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Structural and Biochemical Basis for Intracellular Kinase Inhibition by Src-specific Peptidic Macrocycles

Graphical Abstract

Highlights

- Peptidic macrocycles selectively inhibit Src kinase
- Compounds block ATP and peptide binding site
- Compounds inhibit activity of Src kinase in cells
- Migration of breast tumor-derived cells is inhibited

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In Brief

Aleem et al. show the structural basis for the selective inhibition of c-Src kinase by a macrocycle with activity in cells. This demonstrates that synthetic macrocycles can be engineered to become potent inhibitors of intracellular enzymes.

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Structural and Biochemical Basis for Intracellular Kinase Inhibition by Src-specific Peptidic Macrocycles

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SUMMARY

Protein kinases are attractive therapeutic targets because their dysregulation underlies many diseases, including cancer. The high conservation of the kinase domain and the evolution of drug resistance, however, pose major challenges to the development of specific kinase inhibitors. We recently discovered selective Src kinase inhibitors from a DNA-templated macrocycle library. Here, we reveal the structural basis for how these inhibitors retain activity against a disease-relevant, drug-resistant kinase mutant, while maintaining Src specificity. We find that these macrocycles display a degree of modularity: two of their three variable groups interact with sites on the kinase that confer selectivity, while the third group interacts with the universally conserved catalytic lysine and thereby retains the ability to inhibit the “gatekeeper” kinase mutant. We also show that these macrocycles inhibit migration of MDA-MB-231 breast tumor cells. Our findings establish intracellular kinase inhibition by peptidic macrocycles, and inform the development of potent and specific kinase inhibitors.

INTRODUCTION

Selective inhibition of protein kinases is an effective clinical strategy for the treatment of diseases caused by aberrant kinase signaling (Cohen and Alessi, 2013; Levitzki, 2013). However, we lack selective small-molecule inhibitors for many disease-associated protein kinases, and the limited kinase selectivity of available inhibitors leads to dose-limiting off-target toxicity (Davis et al., 2011).

The Src family of protein tyrosine kinases (SFKs) consists of eight non-receptor tyrosine kinases that share high sequence homology, domain architecture, and regulation (Parsons and Parsons, 2004). SFKs regulate fundamental cellular processes such as cell migration, differentiation, growth, and survival (Parsons and Parsons, 2004). Src kinase, the prototypical SFK, is overexpressed or constitutively activated in many solid tumors types (Summy and Gallick, 2003; Yeatman, 2004), and inhibition of Src decreases metastasis and tumor growth in both cellular and animal cancer models. Therefore, Src is considered a pharmacological target for cancer therapy (Gargalionis et al., 2014; Krishnan et al., 2012; Nagaraj et al., 2011; Tang et al., 2011; Tsai et al., 2013; van Oosterwijk et al., 2013; Zhang and Yu, 2012). However, selective pharmacologic inhibition of Src kinase is challenging because the eight members of the Src kinase family are highly conserved, and few small-molecule kinase inhibitors can distinguish between them (Anastassiadis et al., 2011; Blake et al., 2000; Brandvold et al., 2012, 2015; Georgiou et al., 2012; Gushwa et al., 2012; Kwarcinski et al., 2012). Achieving specificity among different Src kinase family members is crucial because off-target inhibition can create significant clinical problems, such as immunosuppression and impaired T cell function through inhibition of the hematopoietic SFKs Lck and Hck (Lowell, 2004; Palacios and Weiss, 2004).

Most small-molecule kinase inhibitors were discovered in high-throughput screens and their optimization was guided by Lipinski’s “rule of five” (RO5) that describes features of some orally bioavailable drugs (Lipinski et al., 2001). More recently, compounds such as macrocycles that explore chemical space beyond RO5-compliant compounds have received attention as selective enzyme inhibitors (Driggers et al., 2008; Heinis, 2014; Villar et al., 2014). Macrocycles are typically larger and can possess more rotatable bonds than typical RO5 compounds. This plasticity is balanced by the conformational restriction through macrocyclization and allows macrocycles to adopt conformations that precisely complement a binding site (Villar et al., 2014). In addition, functional groups can be displayed from the macrocycle backbone and engage multiple interaction sites on the receptor independently. Macro cyclic kinase inhibitors therefore have the potential to be highly specific by exploiting multiple small differences in the structure and sequence of the conserved kinase domain.
Approximately 70 macrocycles are currently used in the clinic. Most of these are either natural products or natural product derivatives, such as the immunosuppressant cyclosporine A and the antibiotic vancomycin (Heinis, 2014). The challenges associated with synthesizing macrocycles and understanding their pharmacological properties has deterred studies of these compounds, and many questions concerning the molecular basis of macrocycle-target interactions remain (Driggers et al., 2008; Heinis, 2014; Rezai et al., 2006; Villar et al., 2014). In particular, surprisingly little structural information is available for macrocycle-protein complexes. A recent comprehensive study listed only 22 distinct X-ray cocrystal structures of macrocycles bound to proteins (Villar et al., 2014); in contrast, 3,000 structures of proteins (Villar et al., 2014); in contrast, 3,000 structures of protein kinase domains have been determined in complex with acyclic kinase inhibitors.

Recently, we discovered two families of Src-specific kinase inhibitors by in vitro selection of a 13,824-membered DNA-templated macrocyclic peptide library (Kleiner et al., 2010). The two families of Src-specific kinase inhibitors are classified as nitrophenylalanine macrocycles and how they achieve specificity for Src kinase. These structures provide important insights into the binding mode of the pyrazine macrocycles and how they achieve specificity for Src kinase. However, the structural basis of the pyrazine macrocycles’ ability to inhibit Src kinase as well as the activating “gatekeeper” mutant (Thr338Ile) of Src (Azam et al., 2008; Foda et al., 2015) while maintaining selectivity has remained unknown. Pharmacological inhibition of the Src gatekeeper mutant is of particular interest, since the analogous mutation in other kinases frequently underlies drug resistance in the clinic, and few kinase inhibitors are active against this mutation. Moreover, inhibitors that are active against the gatekeeper mutation typically show reduced kinase selectivity (Kwarcinski et al., 2016; Zhao et al., 2014).

Here we present the X-ray cocrystal structure of a nitrophenylalanine-based macrocycle with Src kinase. This structure reveals the molecular basis of Src kinase inhibition by the nitrophenylalanine macrocycles, and identifies a novel interaction between the nitrophenyl group and the catalytically essential and universally conserved Lys 295, thereby demonstrating how these compounds retain potency against Src containing the gatekeeper T338I mutation. We also demonstrate that the macrocycles retain activity against disease-related inhibitor-resistance mutations in the activation loop of the kinase. Finally, we show that the macrocycles inhibit Src-dependent cell migration in a breast tumor-derived cell line. Taken together, our findings establish that macrocycles can be effective and highly selective kinase inhibitors in mammalian cells and reveal the molecular basis of their activity against wild-type and mutant forms of Src kinase. Our findings inform the future development of macrocycles as research tools that selectively modulate Src-dependent cell signaling without inhibiting hematopoietic SFKs.

RESULTS

The Nitrophenylalanine Group of MC25b Forms Unique Interactions in the ATP Binding Pocket of Src Kinase

To elucidate the structural basis for the differences in potency, selectivity, and response to kinase mutations observed between the pyrazine (MC4) and nitrophenylalanine series of macrocycles (MC25) (Figure 1), we determined the X-ray crystal structure of MC25b bound to Src (Figures 2A and S1) and compared it with our previously reported crystal structure of the Src:MC4b complex (Georghiou et al., 2012). MC25 consists of a 19-atom macrocycle, an ornithine scaffold and a trans-olefin, while MC4 consists of an 18-atom macrocycle, a diaminobutyric acid scaffold, and cis-olefin (Figure 1). The site of DNA attachment in the DNA-templated library is a carboxamide in MC4a/MC25a and a carboxylate in MC4b/MC25b (Figure 1).

Despite their different A building blocks and different macrocyclic scaffolds, MC25b and MC4 bind to Src in similar conformations and occupy the same three binding pockets in the active site of Src (Figure 2C). The A, B, and C building blocks occupy the adenine binding pocket, a hydrophobic pocket underneath the β3-αC loop, and an amphipathic pocket near the DFG motif, respectively (Figures 2A and 2B). The peptidic macrocycle backbone form almost identical hydrogen bonding patterns with the
kinase. MC25b forms five direct hydrogen bonds with the peptide backbone of the kinase, and one water-mediated hydrogen bond (Figure 2D) compared with the five direct and four water-mediated hydrogen bonds between MC4b and Src. Four of the five hydrogen bonds between the macrocycles and Src are identical. The water-mediated hydrogen bond between MC4b and Met341 is replaced by a water-mediated hydrogen between MC25b and Arg388. The importance of the backbone interactions in MC25b is highlighted by the loss of potency seen in several of the N-methylated amide variants of MC25 (MC28–31; Table S2) (Georghiou et al., 2012). This loss is most likely due to changes in backbone conformations caused by the methyl groups, since only one of the hydrogen bonds is made by an amide in MC25b, while six of the hydrogen bonds are formed by carbonyl groups, including the C-terminal carboxylic acid. We speculate that N-methylation results in the formation of cis peptide bonds instead of trans peptide bonds (Chatterjee et al., 2008). This altered conformation may impair hydrogen bonding of macrocycle backbone carbonyls or interactions of macrocycle side chains with their respective binding pockets (Chatterjee et al., 2008; Georghiou et al., 2012).

The key difference in the interaction between MC4 and MC25 with Src occurs in the A building-block binding site. The nitrophenylalanine occupies the adenine binding pocket, but does not mimic the adenine hydrogen bonding pattern observed for MC4 and most ATP-competitive kinase inhibitors (Krishnamurty and Maly, 2010). When compared with the positioning of the pyrazine group in MC4b, the nitrophenylalanine building block does not bind as deeply into the pocket (Figure 2C). Instead, the phenyl ring of this group occupies a hydrophobic space formed by the side chains of Leu273, Val281, Ser345, and Leu393.

The remaining interactions between the variable groups in the B and C positions of the macrocycle are very similar between the pyrazine family (MC4) and the nitrophenylalanine family (MC25). The fluorophenylalanine in position B occupies the same hydrophobic pocket underneath the β3–αC loop as the phenylalanine in MC4b. However, MC25b binds 1.1 Å deeper into this hydrophobic pocket. Consistent with the structure, replacement of fluorophenylalanine (half maximal inhibitory concentration [IC50] = 0.004 μM) with tyrosine (IC50 = 0.79 μM) or methylphenylalanine (IC50 = 0.37 μM) resulted in a 100- to 200-fold loss of potency.
static interactions across 4.6 Å with the nitrophenylalanine of favorable van der Waals interactions and longer-range electrostatic interactions (Lys295) of Src is a key residue for MC25 binding, contributing to conserved catalytic lysine.

The Nitrophenylalanine Interacts with the Universally Conserved Catalytic Lysine

The crystal structure of MC25b suggests that the catalytic lysine (Lys295) of Src is a key residue for MC25 binding, contributing favorable van der Waals interactions and longer-range electrostatic interactions across 4.6 Å with the nitrophenylalanine of MC25 (Figures 2D and S2). These electrostatic interactions between the negatively charged oxygen of the zwitterionic nitro group and Lys295 would not be expected if the nitrophenyl group was replaced with pyrazine, as in MC4. Furthermore, the importance of this interaction is supported by the observed loss in potency when the nitrophenylalanine group was substituted with other functionalities (in MC17–MC23) (Table S2). Only the cyanophenylalanine containing MC21 retained its potency for Src, possibly because it is capable of forming an interaction between a negative partial charge at the cyano nitrogen and the positive charge of Lys295.

To probe the putative electrostatic interaction between the nitrophenyl group found in MC25b and the catalytic lysine (Lys295) of Src, we used two macrocycles that were fluorescein labeled at the A position at 0 mM NaCl (low salt) or 500 mM NaCl (high salt). Experiments were performed in triplicate. Error bars represent mean values ± SD. (B) $K_d$ values from (A) are plotted. Error bars represent mean values ± SD.

MC25 and MC4 Have Differential Activity against Drug-Resistance Mutations

Tyrosine kinases (such as Abi kinase) commonly present mutations in the clinic that confer drug resistance. The gatekeeper residue (Abi Thr315, Src Thr338) is the most common clinical imatinib-resistance mutation, followed by mutations in the P loop and the activation loop (Bikker et al., 2009). Replacement of the gatekeeper residue with a hydrophobic residue (Abi T315I, Src T338I/M, EGFR T790M) activates the kinase (Azam et al., 2008; Foda et al., 2015). A single nucleotide exchange in the human gene for Src kinase results in the T338M mutation, while the T338I mutation is present in approximately half of all chicken Rous sarcoma strains of viral Src kinase. Since Src is currently not a primary target of clinical kinase inhibitors, no clinical resistance mutations have emerged.

The crystal structure of Src-MC25b presented here demonstrates why the nitrophenylalanine family of macrocycles is active against the T338I mutation (inhibition constant $K_i = 0.016 \mu M$) while the pyrazine family is not (Table S2) (Georgiou et al., 2012). The distance between pyrazine and the Thr338 side chain is only 4 Å (Figure 2C). Replacement of threonine with the larger isoleucine side chain would clash with the pyrazine (Georgiou et al., 2012). In contrast, the distance between the nitrophenyl MC25b and Thr338 is 6.7 Å (Figure 2C), and the T338I mutation is not predicted to cause steric clashes (Figure S3). Introduction of the T338M mutation causes resistance to MC25b ($K_i = 5.3 \mu M$) (Table S3), and the mutation is predicted to reduce potency for Src, since the C terminus was linked to DNA during the macrocycle library selection and our macrocycle Src structures indicate that the C terminus is solvent exposed. We measured how the ionic strength of the solvent and mutation of the catalytic Lys295 affected binding affinity using fluorescence anisotropy. We found that high salt concentrations lowered the affinity of the nitrophenylalanine compound (fluorescein-MC9) to Src by 4-fold (Figures 3A and 3B). When the catalytic lysine was mutated to methionine (K295M), the binding affinity for the nitrophenylalanine compound decreased more than 100-fold and became independent of ionic strength (Figures 3A and 3B). In contrast, there were no significant differences in the binding affinities of the pyrazine compound (fluorescein-MC2) toward Src, or the K295M mutant, at low or high salt concentrations (Figures 3A and 3B). Taken together, these results suggest that the nitrophenyl group of MC25 forms an ionic interaction with the catalytic lysine (Lys295) of Src kinase, whereas the pyrazine-based MC4 does not. Since Lys295 is essential for kinase activity, resistance mutations at this position that disrupt this ionic interaction are unlikely.

The cyclohexylalanine at position C occupies an amphipathic pocket in both structures of MC4 and MC25, which is generated by the outward rotation of helix aC into the Cdk/Src-like inactive conformation.

Drug-Resistance Mutations

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Figure 4. A Bcr-Abl Activation-Loop Inhibitor-Resistance Mutation Modeled in Src(R419P) Results in a Loss of Potency for MC25b
(A) Inhibitory potency of macrocycles in biochemical kinase assay. Experiments were performed in triplicate, and data represent mean values ± SD. (B) Destabilization of Src/Cdk-like inactive conformation results in loss of macrocycle potency. Src autophosphorylation (pTyr-416) or the L407G mutation cause a loss of potency for both MC4b and MC25b. Experiments were performed in triplicate, and data represent mean values ± SD.

Both Classes of Macrocycles Bind Selectively to the Src-like Inactive Conformation

The crystal structures of MC4 and MC25 both demonstrate that the macrocyclic inhibitors bind to the Src/Cdk-like inactive conformation of Src. In particular, the binding pocket for the B group underneath the β3-αC loop is enlarged by the outward rotation of helix αC in the Src/Cdk-like inactive conformation. To verify that the macrocycles are selective for the inactive conformation, we tested their potency against the autophosphorylated form of Src that is locked in the active conformation. We found that neither MC4b nor MC25b could inhibit Src when the kinase was autophosphorylated (IC50 values >20 μM) (Figure 4B). We also tested the potency of MC4b and MC25b against a mutant form of Src (L407G) that is proposed to destabilize the Src/Cdk-like inactive conformation (Seeliger et al., 2007). The L407G mutation decreased the potency of MC4 and MC25b by 200-fold and 2,000-fold, respectively (Figure 4B).

These results emphasize that the macrocycle Src inhibitors have a strong preference for a specific inactive kinase conformation, and that mutations or posttranslational modifications that destabilize this conformation reduce the activity of the inhibitor.

MC25 Inhibits Migration of Breast Cancer Cells

Our structural data explain how the macrocycles achieve potency, kinase selectivity, and activity against certain mutations while recognizing the Src/Cdk-like inactive conformation. We next investigated how the in vitro inhibition data would translate to the cellular context. We previously showed that high micromolar concentrations of MC25a decreased global tyrosine phosphorylation in NIH 3T3 Src−/− cells overexpressing a constitutively activated form of Src (Y529F) (Georgiou et al., 2012). While the modest cellular potency of MC25a against the constitutively active Src Y529F mutant may be a result of limited membrane permeability and cellular uptake, as has been observed for other macrocycles (Bockus et al., 2015), we also speculated that the Y529F mutation increases intracellular autophosphorylation of Src on Tyr416 (chicken c-Src numbering) (Hunter, 1987), locking the kinase in the active conformation and disfavoring macrocycle binding. Indeed, our biochemical data indicate that MC4 and MC25a were more than 1,000-fold less potent against autophosphorylated Src kinase (Figure 4B). We therefore tested compound potency against endogenous levels of Src in breast tumor-derived MDA-MB-231 cells. To quantify the effect of inhibitor of cellular kinase activity, we treated cells with inhibitor or vehicle control for 24 hr, washed the cells, and immunopurified Src from cells. Kinase activity of the Src-MC25 complexes was quantified in radioactive kinase assays. MC25a decreased the activity of Src from MDA-MB-231 in a dose-dependent manner with an IC50 of 0.06 μM (Figure 5A). Dasatinib as a control at 1 μM inhibited Src kinase activity completely. The levels of immunopurified Src were unaffected by inhibitor concentration (Figure 5B).

MDA-MB-231 cells depend upon Src activity for migration, which is necessary for metastasis (Sanchez-Bailon et al., 2012,
Treatment of cells with 20 μM MC25 decreased the rate of cell migration and the closure of an artificial wound. After 17.5 hr of treatment with MC25 (or 45 hr, when cells had been serum starved), the remaining wound size doubled compared with vehicle control (Figures 5C and 5D). Proliferation of MDA-MB-231 cells was not affected by MC25 as observed previously for other Src inhibitors (Tsai et al., 2013). These results show that the macrocycle Src inhibitors are active in cells and can be used to inhibit a metastasis-related phenotype in a breast tumor-derived cell line.

**DISCUSSION**

Previously we reported two potent and unusually selective macrocyclic inhibitors of Src kinase, MC4 and MC25, and elucidated the structural and biochemical basis of Src-specific inhibition by MC4 (Georghiou et al., 2012). While these two macrocycles share structural similarities, MC25 demonstrated several unique features over MC4 including cellular efficacy, insensitivity to the activating and drug-resistant T338I gatekeeper mutation, and a higher degree of selectivity for Src kinase. Here we have characterized the structural basis of Src kinase inhibition by MC25. Our structural data indicate that this compound makes unique interactions in the adenine binding pocket that enable retention of activity against T338I Src without compromising inhibitor specificity. Furthermore, we demonstrate that MC25 inhibits Src-dependent cell migration in a metastatic breast tumor-derived cell line. These studies will be useful for developing the next generation of macrocyclic kinase inhibitors.

Comparison of the structures of pyrazine-based MC4 and nitrophenylalanine-based MC25 bound to Src kinase allowed us to rationalize their Src selectivity and differential activity against drug-resistant kinase mutations. We hypothesize that the excellent kinase selectivity of these inhibitors is tied to their preference for the inactive kinase conformation as well as the engagement of multiple binding sites. Kinase inhibitors that target a distinct kinase conformation are often more selective, since the relevant conformational states may be more accessible to the target kinase or may reveal target-specific structural features exploited by the inhibitor (Muller et al., 2015; Tong and Seeliger, 2015; Wang et al., 2014). For example, the highly selective kinase inhibitors imatinib and lapatinib bind to their targets in distinct inactive kinase conformations. However, inhibition of specific kinase conformations can also represent an Achilles heel for inhibitors, as destabilization of the targeted conformation can render the inhibitor inactive. The macrocycles presented here and the Abl kinase inhibitor, imatinib, target specific inactive conformations that are less accessible upon phosphorylation of the kinase activation loop (Schindler et al., 2000). Consequently, phosphorylation of Abl kinase domain increases the dissociation constant (Kd) for imatinib 20-fold (Davis et al., 2011) and the enzymatic Ki 100-fold (Seeliger et al., 2007). Despite this limitation, imatinib is highly successful in the clinic, likely because activation-loop phosphorylation is in a dynamic equilibrium mediated by phosphatases. Other examples of clinically successful kinase inhibitors that lose potency against their activated kinase target include the vascular endothelial growth factor receptor inhibitor axitinib (50-fold increase in Ki) (Solowiej et al., 2009) and the c-Kit inhibitor sunitinib (300-fold increase in IC50) (Di Nitto et al., 2010). The clinical success of imatinib, axitinib, and sunitinib indicates that resistance of activation-loop phosphorylated kinases to inhibitors can be tolerated and should not diminish.

![Figure 5. MC25a Inhibits Cell Migration in a Metastatic Breast Cancer Cell Line](image-url)
the potential of the macrocycles from being useful kinase inhibitors.

MC25b buries almost 70% more solvent-accessible surface area on the kinase (537 Å²) than dasatinib (316 Å²). Thus, the larger size of MC25b compared with dasatinib may explain the ability to connect multiple distinct binding sites and to spread the binding energy over multiple and potentially lower-affinity interactions.

Our macrocycle Src structures show that the main differences between MC25 and MC4 interactions with Src occur in the adenine binding site and the kinase activation loop. These two regions are highly conserved among tyrosine kinases and are of particular interest in the clinic as they are frequent sites of drug resistance to small-molecule inhibitors. We find that while MC25 extends toward the activation loop, the smaller scaffold of MC4 does not extend into this region and MC4 binding is therefore insensitive to the R419P mutation, which is analogous to the clinically relevant H396P mutation in c-Abl that confers resistance to imatinib in chronic myeloid leukemia patients. Conversely, while MC4 inserts deep into the adenine binding pocket and forms an adenine-like hydrogen bond with Thr338, MC25 does not. Rather, our structural and biochemical data indicate that MC25 resides 6.7 Å from Thr338 and that its affinity for Src derives from additional electrostatic interaction between the nitrophenyl group and catalytic Lys295, thereby explaining how MC25 binding is not perturbed by the T338I mutation. This shallow positioning in the nucleotide binding site and interaction with Lys295 are unusual, as most small-molecule kinase inhibitors make a characteristic set of hydrophobic and hydrogen bond interactions that mimic the adenine ring of ATP (Krishnamurty and Maly, 2010). Exceptions are inhibitors such as the 5'-fluorosulfonylbenzoate derivatives of the pan-SFK inhibitor PP1 that covalently attach to the catalytic lysine (Gushwa et al., 2012).

Notably, first- and second-generation small-molecule inhibitors of c-Abl that are used in the clinic, such as imatinib and dasatinib, respectively, form hydrogen bonds with Thr315 (the analogous gatekeeper residue in c-Abl) and are inactive against c-Abl containing a T315I mutation, the most common drug-resistant mutation occurring after imatinib treatment (Soverini et al., 2006). While c-Abl inhibitors that can inhibit this mutant protein (e.g., ponatinib) have been described, these compounds typically have compromised target specificity (Liu et al., 2012). In this regard, MC25 may serve as a promising template for the development of future Abi/Src kinase inhibitors, since it is both highly selective and insensitive to mutation of the gatekeeper residue. Moreover, we predict that interactions with Lys295 (or the analogous Lys290 in c-Abl), a residue essential for catalysis, are less likely to be perturbed by resistance mutations. Overall, the structural basis for the inhibition of wild-type Src kinase and c-Abl-related drug-resistant mutants by these two families of macrocycles provides a strategy to develop specific and potent macrocyclic kinase inhibitors that would be insensitive to mutations known to arise in the clinic. Such an inhibitor would benefit from an aromatic group with polar functionality in the A position to form long-distance interactions with Lys295 while maintaining distance from Thr338, and a smaller MC4-based macrocycle backbone that would be insensitive to mutations in the activation loop. We speculate that such a compound would retain selectivity due to the B- and C-position building blocks.

Finally, we show that MC25 inhibits endogenous Src activity in the MDA-MB-231 breast tumor cell line (Tang et al., 2011; Tsai et al., 2013) and that Src inhibition in MDA-MB-231 cells correlates with a reduction of cell migration, a disease-relevant phenotype of this metastatic breast tumor-derived cell line that is known to depend on Src activity. The observation that MC25 inhibits migration of MDA-MB-231 cells, which depends on Src activity but does not inhibit their proliferation, which depends on Abi activity (Srinivasan and Plattner, 2006; Srinivasan et al., 2008), can be attributed to MC25’s excellent selectivity for Src over Abi kinase.

Metastasis is a marker of advanced stage disease clinically, and represents an important target for drug inhibition. Notably, MC25 inhibited wound healing at concentrations not expected to inhibit the hematopoietic kinases Hck and Lck. At present, therapeutic strategies to inhibit Src are limited by off-target effects that cause immunosuppression and inhibitor toxicity (Levitzki, 2013). Therefore, the biological potency and selectivity of MC25 may provide a tool to help evaluate Src-dependent signaling in tumor cell lines.

**SIGNIFICANCE**

Protein kinases are involved in virtually all cellular signaling pathways, and their dysregulation underlies many diseases. Kinases are therefore attractive drug targets if specific and potent inhibition can be achieved. The high level of conservation of structure and sequence within the catalytic kinase domain complicates the development of specific kinase inhibitors. In addition, pharmacologic kinase inhibition is made challenging by drug-resistant mutations in the target that arise after treatment. All small-molecule kinase inhibitors currently in clinical use bind to the conserved ATP binding site in the protein kinase domain. We have previously identified a series of peptidic macrocycles that are unusually specific for Src kinase over other related Src family kinases, and interfere both with ATP and peptide substrate binding. Here we characterize the structural and biochemical mechanisms of Src kinase inhibition by one such compound, MC25, which retains activity against a common disease-relevant inhibitor-resistance mutation at the so-called gatekeeper residue, T338I. Our study reveals a unique mode of Src kinase inhibition and provides a mechanistic rational for MC25’s unusual kinase selectivity as well as activity against T338I Src. In addition, we show that this compound is active in cells. Many natural product-derived drugs are macrocycles, suggesting that the macrocyclic structure imbues these molecules, which lie outside conventional drug-like chemical space, with favorable properties such as high specificity and bioavailability. However, the design of synthetic macrocycles has been hampered by a paucity of structural data and lack of macrocycles with cellular activity. MC25 could serve as a tool compound informing the future development of kinase inhibitors active against Src kinase containing mutations at the gatekeeper residue, including the T338M mutation. Furthermore, the compounds
presented here could motivate future systematic studies to derive empirical rules for bioactive macrocycles.

**EXPERIMENTAL PROCEDURES**

**Constructs and Mutagenesis**

Kinase domain constructs of chicken c-Src (residues 251–533) were purified as previously described (Seelig et al., 2005, 2007). Mutations were introduced into Src kinase domain (K295M, L407G, and R419P) by site-directed mutagenesis and were verified by DNA sequencing.

**Protein Crystalization**

The complex between MC25b (500 μM) and c-Src kinase domain (200 μM) was formed in 20 mM Tris (pH 8.0), 125 mM NaCl, 2.5% DMSO, and 2.5% glycerol. Src-MC25b crystallized in a hanging-drop format at room temperature when mixed in a 1:1 ratio with mother liquor consisting of 14% polyethylene glycol 5000 monomethyl ether and 0.3 M NaH2PO4. Crystals were cryoprotected in 5000 monomethyl ether and 0.3 M NaH2PO4. Crystals were cryoprotected in 20 mM Tris (pH 8.0), 125 mM NaCl, 2.5% DMSO, and 2.5% glycerol.

**Immunoprecipitation and Radioactive Kinase Assay**

liger et al., 2007).

**Data Collection and Processing**

X-ray diffraction data were collected on beamline X29 at Brookhaven National Laboratory at 100 K using a wavelength of 1.075 Å. Phases were obtained by molecular replacement using the kinase domain of c-Src (PDB: 3U4W) (Georgiou et al., 2013) in Phaser (McCoy et al., 2007). The diffraction data were strongly anisotropic, and an anisotropic correction was carried out using the Anisotropy Diffraction Server (Strong et al., 2006). These anisotropically scaled data were then refined in Phenix (Adams et al., 2002). The model was built in Coot. See Supplemental Experimental Procedures for further details and refinement statistics.

**Fluorescence Anisotropy**

The change in fluorescence anisotropy of fluorescein-labeled MC2 and MC9 at 518 nm upon excitation at 492 nm was monitored with a Jobin Yvon FluoroMax-4 spectrorfluorometer (Horiba). Src kinase domain (residues 251–533) was titrated to 0.5 μM of the fluorescein-labeled macrocycle, in 100 mM Tris (pH 8.0), 10 mM MgCl2, at 25°C. For the salt titrations, 500 mM NaCl was added to the buffer prior to the addition of kinase. After equilibration, the increase in anisotropy of the fluorescently labeled ligand was recorded and fitted against a quadratic binding equation utilizing the GraphPad Prism software to yield the KD.

**In Vitro Autophosphorylation of Src Kinase Domain**

Src autophosphorylation experiments were performed by incubating 10 μM Src kinase domain with 1 mM ATP in buffer containing 20 mM Tris (pH 8.0) and 10 mM MgCl2 for 2 hr at 25°C.

**Kinase Assays**

For Src inhibition assays (Barker et al., 1995), 300 μM of an Src-optimal substrate peptide (AEEYEGFAKKK) (Songyang et al., 1999) was combined with 250 μM ATP. The concentrations of kinase used in these assays were as follows: 0.0125 μM Src kinase domain, 0.025 μM R419P, 0.025 L407G. Titrations of MC4b and MC25b (ranging from 0 to 83.3 μM) were performed at 30°C as described previously for imatinib to determine the IC50 and the Kd (See liger et al., 2007).

**Immunoprecipitation and Radioactive Kinase Assay**

MDA-MB-231 cells were grown to 90% confluence and treated with dasatinib, MC25a, or DMSO vehicle. Cells were harvested 24 hr following drug treatment, washed once with PBS, and lysed with RIPA lysis buffer. Lysates were cleared MC25a, or DMSO vehicle. Cells were harvested 24 hr following drug treatment, washed once with PBS, and lysed with RIPA lysis buffer. Lysates were cleared

**Wound-Healing Assay**

MDA-MB-231 cells were grown to confluence in 12-well plates in DMEM/ Ham’s F12 (50:50) supplemented with 10% fetal bovine serum at 37°C in 5% CO2. In some experiments, the cells were then serum starved for 12 hr as indicated. Multiple wounds were generated in each well by scratching with a sterile P200 pipette tip. The cells were then washed twice with PBS, and treated with complete media supplemented with DMSO vehicle control or 20 μM MC25a. Each condition was repeated in triplicate. Photographs were taken per well at four different wound locations and at different time points. The percentage of wound closure was calculated with ImageJ by measuring the area unoccupied by cells at each time point, and normalizing to the zero time point.

**ACCESSION NUMBERS**

Atomic coordinates and structure factors for Src-MC25b have been deposited in the PDB with accession code PDB: 5BMM.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, four figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2016.07.017.

**AUTHOR CONTRIBUTIONS**

B.P.C., S.U.A., and W.T.M. performed all the cellular experiments. S.U.A. performed the fluorescence anisotropy binding experiments. G.G., K.E.G., and S.U.A. determined the structure of the Src-MC25b complex. G.G. performed the in vitro kinase assays. R.E.K., A.I.C., and D.R.L. developed and synthesized all the macrocyclic inhibitors utilized in this work. M.A.S. contributed to the design of all the experiments and directed the study. All authors contributed to the writing of the manuscript.

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Irreversible inhibitors of c-Src kinase that target a nonconserved cysteine.


