Complementary optical and nuclear imaging of caspase-3 activity using combined activatable and radio-labeled multimodality molecular probe

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Abstract. Based on the capability of modulating fluorescence intensity by specific molecular events, we report a new multimodal optical-nuclear molecular probe with complementary reporting strategies. The molecular probe (LS498) consists of tetraazacyclododecanetetraacetic acid (DOTA) for chelating a radionuclide, a near-infrared fluorescent dye, and an efficient quencher dye. The two dyes are separated by a cleavable peptide substrate for caspase-3, a diagnostic enzyme that is upregulated in dying cells. LS498 is radiolabeled with $^{64}$Cu, a radionuclide used in positron emission tomography. In the native form, LS498 fluorescence is quenched until caspase-3 cleavage of the peptide substrate. Enzyme kinetics assay shows that LS498 fluorescence in response to a specific molecular process.

2 Methods

The peptide backbone, metal chelating group DOTA, and NIR fluorescent dye$^6$ used in LS498 (Fig. 1), were assembled as described previously.$^2$ The NIR fluorescent quencher, IR dye QC-1 (Licor, Lincoln, Nebraska), was incorporated into the multifunctional peptide at room temperature in phosphate buffered saline (PBS) for 24 h. The purified LS498 (5 μg, 1.3 nmol) was radio-labeled by heating (60 °C, 0.5 h) with $^{64}$Cu (470 μCi) in aqueous buffer (100-mM NH4OAc, pH 5.5). The caspase-3 enzyme kinetic parameters were determined as previously described.$^4,5$

As a model of tumor-related caspase activity, plastic tubes containing $^{64}$Cu-LS498 (50 μL, 1 μM) and either 260-pM caspase-3 or 5-μM bovine serum albumin (BSA) in assay buffer were implanted subcutaneously in opposite flanks of the mouse. Multimodal imaging and region of interest (ROI) analysis of fluorescence (755-nm excitation, 830-nm emission), x-ray, and scintigraphy were performed with the IS4000MM (Carestream Health, New Haven, Connecticut) as previously described.$^5$
3 Results and Discussion

3.1 Development of Caspase-3 Activatable Probe for Dual Optical-Nuclear Imaging

A multifunctional peptide-based molecular probe LS498 (Fig. 1) was designed and prepared for use in this study. Because of the need to monitor the response of diseased tissue to treatment and the implication of caspase-3 in early cell death, LS498 was specifically developed to report the activity of this diagnostic enzyme. To accomplish this goal, we used a fluorescence resonance energy transfer (FRET) system, where the fluorescence of a NIR dye was efficiently quenched with wide-spectrum quencher dye. Since the tetrapeptide sequence, aspartic acid-glutamic acid-valine-aspartic acid (DEVD), is an established substrate for caspases-3, we incorporated this peptide sequence between the two dyes. Cleavage of the DEVD peptide results in fluorescence enhancement that can be used to monitor enzyme activity.

In the quenched state, it is not feasible to image the distribution of the molecular probe in tissue prior to enzyme cleavage. Moreover, lack of fluorescence enhancement may be due to inadequate delivery of the molecular probe to the target tissue, a situation that could result in false-negative outcomes. To address this issue, LS498 was labeled with $^{64}$Cu, a positron emitter with half-life of 12.8 h. This radionuclide is widely used in PET imaging of molecular processes in small animals and humans. After HPLC purification, LS498 was labeled with $^{64}$Cu at high specific activity (360 Ci/mmol) and purity (>99%). In previous work with $^{64}$Cu-DOTA-c(RGDyK), specific activities ranged from 200 to 500 Ci/mmol and receptor-specific tumor accumulation allowed for $\alpha_\beta$-positive tumor visualization by small animal PET. Therefore, the observed specific activity in LS498 is adequate for receptor targeted tumor imaging in vivo.

3.2 Caspase-3 Enzyme Kinetics

The feasibility of applying a reporter of proteolytic activity to in vivo imaging depends on how fast the substrate is processed by the enzyme before being washed away from the target site. The kinetic parameters, $k_{\text{cat}}$ and $K_M$, are measurable indicators of how well a substrate is processed by an enzyme. Our study shows that LS498 was readily cleaved by caspase-3 and displayed classic Michaelis-Menten kinetics (Fig. 2) with enzyme kinetic parameters $k_{\text{cat}}$ and $K_M$ of $0.55 \pm 0.01 \text{ s}^{-1}$ and $1.12 \pm 0.06 \text{ M}$. Respectively. The observed $k_{\text{cat}}$ and $K_M$ compares favorably with standard substrates Ac-DEVD-AMC ($k_{\text{cat}} = 0.75 \text{ s}^{-1}$, $K_M = 9.7 \text{ M}$) and Ac-DEVD-pNA ($k_{\text{cat}} = 0.55 \pm 0.01 \text{ s}^{-1}$, $K_M = 11 \text{ M}$). The $k_{\text{cat}}/K_M$ ratio, which measures the performance constant of an enzyme for a substrate, was found to be $4.91 \times 10^2 \text{ s}^{-1}$.

3.3 In-Vivo Imaging of LS-498 Distribution and Model of Caspase-3 Activation

LS498 was designed to prevent fluorescence emission prior to activation by caspase-3. To assess if this goal was met, we compared the fluorescence emission of LS498 to a control analog. The control peptide lacks a quencher dye, thereby reporting the maximum fluorescence intensity of a completely cleaved LS498. Intravenous injection of the two molecular probes in healthy mice showed that fluorescence was hardly detectable in the mouse injected with LS498 and remained lower than that of the control probe up to 24-h postinjection. Immediately after injection (30 min), the fluorescence intensity was at least ten-fold less for LS498 relative to control [Fig. 3(a)]. After 24 h, both molecular probes had similar low fluorescence intensity (data not shown), approaching the detection limit of our imaging system. Interestingly, the kidneys were visible at 24 h in both mice, suggesting a possible degradation of LS498 after prolonged retention in this organ.  

![Fig. 1](image1.png)  
**Fig. 1** Structure of caspase-3 activatable optical-nuclear molecular probe (LS498).

![Fig. 2](image2.png)  
**Fig. 2** Nonlinear fit of initial velocity with respect to substrate concentration and the corresponding Lineweaver-Burk plot (inset). Substrate concentrations varied from 270 nM to 35 \mu M.
achieved by incorporating cell-permeating peptides to LS498 or via a receptor-mediated endocytosis mechanism that is coupled with endosomal disrupting peptides.

In conclusion, this work summarizes our ongoing efforts toward the development of multimodality imaging agents for combined optical and nuclear molecular imaging of diseased tissues. In this study, the always “on” nuclear signal is useful for quantifying and localizing the distribution of the probe, while the optical imaging reports the functional status of a target molecular event. Both in-vitro and in-vivo results demonstrate the feasibility of using this approach to image molecular processes. Although we used caspase-3 as a model for this study, the complementary contrast strategy is applicable to imaging the functional status of most enzymes. Studies are in progress to demonstrate the utility of this new imaging strategy in animal disease models.

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References