SUPPORTING INFORMATION

Structural basis of Wee kinases functionality and inactivation by diverse small molecule inhibitors

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Supplementary Table S1: Phosphorylated residues in recombinantly expressed and purified Wee kinases. Mass spectrometric detection and analysis was performed by MS Bioworks. Only residues with Ascore > 20 and localization probability > 0.5 are listed. Bold formatted residues have been reported as phosphorylation sites in UniProt. Grey highlighted residues are sequence conserved, cyan highlighted residues are conserved phosphorylation sites. The kinase domains were expressed and purified from E. coli, the full-length enzymes from Expi293 mammalian cell cultures as described under Methods.

Wee1 full-length

Phosphorylation site	Best Ascore	Localization probability	Corresponding residue in Wee2	Corresponding residue in Myt1
T69	29.21	0.727246	L60	N/A
S139	66.42	1	H87	N/A
S150	1,000.00	1	N/A	N/A
S165	1,000.00	1	N/A	L2
T173	1,000.00	1	Q97	M10
T190	168.61	1	T112	I26
S193	74.63	1	T115	P29
S196	53.69	1	S118	F32
S204	20.98	0.984283	G126	S40
S205	24.95	0.993646	K127	L41
S211	30.97	0.999994	P133	L47
S212	24.95	0.996813	S134	S48
S262	51.21	0.999985	N/A	A88
S270	72.02	1	A183	P95
S312	52.6	0.999995	T225	E123
Y325	1,000.00	1	Y238	Y136
S337	1,000.00	1	L250	P148
S444	70.21	1	E363	N/A
S471	21.95	0.999898	N388	G259
S472	23.19	0.995229	K389	T260
S480	84.28	1	S397	P271
S559	1,000.00	1	S476	T349
S573	18.44	0.985879	L490	R363
S576	41.59	0.999861	T493	A375
S642	41.11	0.999845	S557	R459

Wee1 kinase domain

Phosphorylation site	Best Ascore	Localization probability	Corresponding residue in Wee2	Corresponding residue in Myt1
None				

Supplementary Table S1 (continued)

Wee2 full-length

Phosphorylation site	Best Ascore	Localization probability	Corresponding residue in Wee1	Corresponding residue in Myt1
S123	98.4	1	I201	P37
Y227	60.18	1	F314	F125
S522	71.68	1	A606	T412
S552	1,000.00	1	M637	S454
S557	61.94	1	S642	R459

Wee2 kinase domain

Phosphorylation site	Best Ascore	Localization probability	Corresponding residue in Wee1	Corresponding residue in Myt1
Y227	60.18	1	F314	F125

Myt1 kinase domain

Phosphorylation site	Best Ascore	Localization probability	Corresponding residue in Wee1	Corresponding residue in Wee2
S89	24.95	0.999899	C263	N/A
Y97	46.13	0.999999	Y272	P185
S120	1,000.00	1	E309	E222
Y121	1,000.00	1	F310	F223
Y136	1,000.00	1	Y325	Y238
S141	59.45	1	S330	S243
S160	1,000.00	1	A349	A262
T260	1,000.00	1	S472	K389
Y319	273.9	1	R530	N447
T325	83.7	1	P535	P452

Supplementary Table S2: ITC experimental conditions and thermodynamic parameters of Wee kinases interactions with small molecule inhibitors.

Protein	[P] (μM)	Ligand	[L] (μM)	T (°C)	Kd (nM)	N	ΔH (kcal/mol)	-TΔS	ΔG (kcal/mol)
Wee1	30	PD166285	300	15	11.0 ± 1.6	0.91	-4.50	-5.99	-10.50
	30	MK1775	300		13.4 ± 1.2	0.85	-4.72	-5.65	-10.36
	30	PHA-848125	300		13.6 ± 2.0	0.91	-6.99	-3.37	-10.36
	30	Bos.isomer	300		43.7 ± 10.0	0.99	-6.07	-3.63	-9.70
	30	Bosutinib	300		77.5 ± 6.0	0.95	-5.71	-3.69	-9.39
	30	PF-03814735	300		78.7 ± 8.3	1.08	-4.07	-5.30	-9.37
	30	Pelitinib	300		172 ± 22.3	0.98	-2.65	-6.25	-8.91
	30	Dasatinib	750		5700 ± 314	1.14	-2.25	-4.67	-6.91
	30	Saracatinib	300		NB	NB	NB	NB	NB
Wee2	20	PD166285	200	25	5.0 ± 0.8	0.66	-14.24	2.91	-11.33
	20	MK1775	200		27.0 ± 3.6	0.89	-10.38	0.06	-10.32
	20	PHA-848125	200		26.6 ± 4.2	0.87	-7.25	-3.10	-10.35
	20	Bos. isomer	200		4.7 ± 2.3	0.86	-12.89	1.53	-11.36
	20	Bosutinib	200		106 ± 4.3	0.88	-12.40	2.89	-9.51
	20	PF-03814735	200		128 ± 17.3	0.91	-6.90	-2.51	-9.41
	20	Pelitinib	300		498 ± 48.3	0.98	-8.03	-0.57	-8.60
	20	Dasatinib	200		481 ± 30.5	0.77	-10.16	1.55	-8.61
	20	Saracatinib	300		NB	NB	NB	NB	NB
Myt1	20	PD166285	200	25	247 ± 58.1	0.94	-8.76	-0.25	-9.01
	20	MK1775	200		324 ± 27.3	0.75	-15.95	7.10	-8.85
	20	PHA-848125	300		990 ± 55.2	0.85	-7.92	-0.27	-8.19
	20	Bos. isomer	300		444 ± 41.0	0.93	-10.21	1.54	-8.67
	20	Bosutinib	200		270 ± 17.2	0.78	-15.56	6.59	-8.97
	20	PF-03814735	200		NB	NB	NB	NB	NB
	20	Pelitinib	300		1105 ± 82.9	0.96	-7.91	-0.22	-8.13
	20	Dasatinib	200		150 ± 9.1	0.81	-9.26	-0.05	-9.31
	20	Saracatinib	300		2817 ± 144.4	0.65	-12.20	4.62	-7.58

Supplementary Table S3: Co-crystallization conditions for the kinase domains of Wee1, Wee2 and Myt1 with small molecule inhibitors.

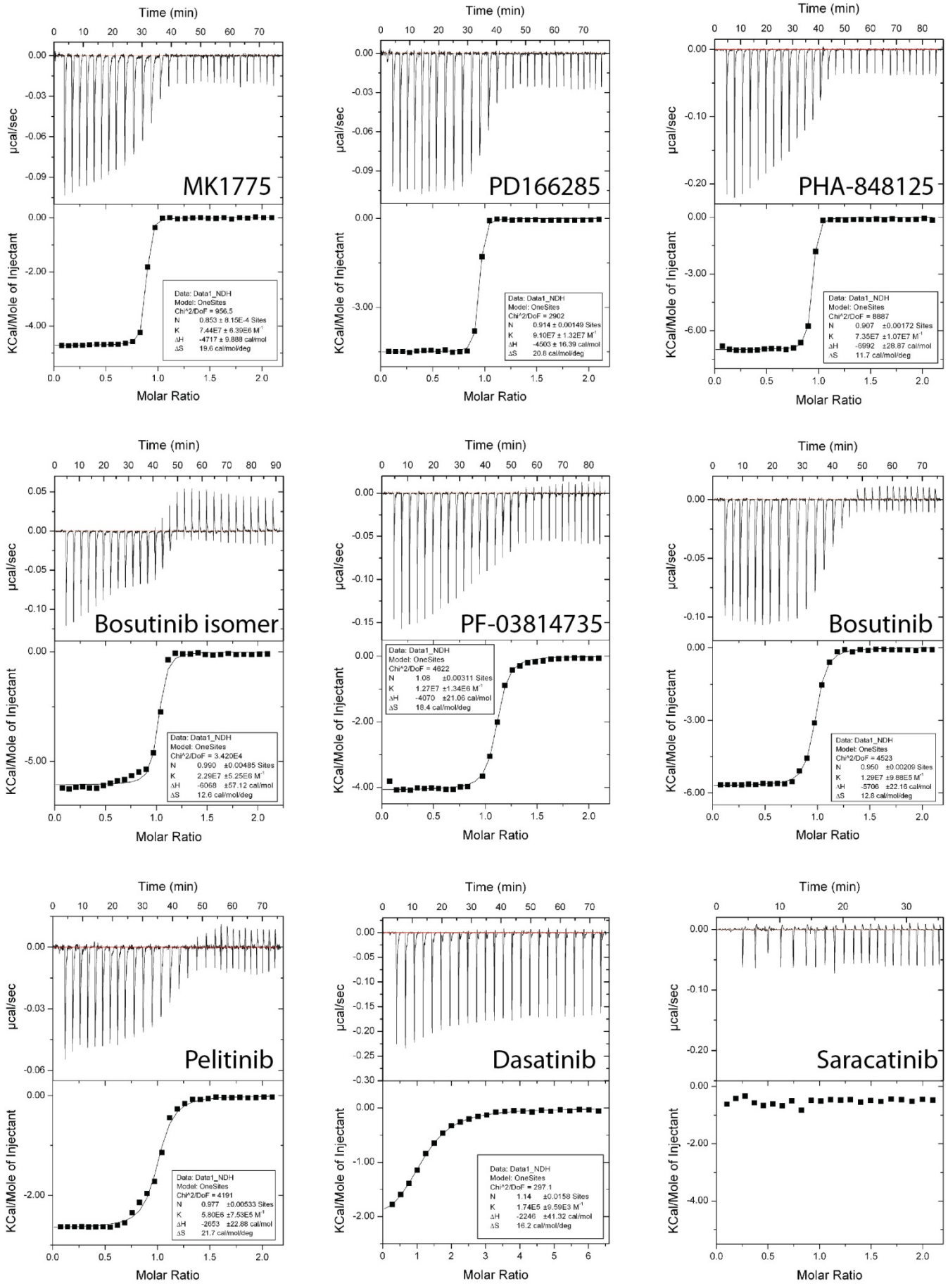
Protein	Protein Buffer	[Protein] (mg mL^{-1})	Ligand	Reservoir solution
Wee1	50 mM Na/K phosphate (pH 6.9), 2 mM DTT	18.0	MK1775 2 mM	0.2 M ammonium sulfate, 0.1 M Bis-tris (pH 5.5), and 25 % PEG 3350
		10.0	PD166285 1 mM	0.1 M ammonium sulfate, 0.1 M Bis-tris (pH 5.5), and 15 % PEG 3350
			PHA-848125 1 mM	
			Bosutinib isomer, 1 mM	
			Bosutinib 1 mM	
			PF-03814735 1 mM	
Wee2	50 mM Tris (pH 8.0), 2 mM DTT		MK1775 2 mM	0.4 M ammonium phosphate monobasic
Myt1	50 mM HEPES (pH 7.5), 300 mM NaCl and 1 mM DTT	13.8	Dasatinib 1 mM	0.2 M calcium chloride, 0.1 M Tris (pH 8.5), and 20% PEG 4,000
			Saracatinib 2 mM	
			Pelitinib 2 mM	0.1 M MES (pH 6.5), and 1.6 M magnesium sulfate
LAMBD treated Myt1	50 mM HEPES (pH 7.5), 2 mM DTT	23.5	MK1775 2 mM	0.1 M potassium chloride, 0.05 M HEPES (pH 7.5), and 35% (v/v) pentaerythritol propoxylate (5/4 PO/OH)
			PHA-848125 1 mM	
			Bosutinib isomer, 1 mM	
			Bosutinib 1 mM	
			Saracatinib 2 mM	

Supplementary Table S4: X-ray crystal structure data collection and refinement statistics.

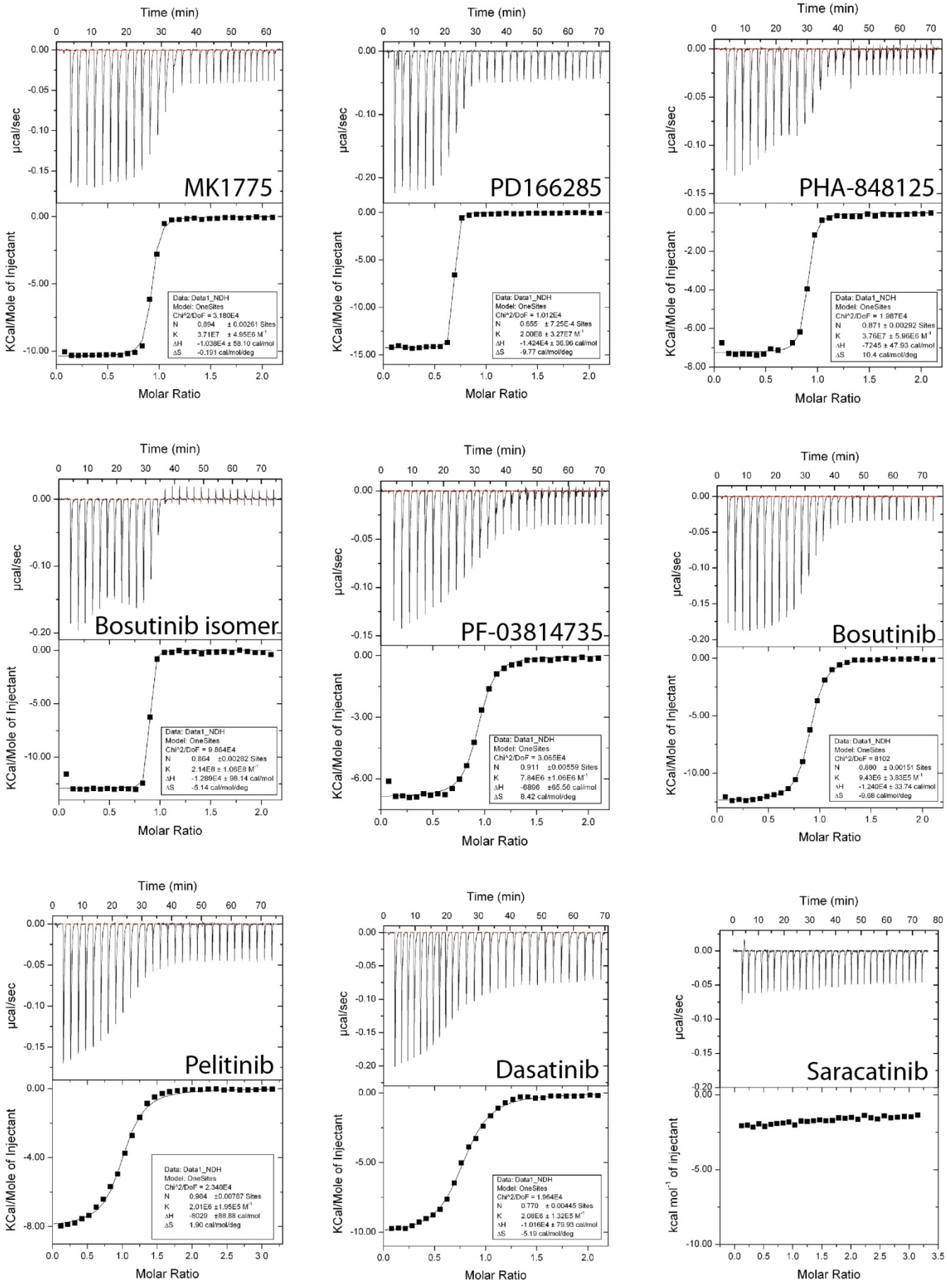
See attached excel sheet

Supplementary Table S5: Kinome profiling results of MK1775 (500 nM single concentration) determined by Discoverx Corp.

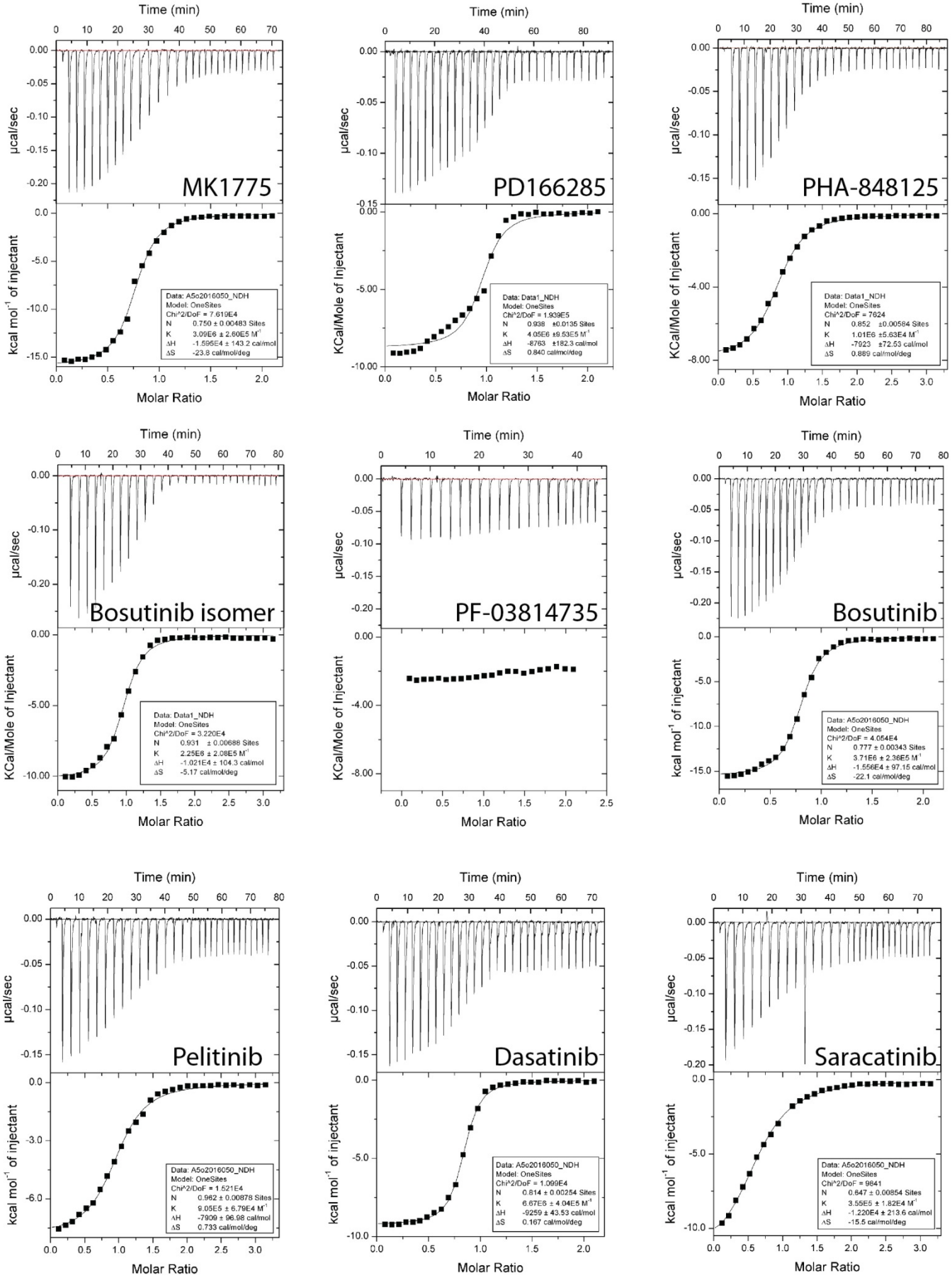
See attached excel sheet



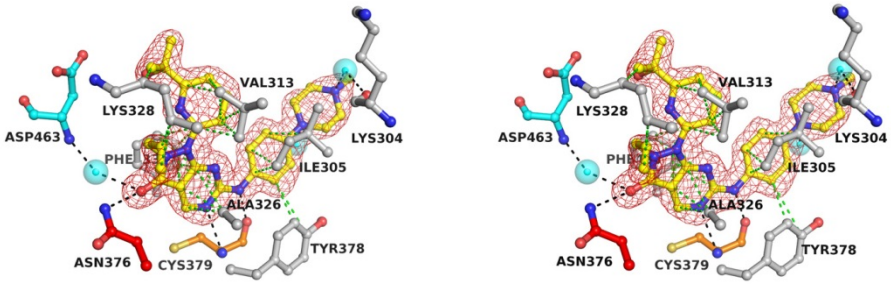
Supplementary Fig. S1: ITC data of Wee1 kinase domain interaction with inhibitors.



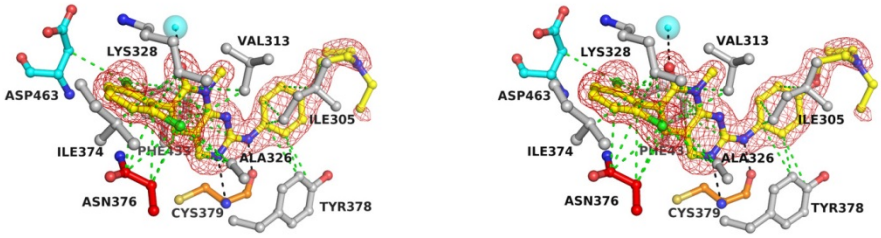
Supplementary Fig. S2: ITC data of Wee2 kinase domain interaction with inhibitors.



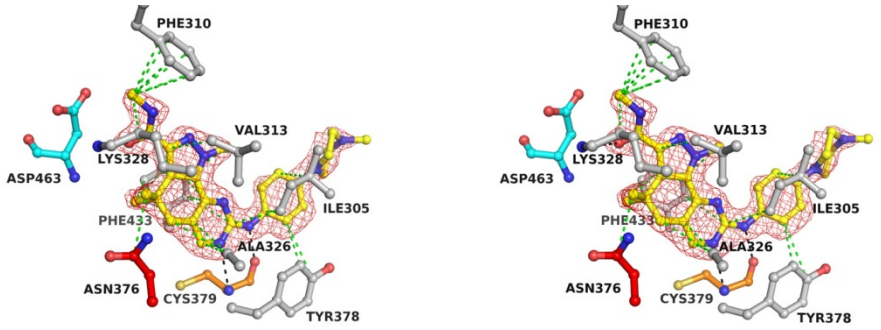
Supplementary Fig. S3: ITC data of Myt1 kinase domain interaction with inhibitors.



MK1775



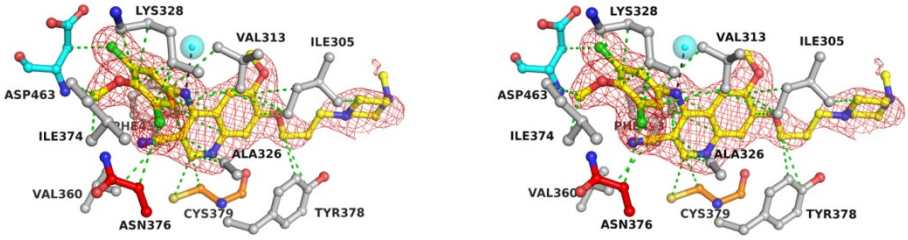
PD-166285



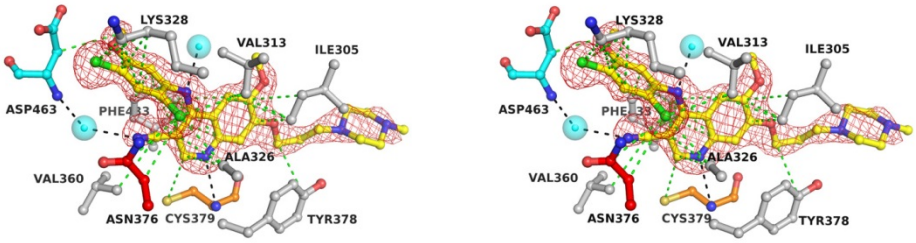
PHA-848125

Supplementary Figure S4: Detailed binding interactions of inhibitors with the ATP binding site of Wee1. Stereo-presentations of the ATP binding site with ligand shown in yellow and the corresponding Fo-Fc electron density map (contoured at 3σ) in red. Protein residues are shown in gray, hinge residue Cys379 in orange, gatekeeper residue Asn376 in red, Asp463 of DLG in cyan and water molecules as cyan spheres. Hydrogen bonding interactions are indicated as black dotted lines and VDW interactions (hydrophobic) interactions as green dotted lines.

Supplementary Fig. S4 (continued)



Bosutinib isomer



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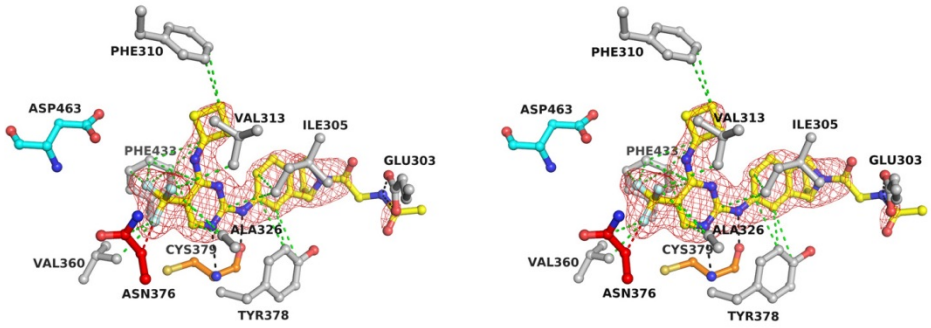

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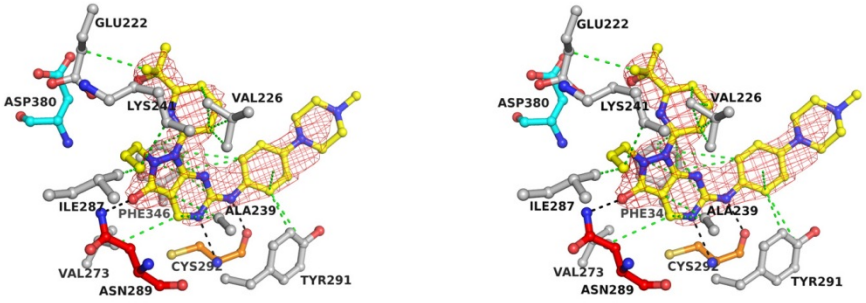
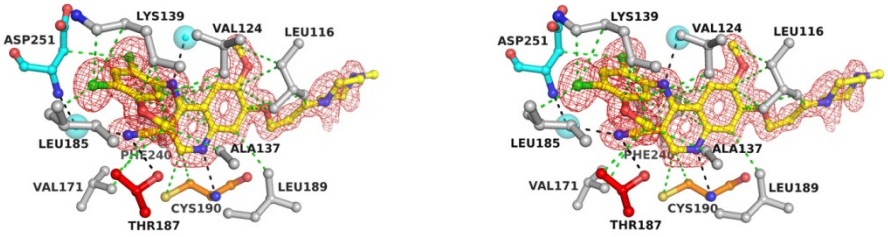
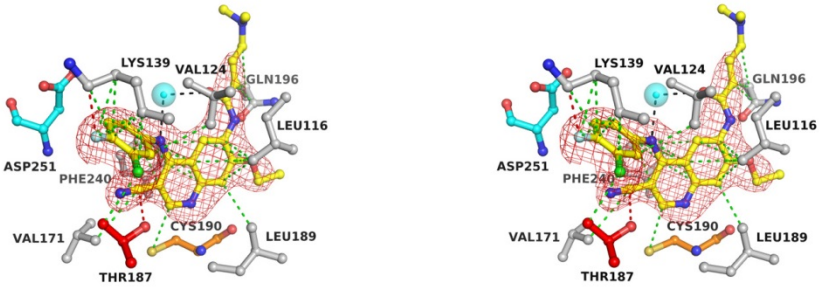


MK1775

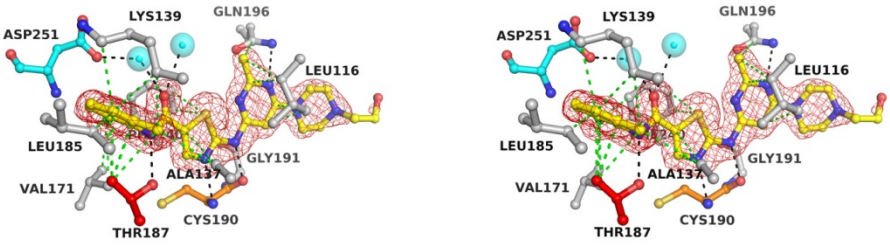
Supplementary Figure S5: Detailed binding interactions of inhibitors with the ATP binding site of Wee2. Stereo-presentations of the ATP binding site with ligand shown in yellow and the corresponding Fo-Fc electron density map (contoured at 3σ) in red. Protein residues are shown in gray, hinge residue Cys292 in orange, gatekeeper residue Asn289 in red, Asp380 of DLG in cyan and water molecules as cyan spheres. Hydrogen bonding interactions are indicated as black dotted lines and VDW interactions (hydrophobic) interactions as green dotted lines.



Bosutinib



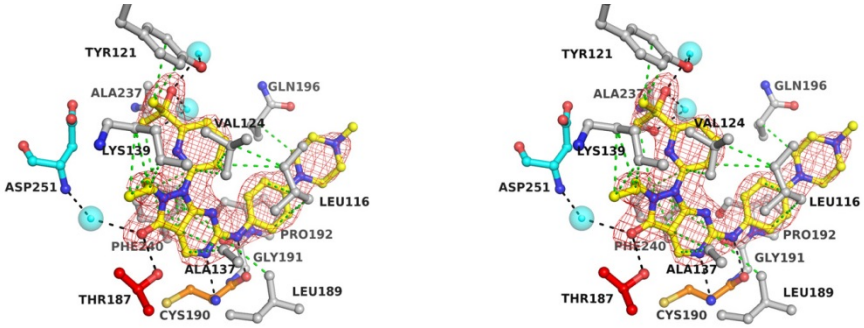
Pelitinib



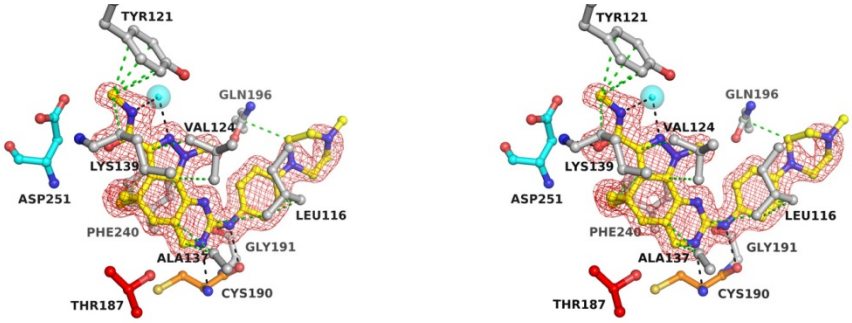
Dasatinib

Supplementary Figure S6: Detailed binding interactions of inhibitors with the ATP binding site of Myt1. Stereo-presentations of the ATP binding site with ligand shown in yellow and the corresponding Fo-Fc electron density map (contoured at 3σ) in red. Protein residues are shown in gray, hinge residue Cys190 in orange, gatekeeper residue Thr187 in red, Asp251 of DFG in cyan and water molecules as cyan spheres. Hydrogen bonding interactions are indicated as black dotted lines and VDW interactions (hydrophobic) interactions as green dotted lines.

Supplementary Fig. S6 (continued)



MK1775



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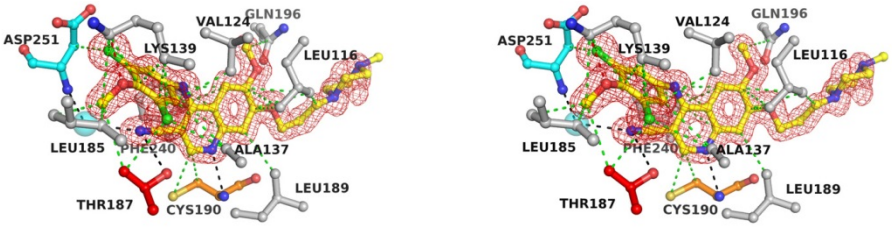
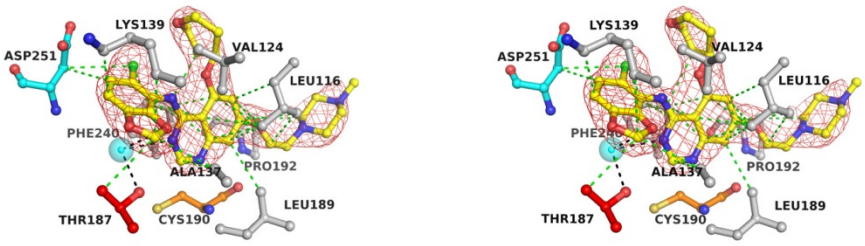
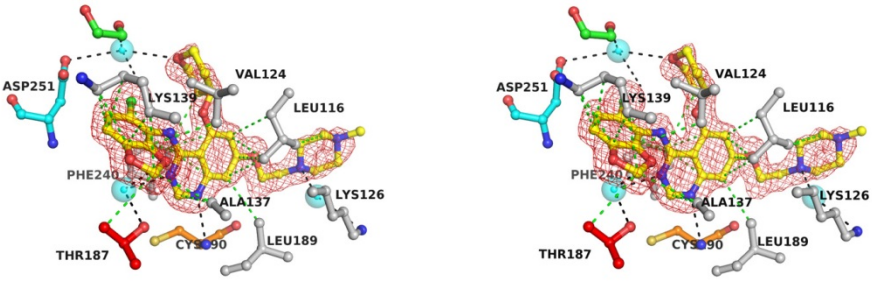


Bosutinib isomer

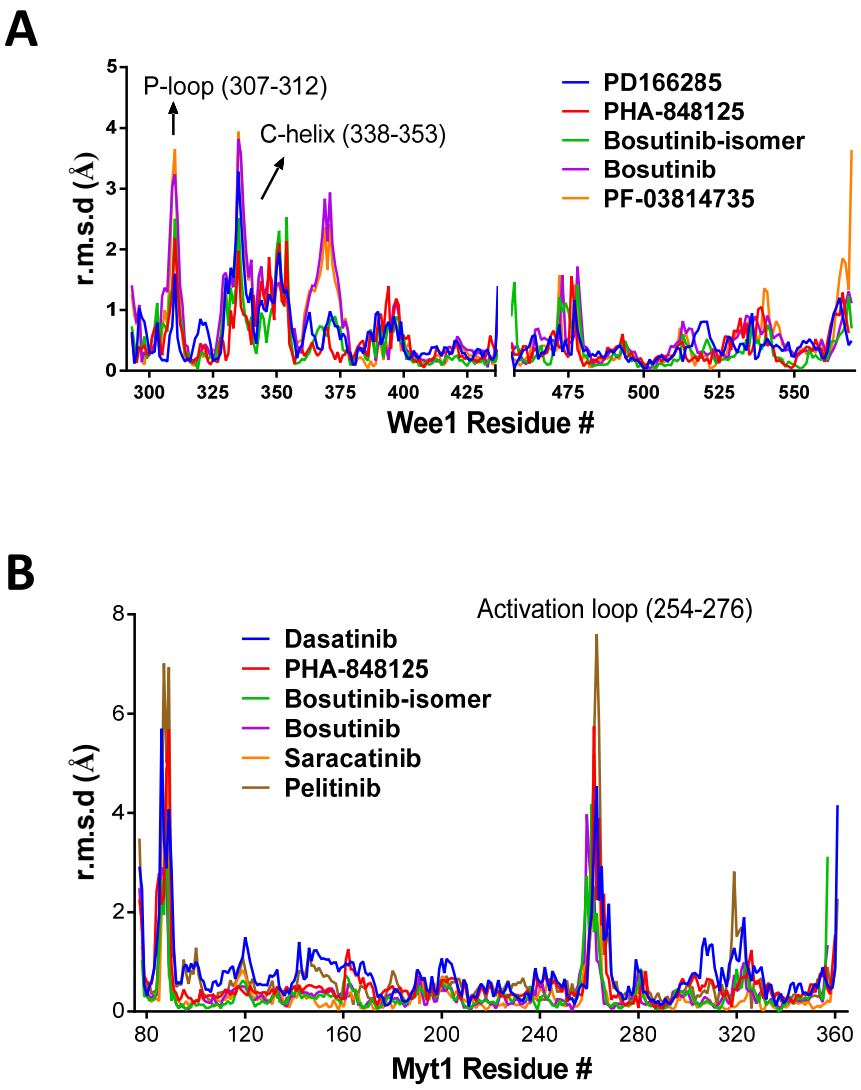
Supplementary Fig. S6 (continued)



Saracatinib with untreated Myt1



Saracatinib with LAMBD-treated Myt1



Supplementary Figure S7: Root mean square deviation plots of Wee1 (A) and Myt1 (B) inhibitor complexes. Shown are the rmsd values of Cα atoms upon superimposition using LSqKabsch of the CCP4 suite. The reference structures were those of the proteins liganded with MK1775.