Article:
Wright, MH orcid.org/0000-0003-2731-4707, Tao, Y, Drechsel, J et al. (6 more authors) (2017) Quantitative chemoproteomic profiling reveals multiple target interactions of spongiolactone derivatives in leukemia cells. Chemical Communications, 95. pp. 12818-12821. ISSN 1359-7345

https://doi.org/10.1039/C7CC04990K

ChemComm

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

rsc.li/chemcomm

View Article Online
DOI: 10.1039/C7CC04990K

Chemical Communications

COMMUNICATION

Quantitative Chemoproteomic Profiling Reveals Multiple Target Interactions of Spongiolactone Derivatives in Leukemia Cells

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

M. H. Wright,[a,d] Y. Tao,[c] J. Drechsel,[a] J. Krysiak,[a] S. Chamni,[b,Δ] A. Weigert-Munoz,[a] N. Harvey,[b] D. Romo[c] * and S. A. Sieber[a] *

The spongiolactones are marine natural products with an unusual rearranged spongiane skeleton and a fused β-lactone ring. These compounds have potential anticancer properties but their mode of action has yet to be explored. Here we employ activity-based protein profiling to identify the targets of a more potent spongiolactone derivative in live cancer cells, and compare these to the targets of a simpler β-lactone. These hits provide the first insights into the covalent mechanism of action of this natural product class.

The spongiolactones are a class of natural products with anti-proliferative and potentially immunosuppressive properties.[1] These compounds contain a unique cyclopentane fused β-lactone, a potentially protein-reactive moiety that targets active site nucleophiles in a range of cell types from cancer cells to bacteria.[2-4] However, the spongiolactone mechanism of action is yet to be elucidated. Previously we reported the first total synthesis of (+)-spongiolactone (**1**) and the discovery of a more potent, isomeric derivative, regio ($\Delta^{9,12}$), C6, C15-bis-epi-spongiolactone (±)-**2** (Fig. 1a).[5] This derivative, even as a racemate, exhibited greater potency toward a human chronic mylogenous leukemia cell line (K562) in comparison to the natural product (IC_{50} 29 vs. 129 μM, respectively). To further understand its mechanism of action we developed an enantioselective synthesis of the isomeric spongiolactone derivative (+)-**2** in addition to a derived alkynyl probe (+)-**3.** This probe, along with the racemic version (±)-**3**, was then applied in activity-based protein profiling (ABPP)[6, 7] to elucidate the targets of this natural product derivative in live cells (Fig. 1b).

Based on our previously established synthetic route to spongiolactone, we utilized a kinetic resolution of a previously described advanced, racemic intermediate, (±)-**8**, obtained in 3 steps from 1,3-cyclohexanedione (Scheme 1).[5] Under standard CBS reduction conditions the corresponding alcohol (+)-**9** was isolated in 37% yield[8, 9] (92% ee). The relative stereochemistry of cyclohexane (+)-**9** was assigned based on coupling constant analysis while the absolute stereochemistry was assigned based on the CBS model (see ESI).[10] Subsequent Swern oxidation delivered the optically active cyclohexanone (+)-**8**, previously described in racemic form. This cyclohexanone (+)-**8** was then converted to alcohol (+)-**11** employing our previously established synthetic route via the tricyclic β-lactone (+)-**10** to

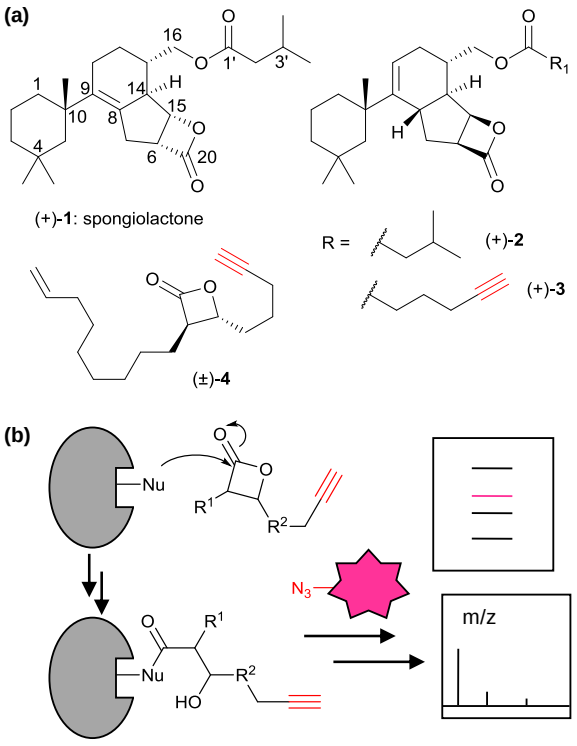
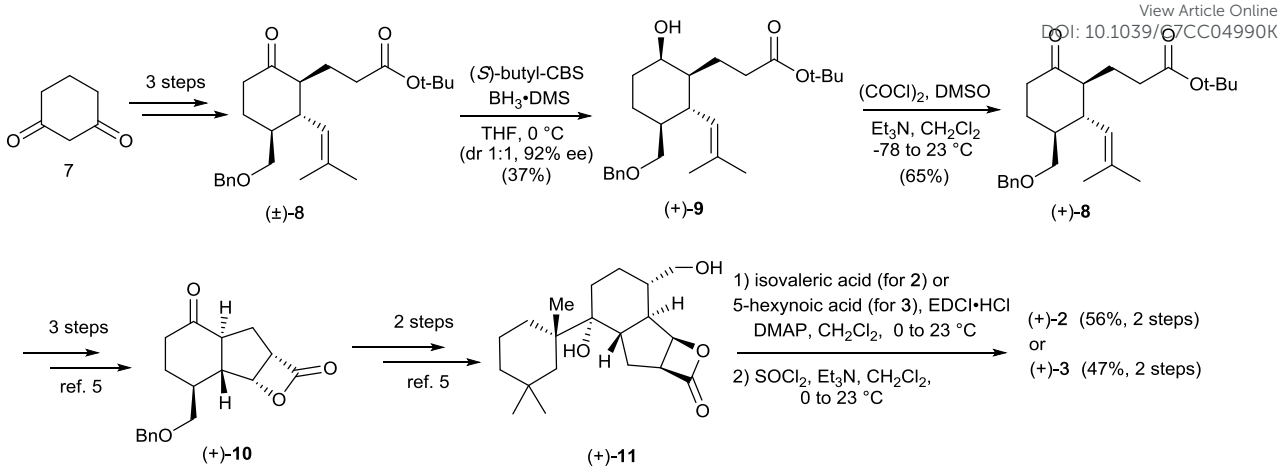


Fig. 1. (a) Structures of spongiolactone (+)-**1**, regio bis-epi spongiolactone (+)-**2**, probe (+)-**3**, and previous β-lactone probe (±)-**4**. **(b)** β-lactone containing compounds potentially react with active site nucleophiles on proteins; compounds containing an alkyne can be clicked to functionalized azides for ABPP experiments to identify protein targets via fluorescent SDS-PAGE or mass spectrometry.

[a] *Center for integrated Protein Science (CIPSM), Department of Chemistry, Technical University of Munich, Lichtenbergstr. 4, D-85748, Garching, Germany.*
[b] *Department of Chemistry, Texas A&M University, P.O. Box 30012, College Station, TX 77842 (USA).*
[c] *Department of Chemistry & Biochemistry, Baylor University, One Bear Place 97348, Waco, TX 76798 (USA).*
[d] *School of Chemistry, University of Leeds, Leeds, LS2 9JT, UK*
[Δ] Current address: Department of Pharmacognosy and Pharmaceutical Botany, Chulalangkorn University, Bangkok (Thailand).
Electronic Supplementary Information (ESI) available: Methods; Supp. Figures S1-S7; Tables S1-S4 (Excel files). Proteomic data are available via ProteomeXchange with identifier PXD006811. See DOI: 10.1039/x0xx00000x

View Article Online
DOI: 10.1039/C7CC04990K



Scheme 1. Synthesis of compound (+)-**2** and probe (+)-**3**. See also Scheme S1 in Supplementary Information.

deliver both regio, bis-epi spongiolactone (+)-**2** and the alkynyl probe (+)-**3** through esterification with isovaleric acid and 5-hexynoic acid, respectively. The racemic version of the alkynyl probe, (±)-**3** was prepared for initial protein profiling in an analogous fashion to the optically active probe (+)-**3** (Scheme S1).

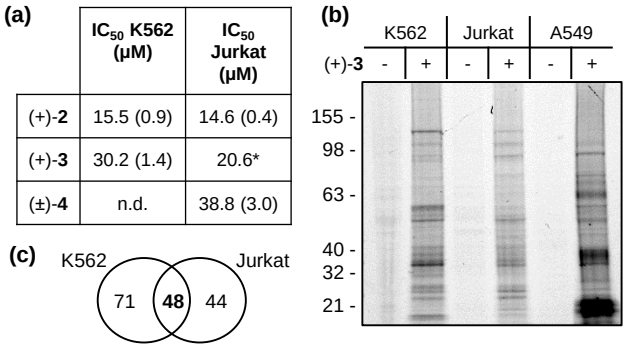
Both parent compound (±)-**2** and probes (±)-**3** and (+)-**3** exhibited micromolar activity (14-30 μM) in a cell cytotoxicity assay against K562 cells and comparable activity in another leukemic cell line, Jurkat (Fig. 2a, Fig. S1). Enantiomerically pure (+)-**2** showed an increase in potency (~2-fold) compared to the racemic compound. Simple racemic, β-lactone (±)-**4**, which was previously utilized to label enzymes in bacteria,[2, 3] also inhibited growth in Jurkat cells; potency was weaker but nonetheless in the same range as the spongiolactones.

We next sought to determine whether these β-lactone-containing compounds covalently labelled proteins in live cells. The terminal alkyne handle of probe **3** enables downstream capture of probe-labelled proteins by copper-catalysed click chemistry (CuAAC) and analysis by in-gel fluorescence and proteomics (Fig. 1b).[11] In initial studies, K562 cells were incubated with 10 μM racemic probe (±)-**3** (or vehicle, DMSO) to obtain an absolute stereochemistry-independent labeling pattern. Cells were lysed and proteins ligated to rhodamine-azide (RhN_3) via CuAAC. Gel separation and fluorescence imaging revealed multiple bands, which were dose-dependently outcompeted by pre-incubation of cells with racemic (±)-**2** and, to a lesser extent, spongiolactone (+)-**1** (Fig. S2), suggesting that the probe (±)-**3** labels the same proteins as these parent compounds. Enantiomerically pure probe (+)-**3** gave a similar pattern to racemic (±)-**3** in both K562 and Jurkat cells (Fig. 2b), with dose-dependent labelling down to 3 μM (Fig. S2). To identify labelled proteins, samples from K562 cells incubated with 10 μM (+)-**3** were ligated to biotin-N_3, enriched on avidin beads, digested by trypsin and peptides analysed by gel-free LC-MS/MS. Label free quantification was performed to compare DMSO with probe-incubated samples. We observed high reproducibility across four biological replicates (Fig. S3).

Consistent with the large numbers of bands on-gel, 104 proteins were significantly enriched in samples treated with (+)-**3** over controls (*t*-test, Fig. 2d, Table S1). Gene Ontology (GO)[12] enrichment analysis revealed that hits are typically involved in lipid metabolism and transport (Table S1). Predicted endoplasmic reticulum (ER) and mitochondria-localised membrane proteins dominated. Indeed, fractionation and gel analysis confirmed that many hits are insoluble (Fig. S2).

(a)

	IC_{50} K562 (μM)	IC_{50} Jurkat (μM)
(+)-**2**	15.5 (0.9)	14.6 (0.4)
(+)-**3**	30.2 (1.4)	20.6*
(±)-**4**	n.d.	38.8 (3.0)



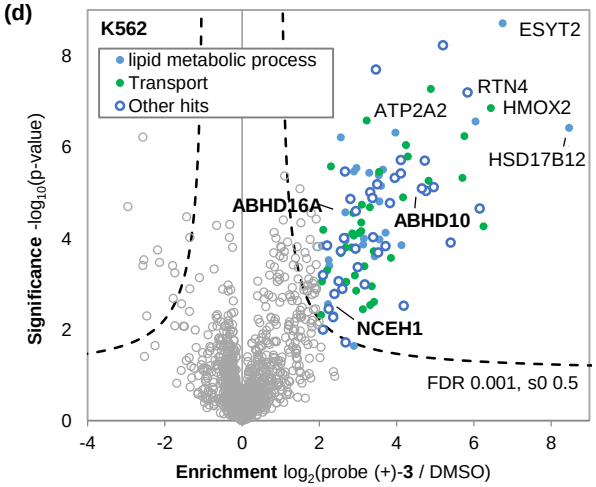


Fig. 2. (a) Cytotoxicity of compounds in K562 and Jurkat cells (MTT assay, 24 h). Biological triplicate, except for * (single experiment, technical triplicate). n.d. = not determined. Weighted mean (error). See Supp. Fig. S1 for curves. **(b)** In-gel fluorescence analysis after 2 h labelling with 10 μM (+)-**3** in K562, Jurkat and A549 cells. **(c)** Overlap of hits in K562 and Jurkat cell lines. **(d)** Volcano plot depicting enrichment and significance of enrichment in chemical proteomics experiments with K562 cells (2 h, 10 μM (+)-**3**). See Tables S1 and S2.

View Article Online
DOI: 10.1039/C7CC04990K

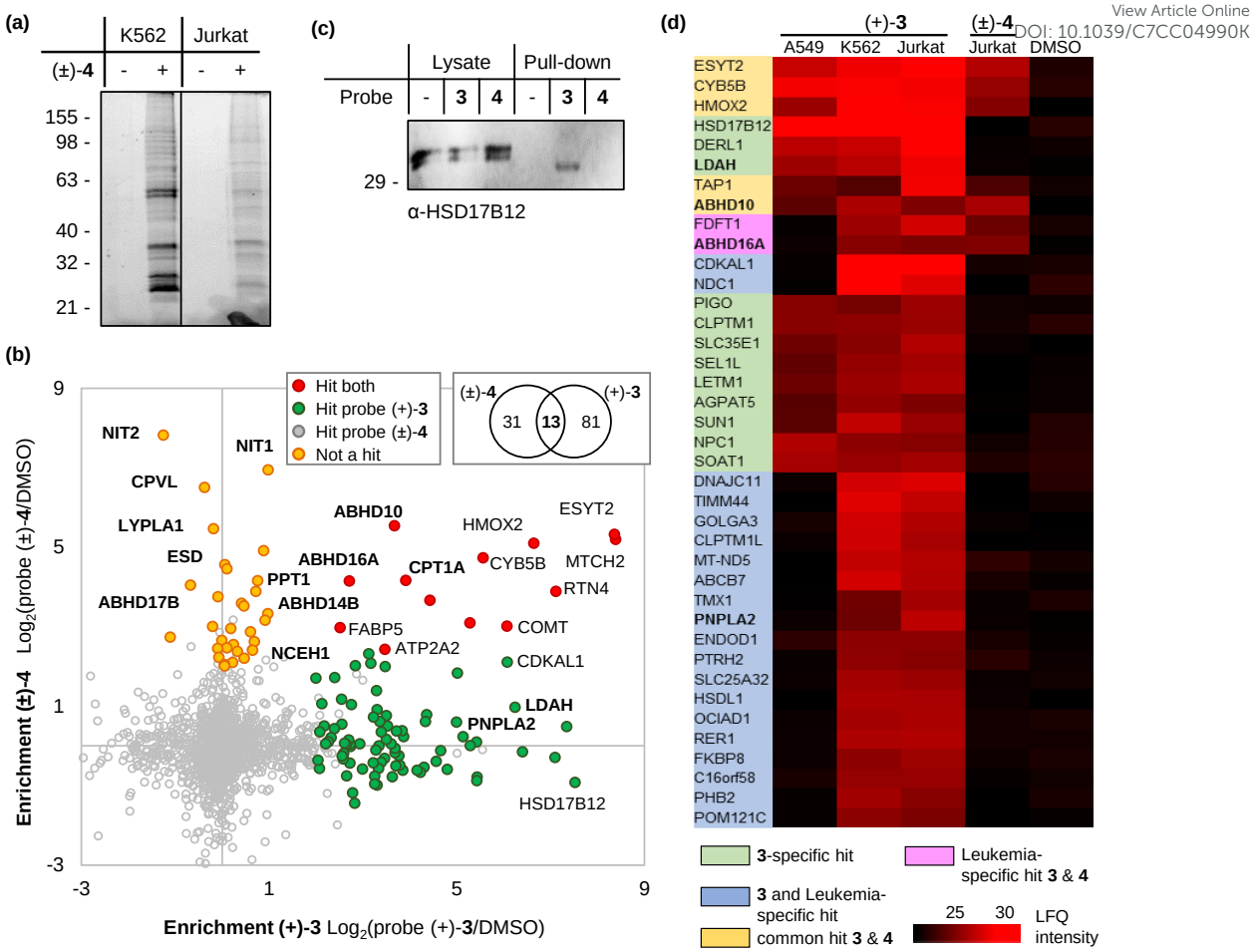


Fig. 3. (a) Gel-based analysis after labelling for 2 h with 10 μM probe (±)-**4** in K562 and Jurkat cells. **(b)** Overlap of proteomic hits with probes (+)-**3** and (±)-**4** in Jurkat cells. Inset: Venn diagram overlap. Hits here were defined based on significance in the *t*-tests (Table S3). **Bold** = hydrolase. **(c)** Western blot for HSD17B12 in lysate and after pull-down following labelling (2 h) with (+)-**3** or (±)-**4** in K562 cells. Full gel and blot shown in Fig. S6. **(d)** Heat-map analysis of LFQ intensities (mean across replicates) for proteins defined as hits in K562 and Jurkat cells. **Bold** = hydrolase. See Table S4.

We repeated the chemical proteomic experiments with Jurkat cells; a large number of hits was again observed (Table S2, Fig. S4) with relatively high overlap between the two leukemic cell lines (Fig. 2c). Gene Ontology enrichment performed for the 48 high confidence overlapping hits revealed that they are preferentially involved in transport processes, lipid metabolism and localisation (Fig. S4). Surprisingly, only a few proteins with a predicted active site nucleophile (e.g. serine hydrolases) were amongst the hits; these included esterase ABHD10, phosphatidylserine hydrolase ABHD16A,[13] and sterol hydrolase NCEH1. Thus at concentrations concurrent with cytotoxicity probe (+)-**3** labels a more diverse set of proteins than hydrolases.

We next asked whether the simple β-lactone (±)-**4**, which binds hydrolases as well as other proteins in bacteria,[2, 3] labels a similar profile of targets to (+)-**3** in mammalian cells. Probe (±)-**4** labelled proteins in K562 cells in a dose-dependent manner (Fig. S5) and gave a similar profile in Jurkat cells (Fig. 3a). Interestingly, probe (±)-**4** exhibited a markedly distinct labelling pattern compared to the regio bis-epi spongiolactone-derived probe (+)-**3** (Fig. 2), suggesting that the scaffold in which the β-lactone warhead is embedded, not surprisingly, has a dramatic effect on preferences for protein binding partners. Chemical proteomic analysis of labelled proteins in Jurkat revealed that, in contrast to (+)-**3**, ~50% of (±)-**4** hits have annotated hydrolase activity (Fig. S5). GO analysis classifies many hits as involved in lipid binding or metabolism (Table S3, Fig. S5).

Comparison with the data from labelling with (+)-**3** shows overlapping and distinct targets of the two probes in Jurkat (Fig. 3b, Table S3). Common non-hydrolase hits include heme-binding oxidoreductases HMOX2 and CYB5B, and membrane proteins with transport or structural roles in the ER and mitochondria (e.g. RTN4, MTCH2). The enrichment of these, mostly abundant, proteins may be due to promiscuous reactivity of the probes in the membrane. However, acrylamide probes have recently been shown to selectively label a specific cysteine residue on RTN4, impairing cancer cell growth.[14] Common hydrolase hits of both probes include ABHD16A, a lipase important for phosphatidylserine metabolism that has previously been shown to be covalently inhibited by a β-lactone probe in K562 and brain cells.[13]

We previously observed (±)-**2** to be >4-fold more toxic towards leukemia K562 than other cell lines.[5] Consistent with this, (+)-

2, its corresponding probe (+)-**3,** and simple β-lactone (±)-**4** all showed greatly reduced activity against lung cancer cell line A549 (>100 μM, Fig. S1). We hypothesised that such selective toxicity could be due to differences in either cellular targets, the susceptibility of different cells towards perturbation of specific pathways via the same targets, or compound metabolism, uptake or efflux. ABPP with spongiolactone-derived probe (+)-**3** in A549 cells gave a different pattern (Fig. 2b, S6) but still strong labelling, suggesting efficient uptake and protein labelling. Chemical proteomics revealed moderate overlap of potential hits with leukemia cell lines (Fig. S7, Table S4). Proteins classified as strong hits in both K562 and Jurkat cells were compared across both probes and all cell lines via clustering of mean LFQ intensities (Fig. S7). Proteins with intensity in DMSO controls were removed to further refine the list down to high confidence binders (shown in Fig. 3d).

This analysis revealed hits with differing cell line- and probe-specificity. Some proteins are global targets, such as HMOX2, which has multiple cysteines that may react with small molecules.[15] Others are probe specific: for example, broadly expressed dehydrogenase HSD17B12 is a strong hit of probe (+)-**3**, as confirmed by Western blot analysis following pull-down of labelled proteins (Fig. 3c). HSD17B12 acts on very long chain fatty acyl chains as well as estrogen,[16, 17] and the fused ring scaffold of (+)-**3** may explain why it strongly labels this protein whilst the simple dialkyl β-lactone probe (±)-**4** does not. A large number of hits (22) appear to be labelled by (+)-**3** only in leukemia cells, with only two of these also labelled by (±)-**4**. However, target profiles cannot be simply explained by differing protein abundance across cell lines, which shows no clear correlation with enrichment (Table S4, Fig. S7). These data suggest that interaction of (+)-**3** with multiple targets is responsible for its cancer cell cytotoxicity. Likely most of these interactions are weak, as suggested by modest competitive labelling (Fig. S2) and incomplete labelling of HSD17B12 (Fig. S6). We performed a global comparative quantitative proteome analysis following 24 h treatment of K562 cells with 30 μM (+)-**3** (the approximate IC_{50} in cytotoxicity assays) or DMSO (Table S5, Fig. S8). This revealed significant probe-induced upregulation of proteins involved in lipid (especially fatty acid and sterol) metabolism, as well as reactive oxygen and nitrogen processes (Fig. S9), consistent with the chemical proteomic data and suggesting that broad perturbations to lipid metabolism result in cytotoxicity.

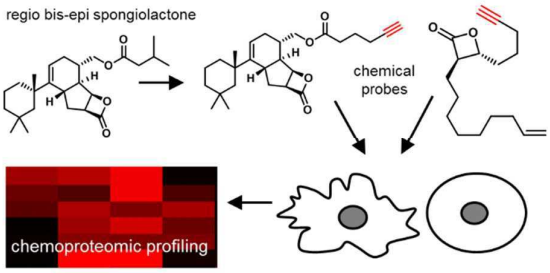
In conclusion, we synthesised a spongiolactone-derived probe and performed quantitative chemical proteomic profiling to identify the potential protein targets in live cells. Probe (+)-**3** based on (+)-**2** addresses a broad array of targets involved in various pathways, suggesting that its bioactivity stems from polypharmacology. Comparison with the target profile of a simpler β-lactone adds to the growing body of evidence that the proteome reactivity of electrophilic moieties, not surprisingly, is heavily influenced by other structural features. Probe (±)-**4**, which mimics natural products such as lipstatin, preferentially labels hydrolases, whereas the spongiolactone-derived probe, embedded in a more rigid and complex scaffold, addresses a distinct spectrum of targets. This is an important observation given the continued exploitation of electrophilic natural products, and in particular β-lactones, as bioactive compounds and chemical probes.[11] Finally, the proteomic hits presented here are valuable starting points for further understanding the mode of action and differing potencies of spongiolactones in diverse cellular contexts.

This work was supported by the European Union's Seventh Framework Programme (PIEF-GA-2013-625528 to MHW), Deutsche Forschungsgemeinschaft FOR1406, CIPSM (SAS), the National Science Foundation (CHE-1546973 to DR) and Welch Foundation (AA-1280 to DR).

Notes and references

1. M. E. Rateb, W. E. Houssen, M. Schumacher, W. T. Harrison, M. Diederich, R. Ebel and M. Jaspars, *J Nat Prod*, 2009, **72**, 1471-1476.
2. T. Bottcher and S. A. Sieber, *Angew Chem Int Ed*, 2008, **47**, 4600-4603.
3. T. Bottcher and S. A. Sieber, *J Am Chem Soc*, 2008, **130**, 14400-14401.
4. M. Zhu, W. D. Harshbarger, O. Robles, J. Krysiak, K. G. Hull, S. W. Cho, R. D. Richardson, Y. Yang, A. Garcia, L. Spiegelman, B. Ramirez, C. T. Wilson, J. A. Yau, J. T. Moore, C. B. Walker, J. C. Sacchettini, W. R. Liu, S. A. Sieber, J. W. Smith and D. Romo, *Bioorg Med Chem*, 2017, **25**, 2901-2916.
5. N. L. Harvey, J. Krysiak, S. Chamni, S. W. Cho, S. A. Sieber and D. Romo, *Chem Eur J*, 2015, **21**, 1425-1428.
6. M. J. Niphakis and B. F. Cravatt, *Annu Rev Biochem*, 2014, **83**, 341-377.
7. L. E. Sanman and M. Bogyo, *Annu Rev Biochem*, 2014, **83**, 249-273.
8. E. J. Corey, R. K. Bakshi and S. Shibata, *J Am Chem Soc*, 1987, **109**, 5551-5553.
9. L. Deloux and M. Srebnik, *Chem Rev*, 1993, **93**, 763-784.
10. E. J. Corey and C. J. Helal, *Angew Chem Int Ed*, 1998, **37**, 1987-2012.
11. M. H. Wright and S. A. Sieber, *Nat Prod Rep*, 2016, **33**, 681-708.
12. GeneOntologyConsortium, *Nucleic Acids Res*, 2015, **43**, D1049-1056.
13. S. S. Kamat, K. Camara, W. H. Parsons, D. H. Chen, M. M. Dix, T. D. Bird, A. R. Howell and B. F. Cravatt, *Nat Chem Biol*, 2015, **11**, 164-171.
14. L. A. Bateman, T. B. Nguyen, A. M. Roberts, D. K. Miyamoto, W. M. Ku, T. R. Huffman, Y. Petri, M. J. Heslin, C. M. Contreras, C. F. Skibola, J. A. Olzmann and D. K. Nomura, *Chem Commun (Camb)*, 2017, DOI: 10.1039/c7cc01480e.
15. L. Yi, P. M. Jenkins, L. I. Leichert, U. Jakob, J. R. Martens and S. W. Ragsdale, *J Biol Chem*, 2009, **284**, 20556-20561.
16. Y. A. Moon and J. D. Horton, *J Biol Chem*, 2003, **278**, 7335-7343.
17. V. Luu-The, P. Tremblay and F. Labrie, *Mol Endocrinol*, 2006, **20**, 437-443.

View Article Online
DOI: 10.1039/C7CC04990K



Identifying the covalent targets of a natural product-derivative in cancer cells.