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Design, synthesis, and evaluation of a selective chemosensor for leucine-rich repeat kinase 2

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ABSTRACT

We describe the design, synthesis, and evaluation of a selective activity probe for leucine-rich repeat kinase 2 (LRRK2), a possible molecular target for the treatment of Parkinson's disease. Our optimal chemosensor design, termed Nictide-S2, incorporates a phosphorylation-sensitive sulfonamido-oxine fluorophore at an engineered cysteine within the substrate sequence. This design allows for the direct, real-time analysis of LRRK2 kinase activity with a detection limit of 2.5 nM. Under optimized conditions, we measured a Z′ factor of 0.7 demonstrating the potential utility of this assay for inhibitor screening. Off-target kinases capable of phosphorylating Nictide-S2 are identified and an optimized inhibitor cocktail for suppressing background signal is provided. The resulting chemosensor could be utilized to identify LRRK2 inhibitors as well as selectively report on LRRK2 activity in the presence of off-target kinases.

Mutations that increase the catalytic activity of the serine/threonine kinase LRRK2 have been identified in both familial and sporadic forms of Parkinson's disease (PD).[1–4] Inhibition of LRRK2 kinase activity has been demonstrated to reverse the neurodegenerative phenotype observed in PD models,[5] indicating that LRRK2 is a potential molecular target for the treatment of PD. Current approaches for assessing inhibition of LRRK2 kinase activity in heterogeneous samples, such as cell lysates, rely on phosphorylation of S910, S935, or S1292 as a proxy for LRRK2 activity.[6–8] As predicted, exposure of cells to LRRK2 inhibitors causes a decrease in the phosphorylation of S910 and S935. This strategy has been utilized with great success to characterize LRRK2 inhibitor efficacy in biological systems.[9,10] However, S910 and S935 are not directly phosphorylated by LRRK2 and the regulatory enzymes that control phosphorylation at these sites have not been identified. Consequently, selective activity assays for LRRK2 could provide a starting point to develop approaches to directly monitor LRRK2 activity in heterogeneous samples, avoiding potential difficulties with currently available LRRK2 proxies.[11]

Towards the goal of developing a selective LRRK2 activity assay, we set out to design and evaluate the selectivity of potential chemosensors for LRRK2 in vitro. Recently, a variety of discontinuous LRRK2 activity assays have been described that utilize radioactivity,[12] time-resolved fluorescence resonance energy transfer,[13] or amplified luminescent proximity homogeneous assay[14] formats. In contrast, fluorescence-based activity sensors for protein kinases are capable of providing a real-time measure of enzyme function.[15–17] Importantly, fluorescence-based activity assays can also be utilized to quantify kinase activity in biological samples, such as cell lysates or tissue homogenates.[18–20] Accordingly, we chose to employ a phosphorylation-sensitive sulfonamido–oxine fluorophore, known as Sox,[21] to directly report on LRRK2 kinase activity in real-time. First generation Sox-based kinase activity sensors require the removal of either N- or C-terminal recognition sequences to afford efficient activity probes. Using this approach, a first generation Sox-based activity sensor for LRRK2 was recently developed by Silva et al.[22] This sensor was capable of detecting 10.5 nM LRRK2 and was employed to assess several biochemical properties of LRRK2 including the effects of GTP, GDP, and autophosphorylation on kinase enzymatic activity. However, the ability of this first generation Sox-based probe to selectively report on LRRK2 activity in presence of closely related enzymes was not demonstrated. To improve upon the selectivity of Sox-based chemosensors, the Imperiali laboratory has described a second generation Sox-based probe design that relies on the alkylation of a single engineered cysteine residue within a kinase substrate.[23] These so-called CSox-based substrates allow for the incorporation of both N- and C-terminal recognition sequences into a peptide substrate and can increase chemosensor selectivity (Fig. 1a).

We hypothesized that a LRRK2 substrate containing both N- and C-terminal recognition elements may afford a selective activity sensor. Accordingly, we synthesized two potential CSox-based substrates using the sequence of an efficient LRRK2 peptide substrate

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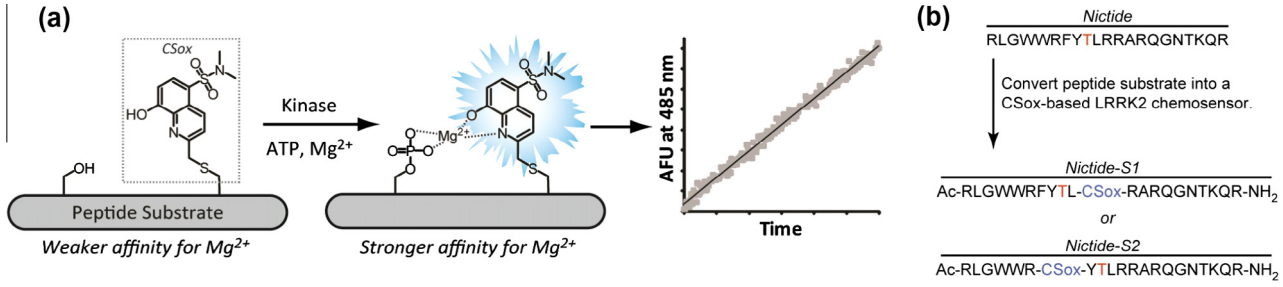

Figure 1. Design of a CSox-based activity sensor for LRRK2. (a) A single amino acid in a peptide substrate is replaced with the CSox unnatural amino acid. Addition of the target kinase, in the presence of ATP and Mg^{2+}, leads to phosphorylation of the substrate and a concurrent increase in fluorescence due to chelation of Mg^{2+} (ex. = 360 nm, em. = 485 nm). This increase in fluorescence can be monitored in real-time and is directly proportional to kinase enzymatic activity. (b) Conversion of Nictide into a CSox-based chemosensor by replacement of the +2 (Nictide-S1) or −2 (Nictide-S2) residues, relative to the site of phosphorylation (red) with CSox (blue). Peptides are capped with an acetyl group (Ac) at the N-terminus and an amide (NH_2) at the C-terminus.

known as Nictide[24] as a template. These chemosensors contain CSox at the +2 or −2 position relative to the site of phosphorylation, termed Nictide-S1 and Nictide-S2 respectively (Fig. 1b). In addition, we synthesized the corresponding phosphorylated peptides as controls, Nictide-P1 and Nictide-P2, respectively, which contain a phospho–threonine at the site of phosphorylation. Since the signal generation of CSox-based activity sensors is a result of chelation-enhanced fluorescence induced by binding of Mg^{2+}, it is important to measure the affinities of corresponding pairs of nonphosphorylated and phosphorylated peptides for Mg^{2+}. This information allows one to tune the concentration of Mg^{2+} in the assay in order to obtain maximal fluorescence enhancements upon phosphorylation. In general, the affinities of nonphosphorylated peptides for Mg^{2+} are >100 mM while phosphorylated peptides bind Mg^{2+} with a K_D of ~10 mM. However, the affinities of each newly designed CSox-based sensor for Mg^{2+} should be investigated as the sequence context can influence Mg^{2+} affinities for both nonphosphorylated and phosphorylated constructs. To investigate this parameter with our Nictide-based chemosensors, we measured the affinity of each substrate, and corresponding product, peptide for Mg^{2+} (Fig. 2). As expected each substrate peptide displayed a decreased affinity for Mg^{2+}, relative to the corresponding product. However, these data also indicated that Nictide-P2 had a relatively weak affinity for Mg^{2+} (K_D = 69 mM) compared to Nictide-P1 (K_D = 10 mM). Based on these results we reasoned that higher concentrations of Mg^{2+} than are typically employed with CSox-based activity sensors may be required to effectively discriminate between the phosphorylated and nonphosphorylated version of

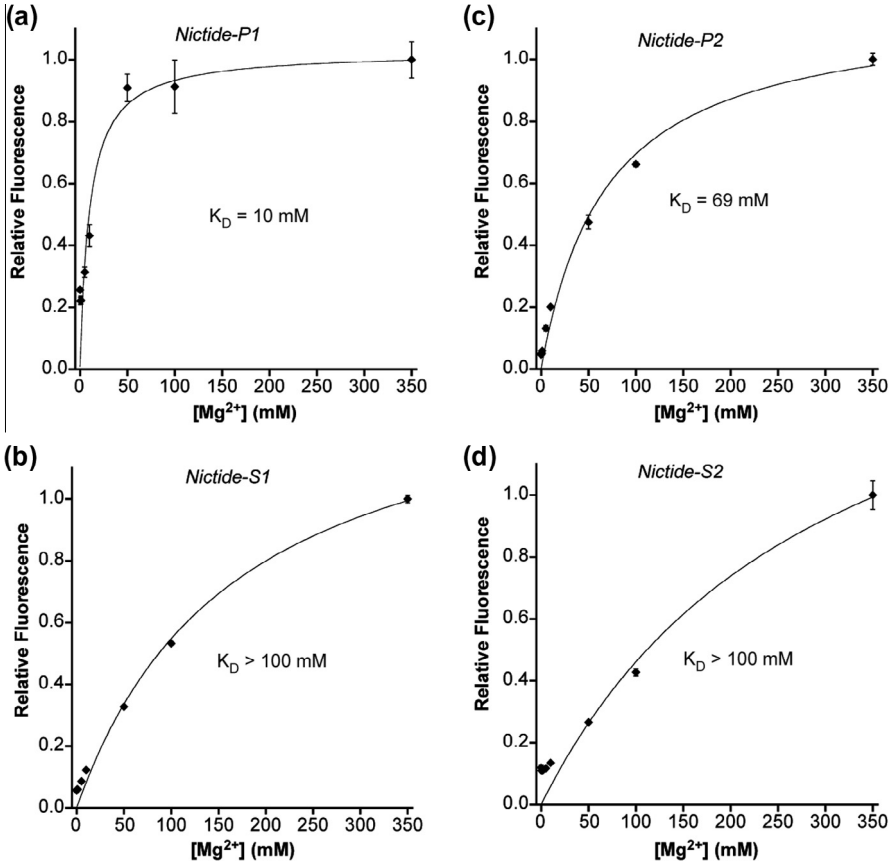

Figure 2. Binding affinities of phosphorylated (a and c) versus nonphosphorylated (b and d) CSox-based Nictide constructs. Phosphorylated peptides (a and c) display tighter affinities for Mg^{2+} than the corresponding nonphosphorylated peptides (b and d).

Nictide-S2. This hypothesis was validated when we investigated the fold increase in fluorescence between each pair of substrate and product peptides under varying concentrations of Mg^{2+} (Table 1). Indeed, a 3.3-fold increase in fluorescence for the Nictice-S1 constructs was observed at 15 mM Mg^{2+}, while the maximal fold fluorescence enhancement (3.6-fold) for the Nictide-S2 constructs required 48 mM Mg^{2+}. These concentrations of Mg^{2+} were employed for all subsequent assays, and highlight the necessity for the careful optimization of CSox-based assays in light of context dependent effects on Mg^{2+} affinity.

We next asked whether LRRK2 could utilize our designed probes as substrates for phosphorylation. Each chemosensor displayed Michaelis–Menten kinetics for recombinant LRRK2 (Figs. S1 and S2). Moreover, comparison of the kinetic parameters for each substrate (Table 2), demonstrated that Nictide-S2 was a 3-fold more efficient substrate compared to Nictide-S1. The K_M of Nictide-S2 (9.1 μM) corresponded to that of the parent sequence[24] and is 3.6-fold tighter than the previously described Sox-based sensor.[22] Importantly, K_M is often a critical parameter when designing selective chemosensors for protein kinases. In particular, we have found that decreasing the amount of sensor in an assay can reduce off-target effects.[17] In addition, tighter K_M values allow for the use of less substrate during experiments, mitigating the cost associated with large scale screening initiatives. In terms of preference for the placement of the CSox unnatural amino acid, these data indicate that insertion of CSox at the +2 position, relative to the site of phosphorylation, interferes with substrate binding. Accordingly, we chose to employ Nictide-S2 for all further experiments.

Table 1
Fold fluorescence enhancements of peptide constructs

Nictide-P1/Nictide-S1		Nictide-P2/Nictide-S2	
Mg^{2+} (mM)	Fold Inc.[a]	Mg^{2+} (mM)	Fold Inc.[a]
5	1.7	24	2.6
10	2.9	48	3.6
15	3.3	72	3.3

[a] Fluorescence of the phosphorylated peptide was divided by the corresponding nonphosphorylated peptide. Measurements were performed in the presence of 1 mM ATP.

Table 2
Kinetic constants for CSox-based Nictide substrates

Substrate	K_M (μM)	k_{cat} (min^{-1})	Efficiency[a]
Nictide-S1	100	5.8	1.0
Nictide-S2	9.1	1.6	3.0

[a] Efficiency was determined by comparing k_{cat}/K_M for each substrate.

Defining the sensitivity of an assay is in important parameter for both inhibitor screening as well as biological studies. Accordingly, we investigated the signal response of Nictide-S2 in the presence of decreasing amounts of LRRK2. Under our optimized conditions, using 20 μM Nictide-S2, we could observe LRRK2-depenent phosphorylation of our sensor within 60 min (Fig. S3). In addition, we could detect LRRK2 concentrations as low as 2.5 nM in a 384-well plate format, corresponding to 20 ng of LRRK2 (Fig. S4). This represents a 4-fold increase in sensitivity compared to the previously described Sox-based sensor.[22]

Due to the relevance of LRRK2 in PD, there is keen interest in the development of inhibitors to block LRRK2 kinase activity.[9,10] To assess whether Nictide-S2 would be capable of monitoring LRRK2 inhibition, we interrogated the phosphorylation of Nictide-S2 in the presence of two known LRRK2 inhibitors, staurosporine and LRRK2-IN-1 (Fig. 3). Taking into account the relatively high ATP concentration used in these assays (1 mM), we obtained IC_{50} values for staurosporine (5.0 nM) and LRRK2-IN-1 (340 nM) that correspond with previous literature data.[9,25] Importantly, recent reports have demonstrated the utility of CSox-based probes for high-throughput screening.[26] In particular, the kinetic assay format of these sensors reduces false positives observed from autofluorescent compounds, indicating that Nictide-S2 could provide a platform for screening LRRK2 inhibitors. In support of this hypothesis, we observed a Z′ factor of 0.7 for the Nictide-S2 assay using 10 nM LRRK2 in a 384-well plate format. These results indicate that Nictide-S2 could provide a powerful tool for LRRK2 inhibitor screening.

The long-term goal of our laboratory is to leverage CSox-based activity sensors to interrogate LRRK2 activity perturbations in unfractionated cell lysates and tissue homogenates. Under these demanding conditions, an activity probe must selectively report on the target enzyme. However, closely-related kinases can often share overlapping peptide substrate specificity, leading to off-target effects. Consequently, we asked whether Nictide-S2 could selectively report on LRRK2 activity among a panel of closely related kinases including RIPK2, IRAK1, MLK1, LIMK1, and ALK1.[27] Within this panel, clear off-target phosphorylation was observed from RIPK2 (90%) and IRAK1 (29%), while background level activity was observed for MLK1, LIMK1, and ALK1 (Fig. 4a). In order to suppress off-target kinase activity from RIPK2 and IRAK1, we searched an existing dataset delineating the binding affinities of 72 small molecule inhibitors to 442 kinases.[28] Using this approach, we identified dasatinib, SB203580, and gefitinib as potential off-target suppressors with no affinity for LRRK2. Indeed,

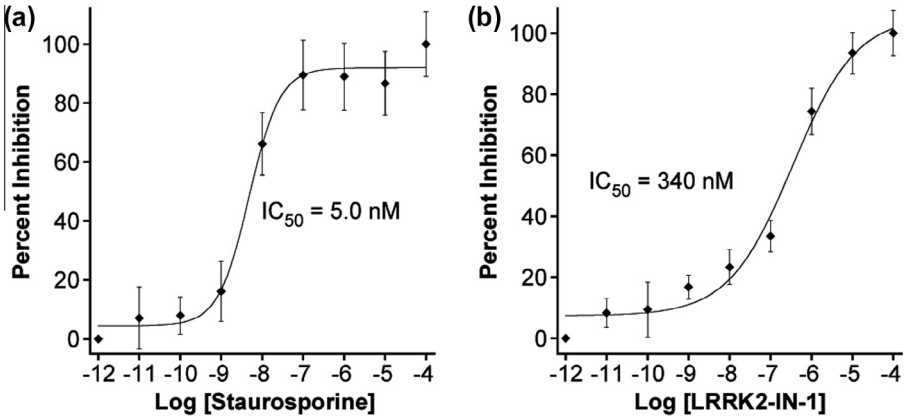

Figure 3. Nictide-S2 can report on the inhibition of LRRK2 by staurosporine (a) or LRRK2-IN-1 (b). Assays contained 20 μM Nictide-S2 and 1 mM ATP. Reactions were initiated by the addition of 10 nM LRRK2.

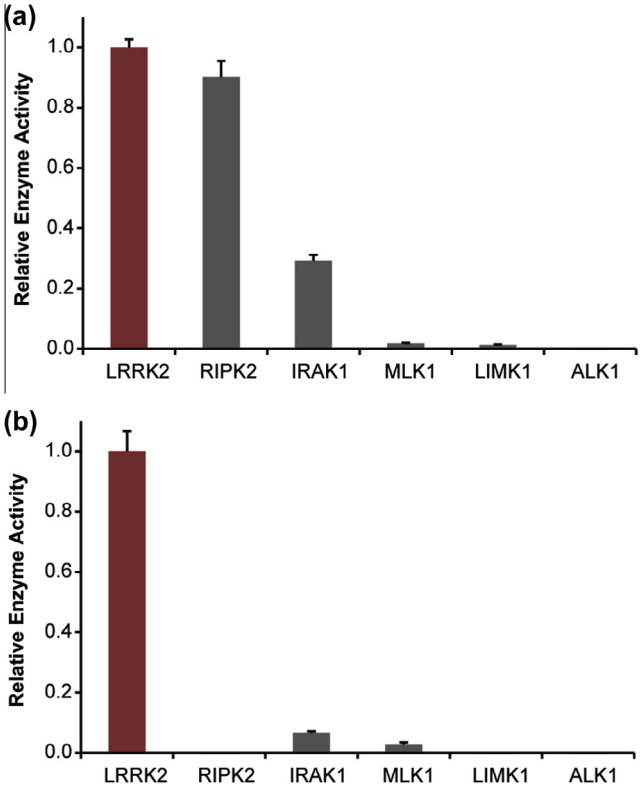

Figure 4. Nictide-S2 (20 μM) can be utilized to selectively detect LRRK2 kinase activity. (a) Significant off-target phosphorylation of Nictide-S2 is observed from RIPK2 and IRAK2, while minimal activity is observed from MLK1, LIMK1, and ALK1. (b) In the presence of off-target inhibitors (10 μM dasatinib, 10 μM SB203580, and 1 μM gefitinib) the activity of closely related enzymes is reduced to background levels. Assays were initiated by the addition of the indicated enzyme at a final concentration of 10 nM.

we tested the effect of these inhibitors on LRRK2 activity and found that these inhibitors do not interfere with LRRK2 kinase activity (Fig. S5). Moreover, these inhibitors were able to suppress off-target phosphorylation of Nictide-S2 by RIPK2 and IRAK1 (Fig. 4b). These results demonstrate the ability to fine tune Nictide-S2 selectivity with small molecule inhibitors. Although these results do not rule out the possibility of off-target phosphorylation by enzymes that are not represented in our panel, this approach could be extended to potentially afford a selective activity assay for LRRK2 in heterogeneous samples by inhibiting the relevant enzymes.

In conclusion we have described a selective, direct activity assay for LRRK2. By optimization of Mg^{2+} concentrations we were able to efficiently detect LRRK2 kinase activity using a CSox-based probe. Moreover, the sensitivity of this assay format will allow for screening of small molecule libraries for potential LRRK2 inhibitors. Our laboratory is currently optimizing this assay to delineate LRRK2 activity perturbations in biological samples, such as cell lysates.[17]

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Supplementary data

Supplementary data (experimental methods and supplementary figures) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.10.079.

References and notes

1. Di Fonzo, A.; Rohe, C. F.; Ferreira, R. J.; Chien, H. F.; Vacca, L.; Stocchi, F.; Guedes, L.; Fabrizio, E.; Manfredi, M.; Vanacore, N.; Goldwurm, S.; Breedveld, G.; Sampaio, C.; Meco, G.; Barbosa, E.; Oostra, B. A.; Bonifati, V.; Network, I. P. G. *Lancet* **2005**, *365*, 412.
2. Gilks, W. P.; Abou-Sleiman, P. M.; Gandhi, S.; Jain, S.; Singleton, A.; Lees, A. J.; Shaw, K.; Bhatia, K. P.; Bonifati, V.; Quinn, N. P.; Lynch, J.; Healy, D. G.; Holton, J. L.; Revesz, T.; Wood, N. W. *Lancet* **2005**, *365*, 415.
3. Nichols, W. C.; Pankratz, N.; Hernandez, D.; Paisan-Ruiz, C.; Jain, S.; Halter, C. A.; Michaels, V. E.; Reed, T.; Rudolph, A.; Shults, C. W.; Singleton, A.; Foroud, T.; Invest, P. S. G. P. *Lancet* **2005**, *365*, 410.
4. West, A. B.; Moore, D. J.; Biskup, S.; Bugayenko, A.; Smith, W. W.; Ross, C. A.; Dawson, V. L.; Dawson, T. M. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 16842.
5. Lee, B. D.; Shin, J. H.; VanKampen, J.; Petrucelli, L.; West, A. B.; Ko, H. S.; Lee, Y. I.; Maguire-Zeiss, K. A.; Bowers, W. J.; Federoff, H. J.; Dawson, V. L.; Dawson, T. M. *Nat. Med.* **2010**, *16*, 998.
6. Dzamko, N.; Deak, M.; Hentati, F.; Reith, A. D.; Prescott, A. R.; Alessi, D. R.; Nichols, R. J. *Biochem. J.* **2010**, *430*, 405.
7. Reynolds, A.; Doggett, E. A.; Riddle, S. M.; Lebakken, C. S.; Nichols, R. J. *Front. Mol. Neurosci.* **2014**, *7*, 54.
8. Sheng, Z. J.; Zhang, S. O.; Bustos, D.; Kleinheinz, T.; Le Pichon, C. E.; Dominguez, S. L.; Solanoy, H. O.; Drummond, J.; Zhang, X. L.; Ding, X.; Cai, F.; Song, Q. H.; Li, X. T.; Yue, Z. Y.; van der Brug, M. P.; Burdick, D. J.; Gunzner-Toste, J.; Chen, H. F.; Liu, X. R.; Estrada, A. A.; Sweeney, Z. K.; Scearce-Levie, K.; Moffat, J. G.; Kirkpatrick, D. S.; Zhu, H. T. *Sci. Transl. Med.* **2012**, *4*(164r), a161.
9. Deng, X. M.; Dzamko, N.; Prescott, A.; Davies, P.; Liu, Q. S.; Yang, Q. K.; Lee, J. D.; Patricelli, M. P.; Nomanbhoy, T. K.; Alessi, D. R.; Gray, N. S. *Nat. Chem. Biol.* **2011**, *7*, 203.
10. Choi, H. G.; Zhang, J. W.; Deng, X. M.; Hatcher, J. M.; Patricelli, M. P.; Zhao, Z.; Alessi, D. R.; Gray, N. S. *ACS Med. Chem. Lett.* **2012**, *3*, 658.
11. Ito, G.; Fujimoto, T.; Kamikawaji, S.; Kuwahara, T.; Iwatsubo, T. *PloS One* **2014**, *9*, e97988.
12. Lee, B. D.; Li, X. J.; Dawson, T. M.; Dawson, V. L. *Methods Mol. Biol.* **2012**, *795*, 45.
13. Liu, M.; Poulose, S.; Schuman, E.; Zaitsev, A. D.; Dobson, B.; Auerbach, K.; Seyb, K.; Cuny, G. D.; Glicksman, M. A.; Stein, R. L.; Yue, Z. Y. *Anal. Biochem.* **2010**, *404*, 186.
14. Pedro, L.; Padros, J.; Beaudet, L.; Schubert, H. D.; Gillardon, F.; Dahan, S. *Anal. Biochem.* **2010**, *404*, 45.
15. Gonzalez-Vera, J. A. *Chem. Soc. Rev.* **2012**, *41*, 1652.
16. Lawrence, D. S.; Wang, Q. Z. *ChemBioChem* **2007**, *8*, 373.
17. Beck, J. R.; Peterson, L. B.; Imperiali, B.; Stains, C. I. *Curr. Protoc. Chem. Biol.* **2014**, *6*, 135.
18. Stains, C. I.; Tedford, N. C.; Walkup, T. C.; Lukovic, E.; Goguen, B. N.; Griffith, L. G.; Lauffenburger, D. A.; Imperiali, B. *Chem. Biol.* **2012**, *19*, 210.
19. Stains, C. I.; Lukovic, E.; Imperiali, B. *ACS Chem. Biol.* **2011**, *6*, 101.
20. Wang, Q. Z.; Zimmerman, E. I.; Toutchkine, A.; Martin, T. D.; Graves, L. M.; Lawrence, D. S. *ACS Chem. Biol.* **2010**, *5*, 887.
21. Shults, M. D.; Imperiali, B. *J. Am. Chem. Soc.* **2003**, *125*, 14248.
22. Silva, R. G.; Geoghegan, K. F.; Qiu, X. Y.; Aulabaugh, A. *Anal. Biochem.* **2014**, *450*, 63.
23. Lukovic, E.; Gonzalez-Vera, J. A.; Imperiali, B. *J. Am. Chem. Soc.* **2008**, *130*, 12821.
24. Nichols, R. J.; Dzamko, N.; Hutti, J. E.; Cantley, L. C.; Deak, M.; Moran, J.; Bamborough, P.; Reith, A. D.; Alessi, D. R. *Biochem. J.* **2009**, *424*, 47.
25. Anand, V. S.; Reichling, L. J.; Lipinski, K.; Stochaj, W.; Duan, W. L.; Kelleher, K.; Pungaliya, P.; Brown, E. L.; Reinhart, P. H.; Somberg, R.; Hirst, W. D.; Riddle, S. M.; Braithwaite, S. P. *FEBS J.* **2009**, *276*, 466.
26. Devkota, A. K.; Warthaka, M.; Edupuganti, R.; Tavares, C. D. J.; Johnson, W. H.; Ozpolat, B.; Cho, E. J.; Dalby, K. N. *J. Biomol. Screen.* **2014**, *19*, 445.
27. Manning, G.; Whyte, D. B.; Martinez, R.; Hunter, T.; Sudarsanam, S. *Science* **1912**, *2002*, 298.
28. Davis, M. I.; Hunt, J. P.; Herrgard, S.; Ciceri, P.; Wodicka, L. M.; Pallares, G.; Hocker, M.; Treiber, D. K.; Zarrinkar, P. P. *Nat. Biotechnol.* **2011**, *1046*, 29.