Carbobenzoxy-capped Phe-Lys(Cy5)-acyloxymethyl ketone

GB123

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Chemical name:	Carbobenzoxy-capped Phe-Lys(Cy5)-acyloxymethyl ketone	
Abbreviated name:	GB123	
Synonym:	Activity-based probe (ABP)	
Agent Category:	Compounds	
Target:	Cysteine cathepsin	
Target Category:	Enzyme	
Method of detection:	Optical imaging	
Source of signal / contrast:	Cy5	
Activation:	No	
Studies:	• *In vitro* • Rodents	Click on the PubChem for additional information of the relevant substances.

Background

[PubMed]

The human cysteine cathepsins have 11 members (cathepsins B, C, H, F, K, L, O, S, V, W, and X/Z) and share a conserved active site that is formed by cysteine, histidine, and asparagine residues (1-5). Cathepsins B, L, H, F, O, X/Z, and C are expressed ubiquitously, whereas the expression of cathepsins S, K, W, and V are relatively organ-limited. Cysteine cathepsins interact with other proteases (aspartic, metallo, serine, and threonine) in a cascade-like manner, involving various physiological processes, including protein degradation, precursor protein activation, MHC-II–mediated antigen presentation, bone

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remodeling, keratinocytes differentiation, hair follicle cycle, reproduction, and apoptosis (6, 7). Increased expression and activity, and relocalization to the plasma membrane of cysteine cathepsins are associated with the pathogenesis of a number of human diseases such as cancer, atherosclerosis, and neurodegenerative diseases, and changes related to cysteine cathepsins have been shown to be of diagnostic and prognostic value for the diseases (2, 8-11).

Significant efforts have been made in developing optical molecular probes of protease activity (4, 7, 12, 13). These probes are either substrate-based or activity-based, providing a readout of the enzyme activity rather than simple protein abundance. Substrate-based probes (SBPs) are designed using fluorescent peptide sequences tethered to a large polymer or dendramer backbone, and these SBPs are internally quenched by the high density of fluorophores loaded onto the backbone structure. Similar to conventional enzymatic tests, SBPs appear to be less reliable because of overlapping substrate specificities, enzymatic activity instability, and interactions with endogenous inhibitors. Activity-based probes (ABPs) label target proteases through the formation of a covalent bond with the active site cysteine. The selectivity of an ABP is controlled by both its peptide selectivity sequence and reactive functional group. The fluorescent reporter allows probe-labeled cathepsins to be directly visualized. Because ABPs tend to be small molecules, the *in vivo* half-lives of ABPs are relatively short, which results in the production of high-contrast images. A drawback of using a covalent probe is the lack of signal amplification because the target proteases are inactivated upon binding the probe. However, sufficient levels of the active proteases have been found to exist in tumor tissues, which allows the generation of reasonable contrast images using noninvasive methods (4, 7, 12).

Blum et al. synthesized a group of near-infrared fluorescent activity-based probes (NIRF-ABPs) for noninvasive optical imaging of cysteine protease activity (4, 7, 12, 14). These probes can be subgrouped into quenched ABPs (qABPs) (e.g., GB117, GB119, GB135, and GB137) and nonquenched ABPs (e.g., GB111, GB123, and GB138). In short, the probes consist of a reactive group of peptide acyloxymethyl ketone (AOMK) that targets diverse members of cysteine cathepsins. The ketone in AOMK reacts with the cysteine in the enzyme active site and produces a stable thiomethyl ketone adduct. The covalent binding involves the loss of the acyloxy group of AOMK. Thus, a probe carrying a fluorescent reporter group on its peptide scaffold and a highly efficient quenching molecule attached to the acyloxy-losing group should result in a quenched probe that only becomes fluorescent upon covalent binding with an enzyme. In addition, a spacer is designed to reduce the steric congestion between the reporter and the fluorescence quencher. Blum et al. have shown that the NIRF-ABPs are nontoxic to cells, reasonably water soluble, potentially valuable as imaging agents for disease diagnosis, and powerful tools for *in vivo* preclinical and clinical testing of small-molecule therapeutic agents. Although NIRF-ABPs present different features regarding stability, specificity, and kinetics, the quenched probes can be used to image specific protease activity at considerably earlier time points than can be used for substrate-based methods or nonquenched ABPs. However, high levels of signal in large organs with high cathepsin activity such as liver, kidney, and

spleen make activity-based imaging of specific locations within the central body cavity difficult. In this chapter, the synthesis and analytic results of nonquenched ABP GB123 were introduced. GB123 is the Cy5 version of the first generation of nonquenched ABP GB111.

Related Resource Links:

1) Cysteine cathepsin imaging-related chapters in MICAD:

- Substrate-based probes: Cy5.5-PL-MPEG, Cy5.5-GHPGGPQK(Fitc)C-PL-MPEG (Cy5.5-CatK-PGC),
- Activity-based probes: GB137

2) Gene and protein information of human cathepsins

3) Cathepsin-related resource in OMIM

4) Cathepsin-related substances in PubChem

Synthesis

[PubMed]

GB123 is the Cy5 version of the first-generation nonquenched ABP GB111, which has been introduced in another chapter of MICAD (4, 12, 14). Briefly, the fully protected carbobenzoxy-capped Phe-Lys dipeptide was synthesized (85% purity and 99% yield relative to resin loading) and then converted to bromo-methyl ketone (BMK, 91% yield). AOMK was obtained by converting the BMK *via* *N*-trityl–protected glycine (25% yield). The final product GB123 was generated by coupling Cy5 fluorophore on the side chain of lysine after removal of the *t*-butoxy carbonyl protecting group (48% yield). GB125 (Cy5-*N*-benzyloxycarbonyl-Phe-Lys) was also generated as a control probe in a similar manner; however, GB125 lacked the AOMK reactive group and the Cy5 was coupled to the *N*-ε-free amine (69.3% yield). The molecular weights were 1,256.5 and 1,109.4 for GB123 and GB125, respectively (4).

In Vitro Studies: Testing in Cells and Tissues

[PubMed]

Blum et al. tested the probes for their sensitivity to serum exposure by preincubating the probes for either 1 h or 4 h before adding them to the monolayer of NIH-3T3 cells to label target proteases (4). Cell labeling showed that GB123 was insensitive to serum even after preincubation for 4 h, but GB119 (the quenched version of the GB123 probe) completely lost its ability to label the target proteases even after preincubation for 1 h. The increased serum sensitivity of GB119 has been confirmed to be caused by the presence of a glycine spacer adjacent to the reactive carbon methylene group of the AOMK.

To verify the cell permeability and labeling specificity of GB123, intact C2C12/Hras1 cells were preincubated with either JPM-OEt, a general papain-family inhibitor, or vehicle control before cell labeling with GB123 or GB125. The specific probe GB123 showed punctate labeling of lysosomal compartments that was overlapped with the acidotropic lysosomal marker LysoTracker. Pretreatment with JPM-OEt completely blocked GB123 staining, which suggested that the signal was due to a specific modification of cysteine cathepsins. On the contrary, while the control probe GB125 accumulated in cells without washout (data not shown), after washout there was no labeling with GB125, which confirmed that the lack of labeling was not caused by a loss of cell permeability. These data indicate that both probes freely penetrated cells, but only GB123 was retained as a result of the irreversible modification of lysosomal cysteine cathepsins (4).

Animal Studies

Rodents

[PubMed]

To decide on an optimal cell line for use in tumor imaging studies, Blum et al. profiled the cysteine cathepsin activities in a number of commonly used cancer cell lines (4). In all cell lines tested, specific labeled proteases in the 20–30 kDa size range were detected with SDS-PAGE gel analysis. The highest cathepsin activity was observed in the mouse skeletal myoblast cell line (C2C12) retrovirally transduced with the *Hras1* oncogene (C2C12/Hras1). A similar labeling pattern, although with reduced intensity, was observed in the human breast cancer cell lines MDA-MB-231, MDA-MB-435, and MDA-MB-231 MFP. These lines were selected for imaging studies because they express similar cathepsins but show marked differences in overall activity.

Imaging studies were performed in BALB/c nude mice bearing subcutaneous MDA-MB-231, MDA-MB-435, or C2C12/Hras1 tumor grafts (n = 2–4 mice/group) (4). After tail vein injection of GB123, the fluorescent probe rapidly circulated throughout the animal, and high fluorescent signals were seen in virtually all tissues, including the tumors. Thereafter, the investigators observed that the signal faded continuously over most regions of the body but remained elevated in the tumors. The control probe GB125, on the other hand, was rapidly cleared and no fluorescent signal above background was observed by the 5-h time point. After imaging, tissues were collected for biochemical analysis. With SDS-PAGE gel analysis, three predominant labeled proteins were observed in the 20–30 kDa size range, which were similar to the labeled cathepsins observed in the parent cell lines. The 30- and 23-kDa proteins were confirmed to be the cathepsin B and the heavy chain form of cathepsin L, respectively. Although Blum et al. cannot determine the identity of the 25-kDa protein with available antibodies, they consider it to be a cysteine cathepsin given that its labeling can be blocked by treatment of animals with specific inhibitors. The overall labeling intensity of the cathepsins observed with gel analysis strongly correlated with the intensity of the signal observed in the whole-body

images. These results suggest that the signal observed in tumors and other tissues results from specific probe modification of active cysteine cathepsins.

To further confirm that the signals observed in whole-body images were due to protease activity, Blum et al. pretreated the animals with intraperitoneal injection of JPM-OEt to reduce protease activity before imaging (4). However, all of the attempts to reduce cysteine cathepsin activity in tumors failed in both imaging and SDS-PAGE analysis of the tissue extracts. Lack of inhibition is explained by the nature of grafted tumors, which may generally be less accessible to this class of small-molecule inhibitors than tumors that develop *in situ* in their native microenvironment. Therefore, the investigators switched to the MDA-MB-231 MFP cells and a broad-spectrum inhibitor K11777, which has more favorable cell permeability properties. In addition, the mice were pretreated for 5 days before imaging. Imaging showed that the specific signal in tumors at the 10-h time point was reduced by 60–80% in mice treated with K11777 relative to control mice. SDS-PAGE analysis of the tissue extracts also showed reduced signal intensity by 50–80% in the K11777-treated mice. Labeling profiles in all tissues from both treated mice and control mice demonstrated the same pattern of active cathepsins that was observed previously.

These data support the notion that NIRF-ABPs provide a fluorescent readout of protease activity in a living animal and that the modulation of this activity using small-molecule therapeutics can be monitored with direct, whole-body imaging methods (4, 7, 12, 14).

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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