ChemComm

A General Approach for Development of Fluorogenic Probes Suitable for No-Wash Imaging of Kinases in Live Cells

Journal:	*ChemComm*
Manuscript ID:	CC-COM-09-2014-007429
Article Type:	Communication
Date Submitted by the Author:	20-Sep-2014
Complete List of Authors:	ZHANG, QING; Peking University, LIU, HUI; Peking University, PAN, ZHENGYING; Peking University, Shenzhen Graduate School

Journal Name

RSCPublishing

COMMUNICATION

A General Approach for Development of Fluorogenic Probes Suitable for No-Wash Imaging of Kinases in Live Cells

Cite this: DOI: 10.1039/x0xx00000x

Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

Qing Zhang,[a] Hui Liu[a] and Zhengying Pan[a,*]

A general approach was presented to develop small molecule-based fluorogenic probes suitable for no-wash imaging of endogenous kinase in live cells. Probe 1, including a fluorophore-quencher system, was only "turned on" upon reacting with its target kinase-Btk, and disclosed Btk's cellular location in live cells without any washing.

Kinases have attracted a great deal of attention due to their indispensable roles in signal transduction and extensive involvement in human diseases.[1] Because most kinases are low abundance with similar catalytic functions, and their cellular activities are often transient under strictly temporal and spatial controls, several ingenious methods have been developed to study their functions through direct visualization. In particular, protein-based fluorescent probes, such as fluorescent proteins,[2] SNAP-tag[3] and the biarsenical-tetracysteine system,[4] have been widely applied to study subcellular locations and movements of kinases during cellular signal transduction events. This type of technology is highly specific to the target kinases, but it requires to introduce non-native proteins into cells and has slow labelling rates.[5] Small molecule-based probes could potentially overcome the drawbacks of genetic methods and provide a faster and non-intrusive means of studying endogenous kinases directly in their native environment.[6,7]

The classic approach to develop kinase-targeting small molecule fluorescent probes is to directly link a fluorophore onto a scaffold based on a covalent kinase inhibitor (Figure 1).[8] A two-step procedure was also developed to label kinases by firstly incubating an inhibitor with its kinase target, followed by a bioorthogonal coupling step to link a fluorophore onto the kinase-bound inhibitor.[8a,9] Alternatively, a "pro-fluorophore" linked to an inhibitor could be activated by a bioorthogonal coupling after the compound binds the protein.[10] When these methods are applied in live cell

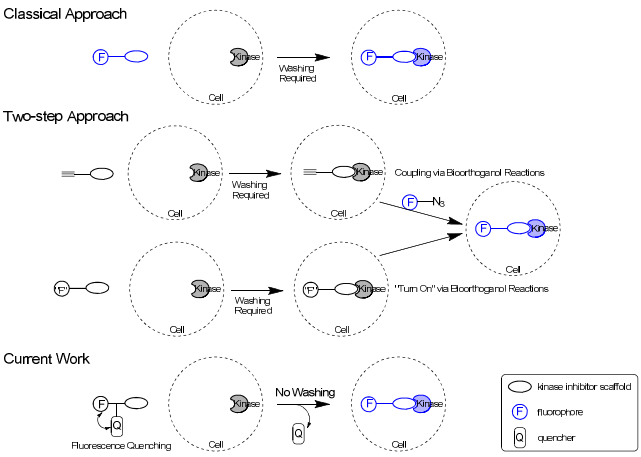


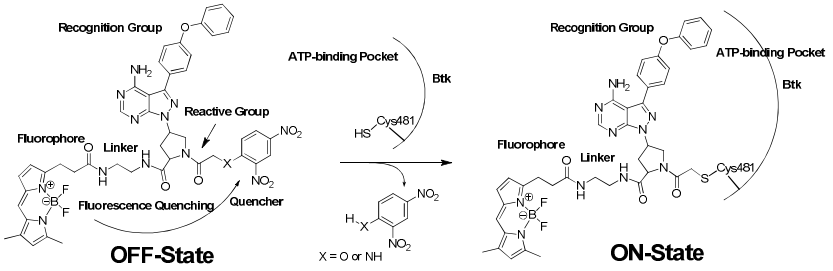
Fig. 1 Approaches to image kinases in live cells by small molecule fluorescent probes.

imaging studies, repeated washes of cells to remove excessive probe molecules are necessary to obtain high contrast signals. Fluorogenic probes[11] and quenched activity-based probes[12] may provide an elegant solution to this problem. Probes that become fluorescent only upon kinase labelling could increase the signal-to-background ratio and facilitate observations of the target proteins.[11c,13]

Bruton's tyrosine kinase (Btk) is a non-receptor tyrosine kinase critically involved in multiple signalling pathways in hematopoietic cells,[14] and has recently been validated as a drug target for B cell lineage cancers.[15] A fluorescent probe for Btk, PCI-33380[8c] was developed using the classic approach based on the scaffold of an approved Btk drug ibrutinib[16] (structures of PCI-33380 and ibrutinib are shown in supporting information). Cellular imaging studies with this probe require repetitive washing to remove excess probe molecules that would impair its use in real-time imaging. Here, we present a general approach for developing small molecule

Journal Name RSCPublishing

COMMUNICATION

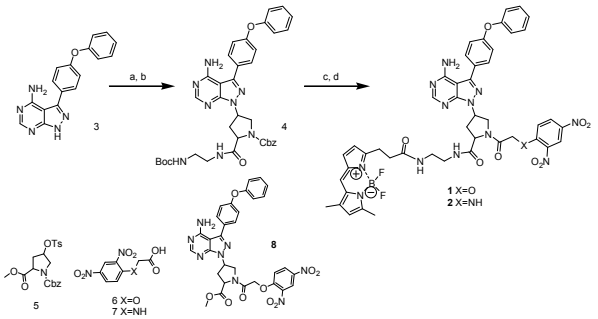


Scheme 1 Modular design of fluorogenic Btk probes. X = O, probe **1**; X = NH, probe **2**.

fluorogenic probes for kinases and demonstrate its feasibility by devloping a "smart" probe suitable for no-wash imaging of Btk in live cells.

A modular approach was adopted to design fluorogenic probes for Btk (Scheme 1). The ibrutinib scaffold was chosen as the recognition group because it had been optimized to preferentially bind to the ATP pocket of Btk. BODIPY®FL was selected as the fluorophore due to its relatively small size, high quantum yield and relative insensitivity to environmental changes.[17] A dinitrophenyl group served as the fluorescence quencher, and was positioned at the alpha carbon of a carbonyl group to facilitate its cleavage by the sulfhydryl group of Cys481 of Btk. This BODIPY® FL-dinitrobenzene system has been utilized in an elegant study of phospholipase A_2 activity in zebrafish.[18] Two types of linkage (X = O or NH) were tested to determine a suitable reactivity.

The syntheses of probes **1** and **2** are depicted in Scheme 2. Beginning with compound **3**, a base-mediated substitution reaction established the ibrutinib scaffold, which underwent a series of functional group manipulations to yield intermediate **4**. The carboxy benzyl group was removed by catalytic hydrogenation, and the resulting free amino group was coupled with acids **6** or **7** under common conditions of an amide bond formation. Lastly, the *tert*-butoxycarbonyl protection group was removed under acidic conditions, and condensation with the BODIPY® FL acid produced the desired probes **1** and **2** with good yields.



Scheme 2 Synthesis of probes **1** and **2**. *Conditions:* a) K_2CO_3, compound 5, DMF, 80 ℃; b) 1N LiOH, THF:MeOH (3:1); 1N HCl to pH 3; N-(2-aminoethyl)carbamic acid *tert*-butyl ester, EDCI, HOBT, DIPEA, DCM; c) H_2, $Pd(OH)_2/C$, EtOAc; compound 6 or 7, HATU, HOBT, DIPEA, DMF; d) TFA, DCM; BODIPY® FL acid, HATU, HOBT, DIPEA, DMF.

We first examined fluorescence properties of probes **1** and **2** (Figure 2 and Supporting Figure 1). Comparing to PCI-33380, which contains the identical BODIPY®FL fluorophore, probes **1** and **2** exhibited minimal fluorescence intensity (Figure 2a), confirming the excellent quenching efficiency of this BODIPY®FL- dinitrobenzene system. When the probes were incubated with the kinase domain of Btk (amino acids 382-659), the fluorescence of probe **1** increased gradually, peaking after 10 hours with an intensity approximately 17-fold greater than that of the probe alone (Figure 2b). Probe **2** exhibited a much slower increase in fluorescence, which reached only about 5-fold over the same period of time. This difference is most likely due to the poorer reactivity of the anilino group in probe **2** compared with the phenoxy group in probe **1**. The fluorescence could be effectively competed off by adding ibrutinib (Figure 2c). Bovine serum albumin (BSA) and glutathione (GSH) were used as surrogates for free thiols to examine the stability of our probes. Both probes (0.5 μM) exhibited small or very little reactivity with 25 μM BSA and no discernible reactivity with 15 mM GSH, suggesting an excellent stability of our probes in native cellular environment (Figure 2d).

Next we proceeded to label full-length Btk with probe **1**. One-hour incubation generated a sufficiently strong fluorescent signal (Figure 3a). The apparently faster labelling rate is likely due to the higher

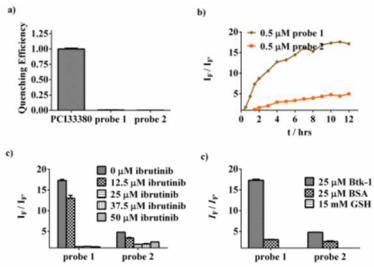


Fig. 2 Fluorescent properties of probes **1** and **2**. a) Probes **1** and **2** alone have indiscernible fluorescence; b) Time course of fluorescence increase of probes **1** and **2** reacting with 25 μM Btk-1 (kinase domain 382-659); c) Ibrutinib effectively blocked the increase of fluorescence (probe **1**: 0.5 μM, Btk: 25 μM); d) Probes **1** and **2** showed minimal reactivity towards BSA and GSH. The fluorescence intensity was calculated as IF/IF'; IF: fluorescence intensity of probe **1** or **2** with Btk-1; IF': fluorescence intensity of probe **1** or **2** alone in reaction buffer.

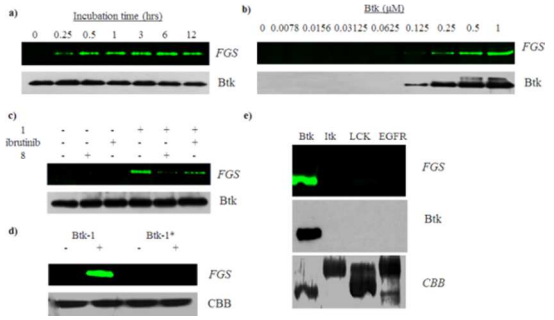


Fig. 3 Probe **1** is a potent and selective probe for Btk. a) Time course of labelling of Btk protein by probe **1**; b) Concentration course of labelling by probe **1**; c) Labelling of Btk can be competed off by ibrutinib and compound **8**; d) Probe **1** labelled Btk-1 but not C481A mutant Btk-1*; e) Probe **1** selectively labelled recombinant Btk (79 kDa), not Itk (99 kDa), Lck (85 kDa) or EGFR (aa. 668-1210, 90.5 kDa).

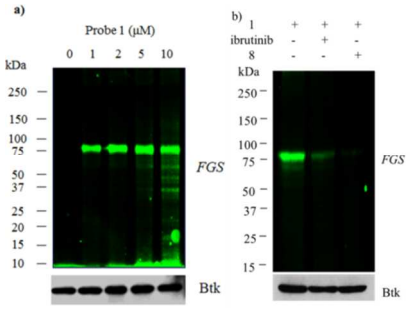


Fig. 4 Labelling studies of probe **1** in live cells. a) Probe **1** predominantly labelled endogenous Btk in live cells; b) Ibrutinib and compound **8** blocked the labelling of probe **1** in cells.

structural integrity of the full-length protein. The detection limit of the probe 1 labelling was close to that of western blotting (Figure 3b).[19] Not surprisingly, the labelling could also be effectively competed off by either ibrutinib or compound **8**, a precursor to probe **1** without the fluorophore (Figure 3c). Since ibrutinib covalently binds to Cys481 in the ATP site of Btk, the competition experiments suggest that probe **1** targets the same site as ibrutinib. Mass spectrometry provided additional evidence since probe **1** labelled the peptide fragment QRPIFIITEYMANGC^{481}LLNYLR (Supporting Figure 2), which contains a single cysteine residue, Cys481. Finally, we generated a C481A mutant of the kinase domain of Btk, probe **1** did not label this mutant Btk-1* (Figure 3d). To further investigate the selectivity of probe **1**, we performed labelling experiments with kinases that are structurally similar to Btk: EGFR, Itk and Lck. EGFR and Itk both contain structurally identical cysteine residues as Cys481 of Btk, and Lck has an ATP site pocket very similar to that of Btk, but with a serine residue replacing Cys481 at the same position. The gel image clearly demonstrated that probe **1** had excellent selectivity among the kinases tested; none of EGFR, Itk or Lck showed appreciable labelling by probe **1** (Figure 3e).

Then we proceeded to address the critical question whether probe **1** could efficiently and selectively label endogenous Btk in native cells (Figure 4). Since Btk is expressed in most hematopoietic cells, but not natural killer cells and T cells, we first incubated Namalwa cells, a B lymphocyte line, with increasing concentrations of probe **1** at 37°C for 1 hour. Following cell lysis, the supernatant was directly loaded onto an SDS-PAGE gel WITHOUT the removal of excess probe molecules. The fluorescence image showed a clear dominant band at the expected molecular weight of Btk. The labelling could be effectively diminished by pre-treating the cells with 2 μM of either ibrutinib or compound 8. The band was confirmed to be Btk using immuno-blotting with an anti-Btk antibody. It showed that 1 μM of probe **1** provided a sufficiently clean readout, whereas higher concentrations (5 or 10 μM) started to show off-target labelling. Similarly results were also observed in other B-cell lines, Raji and K562 cells (Supporting Figure 4). As expected, no labelling was observed in Jurkat cells (Supporting Figure 3), a T lymphocyte line that does not express Btk but does express Itk and Lck. Cell viability assays indicated that all of the tested cells grew normally in the presence of probe **1** at concentrations as high as 100 μM (Supporting Figure 4). Clearly, probe **1** can permeate into live cells and selectively labels Btk without hampering cell viability.

Btk is a critical component in the B-cell receptor (BCR) signalling pathway. Early studies have used *in situ* antibody staining to examine sub-cellular localizations of Btk in fixed and permeabilized cells, and a very recent one used a small molecule probe similar to PCI-33380 and visualized Btk in live cells AFTER washing-out unbound probes.[8d,20] We performed the very first no-wash, real-time imaging study of Btk in live cells to observe the localization of endogenous Btk after BCR activation (Figure 5a and a brief video in the supporting information). Namalwa cells were stimulated with anti-IgM, probe **1** was then added, and the cellular fluorescence was immediately monitored with a confocal fluorescence microscope. The initial dark background suggested that the probe was intact at the "OFF" state. As they permeated into cells and encountered Btk, more and more probes became at the "ON" state, and Btk proteins were clearly visible in just a few minutes. The uneven distribution of the fluorescence signal along the cell membrane confirms a previous report[20a] suggesting that upon activation Btk accumulates along the cell membrane and associates with BCR where the receptors aggregate and cytoskeletal components restructure.

The images also revealed a stark contrast between regions close to the cell membrane and the rest of the cytosol, as well as the absence of fluorescence in the medium, clearly indicating that the washing step required by classic small molecule-based fluorescent kinase probes was unnecessary for our approach. Co-localization experiments were also performed (Figure 5b). Fluorescent signals from protein-bound probe **1** (green) and Btk antibody (red) overlaid nicely. Competition experiments showed that no labelling was observed in live cells upon pre-treatment with either compound **8** or ibrutinib, further indicating that probe **1** exhibited a specific activity towards cellular Btk (Supporting Figure 7). And as expected, in Jurkat cells that do not express Btk, probe **1** did not cause any significant fluorescence (Supporting Figure 8).

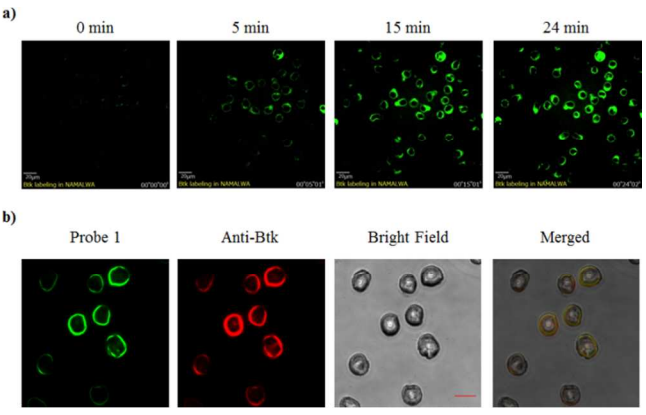


Fig. 5 a) No-wash imaging of Btk by probe **1** in live Namalwa cells. Images were captured at 0, 5, 15 and 24 minutes after addition of probe **1** into the medium. A minute-by-minute short video is available in the supporting information. Scale bar: 20 μm. b) Co-localization study of probe **1**-bound protein (green) and anti-Btk antibody (red), Pearson's correlation coefficient is 0.90. Scale bar: 10 μm.

Conclusions

In summary, we have developed the first fluorogenic probe for Btk. Probe **1** targets Cys481 in the ATP binding pocket of Btk and becomes fluorescent only upon reacting with the target kinase. It efficiently and selectively labelled endogenous Btk in live cells, and was only "turned on" by Btk in a real-time live cell imaging study without extra washing. This probe should be of great use to understand the roles of native Btk at the molecular level in multiple human diseases. More importantly, by adjusting the recognition group, this modular design approach can be easily adapted to develop fluorogenic probes for over 200 kinases that possess a cysteine near the ATP binding pocket.[21] This type of probes offers a high signal-to-background ratio, and is cell-permeable and highly stable in complex biological environment, thus providing a new set of valuable tools for studying the temporal and spatial controls of kinases in real time in live native cells and even directly in patient samples.

We acknowledge funding support from 973 Program (2013CB910700), National Foundation of Natural Science (81373270), Shenzhen Science and Technology Innovation Commission (ZDSYZ20130331145112855, JC201005270281A, KQTD201103), Guangdong Province (S2012010008741) and Peking University. We also thank Dr. Xitao Li for helpful discussions.

Notes and references

[a] Key Laboratory of Chemical Genomics, Key Laboratory of Structural Biology, School of Chemical Biology and Biotechnology Shenzhen Graduate School, Peking University Xili University Town, PKU Campus, F-311, Shenzhen, China 518055. Email: panzy@pkusz.edu.cn..

Electronic Supplementary Information (ESI) available: Synthesis and spectropic data of compounds and detailed procedures of biological assays. See DOI: 10.1039/c000000x/

1 G. Manning, D. B. Whyte, R. Martinez, T. Hunter and S. Sudarsanam, *Science,* 2002, **298**, 1912; L. M. Graves, J. S. Duncan, M. C. Whittle and G. L. Johnson, *Biochem. J.,* 2013, **450**, 1.

2 R. Y. Tsien, *Annu. Rev. Biochem*, 1998, **67**, 509; D. M. M. Chudakov, V. Matz, S. Lukyanovand, K. A. Lukyanov, *Physiol. Rev.*, 2010, **90**, 1103.

3 F. Kampmeier, J. Niesen, A. Koers, M. Ribbert, A. Brecht, R. Fischer, F. Kießling, S. Barthand, T. Thepen, *Eur. J. Nucl. Med. Mol. Imaging*, 2010, **37**, 1926; T. Komatsu, K. Johnsson, H. Okuno, H. Bito, T. Inoue, T. Naganoand, Y. Urano, *J. Am. Chem. Soc.*, 2011, **133**, 6745; X. Sun, A. Zhang, B. Baker, L. Sun, A. Howard, J. Buswell, D. Maurel, A. Masharina, K. Johnsson, C. J. Noren, M. Q. Xu and I. R., CorrÞa, Jr., *ChemBioChem*, 2011, **12**, 2217.

4 P. Kapahi, T. Takahashi, G. Natoli, S. R. Adams, Y. Chen, R. Y. Tsien and M. Karin, *J. Biol. Chem.*, 2000, **275**, 36062.

5 J. Zhang, R. E. Campbell, A. Y. Ting and R. Y. Tsien, *Nat. Rev. Mol. Cell Biol.*, 2002, **3**, 906.

6 L. M. Wysocki and L. D. Lavis, *Curr. Opin. Chem. Biol.*, 2011, **15**, 752.

7 C. H. S. Lu, K. Liu, L. P. Tan and S. Q. Yao, *Chem. Eur. J.*, 2012, **18**, 28.

8 M. S. Cohen, H. Hadjivassiliou and J. Taunton, *Nat. Chem. Biol.*, 2007, **3**, 156; J. A. Blair, D. Rauh, C. Kung, C. H. Yun, Q. W. Fan, H. Rode, C. Zhang, M. J. Eck, W. A. Weiss and K. M. Shokat, *Nat. Chem. Biol.*, 2007, **3**, 229; L. A. Honigberg, A. M. Smith, M. Sirisawad, E. Verner, D. Loury, B. Chang, S. Li, Z. Pan, D. H. Thamm, R. A. Millerand, J. J. Buggy, *Proc. Natl. Acad. Sci. USA*, 2010, **107**, 13075; A. Turetsky, E. Kim, R. H. Kohler, M. A. Miller and R. Weissleder, *Sci. Rep.*, 2014, **4**, 4782.

9 K. A. Kalesh, D. S. B. Sim, J. Wang, K. Liu, Q. Lin and S. Q. Yao, *Chem. Commun.*, 2010, **46**, 1118; H. Shi, C. J. Zhang, G. Y. J. Chen and S. Q. Yao, *J. Am. Chem. Soc.*, 2012, **134**, 3001; N. N. Gushwa, S. Kang, J. Chen and J. Taunton, *J. Am. Chem. Soc.*, 2012, **134**, 20214; C. Zambaldo, K. K. Sadhu, G. Karthikeyan, S. Barluenga, J. P. Daguer and N. Winssinger, *Chem. Sci.*, 2013, **4**, 2088.

10 G. Budin, K. S. Yang, T. Reiner and R. Weissleder, *Angew. Chem. Int. Ed.*, 2011, **50**, 9378; K. S. Yang, G. Budin, T. Reiner, C. Vinegoni and R. Weissleder, *Angew. Chem. Int. Ed.*, 2012, **51**, 6598.

11 M. J. Hangauer and C. R. Bertozzi, *Angew. Chem. Int. Ed.*, 2008, **47**, 2394; S. Tsukiji, H. Wang, M. Miyagawa, T. Tamura, Y. Takaoka and I. Hamachi, *J. Am. Chem. Soc.*, 2009, **131**, 9046; A. Nadler and C. Schultz, *Angew. Chem. Int. Ed.*, 2013, **52**, 2408.

12 G. Blum, S. R. Mullins, K. Keren, M. Fonovic, C. Jedeszko, M. J. Rice, B. F. Sloane and M. Bogyo, *Nat. Chem. Biol*., 2005, **1**, 203; M. Hu, L. Li, H. Wu, Y. Su, P. Y. Yang, M. Uttamchandani, Q. H. Xu and S. Q. Yao, *J. Am. Chem. Soc.,* 2011, **133**, 12009; M. Verdoes, K. O. Bender, E. Segal, W. A. Linden, S. Syed, N. P. Withana, L. E. Sanman and M. Bogyo, *J. Am. Chem. Soc.,* 2013, **135**, 14726.

13 Y. Hori and K. Kikuchi, *Curr. Opin. Chem. Biol.*, 2013, **17**, 1.

14 J. M. Lindvall, K. E. Blomberg, J. Väliaho, L. Vargas, J. E. Heinonen, A. Berglöf, A. J. Mohamed, B. F. Nore, M. Vihinen and C. I. Smith, *Immunol. Rev.*, 2005, **203**, 200.

15 A. Aalipour, R. H. Advani, *Brit. J. Haematol.*, 2013, **163**, 463; L. Vargas, A. Hamasy, B. F. Nore, C. I. E. Smith, *Scand. J. Immunol.*, 2013, **78**, 130.

16 Z. Pan, H. Scheerens, S. J. Li, B. E. Schultz, P. A. Sprengeler, L. C. Burrill, R. V. Mendonca, M. D. Sweeney, K. C. K. Scott, P. G. Grothaus, D. A. Jeffery, J. M. Spoerke, L. A. Honigberg, P. R. Young, S. A. Dalrymple and J. T. Palmer, *ChemMedChem*, 2007, **2**, 58 .

17 G. Ulrich, R. Ziessel and A. Harriman, *Angew. Chem. Int. Ed.*, 2008, **47**, 1184.

18 H. S. Hendrickson, E. K. Hendrickson, I. D. Johnson and S. A. Farber, *Anal. Biochem.*, 1999, **276**, 27; S. A. Farber, M. Pack, S. Y. Ho, I. D. Johnson, D. S. Wagner, R. Dosch, M. C. Mullins, H. S. Hendrickson, E. K. Hendrickson and M. E. Halpern, *Science*, 2001, **292**, 1385.

19 The diastereomeric mixture and chiral compounds exhibited similar labelling abilities (Supporting Figure 5).

20 S. Nisitani, R. M. Kato, D. J. Rawlings, O. N. Witte and M. I. Wahl, *Proc. Natl. Acad. Sci. USA*, 1999, **96**, 2221; C. Brunner, A. Avots, H. W. Kreth, E. Serfling and V. Schuster, *Immunobiology*, 2002, **206**, 432; A. M. Bogusz, R. H.G. Baxter, T. Currie, P. Sinha, A. R. Sohani, J. L. Kutok and S. J. Rodig, *Clin. Cancer Res.*, 2012, **18**, 6122.

21 J. Zhang, P. L. Yang and N. S. Gray, *Nature Rev. Cancer*, 2009, **9**, 28; E. Leproult, S. Barluenga, D. Moras, J. M. Wurtz and N. Winssinger, *J. Med. Chem.* 2011, **54**, 1347.

