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Accepted Article

Title: Intrinsically quenched fluorogenic probe provides selective and rapid detection of cathepsin L activity

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To be cited as: *ChemMedChem* 10.1002/cmdc.202000823

Link to VoR: https://doi.org/10.1002/cmdc.202000823

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COMMUNICATION

Dual-mechanism quenched fluorogenic probe provides selective and rapid detection of cathepsin L activity

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Abstract: Cathepsin L (CTL) is a cysteine protease demonstrating upregulated activity in many disease states. Overlapping substrate specificity makes selective detection of CTL activity difficult to parse from its close homologue CTV and the ubiquitous CTB. Current probes of CTL activity have limited applications due to either poor contrast or extra assay steps required to achieve selectivity. We have developed a fluorogenic probe, **CTLAP**, which displays good selectivity for CTL over CTB and CTV while exhibiting low background fluorescence attributed to dual quenching mechanisms. **CTLAP** achieves optimum CTL selectivity in the first 10 min of incubation suggesting that it is amenable for rapid detection of CTL, even in the presence of competing cathepsins.

The cathepsin family of lysosomal proteases includes 11 members associated with various pathological conditions[1] including cancer[2]. Although their redundancy in certain contexts has been described,[3] they exhibit distinct tissue distribution[4] and singular cathepsins serve as useful biomarkers for cancers[5] or other diseases.[6] Among many roles, CTL is involved in cancer progression by directly degrading the extracellular matrix (ECM) components[7] (including laminin[8] and some forms of collagen[9]) while also activating other ECM-degrading enzymes, such as heparanase, to promote an aggressive phenotype.[4d, 10] Cathepsin L levels in serum and urine have been shown to increase in cancer patients compared to healthy patients and tend to correlate with tumor grade, invasive potential, and metastatic spread.[11] Likewise, CTL contributes to atherosclerosis by degrading the medial elastica laminae and enabling smooth muscle cell and leukocyte migration into atherosclerotic plaques,[12] and serum CTL levels correlate with the presence of coronary artery stenosis.[13]

Since enzymatic activity does not always correlate with expression at the protein[14] or mRNA level,[15] it is equally critical to detect the enzymatic activity of CTL.[16] However, detection of a single cathepsin is necessary to parse relative contribution in contexts involving multiple cathepsins. For instance, CTB, CTL, and CTS are simultaneously involved in atherosclerosis,[17] but each contributes to the process through different mechanisms.[12] It is also crucial in contexts where specific cathepsins may have opposing effects; for example, in some contexts CTL deficiency can be tumorigenic,[18] a correlation believed to be unique to this cathepsin.[19] Activity of CTL should be considered distinct even from that of its close homologue CTV, as such closely related cathepsins exhibit markedly different activity against certain endogenous substrates.[20]

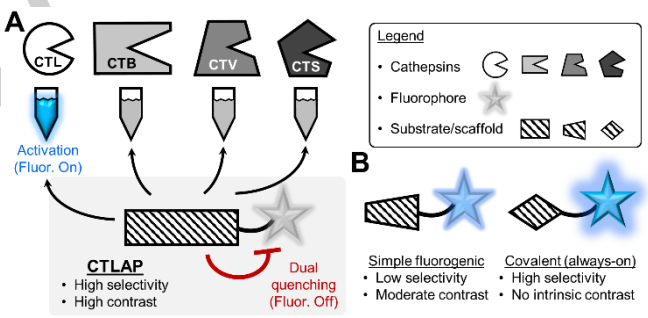

Figure 1. A) **CTLAP** has intrinsically quenched emission, leading to high contrast. leading to selective detection of CTL over related cathepsins (CTB, CTV, CTS). **B**) Alternative designs of fluorescent probes show reduced selectivity or contrast.

The development of selective cathepsin probes is challenging due to overlapping substrate selectivity among cathepsins. Detection of CTL activity commonly uses simple fluorogenic substrates such as **Z-FR-AMC**, however this probe exhibits off-target activation by CTB and other proteases.[21] This type of probe, in which the fluorophore is masked by a substrate sequence or quenching group, can provide high contrast when such groups are removed by the target enzyme.[22] However, its low selectivity often requires inclusion of an exogenous inhibitor[5a, 6b, 12, 23] or pre-incubation under harsh conditions (4 M urea for 30 min)[24] to deactivate competing enzymes (namely CTB) to achieve selectivity for CTL. A probe with greater selectivity for CTL could eliminate the need for such steps, enabling less intrusive and more convenient real-time detection of CTL activity. These concerns are mitigated with highly selective probes, which have been discovered for CTL activity by screening combinatorial substrate libraries,[14c, 25] typically requiring generation and testing of thousands to hundreds of thousands of compounds.[26] While highly selective, these probes often lack fluorescence quenching mechanisms[22a-c] and therefore provide bright labeling but poor contrast until inactive

probes are washed out of the sample,[17, 25a, 27] compromising their use in rapid and single-step detection.

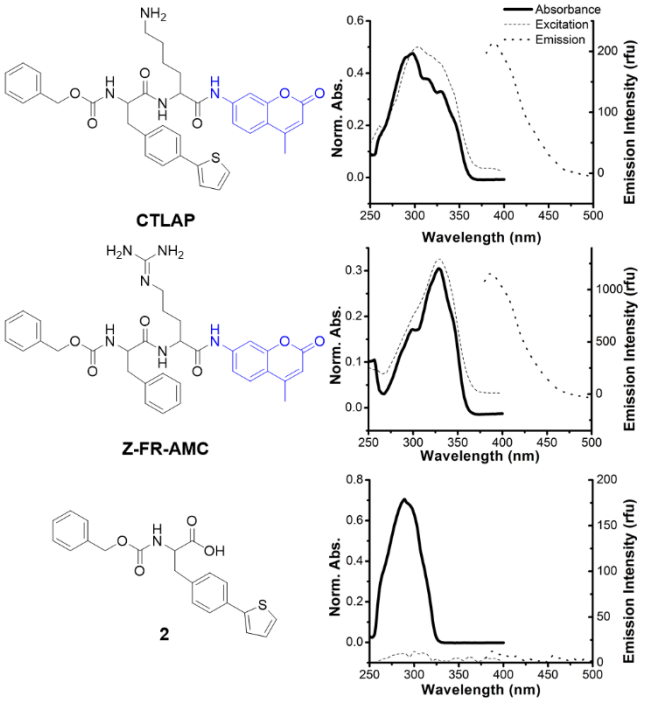

Figure 2. Structures of **CTLAP**, **Z-FR-AMC**, and compound **2**, along with their absorption (solid line), emission, (large dot), and excitation (small dot) spectra. Emission spectra collected with excitation at 350 nm. Excitation spectra collected with emission at 445 nm.

We report a cathepsin-L activable probe (**CTLAP**) that experiences dual quenching[28] by its own substrate structure, precluding the need to incorporate exclusive quenching groups in order to enhance emission turn-on (Figure 1).[29] The probe exhibits low background signal and over 120-fold turn-on ratio while demonstrating high selectivity for CTL over competing cathepsins within the first 10 min of incubation. These attractive characteristics bridge the gap in current CTL probes, providing a combination of high selectivity and high contrast.

To design **CTLAP**, we selected inhibitor scaffolds providing selectivity for CTL over multiple other cathepsins.[29a, 30] Selectivity against multiple cathepsins is desirable because it increases the potential applications of a CTL probe, as each pathological model will exhibit a unique set of competing cathepsins.[7c, 31] In seeking attractive inhibitor scaffolds, one notable result was a 2-phenylthiophene residue that provided selectivity for CTL over multiple related cathepsins[30b]. While examination of the cathepsin binding pockets elicits no obvious explanation for the selectivity provided by this residue, it appears to be a privileged CTL-selective scaffold even among extended aromatic groups.[29g, 30b] We therefore constructed **CTLAP** (Figure 2) by incorporating the 7-amino-4-methylcoumarin (AMC) fluorophore with the peptide sequence containing this 2-phenylthiophene residue. Inclusion of a labile linker between the fluorophore and inhibitor scaffold was avoided, as such linkers have been shown to shift selectivity away from CTL and toward CTB.[32] The probe was accessed by solution-phase peptide synthesis using an Fmoc/Boc strategy (see Supplementary Information and Scheme S1). The novel compound **2** was synthesized from 4-iodophenylalanine by *N*-carboxybenzylation followed by Suzuki coupling with thiophene-2-boronic acid. Intermediate **2** underwent amide coupling with H-Lys(Boc)-AMC and subsequent Boc deprotection afforded CTLAP.

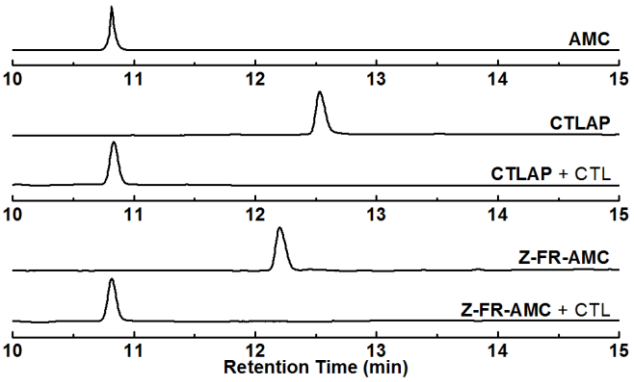

Figure 3. HPLC traces of CTL activation of **CTLAP** and **Z-FR-AMC** to generate **AMC**. Detection wavelength = 325 nm.

We examined the spectral characteristics of **CTLAP** (Figure 2). The absorption spectrum (Figure 2, solid) exhibits three local maxima at 298 nm, 316 nm, and 330 nm. The 330 nm peak is attributed to the attached AMC group, consistent with the reported λ_{max} value for 7-amido-4-methylcoumarin.[33] The peak at 298 nm is attributed to the peptide backbone, with its greater prominence reasonably achieved if the absorbance curves of **Z-FR-AMC** and **2** are summed. The peak at 316 nm is likely also the result of spectral overlap between these two portions of the probe. The emission curve (Figure 2, dotted) has a maximum around 390 nm, again consistent with literature.[33] The excitation curve at 445 nm emission (Figure 2, dashed) mirrors the absorbance curve almost identically; this suggests that all portions of the molecule contributing to the absorption in this range will also contribute to emission. The excitation spectrum of masked AMC alone (in the form of **Z-FR-AMC**) lacks the prominent band at 290 nm, while the excitation of compound **2** under identical conditions shows no emission. This suggests that higher-energy absorbance by 2-phenylthiophene can also contribute to masked AMC emission, although this is not likely under the conditions typically used for AMC-based fluorescence assays (excitation around 350 nm). We next tested whether cathepsins could activate **CTLAP** and release the AMC reporter (Figure 3). Incubation with cathepsins resulted in consumption of **CTLAP** and generation of a new peak with retention time of 10.7 min, consistent with that of AMC, while the emission spectrum shifted to a maximum of 450 nm, also consistent with the generation of AMC (Figure S1).

We investigated whether **CTLAP** would retain selectivity for CTL against the most similar competing cathepsins: CTB, CTV, and CTS (Figure 2). These cathepsins were selected based on their similarity to CTL in substrate specificity or in their common presence in applications of CTL detection. CTV is a close homologue of CTL (being initially characterized as "cathepsin L2"[4b]) and the challenge of its remarkably overlapping substrate specificity is mitigated only by its isolated expression to a few tissues. CTB is the most notable competitor of CTL, being the most highly and ubiquitously expressed cathepsin.[4d] CTS was also examined due to its organization close to CTL in sequence homology.[25d] We incubated **CTLAP** with each cathepsin and monitored the increase in fluorescence emission over time to determine selectivity. Activation of **CTLAP** by CTL completed the initial velocity phase within the first 10 min, followed by formation of a plateau (Figure S2). In contrast, the other cathepsins remained in their initial velocity phase throughout the first 1 h of the assay. The selectivity for CTL was determined from the relative signal intensity generated by each cathepsin, compared to that from CTL (Figure 4A,B). **CTLAP** generated low off-target signal from each competing cathepsin tested: after the first 10 min

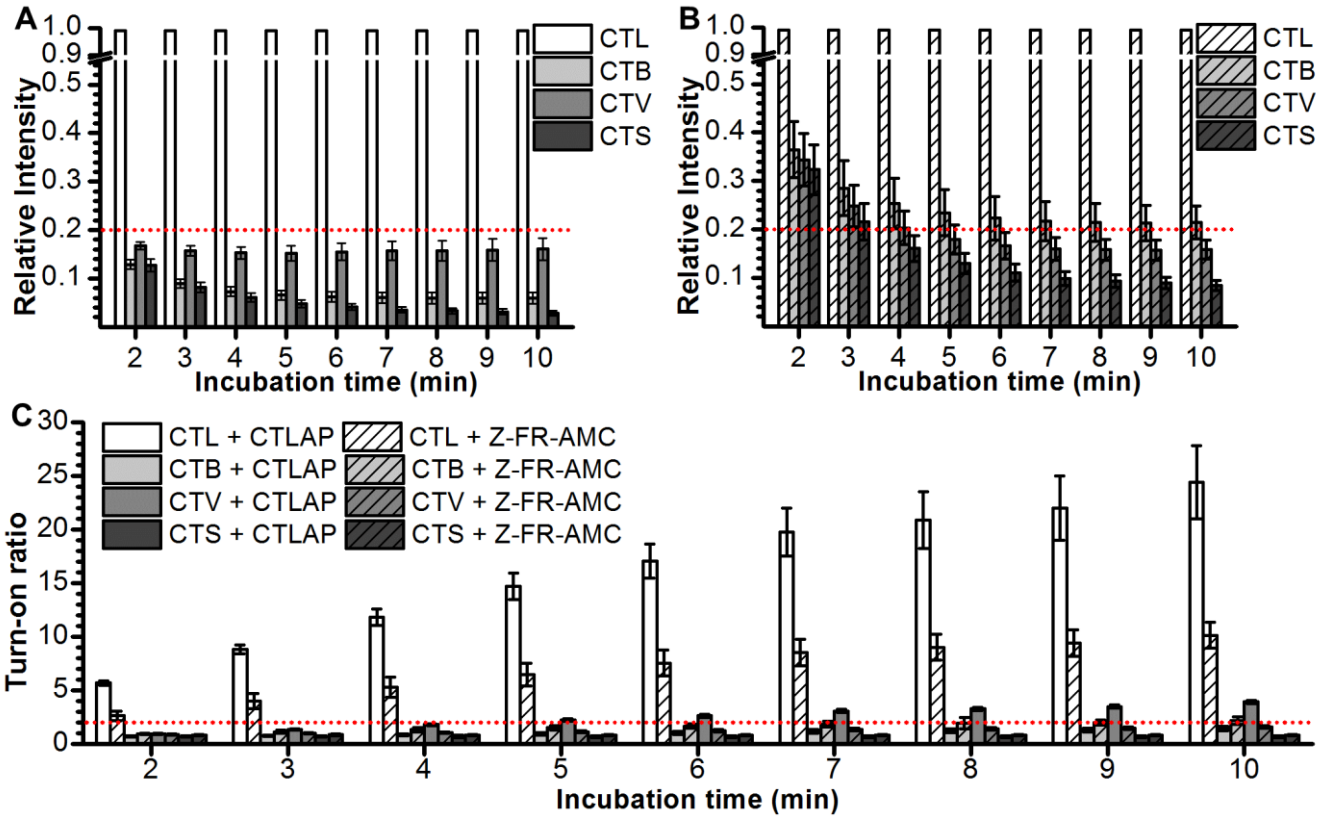

Figure 4. Selectivity of CTLAP and Z-FR-AMC for cathepsins. Relative signal intensity generated by cathepsins with (**A**) **CTLAP** and (**B**) **Z-FR-AMC** at pH 6.5. Horizontal red line marks 0.2 (20%) relative signal. (**C**) Turn-on ratio of probes within 10 min, pH 6.5.

of incubation, the ubiquitous CTB generated only 6% relative signal, while the highly homologous CTV generated only 15% relative signal (Figure 4A, Table S1). This translates to a selectivity profile of 20-fold for CTB and 6-fold for CTV. When **Z-FR-AMC** was used, CTB generated at least 20% relative signal during this time window, while CTV maintained at least 16% signal (Figure 4B, Table S1), giving a selectivity profile of 6-fold for CTV but only 5-fold against CTB, the more common competitor. This suggests **CTLAP** is more suitable for applications similar to those of **Z-FR-AMC**, including determination of CTL activity in clinical samples.[5a, 6b] While this selectivity profile is eclipsed by highly selective CTL probes,[34] few of these contain fluorescence contrast mechanisms, again revealing that **CTLAP** strikes a balance between favorable CTL selectivity and detection contrast.

Cathepsins are typically inactive at pH 7.4 but active in a broad range of acidic pH,[4c, 29b, 35] consistent with their localization within lysosomes and sustained activity in the acidic extracellular space in tumors. Therefore, we tested the response of **CTLAP** to cathepsin activity at pH 5.0 and 6.5, reproducing common assay conditions for cathepsin activity while probing for possible differences in activity under these conditions (Figure S2). CTL exhibited faster turnover of **CTLAP** at pH 6.5 than at pH 5.0, suggesting 6.5 to be the optimal pH for detection with **CTLAP** (Figure S2A,C). Significantly, **CTLAP** also demonstrated greater selectivity for CTL at pH 6.5, as CTB activity was greatly reduced at this pH value. These results support the use of pH 6.5 for optimal selectivity with **CTLAP**.

Strikingly, **CTLAP** maintained a significantly greater turn-on ratio with CTL compared to other cathepsins across the entire assay (Figure S3), most notably within the first 10 min of incubation (Figure 4C, Table S1). After 24 h incubation with cathepsins, **CTLAP** exhibits a final turn-on ratio greater than 120-fold, likely due to its low background fluorescence (Figure S4), resulting in much greater contrast upon activation by CTL. This could possibly be due to interference in either the absorption or emission processes of **CTLAP**. The absorbance of **CTLAP** is linear within the concentration range used (Figure S5), and the 2-phenylthiophene residue does not absorb at this wavelength (Figure 2). We thus hypothesized that **CTLAP** emission was attenuated somehow, resulting in reduced background emission (Figure S4B). The emission of some fluorophores is altered by assay pH, often due to the existence of a non- or low-emissive conjugate acid or base form of the fluorophore,[36] but no such effect was observed for AMC (Figure S6).

While exploring the causes of the low background fluorescence of **CTLAP**, we considered the possibility that the unique 2-phenylthiophene residue of **CTLAP** was quenching the attached AMC.[37] To examine this, we performed density functional-theory (DFT) calculations to determine the relative HOMO and LUMO energy levels of AMC and the 2-phenylthiophene moiety of **CTLAP** (Figure 5). Quenching of AMC emission by benzene or 2-phenylthiophene through energy transfer is unlikely, because the bandgap of AMC is smaller than that of the other moieties. Electron transfer from AMC to benzene in **Z-FR-AMC** is unlikely because of the high LUMO and deep HOMO of benzene. In **CTLAP**, electron transfer (hole transfer) between AMC and 2-phenylthiophene might be possible due to the comparable HOMO levels, which could quench the emission of AMC. To test this hypothesis, the quantum yield and molar attenuation coefficient of **CTLAP** were measured (Table 1, Figure S7). **CTLAP** has a higher molar attenuation coefficient than **Z-FR-AMC** but a markedly lower quantum yield (Table 1), supporting the hypothesis that AMC emission is reduced in **CTLAP** by quenching mechanisms that are not present in **Z-FR-AMC**. This hole transfer mechanism likely does not influence the emission of the probe after activation by CTL, as the enzymatic reaction cleaves the fluorophore from the substrate and allows diffusion outside of the range of quenching. Complementing this, conversion to free AMC from the respective 7-acetamido-4-methylcoumarin increases the quantum yield nearly four-fold.[33]

Thus, the 120-fold turn-on ratio of CTLAP is attributed to the compounding effect of two quenching mechanisms, the first being a standard off-on mechanism caused by release of AMC[21] while the second is a unique quenching event caused by the 2-phenylthiophene residue that also endows the probe with high selectivity for CTL over competing cathepsins. This could be linked to the effect of adding a quenching dye to the probe to reduce the background of always-on probes[26d, 38] (a common strategy for CTL imaging probes) without the need to actually add such a moiety. The idea of probe components (such as a self-immolating aromatic[32] or aliphatic[25a] spacer, or reporter and quencher location[39]) influencing selectivity has been demonstrated, but here we show the reverse, that probe components included to increase selectivity can influence the photophysical properties of the probe. This dual-purpose design is attractive in theory as it reduces the molecular complexity of the designed probe. To translate this to longer emission wavelengths ideal for fluorescent biological probes, a different substrate moiety with a HOMO-LUMO gap matching that of the new red-shifted fluorophore must be identified which still provides a favorable selectivity profile among cathepsins.

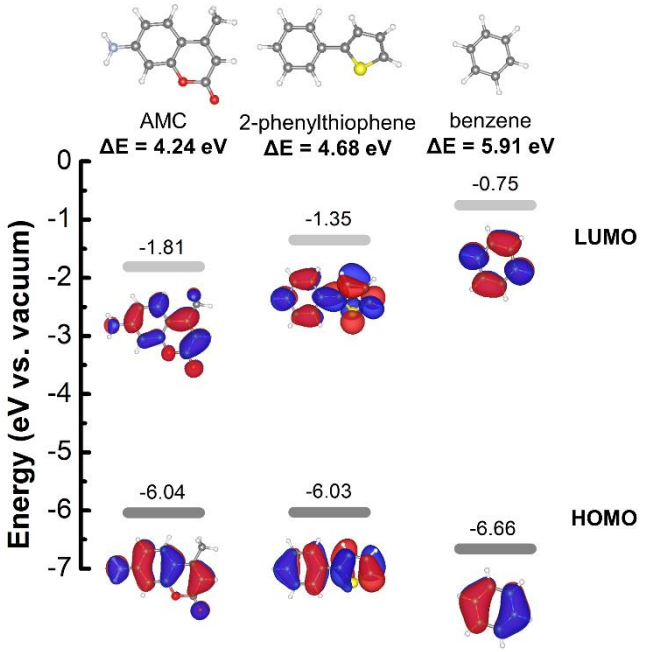

Figure 5. DFT calculations of frontier molecular orbitals of AMC and aromatic residues of **CTLAP** (2-phenylthiophene) and **Z-FR-AMC** (benzene).

Table 1. Molar attenuation coefficient (ε) and fluorescence quantum yield (Φ_F).

Compound	ε x [10^{-3}] [a, b]	Φ_F [a, c]
CTLAP	11.0	0.02
Z-FR-AMC	7.5	0.32

[a] Values standardized against methylumbelliferone (see Supporting Information). [b] Reported in $M^{-1}{\cdot}cm^{-1}$. [c] Quantum yield, determined in cathepsin reaction buffer, pH 6.5.

In summary, we report **CTLAP**, a novel CTL probe that provides high selectivity over closely related cathepsins within 10-15 min while generating high signal contrast, making it amenable to rapid detection of CTL activity in assays. The probe exhibits upwards of 120-fold turn-on ratio, due to dual quenching mechanisms. The presence of extensive quenching eliminates the need to add other quenching groups, reducing molecular complexity while indicating that probe design can take into consideration other possible dual-purpose structural components. Our work reveals that while inhibitor-based probe design can retain the selectivity of the parent inhibitor, it can also result in the discovery of emergent properties that further improve the nature of the probe created. Current work is examining clinical applications of CTLAP that take advantage of these attractive characteristics.

Acknowledgements

This work was supported by research grants to Prof. L. Cui from the National Institute of General Medical Sciences of National Institutes of Health (Maximizing Investigators' Research Award for Early Stage Investigators, R35GM124963), and the Department of Defense Career Development Award (W81XWH-17-1-0529). We are grateful to the support from the University of New Mexico (UNM), the UNM Comprehensive Cancer Center and the National Cancer Institute of the United States (P30CA118100). NMR spectra were collected in part from the NMR Facility, Department of Chemistry and Chemical Biology, UNM, and from the Department of Medicinal Chemistry, College of Pharmacy, University of Florida (UF). Mass spectrometry services were provided in part by the Mass Spectrometry Facility, Department of Chemistry and Chemical Biology, UNM, and from the Mass Spectrometry Research and Education Center, Department of Chemistry, UF (NIH S10 OD021758-01A1).

Keywords: cathepsin • enzymes • fluorescent probes • medicinal chemistry • quenching

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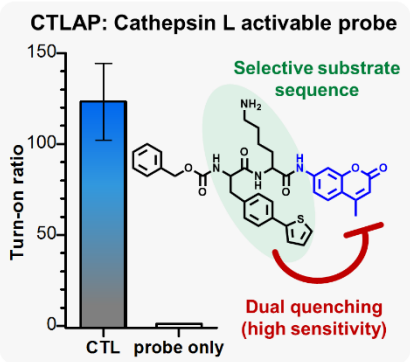

A novel cathepsin L activable probe (CTLAP) bears a chemical structure that simultaneously provides high selectivity for its target while quenching its own background emission, resulting in superior contrast within 10 min of incubation time and 120-fold turn-on fluorescence upon consumption. Along with being a novel detection tool, CTLAP reveals that chemical structure can influence photophysical properties as well as the selectivity of such tools.