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Activity- and Affinity- based assay of FMS tyrosine kinase: the impact of kinase phosphorylation state on the activities of various kinase inhibitory compounds.

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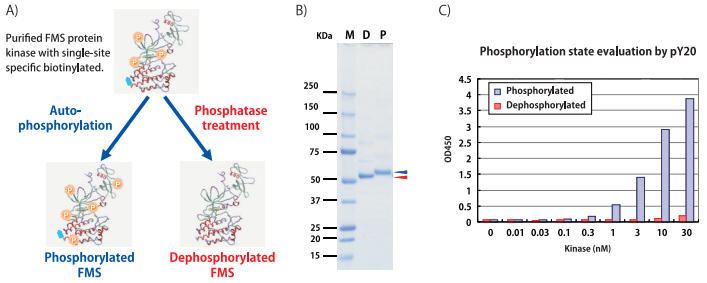
1. Introduction

It is well-known that the sensitivity of certain kinase inhibitors is influenced by the degree at which the kinase is phosphorylated (phosphorylation state). Although it is clear that such inhibitory compounds can discriminate between phosphorylated and nonphosphorylated protein, the biological consequences of this phosphorylation sensitivity are poorly defined. To further understand the relationship between selectivity, efficacy and safety of kinase inhibitors, we investigated effect of kinase protein phosphorylation state on various kinase inhibitors. We generated FMS (also known as CSF1R) tyrosine kinase protein to yield two differently phosphorylated batches; one was highly phosphorylated by its autophosphorylation and the other was dephosphorylated by phosphatase treatment. These two forms were then subjected to evaluation using nine kinase inhibitors in both activity-based and affinity-based assays.

2. Preparation of FMS protein

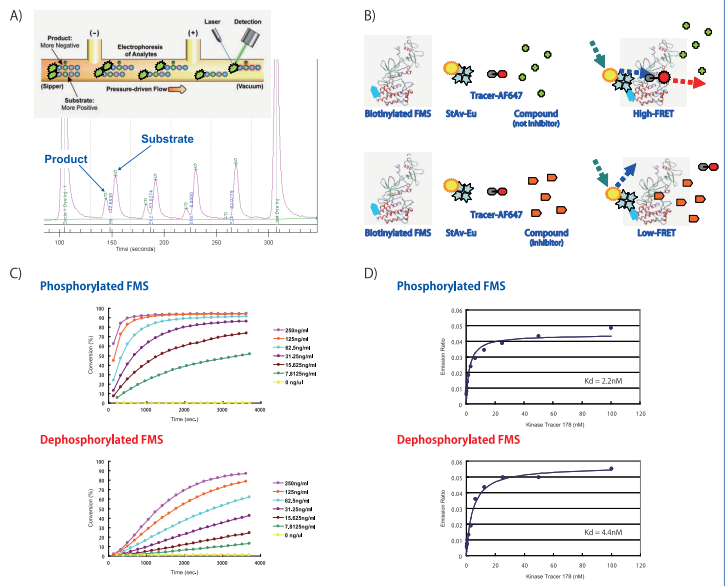


A) **Preparation of FMS kinase.** Cytoplasmic domain of human FMS kinase gene was cloned and expressed as N-terminal His6-tag and C-terminal biotin donor sequence fusion protein using baculovirus expression system. The FMS protein was co-expressed with biotin ligase to add the biotin to the specific donor site in Sf21 cells and purified by metal chelation column chromatography. Resulting specifically biotinylated FMS kinase was divided into two aliquots. One was autophosphorylated and the other was dephosphorylated by treatment with λ-phosphatase.

B) **SDS-PAGE followed by CBB staining.** The band of FMS kinase was slightly shifted upward by autophosphorylation. Lane M, Molecular weight marker. Lane D, phosphatase treated (dephosphorylated) FMS kinase. Lane P, autophosphorylated (highly phosphorylated) FMS kinase.

C) **Determination of phosphotyrosine by ELISA.** Autophosphorylated and λ-phosphatase-treated FMS kinase were immobilized on streptavidin-coated plates. Phosphorylated tyrosine was detected using anti-phospho tyrosine antibody pY20.

3. Assay platform



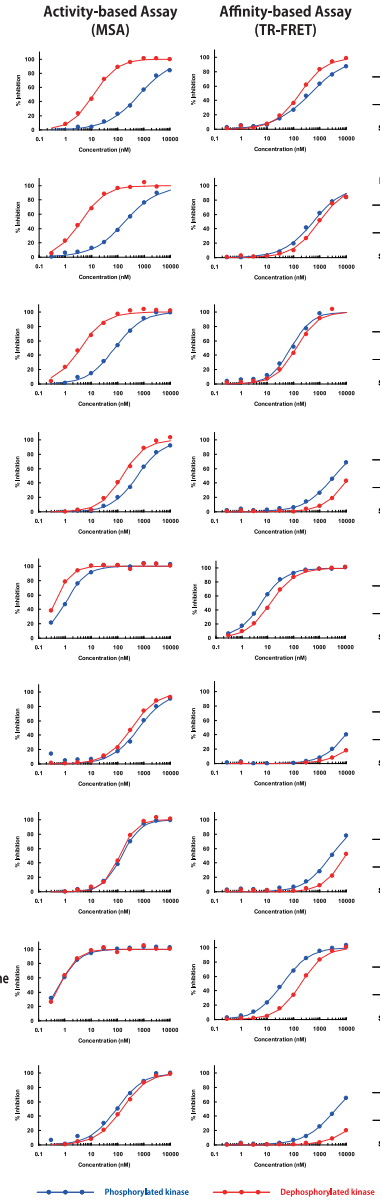
A) **Activity-based assay:** FMS kinase activity was determined by ability to phosphorylate a peptide substrate. The peak ratio of phosphosubstrate to substrate (P/(P+S)) was measured by off-chip mobility shift assay (MSA) using LabChip®3000 (Caliper Life Science).

B) **Affinity-based assay:** Europium-labeled streptavidin (StAv-Eu, FRET donor) is coupled to the FMS kinase through an interaction with its C-terminal biotin. Alexa-Fluor 647-conjugated compound (kinase tracer 178, FRET acceptor) is used as tracer. Binding of the tracer to FMS kinase results in increased TR-FRET signal between the fluorophores and the tracer.

C) **Kinase activity of autophosphorylated and dephosphorylated FMS.** The activity of the autophosphorylated FMS was about 5-times stronger than that of dephosphorylated FMS by continuous monitoring the production of phospho-peptides. The assay was performed at the ATP concentration of 1mM.

D) **TR-FRET signal of autophosphorylated and dephosphorylated FMS.** Both types of FMS kinase showed the similar TR-FRET signal.

4. Results



	Activity		Affinity	
GW2580	Phos-phorylated	Dephos-phorylated	Phos-phorylated	Dephos-phorylated
IC_{50} (nM)	676	12.0	445	175
Standardized by Staurosporine	1060	18.2	11.4	0.92

	Activity		Affinity	
Pazopanib	Phos-phorylated	Dephos-phorylated	Phos-phorylated	Dephos-phorylated
IC_{50} (nM)	189	3.95	570	957
Standardized by Staurosporine	295	5.98	14.6	5.04

	Activity		Affinity	
Sunitinib	Phos-phorylated	Dephos-phorylated	Phos-phorylated	Dephos-phorylated
IC_{50} (nM)	78.9	3.96	79.7	125
Standardized by Staurosporine	123	6.0	2.04	0.66

	Activity		Affinity	
Imatinib	Phos-phorylated	Dephos-phorylated	Phos-phorylated	Dephos-phorylated
IC_{50} (nM)	563	151	3690	>10000
Standardized by Staurosporine	880	229	94.6	N.D.

	Activity		Affinity	
Dasatinib	Phos-phorylated	Dephos-phorylated	Phos-phorylated	Dephos-phorylated
IC_{50} (nM)	1.06	0.42	5.70	12.8
Standardized by Staurosporine	1.66	0.64	0.15	0.067

	Activity		Affinity	
PP2	Phos-phorylated	Dephos-phorylated	Phos-phorylated	Dephos-phorylated
IC_{50} (nM)	640	347	>10000	>10000
Standardized by Staurosporine	1000	526	N.D	N.D.

	Activity		Affinity	
Sorafenib	Phos-phorylated	Dephos-phorylated	Phos-phorylated	Dephos-phorylated
IC_{50} (nM)	142	118	2730	9380
Standardized by Staurosporine	222	179	70	49.4

	Activity		Affinity	
Stauro-sporine	Phos-phorylated	Dephos-phorylated	Phos-phorylated	Dephos-phorylated
IC_{50} (nM)	0.64	0.66	39.0	190
Standardized by Staurosporine	1	1	1	1

	Activity		Affinity	
PD173074	Phos-phorylated	Dephos-phorylated	Phos-phorylated	Dephos-phorylated
IC_{50} (nM)	92.1	145	4330	>10000
Standardized by Staurosporine	144	220	111	N.D.

A) ~ I) Potency of inhibitors toward two types of FMS tyrosine kinase was assessed by MSA and TR-FRET competition assay. IC_{50} values were calculated from concentration vs. %Inhibition curves by fitting to a four-parameter logistic curve. Each point is an average value of at least two independent experiments.

Some compounds (GW2580, pazopanib, and sunitinib) showed different potency between phosphorylated and dephosphorylated FMS in MSA.
TR-FRET assay was insensitive to the difference of phosphorylation state of FMS.

5. Conclusions

We have successfully generated single-site specific biotinylated FMS kinase using the baculovirus expression system, that can be easily adapted for use in affinity-based assay systems.

Dephosphorylation of FMS resulted in an inactive form that possessed approximately one fifth activity of phosphorylated FMS.

Surprisingly, GW2580, pazopanib, and sunitinib showed strong inhibitory activity toward the dephosphorylated FMS compared to phosphorylated FMS, however, such a difference was not observed in TR-FRET.

Further study on the effects of phosphorylation state on potency using SPR methods will be in due course.

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