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JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

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Examining the Mechanism of Action of a Kinesin Inhibitor Using Stable Isotope Labeled Inhibitors for Cross-Linking (SILIC)

Sarah A. Wacker, Sudhir Kashyap, Xiang Li,* and Tarun M. Kapoor*

Laboratory of Chemistry and Cell Biology, The Rockefeller University, New York, New York, 10065

Supporting Information

ABSTRACT: It is difficult to determine a chemical inhibitor's binding site in multiprotein mixtures, particularly when high-resolution structural studies are not straightforward. Building upon previous research involving photo-cross-linking and the use of mixtures of stable isotopes, we report a method, **S**table **I**sotope **L**abeled **I**nhibitors for **C**ross-linking (SILIC), for mapping a small molecule inhibitor's binding site in its target protein. In SILIC, structure−activity relationship data is used to design inhibitor analogues that incorporate a photo-cross-linking group along with either natural or 'heavy' stable isotopes. An equimolar mixture of these inhibitor analogues is cross-linked to the target protein to yield a robust signature for identifying inhibitor-modified peptide fragments in complex mass spectrometry data. As a proof of concept, we applied this approach to an ATP-competitive inhibitor of kinesin-5, a widely conserved motor protein required for cell division and an anticancer drug target. This analysis, along with mutagenesis studies, suggests that the inhibitor binds at an allosteric site in the motor protein.

Bioactive small molecules can be important chemotherapeutic drugs as well as valuable tools to elucidate the cellular functions of their target proteins.[1,2] In both contexts, the value of the small molecule can be limited by a lack of understanding of its mechanism of inhibition and mode of target protein binding. Without these data, it can be difficult to improve potency, evaluate specificity, and fully explain cellular phenotypes resulting from drug treatment. A precise understanding of how a bioactive molecule interacts with its target can address these issues,[3] but in many cases a crystal of the drug bound to the protein is difficult to obtain. Moreover, when the small molecule's target is part of a multiprotein complex, analyzing the mechanism of inhibition using structural approaches can be challenging.

Photo-cross-linking of small molecules to proteins has been used to trap drug−target protein interactions in complex protein mixtures.[4−6] Identifying drug targets and mapping drug binding sites after photo-cross-linking typically relies on systematic mass spectrometry-based analyses of digested protein fragments to identify those with a small-molecule adduct.[7,8] While there are examples of the successful use of this approach, the general applicability of the method has been limited as cross-linking is often substoichiometric[9] and the different possible inhibitor−peptide adducts can be difficult to detect in complex mass spectra. One strategy to address this involves generating inhibitor analogues with an affinity tag for capturing the inhibitor−peptide adducts.[10]

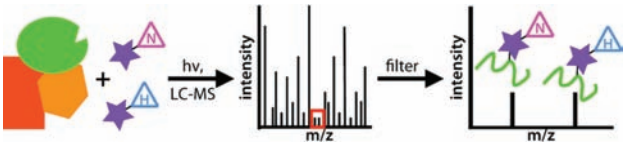

Figure 1. Schematic for SILIC. Inhibitors, with a photo-cross-linking substituent and natural (N) or heavy (H) isotopes, are mixed with a complex of proteins, including the target (green). After UV-cross-linking, protein digestion, and LC−MS one obtains complex mass spectra that can be filtered based upon a 'signature' of peaks with the expected mass difference and equal signal intensities.

In many cases, however, the inhibitor's dual modifications, for photo-cross-linking and affinity-capture, can alter the compound's mechanism of action. As an alternative approach to identify inhibitor−protein adducts within complex mass spectra, inhibitor analogues can be generated such that they carry a unique isotope pattern.[11,12] The incorporation of natural and 'heavy' stable isotopes into a benzophenone photo-cross-linker moiety appended to the inhibitor of interest has been shown to aid the identification of its target in a proof of concept study.[13] However, the method is not likely to be useful for mapping an inhibitor's binding site. This is, in large part, due to the cross-linking group being incorporated via a linker, so that it is a significant distance from the functional groups that are likely to make key contacts with the target's binding site.

Here, building on these studies, we have developed a method, named stable isotope labeled inhibitors for cross-linking (SILIC), for mapping small-molecule−protein binding sites. In the first step of this approach we incorporate a photo-cross-linking group (e.g., azide) into the inhibitor of interest (Figure 1), guided by available structure−activity relationship (SAR) data. The photo-cross-linking group is appended at a site that does not change the inhibitor's mechanism of action, but is in the closest proximity possible to the inhibitor's activity-conferring functionality, so as to increase the probability that cross-links are at, or near, the protein's inhibitor-binding pocket. Natural and heavy isotope inhibitor analogues, which have a mass difference of a few daltons but otherwise identical physical properties, are then generated. The multiprotein complex to be analyzed is then incubated with a 1:1 mixture of natural and heavy inhibitor. After photo-cross-linking and protein digestion, the resulting mixture of peptide fragments is separated by HPLC and analyzed using high-resolution mass spectrometry. The resulting mass spectra can comprise thousands of peaks, and the peptide−inhibitor adduct

Received: May 18, 2011
Published: July 15, 2011

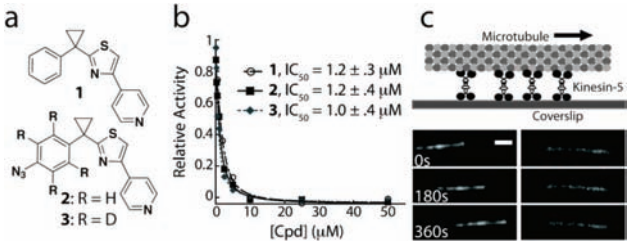

Figure 2. (a) Chemical structure of an ATP-competitive kinesin-5 inhibitor and analogues generated for SILIC. (b) Potency of compounds **1**, **2**, and **3** in inhibiting kinesin-5 activity, as examined using a steady-state microtubule-stimulated ATP hydrolysis assay. (c) In the presence of MgATP (1 mM) and DMSO, homotetrameric kinesin-5 drives microtubule gliding at an average rate of 19.6 ± 4.5 nm/s (left panel, $n > 25$). **1** (50 nM) inhibits this activity (right panel, $n > 30$). Scale bar is 2 μm.

is likely to be of low abundance due to substoichiometric labeling. The peptide−inhibitor adduct is identified when a pair of peptides that coelute in the LC have the expected mass difference and essentially equal signal intensity. Finally, guided by these data, site-directed mutagenesis experiments can be designed to further examine the inhibitor-binding sites identified by SILIC.

As a proof of concept, we focused on compound **1**, an inhibitor of kinesin-5 (Figure 2a).[14] Kinesins, which comprise a family of over 40 proteins, are motor proteins that move cargo along microtubules, polymers of the cytoskeletal protein tubulin.[15,16] The kinesin-5 family is required for the assembly of the microtubule-based apparatus necessary for cell division.[17] Inhibitors of kinesin-5 have provided valuable insight into mechanisms of cell division and have entered clinical trials as anticancer drugs.[18,19] Kinesin-5 inhibitors that are in clinical trials, and have been used for cytological experiments, bind an allosteric site not conserved in other kinesins.[20,21] These inhibitors are not competitive with respect to ATP.[22] Recently, ATP-competitive inhibitors of kinesin-5 have been reported, including compound **1**.[14,23] As the ATP-binding site is the most conserved feature in kinesins, the possibility arises that these inhibitors may provide valuable starting points for developing new inhibitors for other kinesins. However, the binding site of **1** in kinesin-5 is not known, and structural data have been difficult to obtain.[14]

To map the binding site of **1**, we first analyzed its mechanism of action. We find that **1** inhibits steady state ATP hydrolysis by human kinesin-5′s ATPase domain (residues 1−368, expressed in bacteria) 25-times more potently when microtubules, the motor protein's tracks, are present in the reaction (IC_{50}: **1** + microtubules + kinesin-5 = 1.2 ± 0.3 μM; **1** + kinesin-5 = 30 ± 7 μM; Figure 2b, Table S1). We next examined inhibition of kinesin-5 driven microtubule gliding. These assays require protein constructs that are larger than those consisting of the monomeric ATPase domain. We generated full-length homotetrameric kinesin-5 (*Xenopus laevis*, expressed in insect cells as published previously[24]) and found that it drives microtubule gliding at 19.6 ± 4.5 nm/s (1 mM MgATP). Remarkably, even at 50 nM of **1**, the motor activity was completely inhibited (Figure 2c), suggesting that tightly bound motor protein−microtubule complexes are formed in the presence of **1** and these complexes act as 'brakes' against other active motor protein molecules to stop microtubule motion.[25] Together, these ATPase and microtubule gliding assay data suggest that the binding mode of **1** to kinesin-5 should be examined in the presence of microtubules. Analyzing the inhibitor−kinesin interaction in this context provided an interesting case for developing and applying our approach.

Guided by available SAR data,[14] we designed analogues of **1**, which contain an azide substituent on the phenyl ring as a photoreactive group and have natural (hydrogen, H; compound **2**) and heavy (deuterium, D; compound **3**) isotopes (Figure 2a). Synthesis of compound **2** was based on the published procedure for **1**.[14] To incorporate deuterium atoms that generate a 4 Da mass difference compared with **2**, toluene-d_8 was used as a starting material to synthesize the substituted phenyl ring moiety in **3** (see Supporting Information). Notably, the introduction of the cross-linking group in **1** did not affect the potency of the compounds and, as expected, the natural and heavy analogues have similar activities against kinesin-5 (Figure 2b, IC_{50}: **2** = 1.2 ± 0.4 μM; **3** = 1.0 ± 0.4 μM). A 1:1 mixture of **2** and **3** (3 μM each) was incubated with kinesin-5 ATPase domain and microtubules. After UV irradiation at 254 nm for 30 min, the reaction mixture was resolved by SDS-PAGE. Following in-gel digestion, the peptide mixture was separated and analyzed by LC−MS/MS. Computer-based analysis (MaxQuant[26]) was used to efficiently detect peaks with a mass difference of 4 Da that eluted at the same time from the LC column. In independent experiments ($n = 3$), we found equal intensity peaks corresponding to a single peptide (Figure 3a), indicating that this was likely to be the major inhibitor−peptide adduct. Further analysis by MS/MS identified this peptide as a fragment corresponding to Ser^{120}−Arg^{138} of kinesin-5 and the cross-linking site of the inhibitors is very likely at one of three amino acid residues (Tyr125, Thr126, or Trp127) (Figure 3b).

We next examined whether excess **1** can suppress the cross-linking of **2** (or **3**) to kinesin-5. For this experiment, we cross-linked kinesin-5 with **2** (3 μM) in the presence of excess **1** (45 μM) and, in parallel, cross-linked kinesin-5 with **3** (3 μM) alone. These samples were then mixed and processed for mass spectrometry analysis. This protocol provided a quantitative readout of the competition, as only one set of peaks in the mass spectrum should be suppressed. As shown in Figure 3c, **1** competes with **2**, suggesting that **1** also binds at a site proximal to residues Tyr125, Thr126, and Trp127.

In the three-dimensional structure of the kinesin-5 ATPase domain (Figure 4a, PDB: 2WOG[27]), residues Y125, T126, and W127 map to a portion of loop-5. This loop is part of the allosteric binding site of other kinesin-5 inhibitors,[21,27] such as S-Trityl-L-cyteine (STLC, IC_{50} = 1 μM, steady-state ATPase assay without microtubules[28]). As these inhibitors bind this pocket in the absence of microtubules, we examined if **1** did the same. To test this, we repeated the cross-linking and competition experiments in the absence of microtubules and found the same single cross-linking site (Figure S1). Furthermore, we found that addition of excess STLC (50 μM) prevented cross-linking of **2** (5 μM) to kinesin-5, relative to the reference sample (kinesin-5 cross-linked to **3**, Figure 4b). Together, these data suggest that **1**, like STLC, binds in an allosteric site in kinesin-5.

We next used site-directed mutagenesis to analyze whether inhibition of kinesin-5 by **1** was sensitive to changes in the allosteric pocket. A leucine-214 to alanine (L214A) mutation, in this allosteric binding site of kinesin-5, is known to suppress STLC inhibition (Figure 4a).[29] Using the steady-state ATP hydrolysis assay, both with and without microtubules, we found that this mutation led to a 10-fold increase in the potency of **1** (Figure 4c, d, Table S1). Importantly, cross-linking of **2** and **3** to kinesin-5, which had the L214A mutation, resulted in identification of the same binding site (Figure S2). In addition, we found that **1** is an ATP-competitive

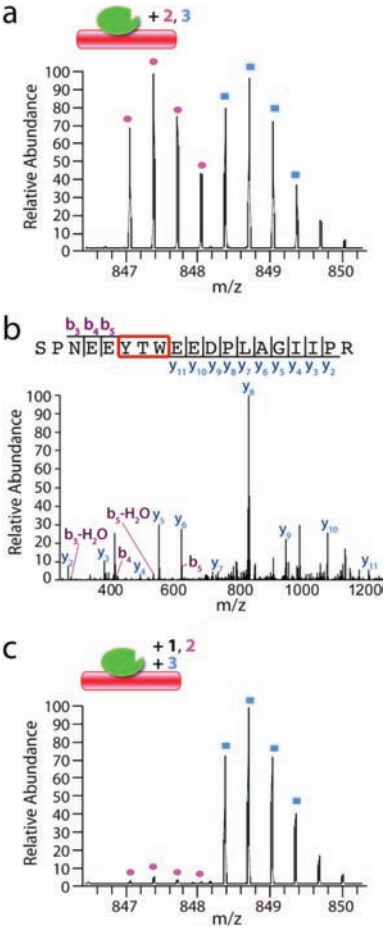

Figure 3. (a) A representative mass spectrum of the 'signature' peaks that denote peptide fragments of kinesin-5 cross-linked by equimolar **2** (pink circles) and **3** (blue squares) in the presence of microtubules. (b) The MS/MS spectrum and summary of fragmented ions of the cross-linked peptide. (c) When **2** (3 μM) is cross-linked in the presence of **1** (45 μM), there is near complete loss of cross-linked peptide, relative to that observed in the presence of **3** (3 μM) alone. Cartoon shows kinesin-5 (green) and microtubules (red) with cross-linking compounds.

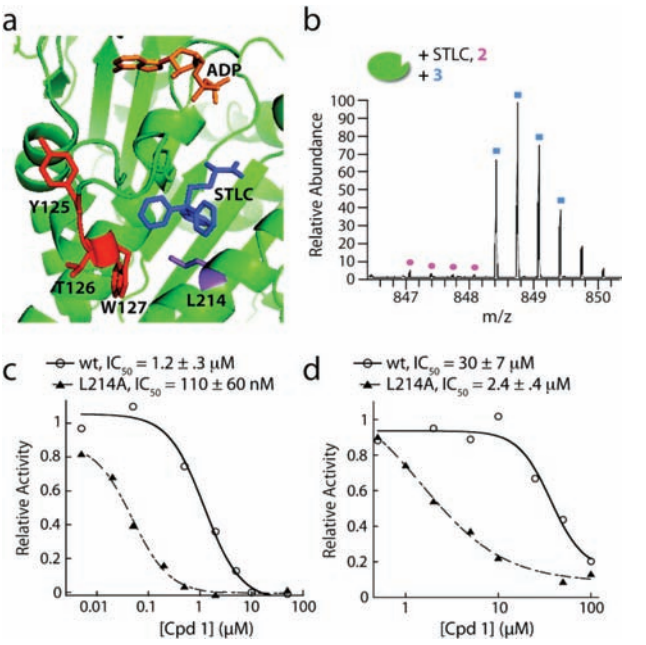

Figure 4. (a) Kinesin-5 ATPase domain (green, PDB: 2WOG[27]) in complex with ADP (orange) and STLC (blue). The cross-linked residues Y125, T126, and W127 are shown in red, while residue L214 is in purple. (b) Cross-linking of **2** (5 μM) to kinesin-5 in the presence of STLC (50 μM). (c) Representative curve of dose-dependent inhibition of steady-state microtubule-stimulated ATP hydrolysis by wildtype (wt) kinesin-5 and the L214A mutant protein. (d) Representative curve of dose-dependent inhibition of steady-state ATP hydrolysis by wildtype (wt) kinesin-5 and the L214A mutant protein, in the absence of microtubules. IC_{50} values are averages $\pm$ s.d. (n = 3).

inhibitor of the mutant kinesin-5 (L214A) (Figure S3), indicating that the mode of inhibition is not altered by this mutation. Together, our data are consistent with **1** binding at, or close to, the site bound by other kinesin-5 inhibitors that are not ATP-competitive.

It is relatively uncommon for nucleotide-competitive inhibitors to bind at an allosteric site in an ATPase.[30] Interestingly, another example of this mechanism of inhibition has been described for another kinesin-5 inhibitor, GSK-1.[23] This compound has been proposed to bind to an allosteric site that is distinct from the binding site of compound **1** and STLC. Further studies with both kinesin-5 ATP-competitive inhibitors will be needed to reveal how binding at allosteric sites results in ATP-competitive inhibition that leads to a tightly bound microtubule−motor protein complex.

In summary, we demonstrate that SILIC can be used to map the binding site of an inhibitor to its target. We believe this approach should be effective in analyzing inhibitor−target interactions, particularly when structural studies are intractable. A potential limitation of the approach is that the incorporation of a photo-cross-linking moiety, such as an aryl azide, may not always be feasible. However, aryl groups appear at high frequency in bioactive small molecules. In these cases, SAR studies could allow the inclusion of an azide moiety such that it does not alter the inhibitor's mechanism of action and yet is proximal to the target's binding site residues. In the future we wish to extend this analysis to more complex cellular contexts to examine whether cellular physiology alters the mode of drug−target interaction and mechanism of drug action.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Details of the synthesis of compounds **2** and **3**, experimental findings from cross-linking without microtubules and with the L214A mutant, ATP-competition results, experimental procedures, and complete refs 5,8,23. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author
xiangli@hku.hk; kapoor@rockefeller.edu

■ ACKNOWLEDGMENT

We thank Brian Chait, Kelly Molloy, and Yinyin Li for reagents, equipment use, technical assistance, and discussions. This work was supported by the NIH (GM65933).

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