DOI: 10.1002/ange.201300683

Kinase Proteomics

Design and Synthesis of Minimalist Terminal Alkyne-Containing Diazirine Photo-Crosslinkers and Their Incorporation into Kinase Inhibitors for Cell- and Tissue-Based Proteome Profiling**

*Zhengqiu Li, Piliang Hao, Lin Li, Chelsea Y. J. Tan, Xiamin Cheng, Grace Y. J. Chen, Siu Kwan Sze, Han-Ming Shen, and Shao Q. Yao**

Photoaffinity labeling (PAL) is a powerful technique in chemical biology and chemical proteomics, and has been successfully demonstrated on a variety of non-covalent molecular interactions.[1] Recently, we and others have shown that this approach is suitable for interrogating non-covalent protein–drug interactions under native cellular conditions.[2,3] To do so, typically the drug is derivatized with a photo-reactive group and a reporter group (referred to herein as the linker), to make the corresponding probe.[2b] Upon administration into live cells and photo-irradiation, a highly reactive intermediate is generated from the probe, which can covalently capture its binding proteins in a distance-dependent manner. The chemically stable, cross-linked protein–probe complexes thus become amenable to various downstream biochemical analyses, including pull-down (PD)/LC-MS/MS analysis, for large-scale identification of potential cellular protein targets of the unmodified drug. One of the most essential considerations in PAL is that the probe, which is a derivative of the drug, must retain most, if not all, of the original biological activities of the drug after derivatization with the linker. This thus calls for the development of what we now call "minimalist" linkers in which both the photoreactive and reporter groups are made as small as possible, so as to minimize interference upon binding to the target proteins.

Based on the literature and our previous experience,[1,2,4] we chose aliphatic diazirines over aromatic diazirines, benzophenones, and other common photoreactive groups, owing to their smaller size (to minimize interference with protein binding) and short irradiation time needed to generate the highly reactive carbene species (to minimize non-specific protein labeling). We used a small terminal alkyne as the reporter, which can be used for subsequent ex vivo PD/target identification by conjugation to suitable reporters (rhodamine-N_3 or biotin-N_3; see the Supporting Information) using well-established bioorthogonal click chemistry.[5,6] The resulting minimalist linkers are shown in Figure 1 A (**L1**–**L3**), each containing a functional group (-CO_2H, -NH_2, and -I) that could be conjugated to drug molecules through robust acylation and alkylation reactions. Our expectation was that, with such linker designs, a variety of bioactive small molecules (for example, compounds **1**–**12** in Figure 1 A) could be readily modified without significant changes in both their size and structure, and at the same time providing a chemically tractable modality to capture non-covalent protein–drug interactions in live cells.

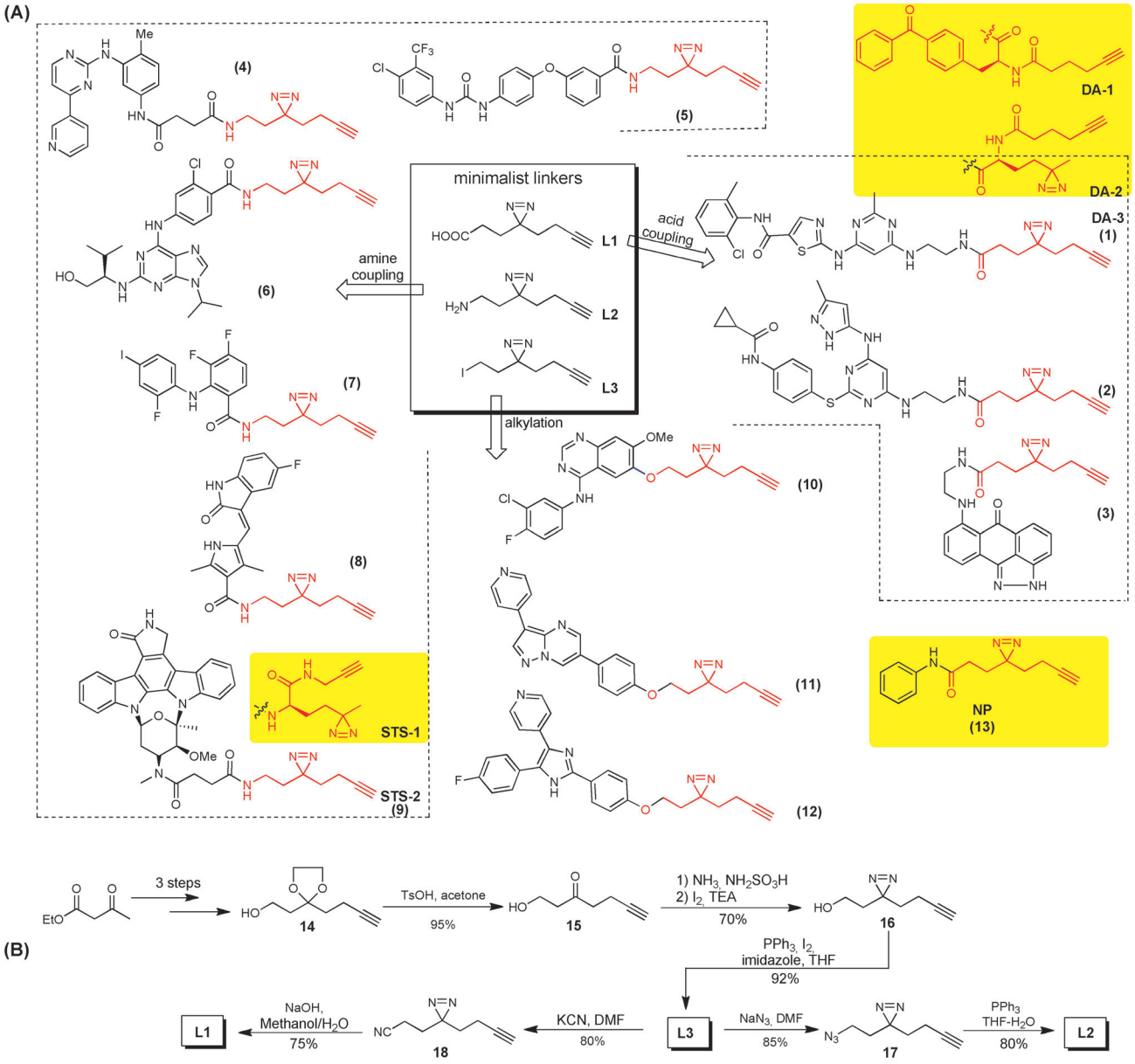
Protein kinases (PKs) are one of the most important classes of enzymes in human cells. Of the more than 500 known PKs, many are potential therapeutic targets.[7] Currently there are more than a dozen FDA-approved drugs based on kinase inhibitors, with many more in various stages of clinical trials.[8] Despite intensive research efforts, most kinase inhibitors tend to inhibit multiple cellular targets because they normally target the highly conserved ATP-binding site of the enzyme. To study potential cellular off-target effects of kinase inhibitors, recent efforts have focused on high-throughput screening (HTS) using a large panel of recombinant kinases as well as mass spectrometry (MS)-based chemical-profiling methods.[9] We previously developed cell-permeable kinase probes (for example, **DA-1**/**DA-2** in Figure 1 A) based on PAL for proteome-wide profiling of potential cellular targets of dasatinib (an FDA-approved kinase inhibitor targeting several types of cancer[10]).[2b] This affinity-based protein profiling (AfBP) approach differs from other strategies, in that it may be capable of interrogating kinase–drug interactions under native cellular conditions (in live cells, not cell lysates). The linker unit in **DA-1**/**DA-2** (highlighted in yellow), however, are not considered minimalist linkers, and could potentially affect cellular protein identification and the general application of this linker to other inhibitors. Herein, with improved linkers (**L1**–**L3**), we have demonstrated for the first time, incorporation into a variety of kinase inhibitors for cell-based proteome profiling of potential cellular targets under native conditions.

[*] Dr. Z. Li, Dr. L. Li, C. Y. J. Tan, X. Cheng, G. Y. J. Chen, Prof. Dr. S. Q. Yao
Department of Chemistry
National University of Singapore
3 Science Drive 3, Singapore 117543 (Singapore)
E-mail: chmyaosq@nus.edu.sg
Homepage: http://staff.science.nus.edu.sg/~syao
P. Hao, Prof. Dr. S. K. Sze
School of Biological Sciences
Nanyang Technological University
60 Nanyang Drive, Singapore 637551 (Singapore)
Prof. Dr. H.-M. Shen
Department of Physiology
National University of Singapore
16 Medical Drive, Singapore 11794 (Singapore)

[**] Funding was provided by the National Medical Research Council (NMRC/1260/2010) to H.-M.S. and S.Q.Y. and the Ministry of Education (MOE2012-T2-1-116). We thank Dr. Andrew Emili (Toronto) for helpful discussions of MS results.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201300683.

© 2013 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim



(C)

Compound (#)	IC_{50}/nM[a]		XTT/% Cell Death[b]		Compound (#)	IC_{50}/nM[a]		XTT/% Cell Death[b]	
	Drug	Probe	Drug	Probe		Drug	Probe	Drug	Probe
DA-3 (1)	4.6±0.5	16.5±0.7	79.5±0.2	69.7±0.4	**PD-1 (7)**	ND	ND	43.5±0.4	37.5±0.2
VX-1 (2)	10.9±0.5	38.8±0.2	69.5±0.5	64.5±0.4	**SU-1 (8)**	88.3±0.5	124.9±0.1	64.5±0.5	59.6±0.4
SP-1 (3)	48.8±0.4	118.3±0.2	33.4±0.9	29.5±0.8	**STS-2 (9)**	33.2±0.3	50.3±0.1	86.3±0.2	83.5±0.2
IM-1 (4)	32.5±0.3	91.8±0.5	61.9±0.5	56.1±0.5	**GE-1(10)**	24.3±0.1	37.5±0.5	69.1±0.4	57.6±0.6
SO-1 (5)	96.5±0.3	209.7±0.4	74.2±0.4	69.3±0.5	**CC-1 (11)**	106.3±0.1	194.9±0.3	34.5±0.3	29.5±0.5
PU-1 (6)	20.5±0.5	33.5±0.3	24.3±0.8	20.9±0.5	**SB-1 (12)**	35.1±0.8	131.5±0.5	64.5±0.7	54.1±0.5

Figure 1. A) Formulas of the 3 "minimalist" linkers and 12 corresponding kinase probes. See Table S1 for the unmodified inhibitor formulas. A control probe (**NP**) and three previously published probes (**DA-1**/**DA-2** and **STS-1**) are also shown. B) Synthetic scheme of the three linkers. C) IC_{50} values and XTT assay antiproliferative effects of the probes against the corresponding recombinant kinases and HepG2 cells. [a] The following kinases were used in the IC_{50} assays: c-Src (**DA-3**,**PU-1**), Aurora A (**VX-1**), JNK1 (**SP-1**), Abl (**IM-1**), KDR (**SO-1**,**SU-1**), PKA (**STS-2**), EGFR (**GE-1**), AMPK (**CC-1**), p38α (**SB-1**). [b] XTT assays were done using different doses of the drug/probe (percent cell death at 20 μM is shown). For **STS**/**STS-2**, percent cell death at 1000 nM is shown. See Supporting Information for details. ND = not determined.

© 2013 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

As shown in Figure 1 A,B, **L1**–**L3** possess two carbons on each side of the diazirine, flanked by the terminal alkyne and functional group (CO_2H/NH_2/I). The length of the carbon chains was carefully chosen based on both the availability of starting materials and to avoid potential side reactions. Shorter linkers were tested but synthetic difficulties were encountered. Starting from the readily available ethyl acetoacetate, **14** was obtained in 3 steps. Upon acetal deprotection, ketone **15** was converted to **16** in two steps (70% overall yield) using standard conditions.[11] Subsequent -OH to -I conversion gave **L3** in 92% yield. **L1** was obtained from **L3** in two steps through **18** (60% overall yield). **L2** was obtained from **L3** in two steps through **17** (68% overall yield). With all three linkers in hand, they were incorporated into 12 well-known kinase inhibitors, making the corresponding probes **1**–**12** (Figure 1 A). These 12 kinase inhibitors were chosen because 1) they cover a variety of important PKs in the human kinome (for example, Src, Aurora A, JNK1, Abl, Raf1, CDK1, MEK1, EGFR, KDR, PKA, AMPK, p38); 2) the kinase-inhibitor structural information and structure-activity relationships (SAR) are well-established. We noticed that water-soluble structural motifs (for example, the hydroxyethylpiperazinyl group in dasatinib; Supporting Information, Table S1), are common features in many kinase inhibitors, but do not participate in kinase–drug interactions (Figure S2). They thus could be replaced with our linkers without compromising the kinase-binding properties of the probe. Synthesis of the kinase-inhibitor cores in **1**–**12** (shown in black; Figure 1 A) was largely based on published methods (Supporting Information). The final coupling steps normally involved simple, yet highly robust, chemistries such as amine/acid coupling and alkylation reactions, making the strategy potentially applicable to many other types of bioactive small molecules. All probes were fully characterized before being evaluated in subsequent biological experiments.

We first confirmed that the biological activities of the probes (**1**–**12**) were not significantly compromised when compared to their parental inhibitors. This was done by standard in vitro kinase inhibition assays and cell-based, XTT anti-proliferation assays with HepG2 cells (Figure 1 C). Results showed that both the IC_{50} values and percentage of cell death of the probes were in most cases within twofold of the parental kinase inhibitors under identical assay conditions, indicating that the minimalist design in our probes had indeed effectively minimized the influence of the linker on protein binding, under both in vitro and cellular conditions. For convenience, HepG2 cells were used as the cell line for all our XTT assays, though we note that it may not be the most suitable cell line for certain kinases. In our experiments, we were mostly concerned about the difference in biological activities between the probe and the parental inhibitor, rather than the absolute effect of the probe/inhibitor against the tested kinases/cells.

We next carried out comprehensive biological and proteomics experiments to assess whether these minimalist probes performed better than our published probes, **STS-1** and **DA-1**/**DA-2**.[2] **STS-2** and **DA-3**, two probes that contain the minimalist linker, were compared side-by-side with **STS-1** and **DA-1**/**DA-2** which contain a significantly bulkier linker unit (shown in yellow, Figure 1 A). A kinase inhibition assay with PKA and c-Src showed that both **STS-2** and **DA-3** consistently performed better than their counterparts, **STS-1** and **DA-1**/**DA-2**, although in the cell-based XTT assay, the

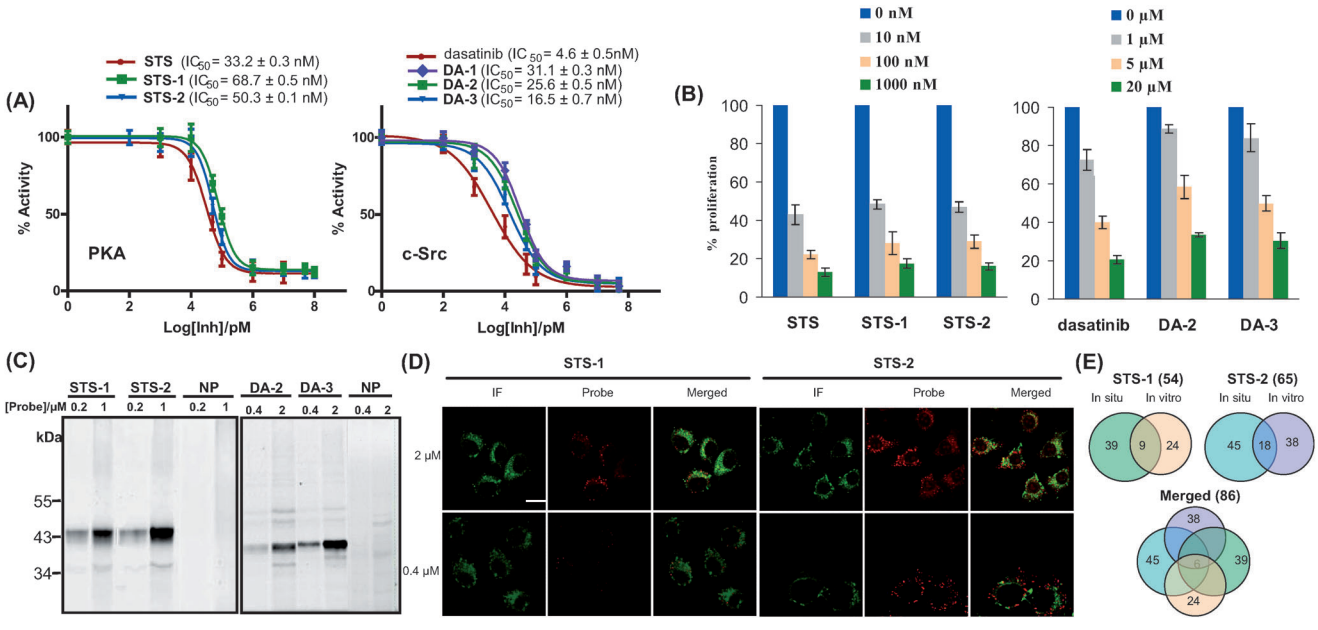


Figure 2. A) IC_{50} plots of staurosporine/**STS-1**/**STS-2** and dasatinib/**DA-1**/**DA-2**/**DA-3** against recombinant PKA and c-Src, respectively. B) XTT assay results of HepG2 cells treated with staurosporine/**STS-1**/**STS-2** and dasatinib/**DA-1**/**DA-2**/**DA-3** (72 h) at different concentrations. C) In-gel fluorescence scans of PKA (left) and c-Src (right) overexpressed bacterial proteomes labeled by **STS-1/2**,**DA-2/3** and **NP**. D) Live cell imaging of HepG2 cells with **STS-1/2**. Immunofluorescence (IF) staining using anti-PKA antibodies. Scale bar = 10 μm. E) Venn diagram analysis of the number of kinases enriched by **STS-1/2** upon proteome labeling/PD/LC-MS/MS using HepG2 cells.

© 2013 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

effect was not as obvious (Figure 2A,B). We next assessed the performance of these probes for labeling complex cellular proteomes. We used PKA and c-Src overexpressed bacterial proteomes as model systems. In-gel fluorescence scanning results of both labeled bacterial proteomes clearly showed positive and highly specific labeling of PKA and c-Src by **STS-1/2** and **DA-2/3**, respectively (Figure 2C). **DA-1** was previously shown to be less effective than **DA-2** in such experiments.[2b] Again, by direct comparison of the labeled kinases under different labeling conditions, both **STS-2** and **DA-3** appeared to consistently give a stronger labeled-protein band than **STS-1** and **DA-2**. We previously showed small-molecule probes such as **STS-1** were suitable bioimaging probes to detect endogenous kinase activities.[2a] Herein, we determined whether **STS-2**, by virtue of its improved minimalist linker design, might confer better cellular imaging activities than **STS-1**. Live HepG2 cells were first treated with the probe followed by UV irradiation to initiate photo-crosslinking. Subsequently, cells were fixed, permeabilized, treated with rhodamine-N_3 under previously optimized click chemistry conditions,[2] then imaged. Immunofluorescence (IF) was performed on the same cells using anti-PKA antibodies. The resulting images were then merged (Figure 2D); for cells treated with **STS-2**, strong fluorescence signals were observed throughout the whole cell excluding the nucleus, even at 0.4 μM probe concentration. On the other hand, fluorescence was observed for cells treated with **STS-1** at a higher probe concentration (2 μM), but not at 0.4 μM. This indicated that **STS-2** either entered the cells more readily or bound to its intended cellular targets more efficiently. The other 11 probes were also shown to be effective bioimaging reagents as well (Figure S7). Control imaging experiments with a negative probe **13** (**NP** in Figure 1A) under similar conditions showed it produced minimal background fluorescence compared to labeled cells (Figure S7). Finally, we carried out endogenous proteome labeling and PD/LC-MS/MS target identification with **STS-1/2** in both lysates and live HepG2 cells. **NP** was used throughout the experiments to eliminate false hits from non-specific photo-crosslinking. We optimized the number of potential positive protein hits we could identify. Briefly, probe-labeled proteomes (upon UV irradiation) were clicked with biotin-N_3, enriched using avidin-agarose beads, separated on SDS-PAGE, followed by LC-MS/MS analysis, as previously reported.[2b] Selected results are summarized in Figure 2E, while the complete list of proteins are found in the Supporting Information (Table S2, S3, and SI_3). Because staurosporine (STS) is a pan-kinase inhibitor, we focused on kinases that were positively identified from our experiments. As shown in Figure 2E. **STS-2** consistently identified more kinases in both in vitro (cell lysates) and in situ (live cells) than **STS-1**. A total of 65 kinases were identified by **STS-2** (45 from in situ and 38 from in vitro experiments, with 18 overlapping targets). These numbers are significantly higher than those from a previous study using a different photo-crosslinking approach.[12] Notably, the in situ and in vitro experiments yielded different sets of unique protein hits with some degree of overlap. For example, 27 and 20 unique kinases were identified in **STS-2** experiments under in situ and in vitro settings, respectively. Similar phenomenon had been observed previously.[2] This underscores the importance of our current affinity-based protein-profiling approach and its unique capability of interrogating kinase–drug interactions in living cells. To summarize, we attributed the improved performance of our new probes (**STS-2** and **DA-3**) to the minimalist linkers, which could have minimized interference with the genuine kinase–probe binding.

We next carried out endogenous proteome labeling followed by PD/Western blotting (WB) to confirm the ability of the 12 probes for labeling their known kinase targets (Figure 3). Both in vitro and in situ labeling were carried out,

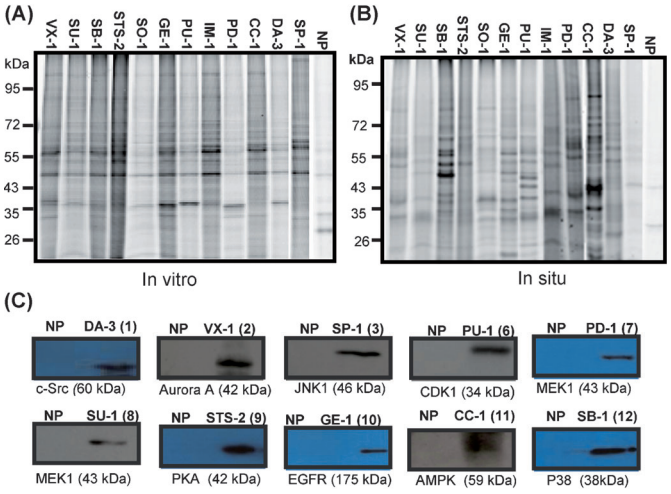


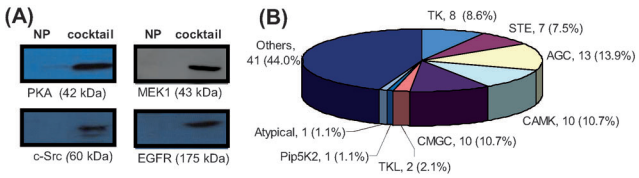
Figure 3. A,B) Proteome reactivity profiles in HepG2 cell lysate with the 12 individual probes and **NP** (10 μM) in vitro (A) and in situ (B). C) PD/WB results for target validation of 10 of the 12 probes in live cells (in situ). See Figures S6,S8 for details.

followed by rhodamine-N_3 click chemistry, and the resulting proteome reactivity profiles were compared (Figure 3A,B). Although there were some similarities in the labeling profiles, obvious differences were evident, clearly indicating the presence of different protein targets under the two experimental settings. Within the same setting, however, the 12 probes also labeled both common and unique protein targets as well, likely indicating their parental kinase inhibitors also possess both common and unique cellular (on and off) targets. Subsequently, the same probe-labeled proteomes were clicked with biotin-N_3, pulled-down, and immunoblotted with the respective antibodies (Figure 3B; Figure S8); with HepG2 cells alone, 10 out of the 12 probes were shown to successfully label their known kinase targets both in vitro and in situ.[13]

While each of these 12 kinase probes might be useful for large-scale identification of the cellular targets (on and off) of the parental inhibitor, we felt a "cocktail" approach containing a mixture of all 12 probes in equal concentrations might offer better throughput and lower operational costs for testing multiple mammalian cell lines. Previously, similar approaches have been demonstrated for both activity- and affinity-based probes.[14] We also thought the cocktail approach, when coupled with quantitative mass spectrometry, could be used for simultaneous measurement of the quantitative binding

 © 2013 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

interaction of a novel kinase inhibitor with multiple cellular kinases in live cells.[15] We first confirmed that the probe cocktail worked as expected to label multiple endogenous kinases in live HepG2 cells (Figure 4A); upon PD/WB with four representative antibodies against PKA, MEK1, c-Src, and EGFR, we observed the labeling of all four kinases. Next, we extended this cocktail approach to the labeling/enrichment of endogenous kinases present in rat kidney tissues. Following standard proteome labeling/PD/LC-MS/MS protocols as earlier described, we obtained the full list of potential protein hits (Supporting Information, SI_3). Control experiments were done concurrently with the negative probe **NP**. Selected enriched proteins are listed in Tables S4,S5. We identified a total of 94 kinases (from 1050 enriched proteins; Figure 4B), representing nearly 10% of all potential protein hits. This clearly indicates that our probe cocktail was able to significantly enrich the concentration of endogenous kinases from the total proteome. Some of the enriched proteins were undoubtedly artifacts, likely due to intrinsic non-specific labeling by photo-crosslinking, especially toward those high-abundance endogenous proteins. This was in spite of our optimization of the linker design and the proteomic methods. Other enriched proteins were likely genuine off-targets of our probes but need to be further validated by independent experiments. Among the 94 enriched kinases, we found representatives from almost all major groups of kinases. We also identified 41 kinases other than protein kinases, including lipid kinases, phosphofructokinases, pyruvate kinases, and others. Figure 4C shows representatives of the kinases identified from our experiments, most of which are known targets of our probes, including c-Src, PKAC-α, AMPKα1/2, JNK2/3, CDK5, P38, VEGFR, MEK, and LRRK2. We also identified unknown kinase targets of these probes, including Grk1, CKB, PRPS2, MRKCB, Nek1, Nek7, ILK, Gsk3a, TLK2, TAOK3, WNK1, and WNK4, which might be interesting off-targets. Three of these newly identified targets, Grk1 (a G-protein-coupled receptor kinase), CKB (a B-type creatine kinase), PRPS2 (isoform 2 of ribose-phosphate pyrophosphokinase), were further validated by preliminary PD/WB experiments (Figure 4D). Owing to the unavailability of recombinant versions of these kinases and/or an assay of their enzymatic activity, no further in vitro kinase inhibition experiment was carried out to delineate which compounds in the probe cocktail might directly inhibit these kinases.



(C)

Kinase	Protein Mass	Protein score	Peptides matched	emPAI ratio	Kinase group
FLT4	124610	30	2	∞	TK
Yes1	61032	32	2	∞	TK
Fyn	61177	33	3	∞	TK
Src	60553	34	3	∞	TK
HCK	59554	33	2	∞	TK
Grk1	64250	26	1	∞	AGC
CKB	42983	123	3	∞	others
PRPS2	35190	44	2	∞	others
AMPK α 2	64560	78	2	∞	CAMK
Hck	59554	33	2	∞	TK
LRRK2	288583	28	3	∞	TKL
RSK1	83230	130	4	∞	AGC
AKT2	55850	31	2	∞	AGC
MST3	48245	69	1	∞	STE
PKCI	69259	25	1	∞	AGC
PAK1	60825	102	1	∞	STE
CDK5	33689	32	3	∞	CMGC
ERK2	33734	71	4	∞	CMGC
TAO3	103669	32	1	∞	STE
JNK3	48610	55	2	∞	CMGC
MEK1	43779	64	7	1.42	STE

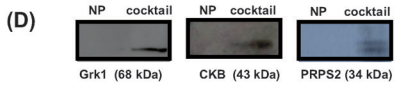


Figure 4. A) PD/WB for target validation of the probe cocktail (10 μM) in live HepG2 cells. B) The number and percentage of each kinase subgroup enriched from rat kidney tissues. C) Representative kinase hits identified from rat kidney tissues labeled by the probe cocktail. ∞ signals detected only in positive PD/LC-MS/MS. See Tables S2-S5 and SI_3 for details. D) Validation of three off-targets by PD/WB in probe cocktail-treated tissue samples. See Figure S8 for details.

In conclusion, we have developed three minimalist terminal alkyne-containing diazirine photo-crosslinkers. Their application in chemical proteomics was demonstrated through the synthesis of 12 linker-modified kinase inhibitors, which were subsequently used for cell-based proteome profiling of potential cellular kinase targets. We demonstrated that these linkers performed better than previously reported methods.[2,12] The probes could be used under various formats in different biological systems. Numerous potential off-targets of these probes were identified, some of which were validated by preliminary PD/WB experiments. Extensive further biochemical and cell-based experiments will be needed to unambiguously confirm the biological relevance of these off-targets. In the future, we will extend this approach to cell-based proteome profiling of other classes of bioactive small molecules.

Received: January 26, 2013
Revised: April 26, 2013
Published online: June 10, 2013

Keywords: affinity-based probes · fluorescent probes · kinases · photo-crosslinking · proteomics

[1] For recent reviews, see: a) F. Kotzyba-Hibert, I. Kapfer, M. Goeldner, *Angew. Chem.* **1995**, *107*, 1391 – 1408; *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 1296 – 1312; b) B. J. Leslie, P. J. Hergenrother, *Chem. Soc. Rev.* **2008**, *37*, 1347 – 1360; c) J. Das, *Chem.*

 © 2013 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

Rev. **2011**, *111*, 4405 – 4417; d) L. Dubinsky, B. P. Krom, M. M. Meijler, *Bioorg. Med. Chem.* **2012**, *20*, 554 – 570.
[2] a) H. Shi, X.-M. Cheng, S. K. Sze, S. Q. Yao, *Chem. Commun.* **2011**, *47*, 11306 – 11308; b) H. Shi, C.-J. Zhang, G. Y. J. Chen, S. Q. Yao, *J. Am. Chem. Soc.* **2012**, *134*, 3001 – 3014.
[3] a) P. Ranjitkar, B. G. K. Perera, D. Swaney, S. B. Hari, E. T. Larson, R. Merritt, E. A. Krishnamurty, J. Villén, D. J. Maly, *J. Am. Chem. Soc.* **2012**, *134*, 19017 – 19025; b) R. Krishnamurty, J. L. Brigham, S. E. Leonard, P. Ranjitkar, E. J. Dale, E. T. Larson, E. A. Merritt, D. J. Maly, *Nat. Chem. Biol.* **2013**, *9*, 43 – 50.
[4] a) E. W. Chan, S. Chattopadhaya, R. C. Panicker, X. Huang, S. Q. Yao, *J. Am. Chem. Soc.* **2004**, *126*, 14435 – 14446; b) K. Liu, H. Shi, H. Xiao, A. G. L. Chong, X. Bi, Y. T. Chang, K. Tan, R. Y. Yada, S. Q. Yao, *Angew. Chem.* **2009**, *121*, 8443 – 8447; *Angew. Chem. Int. Ed.* **2009**, *48*, 8293 – 8297.
[5] a) H. C. Kolb, K. B. Sharpless, *Drug Discovery Today* **2003**, *8*, 1128 – 1137; b) M. Meldal, C. W. Tornøe, *Chem. Rev.* **2008**, *108*, 2952 – 3015; c) E. M. Sletten, C. R. Bertozzi, *Angew. Chem.* **2009**, *121*, 7108 – 7133; *Angew. Chem. Int. Ed.* **2009**, *48*, 6974 – 6998; d) K. A. Kalesh, H. Shi, J. Ge, S. Q. Yao, *Org. Biomol. Chem.* **2010**, *8*, 1749 – 1762.
[6] P.-Y. Yang, K. Liu, M. H. Ngai, M. J. Lear, M. R. Wenk, S. Q. Yao, *J. Am. Chem. Soc.* **2010**, *132*, 656 – 666.
[7] J. Dancey, E. A. Sausville, *Nat. Rev. Drug Discovery* **2003**, *2*, 296 – 313.
[8] T. Force, K. L. Kolaja, *Nat. Rev. Drug Discovery* **2011**, *10*, 111 – 126.
[9] a) T. Anastassiadis, S. W. Deacon, K. Devarajan, H. Ma, J. R. Peterson, *Nat. Biotechnol.* **2011**, *29*, 1039 – 1045; b) M. I. Davis, J. P. Hunt, S. Herrgard, P. Ciceri, L. M. Wodicka, G. Pallares, M. Hocker, D. K. Treiber, P. P. Zarrinkar, *Nat. Biotechnol.* **2011**, *29*, 1046 – 1051; c) U. Rix, G. Superti-Furga, *Nat. Chem. Biol.* **2009**, *5*, 616 – 624.
[10] E. Weisberg, P. W. Manley, S. W. Cowan-Jacob, A. Hochhaus, J. D. Griffin, *Nat. Rev. Cancer* **2007**, *7*, 345 – 356.
[11] L. Dubinsky, L. M. Jarosz, N. Amara, P. Krief, V. V. Kravchenko, B. P. Krom, M. M. Meijler, *Chem. Commun.* **2009**, 7378 – 7380.
[12] J. J. Fischer, O. Y. Graebner, C. Dalhoff, S. Michaelis, A. K. Schrey, J. Ungewiss, K. Andrich, D. Jeske, F. Kroll, M. Glinski, M. Sefkow, M. Dreger, H. Koester, *J. Proteome Res.* **2010**, *9*, 806 – 817.
[13] We failed to confirm whether the remaining two probes, **IM-1** (**4**) and **SO-1** (**5**) labeled their intended kinases, Kit and VEGFR2, respectively, because these two kinases were undetectable even in total HepG2 lysates, indicating either they were not expressed in HepG2 or their expression level was extremely low.
[14] a) S. A. Sieber, S. Niessen, H. S. Hoover, B. F. Cravatt, *Nat. Chem. Biol.* **2006**, *2*, 274 – 281; b) H. Shi, M. Uttamchandani, S. Q. Yao, *Chem. Asian J.* **2011**, *6*, 2803 – 2815.
[15] M. Bantscheff, D. Eberhard, Y. Abraham, S. Bastuck, M. Boesche, S. Hobson, T. Mathieson, J. Perrin, M. Raida, C. Rau, V. Reader, G. Sweetman, A. Bauer, T. Bouwmeester, C. Hopf, U. Kruse, G. Neubauer, N. Ramsden, J. Rick, B. Kuster, G. Drewes, *Nat. Biotechnol.* **2007**, *25*, 1035 – 1044.

 © 2013 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim