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Characterization of kinase inhibitors using different phosphorylation states of colony stimulating factor-1 receptor tyrosine kinase

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It is known that some kinase inhibitors are sensitive to the phosphorylation state of the kinase, and therefore those compounds can discriminate between a phosphorylated and unphosphorylated protein. In this study, we prepared two colony stimulating factor-1 receptor (CSF-1R) tyrosine kinase proteins: one highly phosphorylated by autophosphorylation and the other dephosphorylated by phosphatase treatment. These kinases were subjected to an activity-based assay to investigate the effect of their phosphorylation state on the potency of several kinase inhibitors. Dasatinib, sorafenib, PD173074 and staurosporine showed similar inhibition against different phosphorylation states of CSF-1R, but pazopanib, sunitinib, GW2580 and imatinib showed more potent inhibition against dephosphorylated CSF-1R. Binding analysis of the inhibitors to the two different phosphorylation forms of CSF-1R, using surface plasmon resonance spectrometry, revealed that staurosporine bound to both forms with similar affinity, but sunitinib bound to the dephosphorylated form with higher affinity. Thus, these observations suggest that sunitinib binds preferentially to the inactive form, preventing the activation of CSF-1R. Screening against different activation states of kinases should be an important approach for prioritizing compounds and should facilitate inhibitor design.

Keywords: CSF-1R/drug design/kinase inhibitor/phosphorylation/tyrosine kinase.

Abbreviations: CML, chronic myeloid leukaemia; CSF-1, colony stimulating factor-1; CSF-1R, colony stimulating factor-1 receptor; D-CSF-1R, dephosphorylated CSF-1R; P-CSF-1R, phosphorylated CSF-1R; RTK, receptor tyrosine kinase; Sf, *Spodoptera frugiperda*; VEGFR, vascular endothelial growth factor receptor; λPPase, lambda phosphatase.

There are 518 kinases encoded in the human genome including serine/threonine, tyrosine and dual specific kinases (*1*, *2*). Due to the pivotal roles in virtually all aspects of cellular physiology, the dysregulation of kinase activity-related signalling is involved in many kinds of diseases, such as cancer, inflammation and neurodegeneration (*3*, *4*). Therefore, protein kinases have become one of the most important target classes for drug discovery (*5*). So far, 10 small-molecular weight tyrosine kinase inhibitors have been approved for cancer treatment and >100 kinase inhibitors are at present in clinical development (*6–8*). The majority of these inhibitors compete with ATP on binding to the ATP-binding pocket. Due to the entity of over 500 protein kinases in the human genome and the structural similarity of the kinase ATP sites, generally they inhibit multiple kinases simultaneously (*2*).

To understand the efficacy and side effects of the kinase inhibitors, it is important to know their target and off-target kinases (*9*, *10*). Many tyrosine kinases sample diverse conformations between active and inactive forms, which are regulated by phosphorylation and dephosphorylation in physical or pathological conditions. It is known that some kinase inhibitors are sensitive to the phosphorylation state of the kinase, and therefore those compounds can discriminate between phosphorylated and dephosphorylated proteins (*11*, *12*). However, the biological consequences of this phosphorylation sensitivity are poorly defined. Profiling against different phosphorylation states of kinases should be an important approach for better understanding the efficacy of kinase inhibitors and prioritizing them. In this study, we addressed colony stimulating factor-1 receptor (CSF-1R) for the profiling.

CSF-1R is a type III receptor tyrosine kinase (RTK), encoded by the *c-fms* proto-oncogene, and it is the exclusive receptor for macrophage colony stimulating factor 1 (CSF-1) (*13*). Together with CSF-1, CSF-1R regulates proliferation, differentiation and survival of cells of the mononuclear phagocyte lineage and prostate development (*14–17*). CSF-1 binding to the CSF-1R extracellular domain induces dimerization and trans-autophosphorylation of the intracellular CSF-1R kinase domain on several tyrosine residues (*18*). RTKs, including CSF-1R contain activation loop,

comprise a string of ~22 amino acids that begins with a protein kinase-conserved Asp-Phe-Gly (DFG) motif and ends with a Pro that is conserved among tyrosine kinases (*19*). At least one phosphorylation site resides at centre of activation loop, and its phosphorylation stimulates an outward movement of the activation loop. This movement induces the rotating of the DFG motif into proper orientation (DFG-in) for catalysis (*20*). Tyr-809 is a unique tyrosine residue in the activation loop of CSF-1R, and its phosphorylation is known to be important for kinase activity (*21*). The protein structures of active (DFG-in) and inactive (DFG-out) forms of the CSF-1R kinase domain have been reported (*22–24*). In order to evaluate the inhibitory potencies of protein inhibitors against the active and inactive forms of CSF-1R, we prepared two different CSF-1R tyrosine kinase proteins: one highly phosphorylated by autophosphorylation and the other dephosphorylated by phosphatase treatment. And then we established the kinase assay using these two types of CSF-1R.

The clinically relevant RTK inhibitors imatinib, dasatinib, pazopanib, sorafenib and sunitinib, and the well known kinase inhibitors staurosporine, GW2580 and PD173074 (*25*) are known to associate with CSF-1R (*9*). Imatinib and dasatinib are used for treatment of Philadelphia chromosome-positive chronic myeloid leukaemia (CML) with the chimeric gene BCR-ABL and KIT-positive gastrointestinal stromal tumour through inhibition of tyrosine kinases (*26*, *27*); dasatinib is used for treatment of imatinib-resistant or -intolerant CML patients (*28*). Imatinib has been demonstrated to have potent inhibition only against the inactive (dephosphorylated) form of ABL (*29*), but dasatinib shows potent inhibition also against the active form of ABL (*30*). Sorafenib, sunitinib and pazopanib are used for treatment of patients with advanced renal cell carcinoma through inhibition of multiple RTKs, including vascular endothelial growth factor receptor (VEGFR) tyrosine kinases, which are involved in aberrant tumour angiogenesis (*31–33*). In this study, we investigated the effects of these kinase inhibitors on dephosphorylated and hyperphosphorylated forms of CSF-1R.

Materials and Methods

Reagents

Staurosporine and GW2580 were purchased from Calbiochem (San Diego, CA, USA), PD173074 was from Tocris (Bristol, UK), and pazopanib was from LC laboratories (Woburn, MA, USA). Sunitinib (*34*), dasatinib (*35*) and sorafenib (*36*) were synthesized at Carna Biosciences, Inc (Kobe, Japan). Imatinib mesylate (imatinib) was extracted from its pharmaceutical capsule. Triton X-100 and HEPES were purchased from Sigma-Aldrich (MO, USA), and the other reagents were from Wako Pure Chemical Industries (Osaka, Japan). FITC-labelled peptide substrate was purchased from Peptide Institute (Osaka, Japan).

Plasmid construction

The regions encoding the cytoplasmic domain of human CSF-1R (NM_005211.3, amino acids 538-972) fused with N-terminal $His_{\times 6}$-tag and C-terminal biotin-accepting peptide, and BirA biotin-protein ligase (*37*) were subcloned into pFastBAC dual (Invitrogen, Carlsbad, CA, USA). The recombinant bacmid DNA was prepared according to the instructions for the Bac-to-Bac baculovirus expression system (Invitrogen) and transfected to *Spodoptera frugiperda* (Sf) 9 insect cells to amplify the recombinant baculovirus. The titre of amplified baculovirus was determined by BacPAK Baculovirus Rapid Titer Kit (Takara, Shiga, Japan).

Protein expression and purification

To express CSF-1R, Sf21 cells (2×10^6 cells/ml) in Grace's insect media supplemented with 10% FCS were infected with the recombinant baculovirus at a multiplicity of infection of three and cultured for 48 h at 27°C. The cells were harvested, washed with cold PBS buffer and stored at −80°C until purification. The frozen cells were thawed and lysed in lysis buffer (50 mM Tris–HCl, pH 7.5, 300 mM NaCl, 10 mM imidazole, 1% Nonidet P-40, 5 mM DTT, 1 mM phenylmethanesulfonyl fluoride, 2 μg/ml leupeptin and 2 μg/ml aprotinin) on ice. All purification procedures thereafter were carried out at 4°C. The cell lysate was clarified by centrifugation at 9,000 *g* for 20 min and mixed with Ni-NTA Superflow resins (Qiagen, Tokyo, Japan). The lysate–resin mixture was packed in a column and washed with five volumes of wash buffer (50 mM Tris–HCl, pH 7.5, 1 M NaCl, 20 mM imidazole and 10% glycerol). CSF-1R was eluted with elution buffer (50 mM Tris–HCl, pH 7.5, 300 mM NaCl, 250 mM imidazole and 10% glycerol), and the CSF-1R-containing fractions were pooled. The eluted protein was divided into aliquots: one was autophosphorylated by incubation with 3 mM ATP and 10 mM $MgCl_2$ at 4°C overnight, and another was dephosphorylated by incubation with 10 U/μg·protein lambda phosphatase (λPPase; New England Biolabs, MA, USA) at 4°C overnight. The autophosphorylated CSF-1R (P-CSF-1R) and dephosphorylated CSF-1R (D-CSF-1R) were separated from the ATP and λPPase by chromatography, respectively.

Protein identification

The CSF-1R protein was applied to SDS–PAGE followed by Coomassie brilliant blue (CBB) staining. The band corresponding to CSF-1R was excised and analysed by matrix-assisted laser desorption/ionization reflection time-of-flight (MALDI-TOF) mass spectrometry (MS). The peptide mass analysis of trypsin-digested peptide was performed on autoflex III MALDI-TOF MS (Bruker, Yokohama, Japan) as previously described (*38*). Peptide identification was accomplished using MASCOT Peptide Mass Fingerprinting.

Protein phosphorylation analysis

To analyse the phosphorylation state, CSF-1R protein was digested with Lys-C and trypsin as previously described (*39*, *40*), and the following NanoLC-MS/MS analyses were conducted using an Orbitrap system (LTQ-Orbitrap XL; Thermo Fisher Scientific, Bremen, Germany), Dionex Ultimate3000 pump with FLM-3000 flow manager (Germering, Germany), HTC-PAL autosampler (CTC Analytics, Zwingen, Switzerland), an analytical column needle with 'stone-arch' frit and a PTFE-coated column holder (Nikkyo) (*41*). The digested sample (5 μl) was injected and separated by a three-step gradient (*41*). The spray voltage was 2400 V, the MS scan range was m/z 300–1400 and the top 10 precursor ions were selected for subsequent MS/MS scans. A lock mass function was used for the LTQ-Orbitrap to obtain constant mass accuracy during gradient analysis (*42*). Peptides and proteins were identified by means of automated database searching using Mascot v2.2 (Matrix Science, London, UK) against SwissProt release 2010_06 (18-May-2010) (*43*). Phosphorylation sites were unambiguously determined when b- or y-ions, which were between the existing phosphorylated residues, were observed in the peak list of fragment ions. Quantitative analyses of phosphorylated and non-phosphorylated peptides derived from CSF-1R were carried out by a label-free approach (*44–46*). Phosphorylation stoichiometry was calculated based on relative peak areas of phosphorylated peptides and non-phosphorylated peptides according to the literature (*47*), with the modification that the peak area ratio was estimated with the label-free approach instead of stable isotope labelling by amino acids in cell culture (SILAC) method (*48*).

Activity-based kinase assay

CSF-1R kinase activity was determined by off-chip mobility shift assay (MSA) using LabChip^{TM}3000 (Caliper Life Sciences, Hopkinton, MA, USA). The kinase, FITC-labelled peptide substrate (1 μM) named Srctide (*49*), and compound or vehicle (1% DMSO) were incubated in assay buffer (20 mM HEPES, pH 7.5, 0.01% Triton X-100, 2 mM DTT, 5 mM $MgCl_2$ and 1 mM ATP) at 25°C. The amounts (peak height) of phosphorylated (P) and non-phosphorylated (S) peptide substrates were measured and the

phosphorylation rate of the substrate was defined by P/(P+S). To determine the IC_{50} value, each compound was diluted in DMSO in half-log scale and incubated with CSF-1R kinases for 10 min before the kinase reaction. The kinase reaction (20 µl) was terminated by the addition of 60 µl of termination buffer (127 mM HEPES, pH 7.5, 0.01% Triton X-100, 26.7 mM EDTA-2Na, 1% DMSO and 0.13% Coating-3 Reagent). The inhibition percentage of each compound against kinase activity was determined from the phosphorylation percentage of the substrate and the IC_{50} value was calculated by interpolation on a log-concentration-response curve fitting for four-parameter logistic equation.

Interaction between CSF-1R and kinase inhibitors

The interaction between CSF-1R and the kinase inhibitors was determined by surface plasmon resonance (SPR) using Biacore T100 (GE Healthcare, UK). The instrument running buffer was composed of 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 0.05% Tween-20 and 2% DMSO, which was also used as sample dilution buffer. Immobilization of CSF-1R protein onto a streptavidin-coated sensor chip SA (GE Healthcare) was performed according to the immobilization wizard in the Biacore instrument control software, including the following steps: (i) wash with 50 mM NaOH/1 M NaCl for 30 s, three times; (ii) injection of kinases for 15–20 min at 30 µg/ml in running buffer and (iii) surface blockage with 10 µg/ml EZ-Link™ Biocytin (Thermo Fisher Scientific, Yokohama, Japan). Compounds were dissolved in DMSO at 10 mM, diluted with running buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 0.05% Tween-20 and 2% DMSO) and analysed using a 2-fold dilution series. Interaction analysis cycles consisted of a 60 s sample injection (30 µl/min) followed by 300 s of buffer flow (dissociation phase). All of the bound complexes dissociated back to baseline within a reasonable time frame, and regeneration was required. All sensorgrams were processed by subtracting the binding response recorded from the control surface (centre reference spot), followed by subtracting an average of the buffer blank injections from the reaction spot. To determine the kinetic rate constant, all data sets were fit to a simple 1:1 interaction model, including a term for mass transport using numerical integration and nonlinear curve fitting. Equilibrium analysis was performed by fitting the response at the end of the association phase to a single-site binding isotherm.

Results

Phosphorylation state of CSF-1R

Human recombinant CSF-1R protein was expressed and purified from insect cells, and was subsequently either autophosphorylated by treatment with ATP or dephosphorylated by λPPase. CBB staining of these CSF-1R proteins is shown in Fig. 1A. A prominent band of CSF-1R was apparent at ~55 kDa, and the band of P-CSF-1R showed a slight upward shift from the position of non-treated CSF-1R and D-CSF-1R. To quantify the phosphorylation state of these CSF-1R proteins, LC-MS/MS analysis of tryptic digest of these proteins was carried out. As shown in Table I, the phosphorylation rates of five tyrosine residues (Y561, Y699, Y809, Y873 and Y923) were determined. Y809 is a unique tyrosine residue in the activation loop and considered to be one of the key residues for the kinase activity of CSF-1R (*21*). The phosphorylation rate of this residue in non-treated CSF-1R was 2.2%. In P-CSF-1R, the phosphorylation rate of Y809 was dramatically increased to 61.4%, and in D-CSF-1R it was negligible (0.2%). Tyrosine residues, Y561, Y699 and Y923, had high phosphorylation rates (>90%) in P-CSF-1R and low (<5%) phosphorylation rates in D-CSF-1R. In contrast, the phosphorylation rate of Y873 was increased by only 7% after autophosphorylation.

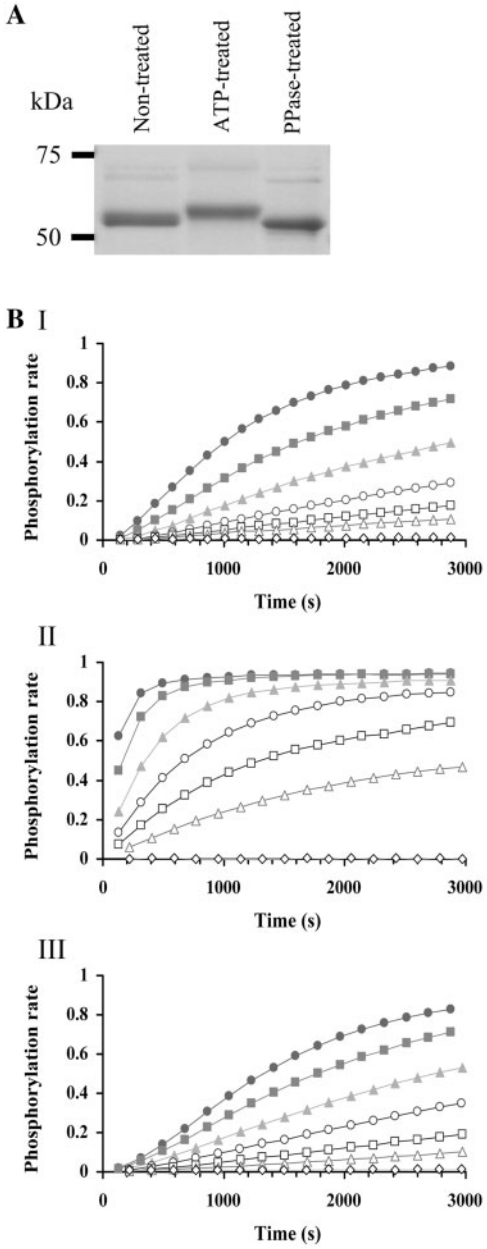

Fig. 1 Phosphorylation state and activity of different preparations of CSF-1R: (A) non-treated, autophosphorylated and λPPase-treated CSF-1R proteins were subjected to SDS–PAGE followed by CBB staining. Migration positions of protein molecular mass standards (kDa) and bands corresponding to the expected protein size are indicated. The autophosphorylated CSF-1R protein showed a slight upward shift compared with the non-treated and λPPase-treated proteins. (B) The kinase activity of (I) non-treated, (II) P- and (III) D-CSF-1R towards the Src peptide was evaluated using the activity-based assay. The ratio of the phosphorylated peptide was measured in a time-series at 1 mM ATP. Regarding non-treated and D-CSF-1R, a slight lag time in the phosphorylation of the peptide was observed at the beginning of the assay. The concentrations of CSF-1R kinases were 250 ng/ml (filled circle), 125 ng/ml (filled square), 62.5 ng/ml (filled triangle), 31.3 ng/ml (open circle), 15.6 ng/ml (open square) and 7.8 ng/ml (open triangle).

Kinase activity of CSF-1R

Analysis of the phosphorylation state of Y809 revealed that P-CSF-1R could be an active form and D-CSF-1R could be an inactive form of the kinase. The kinase activity of these CSF-1R proteins, determined by their

ability to phosphorylate the substrate peptide, was measured using the off-chip MSA. The phosphorylation rate of the substrate was monitored for 50 min from initiation of the kinase reaction (Fig. 1B). P-CSF-1R showed a time- and dose-dependent increase in the phosphorylation rate of the substrate, and its activity was ~10-fold higher than non-treated CSF-1R (Fig. 1B I and II). In contrast, D-CSF-1R showed slightly weaker kinase activities than the non-treated CSF-1R and had a short lag of about 10 min, indicating that D-CSF-1R might be activated to some degree during the kinase reaction (Fig. 1B III). The plots of the initial velocities versus ATP concentrations and fitting to the Michaelis–Menten equation revealed that the K_m value for ATP of P-CSF-1R was 37 μM (Fig. 2A). On the other hand, the velocity of D-CSF-1R could not reach the maximum even at 1,000 μM ATP (Fig. 2B).

CSF-1R kinase inhibition by tyrosine kinase inhibitors

To clarify the preference of eight tyrosine kinase inhibitors for the phosphorylation states of CSF-1R, their inhibitory effects on P- and D-CSF-1R were evaluated in the presence of 1 mM ATP (physiological concentration). P-CSF-1R (8 ng/ml) and D-CSF-1R (40 ng/ml), which could reach ~30% of substrate phosphorylation rate 1 h after initiation of the assay, were used to determine the IC_{50} values. All compounds exhibited concentration-dependent inhibition of both forms of CSF-1R (Fig. 3, Table II). GW2580, pazopanib and sunitinib exhibited a clear preference (>10-fold) for D-CSF-1R, and imatinib tended to prefer D-CSF-1R to P-CSF-1R. Dasatinib, sorafenib, staurosporine and PD173074 inhibited both forms of CSF-1R in a similar manner. These findings indicate that GW2580, pazopanib, sunitinib and imatinib could easily bind to D-CSF-1R and in turn inhibit its activation; however, dasatinib, sorafenib, staurosporine and PD173074 could bind both forms of CSF-1R and inhibit its activity.

Table I. Phosphorylation rate of CSF-1R protein kinase was determined using LC-MS/MS.

	Phosphorylation rate (%)[a]		
	Non-treated	Autophosphorylated	λPPase treated
Y561	15.1	95.8	3.2
Y699	2.3	93.5	0.1
Y809	2.2	61.4	0.2
Y873	2.6	9.7	0
Y923	0.6	94.2	0.1

[a]To quantify the phosphorylation rate of differently prepared CSF-1R proteins, LC-MS/MS analysis of tryptic digest of these proteins was carried out. The phosphorylation rates of five tyrosine residues are indicated.

Binding affinity of sunitinib and staurosporine for CSF-1R

To investigate whether sunitinib has a higher affinity for D-CSF-1R than P-CSF-1R, the interaction of the compounds with CSF-1R protein was determined using SPR-based Biacore T100 technology. As the CSF-1R proteins have a biotinylated peptide sequence at the carboxyl-terminal, it is possible to immobilize the proteins readily onto a sensor chip utilizing the high affinity biotin-streptavidin system. Both D-CSF-1R and P-CSF-1R proteins were immobilized on a streptavidin-coated sensor chip and washed until a stable baseline was achieved. The qualitative plots of the association and dissociation patterns of sunitinib and staurosporine with the CSF-1R protein are shown in Fig 4. To obtain the association and dissociation rate constants, various concentrations of the inhibitors were injected over the CSF-1R surface, ranging from 47 to 3,000 nM for sunitinib and from 3.1 to 300 nM for staurosporine. From the sensorgrams for each compound, association and dissociation rates were determined and K_D values were calculated for sunitinib and staurosporine (Table III). Two independent experiments showed that the average dissociation constant (K_D) of sunitinib was significantly lower for D-CSF-1R (87 nM) compared with P-CSF-1R (320 nM), whereas the K_D value of staurosporine for

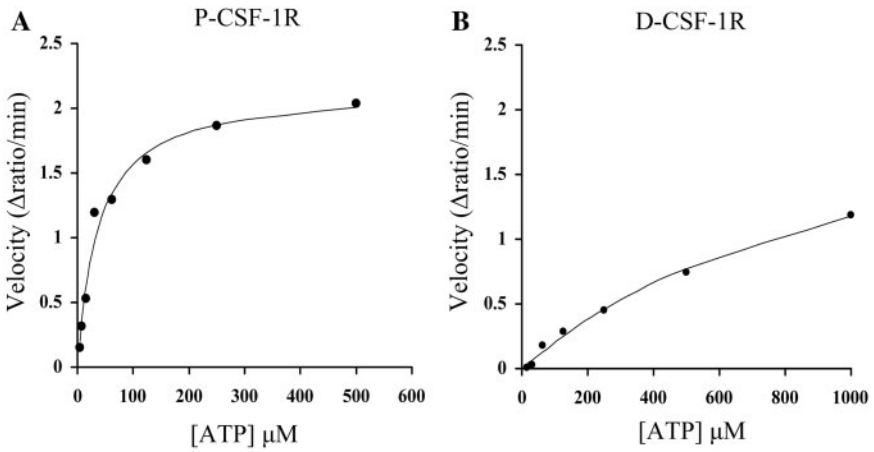

Fig. 2 Michaelis–Menten curves for ATP. (A) P-CSF-1R kinase was assayed at various concentrations of ATP, the initial velocity (Vi) was plotted against the concentration of ATP, and the K_m value was calculated. (B) D-CSF-1R kinase was assayed as described for (A). The concentrations of the CSF-1R proteins were 8 ng/ml for P-CSF-1R and 50 ng/ml for D-CSF-1R.

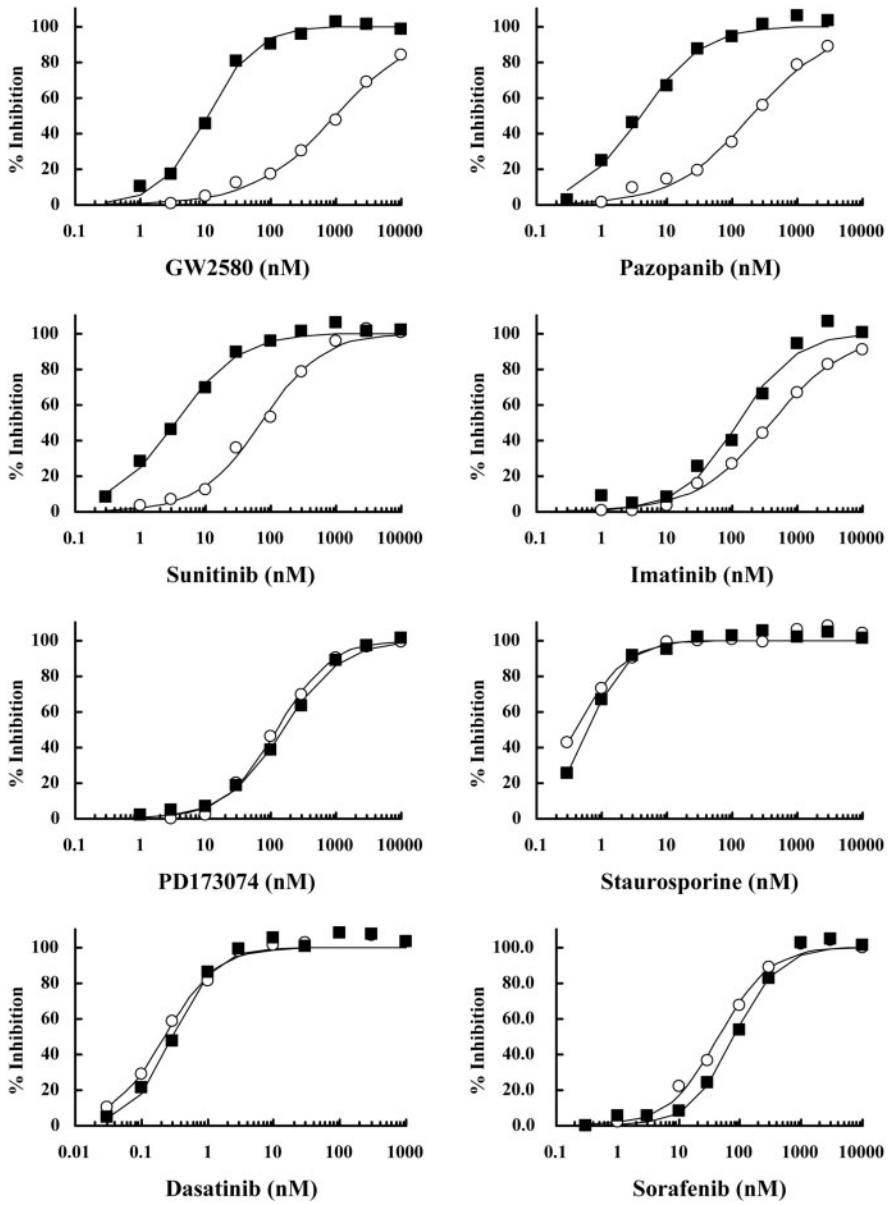

Fig. 3 Potency of tyrosine kinase inhibitors on different phosphorylation states of CSF-1R protein kinases. The kinase reaction was performed in the presence of the indicated concentrations of the inhibitors. Shown here is the inhibition percentage as a function of compound concentration. Data for P-CSF-1R are shown in open circles, and D-CSF-1R in filled squares. Representative curves are shown, and average IC_{50} values from at least two independent experiments are shown in Table II.

Table II. Potency of tyrosine kinase inhibitors measured by the activity-based assay.

	IC_{50} (nM) against CSF-1R[a]	
Compound name	**P-CSF-1R**	**D-CSF-1R**
GW2580	1100	11
Pazopanib	230	4.2
Sunitinib	79	3.8
Imatinib	370	110
PD173074	120	170
Staurosporine	0.44	0.74
Dasatinib	0.18	0.33
Sorafenib	43	91

[a]IC_{50} values (nM) of eight tyrosine kinase inhibitors against two different phosphorylation forms of CSF-1R tyrosine kinase are reported. The values indicate the IC_{50} values from experiments with a 10-min pre-incubation of compound and kinase. Each value is an average of at least two independent experiments.

D-CSF-1R (43 nM) was barely different from that of P-CSF-1R (41 nM). These results indicate that sunitinib has a higher affinity for D-CSF-1R, which is consistent with the results from the activity-based kinase assay.

Discussion

It is known that some kinase inhibitors are sensitive to the phosphorylation state of the kinase, and therefore those compounds can discriminate between a phosphorylated and unphosphorylated protein. Imatinib has been reported to preferentially inhibit the unphosphorylated form of ABL (*50*). This observation is explained by the fact that imatinib preferentially binds to the DFG-out conformation of ABL due to association

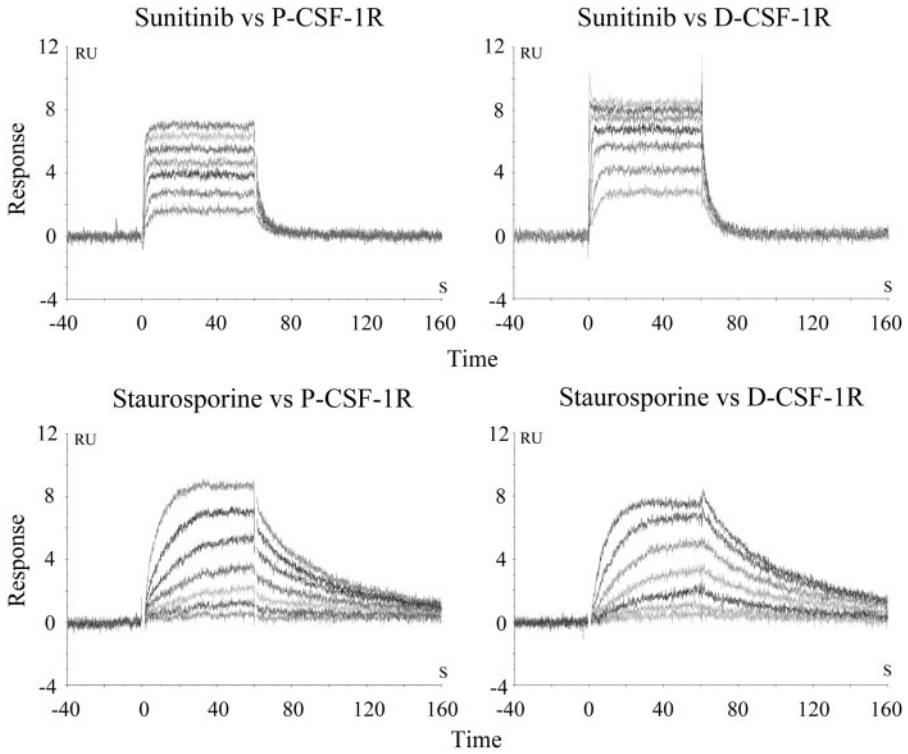

Fig. 4 Representative Biacore sensorgrams of sunitinib binding to D-CSF-1R and P-CSF-1R. To determine the affinities and kinetic constants, inhibitor concentrations above and below the K_D value were injected over immobilized CSF-1R. Sensorgrams of P-CSF-1R are shown in the left column and of D-CSF-1R in the right column. Staurosporine (3.1–300 nM) and sunitinib (47–3000 nM) were injected.

Table III. K_D values (nM) and kinetic rate constants of compounds were determined using Biacore T100.

Kinase	Inhibitor	k_a (1/Ms)	k_d (1/s)	K_D (nM)[a]	Assay conc. (nM)
P-CSF-1R	Sunitinib	1.5×10^6	0.47	320 (420)	47–3000
P-CSF-1R	Staurosporine	6.1×10^5	0.018	30 (53)	3.1–200
D-CSF-1R	Sunitinib	3.0×10^6	0.20	68 (100)	47–3000
D-CSF-1R	Staurosporine	3.6×10^5	0.016	44 (62)	4.7–300

[a]K_D values (nM) were calculated by kinetics analysis of the sensorgrams shown in Fig. 3. The values in parenthesis indicate the K_D values from affinity analysis.

with the allosteric pocket adjacent to the ATP binding site. This pocket is made accessible by the activation-loop rearrangement that is characteristic of kinases in the inactive conformation (*11*). Kinase inhibitors such as imatinib and sorafenib are called type II inhibitors. On the other hand, staurosporine is classified into type I inhibitors that bind to the ATP binding site of the kinase in its active conformation. Although imatinib is known to bind the inactive forms of ABL, KIT, SRC and others (*23*), it binds an active conformation of SYK (*51*). The information provides a precedent that a tyrosine kinase inhibitor adopts different binding modes against different target kinase.

CSF-1R is a class III RTK and is the exclusive receptor for CSF-1. Binding of CSF-1 to the CSF-1R extracellular domain induces CSF-1R dimerization and trans-autophosphorylation of the intracellular CSF-1R kinase domain on several tyrosine residues, including tyrosine 561, 699, 708, 723 and 809 (*52–54*). In this study, we prepared two CSF-1R kinases: one was highly phosphorylated by autophosphorylation and the other was dephosphorylated by λPPase treatment. P-CSF-1R was phosphorylated on several tyrosine residues, including tyrosine 561, 699, 809 and 923. Tyrosine 561 in the juxtamembrane domain has been indicated to be engaged in the auto-inhibitory function (*23*), and the phosphorylated tyrosine acts as a binding site for SRC family tyrosine kinases (*55*). The phosphorylated Y699 is known as the binding site for Grb2 (*56*). Y809 is conserved in most tyrosine kinases, and its phosphorylation results in increased kinase activity (*57*). The phosphorylation of Y561 suggests that P-CSF-1R is relieved of the auto-inhibition, and the phosphorylation of Y809, the only tyrosine residue in the activation loop, indicates that the P-CSF-1R was highly activated. On the other hand, λPPase treatment almost completely dephosphorylated all these tyrosine residues, indicating that D-CSF-1R was in an inactive form. The P-CSF-1R had a high catalytic activity and a higher affinity for ATP (lower K_m value). In contrast, D-CSF-1R was less active and had a lower affinity for ATP

(higher K_m value), confirming that P-CSF-1R was in the active form and D-CSF-1R was in the inactive form (Figs. 1 and 2).

The inhibitory potency of eight CSF-1R inhibitors on two different phosphorylation states of CSF-1R was determined in the presence of the physiological concentration (1 mM) of ATP. GW2580, pazopanib and sunitinib clearly showed higher inhibitory potencies on D-CSF-1R than P-CSF-1R. In the binding analysis using SPR, the K_D value of sunitinib for D-CSF-1R was lower than for P-CSF-1R (Fig. 4, Table III). GW2580 has been demonstrated to be an extremely selective inhibitor for CSF-1R and has been hypothesized to bind to the DFG-out mode of CSF-1R, which might lock it into an inactive conformation (*24*). Although sunitinib is known to be a type I inhibitor of various kinases, including VEGFR2 and PDGFRβ (*58*, *59*), it binds and inhibits inactive KIT that has similar amino acid sequences to CSF-1R around the ATP binding pocket (*60*). Sunitinib cannot be docked into the ATP binding pocket in the active CSF-1R model (*61*). The information supports the idea that GW2580 and sunitinib bound preferentially to the inactive form of CSF-1R and prevented its activation. Like GW2580 and sunitinib, pazopanib is also likely to bind and inhibit the inactive form of CSF-1R. Imatinib showed slightly potent inhibition against D-CSF-1R. It may be consistent with the previous report that imatinib has some steric binding clash in the autoinhibited conformation of CSF-1R (*23*). Thus, imatinib may not have such a strong preference for the CSF-1R inactive form.

Dasatinib, sorafenib, staurosporine and PD173074 inhibited both phosphorylation states of CSF-1R with similar IC_{50} values (Fig. 3 and Table II). Binding analysis of staurosporine to CSF-1R revealed that staurosporine had similar K_D values for the active and inactive forms (Fig. 4 and Table III). Staurosporine is a typical type I inhibitor that is supposed to bind and inhibit the active form of kinases. The docking analysis revealed that dasatinib was docked into the CSF-1R model with the active conformation (*61*). Although the active CSF-1R had higher affinity for ATP than the inactive form, dasatinib, sorafenib, staurosporine and PD173074 are likely to bind both active and inactive forms of CSF-1R and inhibit the kinase activity.

In summary, we established the activity-based assay by which potency of inhibitors against plural activation states of CSF-1R can be estimated. Such an approach should be applicable to other kinases, which may be important to assess efficacy of inhibitors and should facilitate kinase inhibitor design.

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Conflict of interest
None declared.

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