Published in final edited form as:

J Am Chem Soc. 2019 April 10; 141(14): 5602–5606. doi:10.1021/jacs.8b13257.

Designing Allele-Specific Inhibitors of Spastin, a Microtubule-Severing AAA Protein

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Abstract

The bump-hole approach is a powerful chemical biology strategy to specifically probe the functions of closely related proteins. However, for many protein families, such as the ATPases associated with diverse cellular activities (AAA), we lack structural data for inhibitor-protein complexes to design allele-specific chemical probes. Here we report the X-ray structure of a pyrazolylaminoquinazoline-based inhibitor bound to spastin, a microtubule-severing AAA protein, and characterize the residues involved in inhibitor binding. We show that an inhibitor analogue with a single-atom hydrogen-to-fluorine modification can selectively target a spastin allele with an engineered cysteine mutation in its active site. We also report an X-ray structure of the fluoro analogue bound to the spastin mutant. Furthermore, analyses of other mutant alleles suggest how the stereoelectronics of the fluorine-cysteine interaction, rather than sterics alone, contribute to the inhibitor-allele selectivity. This approach could be used to design allele-specific probes for studying cellular functions of spastin isoforms. Our data also suggest how tuning stereoelectronics can lead to specific inhibitor-allele pairs for the AAA superfamily.

Graphical Abstract

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The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.8b13257.

• Methods, biochemical characterization of spastin constructs, compound synthesis and characterization, binding assays, and X-ray data collection and refinement tables (PDF)

The authors declare no competing financial interest.

Chemical inhibitors that specifically block the function of a selected protein allele can be useful tools to examine complex and dynamic cellular processes.(1) Early examples of engineering of allele–ligand pairs focused on modifications of nucleotide analogues to specifically bind a mutant GTPase or ATPase.(2–5) In parallel, a similar strategy called the bump-hole approach was developed that introduced mutations in the protein’s active site to create a pocket (“hole”) that can accommodate an inhibitor with a modification (“bump”).(6,7) The shape complementarity between the inhibitor and the mutant allele can be achieved by even subtle compound modifications (e.g., hydrogen to methyl substitution) that are sufficient to provide substantial improvements in selectivity toward the mutant protein allele.(6,8) For several protein families, including cyclophilins,(6) kinases,(9) GPCRs,(10) PARPs,(11) and BET proteins,(8) we have a wealth of high-resolution structural data for how different chemical scaffolds bind active sites, and as a result, the design of allele-selective inhibitors of these proteins has been possible. However, for many enzyme families, such as the ATPases associated with diverse cellular activities (AAA) superfamily, we lack these critical data, and designing allele-specific chemical inhibitors has been difficult.

There are ~100 AAA proteins in humans, and several of these enzymes have essential functions.(12) The improper function of AAA proteins has also been linked to a wide range of diseases, including Alzheimer’s disease and related dementias(13,14) and cancer.(15,16) Additionally, therapeutic agents targeting AAA proteins have recently entered clinical trials.(15) Therefore, selective chemical inhibitors would be useful probes for normal physiology and disease. These proteins typically function as oligomeric assemblies (e.g., hexamers) of conserved AAA domains (Figure 1), which bind ATP at their interface.(17) Furthermore, the AAA active site can undergo large-scale nucleotide-dependent rearrangements,(18) and as a result, it is unclear which of the different conformational states of the active site can contribute to chemical inhibitor binding and specificity.

Here we focus on spastin, a microtubule-severing AAA protein needed for cell division and organelle transport.(19–21) We report high-resolution structures of wild-type and mutant alleles of spastin bound to chemical inhibitors that we have developed.(22) Our structural data and activity analyses of spastin mutants reveal that the interaction between an engineered cysteine residue in the active site and a fluoro-substituted pyrazolylaminoquinazoline-based compound can be leveraged for the design of allele-specific inhibitors of spastin.

As high-resolution structural data can facilitate the rational design of selective chemical probes, we first sought to obtain an X-ray crystal structure of an inhibitor-bound AAA protein. We characterized a truncated construct including spastin’s AAA domain (amino acids 445–758, *Drosophila melanogaster* spastin, hereafter denoted as spastin-AAA-WT; Figure S1a,b), as only this portion of spastin is well-resolved in the structures of its apo form.(23,24) This construct is not an active ATPase, but gel filtration and differential scanning fluorimetry revealed that it is a stable monomer in solution that retains the ability to bind nucleotides (Figure S1c–f).

We recently described the development of spastazoline, a potent and selective inhibitor of spastin.(22) Here we examined the binding of the spastin-AAA-WT construct to compound

1 (Figure 2a), a pyrazolylaminoquinazoline-based analogue of spastazoline. We found that compound **1** binds this construct with submicromolar affinity in an isothermal titration calorimetry assay ($K_d = 312 \pm 300$ nM, $n = 4$; Figure 2b). In addition, differential scanning fluorimetry revealed concentration-dependent stabilization of the spastin-AAA-WT construct against heat-induced denaturation by compound **1** (Figure S2a).

Next, we set up cocrystallization trials of spastin-AAA-WT with compound **1**. We obtained diffracting crystals and determined the structure of the spastin-AAA-WT–compound **1** complex at ~ 2.4 Å resolution using molecular replacement (Table S1). This structural model contains a clear density that can be assigned to compound **1** in a pocket between the $\alpha\beta\alpha$ core of the large AAA subdomain and the α -helical bundle of the small AAA subdomain (Figures 2c and S2b,c).

In this structural model, the 4-aminopyrazolyl moiety of compound **1** is positioned to form two hydrogen-bonding interactions with Ala-486's carbonyl oxygen and nitrogen atoms in spastin's N-loop. These interactions likely mimic the hydrogen bonds made by adenine in the active site. The pyrazole heterocycle is also aligned to form a third potential hydrogen-bonding interaction with the Asp-484 backbone carbonyl oxygen (Figure 2d). The pyrazolylaminoquinazoline core of compound **1** is sandwiched between the hydrophobic residues projecting from spastin's large and small AAA subdomains (Ile-485, Leu-531, and Leu-659; Figure 2d). In addition, the quinazoline is positioned to interact with residues in the N-loop and P-loop motifs (e.g., Gln-488 and Asn-527; Figure 2d). These data reveal how a pyrazolylaminoquinazoline-based compound interacts with residues in the AAA active site.

Next, we examined whether our structure of the spastin-AAA-WT–compound **1** complex could guide the design of allele-specific inhibitors. The structure reveals that the side-chain amide of Asn-527 in the P-loop is proximal to the quinazoline's C8 hydrogen (the distance between the inhibitor's C8 and the Asn-527 carbonyl oxygen is 3.8 Å; Figures 3a and S2d). We have reported that modifications of the quinazoline's C8 led to inhibitors with improved potency and selectivity toward human spastin.(22) Therefore, to probe the inhibitor interaction with the side-chain amide of Asn-527, we synthesized three analogues of compound **1** with modifications at C8 (compounds **2–4**; Figure 3b). These compounds were synthesized using established procedures.(25) In compound **2**, we replaced the benzene ring of the quinazoline with a pyrrole so that the C–H bond is replaced by the N–H hydrogen-bond donor. In compound **3**, a pyridopyrimidine places a hydrogen-bond acceptor at C8, and in compound **4** the polarity at C8 is reversed, as fluorine replaces the hydrogen. We generated an ATPase-active spastin construct (amino acids 209–758, hereafter called spastin-WT)(22) and tested its inhibition by these compounds. While the potency of compound **2** did not substantially change, compounds **3** and **4** inhibited the wild-type spastin nearly 100-fold less potently (Table S2 and Figure 3c, black traces), indicating the importance of interactions between the quinazoline's C8 hydrogen and the side-chain amide of the Asn-527 residue.

We have found that the asparagine-527 can be mutated to a cysteine without disrupting spastin's ATPase activity.(22) Therefore, we reasoned that it could be suitable for the

development of allele-specific inhibitors and tested analogues of compound **1** against the spastin-N527C construct. Compound **2** does not potently inhibit the ATPase activity of this spastin allele (Table S2 and Figure 3c, orange traces). However, compounds **3** and **4** more potently inhibit the spastin-N527C allele in comparison with the wild-type protein, with compound **4** being ~20-fold selective for the mutant allele (Figure 3c and Table S2).

To examine the structural basis for the selectivity of compound **4** for the spastin-N527C allele, we cocrystallized the spastin-AAA-N527C construct with compound **4** and determined the structure at ~2.2 Å resolution (Figure 4a and Table S1). The quinazoline core of compound **4** is positioned in the pocket with an orientation nearly identical to that of compound **1** (Figure S3a). The side-chain β-carbon and sulfur atoms of the Cys-527 residue also adopt similar positions as the β- and γ-carbon atoms of the Asn-527 residue in the structure of the wild-type protein with bound compound **1** (Figure S3a). The structure of the spastin-AAA-N527C–compound **4** complex shows clearly resolved density around the 8-fluoro atom that points toward the Cys-527 residue (Figures 4a and S3b). In principle, the cysteine side chain can form a covalent bond with the fluoro-substituted heterocycle. Our structural model indicates that this is not the case.

Organic fluorines can engage in n–π* interactions with carbonyl groups in protein-backbone amides as well as with carbonyls in the side chains of asparagine and glutamine residues. (26,27) In addition, interactions of fluorine with sulfur-containing hydrophobic side chains, such as cysteine and methionine, have also been shown to contribute to compound binding. (28–30) To examine the interactions of compound **4** with residue 527, we generated three spastin constructs with mutations at this site (spastin-N527A, -S, and -T) and tested their ATPase activities (Figure S4). The catalytic turnover number (k_{cat}) and the ATP concentration required for half-maximal enzyme rate ($K_{1/2}$) of the N527T construct are within ~1.5-fold of those for the wild-type protein (Table S3). In contrast, the N527A and N527S mutant constructs have lower k_{cat} (~3-fold) and higher $K_{1/2}$ (~5–7-fold) compared with wild-type spastin (Table S3). Next, we tested the inhibition of these mutant alleles by compound **4**. We found that the N527A, -S, and -T mutant constructs are inhibited ~15–100-fold less potently than the N527C allele, consistent with the importance of the fluorine–cysteine interaction (Table S4). As the N527S and N527A mutants have substantially lower enzyme activities than the wild-type construct, we also tested binding of compound **4** to the mutant alleles using differential scanning fluorimetry. We observed dose-dependent stabilization of the N527C mutant allele by compound **4**. Gratifyingly, we observed substantially reduced stabilization of the N527A, -S, and -T constructs (Figure 4c, Figure S5 and Table S5). Taken together, these data suggest that binding of compound **4** to the N527C mutant protein is likely due to favorable stereoelectronic interactions of the fluorine atom with the Cys-527 residue.

Designing the bump–hole approach involves two steps. First, binding sites of proteins need to be modified using mutations that do not disrupt activity. Our structural and biochemical data show how a spastin mutant allele alters the inhibitor's binding site while retaining ATPase activity. In principle, the P-loop residue at an equivalent site in other AAA proteins may also be similarly mutated to generate active alleles. Second, complementary modifications of an inhibitor need to be designed. Our studies reveal how a fluorine

modification of a spastin inhibitor can generate a mutant-allele-selective compound. However, we find that compounds **1** and **4** are selective for spastin and do not inhibit the activity of four other closely related AAA proteins (i.e., Fidgetin-like 1, katanin, VPS4B, and VCP/p97; Figure S6). Therefore, without substantial additional structure–activity analyses, this compound cannot be used to develop the bump–hole approach for other AAA proteins. As other chemical scaffolds that target the active site of AAA proteins have now been identified and more X-ray data are becoming available,(15,31) structure-guided approaches may be used to identify suitable scaffolds. Together, our data suggest that tuning stereoelectronics may be effective for designing allele-specific chemical probes for AAA proteins based on these other scaffolds.

As mammalian cells express at least two isoforms of spastin that share a common AAA domain,(32) allele-specific inhibitors will be powerful tools to dissect spastin's isoform-specific functions. Errors in spastin function have been linked to essential processes such nuclear envelope reformation during cell division and ER–endosome organization.(20,33) In addition, protein knockdown studies indicate that amyloid- β toxicity can be suppressed by loss of spastin function.(14) The allele-specific inhibitors reported here could be valuable starting points to develop chemical tools to evaluate the roles of spastin isoforms in cellular context.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

R.P. was supported by the Tri-Institutional Ph.D. Program in Chemical Biology and the Rockefeller University Graduate Program. T.C. was supported by the EMBO Long-Term Fellowship for Postdoctoral Studies and the Kestenbaum Award for Research in Neurodegenerative Diseases. T.M.K. is grateful to the NIH/NIGMS for funding (GM98579 and R35 GM130234–01). This research used beamlines 17-ID-1 and –2 at the National Synchrotron Light Source II, a U.S. Department of Energy (DOE) Office of Science User Facility operated for the DOE Office of Science by Brookhaven National laboratory under Contract DE-SC0012704. We thank Seth Darst, Elizabeth Campbell, and Michael Oldham for their generous help and advice with crystallographic data processing and refinement. We thank Antonina Roll-Mecak (NIH) and Rebecca Heald (UC Berkeley) for providing plasmids. We also thank Michael Grasso for help with purification of VPS4B protein. We are grateful to Deena Oren at the Rockefeller University Structural Biology Center and Carolina Adura and the staff at the Rockefeller University High-Throughput and Spectroscopy Resource Center for assistance. The use of the Formulator robot and the Rigaku/MSO microMax 007HF in the Rockefeller University Structural Biology Resource Center was made possible by grants 1S10RR027037–01 and 1S10RR022321–01 from the National Center for Research Resources of the NIH.

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Figure 1.

An active-site mutant allele of a AAA protein can be specifically inhibited by a fluorinated analogue of a pyrazolylaminoquinazoline-based inhibitor.

Figure 2.

X-ray structure of wild-type spastin bound to compound **1**, a pyrazolylaminoquinazoline-based spastin inhibitor. (a) Chemical structure of compound **1**. (b) Isothermal titration calorimetry analysis of compound **1**'s binding to spastin-AAA-WT. (c) Crystal structure of the spastin-AAA-WT-compound **1** complex (stick and ribbon representation). Spastin's large and small AAA subdomains are highlighted in gray and black, respectively. The omit electron density map around compound **1** contoured at 3σ is also shown ($mF_o - DF_c$; green mesh). (d) View of compound **1** from the entrance of spastin's ATP-binding site. Selected residues proximal to the inhibitor ($<4 \text{ \AA}$) and predicted hydrogen-bonding interactions (black dashed lines) are indicated.

Figure 3.

Designing analogues of compound **1** to probe inhibitor interactions with a residue in spastin's P-loop. (a) Schematic of compound **1** in the ATP-binding site of spastin. (b) Compound **1** analogues. (c) Concentration-dependent inhibition of spastin's ATPase activity (wild-type, black; N527C mutant, orange) by compound **1** analogues.

Figure 4.

X-ray structure of the spastin-N527C–compound **4** complex and analyses of compound **4**'s binding to mutant spastin constructs. (a) Crystal structure of the spastin-AAA-N527C–compound **4** complex. The omit electron density map around the inhibitor and the Cys-527 residue contoured at 3σ is shown ($mF_o - DF_c$, green mesh). (b) Concentration-dependent inhibition of N527 mutant alleles of spastin with compound **4**. For comparison, data for the wild-type and N527C mutant alleles (data from Figure 3b) are shown as dashed lines. (c) Concentration-dependent effect of compound **4** on the heat-induced unfolding (T_m) of spastin constructs.