ChemComm

COMMUNICATION

View Article Online
View Journal

Cite this: DOI: 10.1039/c8cc09558b

Received 1st December 2018,
Accepted 21st December 2018

DOI: 10.1039/c8cc09558b

rsc.li/chemcomm

Live-cell imaging and profiling of c-Jun N-terminal kinases using covalent inhibitor-derived probes†

Linghui Qian,*[ab] Sijun Pan,[a] Jun-Seok Lee,[c] Jingyan Ge,[d] Lin Li*[e] and Shao Q. Yao*[a]

c-Jun N-terminal kinases (JNKs) are involved in critical cellular functions. Herein, small-molecule JNK-targeting probes are reported based on a covalent inhibitor. Together with newly developed two-photon fluorescence Turn-ON reporters and chemoproteomic studies, we showed that some probes may be suitable for live-cell imaging and profiling of JNKs.

c-Jun N-terminal kinases (JNKs) consist of isoforms derived from three genes (*i.e.* JNK1/2/3) and each gene is expressed as either 46 or 55 kDa proteins, differing in cellular and tissue distributions. JNKs are involved in many physiological processes and their dysregulation has been associated with various pathological states, making them promising drug targets.[1] As subcellular compartmentalization and JNK activation are closely linked,[2] tools capable of direct imaging and profiling of active JNKs in mammalian cells would be highly valuable.[3] Zhang *et al.* previously developed a genetically engineered biosensor to measure JNK activities in live cells.[4] Its adaptation in cell biology and drug discovery has however been hampered by common limitations associated with protein biosensors.[5] Furthermore, direct visualization of JNK–drug interaction at the proteome-wide level is not possible with such techniques.[6]

Small-molecule probes have emerged as promising tools to elucidate protein functions.[7,8] Among them, bioorthogonal probes are particularly valuable,[8] as in some cases, they could be converted into dual-purpose probes capable of both imaging and profiling with excellent permeability, tunable photophysical properties, good binding kinetics and chemical tractability.[9,10] Notwithstanding, dual-purpose bioorthogonal probes for live-cell imaging and profiling of JNKs are not available at present.

Our previous attempt to develop an affinity-based probe (A*f*BP) for JNKs, **SP-1**, was met with significant challenges (Fig. 1A).[11] **SP-1** was made by attaching an alkyne-containing diazirine linker to SP600125 (a non-covalent inhibitor of JNK[12]). Due to the parental compound's poor target selectivity and the need for UV irradiation, it was not further pursued as a dual-purpose probe. Covalent probes based on irreversible inhibitors are known to possess improved target labeling performance.[13–15] Inspired by the recent discovery of JNK-IN-8,[16] the first irreversible inhibitor capable of selective inhibition of JNK activities at nanomolar concentrations, herein we disclose three new bioorthogonal JNK probes, **JP2-TCO**, **JP2-CP** and **JP3** (Fig. 1B). Together with new fluorogenic two-photon reporters (**QT1**, **QT2**, and **AAN-N$_3$**; Fig. 1C), we have successfully carried out both live-cell imaging and proteome profiling experiments to comprehensively evaluate the probes/reporters.

As shown in Fig. 1A, with a built-in Michael acceptor as the electrophilic trap to label a key cysteine residue in JNKs, JNK-IN-8 possesses good cellular selectivity against other kinases.[16] By chemically grafting bioorthogonal tags, including *trans*-cyclooctene (TCO), methylcyclopropene (CP), and terminal alkyne to the solvent-accessible dimethylamine end of JNK-IN-8 (renamed as WT in the current study), we obtained **JP2-TCO**, **JP2-CP** and **JP3** (Fig. 1B and Scheme S1, ESI†), respectively, representing the two most widely used click reactions (Cu-free tetrazine ligation as well as Cu^{I}-catalyzed azide–alkyne cycloaddition (CuAAC)). In order to minimize background fluorescence in live-cell imaging and ultimately carry out deep-tissue imaging by using two-photon fluorescence microscopy (TPFM),[17,18] we also developed three new fluorogenic two-photon reporters (Fig. 1C); they were designed based on the well-known two-photon dye acedan,[17] and represent the first reported case in which such

[a] *Department of Chemistry, National University of Singapore, 3 Science Drive 3, Singapore 117543, Singapore. E-mail: chmyaosq@nus.edu.sg*
[b] *Institute of Drug Metabolism and Pharmaceutical Analysis, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310058, China. E-mail: lhqian@zju.edu.cn*
[c] *Molecular Recognition Research Center, Bio-Med Program of KIST-School UST, Korea Institute of Science & Technology, Hwarangno 14-gil 5, Seongbuk-gu, Seoul 02792, South Korea*
[d] *Key Laboratory of Bioorganic Synthesis of Zhejiang Province, College of Biotechnology and Bioengineering, Zhejiang University of Technology, Hangzhou 310014, China*
[e] *Key Laboratory of Flexible Electronics (KLOFE) & Institute of Advanced Materials (IAM), Nanjing Tech University (NanjingTech), 30 South Puzhu Road, Nanjing 211800, China. E-mail: iamlli@njtech.edu.cn*

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c8cc09558b

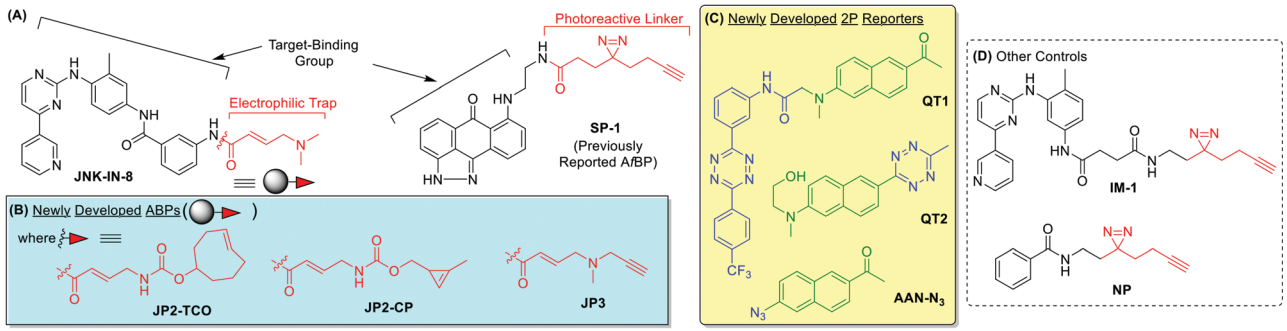

Fig. 1 Small-molecule dual-purpose probes and two-photon (TP) fluorescence Turn-ON reporters for JNKs. (A) Structure of the irreversible JNK inhibitor (JNK-IN-8)[16] and our previously reported affinity-based JNK probe (**SP-1**).[11] (B) Newly developed dual-purpose probes of JNKs and (C) their corresponding TP Turn-ON reporters. (D) Other control probes used in the current study.

molecules are capable of tetrazine ligation-/CuAAC-dependent Turn-ON fluorescence. Although one-photon fluorogenic reporters are available for click reactions, they are ill-suited for TPFM.[19,20] **QT1**/**QT2** and **AAN-N$_3$**, with strategically installed tetrazines and N$_3$ groups to quench intrinsic acedan fluorescence *via* intramolecular energy and charge transfer,[21,22] respectively, would be "Turned-ON" upon ligation to the respective JNK probes (**JP2-TCO**/**JP2-CP** and **JP3**). They may also be adapted to other covalent inhibitor-derived probes containing these clickable tags, enlarging the kinds of proteins capable of TPFM.

The detailed structures and performance of the above JNK probes and reporters, together with two previously published control probes (Fig. 1D),[11] are summarized in Table S1 and Fig. S1 (ESI†). In order to establish the suitability of our new fluorogenic reporters for *in situ* imaging, we confirmed their *in vitro* "click" and photophysical properties (Fig. S1–S4 and Table S2, ESI†); while all three reporters showed negligible background fluorescence prior to click reaction, their ligated products showed >10-fold increases in fluorescence (λ_{ex} = 360 nm; Fig. S4A, ESI†) with reasonable two-photon Turn-ON properties (λ_{ex} = 760 nm; Fig. S4B, ESI†). To better understand the proteome-labeling properties of **JP2-TCO**/**JP2-CP**/**JP3**, previously developed A*f*BPs, **SP-1**, **IM-1** (another A*f*BP sharing the similar core structure with JNK-IN-8[11]) and **NP** (a diazirine-containing photo-labeling control[11]), were compared. As shown in Fig. S5 (ESI†), in addition to maintaining reasonable *in vitro* activities, all three covalent probes potently inhibited cellular c-Jun phosphorylation catalyzed by JNKs;[16] among them, **JP3** provided the strongest inhibition in anisomycin-stimulated HeLa cells (EC_{50}: 0.5 μM), while **JP2-CP** and **JP2-TCO** showed comparatively weaker inhibition. To evaluate whether the drops in cellular activities, when compared to JNK-IN-8 (EC_{50}: 0.2 μM), might affect the probes' covalent engagement towards JNKs, we carried out *in vitro* labeling experiments with recombinant JNK1 (Fig. 2A); all three probes, as well as the two A*f*BPs (**SP-1** and **IM-1**) effectively labeled JNK1. Of note, both A*f*BPs upon UV-initiated labeling produced weaker fluorescent bands than that labeled by **JP3** which was similarly alkyne-tagged, under the same click conditions. This confirms what was originally envisaged; when compared to A*f*BPs, a superior target-labeling performance by our covalent JNK probes could be achieved.

We next evaluated the proteome reactivity profiles of these probes in cellular environments. HeLa cells, which are known to express a low level of endogenous JNKs, were first transfected

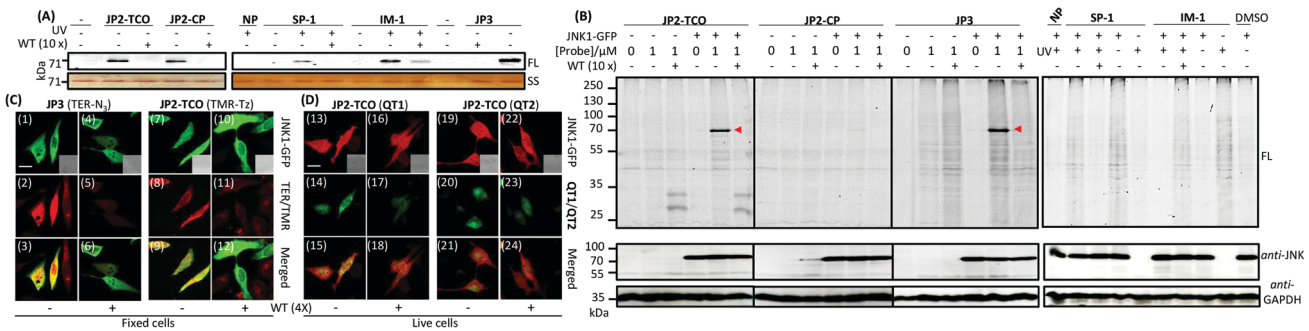

Fig. 2 (A) Labeling of recombinant JNK1 using various probes (1 μM, 1 h), with or without pre-treatment of WT (10×). The labeled protein was clicked with TER-Tz1 (for **JP2-TCO**/**JP2-CP**) or TER-N$_3$ (for **JP3**/**SP-1**/**IM-1**) prior to SDS-PAGE, in-gel fluorescence scanning (FL) and silver staining (SS). (B) *In situ* profiling of various probes in HeLa cells transfected with JNK1-GFP (top: FL). The corresponding western blotting (WB) gels are provided (bottom). The labeled cells (1 μM, 1 h) were lysed and then clicked with a reporter prior to analysis. In the case of A*f*BPs (**NP**/**SP-1**/**IM-1**), probe-treated cells were washed and UV-irradiated (λ_{ex} = 365 nm, 15 min, 4 °C) before lysis. (C) Imaging of JNK1-GFP-transfected HeLa cells using JNK probes (5 μM **JP3**/0.5 μM **JP2-TCO**; 1 h). (panels 1/4/7/10): IF with FITC-labeled *anti*-JNK (green). (Panels 2/5/8/11): probe channel upon click chemistry with TER-N$_3$/TMR-Tz (red). (Panels 3/6/9/12): merged channels. (D) No-wash imaging in live JNK1-GFP-transfected HeLa cells with **JP2-TCO** (5 μM; 1 h). After clicking with **QT1**/**QT2** (10 μM, 1 h), the cells were imaged. Cells were pre-treated with WT (4×) where applicable. Scale bar = 20 μm.

with a plasmid encoding JNK1-GFP, with subsequent expression of JNK1-GFP (70 kDa protein) confirmed by Western blotting (WB in Fig. 2B; bottom). Following *in situ* labeling by directly treating the transfected cells with each probe, and click chemistry of the resulting cell lysates with the corresponding reporters, the labeled proteomes were separated and analyzed by in-gel fluorescence scanning (FL in Fig. 2B; top); both **JP2-TCO** and **JP3** produced a major 70 kDa fluorescent band corresponding to JNK1-GFP. Such labeling was absent in non-transfected cells and in cells pretreated with JNK-IN-8 (10×). In direct contrast, neither **JP2-CP** nor the *Af*BPs produced any noticeable JNK1-GFP labeling. The inability of **JP2-CP** to label JNK1-GFP in a proteome environment, despite its reasonable JNK inhibitory activities (Fig. S5, ESI†), was probably caused by the CP tag which was reported to be less reactive towards tetrazine than TCO.[23] Our results thus highlight the need to carefully tune the size, reactivity and other parameters of bioorthogonal tags when developing fully functional probes.[10,24] Subsequent experiments indicated that both **JP2-TCO** and **JP3** robustly labelled JNK1-GFP even at low probe concentrations (*i.e.* 10 nM; Fig. S6, ESI†). These two probes were thus chosen for further investigations.

Then we evaluated the suitability of **JP2-TCO** and **JP3**, in combination with **QT1**/**QT2** and **AAN-N$_3$**, respectively, for cellular imaging. We first investigated whether **QT1**/**QT2**/**AAN-N$_3$** could be used for no-wash imaging by metabolically incorporating HeLa cells with a TCO- or alkyne-containing metabolite,[25,26] followed by click labeling and imaging *via* confocal microscopy (Fig. S7A, ESI†); the results indicated that both **QT1** and **QT2** were suitable two-photon reporters for no-wash live-cell imaging. Unfortunately, labeling of **AAN-N$_3$** with HPG-treated[26] HeLa cells, even under optimized click conditions (Fig. S3, ESI†), provided relatively poor contrasts, indicating that **AAN-N$_3$** was not suitable for cellular imaging. Next, **JP2-TCO** and **JP3** were used directly in JNK1-GFP-expressing HeLa cells, where overlapped fluorescence signals from JNK1-GFP and probe channels may provide a direct readout of selective target engagement of the probe. Two one-photon reporters, TER-N$_3$ and TMR-Tz (Table S1, ESI†), previously reported to be suitable for cellular imaging of alkyne- and TCO-containing probes, respectively,[25] were used for comparison (Fig. 2C). JNK1-GFP-transfected cells were first treated with **JP3**, followed by fixation and click chemistry with TER-N$_3$. As the native GFP fluorescence was quenched upon cell fixation, immunofluorescence (IF) was performed by using an *anti*-JNK antibody. As shown in Fig. 2C and Fig. S7B (ESI†), merged images showed good overlaps of the JNK1-GFP signal with the TER fluorescence from the probe channel (panel 3), and most probe-derived fluorescence was abolished in WT-pretreated cells (4×; panel 5). Similar results were obtained from **JP2-TCO**-treated cells using TMR-Tz (Fig. 2C and Fig. S7C, ESI†). Under similar conditions, transfected cells treated with **SP-1**/**IM-1** (Fig. S7F, ESI†), when compared to **JP3**-labeled cells side-by-side, failed to display any probe-specific labeling, probably due to their weaker interaction towards the target protein. Different from the always-ON reporter TMR-Tz, which required extensive cell wash prior to imaging, the two-photon fluorogenic reporters **QT1**/**QT2** could be used for no-wash imaging on **JP2-TCO**-treated JNK1-GFP-transfected cells (Fig. 2D and Fig. S7D, E, ESI†); we successfully observed Turn-ON fluorescence from the **QT1**/**QT2** channel which was significantly reduced in WT-pretreated cells, and mostly abolished in non-transfected cells. Such Turn-ON reporters may allow future real-time imaging under undisturbed environmental conditions.[27] It should be noted that, in the above experiments, we detected weak but noticeable probe-derived fluorescence in JNK-IN-8-pretreated cells (Fig. 2C and D, panels 5/11/17/23), indicating the presence of cellular off-targets. This result was consistent with our concentration-dependent labeling experiments (Fig. S6, ESI†), where increasing concentrations of **JP3**/**JP2-TCO** ($\geq$1 μM) introduced additional fluorescent bands.[13] Our results thus suggested that our new covalent JNK probes (**JP2-TCO**/**JP3**), as well as the two-photon Turn-ON reporters (**QT1**/**QT2**), could be used for selective bioimaging of elevated levels of JNKs in consideration of both probe selectivity and detection sensitivity.

We next investigated the proteome-wide selectivity of these covalent probes at lower concentrations (*i.e.*, $\leq$1 μM) towards endogenous JNKs. Cell lines expressing different levels of JNKs (HeLa < HEK293T < SH-SY5Y; Fig. S8A, ESI†) were chosen to assess the labelling performance of **JP3**/**JP2-TCO** (Fig. 3A and Fig. S8B, C, ESI†). Direct visualization of labelled proteomes *via* in-gel fluorescence scanning showed the positive labelling of a ~46 kDa band. To validate whether JNKs were among the labelled proteins, pull-down (PD)/WB was carried out. In all three cell lines, both **JP3** and **JP2-TCO** successfully labelled/enriched endogenous JNKs and such labelling was efficiently abolished by WT (10×; Fig. 3B and Fig. S8D, ESI†), indicating the possibility of directly visualizing JNK–drug interaction at the proteome-wide level.[28] Interestingly, **JP2-CP** which earlier failed to display a distinct fluorescent band from JNK1-GFP-expressing HeLa cells *via* in-gel fluorescence scanning (using TER-Tz1), also showed positively enriched JNKs (using Biotin-Tz2, which showed a stronger reactivity than TER-Tz1[10]). Subsequent large-scale LC-MS/MS experiments on the enriched proteomes from HeLa cells further confirmed that all three probes could label endogenous JNKs (Fig. 3C and Table S3, SI_II, ESI†); among the proteins identified from each labeling reaction, only two hits,

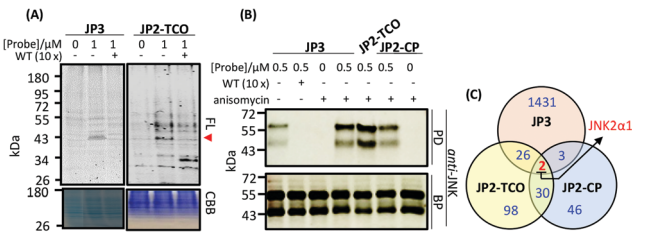

Fig. 3 Cellular performance of small-molecule probes towards endogenous JNKs in anisomycin-stimulated HeLa cells. (A) *In situ* profiling of cells upon labeling by **JP3**/**JP2-TCO** (1 μM, 1 h). Cells were pre-treated with WT (10 μM, 1 h) where indicated. Following lysis, labeled proteomes were clicked with TER reporters and analyzed. (B) The corresponding PD/WB (*anti*-JNK) results of JNK probes (0.5 μM, 1 h). Following cell lysis and click chemistry with biotin-N$_3$ (for **JP3**) or biotin-Tz2 (for **JP2-TCO**/**-CP**), the labeled lysates were analyzed before pull-down (BP) and after pull-down (PD). (C) Standard PD/LC-MS summarizing the number of proteins enriched by **JP3**/**JP2-TCO**/**JP2-CP** (1 μM, 1 h) in HeLa cells.

including an isoform of JNK2 (JNK2α1; 44 kDa), were successfully labeled using all three probes. JNK1 and JNK3 were not detected possibly due to their relatively low expression in HeLa cells (Fig. S8A, ESI†).

Our work herein represents the first reported example in which live-cell imaging and *in situ* proteome profiling were carried out to comprehensively compare covalent and non-covalent probes sharing related target-binding pharmacophores. Taking JNK probes as examples, our current view is that properly designed covalent probes may deliver overwhelmingly better performance in both profiling and imaging of cellular targets compared to affinity-based probes. With the successful development of new JNK probes (**JP2-TCO**/**JP3**) and two-photon fluorogenic reporters (**QT1**/**QT2**), they may be used for *in situ* profiling and imaging of cellular JNK activities in cells expressing moderate/high endogenous JNKs.

We thank Dr Hailong Zhang (NUS) for his help with chemical synthesis. Financial support was provided by the Synthetic Biology Research & Development Programme (SBP) of National Research Foundation (NRF), the GSK-EDB Trust Fund (R-143-000-688-592), MOE-T1 (R-143-000-694-114) of Singapore, the National Natural Science Foundation of China (21877100, 21708034, 81672508 and 61505076) and the Jiangsu Provincial Foundation for Distinguished Young Scholars (BK20170041).

Conflicts of interest

There are no conflicts to declare.

Notes and references

1 A. M. Manning and R. J. Davis, *Nat. Rev. Drug Discovery*, 2003, **2**, 554–565.
2 E. T. Coffey, *Nat. Rev. Neurosci.*, 2014, **15**, 285–299.
3 K. M. Dean and A. E. Palmer, *Nat. Chem. Biol.*, 2014, **10**, 512–523.
4 M. Fosbrink, N. N. Aye-Han, R. Cheong, A. Levchenko and J. Zhang, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 5459–5464.
5 M. Fernandez-Suarez and A. Y. Ting, *Nat. Rev. Mol. Cell Biol.*, 2008, **9**, 929–943.
6 Y. Takaoka, A. Ojida and I. Hamachi, *Angew. Chem., Int. Ed.*, 2013, **52**, 4088–4106.
7 M. Grammel and H. C. Hang, *Nat. Chem. Biol.*, 2013, **9**, 475–484.
8 M. J. Niphakis and B. F. Cravatt, *Annu. Rev. Biochem.*, 2014, **83**, 341–377.
9 Z. Li, D. Wang, L. Li, S. Pan, Z. Na, C. Y. Tan and S. Q. Yao, *J. Am. Chem. Soc.*, 2014, **136**, 9990–9998.
10 S. Pan, S. Y. Jang, D. Wang, S. S. Liew, Z. Li, J. S. Lee and S. Q. Yao, *Angew. Chem., Int. Ed.*, 2017, **56**, 11816–11821.
11 Z. Li, P. Hao, L. Li, C. Y. Tan, X. Cheng, G. Y. Chen, S. K. Sze, H. M. Shen and S. Q. Yao, *Angew. Chem., Int. Ed.*, 2013, **52**, 8551–8556.
12 B. L. Bennett, D. T. Sasaki, B. W. Murray, E. C. O'Leary, S. T. Sakata, W. Xu, J. C. Leisten, A. Motiwala, S. Pierce, Y. Satoh, S. S. Bhagwat, A. M. Manning and D. W. Anderson, *Proc. Natl. Acad. Sci. U. S. A.*, 2001, **98**, 13681–13686.
13 B. R. Lanning, L. R. Whitby, M. M. Dix, J. Douhan, A. M. Gilbert, E. C. Hett, T. O. Johnson, C. Joslyn, J. C. Kath, S. Niessen, L. R. Roberts, M. E. Schnute, C. Wang, J. J. Hulce, B. Wei, L. O. Whiteley, M. M. Hayward and B. F. Cravatt, *Nat. Chem. Biol.*, 2014, **10**, 760–767.
14 M. H. Wright and S. A. Sieber, *Nat. Prod. Rep.*, 2016, **33**, 681–708.
15 L. Li, C. W. Zhang, J. Ge, L. Qian, B. H. Chai, Q. Zhu, J. S. Lee, K. L. Lim and S. Q. Yao, *Angew. Chem., Int. Ed.*, 2015, **54**, 10821–10825.
16 T. Zhang, F. Inesta-Vaquera, M. Niepel, J. Zhang, S. B. Ficarro, T. Machleidt, T. Xie, J. A. Marto, N. Kim, T. Sim, J. D. Laughlin, H. Park, P. V. LoGrasso, M. Patricelli, T. K. Nomanbhoy, P. K. Sorger, D. R. Alessi and N. S. Gray, *Chem. Biol.*, 2012, **19**, 140–154.
17 H. M. Kim and B. R. Cho, *Chem. Rev.*, 2015, **115**, 5014–5055.
18 L. Qian, L. Li and S. Q. Yao, *Acc. Chem. Res.*, 2016, **49**, 626–634.
19 H. Wu and N. K. Devaraj, *Acc. Chem. Res.*, 2018, **51**, 1249–1259.
20 P. Shieh and C. R. Bertozzi, *Org. Biomol. Chem.*, 2014, **12**, 9307–9320.
21 L. G. Meimetis, J. C. Carlson, R. J. Giedt, R. H. Kohler and R. Weissleder, *Angew. Chem., Int. Ed.*, 2014, **53**, 7531–7534.
22 P. Shieh, M. S. Siegrist, A. J. Cullen and C. R. Bertozzi, *Proc. Natl. Acad. Sci. U. S. A.*, 2014, **111**, 5456–5461.
23 D. N. Kamber, L. A. Nazarova, Y. Liang, S. A. Lopez, D. M. Patterson, H. W. Shih, K. N. Houk and J. A. Prescher, *J. Am. Chem. Soc.*, 2013, **135**, 13680–13683.
24 D. S. Tyler, J. Vappiani, T. Caneque, E. Y. N. Lam, A. Ward, O. Gilan, Y. C. Chan, A. Hienzsch, A. Rutkowska, T. Werner, A. J. Wagner, D. Lugo, R. Gregory, C. Ramirez Molina, N. Garton, C. R. Wellaway, S. Jackson, L. MacPherson, M. Figueiredo, S. Stolzenburg, C. C. Bell, C. House, S. J. Dawson, E. D. Hawkins, G. Drewes, R. K. Prinjha, R. Rodriguez, P. Grandi and M. A. Dawson, *Science*, 2017, **356**, 1397–1401.
25 J. Ge, C. W. Zhang, X. W. Ng, B. Peng, S. Pan, S. Du, D. Wang, L. Li, K. L. Lim, T. Wohland and S. Q. Yao, *Angew. Chem., Int. Ed.*, 2016, **55**, 4933–4937.
26 P. Shieh, V. T. Dien, B. J. Beahm, J. M. Castellano, T. Wyss-Coray and C. R. Bertozzi, *J. Am. Chem. Soc.*, 2015, **137**, 7145–7151.
27 Y. Hori and K. Kikuchi, *Curr. Opin. Chem. Biol.*, 2013, **17**, 644–650.
28 C. G. Parker, A. Galmozzi, Y. Wang, B. E. Correia, K. Sasaki, C. M. Joslyn, A. S. Kim, C. L. Cavallaro, R. M. Lawrence, S. R. Johnson, I. Narvaiza, E. Saez and B. F. Cravatt, *Cell*, 2017, **168**(527–541), e529.

