This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the **Information for Authors**.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard **Terms & Conditions** and the **Ethical guidelines** still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

An Intracellularly Activatable, Fluorogenic Probe for Cancer Imaging

Ruisong Tian,[a,b‡] Mingjie Li,[a] Jin Wang,[a] Min Yu,[a] Xiuqi Kong,[a] Yupeng Feng,[a] Zeming Chen,[a]

Yuxi Li,[b] Weiqiang Huang,[c,*] Wenjie Wu[b,*] and Zhangyong Hong[a,*]

[a]State Key Laboratory of Medicinal Chemical Biology, College of Life Sciences,

Nankai University, Tianjin 300071, P. R. China. E-mail: hongzy@nankai.edu.cn; Tel:

+86 022 23498707.

[b]College of Material Science and Chemical Engineering, Tianjin University of

Science and Technology, Tianjin 300457, P. R. China. E-mail: wwjie@tust.edu.cn;

Tel: +86 022 23502875.

[c]PracticaChem-China, Tianjin 300192, P. R. China. E-mail:

practicachem@gmail.com; Tel: +86 022 23003610.

[‡]Electronic Supplementary Information (ESI) available: [syntheses and ESI-HRMS

of peptides]. See DOI:

[†]Ruisong Tian and Mingjie Li contributed equally to this work.

Abstract : A newly designed, dual-functional probe based on intracellular activation

has been successfully developed for the detection of cancer cells. The probe is nearly non-fluorescent in buffer due to its highly efficient FRET quenching, but it can be specifically activated with dramatic fluorescence enhancement upon intracellular cathepsin B cleavage in target cancer cells after selective internalization *via* folate receptor-dependent endocytosis. Therefore, this probe enables "turn-on" visualization of cancer cells with desirable specificity and contrast enhancement. This targeted, intracellularly activatable probe displays low fluorescence-quenched background when compared with "always-on" probes and avoids non-specific activation by non-specifically expressed enzymes in normal tissue, which normally occurs when using common "turn on" probe design strategies. Therefore, this probe can be potentially applied in intraoperative inspection during clinical cancer surgery with higher contrast and sensitivity.

1. Introduction

Biological imaging technology has provided indispensable tools for cancer research and clinical treatment by offering anatomic and physiological information that is useful for diagnosis or during surgical procedures.[1] Noninvasive imaging can clearly show the location and anatomic structure of tumors, allowing for the observation of distinct clinical presentations and tumor diagnosis at much earlier stages.[2] Surgical operation aided by tumor-specific, probe-based imaging enables the discrimination of the malignant tumor from the normal tissues, which is difficult or impossible to

determine by visual observation, and provides real-time feedback on residual malignant tissue and more radical excision of tumor tissues.[3]

The development of sensitive and specific molecular probes using novel techniques is one of the central challenges for noninvasive imaging.[4] Currently, many *in vivo* imaging technologies, such as radiologic approaches (X-ray, CT, PET, MRI) and ultrasound have been intensively tested or used in these fields.[5] However, these methods usually suffer from disadvantages, such as non-tumor specificity, low sensitivity and high expense.[6] Furthermore, they are generally not applicable for intra-operative applications such as surgical inspection and practice. By contrast, fluorescence imaging, as an optical technique, offers superior resolution and sensitivity and accurate, real-time imaging for surgical inspection compared with preoperative radiological imaging and visual inspection and palpation during surgery.[7] In the last decade, many studies have contributed to the development of tumor-specific fluorescent imaging probes for clinical applications,[8] and their potential in imaging-guided surgery processes has been demonstrated in several preclinical[9] and clinical studies.[10]

Fluorescent probes for cancer imaging primarily rely on a strategy based on "always-on" probes, in which the fluorophore-bearing ligands are bound to and accumulate around the target cancer cells, resulting in an elevated signal in reference to the surrounding environment.[11] These types of "always on" probes have constant fluorescence signals, which result in high background and limited signal contrast. In

contrast, "turn on" fluorogenic probes, which are activated only at specific tumor sites using cancer-related enzymes, could significantly reduce *in vivo* background signals, thus improving contrast and sensitivity and offering a more ideal imaging strategy for *in vivo* tumor diagnosis.[12] In this strategy, fluorogenic probes consisting of enzyme-cleavable substrates that are labeled with a fluorescent reporter and a quencher dye are highly sensitive and sequence-specific to the environment of the tumor sites. This strategy has been well demonstrated, and a variety of useful probes have been successfully developed.[13]

Usually, "turn on" probes use a highly expressed protease, such as metal-protease 2 and 9, at tumor sites to cleave and activate the probe.[14] However, these enzymes are not tumor-specific, and large amounts of normal tissue may also express these enzymes, but at a different level.[15] Therefore, these types of probes could be nonspecifically cleaved and activated in normal tissues, which will consume the probes and increase the background signals in the normal tissues. In contrast, cathepsin B is a specific, intracellular enzyme that is only expressed inside the lysosome.[16] It is absent outside all normal cells and derived from the cell matrix, and it is only occasionally expressed on the cell surface of some tumor cells.[17] Cathepsin B substrates show high stability in the plasma and are only cleavable inside the lysosome.[18] Therefore, a "turn on" fluorogenic probe based on activation by the specific, intracellular enzyme cathepsin B can maintain a stably quenched state in circulation before entering into cells, avoid hydrolysis consummation outside cells and reduce the background signal. On the other hand, if such a probe is conjugated to

cell-specific ligands for targeting, this type of dual-functional probe can be selectively internalized inside specific tumor cells *via* ligand-based endocytosis and then activated by cathepsin B inside cells. Because these types of probes exist in a stably quenched state and are not hydrolyzed outside of cells, the detection contrast could increase as long as the ligands have high affinity and selectivity for specific cells.

The folate receptor (FR), which is a glycosylphosphatidylinositol-linked protein that captures folic acid from the extracellular milieu and transports it into the cell *via* a recycling endosomal pathway, is highly expressed on the cell surface of many human malignancies,[19] especially when associated with aggressively growing cancers,[20] but it is absent from healthy cell surfaces. As such, the FR is potentially a good target for therapeutic and imaging purposes.[21] As a ligand of the FR, folic acid has been linked to fluorescein isothiocyanate (FITC) to form a fluorescence probe for use in imaging ovarian cancer during human surgery, and real-time surgical visualization of tumor tissue in patients with the aid of intraoperative, tumor-specific fluorescence imaging was carried out for suspected ovarian cancer.[22] Compared with the antibody-targeting strategy, as non-immunogenic small-molecular probes, folic acid conjugates may exhibit faster tissue penetration and uptake, shorter residence in the blood and non-target organs and a higher ratio of target accumulation. When combined with their inexpensive prices, these probes offer great potential for *in vivo* cancer imaging for clinical use. The number of tumors that overexpress FR is large. Therefore, FR-targeted imaging probes could have broad-spectrum applications for

tumor detection. However, as an "always on" fluorescent probe, a probe based on folic acid conjugated to FITC may suffer from the limitation of relatively low signal contrast due to its constant signal, which would hamper its intensive use in clinical surgery.

To test the idea of a new "turn on" strategy based on intracellular activation, we designed a new, dual-functional, intracellular, cathepsin B-activatable fluorogenic probe, Folate-Lys(FITC)-Val-Cit-Lys(DABCYL) (FA-FITC-CathepsinSubstrate-DABCYL, shortened to FFCD **1**), that uses folic acid as the targeting group and the cathepsin B sequence for cleavage, as illustrated in Figure 1, with the aim to improve the contrast and sensitivity of a ligand-targeted probe in cancer imaging. The designed probe was a peptide conjugate consisting of three fragments: (1) a short peptide sequence linker, Val-Cit-Lys, with the cathepsin B-specific cleavage site at the C-terminus of Cit; (2) a fluorescent dye, FITC, and a quencher, DABCYL, that are covalently linked at either terminus to make this construct fluorescently silent using FRET; and (3) folic acid as the targeting group to direct the selective internalization of the probe through receptor-mediated endocytosis. The dual-functional, activatable probe itself is fluorescently quenched. We postulate that when this probe encounters target cancer cells that overexpress FRs, it binds and enters into cells *via* receptor-mediated endocytosis, and, consequently, cathepsin B in the lysosome should induce specific cleavage of the peptide linker and separate FITC from the quencher, DABCYL, thus restoring its fluorescence. Subsequently, the targeted cells should be fluorescent, while leaving normal cells undetectable. Herein,

6

we describe the synthesis and evaluation of the dual-functional cancer-imaging probe FFCD **1** in enzyme- and cell-based studies.

2. Results and discussion

2.1. Synthesis and characterization

Folic acid displays a high affinity for the FR, which functions to concentrate exogenous folic acid into the cell cytosol by endocytosis. Folic acid conjugates also bind tightly to the FR and trigger cellular uptake *via* endocytosis, which has been intensively studied for the delivery of cargos to FR-positive cells and tissues for cancer treatment and diagnosis.[23] The designed, folic acid-targeted, cathepsin B-triggered, dual-functional, fluorogenic cancer imaging probe FFCD **1** (FA-Lys(FITC)-Val-Cit-Lys(DABCYL)-OH), as well as the quencher-less probe FFC **2 (**FA-Lys(FITC)-Val-Cit-Lys(Ac)-OH, without DABCYL), which serves as an "always on" control, and FCD **3** (Ac-Lys(FITC)-Val-Cit-Lys(DABCYL)-OH, without folic acid) and FC **4** (Ac-Lys(FITC)-Val-Cit-Lys(Ac)-OH, without DABCYL and folic acid), which serve as non-targeting, negative controls, are shown in Figure 1 (see Supporting Information for their syntheses). The UV-Vis spectra of FFCD **1**, FFC **2** and DABCYL in PBS (pH = 7.4) are shown in Figure 2A, and the fluorescence spectra of FFCD **1** and the "always on" control, FFC **2,** in PBS (pH = 7.4) are shown in Figure 2B at the same concentration for comparison. FFCD **1** (1.0 μM) only showed low, intrinsic fluorescence intensity due to the FITC structure being quenched

by DABCYL, and the intensity was approximately 10-fold less than that of FFC **2**, meaning that approximately 90% of the fluorescence emission was quenched in the intact FFCD **1** molecule. This result demonstrated that DABCYL effectively quenches FITC fluorescence. The Absolute fluorescence quantum efficiency of these probes were measured to be 0.08 (FFCD **1**), 0.72 (FFC **2**), 0.10 (FCD **3**) and 0.81 (FC **4**).

2.2. Verification of enzymatic cleavage and stability in mouse plasma using HPLC

We confirmed the specific cleavage of the peptide linker Val-Cit-Lys in FFCD **1** by bovine spleen cathepsin B using HPLC. In principle, FFCD **1** would be cleaved by cathepsin B and yield two products, **5** and **6**, and this cleavage would be accompanied by fluorescence due to the abolishment of FRET quenching, as shown in Figure 3A.

Figure 3B shows the high-performance liquid chromatography (HPLC) profile of the intact FFCD **1** and FFCD **1** after a 4-h incubation with 1 μM cathepsin B at 37 °C. HPLC analysis clearly indicated the disappearance of FFCD **1** (at 16.7 min) and the appearance of the two new products at 8.2 min and at 9.5 min, respectively (Fig. 3B). In addition, using HPLC, no cleavage was observed in the solution containing FFCD **1** without cathepsin B, and only a single peak corresponding to the intact FFCD **1** was found (data not shown). These data demonstrate that cathepsin B is able to specifically cleave FFCD **1** and separate FITC from the quencher DABCYL.

Although cathepsin B is not present outside cells, the stability of FFCD **1** after incubation with plasma was still examined using HPLC. No signs of FFCD 1 degradation or metabolism were observed after 24-h incubation with mouse plasma (HPLC data shown in Fig. 3B), demonstrating the high plasma stability of FFCD **1** and allowing its use *in vivo*.

2.3. Fluorescence activation by cathepsin B

To evaluate the specificity of fluorescence activation by cathepsin B-induced peptide cleavage, we monitored the process of fluorescence enhancement of FFCD **1** treated with cathepsin B in a buffer solution at 37 °C over time using fluorescence spectroscopy. Figure 4A shows the spectra of the fluorescence variation of 2.0 μM FFCD **1** treated with 200 nM cathepsin B at different incubation times. As time elapsed, FFCD **1** exhibited a continuous increase in FITC fluorescence emission. After incubation with cathepsin B for 960 min, the fluorescence of FFCD **1** at 514 nm gradually increased from 190 to 2000, which was an approximately 10-fold increase. Figure 4B shows the fluorescence intensity-time curve of FFCD **1** incubated with different concentrations of cathepsin B. The cleavage reaction was complete in 4 h when 400 nM of cathepsin B was used, as observed by the restoration of fluorescence that reached a plateau after 4 h. The time point of the plateau is delayed with reduced concentrations of cathepsin B because, when 100 nM of enzyme was used, the fluorescence intensity still increased after 16 h and did not reach the plateau.

Therefore, the Lys-Val-Cit-Lys peptide linker can be effectively cleaved by cathepsin B, and the FRET quenching between FITC and DABCYL is subsequently abolished. Complete cleavage of FFCD by cathepsin B resulted in an approximately 10-fold increase in fluorescence and complete fluorescence restoration (i.e., the final fluorescence intensity was the same as that of FFC at the same concentration).

2.4. FACS analysis of fluorescence activation in KB cells treated at 37 °C

After we validated cathepsin B-triggered FFCD **1** activation in solution, we performed flow cytometry assays to investigate the targeting function and fluorescence activation of the probe by target cancer cells overexpressing the FR. Elevated expression of the FR occurs in many malignant cells.[18-19] Approximately 80-90% of ovarian tumors[24] and pediatric ependymal brain tumors, mesothelioma, myeloid leukemia and tumors associated with breast, lung, colon, renal, kidney, brain and endometrium overexpress the FR.[25] We selected human epidermoid carcinoma cells (KB cells) for this aim because KB cells overexpress cell surface FRs, especially when grown in low-folic-acid medium.[26] The cells were treated with different concentrations of the targeted "turn on" probe, and the specific binding and dose dependence of the probes for KB cells was monitored *via* flow cytometry. The "always on" probe FFC **2,** which lacked the quencher DABCYL; the non-targeted "turn on" probe, FCF **3;** and the "always on" probe FC **4,** which lacked folic acid, were used as controls and compared to the targeted "turn on" probe, FFCD **1**.

As shown in Figure 5, the KB cells were incubated with these probes at final concentrations of 10 nM, 50 nM, 100 nM, 200 nM and 300 nM at 37 °C for 4 h. The targeted "turn on" (FFCD **1**) and "always on" (FFC **2**) probes achieved much higher relative fluorescence intensities and showed dose-dependent binding to KB cells, whereas the non-targeted controls (FCD **3** and FC **4**) showed minimal binding. The targeted "turn on" probe, FFCD **1,** showed an MFI profile and dose dependence similar to those of the targeted "always on" probe, FFC **2**, which revealed that FFCD **1** was substantially activated and its fluorescence was restored after binding and entering into the target cancer cell under these incubation conditions.

2.5. FACS analysis of fluorescence activation in KB cells treated at 0 °C

We also performed experiments at 0 °C to investigate ATP-dependent internalization of the probes through the high-affinity FR, which is inactive at this temperature. The targeted "turn on" (FFCD **1**) and "always on" (FFC **2**) probes showed a substantial difference in MFI when we treated KB cells with them for 4 h at 0 °C. As shown in Figure 6, the "turn on" probe, FFCD **1,** has a much lower MFI compared with the "always on" probe, FFC **2**. At 0 °C, the cell endocytosis process was greatly reduced, which blocked the fluorescence activation of the FFCD **1** probe.

2.6. Competition tests with free folic acid

Then, we tested the ability of excess free folic acid to outcompete the uptake of FFCD

1 by preincubating KB cells with 50 μM of free folic acid for 2 h and then incubating the cells with FFCD 1 plus 50 μM of folic acid. As shown in Figure 7, the MFI was significantly reduced, from 150 to 50，in these cells, which indicated that the targeting and activation of the probe is FR-dependent and provided evidence of FFCD **1** binding to the FR with specificity.

2.7. FACS analysis of fluorescence activation using different cells

We also tested the targeting function and activation efficiency of this targeted "turn on" probe in several other cell lines with varied levels of FR expression using flow cytometry. Human cervical cancer cells (HeLa cells, moderate expression of FR), human breast adenocarcinoma cells (MCF-7, negative expression of FR) and mouse embryonic fibroblast cells (NIH-3T3, negative expression of FR) were selected[27] to compare to KB cells. Figure 8 depicts the fluorescence intensity of different cell lines in response to the probe. FFCD **1** showed varied specificities for cell lines having different levels of cell surface FR expression. HeLa cells were able to bind and activate the probe quite effectively, nearly as well as KB cells. MCF-7 and 3T3 cells only showed low fluorescence intensities, indicating that FFCD **1** was not effectively activated by these non-target cells. Therefore, the binding and activation of FFCD **1** are critically dependent on the FR expression patterns of cells.

2.8. Confocal laser scanning microscopy (CLSM) confirmation of KB cells

The specific binding and activation was then further confirmed using confocal microscopic analysis of KB cells. The KB cells were incubated with FFCD **1** or the controls (FCD **2**, FFC **3** and FC **4**) at varied concentrations (10 nM, 50 nM, 200 nM) at 37 °C for 4 h. After washing with PBS, fixing with p-formaldehyde and counter-staining of the nuclei with DAPI, the FITC signal of the cells was detected using 488 nm excitation and 515 nm detection wavelengths.

As shown in Figure 9, confocal images showed a strong fluorescence signal in KB cells incubated with FFCD **1** and FFC **2**, whereas control experiments using the non-targeted probes, FCD **3** and FC **4,** without folic acid showed minimal fluorescence. High FITC fluorescence was even detected at 10 nM FFCD **1** or FFC **2**, which showed the high affinity of the folic acid-type probes. The fluorescence intensity increased with increasing concentrations of the probes, but FCD **3** and FC **4** showed minimal, nonspecific fluorescence when the cells were treated with high concentrations of the probes, as observed at the 200 nM concentration. The structures of folic acid, FITC and DABCYL are hydrophobic, which increases the non-specific interaction with the cell membrane and results in minimal, non-specific uptake at high concentrations. It is possible that more hydrophilic modifications could be useful to reduce these non-specific interactions. The FITC signals of FFCD **1** and FFC **2** could be detected throughout the cytoplasm. These signals were absent from the nucleus and showed a punctuate pattern within the cytoplasm, which indicated that the probes were internalized into the target cells *via* endocytosis and then released into the cytoplasm. Compared with the images for FFC **2**, the images for FFCD **1** lacked the

FITC signal on the cell surface membrane, which indicated that the FFCD **1** probe existed in the quenched state before uptake by the cells.

In Figure 9, the KB cells were incubated with the probes at 37 °C for 4 h, which was sufficient time for FFCD **1** to enter into the cell and then be activated by cathepsin B in the lysosome. However, when we incubated KB cells with the probes at 37 °C for a shorter time, confocal images showed quite different fluorescence signal patterns between the FFCD **1** and FFC **2** probes. As shown in Figure 10, we can see a stepwise internalization process. Confocal microscopy showed that after incubation with the probes for 30 min, a strong membrane distribution pattern of FITC fluorescence was observed when the cells were treated with the "always on" probe, FFC **2**; however, the FITC fluorescence for the "turn on" probe, FFCD **1,** was lacking on the membrane, and nearly no fluorescence was detected inside the cells. After 1 h of incubation, fluorescence began to be emitted from inside the cells for both probes, and the fluorescence signals were still increasing after 4 h of incubation. The different membrane distribution patterns of FITC fluorescence for these two probes were still obvious after 1 h or 4 h of incubation. These data clearly demonstrate that FFCD **1** exists in a quenched state before entering the cells but can be selectively internalized *via* receptor-mediated endocytosis by specific target cells and then undergo an efficient fluorescence activation process inside the cells.

3. Conclusion

14

In summary, a folic acid-targeted, intracellularly activatable, dual-functional probe has been successfully developed for the detection of cancer cells. Due to highly efficient FRET quenching, this probe is nearly non-fluorescent in buffer, but it can be specifically activated upon cathepsin B cleavage in target cancer cells with dramatic fluorescence enhancement. Furthermore, because of the specific affinity of folic acid for cancer cells, the probe showed desirable specificity in discriminating tumor cells with high FR expression. This targeted, intracellularly activatable, dual-functional probe may enable "turn-on" visualization of cancer cells with contrast enhancement by displaying a low-fluorescence-quenched background compared to that of "always-on" probes and avoiding non-specific activation by the non-specifically expressed enzyme in normal tissue that is a common problem in "turn on" probe design strategies. This new, targeted, intracellularly activatable probe may have potential applications for intraoperative inspection in clinical cancer surgery that would offer higher contrast and sensitivity. The design concept can also be widely adapted to other specific targeting probes for *in vivo* molecular imaging of cancer.

4. Experimental section

4.1. Chemicals and materials

Bovine spleen cathepsin B and RPMI-1640 without folic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Penicillin/streptomycin, fetal bovine serum, RPMI-1640 and Trypsin-EDTA were purchased from Thermo Scientific HyClone

(South Logan, USA). 4',6-Diamidino-2-phenylindole (DAPI) was purchased from Roche (Mannheim, Germany). Paraformaldehyde and PBS were purchased from Sangon (Shanghai, China). Deionized water was obtained using the Barnstead Nanopure water purification system from Thermo Scientific (Thermo, USA). Other chemical agents were purchased from Alfa Aesar (Tianjin, China).

4.2. Cells and culture conditions.

Human nasopharyngeal epidermoid carcinoma cells (KB), human cervical cancer cells (HeLa) and human breast adenocarcinoma cells (MCF-7) were obtained from the Cell Bank of the Committee on Type Culture Collection of the Chinese Academy of Sciences. The mouse embryonic fibroblast cells (NIH-3T3) were kind gifts from Dr. Zhimou Yang (Nankai University, China). The cells were continuously cultured in folic acid-free RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. The final folic acid concentration (with FBS as the only source of folic acid) falls in the range of the physiological concentration of human serum.

4.3. Cleavage detection and plasma stability analysis using HPLC

The cathepsin B assay conditions were adapted from a published procedure.[28] Briefly, bovine spleen cathepsin B (10 μL, 20 μM) was activated at room temperature in 10

16

μL of 30 mM DTT/15 mM EDTA for 15 min and then diluted with 160 μL of 25 mM

acetate/1 mM EDTA buffer (pH = 5.0, pre-incubated at 37 °C), followed by addition

of FFCD **1** (20 μL, 500 μM in H$_2$O) to give the following concentrations: [cathepsin

B] = 1 μM and [FFCD **1**] = 50 μM. The mixture was incubated at 37 °C for 240 min

and then injected into the HPLC for analysis (25 cm C-18 column, 7:3 water/CH$_3$CN

at 1.0 mL/min, λ = 450 nm).

For plasma stability analysis, FFCD **1** was incubated in triplicate at 37 °C for 16

h in normal mouse plasma (1.0 mL, 150 μM of FFCD **1**), which was separated from

the mouse blood by centrifugation. Two milliliters of cold CH$_3$CN was added to the

mixture, followed by centrifugation at 12,000 rpm for 10 min. The supernatant was

transferred and injected into the HPLC for analysis.

4.4. Fluorescence activation by cathepsin B

Cathepsin B was first activated as described above and then diluted with FFCD **1** in

25 mM acetate/1 mM EDTA buffer (pH = 5.0) to give the following concentrations:

[nanoprobe 1] = 2 μM, [cathepsin B] = 100 nM, 200 nM or 400 nM, and the mixtures

were incubated at 37 °C. Aliquots (100 μL) were periodically removed and stopped

with 100 μL PBS (pH = 7.4, 10 ×), and the fluorescence intensity was monitored

using a microplate reader (Synergy 4 Hybrid, BioTeK, USA) with an excitation

wavelength at 480 nm and an emission wavelength from 500 nm to 600 nm. Because

the fluorescence of FITC is sensitive to pH, a high-concentration PBS buffer was used

to stop the reaction to diminish the influence of the acidity of the original buffer.

4.5. Flow cytometry measurements

The cells were continuously cultivated at 37 °C and 5% CO_2 in folic acid-deficient RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin, seeded in 12-well cell culture plates at a density of 2×10^5 cells/well and allowed to grow for 24 h at 37 °C before initiating the experiments. Two hours before the experiments, the medium was removed and replaced with 1.0 mL of fresh folic acid-deficient RPMI-1640. Then, the cells were incubated with a series of the prepared probes dissolved in folic acid-free RPMI 1640 medium at either 37 °C or 0 °C for 4 hours. After removal of the supernatants, the cells were trypsinized and collected in FACS tubes, followed by centrifugation at 1000 rpm for 5 min. The pellets were washed three times with PBS using repetitive centrifugation at 800 rpm for 5 min and finally resuspended in PBS with 0.1% FBS. The preparation of FACS samples was performed on ice to inhibit further cellular uptake. The fluorescence intensities of the samples were acquired and analyzed using a BD-FACS Calibur flow cytometer (BD, Franklin Lakes, NJ, USA). For the competitive inhibition experiments with free folic acid, the procedure was the same as above except that free folic acid was added at the specified concentrations to the medium two hours before the experiments and to the medium used for dissolving the FFCD **1** probe.

4.6. Confocal Laser Scanning Microscopy (CLSM) detection

For the microscopy studies, the procedure for cell culture was the same as described above. KB cells that were continuously cultured in folic acid-free RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C and 5% CO_2 were seeded in a glass-bottomed Petri dish and then placed in a 24-well plate at a concentration of 5×10^4 cells/well 24 h before initiating experiments. Two hours before the experiments, the medium was removed and replaced with 1.0 mL of fresh folic acid-deficient RPMI-1640. After incubation with a series of the prepared probes at 37 °C for 30 min, 1 h or 4 h, the cells were washed three times with PBS. The cells were then fixed with 4% formaldehyde for 10 min at room temperature and washed three times with PBS. Then, the cells were stained with 1 μg/mL DAPI for 3-5 min and washed three times with PBS. Confocal images were acquired using a Laser Scanning Confocal Microscope (TCS SP8, Leica, Wetzlar, Germany).

4.7. Statistical Analysis

All data are presented as the mean ± SEM. Two-group comparison was performed using the independent samples t-test. A p value of less than 0.05 was considered statistically significant.

Acknowledgments

References

1. (a) R. Weissleder and M. J. Pittet, *Nature*, 2008, **452**, 580–589; (b) J. V. Frangioni, *J. Clin. Oncol.*, 2008, **26**, 4012–4021; (c) K. Brindle, *Nat. Rev. Cancer*, 2008, **8**, 94-107.

2. (a) M. B. Wallace and R. Kiesslich, *Gastroenterology*, 2010, **138**, 2140–2150; (b) J. von Burstin, S. Eser, B. Seidler, A. Meining, M. Bajbouj, J. Mages, R. Lang, A. J. Kind, A. E. Schnieke, R. M. Schmid, G. Schneider and D. Saur, *Int. J. Cancer*, 2008, **123**, 2138–2147.

3. (a) I. Vergote, T. von Gorp, F. Amant, K. Leunen, P. Neven and P. Berteloot, *Int. J. Gynecol. Cancer*, 2008, **18**, 11–19; (b) M. J. Koppe, O. C. Boerman, W. J. Oyen, and R. P. Bleichrodt, *Ann. Surg.*, 2006, **243**, 212–222.

4. (a) Y. Urano, D. Asanuma, Y. Hama, Y. Koyama, T. Barrett, M. Kamiya, T. Nagano, T. Watanabe, A. Hasegawa, P. L. Choyke and H. Kobayashi, *Nat. Med.*, 2009, **15**, 104–109; (b) N. Thekkek and R. Richards-Kortum, *Nat. Rev. Cancer.*, 2008, **8**, 725–731; (c) G. Blum, G. von Degenfeld, M.-J. Merchant, H.-M. Blau and M. Bogyo, *Nat. Chem. Biol.*, 2007, **3**, 668–677.

5. (a) L. Fass, *Mol. Oncol.*, 2008, **2**, 115–152; (b) P. K. Borjesson, Y. W. Jauw, R. Boellaard, R. de Bree, E. F. Comans, J. C. Roos, J. A. Castelijns, M. J. Vosjan, J. A. Kummer, C. R. Leemans, A. A. Lammertsma and G. A. van Dongen, *Clin. Cancer Res.*, 2006, **12**, 2133-2140; (c) C. R. Divgi, N. Pandit-Taskar, A. A. Jungbluth, V. E.

Reuter, M. Gonen, S. Ruan, C. Pierre, A. Nagel, D. A. Pryma, J. Humm, S. M. Larson, L. J. Old and P. Russo, *Lancet Oncol.*, 2007, **8**, 304-310.

6. K. Hemminki, H. Liu, A. Heminki and J. Sundquist, *Ann Oncol*., 2012, 760-764

7. (a) Q. T. Nguyen, E. S. Olson, T. A. Aguilera, T. Jiang, M. Scadeng and L. G. Ellies, *Proc. Natl. Acad. Sci. USA*, 2010, **107**, 4317-4322; (b) H. Kobayashi, M. Ogawa, R. Alford, P. L. Choyke and Y. Urano, *Chem. Rev.*, 2010, **110**, 2620–2640; (c) G. D. Luker and K. E. Luker, *J. Nucl. Med.*, 2008, **49**, 1–4.

8. (a) M. C. Pierce, D. J. Javier and R. Richards-Kortum, *Int. J. Cancer*, 2008, *123*, 1979–1990; (b) B. J. Tromberg, B. W. Pogue, K. D. Paulsen, A. G. Yodh, D. A. Boas and A. E. Cerussi, *Med. Phys.*, 2008, **35**, 2443–2451.

9. G. Themelis, J. S. Yoo, K. S. Soh, R. Schulz, and V. Ntziachristos, *J. Biomed. Opt.*, 2009, **14**, 064012.

10. (a) S. L. Troyan, V. Kianzad, S. L. Gibbs-Strauss, S. Gioux, A. Matsui, R. Oketokoun, L. Ngo, A. Khamene, F. Azar, and J. V. Frangioni, *Ann. Surg. Oncol.*, 2009, **16**, 2943–2952; (b) W. Stummer, U. Pichlmeier, T. Meinel, O. D. Wiestler, F. Zanella and H. J. Reulen, *Lancet Oncol.*, 2006, **7**, 392–401.

11. (a) H. Shi , Z. Tang, Y. Kim, H. Nie, Y. F. Huang, X. He, K. Deng, K. Wang and W. Tan, *Chem-Asian J.*, 2010, **5**, 2209–2213; (b) B. J. Hicke, A. W. Stephens, T. Gould, Y. F. Chang, C. K. Lynott, J. Heil, S. Borkowski, C. S. Hilger, G. Cook, S. Warren, P. G. Schmidt and B.-J. Hicke, *J. Nucl. Med.*, 2006, **47**, 668–678.

12. H. Kobayashi and P. L. Choyke, *Acc. Chem. Res.*, 2011, **44**, 83–90.

13. (a) M. Ogawa, N. Kosaka, M. R. Longmire, Y. Urano, P. L. Choyke and H. Kobayashi, *Mol. Pharm.*, 2009, **6**, 386–395; (b) M. Ogawa, N. Kosaka, P.-L. Choyke and H. Kobayashi, *ACS Chem. Biol.*, 2009, **4**, 535–546; (c) Y. Hama, Y. Urano, Y. Koyama, M. Kamiya, M. Bernardo, R. S. Paik, I. S. Shin, C. H. Paik, P. L. Choyke and H. Kobayashi, *Cancer Res.,* 2007, **67**, 2791–2799.

14. (a) E. S. Olson, T. Jiang, T. A. Aguilera, Q. T. Nguyen, L. G. Ellies, M. Scadeng, and R. Y. Tsien, *Proc. Natl. Acad. Sci. USA*, 2010, **107**, 4311-4316; (b) R. Weissleder, C.-H. Tung, U. Mahmood and A. Jr Bogdanov, *Nat. Biotechnol.*, 1999, **17**, 375–378; (c) Tao Jiang, E. S. Olson, Q. T. Nguyen, M. Roy, P. A. Jennings, and R. Y. Tsien, *Proc. Natl. Acad. Sci. USA*, 2004, **101**, 17867–17872.

15. (a) M. Toth, A. Sohail and R. Fridman., Methods Mol. Biol., 2012, 878, 121-135; (b) A. Talvensaari-Mattila, P. Paakko, and T. Turpeenniemi-Hujanen, *Br. J. Cancer*, 2003, **89**, 1270–1275; (c) C. Bremer, C.–H. Tung, and R. Weissleder, *Nat. Med.*, 2001, **7**, 743–748.

16. (a) S. O. Doronina, B. A. Mendelsohn, T. D. Bovee, C. G. Cerveny, S. C. Alley, D. L. Meyer, E. Oflazoglu, B. E. Toki, R. J. Sanderson, R. F. Zabinski, A. F. Wahl and P. D. Senter, *Bioconjug. Chem.*, 2006, **17**, 114-124; (b) H. Kirschke, A. J. Barrett and N. D. Rawlings, In Protein Profile - Proteinases 1: Lysosomal Cysteine Proteinases; Sheterline, P., Ed.; Academic: London, 1995; Vol. 2, pp 1587-1643; (c) G. M. Dubowchik and R. A. Firestone, *Bioorg. Med. Chem. Lett.*, 1998, **8**, 3341-3346; (d) J. S. Mort and D. J. Buttle, *Int. J. Biochem. Cell B.*, 1997, **29**, 715-720.

17. S. Yan and B. F. Sloane, *Biol Chem.*, 2003, **384**, 845-854.

18. S. O. Doronina, B. E. Toki, M. Y. Torgov, B. A. Mendelsohn, C. G. Cerveny, D. F. Chace, R. L. DeBlanc, R. P. Gearing, T. D Bovee, C. B. Siegall, J. A. Francisco, A. F. Wahl, D. L. Meyer and P. D. Senter, *Nat. Biotechnol.*, 2003, **21**, 778-784.

19. (a) R. E. Fisher , B. A. Siegel, S. L. Edell, N. M. Oyesiku, D. E. Morgenstern, R. A. Messmann and R. J. Amato, *J. Nucl. Med.*, 2008, **49**, 899–906; (b) N. Parker, M. J. Turk, E. Westrick, J. D. Lewis, P. S. Low and C. P. Leamon, *Anal. Biochem.*, 2005, **338**, 284–293.

20. (a) L. C. Hartmann, G. L. Keeney, W. L. Lingle, T. J. Christianson, B. Varghese, D. Hillman, A. L. Oberg, and P. S. Low, *Int. J. Cancer*, 2007, *121*, 938-942; (b) J. E. Allard, J. I. Risinger, C. Morrison, G. Young, G. S. Rose, J. Fowler, A. Berchuck and G. L. Maxwell, *Gynecol. Oncol.*, 2007, **107**, 52–57.

21. (a) W. Xia and P. S. Low, *J. Med. Chem.*, 2010, **53**, 6811-6824; (b) P. S. Low, and A. C. Antony, *Adv. Drug Deliv. Rev.* 2004, **56**, 1055–1058; (c) D. K. Armstrong, A. Bicher, R. L. Coleman, D. G. Gibbon, D. Glenn, L. Old, N. N. Senzer, A. Schneeweiss, R. H. Verheijen, A. J. White and S. Weil, *J. Clin. Oncol.*, 2008, **26**, 5500; (d) E. I. Sega and P. S. Low, *Cancer Metastasis Rev.*, 2008, **27**, 655–664.

22. G. M van Dam, G. Themelis, L. M. Crane, N. J. Harlaar, R. G. Pleijhuis, W. Kelder, A. Sarantopoulos, J. S. de Jong, H. J. Arts, A. G. van der Zee, J. Bart and P. S. Low, *Nat. Med.*, 2011, **17**, 1315–1319.

23. (a) P. S. Low and S. A. Kularatne, *Curr. Opin. Chem. Biol.*, 2009, **13**, 256–262; (b) C. P. Leamon, *Curr. Opin. Investig. Drugs*, 2008, **9**, 1277–1286.

24. I. G. Campbell, T. A. Jones, W. D. Foulkes and J. Trowsdale, *Cancer Res.*, 1991, **51**, 5329–5338.

25. L. C. Hartmann, G. L. Keeney, W. L. Lingle, T. J. Christianson, B. Varghese, D.

Hillman, A. L. Oberg and P. S. Low, *Int. J. Cancer*, 2007, **121**, 938-942.

26. Y. Wang, R. Guo, X. Cao, M. Shen and X. Shi, *Biomaterials*, 2011, **32**, 3322-3329

27. (a) G. A. Mansoori, K. S. Brandenburg   and A. Shakeri-Zadeh, *Nanotechnol. Sci. Appl.*, 2010, **2**, 1911-1928; (b) J.-J. Yin, S. Sharma, S. P. Shumyak, Z.-X. Wang, Z.-W. Zhou, Y. Zhang, P. Guo, C.-Z. Li, J. R. Kanwar, T. Yang, S. S. Mohapatra, W. Liu, Wei Duan, J.-C. Wang, Q.   Li, X. Zhang, J. Tan, L. Jia, J. Liang, M. Q. Wei, X. Li and S.-F. Zhou, *PLoS ONE*, 2013, **8**, e62289.

28. G. M. Dubowchik, and R. A. Firestone. *Bioorg. Med. Chem. Lett.,* 1998, **8**, 3341-3346.

Figure Legends：

Fig. 1. Chemical structures of the targeted, cathepsin B-activatable fluorescence probe FFCD **1**, the "always on" control, FFC **2**, and the non-targeting negative controls, FCD **3** and FC **4**.

Fig. 2. UV-Vis and fluorescence spectra of FFCD **1** and FFC **2**. For the fluorescence spectra, the excitation wavelength was 480 nm.

Fig. 3. (A) Schematic illustration of fluorescence quenching and dequenching of FFCD **1** before and after cathepsin B cleavage, respectively. The blue arrow indicates the enzyme cleavage site. (B) HPLC trace of FFCD **1** before cathepsin B cleavage (black), after cathepsin B cleavage (red) and after incubation with mouse plasma (blue). Wavelength for detection: 450 nm.

Fig. 4. Time course of the fluorescence emission spectra of FFCD **1** incubated with cathepsin B. (A) 2 μM of FFCD **1** was incubated with 200 nM of cathepsin B at 37 °C for 0 min to 960 min. Excitation wavelength: 480 nm. (B) 2 μM of FFCD **1** was incubated with 100 nM, 200 nM or 400 nM of cathepsin B at 37 °C for 0 min to 960 min. Error bars represent standard deviations (n = 3).

Fig. 5. Uptake and activation of FFCD **1**, FFC **2**, FCD **3** and FC **4** by FR-overexpressing KB Cells measured using FACS. The cells were incubated with different concentrations of each of the conjugates at 37 °C for 4 h, rinsed and measured for their mean fluorescence using a flow cytometer. (A) Uptake and

activation of the probes (300 nM) at 37 °C for 4 h. (B) Concentration-dependent uptake and activation of the probes at 37 °C for 4 h. Error bars represent standard deviations (n = 3).

Fig. 6. Uptake and activation of FFCD **1** and FFC **2** by FR-overexpressing KB cells measured using FACS. The cells were incubated with each of the conjugates at different concentrations at 0 °C for 4 h and then treated as above for measurement by flow cytometry. Error bars represent standard deviations (n = 3).

Fig. 7. The effect of free folic acid on the uptake and activation of FFCD **1** in KB cells. The cells were incubated with FFCD **1** (300 nM) in the presence of free folic acid (50 μM) at 37 °C for 4 h, rinsed and measured for their mean fluorescence using a flow cytometer. Error bars represent standard deviations (n = 3).

Fig. 8. Binding of FFCD **1** to various cell lines, as determined using flow cytometry. The cells (KB, HeLa, MCF-7 or 3T3) were incubated at 37 °C for 4 h with different concentrations of FFCD **1**. Error bars represent standard deviations (n = 3).

Fig. 9. Confocal microscopic images of KB cells after incubation with FFCD **1**, FFC **2**, FCD **3** or FC **4** (10 nM, 50 nM or 200 nM) at 37 °C for 4 h. KB cells were then fixed and nuclear stained with DAPI prior to the measurement of FITC (green) and DAPI (blue) fluorescence using a confocal microscope.

Fig. 10. Confocal microscopic images of KB cells after incubation with 200 nM of FFCD **1** or FFC **2** at 37 °C for 30 min, 1 h or 4 h. The cells were then treated as above

for confocal microscopic observation.