. Ntziachristos has ownership interest (including patents) in iThera Medical GmbH and is a consultant/advisory board member for iThera Medical GmbH. No potential conflicts of interest were disclosed by the other authors.

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Development of methodology: S. Stangl

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Stangl, J. Varga, B. Freysoldt, J.T. Siveke, V. Ntziachristos, G. Multhoff

Writing, review, and/or revision of the manuscript: S. Stangl, J. Varga, J.T. Siveke, V. Ntziachristos, G. Multhoff

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Stangl, V. Ntziachristos

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Selective *In Vivo* Imaging of Syngeneic, Spontaneous, and Xenograft Tumors Using a Novel Tumor Cell–Specific Hsp70 Peptide-Based Probe

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