

Protein Modifications

A Chemical Probe for Lysine Malonylation**

Xiucong Bao, Qian Zhao, Tangpo Yang, Yi Man Eva Fung,* and Xiang David Li*

Protein posttranslational modifications (PTMs) play fundamental roles in regulating normal cell physiology and disease pathogenesis.^[1] Extensive studies on their cellular functions and mechanisms have advanced our understanding on some well-known PTMs such as phosphorylation, methylation, and acetylation. However, the biological roles played by many newly identified PTMs still remain poorly understood.^[2] Lysine malonylation, a covalent modification of the side chain of lysine, with a malonyl group incorporated at the ϵ -amine (Figure 1a), was recently identified as a novel PTM using two different approaches.^[3] One involved an antibody-

otide (NAD)-dependent hydrolase, which had been previously classified as a deacetylase but was found to have much stronger activity toward malonyllysines over acetyllysines (Figure 1a).^[3b] Given the distinct chemical nature of malonyllysine with a negatively charged carboxylate group, it has been proposed that lysine malonylation could result in significant changes in protein structure and function.^[2a,c]

Characterization of the biological functions of malonylation requires identification of malonylated protein substrates. Use of an anti-malonyllysine antibody led to the identification of several malonylated proteins, including metabolic enzymes^[3a] and histones,^[4] which implies a potential role for this PTM in metabolic and epigenetic regulations. Although the antibody has been used to detect malonylated proteins, immunoblotting methods are not ideal for monitoring the dynamics of malonylation. We still lack a more general and unbiased method to profile new malonylated substrates and examine their dynamic regulation. Herein, we present the development of an alkyne-functionalized chemical probe for efficient metabolic labeling, robust fluorescent visualization, and mass-spectrometry-based identification of malonylated proteins.

Alkyne-carrying chemical probes that can be metabolically incorporated into proteins allow for subsequent copper(I) ion-catalyzed click chemistry to conjugate the labeled proteins with azide-fluorescent dyes or affinity purification tags.^[5] They have therefore been used to detect and identify a variety of PTMs, including acetylation,^[6] lipidation,^[7] glycosylation,^[8] and AMPylation.^[9] Isotopic sodium malonate has been used in cell culture to enhance lysine malonylation in bacteria and human cells.^[3a] This result indicated that malonate can be used for metabolic labeling of malonylated proteins. Inspired by these studies, we designed and synthesized a malonate analogue, 2-propargyl malonate (Mal-yne; Figure 1b and Supporting Information, Scheme S1), as a potential chemical probe for lysine malonylation (Figure 1c).

We first examined whether Mal-yne could be metabolically incorporated into cellular proteins. A stock solution of Mal-yne (in PBS at pH 7.4) was used for metabolic labeling of HeLa S3 cells. After harvesting the cells, the whole-cell lysates were subjected to azide-alkyne click chemistry to conjugate the Mal-yne labeled proteins to a rhodamine dye. The labeled proteins were then resolved by SDS-PAGE and visualized by in-gel fluorescent imaging (Figure 1c). Dose- and time-dependent analyses revealed that a wide range of proteins was labeled by Mal-yne at the optimal concentration of 10–20 mM for 4–6 hours (Supporting Information, Figure S1). This concentration is comparable to that used in the

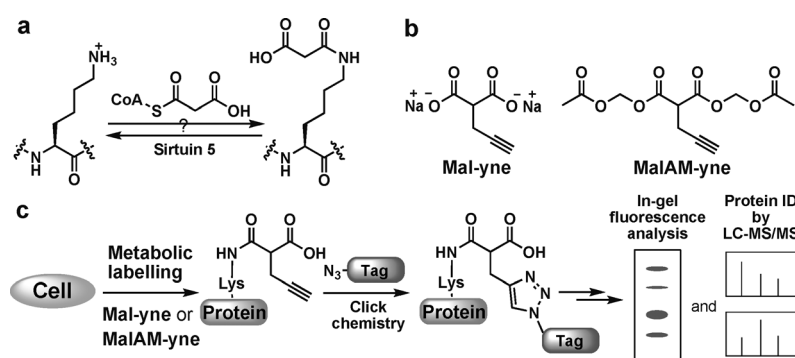


Figure 1. a) The hypothesized enzymatic reactions for lysine (de)malonylation. b) Chemical formulas of Mal-yne and MalAM-yne. c) Strategy for detection and identification of malonylated protein substrates using chemical probes.

based affinity purification of malonylated peptides in conjugation with mass spectrometry.^[3a] The other was through a careful analysis of the enzymatic activity and structural features of sirtuin 5 (Sirt5), a nicotinamide adenine dinucle-

[*] X. Bao,^[†] Q. Zhao,^[†] T. Yang, Dr. Y. M. E. Fung, Prof. X. D. Li
Department of Chemistry,
The University of Hong Kong
Pokfulam Road, Hong Kong (China)
E-mail: eva.fungym@hku.hk
xiangli@hku.hk

[†] These authors contributed equally to this work.

[**] We thank Prof. Chi-Ming Che for generous support on the mass spectrometer, Prof. Dan Yang for advice and support, Prof. Quan Hao for useful discussions, and Di Hu for technical assistance. X.D.L. acknowledges a seed fund from The University of Hong Kong (20111159240) and Hung Hing Ying Physical Science Research Fund (20373739). Y.M.E.F. thanks the Small Project Funding from The University of Hong Kong (201109176193) and the Special Equipment Grant from the University Grants Committee of the Hong Kong Special Administrative Region, China (Project Code: SEG_HKU02).

Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/anie.201300252>.

metabolic labeling of malonylated proteins with isotopic malonate (20 mM for 24 hours),^[3a] as well as acetylated proteins with alkyne-derived chemical reporters (2.5–10 mM for 6–8 hours).^[6] However, we noticed that malonate, with two negative charges at physiological pH, may have limited permeability across cell membranes. As a result, a relatively high concentration of Mal-yne had to be used to achieve optimal metabolic labeling. To test this hypothesis, we synthesized a new probe MalAM-yne (Figure 1b) that masks the carboxylates of Mal-yne with two acetoxymethyl (AM) groups (Supporting Information, Scheme S1). We expected that this uncharged probe could readily permeate the cell membrane, and the subsequent cleavage of AM groups by nonspecific cellular esterases would rapidly release Mal-yne inside living cells for metabolic labeling. When compared with Mal-yne, at a much lower concentration of MalAM-yne (50 μ M), labeled a variety of proteins in as little as one hour (Figure 2), showing an improvement of the

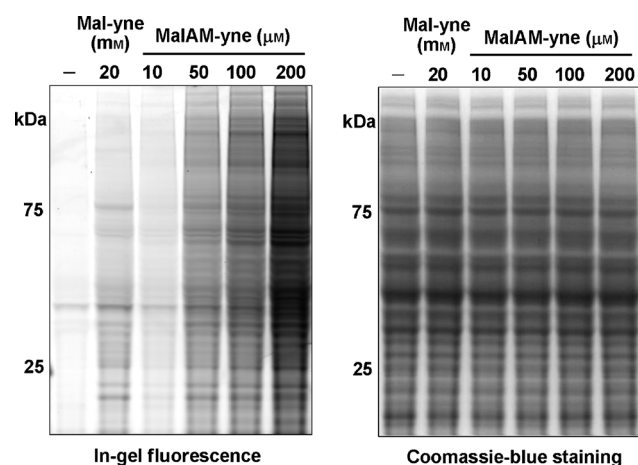


Figure 2. Assessment of chemical probes Mal-yne and MalAM-yne for metabolic labeling of cells. HeLa S3 cells were incubated with 20 mM Mal-yne for six hours or MalAM-yne at the indicated concentrations for one hour. The cell lysates were reacted with rhodamine azide and analyzed by in-gel fluorescent scanning. Coomassie-blue staining shows the protein loading for each lane.

labeling efficiency. Importantly, the profile of the proteins labeled with MalAM-yne was similar to that with Mal-yne (Supporting Information, Figures S1,S2), suggesting the two probes are metabolically equivalent. We therefore focused on MalAM-yne for further studies on lysine malonylation.

Like most PTMs, lysine malonylation is a reversible biochemical process. While the enzymes that place malonyl groups on lysine side chains remain unknown, Sirt5 has been identified as a demalonylase for the removal of this modification.^[3] To determine whether the protein labeling by MalAM-yne is also reversible, we used a pulse-chase assay^[8a] to monitor the removal of a propargyl malonate group. HeLa S3 cells were pulse-labeled with MalAM-yne for one hour, washed, and chased with media containing bis-(acetoxymethyl) malonate (MalAM). The cells were harvested and lysed at different time points. The labeled proteins were conjugated with a rhodamine azide dye through click

chemistry. In-gel fluorescent analysis showed that the fluorescence signal of the probe-labeled proteins faded rapidly (Figure 3a), indicating that the Mal-yne group can be

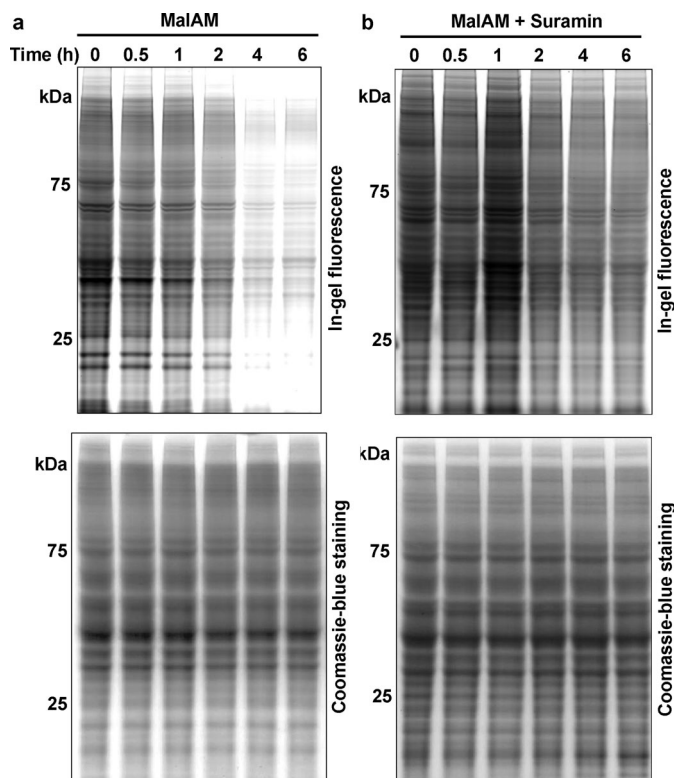


Figure 3. Analysis of the dynamics of malonylation using MalAM-yne. In the a) absence or b) presence of a Sirt5 inhibitor (suramin), HeLa S3 cells were labeled with MalAM-yne (200 μ M) for one hour and chased with bis(acetoxymethyl) malonate (MalAM; 200 μ M) for time indicated. The cell lysates were reacted with rhodamine azide and analyzed by in-gel fluorescent scanning. Coomassie-blue staining shows the protein loading for each lane.

efficiently removed from the protein substrates. To examine whether this removal was regulated by an endogenous demalonylase, we pre-incubated the cells with suramin, a Sirt5 inhibitor.^[10] This treatment not only enhanced the labeling of some proteins by MalAM-yne (Supporting Information, Figure S3), it also significantly reduced the rate of Mal-yne removal from the labeled proteins (Figure 3b). These results suggest that MalAM-yne modified proteins could be substrates for Sirt5. Taken together, the rapid incorporation and removal of the chemical probe reveal the fast dynamics of protein malonylation.

To assess the ability of our chemical probe for identification of malonylation substrates, we first tested if MalAM-yne could selectively enrich known malonylated proteins. HeLa S3 cells were labeled with MalAM-yne (200 μ M) for one hour. The labeled proteins were conjugated to biotin through click chemistry and isolated using streptavidin-coated beads. Western blot analysis using antibodies against five known malonylated substrates,^[3a] including PGK1, HSPA9, HSP90 β , GAPDH, and NSUN2, confirmed their specific enrichment by the probe (Figure 4a). To identify new

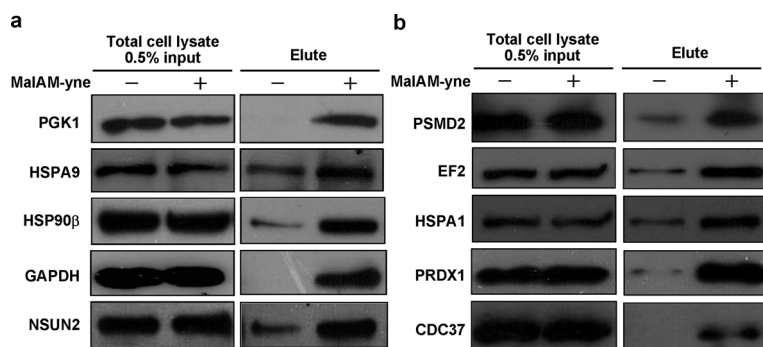


Figure 4. Western-blot analysis shows the enrichment of a) known and b) potential substrates of malonylation. HeLa S3 cells were incubated with MalAM-yne (200 μ M) for one hour. The labeled proteins were then conjugated to biotin through click chemistry. Following incubation with streptavidin beads, the enriched proteins were eluted for western-blotting analysis.

malonylated substrates, we performed a proteomic analysis of the MalAM-yne labeled proteins using an LTQ-Orbitrap mass spectrometer. Out of the 17 currently known malonylated proteins,^[3a] 14 of them were identified by MalAM-yne labeling in both independent experiments (Supporting Information, Table S1). In addition, we identified another 361 MalAM-yne enriched proteins as new candidates of malonylation substrates (Supporting Information, Table S2). We subsequently verified the identification of five new malonylated proteins (Supporting Information, Table S3), including PSMD2, EF2, HSPA1, PRDX1, and CDC37, by Western blot analysis of the affinity-purified proteins (Figure 4b). A bioinformatics survey revealed that the newly identified malonylated proteins are mainly associated with metabolic

processes, but are also involved in other diverse cellular functions (Supporting Information, Figure S4).

To further examine if MalAM-yne modifies the lysine residues of proteins, we focused on HSP90 β that has a known malonylation site on lysine residue 399 (K399).^[3a] We overexpressed streptavidin-tagged HSP90 β in HEK293T cells. The cells were labeled with MalAM-yne (200 μ M) for one hour, and the tagged protein was then purified with Strep-Tactin resin. Following digestion with trypsin in solution, the resulting peptides were separated by HPLC and analyzed with an LTQ-Orbitrap mass spectrometer. To identify the peptides that were modified with MalAM-yne, the MS/MS spectra were analyzed by protein sequence alignment using the SEQUEST search engine, followed by manual verification to ensure the

correct assignments. As shown in Figure 5, we identified a peptide with a 2-propargylmalonylated lysine (K^{Malyne}) residue, ³⁹³EMLQQSK^{Malyne}ILKVIR⁴⁰⁵, with a mass shift of 124.0160 Da, corresponding to the addition of a Mal-yne group at K399. In the MS/MS spectrum of this peptide, we also found that many of the peptide fragments with the K^{Malyne} residue have satellite peaks, with a mass loss of 44 Da (Figure 5), which corresponds to the loss of CO₂. This unique mass signature verified the identification of the K^{Malyne} site, because this thermal decarboxylation process has also commonly been observed in the collision-induced dissociation (CID) MS/MS spectra of lysine-malonylated peptides.^[3a] This result indicated that MalAM-yne can be used to identify malonylation sites (Supporting Information, Figure S5) in live cells under physiological conditions.^[3a] The validation of this known malonylated peptide also inspired us to use the probe to identify novel malonylation sites. We thus studied one of the newly identified malonylated protein candidates, PRDX1. LC-MS/MS analysis of this protein revealed a K^{Malyne} containing peptide, ¹⁶⁹HGEVCPAGWK^{Malyne}PG-SDTIKPDVQK¹⁹⁰ (Supporting Information, Figure S6), indicating that a novel malonylation site had been identified on this protein. These experiments collectively demonstrate that MalAM-yne can directly target Lys residues for the detection and identification of lysine malonylation.

To explore the scope of MalAM-yne to study protein malonylation, we performed metabolic labeling of different cell types with MalAM-yne. In-gel fluorescent analysis showed robust labeling of a wide variety of proteins in all the cell types that were tested (Supporting Information, Figure S6), ranging from bacteria to human cells, indicating that lysine malonylation is evolutionarily conserved in these species. Additionally, the different protein-labeling patterns of various human cell lines suggest a diverse role and the dynamic nature of lysine malonylation in mammalian cells.

In summary, we have developed the first chemical probe, MalAM-yne, to study protein lysine malonylation. MalAM-yne is permeable across the cell membrane and allows for efficient metabolic labeling, robust fluorescent detection, and proteomic profiling of malonylated proteins in a variety of cells. MalAM-yne based fluorescent detection also enabled

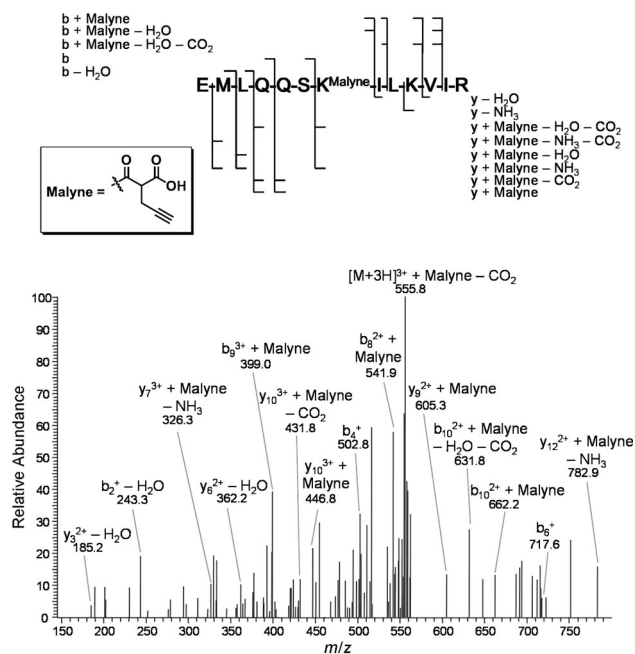


Figure 5. The CID MS/MS spectrum of a triply charged tryptic-digest peptide, ³⁹³EMLQQSK^{Malyne}ILKVIR⁴⁰⁵, from MalAM-yne modified HSP90 β .

the dynamic analysis of malonylation levels and patterns, which therefore is a complementary method to the antibody-based methods. Moreover, when combined with methods to inhibit or activate the functions of a protein, such as RNA interference or the use of chemical inhibitors or activators, our probe that unbiasedly labels malonylated proteins could be used to determine cellular substrates of the demalonylase, Sirt5. It is also worth mentioning that the probe, as a mimic of malonate, could be subjected to a variety of metabolic pathways. Therefore, the further identification of malonylation sites and functional validation of these substrates is an important next step, and will be reported in due course.

Received: January 11, 2013
Revised: February 4, 2013
Published online: March 26, 2013

Keywords: click chemistry · lysine malonylation · metabolic labeling · posttranslational modifications · protein modifications

-
- [1] C. T. Walsh, *Posttranslational Modifications of Proteins: Expanding Nature's Inventory*, Roberts and Co. Publishers, Greenwood Village, CO, **2006**.
- [2] a) H. Lin, X. Su, B. He, *ACS Chem. Biol.* **2012**, *7*, 947–960; b) C. A. Olsen, *Angew. Chem.* **2012**, *124*, 3817–3819; *Angew. Chem. Int. Ed.* **2012**, *51*, 3755–3756; c) W. He, J. C. Newman, M. Z. Wang, L. Ho, E. Verdin, *Trends Endocrinol. Metab.* **2012**, *23*, 467–476.
- [3] a) C. Peng, Z. Lu, Z. Xie, Z. Cheng, Y. Chen, M. Tan, H. Luo, Y. Zhang, W. He, K. Yang, B. M. Zwaans, D. Tishkoff, L. Ho, D. Lombard, T. C. He, J. Dai, E. Verdin, Y. Ye, Y. Zhao, *Mol. Cell. Proteomics* **2011**, *10*, M111 012658; b) J. Du, Y. Zhou, X. Su, J. J. Yu, S. Khan, H. Jiang, J. Kim, J. Woo, J. H. Kim, B. H. Choi, B. He, W. Chen, S. Zhang, R. A. Cerione, J. Auwerx, Q. Hao, H. Lin, *Science* **2011**, *334*, 806–809.
- [4] Z. Xie, J. Dai, L. Dai, M. Tan, Z. Cheng, Y. Wu, J. D. Boeke, Y. Zhao, *Mol. Cell. Proteomics* **2012**, *11*, 100–107.
- [5] E. M. Sletten, C. R. Bertozzi, *Angew. Chem.* **2009**, *121*, 7108–7133; *Angew. Chem. Int. Ed.* **2009**, *48*, 6974–6998.
- [6] Y. Y. Yang, J. M. Ascano, H. C. Hang, *J. Am. Chem. Soc.* **2010**, *132*, 3640–3641.
- [7] a) B. R. Martin, B. F. Cravatt, *Nat. Methods* **2009**, *6*, 135–138; b) G. Charron, M. M. Zhang, J. S. Yount, J. Wilson, A. S. Raghavan, E. Shamir, H. C. Hang, *J. Am. Chem. Soc.* **2009**, *131*, 4967–4975; c) G. Charron, J. Wilson, H. C. Hang, *Curr. Opin. Chem. Biol.* **2009**, *13*, 382–391.
- [8] a) B. W. Zaro, Y. Y. Yang, H. C. Hang, M. R. Pratt, *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 8146–8151; b) N. J. Agard, C. R. Bertozzi, *Acc. Chem. Res.* **2009**, *42*, 788–797.
- [9] M. Grammel, P. Luong, K. Orth, H. C. Hang, *J. Am. Chem. Soc.* **2011**, *133*, 17103–17105.
- [10] a) A. Schuetz, J. Min, T. Antoshenko, C. L. Wang, A. Allali-Hassani, A. Dong, P. Loppnau, M. Vedadi, A. Bochkarev, R. Sternglanz, A. N. Plotnikov, *Structure* **2007**, *15*, 377–389; b) A. S. Madsen, C. A. Olsen, *J. Med. Chem.* **2012**, *55*, 5582–5590.
-