Bioorganic & Medicinal Chemistry Letters 18 (2008) 5982–5986

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Chemical probes for profiling fatty acid-associated proteins in living cells

Anuradha Raghavan, Guillaume Charron, James Flexner, Howard C. Hang *

The Laboratory of Chemical Biology and Microbial Pathogenesis, The Rockefeller University, 1230 York Avenue, Box 250, New York, NY 10065, USA

ARTICLE INFO

Article history:
Received 3 July 2008
Revised 22 September 2008
Accepted 22 September 2008
Available online 30 September 2008

Keywords:
Click chemistry
Chemical probe
Fatty acid
Mechanism-based probe
Activity-based probe
Bioorthogonal labeling

ABSTRACT

Chemical probes appended with reactive electrophiles afford powerful tools for profiling discrete protein families in living cells. Herein, we have synthesized cell-permeable chemical probes that target fatty acid-associated proteins. These fatty acid-based chemical probes contain acyloxymethylketone or fluorophosphonate functional groups and an alkyne click chemistry tag for visualization of covalently modified proteins by in-gel fluorescence scanning. Our fatty acid-based chemical probe affords new tools to evaluate the activity/expression of lipid-associated proteins that should facilitate their functional characterization and inhibitor discovery.

Published by Elsevier Ltd.

Fatty acids control diverse biological processes in microbes and animals. The metabolism of fatty acids provides a principle carbon source for energy in cells, the mis-regulation of which is at the heart of metabolic disorders. Besides being a source of nutrients, the composition of fatty acids inside cells influences basic processes such as vesicle trafficking and protein activity.[1,2] Moreover, the covalent attachment of fatty acids onto proteins directly modulates their subcellular localization and signaling properties.[3] While some key functions of fatty acids have emerged, the mechanisms that link lipid metabolism to discrete cellular processes is less clear. New tools for fatty acid-associated proteins (transporters, chaperones and enzymes) in cells should facilitate their characterization in physiology and disease.

Chemical probes appended with detection tags have provided new opportunities to analyze complex proteomes.[4–6] These mechanism- or activity-based probes covalently label specific protein families and provide excellent tools to measure the expression and/or activity of proteins in complex mixtures by virtue of the appended detection tag.[4–6] The selectivity of these chemical probes towards various protein families is determined by the reactive functional groups chosen for covalent labeling, and the surrounding chemical scaffold.[4–6] A variety of chemical probes have been generated using covalent electrophilic traps for enzyme classes with active-site nucleophiles such as serine hydrolases, cysteine proteases, glycosidases, kinases, phosphatases and other protein families.[4–6] For enzyme families without reactive nucleophiles, photocrosslinking groups have been appended onto chemical scaffolds to target proteases[7–11] and histone deacetylases.[12,13] Non-directed chemical probes have also been generated to label unanticipated protein activities.[14] Furthermore, these chemical probes also label amino acid residues beyond the active site of enzymes and provide unique pharmacological tools to profile diverse enzyme/protein families.[15–17] Attachment of detection tags such as biotin onto chemical probes enables visualization and identification of labeled proteins with streptavidin reagents. Alternatively, the introduction of fluorophores onto chemical probes provides quantitative means for profiling the expression/activity of protein families by in-gel fluorescence scanning.[4–6] In addition to profiling specific protein families in different cell types, mechanism- or activity-based probes have provided powerful tools for annotating the function of uncharacterized gene products, classification of small molecule inhibitor specificities and even revealed novel regulatory mechanisms of enzymes.[4–6]

The application of chemical probes to living cells should provide new insight into the function of protein families not apparent in cell lysates (Fig. 1A). Direct attachment of detection tags to chemical probes has enabled the labeling of specific proteins in cell lysates, but the pharmacological properties of biotin or fluorescent dyes often precludes their passive diffusion into cells and also influences specificity of protein labeling.[5,6] The emergence of bioorthogonal chemical reactions such as the Staudinger ligation and Huisgen [3 + 2] cycloaddition or click chemistry (Fig. 1B) allows the installation of detection tags onto chemical probes after protein labeling in living cells (Fig. 1A).[18] For example, the introduction of alkynes or azides onto various chemical probes enabled the labeling of diverse protein families in living cells.[19–22] Cell lysates can then be prepared and reacted with phosphine- or

* Corresponding author. Fax: +1 212 327 7276.
E-mail address: hhang@mail.rockefeller.edu (H.C. Hang).

0960-894X/$ - see front matter Published by Elsevier Ltd.
doi:10.1016/j.bmcl.2008.09.083

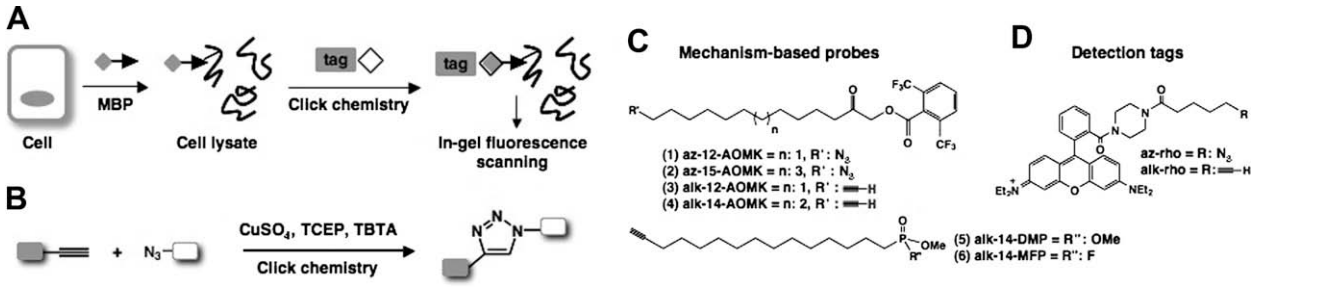
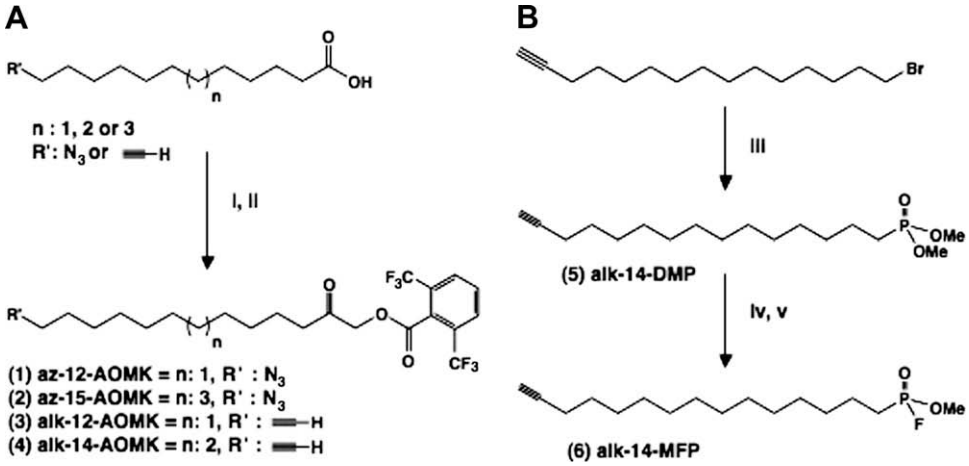


Figure 1. Chemical probes targeted at fatty acid-associated proteins in living cells. (A) Administration of fatty acid-based chemical probes to live cells, followed by click chemistry/in-gel fluorescence detection of labeled proteins. (B) Cu^{I}-mediated Huisgen [3 + 2] cycloaddition or click chemistry. $CuSO_4$, *tris*(2-carboxyethyl)phosphine (TCEP) and *tris*-(triazolyl)benzyl amine (TBTA) reagents for click chemistry. (C) Fatty acid-based chemical probes. (D) Detection tags for click chemistry/in-gel fluorescence scanning.

azide-functionalized detection tags for the visualization of specifically targeted proteins.[19–22] This two-step protein labeling and detection approach has even allowed the imaging of the cysteine protease cathepsin B within the endocytic vacuoles of primary macrophages infected with intracellular bacterial pathogens such as *Salmonella typhimurium*.[21] Application of mechanism-based probes to living cells therefore affords new opportunities to analyze the function of specific protein families in vivo. Herein we describe the synthesis and characterization of cell-permeable chemical probes targeted at fatty acid-associated proteins in living cells and the detection of labeled proteins using click chemistry/in-gel fluorescence scanning.

To explore the diversity of fatty acid-associated proteins in cells, we designed azide/alkyne-modified fatty acid-based chemical probes functionalized with reactive electrophiles (acyloxymethylketone (AOMK) or fluorophosphonate) to target enzymes or proteins that would utilize or bind fatty acids as well as fluorescent detection tags for click chemistry analysis of covalently labeled proteins (Fig. 1C). Fatty acid-based chemical probes containing the AOMK functionality were synthesized to specifically target lipid-associated proteins with reactive cysteines (**1–4**) (Fig. 1C). Chemical probes containing the AOMK moiety have been shown to react with cysteine residues of proteases.[23,24] We also synthesized alkyne-modified fatty acid derivatives bearing alkyl- and fluorophosphonate groups that should target nucleophilic serine residues (**5** and **6**) (Fig. 1C).[25,26] The synthesis of the AOMK probes proceeded by isobutylchloroformate activation of azide/alkyne-fatty acids followed by reaction with diazomethane generated in situ (Scheme 1). The corresponding chloromethylketones formed were then reacted with di-2,6-trifluoromethybenzoate in the presence of potassium fluoride to afford the azide/alkyne-modified fatty acid-AOMKs (**1–4**) (Scheme 1). The alkyne-modified phosphonates were generated by reaction of the terminal alkynyl–alkyl bromide with trimethylphosphine to yield the 16-carbon alkynyl-dimethylphosphonate (**5**) (Scheme 1). Partial hydrolysis of compound **5** followed by fluorination with diethylaminosulfur trifluoride (DAST) afforded the 16-carbon alkynyl-methylfluorophosphonate (alk-14-MFP, **6**) (Scheme 1).

With the fatty acid-based chemical probes in hand, we characterized the ability of the AOMKs (**1–4**) and phosphonates (**5** and **6**) to selectively label proteins in living cells. The compounds (**1–6**) were incubated in HeLa cells, a commonly used tumor cell line, at 50 μM for 1 h. Cell lysates were then prepared and reacted with azide/alkyne-rhodamine detection tags (az-rho or alk-rho) under standard click chemistry conditions,[20] separated by gel electrophoresis and analyzed by in-gel fluorescence scanning. Cells were lysed with buffer containing high detergent to maximize the recovery of proteins from various cellular compartments and in the presence of protease inhibitor cocktail to minimize post-lysis labeling of proteins with chemical probes. A number of proteins are labeled with the fatty acid AOMKs and phosphonate probes (Fig. 2). The profile of polypeptides selectively targeted by azide- and alkynyl-fatty acid AOMKs were similar, but the non-specific labeling of proteins with the alk-rho detection tag is more pronounced (Fig. 2). We therefore proceeded with the alkyne-modified chemical probes and az-rho for optimal click chemistry detection. While the fatty acid AOMK probes have some overlap in the labeled proteins, the shorter chain (**1** and **3**) and long chain (**2** and **4**) fatty acid AOMK probes also labeled unique sets of proteins (Fig. 2).



Scheme 1. Synthesis of fatty acid-based chemical probes. Reagents and conditions: (A) acyloxymethylketones. i—(a) IBCF, NMM, CH_2Cl_2. (b) CH_2N_2. (c) HCl/acetic acid (48–50%). ii—KF, bis-2,6-trifluoromethybenzoic acid (35–45%). (B) Phosphonates. iii—$P(OMe)_3$.(73%). iv—TMSBr, CH_2Cl_2. v—DAST, 0 °C (49%).

These data demonstrate that different profiles of proteins are targeted depending on the chain length of fatty acid AOMK probes. In comparison to the AOMKs, the dimethylfluorophosphonate probe (**5**) did not label proteins under conditions of the assay, whereas the methylfluorophosphonate analog (**6**) targeted a large number of proteins (Fig. 2). The differential reactivity of the dialkylphosphonates compared with fluorophosphonates in our experiments is consistent with their observed inhibitory activities with serine hydrolases in vitro.[27] Time- and dose-dependent analysis of the fatty acid-based probes in cells demonstrates that the AOMK probes (alk-12-AOMK **3** and alk-14-AOMK **4**) protein labeling is optimal at 50 μM and 30 min (Supplementary Fig. 1). The methylfluorophosphonate probe (**6**) protein labeling saturates at approximately 20 μM and labels proteins after 10 min of incubation with cells (Supplementary Fig. 1). These experiments demonstrate our fatty acid-based chemical probes label unique profiles of proteins in cells and that the alkyne-modified probes (**3**, **4** and **6**) provide superior visualization of labeled proteins using click chemistry/in-gel fluorescence scanning as compared to azide-modified probes.

To survey the protein labeling selectivity of our fatty acid-based probes, we performed competition experiments in cells with compounds that react with nucleophilic cysteine and serine residues on proteins or hydrolytic enzymes. Reactive amino acid residues on proteins were blocked with either *N*-ethyl maleimide (NEM) or broad-spectrum cysteine protease inhibitors[27]: E-64 (cathepsins) and z-VAD(OMe)-fmk (caspases). Alternatively, nucleophilic serine residues were blocked with phenylmethylsulfonylfluoride (PMSF) or phospholipase A_2 inhibitors[28]: methyl arachidonyl fluorophosphonate (MAFP) or bromoenol lactone (BEL). HeLa cells were pretreated with cysteine and serine reactive compounds for 1 h and subsequently incubated with our fatty acid-based probes (**3**, **4** and **6**), harvested and evaluated for selective protein labeling (Supplementary Fig. 2). The majority of proteins targeted by fatty acid AOMK (**4**) were blocked by NEM (Supplementary Fig. 2B), whereas the profile of polypeptides labeled with fatty acid AOMK (**3**) was only partially inhibited and altered by NEM (Supplementary Fig. 2A). Proteins targeted by the fatty acid AOMKs (**3** and **4**) were not blocked by serine reactive compounds such as PMSF or MAFP (Supplementary Fig. 2A and B). Interestingly, pre-incubation of cells with BEL did not compete with protein labeling by the AOMKs (**3** and **4**), but altered the profile of proteins that were targeted. The competition experiments with alk-14-MFP (**6**), demonstrated that NEM, PMSF, MAFP and BEL partially abrogated protein labeling and also altered the profile of targeted proteins (Supplementary Fig. 2C). The cysteine protease inhibitors E-64 and z-VAD(OMe)-fmk did not reduce the labeling of any proteins targeted by the fatty acid-based chemical probes (**3**, **4** or **6**) (Supplementary Fig. 2). These competitive labeling experiments in cells suggest that the fatty acid AOMKs (**3** and **4**) label cysteine residues on target proteins that are distinct from known cysteine proteases, whereas alk-14-MFP (**6**) reacts primarily with proteins containing nucleophilic serine residues. Interestingly, the fatty acid-based probes (**3, 4** and **6**) appear to target discrete sets of proteins that are not entirely blocked by known cysteine and serine hydrolase inhibitors.

The generality of the fatty acid-based chemical probes (**3**, **4** and **6**) was determined with a variety of cell lines. Compounds (**3**, **4** and **6**) were incubated with a panel of cell lines and the profiles of selectively labeled proteins were evaluated. In general, unique profiles of proteins were labeled in different cell types for all three chemical probes (Fig. 3). Direct comparison of the fatty acid AOMKs (**3** and **4**) revealed both discrete and overlapping profiles of polypeptides labeled in various cell types, even though these two fatty acid-based chemical probes only differ in structure by two methylene units. The profile of proteins targeted by the fatty acid AOMKs (**3** and **4**) also varies between cell types (Fig. 3), suggesting that these compounds do not just target abundant house keeping proteins but may indeed label specific proteins associated with the function of individual cell types. The profile of proteins targeted by the alk-14-MFP (**6**) in the different cell types was significantly broader than the AOMKs (Fig. 3). Between the various cell types, the alk-14-MFP (**6**) labeled overlapping as well as discrete sets of proteins (Fig. 3). In general, our experiments with alk-14-MFP (**6**) are consistent with other fluorophosphonate probes reported,[29,30] although the exact chemical scaffold and cell types examined are different. Collectively, these experiments demonstrate that our fatty acid-based chemical probes (**3**, **4** and **6**) function in a variety of cell types and label a diverse profile of proteins, which depends upon the fatty acid chain length and the reactive electrophile of the probe.

To determine the reactivity of our chemical probes with a fatty acid-associated protein or enzyme, we evaluated the labeling of cytoplasmic phospholipase A_2 in living cells. Cytoplasmic phospholipase A_2 (cPLA$_2$) is a broadly expressed serine hydrolase that cleaves fatty acids such as arachidonic acid from glycerolipids and is associated with cellular differentiation, cytotoxicity as well as inflammation.[31] HeLa cells were incubated with DMSO or our fatty acid-based chemical probes (**3**, **4** and **6**), lysed and analyzed for cPLA$_2$ labeling after immunoprecipitation with specific anti-cPLA$_2$ polyclonal sera. While the fatty acid AOMK probes (**3** and **4**) do not covalently react with cPLA$_2$, the fluor-

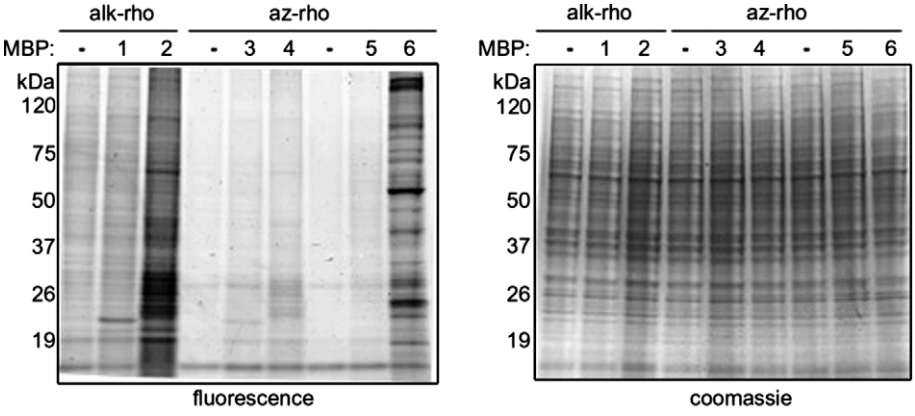


Figure 2. Analysis of fatty acid-based chemical probes in cells. Approximately 20 μg of cell lysate was loaded in each lane. Fluorescently labeled proteins were visualized with Amersham Biosciences Typhoon 9400 variable mode imager with ex/em 532/580 nm.

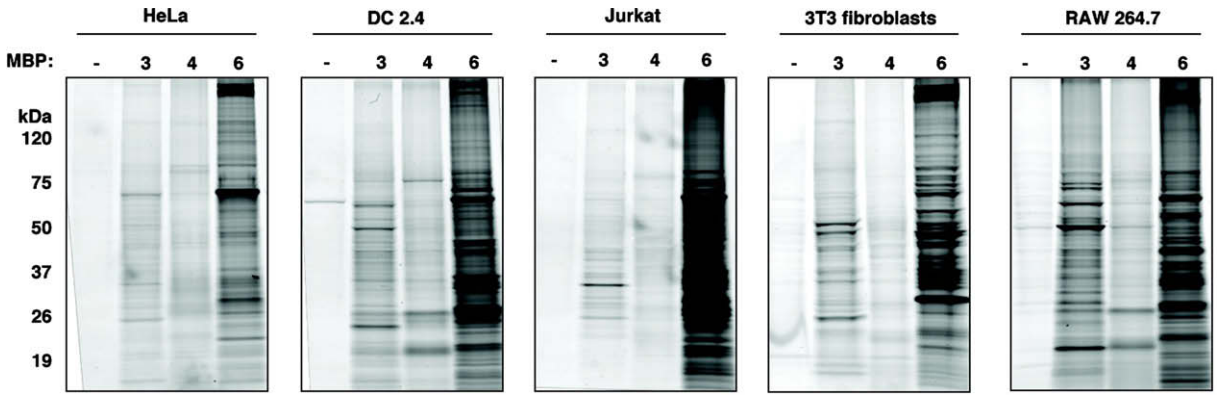


Figure 3. Profile of proteins targeted by fatty acid-based chemical probes in various cell types. Fatty acid probes **3**, **4** and **6** were administered at 50 mM for 1 h. Cell lysates were prepared and analyzed for selective protein labeling as described in Figure 2. HeLa, cervical cancer cells; DC2.4, murine monocytes; Jurkat, human T cell lymphoma; NIH 3T3, murine fibroblasts; RAW 264.7, murine macrophages.

ophosphonate probe alk-14-MFP (**6**) effectively labeled $cPLA_2$ at the expected molecular weight (~96 kDa) as visualized by click chemistry/in-gel fluorescence scanning and Western blot analysis of $cPLA_2$ protein (Fig. 4). These results demonstrate that our chemical probes can efficiently label fatty acid-associated proteins/enzymes in cells.

Our studies with the fatty acid-based chemical probes describe progress towards new chemical tools for targeting lipid-associated proteins in living cells. The global identification of specific proteins labeled by our chemical probes is currently underway and should afford important insight into the reactivity profile of AOMKs and fluorophosphonate group in cells. These studies should complement the analysis of other electrophilic chemical probes[17,5] as well as fatty acid chemical reporters that target fatty-acylated proteins.[32] The ability to profile the expression/activity of fatty acid-associated proteins/enzymes in living cells should provide new opportunities to dissect their functions in physiology and disease.

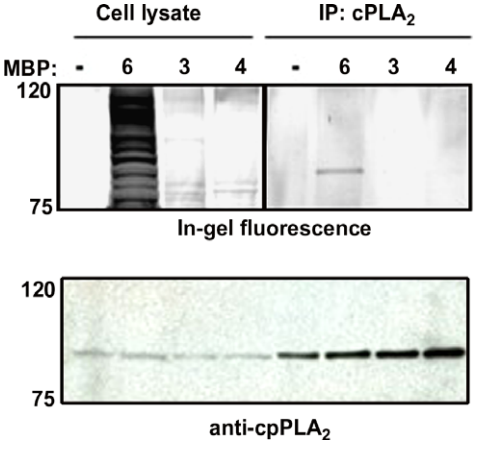


Figure 4. Chemical probe labeling of fatty acid-associated enzyme, $cPLA_2$. Fatty acid-based chemical probes **3**, **4** and **6** were administered at 50 mM for 1 h in HeLa cells. HeLa lysates and $cPLA_2$ immunoprecipitates were analyzed by click chemistry/in-gel fluorescence scanning as described in Figure 2 (upper panel). The amount of $cPLA_2$ in cell lysates and immunoprecipitates were analyzed by Western blot (lower panel).

Acknowledgments

The authors thank Prof. Ben Cravatt and Prof. Dale Boger for the opportunity to participate in this issue honoring Prof. Cravatt as one of the 2008 Tetrahedron Young Investigators. This work was supported by The Rockefeller University and Irma T. Hirschl/Monique Weil-Caulier Trust. A.R. thanks the New York Community Trust-Heiser Grant for a post-doctoral fellowship.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.09.083.

References and notes

1. van Meer, G.; Voelker, D. R.; Feigenson, G. W. *Nat. Rev. Mol. Cell. Biol.* **2008**, *9*, 112.
2. Sprong, H.; van der Sluijs, P.; van Meer, G. *Nat. Rev. Mol. Cell. Biol.* **2001**, *2*, 504.
3. Linder, M. E.; Deschenes, R. J. *Nat. Rev. Mol. Cell. Biol.* **2007**, *8*, 74.
4. Sadaghiani, A. M.; Verhelst, S. H.; Bogyo, M. *Curr. Opin. Chem. Biol.* **2007**, *11*, 20.
5. Cravatt, B. F.; Wright, A. T.; Kozarich, J. W. *Annu. Rev. Biochem.* **2008**, *77*, 383.
6. Evans, M. J.; Cravatt, B. F. *Chem. Rev.* **2006**, *106*, 3279.
7. Kukar, T. L.; Ladd, T. B.; Bann, M. A.; Fraering, P. C.; Narlawar, R.; Maharvi, G. M.; Healy, B.; Chapman, R.; Welzel, A. T.; Price, R. W.; Moore, B.; Rangachari, V.; Cusack, B.; Eriksen, J.; Jansen-West, K.; Verbeeck, C.; Yager, D.; Eckman, C.; Ye, W.; Sagi, S.; Cottrell, B. A.; Torpey, J.; Rosenberry, T. L.; Fauq, A.; Wolfe, M. S.; Schmidt, B.; Walsh, D. M.; Koo, E. H.; Golde, T. E. *Nature* **2008**, *453*, 925.
8. Weihofen, A.; Binns, K.; Lemberg, M. K.; Ashman, K.; Martoglio, B. *Science* **2002**, *296*, 2215.
9. Sieber, S. A.; Niessen, S.; Hoover, H. S.; Cravatt, B. F. *Nat. Chem. Biol.* **2006**, *2*, 274.
10. Qiu, W. W.; Xu, J.; Li, J. Y.; Li, J.; Nan, F. J. *Chembiochem* **2007**, *8*, 1351.
11. Chan, E. W.; Chattopadhaya, S.; Panicker, R. C.; Huang, X.; Yao, S. Q. *J. Am. Chem. Soc.* **2004**, *126*, 14435.
12. Salisbury, C. M.; Cravatt, B. F. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 1171.
13. Salisbury, C. M.; Cravatt, B. F. *J. Am. Chem. Soc.* **2008**, *130*, 2184.
14. Evans, M. J.; Saghatelian, A.; Sorensen, E. J.; Cravatt, B. F. *Nat. Biotechnol.* **2005**, *23*, 1303.
15. Patricelli, M. P.; Szardenings, A. K.; Liyanage, M.; Nomanbhoy, T. K.; Wu, M.; Weissig, H.; Aban, A.; Chun, D.; Tanner, S.; Kozarich, J. W. *Biochemistry* **2007**, *46*, 350.
16. Cohen, M. S.; Zhang, C.; Shokat, K. M.; Taunton, J. *Science* **2005**, *308*, 1318.
17. Weerapana, E.; Simon, G. M.; Cravatt, B. F. *Nat. Chem. Biol.* **2008**.
18. Prescher, J. A.; Bertozzi, C. R. *Nat. Chem. Biol.* **2005**, *1*, 13.
19. Speers, A. E.; Adam, G. C.; Cravatt, B. F. *J. Am. Chem. Soc.* **2003**, *125*, 4686.
20. Speers, A. E.; Cravatt, B. F. *Chem. Biol.* **2004**, *11*, 535.
21. Hang, H. C.; Loureiro, J.; Spooner, E.; van der Velden, A. W.; Kim, Y. M.; Pollington, A. M.; Maehr, R.; Starnbach, M. N.; Ploegh, H. L. *ACS Chem. Biol.* **2006**, *1*, 713.
22. Reddie, K. G.; Seo, Y. H.; Muse Iii, W. B.; Leonard, S. E.; Carroll, K. S. *Mol. Biosyst.* **2008**, *4*, 521.
23. Kato, D.; Boatright, K. M.; Berger, A. B.; Nazif, T.; Blum, G.; Ryan, C.; Chehade, K. A.; Salvesen, G. S.; Bogyo, M. *Nat. Chem. Biol.* **2005**, *1*, 33.

24. Sexton, K. B.; Witte, M. D.; Blum, G.; Bogyo, M. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 649.
25. Liu, Y.; Patricelli, M. P.; Cravatt, B. F. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 14694.
26. Kidd, D.; Liu, Y.; Cravatt, B. F. *Biochemistry* **2001**, *40*, 4005.
27. Powers, J. C.; Asgian, J. L.; Ekici, O. D.; James, K. E. *Chem. Rev.* **2002**, *102*, 4639.
28. Balsinde, J.; Balboa, M. A.; Insel, P. A.; Dennis, E. A. *Annu. Rev. Pharmacol. Toxicol.* **1999**, *39*, 175.
29. Jessani, N.; Liu, Y.; Humphrey, M.; Cravatt, B. F. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 10335.
30. Gillet, L. C.; Namoto, K.; Ruchti, A.; Hoving, S.; Boesch, D.; Inverardi, B.; Mueller, D.; Coulot, M.; Schindler, P.; Schweigler, P.; Bernardi, A.; Gil-Parrado, S. *Mol. Cell. Proteomics* **2008**.
31. Leslie, C. C. *J. Biol. Chem.* **1997**, *272*, 16709.
32. Hang, H. C.; Geutjes, E. J.; Grotenbreg, G.; Pollington, A. M.; Bijlmakers, M. J.; Ploegh, H. L. *J. Am. Chem. Soc.* **2007**, *129*, 2744.