Supporting Information

Structural and Thermodynamic Basis of the Enhanced Interaction between Kinesin Spindle Protein Eg5 and STLC-type Inhibitors

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Table S1. Primers used to construct the Eg5 expression plasmid

Eg5	#	Primer sequence
$Eg5_{17\text{-}369}$	1	5′- CAGCAAATCATATGAAGAACATCCAGGTGGTGGTG -3′
	2	5′- TATCTAGATTAATGATGATGATGATGATGCTGCAGTTTCTGATTCACTTCAGGCTTATT -3′

Table S2. Least-squares fitting between two structures of the Eg5 – inhibitor complexes

		rmsd (Å)			rmsd (Å)
PVEI0138_A ↔	PVEI0138_B	0.19	PVEI0021_*P*2$_1$_A ↔	PVEI0021_*P*2$_1$_B	0.45
	PVEI0021_*P*2$_1$_A	0.30		PVEI0021_*C*2_A	0.31
	PVEI0021_*P*2$_1$_B	0.54		PVEI0021_*C*2_B	0.30
	PVEI0021_*C*2_A	0.33		STLC_A	0.67
	PVEI0021_*C*2_B	0.33		STLC_C	1.30
	STLC_A	0.76			
	STLC_C	1.29	PVEI0021_*C*2_A ↔	PVEI0021_*C*2_B	0.10
				STLC_A	0.70
				STLC_C	1.23

Least-squares fitting between two structures of the Eg5 – inhibitor complexes was performed using PDBeFold,[1] and the resultant root-mean-square deviation (rmsd) values are shown. PVEI0138_A, molecule A of Eg5$_{17\text{-}369}$ – PVEI0138 complex; PVEI0021_*P*2$_1$_A, molecule A of Eg5$_{17\text{-}369}$ – PVEI0021 complex (*P*2$_1$–type); PVEI0021_*C*2_A, molecule A of Eg5$_{17\text{-}369}$ – PVEI0021 complex (*C*2–type); STLC_A, molecule A of Eg5$_{1\text{-}368}$ – STLC complex (PDB code 2WOG).[2] Molecule A superposes well onto molecule B in the three structures; Eg5$_{17\text{-}369}$ – PVEI0138 complex and PVEI0021 complexes (*P*2$_1$– and *C*2–types). These rmsd values are less than 0.5 Å. Molecule A of Eg5$_{17\text{-}369}$ – PVEI0138 complex superposes well with molecules A of PVEI0021 complexes (*P*2$_1$– and *C*2–types). In the structure of Eg5$_{1\text{-}368}$ – STLC complex, molecules A and B reveal final inhibitor-bound states, and molecule C reveals an intermediate state. All molecules A of Eg5$_{17\text{-}369}$ – PVEI0138 complex and PVEI0021 complexes (*P*2$_1$– and *C*2–types) were superposed better with molecule A than molecule C in the STLC complex. Therefore, we use each molecule A of STLC-type inhibitor complexes to discuss the difference in the structures in the main text.

Table S3. Surface area of Eg5 interfacing with inhibitor (A), volume and molecular surface area in the pocket (B), and shape complementarity of the interface (C) of Eg5 – inhibitor complexes.

A AREAIMOL

(Å^2)	**PVEI0138**	**PVEI0021**	**STLC**
T112	12	10	12
E116	41	41	38
G117	16	15	16
E118	16	15	13
R119	34	31	31
W127	7	7	7
D130	2	2	1
A133	5	6	6
I136	5	5	6
P137	18	15	16
L160	9	9	10
Y211	26	25	22
L214	35	40	36
E215	19	15	14
A218	37	39	39
R221	16	17	19
T222	0	1	3
F239	2	3	1
Total	301	294	288

B CASTp

	PVEI0138	**PVEI0021**	**STLC**
MS volume (Å^3)	558	507	474
Pocket MS area (Å^2)	426	394	346

C Shape complementarity (Sc)

	PVEI0138	**PVEI0021**	**STLC**
Sc statistic	0.814	0.811	0.785

(A) The surface area of Eg5 interfacing with each inhibitor was calculated using AREAIMOL in the CCP4 suite. The surface area of each residue interfacing with the inhibitor is shown. The residues that belong to the walls forming the binding pocket are shown in Figure 4B. The other residues, T112, G117, E118, D130, A218, and T222, are located at the corners of the pocket, so they do not belong to any components forming the walls. (B) The volume and molecular surface area of the pocket of Eg5 interfacing with each inhibitor were calculated using CASTp. (C) The shape complementarity of the interface between the Eg5 motor domain and each inhibitor was calculated using Sc. In the program Sc,[3] the shape correlation *S*c is defined as follows: on molecular surfaces P_A and P_B between the interacting molecules A and B, x_A is a point of P_A, n_A is the outwardly oriented unit vector normal to the P_A surface at x_A, and x'_A is a point of P_B nearest to x_A, n'_A is its inwardly oriented unit vector normal to the P_B surface at x'_A. The function $S^{A\rightarrow B}(x_A) = (n_A \cdot n'_A) \exp[- w (| x_A - x'_A |)^2]$ is defined at every point on P_A, where w is a constant. The similar function $S^{B\rightarrow A}(x_B) = (n_B \cdot n'_B) \exp[- w (| x_B - x'_B |)^2]$ is defined at every point on P_B. The shape correlation *S*c is then defined as $Sc = (\{S^{A\rightarrow B}\} + \{S^{B\rightarrow A}\}) / 2$, where the braces denote the median of the distribution of $S^{A\rightarrow B}$ and $S^{B\rightarrow A}$ values over the surfaces P_A and P_B, respectively. *S*c = 1 will mesh

presicely, and *Sc* ~ 0 will be uncorrelated in their topography. In all cases (A), (B), and (C), molecule A was used. PVEI0138, $Eg5_{17\text{-}369}$ – PVEI0138 complex; PVEI0021, $Eg5_{17\text{-}369}$ – PVEI0021 complex ($P2_1$–type); STLC, $Eg5_{1\text{-}368}$ – STLC complex (PDB code 2WOG).[2]

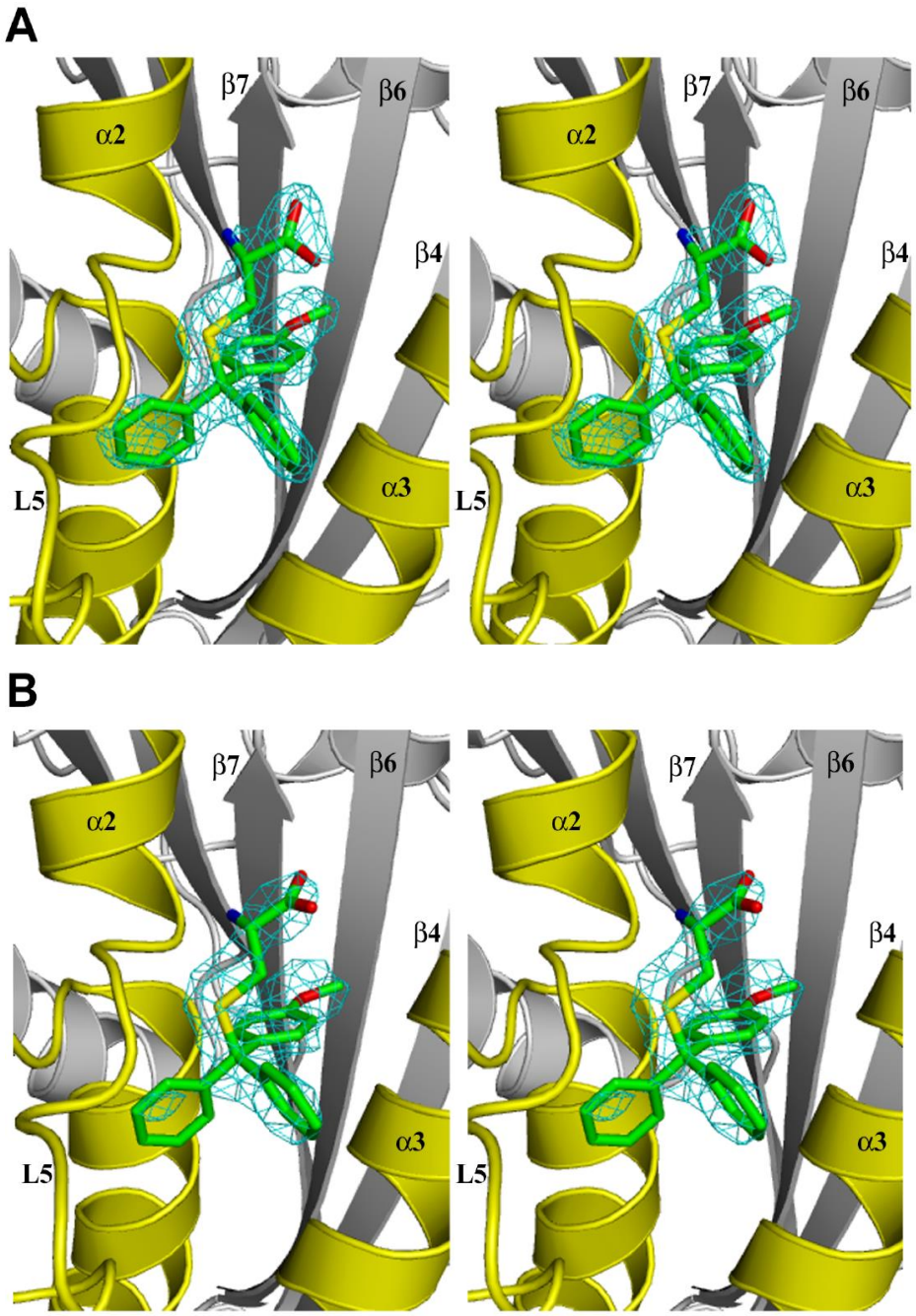


Figure S1. Close-up view of PVEI0021 and its binding pocket in the $Eg5_{17\text{-}369}$ – PVEI0021 complex. Molecule A was used to generate these figures. The F_o-F_c omit map of PVEI0021 was calculated with phases from the model without PVEI0021, contoured at 3 σ, and colored in cyan. (A) $P2_1$–type structure. The values of the real space correlation coefficient of PVEI0021 are 0.912 in chain A and 0.909 in chain B. (B) *C*2–type structure. The values of the real space correlation coefficient of PVEI0021 are 0.870 in chain A and 0.848 in chain B.

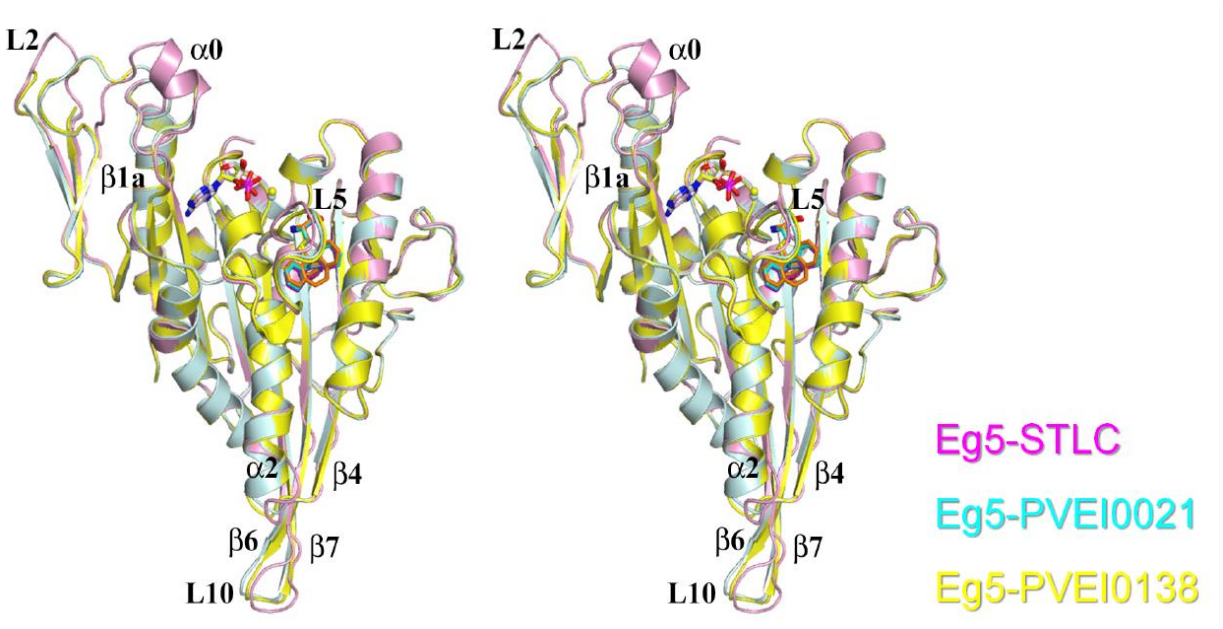
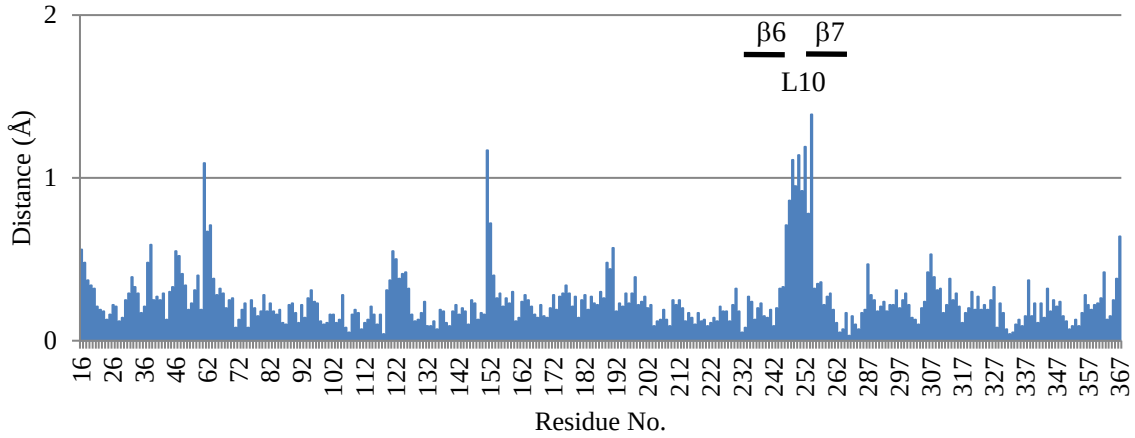


Figure S2. Superposition of three structures of the Eg5 motor domain in complex with STLC-type inhibitors (stereo view). Based on the structure of the $Eg5_{17\text{-}369}$ – PVEI0138 complex (yellow), the structures of PVEI0021 ($P2_1$–type, cyan) and STLC (PDB code 2WOG, pink) complexes were superposed. Molecule A was used to generate these figures. PVEI0138 (orange), PVEI0021 (cyan), and STLC (magenta) are shown as ball-and-stick models. The view is almost the same as that in Figure 2A. It is noted that the images of ADP molecules appear to be a single one, because the positions of ADP molecules are nearly identical to each other in the three structures (Figure S5C, D).

Molecules A of the Eg5 motor domain in complex with PVEI0138, PVEI0021 ($P2_1$–type), and STLC are in similar structures with rmsd values less than 0.8 Å (Table S2). L10 is an outstanding loop, and readily have different conformations among Eg5 structures. L10 and loop regions between α0 and β1a and between α2 and β4 show relatively large deviations between the superposed structures. The difference in the loop region between α0 and β1a may be derived from differences in crystal packing.

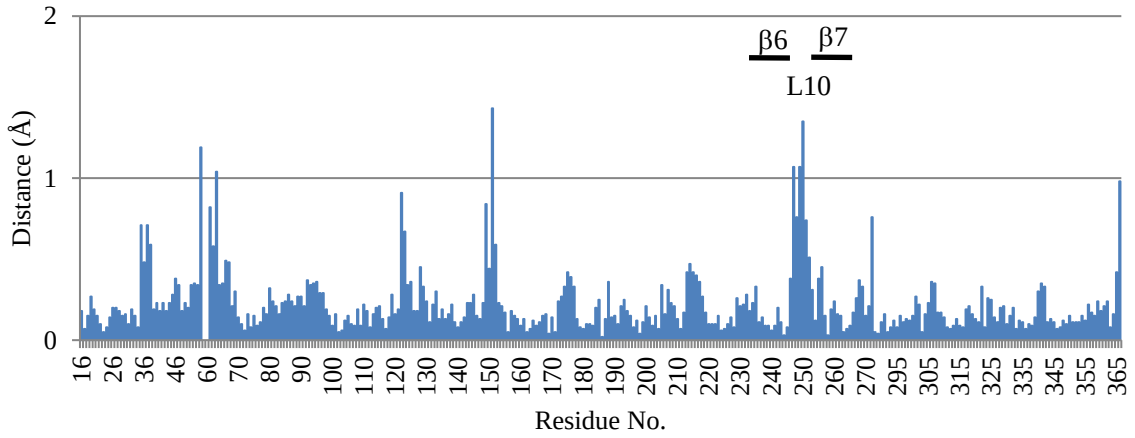
A Eg5–PVEI0021 complex ($P2_1$–type) vs. Eg5–PVEI0021 complex ($C2$–type)

rmsd 0.31 Å



B Eg5–PVEI0138 complex vs. Eg5–PVEI0021 complex ($P2_1$–type)

rmsd 0.30 Å



C Eg5–PVEI0021 complex ($P2_1$–type) vs. Eg5–STLC complex

rmsd 0.67 Å

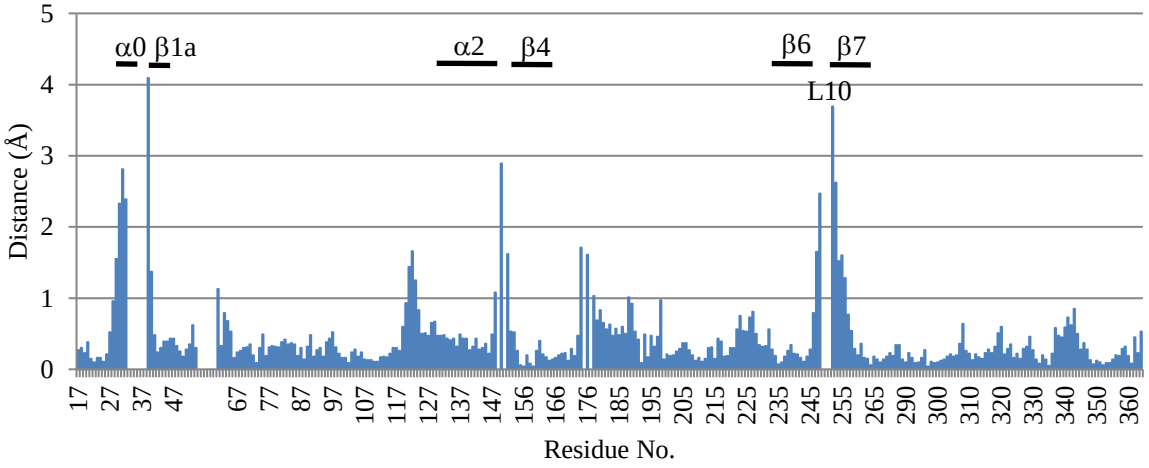


Figure S3. Plots of Cα-atom distances between two structures after least-squares fitting using PDBeFold[1] as described in Table S2. In all cases, molecule A was used. Each rmsd value is also shown. The corresponding secondary structure elements are labeled. (A) Molecule A of $P2_1$–type PVEI0021 complex superposes well with that of *C*2–type PVEI0021 complex, and its rmsd is very small, 0.31 Å. Loop L10 between β6 and β7 shows a relatively large deviation. (B) Molecule A of $Eg5_{17\text{-}369}$ – PVEI0138 complex superposes well with molecule A of the $P2_1$–type PVEI0021 complex, and its rmsd is 0.30 Å. (C) Molecule A of $P2_1$–type PVEI0021 complex superposes well with molecule A of the STLC complex,[2] and its rmsd is 0.67 Å.

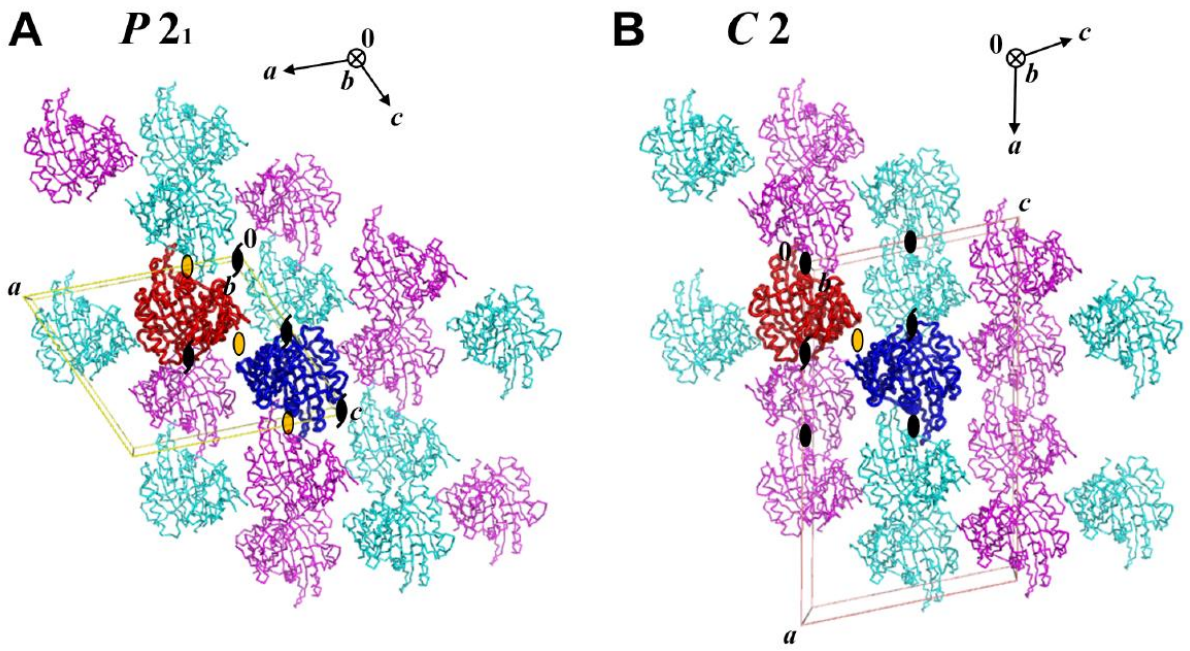
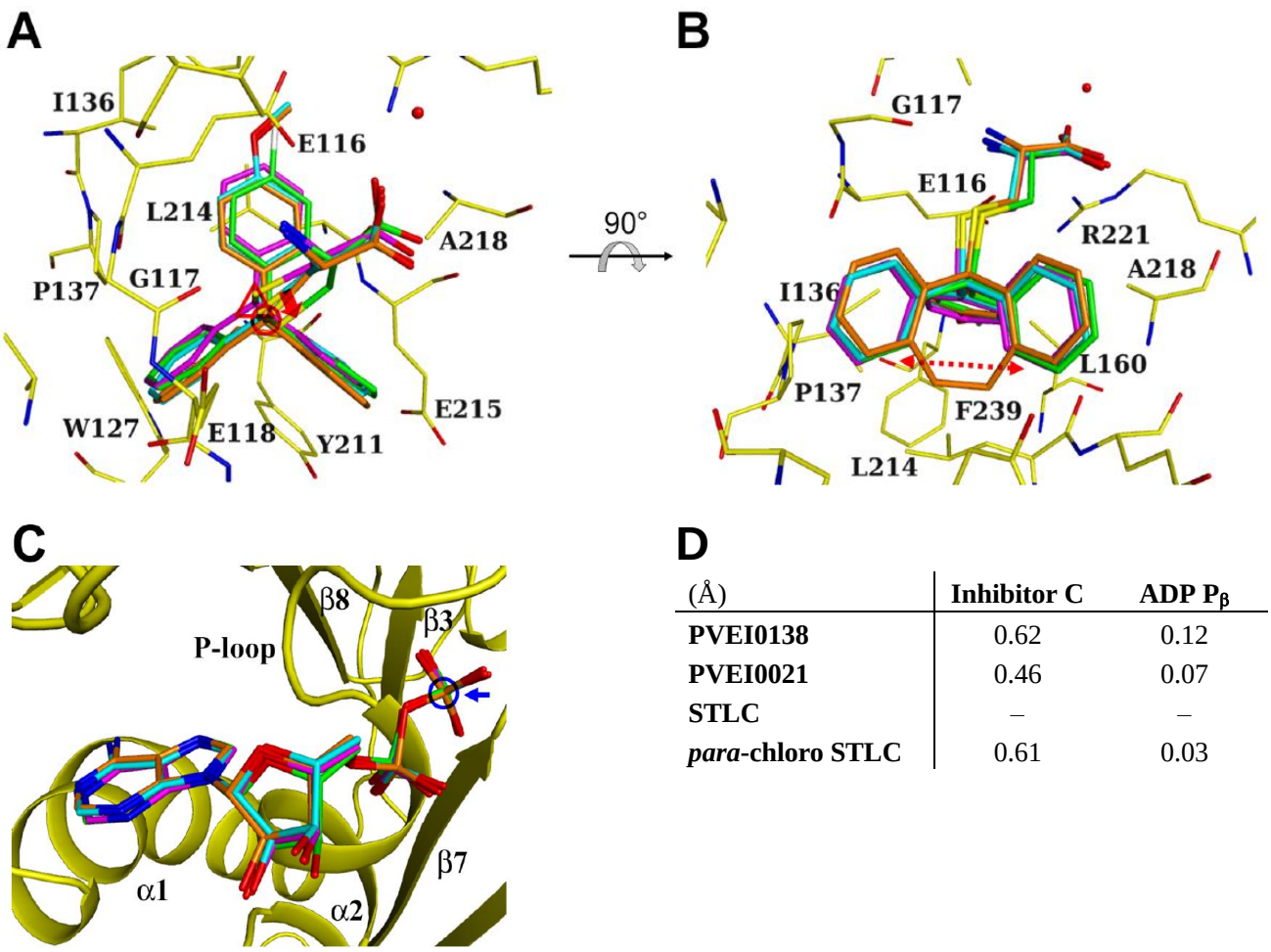


Figure S4. Two types of crystal packing of Eg5–PVEI0021 complexes. These structures contain two molecules in an asymmetric unit. Molecules A (red) and B (blue) in an asymmetric unit are shown as bold ribbons, and other molecules A (magenta) and B (cyan) in symmetry-related molecules are also shown together with unit cell boxes. Both figures are viewed along the *b* axes. Crystallographic two-fold axes and two-fold helical axes run along the *b* axes, and are indicated as black symbols. Non-crystallographic pseudo two-fold axes also run along the *b* axes, and are indicated as orange ellipses. (A) $P2_1$–type structure. Molecules A (red) and B (blue) in an asymmetric unit are related by the non-crystallographic pseudo two-fold axis located in fractional coordinate (*a*, *c*) = (0.25, 0.5). Another pair of molecules A and B are related by non-crystallographic pseudo two-fold axes located in (*a*, *c*) = (0.25, 0) and (0.25, 1), and each L10 loop faces each other in these pairs. Molecules align as AABBAABB⋯ along with a vertical direction in this figure. (B) *C*2–type structure. Molecules A (red) and B (blue) in an asymmetric unit are related by non-crystallographic pseudo two-fold axis located in (*a*, *c*) = (0.25, 0.25). The interactions via each L10 loop are observed between molecules A and A related by the crystallographic two-fold axis located in (*a*, *c*) = (0, 0), and between molecules B and B related by the crystallographic two-fold axis located in (*a*, *c*) = (0.5, 0.5). Molecules align as AAAAA⋯ or BBBBB⋯ along with a vertical direction in this figure.

As described above, in the $P2_1$– and *C*2–type structures, inter-molecular interactions via the L10 loops are observed from different pairs of molecules. However, given that molecules A and B are in nearly the same

structures, the patterns of crystal packing can be regarded as being nearly the same between the structures of the $P2_1$– and $C2$–type PVEI0021 complexes.



D

(Å)	Inhibitor C	ADP P_β
PVEI0138	0.62	0.12
PVEI0021	0.46	0.07
STLC	–	–
***para*-chloro STLC**	0.61	0.03

Figure S5. Comparison of the inhibitor and ADP structures of the Eg5 motor domain in complex with STLC-type inhibitors. Based on the structure of the $Eg5_{17\text{-}369}$ – PVEI0138 complex, the structures of the PVEI0021 ($P2_1$–type), STLC (PDB code 2WOG),[2] and *para*-chloro substituted STLC (PDB code 2XAE)[4] complexes were superposed. Molecule A was used to generate these figures. PVEI0138 (orange), PVEI0021 (cyan), STLC (magenta), and *para*-chloro STLC (green) are shown with the surrounding residues (yellow) of $Eg5_{17\text{-}369}$ – PVEI0138 complex. (A) Displacement of the C atom in the center of the trityl group from STLC (a red triangle) to each other STLC-type inhibitor (a red circle) is shown as a red arrow. (B) Distances between two C atoms attaching ethylene linkers (shown as a double-headed arrow) are 2.9 Å, 3.3 Å, 3.1 Å, and 3.2 Å in the PVEI0138, PVEI0021 ($P2_1$–type), STLC, and *para*-chloro STLC complexes, respectively. Because of the presence of the ethylene linker, the two phenyl rings of PVEI0138 approach each other. (C) ADP molecules (orange for PVEI0138 complex, cyan for PVEI0021 complex, magenta for STLC complex, and green for *para*-chloro STLC complex) are in nearly the same locations in the four structures (Figure S2). A blue circle and arrow indicate the positions of P_β atoms. (D) Based on the structure of the STLC complex, displacement values

(Å) of the central C atoms of the trityl group of STLC-type inhibitors (inhibitor C, shown as the red arrow from the triangle to the circle in (A)) are shown. For comparison, those of the P_β atoms of ADP (ADP P_β, shown as a blue arrow and a circle in (C)) in the structures of PVEI0138, PVEI0021, and *para*-chloro STLC complexes are also shown.

Supplementary Methods

Construction of the Plasmid. The primers used in this study are listed in Supplementary Table S1. The cDNA corresponding to the motor domain of Eg5 (coding for residues 17 to 369 of Eg5) fused to a His_6 tag at the C terminus was prepared by PCR using $pEg5_{1\text{-}369}$ and the primer pair #1 and #2.[5] To construct an expression plasmid of the Eg5 motor domain, the amplified cDNA was inserted between the *Nde* I and *Xba* I sites of pCold III (Takara Bio Inc.) to generate $pEg5_{17\text{-}369}$, which expressed the Eg5 motor domain prolonged by MNHKVHM at the N-terminus and by QHHHHHH at the C-terminus. The N-terminal extra sequence comes from pCold III vector and works to induce high-yield production of recombinant proteins through enhancing translation initiation.[6,7] The sequence of cDNA prepared by PCR was confirmed by DNA sequencing.

Protein Preparation of the Eg5 Construct. $Eg5_{17\text{-}369}$ was expressed in *Escherichia coli* BL21(DE3) CodonPlus RIL (Stratagene). The bacteria strain was transformed with $pEg5_{17\text{-}369}$, and the cells were grown at 37°C in Luria-Bertani media in the presence of 100 μg/mL ampicillin (Nacalai) and induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Nacalai) at 15°C overnight. Harvested cells were resuspended in buffer containing 50 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 1 mM ADP (Sigma), 2 mM $MgCl_2$, 0.2 mM EGTA-NaOH, 5 mM β-mercaptoethanol, 10% (w/v) sucrose, 25 mM imidazole, and the protease inhibitor cocktail (Roche), and disrupted by sonication. The cell lysate was centrifuged and the supernatant was loaded onto a 1 mL Ni-NTA Agarose resin (Qiagen) equilibrated with a buffer containing 20 mM Tris-HCl (pH 8.0), 0.3 M NaCl, 1 mM ADP, 2 mM $MgCl_2$, 5 mM β-mercaptoethanol, 10% (w/v) sucrose, and 20 mM imidazole. After washing with the same buffer, the proteins were eluted with a buffer containing 500 mM imidazole, 50 mM piperazine-1,4-bis(2-ethanesulfonic acid)(PIPES)-NaOH (pH 6.8), 0.1 M NaCl, 1 mM ADP, 2 mM $MgCl_2$, 5 mM β-mercaptoethanol, 10% (w/v) sucrose. The eluted proteins were loaded onto a 1 mL HiTrap SP HP cation-exchange column (GE Healthcare) in an AKTAprime plus system (GE Healthcare) using a buffer containing 50 mM PIPES-NaOH (pH 6.8), 1 mM ADP, 2 mM $MgCl_2$, 1 mM EGTA-NaOH, 1 mM tris(2-carboxyethyl)phosphine (TCEP)-HCl, and 5% (w/v) sucrose, and then eluted with a linear gradient from 0 to 1.0 M NaCl. The resultant protein was concentrated using a Vivaspin-20 centrifugal concentrator (Sartorius) with a 10 kDa molecular-mass cut-off. Samples (about 20 mg/ml) were stored at -20°C until use. The concentration

of the protein was determined by the Bradford assay (Bio-Rad).

Supplementary References

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