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Cell-Permeable ^{99m}Tc(CO₃)-Labeled Fluorogenic Caspase 3 Substrate for Dual-Modality Detection of Apoptosis

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Keywords

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Apoptosis, or programmed cell death, is a specialized form of cell death involved in a wide variety of physiological processes.^[1, 2] Most anticancer therapeutic regimens induce apoptosis. The ability to assess apoptosis using noninvasive imaging techniques would markedly enhance the early evaluation and continuous monitoring of anticancer drug efficacy.

Two strategies have been explored for the noninvasive imaging of apoptosis. The first approach uses annexin V, a 36-KDa protein, as the imaging probe for apoptotic cells.^[3-5] Annexin V binds with high affinity to phosphatidylserine, which is externalized from the inner to the outer leaflet of the plasma membrane during the early stage of apoptosis.^[6] The second approach is to measure the activity of specific intracellular enzymes involved in the apoptotic pathway. Caspases, a family of cysteinyl aspartate-specific proteases, play a central role in the regulation and execution of apoptosis.^[7, 8] In particular, the executioner caspase 3 mediate the initiation and propagation of the apoptosis cascade.^[9] Caspase 3 typically recognizes the 4-amino acid peptide sequence Asp-Glu-Val-Asp (DEVD) and cleaves their substrate at the C-terminal Asp residue. Fluorogenic DEVD conjugates that become fluorescent in the presence of caspase 3 have been used for the *in vitro* detection of apoptosis using fluorescence microscopy, a powerful method routinely used in biomedical research to gain information at the cellular and subcellular level.^[10-12] However, the application of optical imaging in the clinic for the detection of apoptosis is not yet a viable technique, owing to the strong attenuation of fluorescent light and limited penetration depth.

Nuclear imaging offers high detection sensitivity, which makes it especially suited for tracking radiotracers used in the *in vivo* molecular imaging of apoptosis.^[13] However, neither positron emission tomography nor single-photon emission computed tomography can localize radiotracers at the cellular level because of low spatial resolution. An effective and economical imaging strategy is to develop multimodality imaging probes that allow the extraction of as much diagnostic information as possible from each examination.

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We sought to develop a single imaging probe that possesses both fluorescent and radioactive tags to allow both optical and nuclear imaging studies. Such a probe would make it possible to validate *in vivo* imaging findings using *in vitro* fluorescent microscopic examinations.^[14, 15]

The following criteria were considered in our design: (a) the probe should be labelled with both a radionuclide and a fluorogenic compound that becomes activated upon cleavage from DEVD; (b) the probe should be cell permeable—in its intact form, the probe should be able to freely diffuse into and, most important, out of viable cells; (c) the probe should be efficiently cleaved by caspase 3 in apoptotic cells; and (d) the cleaved products, including radionuclide and fluorophores, should be trapped within apoptotic cells.

Technetium-99m (^{99m}Tc) is the most extensively used radiometal in nuclear medicine radiodiagnostics.^[16-18] Rhenium (a mixture of ¹⁸⁵Re and ¹⁸⁷Re) generally produces complexes with physical and biodistribution properties similar to those formed with ^{99m}Tc and is often used as a nonradioactive alternative to ^{99m}Tc for structural characterization.^[19] Both ^{99m}Tc and Re form stable complexes with a lipophilic amino acid derivative, Lys[di-(2-pyridinemethyl)]-CO₂H, also termed single-amino acid chelate (SAAC).^[20, 21] Rhodamine-based fluorogenic substrates have a low background signal in their uncleaved state and the ability to unveil the majority of the fluorescence after cleavage of one of the two amide bonds. We therefore reasoned that the cleavable rhodamine-based DEVD substrate linked to Re-SAAC or ^{99m}Tc-SAAC chelate might be a suitable candidate for detecting caspase activity both *in vitro* using fluorescence microscopy and *in vivo* using nuclear imaging. The lipophilic nature of rhodamine 110 (R110) and Re/^{99m}Tc-SAAC should facilitate the cellular uptake of the DEVD substrate. Once the amide bond between DEVD and rhodamine is cleaved, the amino group on the R110-Re/^{99m}Tc-SAAC fragment in the cytosol is expected to be protonated and to have increased hydrophilicity and increased cellular retention.

To demonstrate the feasibility and implementation of this paradigm, we assembled SAAC to R110 through one of its two aromatic amines, and the other amine in R110 was linked to DEVD peptide at its C terminus. Synthesis of Ac-DEVD-R110-D-SAAC-Fmoc (**2**) was initiated by coupling R110 with 10 equiv in excess of Fmoc-Asp(OBu-*t*)-CO₂H followed by removal of the Fmoc-protecting group. The resulting product [Asp(OBu-*t*)]₂-R110 was obtained with 90% yield. Sequential condensation with 1.5 equiv of Fmoc-protected SAAC (Fmoc-SAAC-CO₂H) and 2 equiv of tripeptide Ac-Asp(OBu-*t*)-Glu(OBu-*t*)-Val-OH followed by removal of *t*-butyl protecting groups with trifluoroacetic acid gave **2** with an overall yield of 8% (Scheme 1). The profluorophore **2** was stable in both PBS and culture medium supplemented with serum (Supporting Information). This is important for **2** and its corresponding ^{99m}Tc-chelate to be useful as imaging agents because premature hydrolysis of the imaging probes can raise background signal levels and deteriorate imaging quality.

Rhenium was chelated to **2** using a stoichiometric amount of the Re (I) tricarbonyl precursor (NEt₄)₂[Re(CO)₃Br₃] in methanol to give **3** in quantitative yield.^[22] The structure of **3** was verified by liquid chromatography-mass spectroscopy (LC-MS).

Compound **2** was completely degraded when it was incubated with caspase 3, as verified by increase in fluorescence signal upon exposure to caspase 3, and by LC-MS analysis showing that the hydrolytic loss of R110-Asp-SAAC-Fmoc fragment occurred selectively at the C-terminus of DEVD peptide (Supporting Information). To ascertain that **2** and its Re-chelate, **3**, could be cleaved by apoptotic cells and activate the fluorescent signal, both compounds were incubated with either viable human colon cancer DLD1 cells or apoptotic DLD1 cells that had been treated with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) for 2 h at 37°C. Fluorescence microscopic images of cells incubated with **3** are shown in Figure 1. TRAIL induced significant apoptosis, as revealed by extensive staining of TRAIL-treated DLD1 cells with annexin V–Alexa Fluor 594 (Fig. 1B, 1C). Cleavage of R110-D-SAAC-Re(CO)₃ and subsequent activation of fluorescent signal from **3** was visualized in apoptotic cells (Fig. 1B) but not in untreated viable cells (Fig. 1A). Treatment with both TRAIL and a selective caspase 3 inhibitor Ac-DEVD-CHO, significantly reduced the fluorescent signal from **3**, although Ac-DEVD-CHO did not inhibit the apoptosis of DLD1 cells, as indicated by their strong positive annexin V staining (Fig. 1C). These results indicated that **3** was specifically cleaved by caspases 3 in apoptotic cells. A Z-stack image of TRAIL-treated DLD1 cells at higher magnification showed that the fluorescent signal was activated in the cytoplasm (Fig. 1D), providing direct evidence that **3** was cell permeable and that the degradation product was retained in the cells. Similar findings were observed for the nonchelated profluorophore **2**.

To test whether **2** and **3** could diffuse out of cells, each compound was exposed to DLD1 cells for 2 h. The cells were then washed and incubated in drug-free culture medium for 2 or 24 h. If the test compound diffused out of the cells during the incubation period, the fluorescent signal resulting from subsequent treatment with TRAIL would decrease with increasing incubation time in drug-free medium owing to the reduced concentration of each compound entrapped in the cells. This is exactly what we observed (Fig. 2). Our data indicated that uptake of **2** and **3** in viable tumor cells was a reversible process, a condition necessary for reduced background signal and satisfactory nuclear imaging applications.

We next labelled **2** with ^{99m}Tc to afford ^{99m}Tc-chelate **4**, which shares chemical and biological properties similar to those of Re-chelate **3**. Compound **2** was treated with [^{99m}Tc-(CO)₃H₂O]⁺, which was generated from ^{99m}TcO₄[−] using commercially available carbonyl labelling kits (Mallinckrodt, St. Louis, MO). The labelling efficiency was >95%. HPLC analysis revealed that ^{99m}Tc-chelate **4** had a retention time (*t_R* = 31.2 min) identical to that of Re-chelate **3** (*t_R* = 30.9 min), indicating that **4** had the same structure as **3** (Supporting Information). The stability of ^{99m}Tc-chelate **4**, Ac-DEVD-R110-DSAAC-^{99m}Tc(CO)₃, was evaluated using a ligand challenge method, in which a large excess of cysteine and histidine was used to compete for the complex's tendency to undergo transchelation. Chelate **4** was stable as no transchelation was observed after samples were incubated at 37°C for up to 5 h.

We attempted to examine whether the ^{99m}Tc-chelate of **2** (**4**) could be retained by TRAIL-treated apoptotic DLD1 cells. However, because a fraction of treated cells were detached from the microwell plate and some late-stage apoptotic cells lost membrane integrity during the time course of the induction, large variations in experimental data were observed. The results of radiotracer uptake study in apoptotic cells were not conclusive. To provide

evidence to differential cellular uptake and wash-out between ^{99m}Tc -chelate **4** and its fragment R110-D-SAAC- $^{99m}\text{Tc}(\text{CO})_3$, each radiotracer was incubated with DLD1 cells, and their retention in the cells was measured. ^{99m}Tc -chelate **4** had significantly higher uptake in DLD1 cells than did R110-D-SAAC- $^{99m}\text{Tc}(\text{CO})_3$ after 1–2 h of incubation. On the other hand, while chelate **4** was gradually washed out, its fragment was trapped in the cells (Supporting Information). These data support the notion that the cleaved fragment R110-D-SAAC- $^{99m}\text{Tc}(\text{CO})_3$ is less permeable to plasma membrane as compared to its parent substrate ^{99m}Tc -chelate **4**.

We used a murine liver apoptosis model to evaluate whether chelate **4** could be used to image apoptosis *in vivo*.^[4, 5] Male Balb/c mice (10–12 weeks old, weight 20–25 g) were injected intravenously with an anti-Fas monoclonal antibody (10 $\mu\text{g}/\text{mouse}$) to induce apoptosis in the liver. Two hours later, ^{99m}Tc -chelate **4** [300 μCi (11.1 MBq), 20 μg peptide in 0.2 mL] was injected intravenously. Radionuclide imaging was acquired 120 min after the injection of **4**. Significantly higher uptake of **4** was seen in anti-Fas-treated apoptotic liver than in the PBS-treated control mice. ^{99m}Tc -chelate **4** was cleared through the renal route as indicated by the distribution of radioactive species into the bladder (Fig. 3)

Quantitative analysis of the biodistribution of ^{99m}Tc -chelate **4** in anti-Fas-treated mice and PBS-treated mice was performed using the cut-and-count method. In both treatment groups, compound **4** was taken up primarily by the liver, spleen, and kidney after intravenous injection. Significantly higher radioactivity was found in livers of mice treated with anti-Fas (19.5 ID%/g) than in livers of mice given PBS (13.9 ID%/g) at 2 h after injection ($p = 0.015$) (Supporting Information).

To confirm that **4** was activated by caspases 3 and retained in apoptotic liver, the liver tissues were dissected and cryosectioned to 5- μm slides after gamma imaging. Activated caspase 3 was immunohistochemically stained with a rabbit anti-mouse monoclonal antibody against activated caspase 3, and cell nuclei were counterstained with Hoechst 33342. As shown in Figure 4, anti-Fas-treated liver but not PBS-treated liver displayed extensive staining for activated caspase 3. Importantly, cleavage of the fluorogenic ^{99m}Tc -chelate **4** was shown only in the apoptotic liver but not in the normal liver, indicating that **4** was cleaved by caspase 3 *in vivo* (Fig. 4).

Cleavage of R110 dye from the DEVD peptide in **4** does not necessarily imply that the radionuclide component SAAC- $^{99m}\text{Tc}(\text{CO})_3$ is associated with the dye. To confirm that SAAC- $^{99m}\text{Tc}(\text{CO})_3$ remains linked to R110 in the apoptotic liver after cleavage of **4** by caspase 3, we analyzed the chromatograms of liver extracts from mice 2 h after intravenous injection of **4**. Whereas only trace amount of the fragment R110-D-SAAC- $^{99m}\text{Tc}(\text{CO})_3$ was detected from the liver of PBS-treated mouse, substantially more R110-D-SAAC- $^{99m}\text{Tc}(\text{CO})_3$ was detected in the liver of anti-Fas-treated mouse (Fig. 5). The ratios of area integrals between peaks representing R110-D-SAAC- $^{99m}\text{Tc}(\text{CO})_3$ and **4** were 0.20 and 1.48 in PBS-treated liver and anti-Fas-treated liver, respectively. These data indicate that ^{99m}Tc -chelate **4** was cleaved selectively at the amide bond between Ac-DEVD and R110-D-SAAC- $^{99m}\text{Tc}(\text{CO})_3$, and that the corresponding degradation product R110-D-SAAC- $^{99m}\text{Tc}(\text{CO})_3$ was retained in the apoptotic liver in its intact form. Because fluorescent

signal from cleaved products of **4** co-localized with activated caspase 3 (Fig. 4), we conclude that the increased radioactivity in the liver of anti-Fas-treated mice observed in the gamma imaging study is attributed to the activation of caspase 3.

In summary, we have developed a novel cell-permeable imaging probe suitable for both fluorescence microscopy and nuclear imaging of caspase 3 activity in apoptotic cells. Both *in vitro* and *in vivo* data support our hypothesis that the underlying mechanism for satisfactory nuclear imaging is attributable to reversible diffusion of the parent substrate in viable cells and increased retention of the radioactive fragment cleaved by activated caspase 3 in apoptotic cells. The combined use of two powerful molecular imaging methods provides the opportunity for a direct correlation between *in vitro* and *in vivo* biological activities, and allows validation of nuclear imaging using *ex vivo* fluorescence microscopy technique. To the best of our knowledge, this is the first demonstration of dual optical and nuclear imaging of enzymatic activity using a single imaging probe. Further studies are needed to define the pharmacokinetics, optimal imaging protocol, and the suitability of **4** for non-invasive detection of apoptosis in various disease models, including apoptotic response of solid tumors to anticancer therapy. Fluorogenic dyes that emits fluorescent signal in the near-infrared region upon activation are also needed for *in vivo* optical imaging applications because of the deep tissue penetration of near-infrared light.

Experimental section

Synthesis of Ac-DEVD-R110-D-SAAC-Fmoc (2). To a solution of Ac-Asp(OBu-*t*)-Glu(OBu-*t*)-Val-CO₂H (288 mg, 0.56 mmol) in anhydrous 1:1 mixture of DMF and pyridine (5 mL) was added *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide (110 mg, 0.56 mmol). The solution was stirred at room temperature for 30 min, and then (Asp(OBu-*t*)-R110-Asp(OBu-*t*)-SAAC-Fmoc (**S2**, Supporting Information) was added (116 mg, 0.113 mmol). The solution was stirred at room temperature for 3 days. After removal of the solvent under vacuum, the residue was dissolved in ethyl acetate and washed with saturated NaHCO₃ (3 times) and brine (3 times), and the organic solution was dried over Na₂SO₄. Ethyl acetate was removed under vacuum, and the solid was treated with 50% TFA in dichloromethane for 30 min to remove the *t*-butyl-protecting groups. The product was purified by preparative HPLC to yield 50 mg (30%) of **2**. ¹H NMR (300MHz, CD₃OD): δ 1.00 (m, 2H), 1.45 (br, 2H), 1.65–2.50 (m, 10H), 3.25–3.35 (m, 20H), 4.09–4.62 (m, 10H), 6.60–6.82 (m, 2H), 7.10–8.10 (m, 22H), 8.62–8.64 (m 2H). High resolution MS calcd. for C₇₇H₈₀N₁₁O₂₀ (M + H) 1478.5503, found 1478.5657. HPLC: *t*_R = 18.1 min (solvent A, 0.1% TFA in water; solvent B, acetonitrile; gradient: 0–90% B in A over 30 min, flow rate 1.0 mL/min, C18 4.6 × 250 mm, 5-μm column).

Synthesis of Re-chelate (3). The Re chelate was prepared in quantitative yield by adding a stoichiometric amount of (NEt₄)₂[Re(CO)₃Br₃] in methanol to **2**. High resolution MS calcd. for C₈₀H₇₉N₁₁O₂₃Re (m/z) 1748.4908, found 1748.5588. HPLC: *t*_R = 20.9 min (solvent A, 0.1% TFA in water; solvent B, acetonitrile; gradient: 0–90% B in A over 30 min, flow rate 1.0 mL/min, C18 4.6 × 250 mm, 5-μm column). *t*_R = 30.9 min (solvent A, 0.1% TFA in water; solvent B, acetonitrile; gradient: 10-50-80% B in A over 36 min, flow rate 1.0 mL/

min, C18 4.6 × 250 mm, 5-μm column). The same chromatographic conditions were used in the analysis of ^{99m}Tc -chelate.

Synthesis of ^{99m}Tc -chelate (**4**). [$^{99m}\text{Tc}(\text{CO})_3(\text{H}_2\text{O})_3$] $^{+1}$ was prepared using the following general procedure: 1.0 mL of $^{99m}\text{TcO}_4^-$ (5 mCi) was added to commercially available Isolink carbonyl kits (Mallinckrodt, St. Louis, MO). The solution was heated in a water bath at 100 °C for 20 min. The solution was then cooled for 5 min and vented. Aliquots of 1N HCl (~120 μL) were added to the above solution to adjust the pH to 6–7 and to decompose any residual boranocarbonate. Into a sealed vial containing 10–20 μg of **2** in distilled water was added 0.5 mL of $^{99m}\text{Tc}-(\text{CO})_3(\text{H}_2\text{O})_3$ (2 mCi). The vial was heated for 30 min at 75°C. After the sample was cooled, the chelate were analyzed using HPLC. The labeling efficiency was greater than 95%. HPLC: t_R = 31.2 min. The following conditions were used for HPLC analysis: solvent A, 0.1% TFA in water; solvent B, acetonitrile; gradient: 10-50-80% B in A over 36 min; flow rate 1.0 mL/min; C18 4.6 × 250 mm, 5-μm column; UV detector: Agilent 1100 210 set at 254 nm; radioactivity detector: Bioscan flow-count radio-HPLC detector.

Fluorescence Microscopy. Human epithelial cells from colorectal adenocarcinoma (DLD1) were seeded (1×10^4 /well) in a 96-well plate 1 day before experiments. Apoptosis was induced by treating cells with TRAIL (100 μL, 250 ng/mL) for 2 h at 37°C. Both apoptotic and viable cells were incubated with **2** or **3** in (final concentration, 50 μM) for 2 h at 37°C. The cells were washed and stained with Annexin V–Alexa Fluor 594. After washing with PBS, the cells were transferred into Lab-Tek II chambered cover glass and visualized under Olympus Fluoview FV1000 confocal laser scanning microscope (FV1-ASW, Olympus, Melville, NY) equipped with fluorescein isothiocyanate (wavelength: 495/519 nm) and Texas red filters (wavelength: 589/615 nm). In separate wells, TRAIL was added in the presence of 50 μM of Ac-DEVD-CHO to inhibit caspase 3 activity.

Gamma Imaging and Biodistribution. For γ-imaging study, two male Balb/c mice (10–12 weeks old, weight 20–25 g) (Charles River, Wilmington, MA) were injected intravenously with a purified anti-Fas monoclonal antibody (10 μg/mouse) (Pharmingen, San Diego, CA). Two mice injected with PBS were used as a control group. Two hours later, ^{99m}Tc -chelate **4** [300 μCi (11.1 MBq), 20 μg peptide in 0.2 mL] was injected intravenously. Radionuclide imaging was acquired 120 min after the administration of ^{99m}Tc -chelate **4** with the following parameters: matrix, 512×512 pixels; energy peak, 140 keV (15%). For biodistribution study, Balb/c mice were divided into 2 groups consisting of 7 mice each. Mice in the first group were subjected to anti-Fas antibody treatment (10 μg/mouse); mice in the second group were injected with PBS solution (200 μL/mouse). Two hours after treatments, the mice were injected with ^{99m}Tc -chelate **4** intravenously through the tail vein at a dose of 2 nmol/mouse (50 μCi/mouse). Mice were killed with CO₂ exposure 2 h after radiotracer injection. The organs of interest were excised and weighed, and radioactivity was counted in a gamma counter. The stomach and intestines were emptied of food contents prior to radioactivity measurements. The percentage of injected dose per gram (% ID/g) was calculated by dividing the %ID/organ by the weight of the tissue.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Scheme 1.

Reaction scheme for the synthesis of Ac-DEVD-R110-D-SAAC-Fmoc (**2**) and its Re- and ^{99m}Tc -chelates **3** and **4**.

Figure 1.

Fluorescence images acquired with (A) untreated DLD1 cells, (B) TRAIL-treated DLD1 cells, and (C) TRAIL-plus-Ac-DEVD-CHO-treated DLD1 cells. Activation of fluorescent signal resulting from R110-D-SAAC-Re(CO)₃ fragment cleaved from **3** (green) was seen only in TRAIL-treated cells. Annexin V–Alexa Fluor 594–labeled apoptotic cells were pseudocolored red. Note that cells treated with both TRAIL and Ac-DEVD-CHO were stained with annexin V but not with **3**. Bar: 200 μ m. (D) Z-stacking of apoptotic cells stained with both annexin V and **3**. Fluorescent signal from cleaved **3** was seen in the cytoplasm of cells with intact membrane (arrows). Early stage apoptotic cells stained only with cleaved **3** but not with annexin V was also observed (arrowhead). Bar: 20 μ m.

Figure 2.

Activation of fluorescent signal in DLD1 cells by TRAIL. The DLD1 cells were sequentially treated with 100 μ L of the following agents at 37°C: (A) 25 μ M **2** or **3** for 2 h; (B) 25 μ M each compound for 2 h followed by TRAIL (150 ng/mL) for 2 h; (C) 25 μ M each compound for 2 h, drug-free culture medium for 2 h, and TRAIL (150 ng/mL) for 2 h; or (D) 25 μ M each compound for 2 h, drug-free culture medium for 24 h, and TRAIL (150 ng/mL) for 2 h. Note the reduced fluorescence activity with increasing incubation time in drug-free medium. Also note the activation of **2** and **3** in apoptotic cells in (B); the fluorescence intensities increased by 27- and 99-fold, respectively, upon TRAIL treatment.

Figure 3.

Gamma images after the administration of ^{99m}Tc -chelate **4** in Balb/c mice pretreated with anti-Fas monoclonal antibody (left) or with PBS as a control (right). The pseudocolor scale on the right side shows the signal intensity range. Arrows: liver; arrowheads: bladder.

Figure 4.

Fluorescence microscopic analysis of livers from mice treated with anti-Fas to induce apoptosis (upper panel) and with PBS as a control (lower panel). Tissues were harvested 4 h after drug treatment and 2 h after the intravenous injection of ^{99m}Tc -chelate **4**.

Activated caspase 3 was stained with anti-caspase 3 antibody (red), and cell nuclei were counterstained with Hoechst 33342 (blue). Signal from cleaved **4** is pseudocolored green. Bar 40 μm

Figure 5.

Comparison of radio-HPLC chromatograms of liver extracts from a PBS-treated mouse (A) and an anti-Fas-treated mouse (B). HPLC condition: solvent A, 0.01M NH_4OAc in water; solvent B, acetonitrile; gradient: 0–80% B in A over 40 min; flow rate 1.0 mL/min; C18 4.6 $\times$ 250 mm, 5- μm column. ^{99m}Tc radioactivity was detected with a NaI crystal radiodetector.