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Chemical Genetics Approach to Engineer Kinesins with Sensitivity towards a Small-Molecule Inhibitor of Eg5

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Due to their fast and often reversible mode of action, small molecules are ideally suited to dissect biological processes. Yet, the validity of small-molecule studies is intimately tied to the specificity of the applied compounds, thus imposing a great challenge to screens for novel inhibitors. Here, we applied a chemical-genetics approach to render kinesin motor proteins sensitive to inhibition by the well-characterized small molecule *S*-Trityl-L-cysteine (STLC). STLC specifically inhibits the kinesin Eg5 through binding to a known allosteric site within the motor domain. Transfer of this allosteric binding site into the motor domain of the human kinesins Kif3A and Kif4A sensitizes them towards STLC. Single-molecule microscopy analyses confirmed that STLC inhibits the movement of chimeric but not wild-type Kif4A along microtubules. Thus, our proof-of-concept study revealed that this chemical-genetic approach provides a powerful strategy to specifically inhibit kinesins in vitro for which small-molecule inhibitors are not yet available.

The application of small molecules to modulate protein function is a powerful approach to reveal the molecular mechanisms of biological processes. Yet, conclusions drawn from these studies are only valid if the applied compounds are specific, and this requirement for stringent specificity constitutes a major obstacle for the identification of novel inhibitors. We therefore sought an alternative approach to inhibit microtubule (MT)-associated kinesin motor proteins with high specificity. Kinesins are molecular ATPases that convert the energy released by ATP hydrolysis into mechanical force.[1] Based on phylogenetic analyses, kinesins are classified into fourteen different families, with highly diverse cellular functions.[2] In line with their shared basic enzymatic activity, the characteristic structure of the motor domain (MD) is well conserved among the more than 40 human kinesins, whereas domains contributing to specialized functions share little—if any—sequence conservation. The mitotic kinesin Eg5, a member of the kinesin-5 family, is a plus-end-directed kinesin, and in most eukaryotes studied, its activity is essential to establish spindle bipolarity.[3] Due to its homotetrameric structure and plus-end-directed motility, Eg5 is thought to crosslink and push apart antiparallel MTs emanating from the two centrosomes, thereby resulting in the formation of a bipolar spindle.[4] Consistent with the function of Eg5, cells treated with Eg5 inhibitors such as monastrol and *S*-trityl-L-cysteine (STLC) fail to assemble bipolar spindles, but rather form monopolar spindles.[5] Monastrol and STLC specifically target an allosteric binding site in Eg5 formed by helices α2, α3 and the insertion loop (L5) of α2.[6] Intriguingly, L5, while being a characteristic feature of all kinesins, differs significantly in length between different kinesins with Eg5's L5 being unusually long (Figure 1 B). Thus, the low degree of conservation of L5 seems to be the key for the specificity of L5-targeting Eg5 inhibitors. Klp61F, the *Drosophila melanogaster* Eg5, is not inhibited by either monastrol or STLC. Yet, replacing its L5 with that of human Eg5 renders the chimera sensitive to inhibition.[7] Intrigued by these findings, we speculated that this approach could be applied to engineer kinesins beyond the kinesin-5 family, which are to be susceptible to L5 pocket inhibitors.

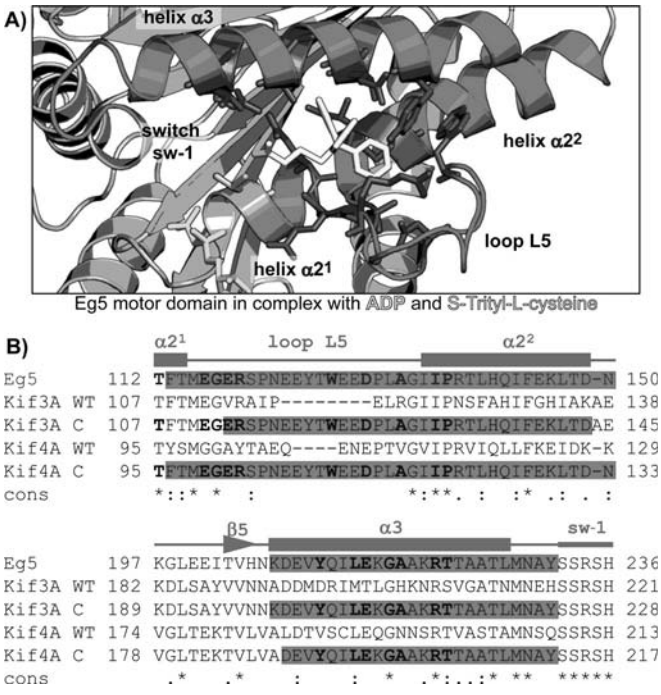

Figure 1. Design of STLC-sensitive kinesin chimeras. A) PyMOL presentation of the STLC (yellow) binding site within the human Eg5 motor domain (gray) bound to ADP (green) according to ref. [6b]. B) Alignment of human Eg5, Kif3A, and Kif4A sequences. Residues important for STLC binding are shown in bold. The transferred STLC binding cluster includes α2, L5, and α3 (red).

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Here, by transferring the STLC-binding cluster we create chimeras of the human kinesins Kif3A and Kif4A that, unlike their wild-type counterparts, are susceptible to inhibition by STLC. Using single-molecule microscopy, we show that the motility of full-length chimeric Kif4A along MTs is efficiently suppressed by STLC, thus demonstrating that this chemical-genetics approach provides a novel strategy to inhibit kinesin function with high specificity in vitro.

For our Eg5 inhibitor-sensitive chimeras, we selected Kif3A and Kif4A from the kinesin-2 and -4 families, respectively, because no inhibitors were available for these kinesins. As L5 targeting inhibitor, we chose STLC given its higher potency than monastrol.[8] To design the inhibitor-binding site, we first analyzed the co-crystal structure[6b] of the human Eg5 MD in complex with STLC (Figure 1A). A previous study suggested that 17 residues within L5, α2, and α3 are critical for STLC binding.[7] Sequence alignment revealed that the key residues in L5 and α3 are poorly conserved between Eg5 and Kif3A or Kif4A, whereas the critical threonine, isoleucine, and proline residues (Eg5: T112, I136, and P137) in α2 are conserved (Figure 1B). Exchange of just L5 was not sufficient to confer STLC sensitivity to Kif3A or Kif4A (data not shown), thus confirming that residues outside of L5 are critical for the inhibitory effect of STLC. We therefore engineered chimeras of Kif3A and Kif4A by introducing the complete Eg5 STLC-binding cluster, including α2, L5, α3, and adjacent residues (red in Figure 1).

Wild-type (WT) and chimeric (C) MD fused at the N terminus to a His_6 tag followed by a tobacco etch virus (TEV) cleavage site were expressed in *Escherichia coli*. Proteins were purified by immobilized metal ion affinity chromatography (IMAC), followed by TEV protease-mediated removal of the His_6 tag, and cation exchange chromatography to remove impurities. Gel filtration chromatography was performed to confirm size homogeneity and avoid potential protein aggregation. Apart from a small shoulder, the chimeric MDs (like the WT proteins) eluted in single peaks, and the retention profiles indicated that the purified MDs were monomeric and did not form aggregates (Figure 2A, E). The purity of recombinant MDs was confirmed by SDS-PAGE. Next, we characterized enzymatic activity by using an enzyme-coupled ATPase assay (ECA, Figure S1 in the Supporting Information). Chimeric Kif3A and Kif4A MDs displayed MT-stimulated ATPase activity in the range of the respective wild-type proteins (Figure 2C and G), thus confirming—in line with the fact that none of the introduced changes was in a catalytic residue or at the MT binding site—that the mechanochemical coupling of ATP hydrolysis, MT binding and conformational changes within the MD is largely intact in the engineered chimeras. Closer analyses revealed that the introduction of α2-L5-α3 into Kif3A MD decreased k_m for ATP from 17.2 ± 1.7 to 9.7 ± 1.0 μM, while $k_{1/2}$ for MTs increased from 7.7 ± 0.5 to 14.2 ± 2.6 μM (Figure 2B, C and Table 1). The ATP hydrolysis rate was reduced from $9.4 \pm 0.2\ s^{-1}$ (at 20 μM MTs) to $5.9 \pm 0.8\ s^{-1}$ (Table 1). Thus, consistent with the reported function of L5 in regulating both ATP hydrolysis and the MT affinity of Eg5,[9] exchange of α2-L5-α3 affected the affinity of the Kif3A MD for ATP as well as for MTs resulting in a decreased ATP hydrolysis rate. In line with a previous report,[10] WT Kif4A

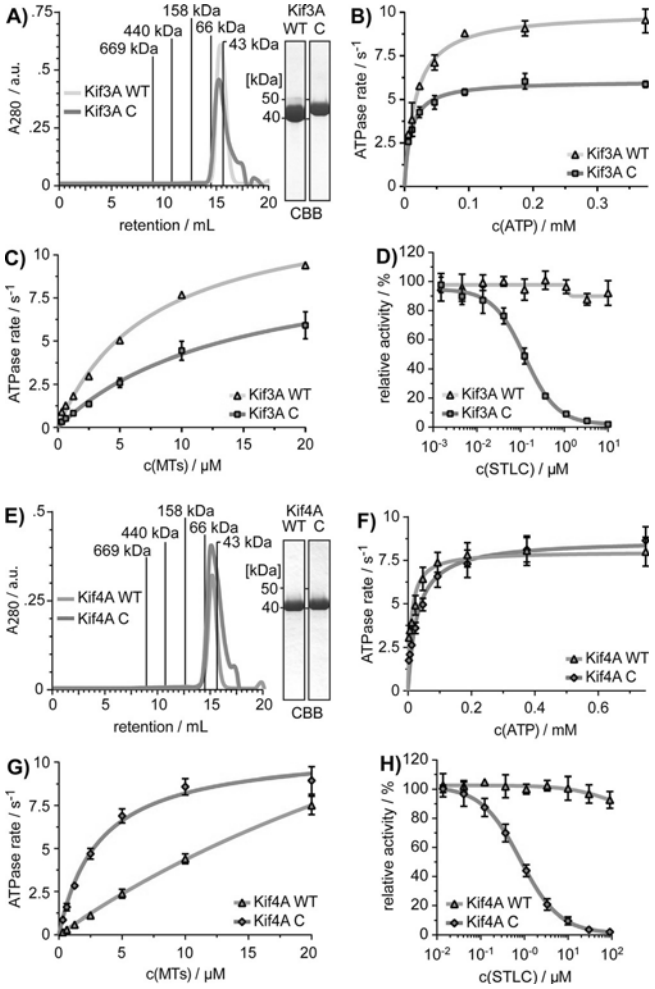

Figure 2. Characterization of chimeric Kif3A and Kif4A MD. A) Gel filtration and SDS-PAGE analyses of WT and C Kif3A MD. (CBB: Coomassie Brilliant Blue). B) The ATPase activity of WT and C Kif3A MD was determined at increasing concentrations of ATP (20 μM MTs). C) Same assay with 1 mM ATP and increasing concentrations of MTs. D) ATPase activity of WT and C Kif3A MD at increasing concentrations of STLC (1 mM ATP, 20 μM MTs). E) Gel filtration and SDS-PAGE analyses of WT and C Kif4A MD. F) ATPase activity of WT and C Kif4A MD at increasing concentrations of ATP (20 μM MTs). G) Same assay with 1 mM ATP and increasing concentrations of MTs. H) ATPase activity of WT and C Kif4A MD at increasing concentrations of STLC (1 mM ATP, 20 μM MTs). All data are mean ± SD ($n = 3$), with fits assuming Michaelis–Menten kinetics (B, C, F, and G) or a four-parameter dose–response curve (D and H).

MD had a low affinity for MTs ($k_{1/2} = 53.6 \pm 11.2$ μM), which was greatly increased by the transfer of α2-L5-α3 ($k_{1/2} = 3.2 \pm 0.3$ μM; Figure 2G and Table 1). While the STLC binding cluster

Table 1. Catalytic data for MDs of Eg5, Kif3A, and Kif4A wild-type (WT) and chimera (C).

motor domain:	Eg5	Kif3A WT	Kif3A C	Kif4A WT	Kif4A C
k_{cat} [s^{-1}]	4.9 ± 0.1	9.4 ± 0.2	5.9 ± 0.8	7.5 ± 0.5	8.9 ± 0.8
k_m (ATP) [μM]	11.3 ± 1.2	17.2 ± 1.7	9.7 ± 1.0	10.2 ± 1.5	29.6 ± 3.2
$k_{1/2}$ (MTs) [μM]	1.3 ± 0.1	7.7 ± 0.5	14.2 ± 2.6	53.6 ± 11.2	3.2 ± 0.3
IC_{50} (STLC) [nM]	170	–	130	–	810

increased the affinity for MTs, it decreased the affinity for ATP ($k_m = 10.2 \pm 1.5$ μM for WT vs. 29.6 ± 3.2 μM for C Kif4A, Figure 2F and Table 1). Because the MT affinity of WT Kif4A MD was much lower than that of chimeric MD, we were not able to saturate the MT concentration for WT Kif4A in the ECA (Figure 2G). At 20 μM MTs, C Kif4A MD was slightly more active than WT Kif4A MD (ATP hydrolysis rate 8.9 ± 0.8 vs. 7.5 ± 0.5 s^{-1}). In summary, these data indicate that—like in the case of Kif3A—implantation of α2-L5-α3 affected the affinity of Kif4A MD for both ATP and MT resulting in an enzymatic activity that is comparable to that of WT Kif4A MD. The affinities for ATP and MT were differently affected in Kif3A and Kif4A, thus suggesting that the molecular context in which the allosteric L5-regulatory element is transferred plays an important role.

After validation of the MDs, we tested if the introduction of α2-L5-α3 rendered them sensitive to STLC. To this end, we first confirmed the inhibitory effect of STLC towards Eg5. Indeed, STLC inhibited the Eg5 MD with a median inhibitory concentration (IC_{50}) of 170 nM (Table 1). As expected, the WT MD of neither Kif3A nor Kif4A was significantly inhibited by STLC at a concentration as high as 10 (Kif3A) or 100 μM (Kif4A; Figure 2D, H, Table 1). Intriguingly, the chimeric MDs of Kif3A and Kif4A were inhibited by STLC in a dose-dependent manner with an IC_{50} value of 130 and 810 nM, respectively (Figure 2D, H, Table 1). Consistently, Kif3A but not Kif4A was inhibited in a dose-dependent manner by the less potent L5 pocket inhibitor monastrol (Figure S2). Thus, transplantation of α2-L5-α3 into Kif3A and Kif4A conferred sensitivity towards L5 pocket inhibitors with an inhibitory efficacy, at least in the case of Kif3A, comparable to the one of Eg5.

Next, we sought to analyze the effect of the STLC-binding cluster in a more physiological context, that is, in the context of full-length (FL) kinesin, which is able to form dimers. In its functional form Kif3A heterodimerizes with Kif3B,[11] thus complicating its in vitro analysis. We therefore focused on homodimeric FL Kif4A. FL Kif4A labeled at its C terminus with monomeric green fluorescent protein (mGFP) and a His_{10} tag was expressed by using the Sf9-baculovirus insect cell expression system and purified by IMAC followed by gel filtration. SDS-PAGE analysis confirmed the purity of recombinant full-length kinesins (Figure 3A). Previous studies revealed that the low processivity of Kif4A—it walks only few steps along MTs before dissociating—can be greatly enhanced by the association of Kif4A with the MT binding protein PRC1.[12] We therefore included PRC1 purified from insect cells (Figure 3A) in our assay. To follow the movement of FL Kif4A along immobilized MTs, we performed total internal reflection fluorescence microscopy (TIRF-M), which allows the visualization of single fluorescent molecules (Figure 3B). The trajectories of GFP-labeled FL Kif4A were analyzed as space-time plots ("kymographs"; Figure 3C). In the presence of PRC1, WT Kif4A FL displayed processive movement with an average run-length of 6.0 ± 4 μm, and moved at an average speed of 350 ± 100 nm s^{-1} (Figure 3C–E). Consistent with the observation that the STLC binding cluster affects the affinity of the Kif4A MD for MT and ATP (Figure 2), TIRF-M analysis revealed that the chimeric full-length Kif4A displayed significantly reduced run-length (2.2 ± 1.7 μm) and speed (60 ± 20 nm s^{-1}). Importantly, 100 μM STLC affected neither the run-length nor the velocity of WT Kif4A FL. In strong contrast, 100 μM STLC completely impaired the ability of chimeric Kif4A FL to move along MTs. Thus, transplantation of α2-L5-α3 confers STLC sensitivity not only to monomeric Kif4A MD, but also to full-length dimeric Kif4A.

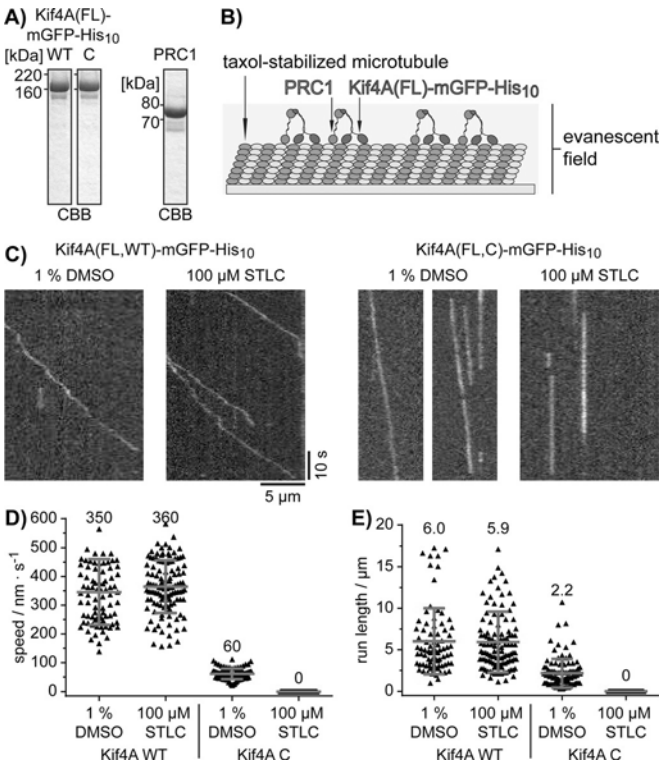

Figure 3. Inhibition of chimeric FL Kif4A by STLC. A) SDS-PAGE analysis of FL Kif4A-mGFP-His_{10} WT and C and FL untagged PRC1. B) Scheme of TIRF microscopy to follow the movement of GFP-tagged Kif4A along MTs. C) Example time (*y*-axis, scale bar: 10 s) versus space (*x*-axis, scale bar: 5 μm) plots (kymographs) of GFP-tagged Kif4A WT and C moving along MTs in the presence of 1% DMSO or 100 μM STLC. D) Speed and E) run-length of individual Kif4A-mGFP-His_{10} WT or C molecules in the presence of DMSO or STLC. Data are mean ± SD in red from $n = 3$ independent experiments, mean values shown above.

In summary, this study demonstrates the feasibility of the binding-site swap approach to render kinesins beyond the kinesin-5 family sensitive to L5-targeting drugs. Similar approaches were recently reported for engineered kinesin variants that could be either inactivated or translocated away from MTs by well-established small molecules such as biarsenical dyes or rapamycin,[13] thus highlighting the demand for chemical-genetic approaches to suppress kinesin function with high specificity. Monastrol and STLC are both highly specific for Eg5 and this specificity stems from the characteristic structure of the L5 binding pocket. Yet, as shown by our in vitro analyses, transplantation of the STLC binding pocket resulted in decreased enzymatic activity; this indicates that the engineered kinesins are not likely to complement the function of the wild-type proteins in cells. Reportedly, allosteric communication between the L5 binding pocket and the binding sites for ATP and MTs seems to be critical for the inhibitory effect of

monastrol and STLC.[7,9a,d,14] Our finding that Kif3A and Kif4A can be sensitized to STLC suggests that the reported mechanism of allosteric communication between the ligand-binding pocket and the binding sites for ATP and MTs in the Eg5 MD is—at least to a certain degree—conserved even within kinesins outside the kinesin-5 family. Thus, our chemical genetics approach not only opens a new strategy to inhibit kinesins in vitro without going through the laborious and challenging process of compound screening, but also allows novel insights into the mechanochemical properties of kinesins.

Experimental Section

Experimental Details are given in the Supporting Information.

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