Title

Selective Targeting of Distinct Active Site Nucleophiles by Irreversible Src-Family Kinase Inhibitors

Journal

Journal of the American Chemical Society, 134(50)

ISSN

0002-7863

Authors

Gushwa, Nathan N
Kang, Sumin
Chen, Jing
et al.

Publication Date

2012-12-19

DOI

10.1021/ja310659j

Peer reviewed

Published in final edited form as:
J Am Chem Soc. 2012 December 19; 134(50): 20214–20217. doi:10.1021/ja310659j.

Selective targeting of distinct active site nucleophiles by irreversible Src-family kinase inhibitors

Nathan N. Gushwa[†], **Sumin Kang**[‡], **Jing Chen**[‡], and **Jack Taunton**[†,*]

[†]Howard Hughes Medical Institute, Department of Cellular and Molecular Pharmacology, University of California, San Francisco; San Francisco, CA 94158

[‡]Winship Cancer Institute, Emory University; Atlanta, GA 30322

Abstract

Src-family tyrosine kinases play pivotal roles in human physiology and disease, and several drugs that target members of this family are in clinical use. None of these drugs appear to discriminate among closely related kinases. However, assessing their selectivity toward endogenous kinases in living cells remains a significant challenge. Here, we report the design of two Src-directed chemical probes, each consisting of a nucleoside scaffold with a 5'- electrophile. A 5'-fluorosulfonylbenzoate (**1**) reacts with the conserved catalytic lysine (Lys295) and shows little discrimination among related kinases. By contrast, a 5'-vinylsulfonate (**2**) reacts with a poorly conserved, proximal cysteine (Cys277) found in three Src-family and six unrelated kinases. Both **1** and **2** bear an alkyne tag and efficiently label their respective endogenous kinase targets in intact cells. Using **1** as a competitive probe, we determined the extent to which ponatinib, a clinical Bcr-Abl inhibitor, targets Src-family kinases. Remarkably, while ponatinib had little effect on endogenous Fyn or Src, it potently blocked the critical Tcell kinase, Lck. Probes **1** and **2** thus enable competitive profiling vs. distinct kinase subsets in living cells.

The protein tyrosine kinase c-Src is the archetypal protooncogene. Identified nearly 30 years ago, Src has recently been pursued as a potential drug target for cancer and related bone disease.[1,2] Nine closely related Src family members carry out both overlapping and nonredundant functions.[3] Src-family kinase domains share 70–90% sequence identity, making it difficult to design inhibitors that show selectivity within the family. Moreover, Src-family kinases show a high degree of structural similarity to several other tyrosine kinases, including Abl/Arg and Tec-family kinases. Clinical Src and Abl inhibitors such as dasatinib,[4] bosutinib,[5] and ponatinib[6] appear to show little selectivity among these related kinases. However, the selectivity of these inhibitors toward endogenous kinases in intact cells has not been well defined. To our knowledge, there are few inhibitors that distinguish among Src-family kinases,[7,8,9,10] and none are in common use for cellular studies.

Targeting nucleophilic amino acid side chains with electrophiles is a powerful strategy for developing both selective inhibitors[11] and activity-based probes.[12] The Src active site contains at least two potentially nucleophilic side chains, Lys295 and Cys277 (Figure 1A).[13] Although Lys295 does not function as a nucleophile during catalysis,[14] this essential lysine reacts with millimolar concentrations of the electrophilic ATP analogue, *p*-fluorosulfonylbenzoyl adenosine (FSBA, Figure 1B).[15] Moreover, the equivalent lysine in

[*]***Corresponding Author***taunton@cmp.ucsf.edu.

ASSOCIATED CONTENT

Supporting Information Detailed experimental procedures and supplementary figures. This material is available free of charge via the Internet at http://pubs.acs.org.

phosphatidylinositol-family kinases and at least two protein kinases is trapped by the electrophilic furan of the natural product wortmannin.[16] Finally, an adenosine acyl phosphate probe reacts with the catalytic lysine of most kinases and has been developed into a powerful chemoproteomics tool.[17] The second nucleophile in the Src active site, Cys277, sits at the tip of a flexible glycine-rich loop (G-loop), proximal to both Lys295 and the 5'-triphosphate of ATP (Figure 1A). This G-loop cysteine is an attractive target for covalent inhibition because it is poorly conserved, exposed to solvent, and readily accessible from the Src active site. Only nine human kinases have an equivalent cysteine, including Src, Yes, Fgr, FGFR1–4, LIMK1, and TNK1. Recent elegant studies have reported covalent inhibitors that exploit this cysteine in Src[9] and/or FGFR[18] kinases.

The close proximity of Lys295 and Cys277 to the γ-phosphate of ATP (Figure 1A) suggested the possibility of targeting both side chains with electrophiles appended to the 5'-hydroxyl group of a nucleoside scaffold. To access Lys295 and Cys277, we designed a hybrid nucleoside, borrowing structural elements from FSBA and the Src-family inhibitor PP1 (Figure 1B,C). A superposition of 5'- (β,γ-imido)triphosphate adenosine (AMP-PNP) and PP1 bound to the Src-family kinase Hck[19] suggested that the affinity of the adenine core, common to AMP-PNP and FSBA, could be increased by adding a *p*-tolyl substituent. In PP1, this group exploits a hydrophobic pocket found in all Src-family kinases.[20] The 2'-hydroxyl group of AMP-PNP bound to Src is solvent exposed. We therefore added a 2'-propargyl ether to monitor covalent binding to proteins using copper-promoted click chemistry (Figure 1C).[21,22]

Starting with the *p*-tolyl nucleoside, we modified the 5'- hydroxyl with a fluorosulfonylbenzoate or a vinylsulfonate to yield **1** and **2**, respectively (Figure 1C). The inhibitors were first characterized by pretreating Src for 30 min in the presence of 0.25 mM ATP, prior to initiating the kinase reaction. Both compounds **1** (IC_{50}: 200 nM) and **2** (IC_{50}: 9 nM) were vastly superior to FSBA, which was inactive up to 10 μM (Figure 1D). The striking difference between FSBA and fluorosulfonylbenzoate **1** can be explained by the enhanced affinity provided by the *p*-tolyl group. Both compounds **1** and **2** displayed time-dependent inhibition of Src, characteristic of covalent inhibitors (Figure S1).

We next evaluated the ability of **1** and **2** to covalently modify Src in a nucleophile-specific manner in intact cells. Cells transfected with an activated FLAG-Src construct (Y527F) were treated with increasing concentrations of **1** for 1 h. Cells were lysed, and rhodamine-azide was conjugated to probe-modified proteins via click chemistry.[21] After SDS-PAGE, probe-labeled FLAG-Src was easily detected as the most intensely fluorescent band (Figure 2A), a surprising result given the complexity of the lysate. Importantly, mutation of Lys295 to Arg abolished labeling of FLAG-Src (Figure 2B), demonstrating the requirement of this lysine for covalent modification. Src has 32 lysines, including three near the ATP binding site; thus, **1** is highly selective for Lys295. We also tested the ability of **1** to inhibit Src autophosphorylation in cells. This experiment revealed a dose-dependent loss of Src kinase activity that closely paralleled labeling by **1** (Figure 2C). Together, these results demonstrate that fluorosulfonylbenzoate **1** can enter cells and inhibit Src via covalent modification of Lys295.

Vinylsulfonate **2** also labeled a prominent band corresponding to FLAG-Src, in addition to labeling several other unidentified proteins. In contrast to **1**, labeling of FLAG-Src by **2** was not affected by the K295R mutation (Figure 3A), implicating Cys277 as the relevant nucleophile. Whereas all Src-family kinases have a lysine corresponding to Lys295, only three have a G-loop cysteine (Figure 3B). Enzymatic assays with a small panel of kinases revealed that Src, Yes, and the unrelated kinase, FGFR3 (all with the G-loop Cys), were

inhibited by **2** with single-digit nanomolar potency, whereas closely related kinases lacking the G-loop Cys were more than 40-fold less sensitive under similar conditions (Figure S2).

To address the requirement for the G-loop cysteine, we evaluated the sensitivity of C277Q Src to our electrophilic inhibitors. Cells were treated with **1** or **2** for 30 min, followed by a brief washout with compound-free media. Covalent modification of FLAG-Src by **2** increased in a dose-dependent manner in parallel with a decrease in autophosphorylation (Figure 3C). By contrast, the C277Q mutant was relatively resistant to vinylsulfonate **2**, yet still sensitive to fluorosulfonylbenzoate **1**. Weak labeling by high concentrations of **2** is likely caused by a slow reaction with Lys295 in the mutant. Finally, using Ba/F3 cells stably expressing wild-type or C482A FGFR3, we demonstrated potent 5'-vinylsulfonate-mediated inhibition of cell proliferation; importantly, mutation of the G-loop cysteine conferred complete resistance (Figure S3).

To test whether the new probes can detect endogenous Src-family kinases, which are typically membrane-associated and expressed at low levels, we developed a protocol based on sequential affinity purification and Western blotting. After treating Jurkat or HeLa cells with **1** (1 μM) or **2** (250 nM) for 10 min, lysates were prepared and subjected to click conjugation with biotin-azide. The resulting probe/biotin-modified proteins were then affinity purified with streptavidin-coated magnetic beads. Finally, eluted proteins were analyzed by Western blotting for five Src-family kinases (Src, Yes, Lck, Blk, and Lyn). Fluorosulfonyl-benzoate **1** labeled all five endogenous kinases (Figure 4A), consistent with its ability to potently inhibit the enzymatic activity of these kinases (Table S1). By contrast, vinylsulfonate **2** labeled Src and Yes, both of which have the G-loop cysteine, but it did not label Lck, Blk, or Lyn (Figure 4A). Pretreatment of cells with PD166326 (20 μM), a well characterized promiscuous tyrosine kinase inhibitor,[23] abolished kinase labeling by both probes. Based on the shared cysteine, we anticipate that endogenous FGFR kinases (and possibly LIMK1 and TNK1) will also be labeled by **2**.

We exploited the unique properties of **1** to test whether ponatinib, a Bcr-Abl inhibitor used to treat refractory chronic myeloid leukemia, indiscriminately targets endogenous Src-family kinases, as suggested by in vitro kinase assays.[6] Jurkat cells were treated with increasing concentrations of ponatinib, followed by brief treatment with **1** (1 μM, 15 min). Strikingly, ponatinib at 300 nM abrogated labeling of endogenous Lck and Blk by **1** (EC_{50}~50 nM), whereas labeling of Src and Fyn was unaffected (Figure 4B). Thus, ponatinib appears to be significantly more potent vs. endogenous Lck and Blk than Fyn or Src, perhaps in part due to slower dissociation from the former kinases. It remains to be seen whether ponatinib, with mean steady-state concentrations of 75–180 nM in humans,[24] has immunosuppressive effects due to Lck inhibition.

In this communication, we have begun to address a critical question that inevitably arises during kinase inhibitor development: when cells (or animals) are treated with a given inhibitor at a defined concentration, which kinases are bound, and to what extent? Our experiments with **1** and **2** demonstrate covalent capture of multiple Src-family kinases in intact cells, along with robust competition by PD166326 and ponatinib. In future studies, quantitative mass spectrometry will be used to define the full set of endogenous kinases targeted by **1** and **2**; based on the known promiscuity of PP1-like inhibitors and the conservation of the catalytic lysine, the set of kinases targeted by **1** is likely to extend well beyond the Src family.

Despite profiling a limited set of kinases, the Western blotting approach reported here is complementary to mass spectrometry approaches based on adenosine acyl phosphate probes[17] and bead-immobilized kinase inhibitors ("kinobeads").[25] The latter probes require

cell lysis and dilution, which may disrupt dynamic signaling complexes and perturb interactions between inhibitors and endogenous kinases. By contrast, probes **1** and **2** are capable of monitoring inhibitor/kinase interactions under native conditions in living cells. Moreover, detection and quantification of probe-modified kinases is much easier with Western blotting as compared to mass spectrometry, although fewer kinases can be monitored in a single experiment. Finally, the ability of the fluorosulfonyl group of **1** to survive the cellular milieu and rapidly engage the catalytic lysine suggests the possibility of pairing this electrophile with additional kinase-directed scaffolds, thereby expanding the range of kinases amenable to this approach.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institutes of Health (GM071434) and the Sandler Center for Drug Discovery. We acknowledge the UCSF Mass Spectrometry Facility (supported by NIH grant P41RR001614).

REFERENCES

1. Courtneidge SA, Smith AE. Nature. 1983; 303:435–439. [PubMed: 6304524]
2. Kim LC, Song L, Haura EB. Nat Rev Clin Oncol. 2009; 6:587–595. [PubMed: 19787002]
3. Benati D, Baldari CT. Curr Med Chem. 2008; 15:1154–1165. [PubMed: 18473810]
4. Remsing Rix LL, Rix U, Colinge J, Hantcschel O, Bennett KL, Stranzl T, Muller A, Baumgartner C, Valent P, Augustin M, Till JH, Superti-Furga G. Leukemia. 2009; 23:477–485. [PubMed: 19039322]
5. Bantscheff M, Eberhard D, Abrahma Y, Basteck S, Boesche M, Hobson S, Mathieson T, Perrin J, Raida M, Rau C, Reader V, Sweetman N, Baur A, Bouwmeester T, Hopf C, Kruse U, Neubauer G, Ramsden N, Rick J, Kuster B, Drewes G. Nat Biotechnol. 2007; 25:1035–1044. [PubMed: 17721511]
6. O'Hare T, Shakespeare WC, Zhu X, Eide CA, Rivera VM, Wang F, Adrian LT, Zhou T, Huang WS, Xu Q, Metcalf CA, Tyner JW, Loriaux MM, Corbin AS, Wardwell S, Ning Y, Keats JA, Wang Y, Sundaramoorthi R, Thomas M, Zhou D, Snodgrass J, Commodore L, Sawyer TK, Dalgarno DC, Deininger MW, Druker BJ, Clackson T. Cancer Cell. 2009; 16:401–412. [PubMed: 19878872]
7. Maly DJ, Choong IC, Ellman JA. Proc Natl Acad Sci. 2000; 97:2419–2424. [PubMed: 10716979]
8. Georghiou G, Kleiner RE, Pulkoski-Gross M, Liu DR, Seeliger MA. Nature Chem Biol. 2012; 8:366–374. [PubMed: 22344177]
9. Kwarcinski FE, Fox CC, Steffey ME, Soellner MB. ACS Chem Biol. 2012; 7:1910–1917. [PubMed: 22928736]
10. Meyn MA, Smithgall TE. Mini Rev Med Chem. 2008; 8:628–637. [PubMed: 18537718]
11. Singh J, Petter RC, Baillie TA, Whitty A. Nat Rev Drug Discov. 2011; 10:307–317. [PubMed: 21455239]
12. Moellering RE, Cravatt BF. Chem Biol. 2012; 19:11–22. [PubMed: 22284350]
13. Xu W, Doshi A, Lei M, Eck MJ, Harrison SC. Mol Cell. 1999; 3:629–638. [PubMed: 10360179]
14. Adams JA. Chem Rev. 2001; 101:2271–2290. [PubMed: 11749373]
15. Kamps MP, Taylor SS, Sefton BM. Nature. 1984; 310:589–592. [PubMed: 6431300]
16. (a) Wymann MP, Bulgarelli-Leva G, Zvelebil MJ, Pirola L, Vanhaesebroeck B, Waterfield MD, Panayotou G. Mol Cell Biol. 1996; 16:1722–1733. [PubMed: 8657148] (b) Liu Y, Shreder KR, Gai W, Corral S, Ferris DK, Rosenblum JS. Chem Biol. 2005; 12:99–107. [PubMed: 15664519]
17. Patricelli MP, Nomanbhoy TK, Wu J, Brown H, Zhou D, Zhang J, Jagannathan S, Aban A, Okerberg E, Herring C, Nordin B, Weissig H, Yang Q, Lee JD, Gray NS, Kozarich JW. Chem Biol. 2011; 18:699–710. [PubMed: 21700206]

18. Zhou W, Hur W, McDermott U, Dutt A, Xian W, Ficarro SB, Zhang J, Sharma SV, Brugge J, Meyerson M, Settleman J, Gray NS. Chem Biol. 2010; 17:285–295. [PubMed: 20338520]
19. Schindler T, Sicheri F, Pico A, Gazit A, Levitzki A, Kuriyan J. Mol Cell. 1999; 3:639–648. [PubMed: 10360180]
20. Liu Y, Bishop A, Witucki L, Kraybill B, Shimizu E, Tsien J, Ubersax J, Blethrow J, Morgan DO, Shokat KM. Chem Biol. 1999; 6:671–678. [PubMed: 10467133]
21. Speers AE, Cravatt BF. Chem Biol. 2004; 11:535–546. [PubMed: 15123248]
22. Rostovtsev VV, Green LG, Fokin VV, Sharpless KB. Angew Chem Int Ed Engl. 2002; 41:2596–2599. [PubMed: 12203546]
23. Kraker AJ, Hartl BG, Amar AM, Barvian MR, Showalter HDH, Moore CW. Biochem Pharmacol. 2000; 60:885–898. [PubMed: 10974196]
24. Gozgit JM, Wong MJ, Moran L, Wardwell S, Mohemmad QK, Narasimhan NI, Shakespeare WC, Wang F, Clackson T, Rivera VM. AACR Annual Meeting Abstract 3560. 2011
25. Bantscheff M, Eberhard D, Abraham Y, Bastuck S, Boesche M, Hobson S, Mathieson T, Perrin J, Raida M, Rau C, Reader V, Sweetman G, Bauer A, Bouwmeester T, Hopf C, Kruse U, Neubauer G, Ramsden N, Rick J, Kuster B, Drewes G. Nat Biotechnol. 2007; 25:1035–1044. [PubMed: 17721511]

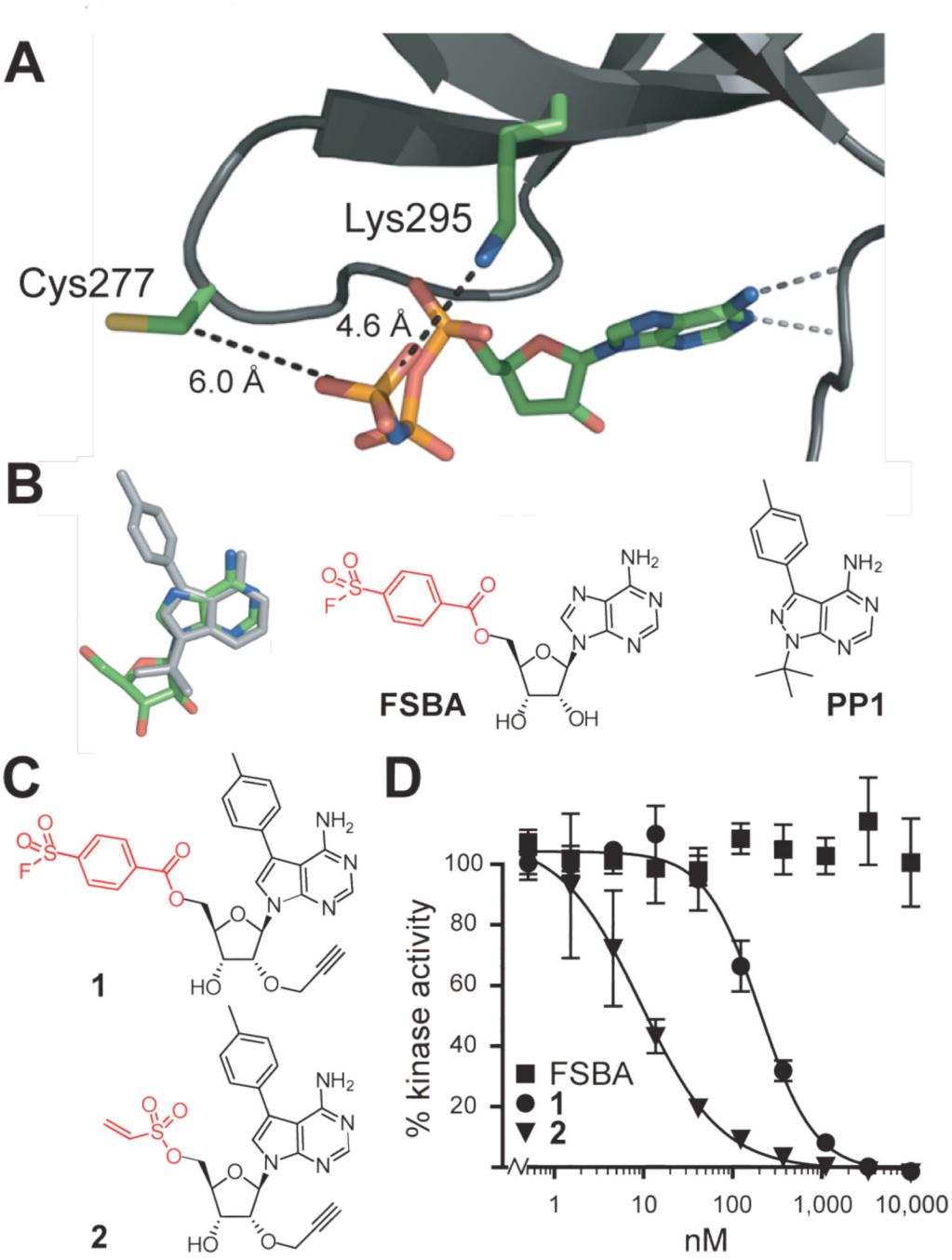

Figure 1.

(A) Structure of AMP-PNP bound to Src (PDB: 2SRC) (B) Superposition of AMP-PNP (adenosine portion) and PP1 (gray) bound to Src-family kinase, Hck (PDB: 1AD5 and 1QCF). (C) Electrophilic inhibitors **1** and **2**. (D) Src kinase assays (± SD, n = 3).

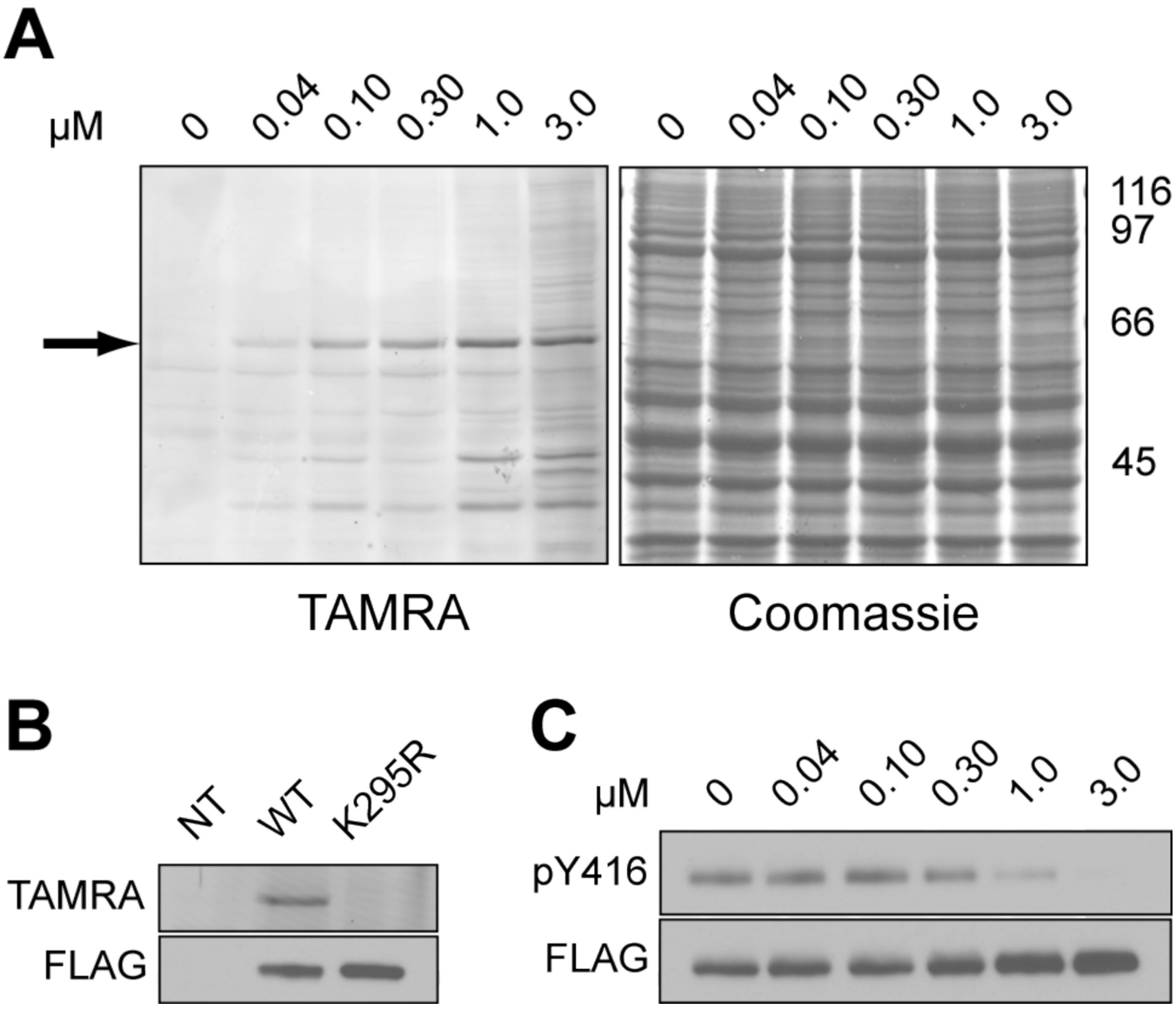

Figure 2.

(A) COS-7 cells expressing activated (Y527F) FLAG-Src were treated with **1** for 1 h. Cell lysates were subjected to click chemistry with rhodamine-azide, resolved by SDS-PAGE, and scanned for fluorescence (TAMRA). (B) Nontransfected (NT) or cells expressing wild type (WT) or K295R FLAG-Src were treated with 1 μM **1** for 1 h, processed as in (A), and analyzed for TAMRA fluorescence and FLAG-Src by Western blot. (C) Lysates from cells treated as in (A) were analyzed for Src autophosphorylation.

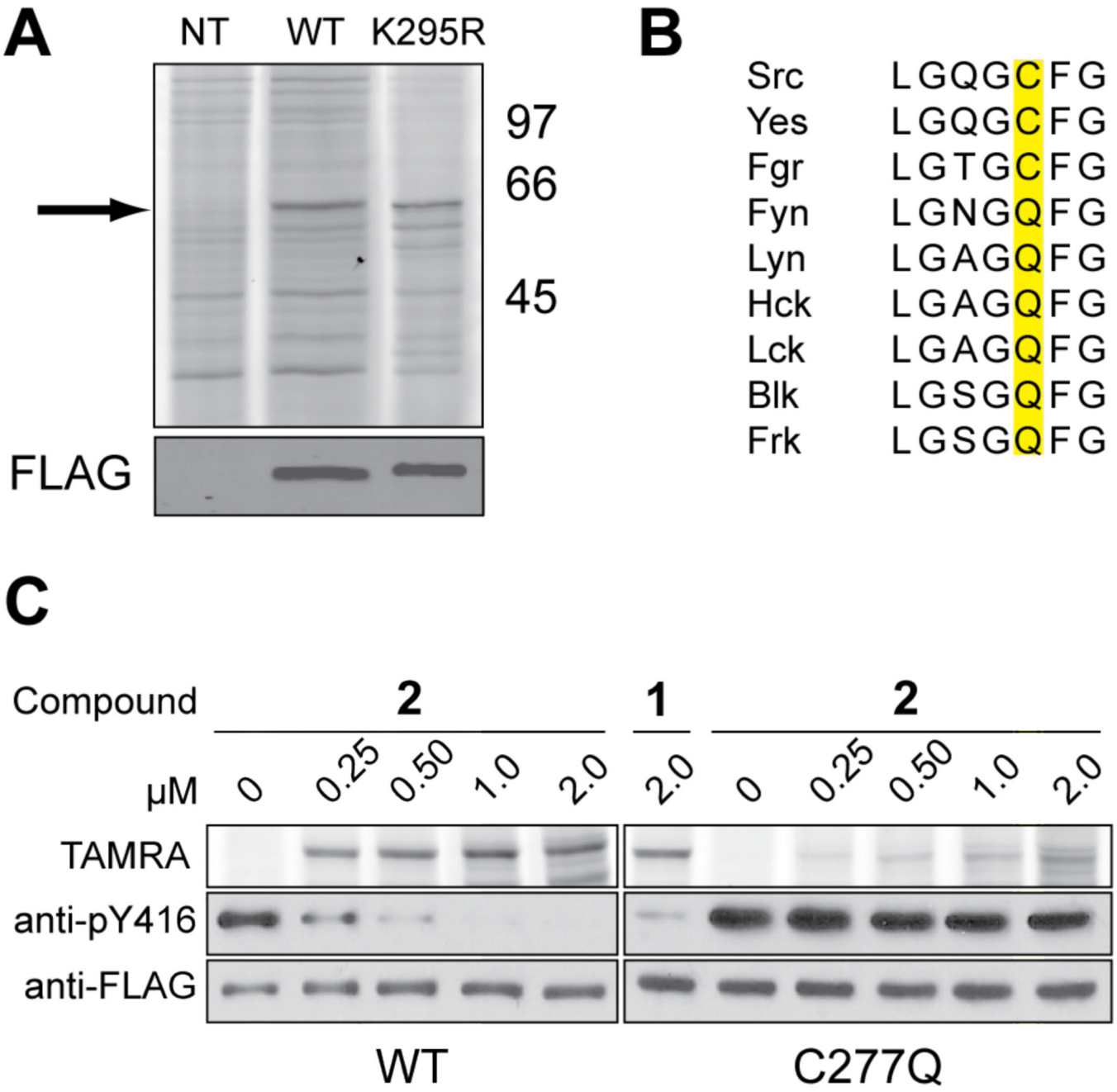

Figure 3.

(A) Nontransfected COS-7 cells (NT) or cells expressing wild type (WT) or K295R FLAG-Src were treated with 1 μM **2** for 20 min. After click chemistry, lysates were analyzed by in-gel fluorescence and Western blot as described in Figure 2. (B) G-loop region of Src-family kinases. (C) Cells expressing WT or C277Q FLAG-Src were treated with **1** or **2** for 30 min and briefly washed with compound-free media. After click chemistry, lysates were analyzed by in-gel fluorescence and Western blot as described in Figure 2.

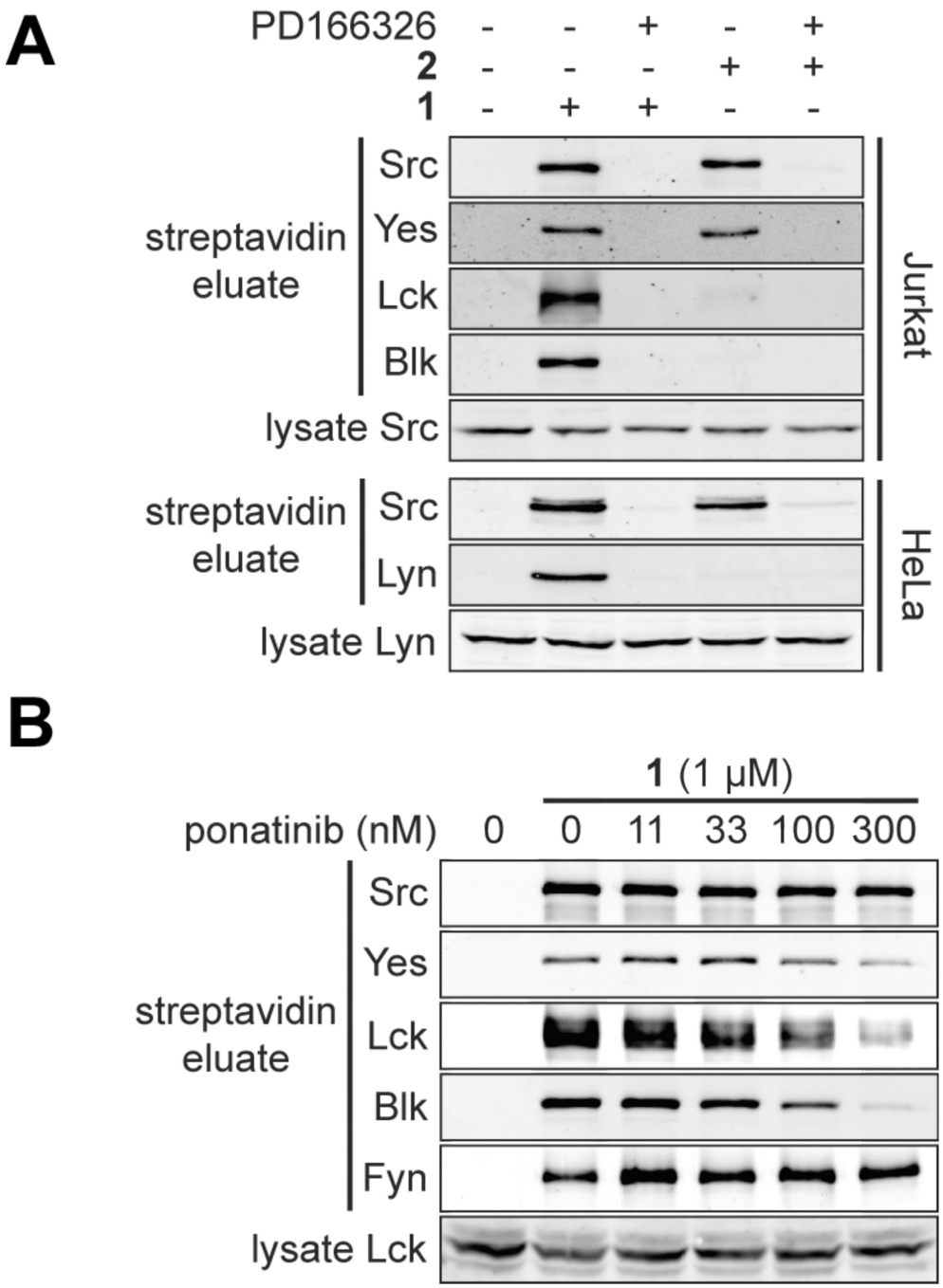

Figure 4.

Competitive profiling vs. endogenous Src-family kinases. (A) Cells were treated with or without PD166326 (20 μM) for 20 min, followed by **1** (1 μM) or **2** (250 nM) for 10 min. Lysates were subjected to click chemistry with biotin-azide, and modified proteins were affinity purified with streptavidin beads. Eluted proteins were resolved by SDS-PAGE and analyzed by Western blotting. (B) Jurkat cells were treated with ponatinib for 30 min, followed by **1** for 15 min. Lysates were processed and analyzed as in (A).