In-vivo imaging of tumor associated urokinase-type plasminogen activator activity

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Abstract. The ability to image tumor associated protease *in vivo* has biological and clinical implications. In the present study, we describe the development and validation of a urokinase-type plasminogen activator (uPA) sensitive fluorescence imaging probe. The activation of our probe is highly specific to uPA in both enzymatic and cellular-based assays. In two distinct *in-vivo* tumor models (human colon adenocarcinoma HT-29 and human fibrosarcoma HT-1080), the observed fluorescence changes correlate well with tumor associated uPA activity. The signal intensities of the tumors are about three-fold higher in animals with probe injections. Our results suggest a direct detection method for uPA activity *in vivo* and the approach can be used for monitoring tumor growth and development. [DOI: 10.1117/1.2204029]

Keywords: urokinase-type plasminogen activator; protease; optical imaging; fluorescence; tumor.

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1 Introduction

A urokinase-type plasminogen activator (uPA) is a serine protease with the molecular weight of approximately 50 kD. It plays important roles in tumor invasion, metastasis, and angiogenesis.[1] The uPA and urokinase-type plasminogen activator receptor (uPAR) system has been reported to regulate downstream proteases such as MMP-2, MMP-9, and other proteases to facilitate basement membrane remodeling and tumor invasion.[2,3] In addition, uPA, either directly or through its action on plasmin, is known to be responsible for the release or activation of various proangiogenic growth factors such as basic fibroblast growth factor (BFGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and transforming factor-ß (TGF-ß).[2]

The urokinase-type plasminogen activator consists of three domains: C-terminal serine protease domain, a kringle domain, and a growth factor N-terminal domain.[4] The original form of uPA secreted from cells is a single-chain zymogen, pro-uPA, which is converted into a two-chain active form by catalyzation of plasmin. The proteolytic activity of uPA is several hundred-fold higher than that of the pro-form.[5] The free uPA is well known for its thrombolytic ability, which has been applied to treat acute ischemic stroke.[6] Although a certain amount of uPA (20 pM) is present in the plasma, it is mostly enzymatically inactive by complexing with its inhibitor, PAI-1.[7]

Clinically, elevated uPA in malignant breast, gastrointestinal and urogenital tumors indicates poor disease prognosis. In primary breast cancer, high tumor levels of uPA are associated with poor overall survival and relapse-free survival.[8,9] For node-negative breast cancer patients, the uPA expression level has been suggested to be a biomarker to guide the necessity of further chemotherapy.[8] Many urokinase inhibitors have shown antimetastatic and antimigratory ability,[10–12] and some have recently entered clinical trials.[13] Thus, a way to directly monitor uPA activity *in vivo* could provide valuable information for tumor behavior, guidance for treatment plan, and drug evaluation.

Previously, we have demonstrated the feasibility of *in-vivo* imaging of protease activity, including cathepsins, matrix metalloproteinases (MMPs), viral proteases, and others, using a series of fluorescent probes.[14] In this study, we study and report the *in-vivo* imaging of uPA activity using a recently synthesized fluorescent probe.[15]

2 Materials and Methods

2.1 *Synthesis of the Probes*

The uPA-sensitive probe was synthesized as previously described.[15] The probe, with the excitation and emission maxima of 675 and 694 nm, respectively, has a low fluorescence signal in its initial state, but becomes strongly fluorescent after uPA proteolysis. The uPA probe has the peptide substrate sequence of GGSGRSANAKC-NH_2, which can be recognized by uPA and cleaved between Arg and Ser residues.

2.2 *Cell Culture*

HT-1080 fibrosarcoma and HT-29 colon adenocarcinoma (ATCC, Rockville, Maryland) were selected from literatures for their distinct uPA expression.[16] Secreted uPA was reported in the culture medium of HT-1080, but not in HT-29. HT-1080

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cells were cultured in Dulbecco's modified eagle medium (DMEM) (Cellgro Herndon, Virginia), supplemented with 10% heat-inactivated fetal bovine serum, penicillin (50 U/ml), and streptomycin (0.05 mg/ml), at 37°C in 5% CO_2. HT-29 cells were cultured similarly, except McCoy's 5A (Cellgro Herndon) was used as the medium.

2.3 *Fluorescence Intensity Measurements*

For specificity of the uPA-sensitive probe, fluorescence intensity measurements with time were recorded on a fluorescence plate reader (GENios Pro, Tecan, Durham, North Carolina). Activation of the probes (1 μM) with MMP-2, MMP-7, MMP-9, cathepsin B, cathepsin D, and uPA (5 μl, 40 ng) was assayed in a 96 well clear bottom plate (Costar, Corning, New York) in phosphate buffered saline (PBS) buffer (200 μl) at 25°C. The sample was excited at 670 nm and the emission was monitored at 720 nm. The fluorescent signals were recorded every 5 min for 135 min.

2.4 *Optical Zynography and Plasminogen-Casein Zymography*

To determine the specificity of an uPA-sensitive probe, optical zymography was performed as described.[17] Briefly, 10% SDS-PAGE gel was freshly prepared with a uPA-sensitive probe (200 pmol). Different biological samples including uPA (Sigma, Saint Louis, Missouri), cultured cell medium, and tumor tissue extracts were loaded onto the gel. Electrophoresis was then carried out at 100 V in Laemmli buffer (BioRad, Hercules, Calfornia). The running buffer was Tris buffer (25 mM, pH 8.3), containing 0.25 M of glycine and 0.1% (w/v) SDS. After electrophoresis, the enzymes were renatured by incubating the gel in 2.5% (v/v) Triton X-100 buffer for 2×30 min at room temperature. It was then washed twice (10 min) with Tris buffer (50 mM, pH 7.4), and immediately imaged by using the near-infrared fluorescence (NIRF) reflectance imaging system.[18]

Plasminogen-casein zymography was performed as described.[17] In short, 10% (v/v) SDS-PAGE gels were casted with Glu-Plg (22 mg/ml) (Calbiochem, La Jolla, California) and ß-casein (30 mg) (Sigma, Saint Louis, Missouri). After electrophoresis, the separated proteins were renatured by two 30-min incubation periods in 2.5% (v/v) Triton X-100, and the gels were incubated overnight at 37°C in glycine (100 mM, pH 8.0). Then it was stained for 2 h with 2% (w/v) comassie blue, destained with 30% (v/v) methanol, 10% (v/v) acetic acid, dried, and analyzed.

To evaluate the specificity of a uPA-sensitive probe, equal amounts of tumor-associated proteases (40 ng), including MMP-2, MMP-7, MMP-9, cathepsin B, cathepsin D (Calbiochem, La Jolla, California), and uPA were analyzed using optical zymography. To acquire secreted uPA proteins, cells were grown to approximately 80% confluent, the culture medium was removed, and the cells were gently rinsed with Hanks' balanced salt solution (HBSS) twice to remove fetal bovine serum. The cells were incubated with serum-free DMEM medium for an additional 24 h, then the medium was collected and centrifuged at 1500 rpm for 5 min to remove cell debris. An equal amount of Laemmli sample buffer was mixed with the cell medium for optical zymography (5 μl) and for plasminogen-casein zymography (2 μl). For preparation of tumor tissue extracts, tumor tissue was homogenized in Tris buffer (50 mM, pH 7.5) containing 0.25% (v/v) of Triton-X 100. Minced tissue was ultrasonicated and centrifuged at 10,000 rpm for 10 min. The supernatant was collected for electrophoresis.

2.5 *Western Blot*

Following electrophoresis, the separated proteins from either the conditioned culture mediums or the tumor extracts were transferred to a polyvinylidene fluoride membrane (Hybond™-P, Amersham, Piscataway, New Jersey). The membrane was then blocked with 5% (w/v) nonfat dry milk (BioRad, Hercules, California) in 0.1% (v/v) Tween 20-TBS buffer for overnight at 4°C. Primary mouse antibody against human urokinase (1:2000 dilution, American Diagnostica, Stamford, Connecticut) was added into the buffer for further incubation for 8 h. The membrane was washed with 0.1% (v/v) Tween 20-Tris-buffered saline (TBS) buffer (3×15 min) and allowed to incubate in 0.1% (v/v) Tween 20-TBS buffer containing secondary goat antimouse IgG (H +L) HRP conjugated (1:1000, ImmunoPure®, Pierce, Rockford, Illinois) for 1.5 h at room temperature. After washing, the uPA was detected with ECL™ Western Blotting Detection Reagents (Amersham) and the image was developed on a Kodak BioMax Light Film.

2.6 *Near-Infrared Fluorescence Reflectance Imaging System*

Imaging of the optical-zymographic gel as well as the animals was performed using a NIRF reflectance imaging system, which has previously been described.[18] A broadband white light from a 150-W halogen bulb was used as an excitation photon source. Bandpass filters (Omega Optical, Brattleboro, Vermont) were employed to adjust the corresponding excitation wavelength for Cy 5.5 (615 to 645 nm). SDS-PAGE gels or the mice were exposed to the excitation light source. The corresponding bandpass emission filter (Omega Optical, Brattleboro, Vermont) was used to filter the fluorescence emission (680 to 720 nm). A 12-bit monochrome CCD camera (Kodak, Rochester, New York) equipped with a 12.5-75-mm zoom lens was used as a detector. Focusing was done manually. Exposure time and aperture were specifically 5 min/f1.2 for optical zymography and 120 s/f1.2 for animal imaging.

2.7 *In-Vivo Imaging*

All animal studies were approved by the institutional animal care committee. Tumor cell suspensions (2×10^6 cells/50 μl) were implanted at both sides of mammary fat pads under intraperitoneal anesthesia (Ketamine, 90 mg/kg, BenVenue, Bedford, Ohio and Xylazine, 10 mg/kg, Phoenix, Saint Joseph, Missouri). Tumor size was monitored daily until its diameter reached 5 to 10 mm, 2 to 3 weeks after implantation. An uPA-sensitive probe (10 nmol, 100 μl) was injected intravenously *via* tail vein under intraperitoneal anesthesia. All mice were imaged 6 and 24 h post probe injection. Arbitrary units, which indicate real tumor signals, were determined by subtracting tumor signal intensities with the background.

2.8 *Histologic Studies*

Tumors were excised, frozen, and sliced into 10-μm sections. Both HT-1080 fibrosarcoma and HT-29 adenocarcinoma were stained with hematoxylin-eosin (Sigma). Immunohistochemistry was performed using a primary goat polyclonal antibody against human uPA (American Diagnostica, Stamford, Connecticut) and biotinylated secondary rabbit antigoat antibody (Vector Laboratories, Burlingame, California). Endogenous peroxidase activity was eliminated using 3% (v/v) H_2O_2. Peroxidase conjugated avidin (Vector Laboratories) was incubated, allowing avidin-biotin complex formation. The activity of peroxidase was visualized using 3,3′—diaminobenzidine substrate, and the sections were counterstained with hematoxylin. Control sections were processed identically, except for the lack of the primary antibody.

To determine the location of the fluorescence signals, NIRF fluorescence microscopy was performed. Tumors were snap frozen and cryosectioned into 10-μm-thick slices. A fluorescence microscope (Nikon, Eclipse 80i, Japan), and a cooled change-coupled device (CCD) camera (Photometrics, Cascade 512B) adapted with a bandpass filter were used for image capture.

2.9 *Statistical Analysis*

Data are presented as means with standard deviation. Statistical analysis of *in-vivo* tumor fluorescence was conducted by using a two-tailed paired Student t test ($n=16$ tumors for uPA-sensitive probe in HT-1080, and $n=10$ tumors for uPA-sensitive probe in HT-29.). P values smaller than 0.05 were considered of statistically significance.

3 Results

3.1 *In-Vitro Urokinase-Type Plasminogen Activator-Sensitive Probe Specificity*

To verify the selectivity of the uPA-sensitive probe, several tumor associated proteases, including MMP-2, MMP-7, MMP-9, cathepsin B, and cathepsin D were tested using newly developed optical zymography.[17] The gel was prepared as in traditional zymography, except casein was replaced with the uPA-sensitive probe. After electrophoresis, the renatured uPA activated/hydrolyzed the quenched probe, resulting in fluorescence signal increase. Except uPA, none of the tested proteases was found to activate the uPA-sensitive probe under the testing condition, as seen by the fluorescent band in optical zymography [Fig. 1(a)]. The specificity of the uPA-sensitive probe was also confirmed by measurement of fluorescent changes with pure proteases in PBS buffer. As shown in Fig. 1(b), only uPA could activate the uPA-sensitive probe, resulting in a ten-fold increase of fluorescence signal. In contrast, the signal intensity of the uPA-sensitive probe incubated with other proteases remained optically silent.

3.2 *In-Vivo Tumor Imaging*

Previously, HT-1080 human fibrosarcoma and HT-29 human colon adenocarcinoma cell lines have been reported as having distinct uPA expression in cell culture.[19] Analyzing their culture medium, a high level of uPA was found in HT-1080, but an extremely low level of uPA was detected in HT-29. Surprisingly, both cell lines expressed a high level of uPAR.[19,20] It is known that uPAR and uPA are strongly associated with tumor progression.[21] For a HT-29 tumor to grow *in vivo*, it may need to recruit uPA expressed by neighboring stroma cells through its surface uPA receptors. We thus explored this possibility by using the developed uPA-sensitive probe.

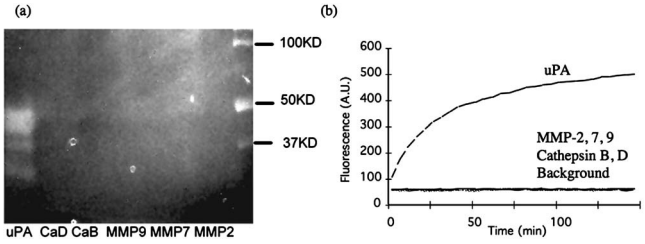

Fig. 1 *In-vitro* selectivity of the uPA probe to tumor associated proteases. (a) Optical zymography of tumor-associated proteases revealed only uPA cleaved the uPA-sensitive probe, yielding a fluorescent band at 48 kD. (b) Fluorescence measurement showed uPA digested uPA-sensitive probe, which generated increased fluorescence signal up to 10 fold. Other proteases did not digest the uPA-sensitive probe.

Prior to the imaging experiments, the difference in uPA expression of these two cell lines was confirmed by using Western blot, traditional plasminogen-casein zymography, and newly developed optical zymography. As previously reported,[19] it was found that only HT-1080 cell line secreted uPA, but not HT-29 cells (Fig. 2). The dark band in Western blot indicated the uPA expression, whereas white bands in both zymographies demonstrated the proteolytic activity of uPA. The bands generated by the HT-1080 cell medium in all assays were identified to be 48-kD uPA.

HT-1080 and HT-29 tumors were implanted in the upper chest of nude mice (HT-1080 $n=16$, and HT-29 $n=10$). Whole body optical imaging showed negligible NIR fluorescence signals (75 ± 1 AU) prior to probe injection. After probe administration, an intense fluorescence signal was observed in the HT-1080 experimental groups, 735 ± 52 AU at 6 h and increased to 1206 ± 107 AU at 24 h (Fig. 3). The average tumor to normal tissue ratio was 2.8 and 3.2, respectively. Interestingly, the fluorescence intensity of the supposedly low uPA expressing HT-29 tumor was about the same as those of the HT-1080 tumor, at 959 ± 106 AU and 1217 ± 87 AU at 6 and 24 h, respectively. The average contrasts of tumor to normal tissue were 3.1 and 3.1 at 6 and 24 h, respectively. No

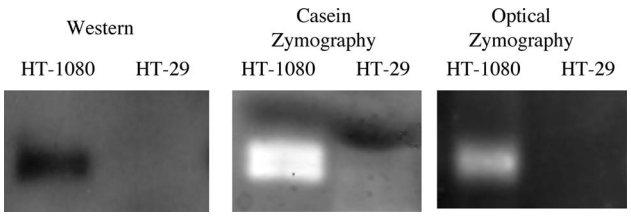

Fig. 2 uPA secretion in HT-1080 human fibrosacoma and HT-29 colon adenocarcinoma cell lines. Western blot, plasminogen-casein zymography, and optical zymography revealed uPA-secretion ability in HT-1080 human fibrosarcoma cells. HT-29 colon adenocarcinoma did not secrete uPA in culture.

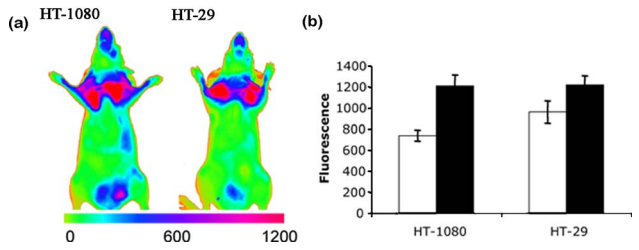

Fig. 3 *In-vivo* imaging of uPA activity. (a) A representative near-infrared fluorescence image of HT-1080 and HT-29 bearing mice 24 h after probe injection. The color map indicated fluorescence intensity (AU). (b) Bar graph shows the NIRF signals in tumors at 6 (white bar) and 24 (black bar) h after probe injection. There were no significant differences in signal intensities in HT-1080 and HT-29 groups at 6 and 24 h after probe administration.

statistical significance was found between HT-1080 and HT-29 tumors. The results suggested that the uPA sensitive probe was activated in both types of tumors.

3.3 *Urokinase-Type Plasminogen Activator Expression in Tumor Tissue Extract*

To correlate the fluorescence signal with uPA expression *in vivo*, proteins were extracted from tumors and analyzed by Western blot. As expected, uPA was found in both tumors [Fig. 4(a)], despite the differences observed in cell culture experiments. Moreover, these results were also confirmed by plasminogen-casein zymography [Fig. 4(b)].

3.4 *Histological Correlations*

Hematoxylin-eosin staining showed increased nuclear cytoplasm ratio as well as the presence of multiple mitotic figures in the both HT-1080 fibrosarcoma and HT-29 colon adenocarcinoma (Fig 5). The expression of uPA in HT-1080 and HT-29 tumors was readily detected by immunohistochemistry. The majority of the enzyme was located pericellularly in both tumors. No significant difference in uPA expression was found in both types of tumors. NIRF signal generation was confirmed by fluorescent microscopy of the tumors. Considerable

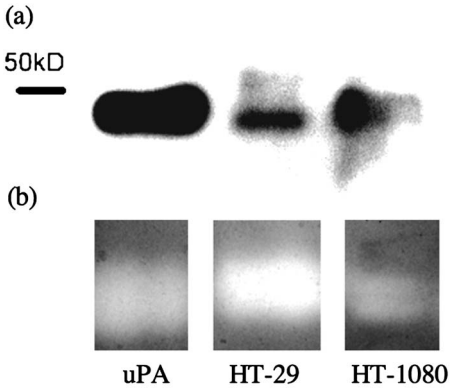

Fig. 4 uPA expression in tumors. The uPA expression revealed by (a) Western blot analysis and (b) plasminogen-casein zymography of tumor extract demonstrated both HT-1080 fibrosarcoma and HT-29 colon adenocarcinoma secreted UPA.

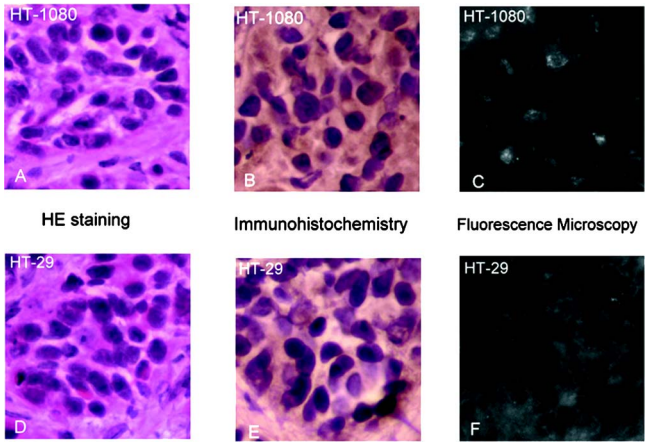

Fig. 5 Histology of tumor sections. Histology of HT-1080 fibrosarcoma (top row) and HT-29 (bottom row). The tumors revealed increased nuclear cytoplasm ratio in H/E staining (a) and (d). Immunohistochemistry targeting on the expression of uPA revealed that both tumors express uPA *in vivo* (b) and (e). Fluorescent microscope showed a strong fluorescence signal in both HT-1080 and HT-29 tumors (c) and (f). The signals are distributed more intracellularly in HT-1080, while HT-29 showed fluorescent signal both intracellularly and in the interstitium.

fluorescence was detected in the interstitium and tumor cells of HT-1080 fibrosarcoma and HT-29 adenocarcinoma.

4 Discussion

Recently, protease-sensitive probes targeting MMP-2,[22] Cathepsin B,[23] Cathepsin D,[24] and other proteases[25] have been developed in our laboratory, and their imaging applications were further demonstrated.[14] We extended our study to another clinically important protease. Unlike other tumor markers, uPA has been reported as an independent prognostic factor for certain cancers.[8] Studies have shown that patients with node-negative breast cancer, whose tumors overexpressed with uPA, would benefit from adjuvant chemotherapy. On the other hand, drug treatments would be optional if the tumors expressed a low level of uPA. Our probe has the potential to provide a powerful diagnostic tool for radiologists as well as therapeutic guidance for clinical physicians. Furthermore, uPA has been reported as an upstream enzyme in the protease activation cascade.[2] A better understanding of this physiological cascade *in vivo* could be achieved by using uPA-sensitive probes in adjuvant with the previously described optical reporters.[14]

The selectivity of the synthesized uPA probe was confirmed *in vitro* with a panel of common tumor-associated proteases. Among the tested proteases, only uPA was able to hydrolyze the probe, resulting in significant fluorescence changes (Fig. 1). To demonstrate that uPA activity can be depicted with imaging, two tumor cell lines were carefully selected and characterized. Using plasminogen-casein zymography and the developed optical zymography, uPA expression was not found in the culture medium of HT-29, but in HT1080 (Fig 2). Previous studies with HT-29 have shown uPA expression in cytoplasm, but the secretion of uPA was found impaired.[26] Nevertheless, our results suggested that HT-29 tumors exhibited uPA activity in live animals (Figs. 4

and 5). Immunohistochemistry showed uPA expression in both the tumoral and stromal cells. Importantly, the fluorescence signal obtained from *in-vivo* animal imaging supported our histological findings. High fluorescence signals were found in both tumors after uPA probe injection (Fig 3). Although secreted uPA was not observed in HT-29 cell culture, uPAR expression in HT-29 colon adenocarcinoma has been reported.[19] In our case, the expressed uPAR might have interacted with the host uPA, thus facilitating uPA expression and secretion from the host or even tumor cells. An early investigation in uPA-knockout mice implanted with murine T241 fibrosarcoma cells supported the importance of interaction of an uPA-uPAR system between tumor and host.[27] Further studies on uPA-uPAR interaction can be conducted by using our uPA-sensitive probe in combination with uPA or uPAR knockout animal models.

Our results indicate that a uPA-sensitive probe could be activated in a HT-29 tumor, a colon adenocarcinoma. Therefore, it should be possible to utilize this probe in conjunction with catheter-based imaging systems for colon diagnosis.[28] Another potential application of the reported uPA probe is to assist therapeutic agent development, since many inhibitors have been developed to interfere with the uPA-uPAR system.[10,11,13]

Acknowledgments

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References

1. S. A. Rabbani and A. P. Mazar, "The role of the plasminogen activation system in angiogenesis and metastasis," *Surg. Oncol. Clin. N. Am.* **10**(2), 393–415 (2001).
2. P. F. Choong and A. P. Nadesapillai, "Urokinase plasminogen activator system: a multifunctional role in tumor progression and metastasis," *Clin. Orthop. Relat. Res.* **415**, suppl. S46–58 (2003).
3. J. S. Rao, "Molecular mechanisms of glioma invasiveness: the role of proteases," *Nat. Rev. Cancer* **3**(7), 489–501 (2003).
4. A. J. Barrett, N. D. Rawlings, and J. F. Woessner, *Handbook of Proteolytic Enzymes*, Academic Press, San Diego (1998).
5. P. A. Andreasen, L. Kjoller, L. Christensen, and M. J. Duffy, "The urokinase-type plasminogen activator system in cancer metastasis: a review," *Int. J. Cancer* **72**(1), 1–22 (1997).
6. J. A. Pettersen, M. E. Hudon, and M. D. Hill, "Intra-arterial thrombolysis in acute ischemic stroke: a review of pharmacologic approaches," *Expert Rev. Cardiovasc. Ther.* **2**(2), 285–299 (2004).
7. P. A. Andreasen, L. Sottrup-Jensen, L. Kjoller, A. Nykjaer, S. K. Moestrup, C. M. Petersen, and J. Gliemann, "Receptor-mediated endocytosis of plasminogen activators and activator/inhibitor complexes," *FEBS Lett.* **338**(3), 239–245 (1994).
8. F. Janicke, A. Prechtl, C. Thomssen, N. Harbeck, C. Meisner, M. Untch, C. G. J. F. Sweep, H. K. Selbmann, H. Graeff, and M. Schmitt, "Randomized adjuvant chemotherapy trial in high-risk, lymph node-negative breast cancer patients identified by urokinase-type plasminogen activator and plasminogen activator inhibitor type 1," *J. Natl. Cancer Inst.* **93**(12), 913–920 (2001).
9. M. P. Look, W. L. J. van Putten, M. J. Duffy, N. Harbeck, I. J. Christensen, C. Thomssen, R. Kates, F. Spyratos, M. Ferno, S. Eppenberger-Castori, C. G. J. F. Sweep, K. Ulm, J. P. Peyrat, P. M. Martin, H. Magdelenat, N. Brunner, C. Duggan, B. W. Lisboa, P. O. Bendahl, V. Quillien, A. Daver, G. Ricolleau, M. E. Meijer-van Gelder, P. Manders, W. E. Fiets, M. A. Blankenstein, P. Broet, S. Romain, G. Daxenbichler, G. Windbichler, T. Cufer, S. Borstnar, W. Kueng, L. V. A. M. Beex, J. G. M. Klijn, N. O'Higgins, U. Eppenberger, F. Janicke, M. Schmitt, and J. A. Foekens, "Pooled analysis of prognostic impact of urokinase-type plasminogen activator and its inhibitor PAI-1 in 8377 breast cancer patients," *J. Natl. Cancer Inst.* **94**(2), 116–128 (2002).
10. S. Ertongur, S. Lang, B. Mack, K. Wosikowski, B. Muehlenweg, and O. Gires, "Inhibition of the invasion capacity of carcinoma cells by WX-UK1, a novel synthetic inhibitor of the urokinase-type plasminogen activator system," *Int. J. Cancer* **110**(6), 815–824 (2004).
11. A. Schweinitz, T. Steinmetzer, I. J. Banke, M. J. Arlt, A. Sturzebecher, O. Schuster, A. Geissler, H. Giersiefen, E. Zeslawska, U. Jacob, A. Kruger, and J. Sturzebecher, "Design of novel and selective inhibitors of urokinase-type plasminogen activator with improved pharmacokinetic properties for use as antimetastatic agents," *J. Biol. Chem.* **279**(32), 33613–33622 (2004).
12. H. Kobayashi, M. Suzuki, Y. Hirashima, and T. Terao, "The protease inhibitor bikunin, a novel anti-metastatic agent," *Biol. Chem.* **384**(5), 749–754 (2003).
13. V. Magdolen, A. Kruger, S. Sato, J. Nagel, S. Sperl, U. Reuning, P. Rettenberger, U. Magdolen, and M. Schmitt, "Inhibition of the tumor-associated urokinase-type plasminogen activation system: effects of high-level synthesis of soluble urokinase receptor in ovarian and breast cancer cells in vitro and in vivo," *Recent Results Cancer Res.* **162**, 43–63 (2003).
14. M. Funovics, R. Weissleder, and C. H. Tung, "Protease sensors for bioimaging," *Anal. Bioanal. Chem.* **377**(6), 956–963 (2003).
15. B. Law, A. Curino, T. H. Bugge, R. Weissleder, and C. H. Tung, "Design, synthesis, and characterization of urokinase plasminogen-activator-sensitive near-infrared reporter," *Chem. Biol.* **11**(1), 99–106 (2004).
16. A. Ginestra, M. D. La Placa, F. Saladino, D. Cassara, H. Nagase, and M. L. Vittorelli, "The amount and proteolytic content of vesicles shed by human cancer cell lines correlates with their in vitro invasiveness," *Anticancer Res.* **18**(5A), 3433–3437 (1998).
17. B. Law, J. K. Hsiao, T. H. Bugge, R. Weissleder, and C. H. Tung, "Novel optical zymography for specific detection of urokinase plasminogen activator in biological samples," *Anal. Biochem.* **338**(1), 151–158 (2005).
18. U. Mahmood, C. H. Tung, A. Bogdanov, Jr., and R. Weissleder, "Near-infrared optical imaging of protease activity for tumor detection," *Radiology* **213**(3), 866–870 (1999).
19. L. S. Reiter, E. K. Kruithof, J. F. Cajot, and B. Sordat, "The role of the urokinase receptor in extracellular matrix degradation by HT29 human colon carcinoma cells," *Int. J. Cancer* **53**(3), 444–450 (1993).
20. M. A. Hudson and L. M. McReynolds, "Urokinase and the urokinase receptor: association with in vitro invasiveness of human bladder cancer cell lines," *J. Natl. Cancer Inst.* **89**(10), 709–717 (1997).
21. A. P. Mazar, "The urokinase plasminogen activator receptor (uPAR) as a target for the diagnosis and therapy of cancer," *Anti-Cancer Drugs* **12**(5), 387–400 (2001).
22. C. Bremer, S. Bredow, U. Mahmood, R. Weissleder, and C. H. Tung, "Optical imaging of matrix metalloproteinase-2 activity in tumors: feasibility study in a mouse model," *Radiology* **221**(2), 523–529 (2001).
23. R. Weissleder, C. H. Tung, U. Mahmood, and A. Bogdanov, Jr., "In vivo imaging of tumors with protease-activated near-infrared fluorescent probes," *Nat. Biotechnol.* **17**(4), 375–378 (1999).
24. C. H. Tung, U. Mahmood, S. Bredow, and R. Weissleder, "In vivo imaging of proteolytic enzyme activity using a novel molecular reporter," *Cancer Res.* **60**(17), 4953–4958 (2000).
25. C. H. Tung, "Fluorescent peptide probes for in vivo diagnostic imaging," *Biopolymers* **76**(5), 391–403 (2004).
26. G. Huet, F. Zerimech, M. C. Dieu, B. Hemon, G. Grard, M. Balduyck, A. Janin, R. Lafyatis, and P. Degand, "The state of differentiation of HT-29 colon carcinoma cells alters the secretion of cathepsin D and of plasminogen activator," *Int. J. Cancer* **57**(6), 875–882 (1994).
27. L. S. Gutierrez, A. Schulman, T. Brito-Robinson, F. Noria, V. A. Ploplis, and F. J. Castellino, "Tumor development is retarded in mice lacking the gene for urokinase-type plasminogen activator or its inhibitor, plasminogen activator inhibitor-1," *Cancer Res.* **60**(20), 5839–5847 (2000).
28. M. A. Funovics, R. Weissleder, and U. Mahmood, "Catheter-based in vivo imaging of enzyme activity and gene expression: Feasibility study in mice," *Radiology* **231**(3), 659–666 (2004).