Cy5-labeled aza-peptidyl Pro-Asn epoxide

LP-1

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Chemical name:	Cy5-labeled aza-peptidyl Pro-Asn epoxide	
Abbreviated name:	LP-1	
Synonym:	Legumain probe 1	
Agent Category:	Compounds	
Target:	Legumain/Asparaginyl endopeptidase	
Target Category:	Enzyme	
Method of detection:	Optical imaging	
Source of signal / contrast:	Cy5	
Activation:	No	
Studies:	• *In vitro* • Rodents	No structure is available.

Background

[PubMed]

Legumain or asparaginyl endopeptidase is a lysosomal cysteine protease that cleaves protein substrates on the C-terminal side of asparagine and, occasionally, aspartic acid residues (1-3). Like other proteases, legumain is synthesized as an inactive zymogen and is activated under acidic conditions in an autocatalytic process (4, 5). Legumain is expressed in diverse cell types and plays a key role in MHC class II–mediated antigen presentation, matrix degradation, and cysteine cathepsin processing (6, 7). Legumain has also been shown to be implicated in various pathological conditions including parasite infection, atherosclerosis, and tumorigenesis (1, 8-10). In mouse models of cancer, knockdown of legumain expression results in a marked decrease in tumor growth and metastasis, while overexpression of legumain leads to increased tumor migration, invasion, and metastasis

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(1, 11). In human tumors such as carcinomas of the breast, colon, and prostate, legumain has been shown to be overexpressed, while in normal organs like the kidney, liver, and spleen, only a limited quantity of legumain is detectable (10, 11).

Activity-based probes (ABPs) provide a highly versatile means to monitor *in vivo* protease function and regulation. ABPs utilize irreversible inhibitors that can covalently modify the active site of a protease in an activity-dependent fashion (3, 12, 13). To date, most ABPs have been designed to target cysteine cathepsins and caspases, and only a few legumain-specific probes have been reported in the literature. A typical legumain-specific probe consists of a peptide scaffold, a reactive functional group, and a reporter (1, 14-18). The Cbz-Ala-Ala-Asn peptide is commonly applied as a scaffold, which is designed on the basis of the sequence of a known substrate of legumain. Reactive functional groups, such as aza-Asn halomethylketones, aza-Asn epoxides, and aza-Asn Michael acceptors, have been used to make irreversible legumain binding. *In vitro* studies have shown that these probes have a high potency against legumain, but they also cross-react with cathepsins and caspases. The *in vivo* potency and specificity of these probes are largely unknown.

Investigators at Stanford University developed a class of aza-Asn epoxide ABPs with fast kinetic properties and increased selectivity for legumain for use in *in vivo* imaging studies (1, 3, 16, 19). Some of the probes were also tagged with a series of cell-permeabilizing carriers. These legumain-specific probes showed a high potential for use in imaging active legumain both in normal tissues and solid tumors. In this chapter, legumain probe-1 (LP-1), a Cy5-labeled aza-Pro-Asn epoxide probe, is introduced (1).

Related Resource Links:

1) Gene information of legumain

2) Legumain-related resource in OMIM

Synthesis

[PubMed]

For comparative analysis, a group of probes have been synthesized and described in detail in several reports (1, 3, 7, 16, 19). These probes included LP-1, LP-1 ctrl, legumain probe-0 (LP-0), legumain inhibitor-1 (LI-1), legumain inhibitor-0 (LI-0), tLP-1, r8 LP-1, penetratin LP-1, and cholesterol LP-1. LP-1 ctrl was the control version of LP-1, which lacks the reactive epoxide group and does not covalently bind to legumain. LP-0 was the Cy5-labeled version of the probe Biotin-PD-AOMK which was developed previously in the same laboratory (18). LI-1 and LI-0 were the acetyl-capped versions of LP-1 and LP-0, respectively. Therefore, LP-1 and LI-1 were aza-epoxide inhibitors, whereas LP-0 and LI-0 were acyloxymethyl ketone (AOMK) inhibitors. The probes of tLP-1, r8 LP-1, penetratin LP-1, and cholesterol LP-1, were the enhanced versions of LP-1, which were generated by conjugating LP-1 with cell-penetrating carriers of tat peptides, cell-penetrating peptides (octa-arginine (r8) and penetratin), and cholesterol, respectively (1). All final products

were >97% pure and characterized by either high-resolution mass spectrometry or matrix-assisted laser desorption ionization time-of-flight mass spectrometry. The amounts of impurities were estimated to be minimal (3-7%) (1).

In Vitro Studies: Testing in Cells and Tissues

[PubMed]

Lee and Bogyo evaluated the potency and selectivity of LI-1 (as a representative of aza-epoxide inhibitors) and LI-0 (as a representative of AOMK inhibitors) against recombinant legumain, cathepsins B and L, and caspase-3 (1). Inhibition studies on the half-maximal inhibitory concentration (IC_{50}) showed that LI-1 (IC_{50} = 11.5 nmol) was 70-fold more potent than LI-0 (IC_{50} = 704 nmol) against legumain, whereas both exhibited very weak activity against cathepsin B (IC_{50} = 390 µmol for LI-1 and >1 mmol for LI-0) and cathepsin L (IC_{50} = 220 µmol for LI-1 and >1 mmol for LI-0). LI-0 demonstrated a significant inhibitory effect (IC_{50} = 2.8 µmol) on caspase-3, whereas LI-1 showed nearly no inhibition (IC_{50} = 890 µmol). LI-1 inhibited legumain ~50-fold faster than LI-0 did. These results suggested that incorporation of P1 Asn *via* an aza-peptidyl scaffold greatly enhanced the efficiency and specificity of aza-epoxide scaffolds compared with that of the P1 Asp AOMK scaffolds. The rapid inhibition kinetics of the aza-Asn epoxide scaffolds may be advantageous for *in vivo* imaging.

Lee and Bogyo then verified the labeling of active legumain with LP-1 and LP-0 probes in intact NIH-3T3 and RAW 264.7 cells (1). After incubation of the cells with LP-1 and LP-0, SDS-PAGE gel analysis of the cell extracts showed that both probes selectively labeled the active legumain in NIH-3T3 fibroblasts. LP-1 labeled active legumain more efficiently than LP-0, showing an overall stronger labeling signal. However, both probes had some degree of cross-reactivity toward lysosomal cathepsins in RAW 264.7 macrophages. These data suggested that even though probes may have very low potency toward a particular protease target *in vitro*, when added to cells that actively accumulate the probes in their lysosomes, such as macrophages, they are able to react with other abundant proteases. Furthermore, when RAW 264.7 cells were pretreated with legumain inhibitors, more intense cathepsin labeling was observed. The investigators explained this finding by the fact that cathepsins are substrates of legumain and legumain inhibition could result in increased levels of cathepsins and, therefore, increased nonspecific labeling by the legumain probes.

On the basis of these results, LP-1 was selected for *in vivo* imaging studies because it has faster kinetics and overall higher potency, resulting in less background labeling of cathepsins and caspases.

Animal Studies

Rodents

[PubMed]

The feasibility of LP-1 for *in vivo* imaging of active legumain has been tested with the C2C12/Hras1 tumor xenograft model (1). After injection of the LP-1 *via* tail vein of mice (n = 3), LP-1 rapidly accumulated in tumor tissues, whereas the control probe LP-1 ctrl did not show such accumulation (n = 2 mice). The control probe LP-1 ctrl lacks the reactive epoxide group and therefore does not covalently bind to legumain. The maximum signal/background ratio for LP-1 was reached at ~90 min after injection. The specific legumain signal declined over time but remained significantly higher than the signal observed for LP-1 ctrl, even at the later time points. These data suggested that LP-1 has fast binding and clearance properties. *Ex vivo* imaging of the tumors and SDS-PAGE analysis of the tumor extracts at the end of the imaging time course confirmed the *in vivo* selective labeling of legumain by LP-1. The levels of active legumain by imaging directly correlated with the intensity of legumain labeling by SDS-PAGE analysis, which suggests that LP-1 is selective toward legumain *in vivo* and has virtually no off-target labeling.

Lee and Bogyo further comparatively analyzed LP-1 and the corresponding versions with cell-penetrating carriers (1). In cultured cells, all the carrier-conjugates showed increased cellular uptake and resulted in stronger labeling, but also showed more cross-reactivity with other proteases compared with LP-1. In animal models, enhanced cellular delivery of the conjugates adversely affected their overall circulation, and caused increased association with tissues other than the target tumors, resulting in less useful imaging reagents. These results indicated that legumain ABPs are more effective as free probes that do not contain a carrier peptide.

Lee and Bogyo concluded that they developed a highly potent and selective probe, LP-1, toward active legumain. The favorable reactivity and clearance of LP-1 could result in a high contrast in tumors soon after probe injection. This probe can be used for noninvasive imaging studies of active legumain in whole tissues or cells (1).

Other Non-Primate Mammals

[PubMed]

No references are currently available.

Non-Human Primates

[PubMed]

No references are currently available.

Human Studies

[PubMed]

No references are currently available.

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