J|A|C|S COMMUNICATIONS

Published on Web 04/24/2009

A Chemical Genetic Method for Generating Bivalent Inhibitors of Protein Kinases

Zachary B. Hill, B. Gayani K. Perera, and Dustin J. Maly*

Department of Chemistry, University of Washington, Box 351700, Seattle, Washington 98195-1700

Received February 4, 2009; E-mail: Maly@chem.washington.edu

Protein kinases are a diverse enzyme family that mediate intracellular protein phosphorylation by catalyzing the transfer of the γ-phosphate of ATP to serine, threonine, or tyrosine residues.[1] Mis-regulation of protein kinase activity has been implicated in a number of diseases including diabetes, chronic inflammation, and many forms of cancer. Despite widespread interest in this important enzyme family, the function of most kinases is still not well understood and the role of specific kinases in the etiology of disease is unclear. For this reason, reagents that allow the function of specific kinases to be dissected are of great utility.[2] Selective inhibitors that block the catalytic activity of protein kinases have emerged as useful tools for this purpose. As most potent kinase inhibitors bind in the highly conserved ATP-binding cleft, obtaining selectivity is extremely challenging.[3] A promising strategy for the identification of kinase inhibitors with increased potency and selectivity is the generation of bivalent ligands that interact with two distinct binding sites.[4] By targeting less conserved interactions outside of the ATP-binding cleft, increased affinity and selectivity can be obtained.

Here we describe a chemical genetic method for generating highly selective, protein-small molecule conjugate inhibitors of protein kinases. These inhibitors consist of two distinct ligands that are displayed from a protein scaffold: (1) a synthetic, small molecule that binds to the ATP-binding cleft and (2) a peptidic ligand that enhances selectivity between kinases by targeting a secondary binding domain (Figure 1A). A key feature of these inhibitors is that they can be assembled with a chemoselective protein labeling technique. Johnsson and co-workers have demonstrated that diverse small molecules that are linked to O^6-benzylguanine (BG) can be selectively transferred to the active site cysteine of O^6-alkylguanine-DNA alkyltransferase (AGT) *in vitro* and in living cells.[5] Here we use this labeling methodology to generate potent and selective bivalent kinase inhibitors from distinct modular components. Importantly, these bivalent conjugates can be assembled in living cells if cell permeable BG-inhibitor derivatives are used.

To test the feasibility of using the AGT labeling methodology for rapidly generating selective ligands of protein kinases, we selected two closely related nonreceptor tyrosine kinases, Src and Abl, as initial targets. While the *N*- and *C*-termini of Src and Abl are unique, both kinases contain highly homologous catalytic (SH1) domains that are regulated by SH2 and SH3 domains.[6] The SH1 domain contains the substrate binding sites (ATP and protein) and all of the catalytic residues necessary for phosphate transfer. The SH2 and SH3 domains are responsible for the regulation, substrate selection, and localization of the catalytic domain. The high degree of sequence homology shared by Src and Abl has made generating ligands that are able to discriminate between these two kinases extremely challenging.

Two components are needed to assemble bivalent kinase inhibitors with our strategy: a fusion protein that contains a ligand for a secondary binding domain of the kinase being targeted and a synthetic, ATP-competitive inhibitor that is linked to O^6-benzylguanine (BG) through a flexible linker. We designed AGT fusion proteins containing short polyproline (PP) motifs (AGT(PP1)-AGT(PP8), Figure 1B) that target the SH3 domains of Src and Abl. These secondary binding domain targeting elements are linked to the *N*- and *C*-terminus of AGT through a flexible serine-glycine linker to ensure that they are accessible to the SH3 domains of Src and Abl. To target the SH3 domain of Src, AGT fusion proteins containing a PP motif (APPLPPRNRPRL) of moderate affinity (K_d = 1−2 μM) for Src-family kinases were generated (AGT(PP1)-AGT(PP4), Figure 1B).[7] For Abl-selective SH3 domain ligands, AGT fusions containing a PP motif (APTYSPPPPP) that has been optimized for Abl (K_d = 0.4 μM) were generated (AGT(PP5)-AGT(PP8), Figure 1B).[8]

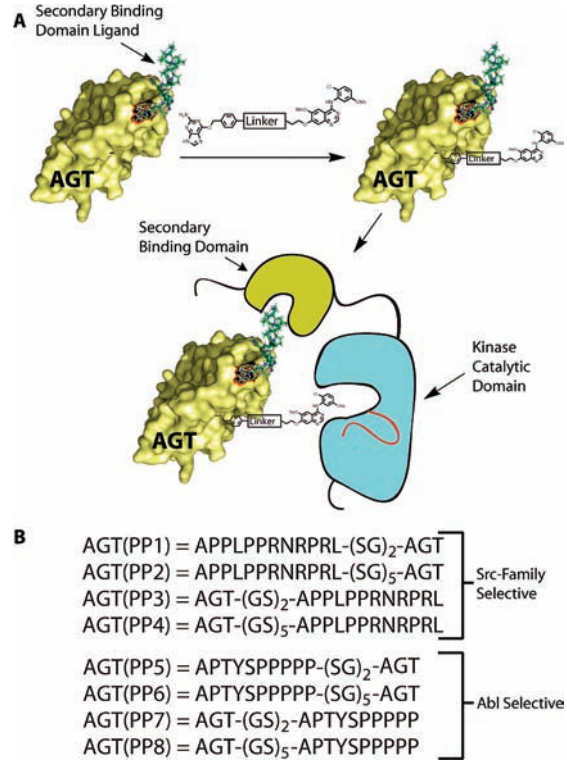


Figure 1. Bivalent AGT-small molecule conjugate inhibitors of Src and Abl. (A) Schematic representation of the protein-small molecule conjugate assembly and bivalent inhibition of a protein kinase that contains a secondary binding domain. (B) His6-tagged polyproline (PP) motif AGT fusions that were generated in this study.

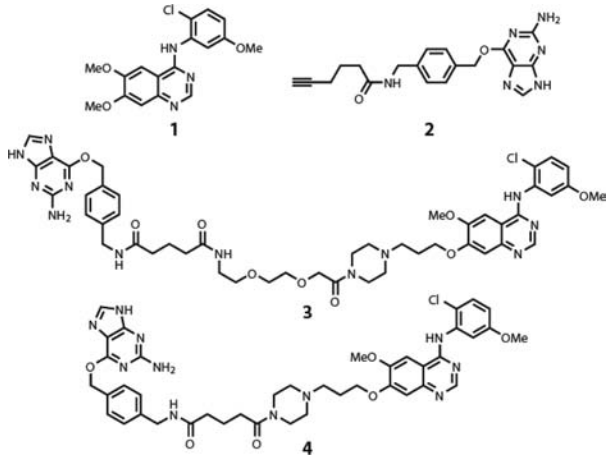
The relative catalytic activities of AGT(PP1)-AGT(PP8) and wild type AGT (AGT(wt)) were quantified *in vitro* with a fluorescein-BG conjugate (Figure S1). As expected, all eight PP fusions were labeled with the same efficiency as AGT(wt), demonstrating that appending a PP-containing peptide to the *N*- or *C*-terminus of AGT

10.1021/ja900871y CCC: $40.75 © 2009 American Chemical Society

does not alter the activity of this enzyme. The ability of the SH3 domain of an Src kinase construct that contains an SH1, SH2, and SH3 domain (Src-3D) to interact with the PP motifs of AGT(PP1)-AGT(PP4) was determined with a pull-down assay. His6-tagged AGT constructs were immobilized on ion metal affinity resin and incubated with Src-3D. Extensive washing of the beads, followed by elution of bound protein, and dot blot analysis demonstrated that Src-3D binds to all four PP-containing AGT fusions and not to AGT(wt) (Figure S2).

We next turned to selecting a suitable ATP-competitive inhibitor that can be displayed from the active site of AGT. Because the 4-anilinoquinazoline scaffold has proven to be an effective pharmacophore for the generation of potent inhibitors of several kinases, including Abl and Src, we decided to generate derivatives of compound **1** for this purpose.[9] Previous structural studies and structure−activity relationship (SAR) analyses have demonstrated that the quinazoline scaffold binds in the ATP-binding cleft and that the C-6 and C-7 positions point out of the active site into solvent. Based on this precedent, we generated inhibitors that are linked to BG through the C-7 position of the 4-anilinoquinazoline scaffold. To ensure that the inhibitor is displayed at a sufficient distance from the active site of AGT, conjugates with two linker lengths were generated (**3** and **4**). BG derivatives **3** and **4** were tested in an *in vitro* activity assay for their ability to inhibit the catalytic activity of Src-3D and Abl-3D (Figure 2A and 2B, row 1). Gratifyingly, **3** and **4** are moderately potent inhibitors of Src-3D and Abl-3D. Importantly, linking the 4-anilinoquinazoline scaffold to BG through a flexible linker does not significantly affect the potency of these compounds.

Encouraged by the ability of our designed modular components to independently interact with the SH1 and SH3 domains of Src-3D and Abl-3D, we determined the effectiveness of the assembled protein-small molecule conjugates as inhibitors. The purified protein-small molecule conjugates shown in Figure 2 were tested for their ability to inhibit Src-3D and Abl-3D with an *in vitro* activity assay. Each bivalent ligand was generated by incubating an AGT construct with a small excess of **3**, **4**, or negative control compound **2** for 1.5 h, followed by removal of noncovalently bound BG derivatives with gel filtration chromatography. The purified AGT-small molecule conjugates were quantified and used directly in activity assays.



The key requirement of targeting the ATP-binding cleft to inhibit Src or Abl was demonstrated by using AGT conjugates that display a small molecule with no affinity for the active site (BG-alkyne **2**). AGT constructs displaying **2** show no inhibition of either kinase at the highest concentration tested (Figure 2A and 2B, column 1),

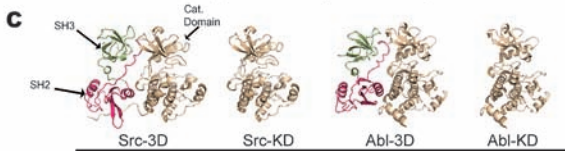
A IC_{50} (nM) Src-3D

	2	3	4
No Protein	N/T	130 ± 4	300 ± 20
AGT(wt)	> 5,000	2000 ± 300	> 5,000
AGT(PP1)	> 5,000	12 ± 1	13 ± 3
AGT(PP2)	> 5,000	15 ± 1	25 ± 6
AGT(PP3)	> 5,000	66 ± 10	72 ± 10
AGT(PP4)	> 5,000	16 ± 1	34 ± 3

B IC_{50} (nM) Abl-3D

	2	3	4
No Protein	N/T	510 ± 50	410 ± 50
AGT(wt)	> 5,000	1300 ± 200	3200 ± 100
AGT(PP5)	> 5,000	< 6	18 ± 6
AGT(PP6)	> 5,000	< 6	15 ± 4
AGT(PP7)	> 5,000	< 6	18 ± 7
AGT(PP8)	> 5,000	< 6	15 ± 3

C



	Src-3D	Src-KD	Abl-3D	Abl-KD
AGT(PP1)-**3**	12 ± 1	2600 ± 300	N/T	3200 ± 300
AGT(PP7)-**3**	N/T	4200 ± 500	< 6	3100 ± 400
AGT(WT)-**3**	2000 ± 300	3700 ± 700	1300 ± 200	3600 ± 200

Figure 2. IC_{50} values of BG derivatives and AGT-small molecule conjugates. (A) *In vitro* activities of BG derivatives and assembled AGT-small molecule conjugates against a Src kinase construct that contains an SH1, SH2, and SH3 domain (Src-3D). (B) *In vitro* activities of BG derivatives and assembled AGT-small molecule conjugates against an Abl kinase construct that contains an SH1, SH2, and SH3 domain. (C) *In vitro* activities of AGT(wt)-**3**, AGT(PP1)-**3**, and AGT(PP7)-**3** against Src-3D, a Src construct that only contains the catalytic domain (Src-KD), Abl-3D, and an Abl construct that only contains the catalytic domain (Abl-KD). All protein-small molecule conjugates were prepared in two independent labeling reactions, and all assays were performed in quadruplicate. N/T = not tested.

while both **3** and **4** are inhibitors of moderate potency when displayed from the active site of AGT(wt) (Figure 2A and 2B, row 2). Importantly, all eight small molecule conjugates that contain a Src-selective PP motif (AGT(PP1)-**3**, AGT(PP2)-**3**, AGT(PP3)-**3**, AGT(PP4)-**3,** AGT(PP1)-**4**, AGT(PP2)-**4**, AGT(PP3)-**4**, and AGT-(PP4)-**4**) are significantly more potent inhibitors of Src-3D than the wild type constructs AGT(wt)-**3** and AGT(wt)-**4**, consistent with a bivalent mode of inhibition (Figure 2A). In addition, these bivalent conjugates have a 2- to 23-fold lower IC_{50} for Src-3D than BG derivatives **3** or **4**. For Abl, an identical increase in potency is observed for bivalent AGT constructs that contain an Abl-selective PP motif, with some PP-containing conjugates demonstrating a >200-fold lower IC_{50} than the AGT(wt)-small molecule conjugates and a >80-fold lower IC_{50} than BG derivatives **3** or **4** (Figure 2B). Notably, changes in the relative display of the PP motif ligand and ATP-competitive inhibitor appear to be well tolerated.

To further determine the contribution of the interaction between the PP motif and the SH3 domain for bivalent inhibition, small molecule conjugates containing a Src-selective PP motif (AGT(PP1)-**3**), an Abl-selective PP motif (AGT(PP7)-**3**), and no secondary binding domain ligand (AGT(wt)-**3**) were tested for their ability to inhibit a Src kinase construct lacking an SH2 and SH3 domain (Src-

KD) and an Abl kinase construct lacking an SH2 and SH3 domain (Abl-KD). Consistent with the SH3 domain−PP motif interaction being responsible for the observed increase in potency of the bivalent ligands, all three AGT conjugates are nearly equipotent inhibitors of Src-KD (Figure 2C). In addition, the IC_{50}'s of all three conjugates for Abl-KD are almost identical. As expected, the potencies of all three bivalent ligands for the catalytic domains of these kinases are similar to AGT(wt)-**3** for Src-3D and Abl-3D.

Having demonstrated that our chemical genetic method is able to generate potent inhibitors of Src and Abl, we next wished to determine how selective these bivalent ligands are for their intended targets. Therefore, AGT constructs containing a Src-selective PP motif (AGT(PP1)-**4**) and an Abl-selective PP motif (AGT(PP7)-**4**) were screened against a panel of 13 protein kinases (Figure 3A).

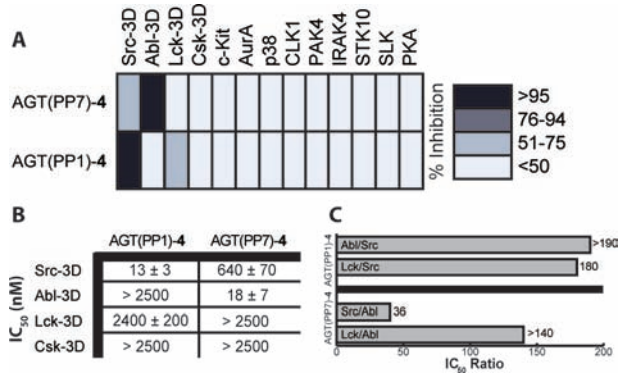


Figure 3. Kinase selectivity profile of AGT(PP1)-**4** and AGT(PP7)-**4**. (A) Percent inhibition of 2.5 μM AGT(PP1)-**4** and 2.5 μM AGT(PP7)-**4** against a panel of thirteen protein kinases. All assays were performed in triplicate and the SEM for all data points is <5%. (B) IC_{50} values of AGT(PP1)-**4** and AGT(PP7)-**4** against SH3 domain-containing tyrosine kinases. All assays were performed in triplicate. (C) The ratio of IC_{50}s against Abl-3D, Src-3D and Lck-3D for AGT(PP1)-**4** and AGT(PP7)-**4**.

Because BG derivative **4**, which contains a shorter linker than **3**, is more likely to be cell permeable, AGT constructs conjugated to this inhibitor were generated. (AGT(PP1)-**4**) and (AGT(PP7)-**4**) were screened at a single concentration (2.5 μM) for each kinase. As expected, kinases that lack an SH3 domain and are insensitive to inhibitors based on the 4-anilinoquinazoline scaffold (AurA, CLK1, PAK4, IRAK4, STK10, SLK, c-Kit, and PKA) are not significantly inhibited by either bivalent inhibitor. Even the serine/threonine kinase p38, which lacks an SH3 domain but is targeted by several potent inhibitors that are based on the 4-anilinoquinazoline scaffold, does not show a measurable decrease in catalytic activity. Significantly, both bivalent ligands show a high degree of selectivity among highly homologous nonreceptor tyrosine kinases that contain SH3 domains. The tyrosine kinase Csk, which contains an SH3 domain and is the target of several 4-anilinoquinazoline inhibitors, is not inhibited by either construct at 2.5 μM. Furthermore, AGT(PP1)-**4** is >150-fold selective for Src ($IC_{50} = 0.013 \pm 0.003$ μM) over Abl ($IC_{50} = >2.5$ μM) and Src-family kinase Lck ($IC_{50} = 2.4 \pm 0.2$ μM). In addition, AGT(PP7)-**4** is selective for Abl ($IC_{50} = 0.018 \pm 0.007$ μM) over Src ($IC_{50} = 0.64 \pm 0.07$ μM) and Lck ($IC_{50} = >2.5$ μM) (Figure 3B).[10] Thus, the same ATP-competitive inhibitor can be used to generate highly selective inhibitors of closely related kinases by varying the secondary binding domain ligand.

In conclusion, we have developed a new chemical genetic method for constructing bivalent kinase inhibitors. These protein-small molecule conjugates are rapidly assembled by combining a genetically encoded fusion protein with a synthetic, ATP-competitive inhibitor. In this study, inhibitors that are able to discriminate between the highly homologous nonreceptor tyrosine kinases Src, Abl, and Lck were generated by tethering an ATP-competitive inhibitor with moderate affinity and selectivity to a ligand that targets a secondary domain with a unique binding specificity. Despite the structural similarities of the kinases targeted, their SH3 domains have distinct PP motif binding preferences. Current efforts are underway to optimize the selectivity of each PP motif and to determine the selectivity of these constructs among other closely related kinases; including additional Abl- and Src-family kinases. As protein kinases are bisubstrate enzymes and often contain multiple binding domains, this strategy should be applicable to a large percentage of the kinome. Furthermore, it should be possible to generate bivalent ligands in living cells through the use of cell-permeable BG-inhibitor derivatives. The cell permeability of BG derivatives **3** and **4** is currently being investigated, and cellular studies with these constructs will be reported in due course.

Acknowledgment. This work was supported by the University of Washington. We thank Markus Seeliger for providing Abl-3D, Lck-3D, and Src-3D. Charles Buser III is acknowledged for synthesizing peptide substrates for Abl and Src.

Supporting Information Available: Experimental details and the complete citation for ref 3c. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Manning, G.; Whyte, D. B.; Martinez, R.; Hunter, T.; Sudarsanam, S. *Science* **2002**, *298*, 1912–1934.
(2) (a) Johnson, S. A.; Hunter, T. *Nat. Methods* **2005**, *2*, 17–25. (b) Knight, Z. A.; Shokat, K. M. *Cell* **2007**, *128*, 425–430. (c) Lawrence, D. S. *Acc. Chem. Res.* **2003**, *36*, 401–409. (d) Shen, K.; Hines, A. C.; Schwarzer, D.; Pickin, K. A.; Cole, P. A. *Biochim. Biophys. Acta* **2005**, *1754*, 65–78.
(3) (a) Knight, Z. A.; Shokat, K. M. *Chem. Biol.* **2005**, *12*, 621–637. (b) Fedorov, O.; Marsden, B.; Pogacic, V.; Rellos, P.; Müller, S.; Bullock, A. N.; Schwaller, J.; Sundström, M.; Knapp, S. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 20523–20528. (c) Karaman, M. W.; et al. *Nat. Biotechnol.* **2008**, *26*, 127–132.
(4) (a) Hah, J. M.; Sharma, V.; Li, H.; Lawrence, D. S. *J. Am. Chem. Soc.* **2006**, *128*, 5996–5997. (b) Profit, A. A.; Lee, T. R.; Niu, J.; Lawrence, D. S. *J. Biol. Chem.* **2001**, *276*, 9446–9451. (c) Meyer, S. C.; Shomin, C. D.; Gaj, T.; Ghosh, I. *J. Am. Chem. Soc.* **2007**, *129*, 13812–13813. (d) Ricouart, A.; Gesquiere, J. C.; Tartar, A.; Sergheraert, C. *J. Med. Chem.* **1991**, *34*, 73–78. (e) Parang, K.; Till, J. H.; Ablooglu, A. J.; Kohanski, R. A.; Hubbard, S. R.; Cole, P. A. *Nat. Struct. Biol.* **2001**, *8*, 37–41. (f) Shen, K.; Cole, P. A. *J. Am. Chem. Soc.* **2003**, *125*, 16172–16173. (g) Schneider, T. L.; Mathew, R. S.; Rice, K. P.; Tamaki, K.; Wood, J. L.; Schepartz, A. *Org. Lett.* **2005**, *7*, 1695–1698.
(5) (a) Keppler, A.; Kindermann, M.; Gendreizig, S.; Pick, H.; Vogel, H.; Johnsson, K. *Methods* **2004**, *32*, 437–444. (b) Keppler, A.; Pick, H.; Arrivoli, C.; Vogel, H.; Johnsson, K. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 9955–9959. (c) Keppler, A.; Gendreizig, S.; Gronemeyer, T.; Pick, H.; Vogel, H.; Johnsson, K. *Nat. Biotechnol.* **2003**, *21*, 86–89. (d) Juillerat, A.; Gronemeyer, T.; Keppler, A.; Gendreizig, S.; Pick, H.; Vogel, H.; Johnsson, K. *Chem. Biol.* **2003**, *10*, 313–317. (e) Kindermann, M.; George, N.; Johnsson, N.; Johnsson, K. *J. Am. Chem. Soc.* **2003**, *125*, 7810–7811.
(6) (a) Boggon, T. J.; Eck, M. J. *Oncogene* **2004**, *23*, 7918–7927. (b) Nagar, B.; Hantschel, O.; Seeliger, M. A.; Davies, J. M.; Weis, W. I.; Superti-Furga, G.; Kuriyan, J. *Mol. Cell* **2006**, *21*, 787–798.
(7) (a) Feng, S.; Kasahara, C.; Rickles, R. J.; Schreiber, S. L. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 12408–12415. (b) Rickles, R. J.; Botfield, M. C.; Zhou, X. M.; Henry, P. A.; Brugge, J. S.; Zoller, M. J. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 10909–10913. (c) Zellefrow, C. D.; Griffiths, J. S.; Saha, S.; Hodges, A. M.; Goodman, J. L.; Paulk, J.; Kritzer, J. A.; Schepartz, A. *J. Am. Chem. Soc.* **2006**, *128*, 16506–16507.
(8) Pisabarro, M. T.; Serrano, L. *Biochemistry* **1996**, *35*, 10634–10640.
(9) (a) Perera, B. G. K.; Maly, D. J. *Mol. BioSyst.* **2008**, *4*, 542–550. (b) Plé, P. A.; Green, T. P.; Hennequin, L. F.; Curwen, J.; Fennell, M.; Allen, J.; Lambert-van der Brempt, C.; Costello, G. *J. Med. Chem.* **2004**, *47*, 871–887.
(10) See page S9 in the Supporting Information for a discussion of the Src-family kinase selectivity of AGT(PP1)-**4**.

JA900871Y