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MALDI-TOF Mass-Spectrometry-Based Versatile Method for the Characterization of Protein Kinases

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Abstract: We describe a MALDI-TOF mass-spectrometry-based method that is rapid and versatile for the characterization of protein kinases and their inhibitors. We have designed new kinase substrates by the modification of common synthetic peptides, such as kemptide (LRRALSG), CaMKII substrate (KRQQSFDLF), erktide (ATGPLSPGPFGRR), abltide (EAIYAAPFAKKK), srctide (AEEEIYGEFEAKKKK), neurogranin (AAAKIQASFRGHMARKK), and casein kinase I (CKI) substrate (RRKDLHDDEEDEAMSITA).

There are two fundamental points on which the proposed method is based to improve the mass-spectrometric response: 1) mass tag technology by N-derivatization through stable isotope labeling and 2) C-terminal conjugation with tryptophanylarginine (WR). It was suggested that C-terminal conjugation with the WR moiety enhances the ionization potency of these new substrates 1.5–13.7 times as much as those of the original peptides. We demonstrated, by using modified abltide (Ac-EAIYAAPFAKKKWR-NH_2), that WR conjugation at the C-terminus in combination with stable-isotope labeling at the N-terminus allowed the quantitative assay of recombinant c-Abl kinase in the presence of adenosine 5′-triphosphate (ATP; $K_{M,ATP}$ = 18.6 μM and V_{max} = 642 pmol min^{-1} μg^{-1}). The present protocol made a simple and reliable inhibition assay of recombinant c-Abl kinase by imatinib possible ($IC_{50(recombinant)}$ = 291 nM; STI571, Gleevec; Novartis Pharma). Moreover, it was also demonstrated that this ATP noncompetitive inhibitor differentiates between two conformers of c-Abl kinases: the phosphorylated active and dephosphorylated inactive forms ($IC_{50(active\ form)}$ = 1049 nM and $IC_{50(inactive\ form)}$ = 54 nM). The merit of this approach is evident because the present protocol can be applied to the direct monitoring of the activities of living cell kinases by using cancer-cell lines, such as mouse B16 melanoma cells and human lung cancer K562 cells. A multiple-kinase assay that uses K562 cell lysate in the presence of seven new synthetic substrates made high-throughput inhibitor profiling possible. It should be emphasized that this radioactive isotope-free quantitative kinase assay will greatly accelerate the discovery of a new generation of potential kinase inhibitors that exhibit highly selective or unique inhibitory profiles.

Keywords: inhibitor assays · isotopes · kinases · mass spectrometry · proteins

Introduction

It has been documented that 518 kinases encoded in the human genome play pivotal functional roles in virtually all aspects of cellular physiology.[1] As these kinases are key enzymes in post-translational modifications to control pathological conditions, which range from neuronal disorders to cellular transformation in leukemias, protein kinases are currently the most important pharmaceutical drug targets.[2] Tremendous effort has been invested in the discovery of protein kinase inhibitors, and at present five distinct kinase inhibitors have been approved for the treatment of specific cancers.[3]

However, it is also known that the majority of disease-related kinases are still not targeted by an inhibitor with a practical level of profiles because of poor selectivity. This is mainly caused by the fact that mostly synthetic kinase inhibitors (type I inhibitors) have been designed by targeting the three-dimensional structures of highly conserved adenosine

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5′-triphosphate (ATP) binding pockets of active kinase conformations.[4] Most kinases are believed to interconvert between at least two structural conformations, that is, active and inactive, and the phosphorylation of key residues can shift the balance between these states.[5] These two states are characterized by conformational changes in flexible activation-loops bearing, for example, a DFG (Asp-Phe-Gly) motif that borders or blocks the ATP-binding site. Therefore, the binding affinity of protein kinases with ATP ($K_{M,ATP}$) strongly depends on active/inactive conformations and it seems likely that the $K_{M,ATP}$ values may be significantly higher for the inactive conformation than for the active conformation.[3] Recently, a growing number of kinase inhibitors that bind selectively to inactive conformations, namely type II inhibitors, has been developed.[6] The merit of this type of inhibitor is evident because the inhibitors that bind selectively to the inactive conformation will not compete with cellular ATP. The rational design of the type II inhibitors such as imatinib (STI571), BIRB796, and sorafeniv (BAY 43-9006) is therefore a promising strategy toward the development of practical kinase inhibitors,[6c] whereas most of the type I inhibitors will face significant competition by cellular ATP as a result of their similar binding affinity to both conformations. Even though the p38α inhibitor SB203580 is known to interact with both the active/inactive conformations, this compound also acts primarily in cells by stabilizing the inactive conformation, thus decreasing the rate of p38α phosphorylation by map kinase kinases (MAPKKs).[7] It seems likely that the actual inhibitory effect in living cells by kinase inhibitors can be predicted by the relationship between the $K_{M,ATP}$ values of active/inactive kinases and the K_i/IC_{50} values of the inhibitors under an intracellular ATP concentration.[3]

The above type II inhibitors and ATP noncompetitive/allosteric kinase inhibitors, such as BMS-345541[8] and PD184352,[9] may also be discovered from reliable kinetic investigations in vitro that predict the mode-of-action in cellular and animal models. In general, the kinetic analysis of kinase inhibitors has been carried out by means of ^{32}P-labeled ATP, and protocols are usually accompanied with some disadvantages, such as time-consuming and tedious procedures for the isolation of short-half-life ^{32}P-labeled products and the generation of hazardous ionizing radiation.[10] There have been alternative tools, such as fluorometric assay, enzyme-linked immunosorbent assay (ELISA) assay, and peptide microarray-based approaches for profiling protein kinase activities.[11] However, we considered that the advent of a facile and much more versatile method that allows a direct and precise kinetic investigation both in vitro and cellular kinases will greatly accelerate the discovery of potential kinase inhibitors that exhibit specific and/or unique binding profiles/mechanisms.

Our interest is now focused on the feasibility of a quantitative approach based on mass spectrometry (MS) to investigate the post-translational modification by protein kinases. MS-based proteomics has become quantitative by using some stable-isotope labeling of peptides/proteins through chemical tagging or metabolic incorporation.[12] Recently, Katayama and co-workers reported a novel assay method for detecting cellular protein kinase C activity by means of mass tag technology.[13] On the other hand, we demonstrated that the stable-isotope labeling technique allows MS-based quantitative glycomics[14] when the reducing carbohydrates are modified with some ion-sensitivity-controlling reagents inspired from highly enhanced MS intensity in the peptides containing arginine residues.[15] We hypothesized that combining a controlled ion-enhancement/suppression strategy and stable-isotope labeling will facilitate the construction of highly sensitive peptide substrates, thus allowing the rapid and quantitative MS-based characterization of protein kinases.

Results and Discussion

General concept

Double-probing of the peptide substrates: New peptide substrates were designed from seven well-known synthetic peptides used as common substrates of serine or tyrosine kinases and synthesized by the fluorenylmethoxycarbonyl (Fmoc) amino acid strategy on Tentagel resin on an automated peptide synthesizer. For example, a tyrosine kinase c-Abl optimal peptide abltide (EAIYAAPFAKKK),[16] in which the C-terminal lysine residue was conjugated with tryptophanyl-arginine (WR) and the N-terminal glutamic acid residue was capped by acetylation with $(CH_3CO)_2O$ or $(CD_3CO)_2O$ to afford new peptide analogues labeled by stable isotopes, namely, abltide–WR(H) and abltide–WR(D) (Ac-EAIYAAPFAKKKWR-NH_2). Figure 1 shows the effect of WR conjugation on the MALDI-TOF MS ion sensitivity of the new substrates. In case of the abltide derivatives, the precursor ion peak observed at m/z 1719.9 from Abltide–WR(H) exhibits a distinctly higher intensity (approximately threefold) than the parent abltide (m/z 1377.8; Figure 1d). Similarly, it was revealed that WR conjugation at the C-terminus of other peptides (kemptide, CaMKII substrate, erktide, srctide, neurogranin, and CKI substrate) significantly enhances their ionization potency. It should be noted that the effect of WR conjugation depends on the sequence rather than the composition of the amino acid residues of the original peptides. In case of kemptide (L**RR**ALSG), a serine kinase protein kinase A (PKA) optimal peptide, the modified peptide (kemptide–WR(H)) showed a 14-fold higher ion intensity than kemptide. However, when the arginine residues are located at/by the C-terminus, such as erktide (ATGPLSPGPFG**RR**) and neurogranin (AAAKIQASFRGHMA**R**KK), WR conjugation gave only 1.5- and 2.0-fold enhanced ion intensity relative to the starting peptides. We can conclude that WR tagging at the C-terminus amino acid residue would become an efficient and general protocol to enhance the ion sensitivity of various peptides, including specific branched structures derived by some post-translational modifications, such as glycosylation, acylation, sulfation, or phosphorylation (Table 1).

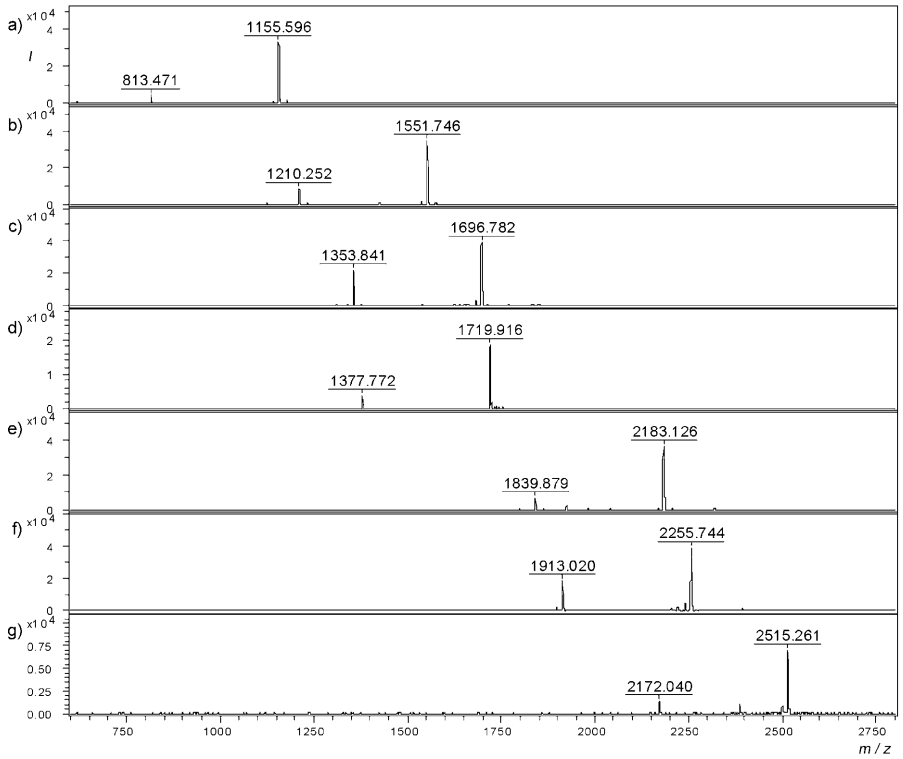

Figure 1. The effect of ion-sensitivity enhancement by novel synthetic substrates. MALDI-TOF mass spectrum of the 1:1 (mol/mol) mixture of the original peptide and peptide–WR(H) were measured in the presence of DHB. a) Kemptide and kemptide–WR(H), b) CaMKII substrate and CaMKII substrate–WR(H), c) erktide and erktide–WR(H), d) abltide and abltide–WR(H), e) srctide and srctide–WR(H), and f) neurogranin and neurogranin–WR(H). g) CKI substrate and CKI substrate–WR(H). I = intensity.

Standard protocol for the kinetic study in vitro: Figure 2 shows a general protocol of the MS-based quantitative kinase assay with recombinant c-Abl and a series of abltide–WR(H)/(D) species. Abltide–WR(D) was used as an internal standard to determine the precise concentration of product phosphorylated abltide–WR(H). In addition, fully phosphorylated abltide–WR(H)/(D) were synthesized by treating abltide–WR(H)/(D) with c-Abl kinase in the presence of excess of ATP. The fully phosphorylated abltide–WR(H)/(D) were employed for the preparation of the calibration curve. Thus, the mass intensity at m/z 1801.3 that corresponds to phosphorylated bltide–WR(H) produced from the starting material was compared with the signal detected at m/z 1804.3 from the standard phosphorylated Abltide–WR(D). Figure 3a exhibits typical profiles of the time-course assay observed for the substrate concentrations of 0.25, 0.50, and 1.00 $\mu g\,mL^{-1}$, and kinetic constants of c-Abl against ATP were determined to be $K_{M,ATP} = 18.6\ \mu M$ and $V_{max} = 642\ pmol\,min^{-1}\,\mu g^{-1}$ by the Lineweaver–Burk plot (Figure 3b). This result clearly suggests that the MS-based protocol permits rapid, highly sensitive, and quantitative analysis and the kinetic constants estimated herein were in good agreement with the data reported previously ($K_{M,ATP} = 4\ \mu M$, $V_{max} = 0.5\ \mu mol\ min^{-1}\,mg^{-}$[16] and $K_{M,ATP} = 17\ \mu M$[17]).

Table 1. Structures and relative ion intensities of novel kinase substrates.

Name	Sequence	Relative intensity[a]
kemptide	Ac-LRRALSG(**WR**)-NH_2	13.7
CaMKII substrate	Ac-KRQQSFDLF(**WR**)-NH_2	2.8
erktide	Ac-ATGPLSPGPFGRR(**WR**)-NH_2	1.5
abltide	Ac-EAIYAAPFAKKK(**WR**)-NH_2	3.4
srctide	Ac-AEEEIYGEFEAKKKK(**WR**)-NH_2	5.4
neurogranin	Ac-AAAKIQASFRGHMARKK(**WR**)-NH_2	2.0
CKI Substrate	Ac-RRKDLHDDEEDEAMSITA(**WR**)-NH_2	4.6

[a] Refers to the increment of the signal with respect to the unlabeled peptides.

Application to inhibition assays in vitro: To demonstrate the feasibility of this simple protocol in the characterization of inhibitors in vitro, the effect by imatinib (STI571, Gleevec; Novartis Pharma) and staurosporine (a nonspecific tyrosine kinase inhibitor) were tested according to the procedure illustrated in Figure 2. The inhibition assay of c-Abl-catalyzed phosphorylation was carried out by comparing the MS intensity of phosphorylated abltide–WR(H) produced in the presence of an inhibitor with that of the internal standard phosphorylated Abltide–WR(D) generated in the absence of an inhibitor. The IC_{50} values of imatinib and staurosporine against c-Abl were determined to be 291 and 83 nM, respectively (Figure 4a,b), thus indicating that the present assay is a promising alternative to conventional methods[11,18] (see for example, imatinib ($IC_{50} = 300$ nM)[18a] and staurosporine ($IC_{50} = 80$ nM)[18b]). We demonstrated that treating recombinant c-Abl with *Yersinia enterocolitica* protein tyrosine phosphatase (YopH) remarkably enhances the inhibitory effect of imatinib ($IC_{50(inactive\ form)} = 54$ nM), known to exhibit much stronger and specific inhibition against an inactive c-Abl conformation, dephosphorylated form of c-Abl[5b,6c,19] (Figure 5). As anticipated, imatinib showed a drastically decreased inhibitory effect against an active c-Abl conformation ($IC_{50(active\ form)} = 1049$ nM) when the recombinant c-Abl was converted into the active conformation by phosphorylation with srctide (Src) in the presence of ATP. Our results clearly suggest that the present protocol is suited for the high-throughput

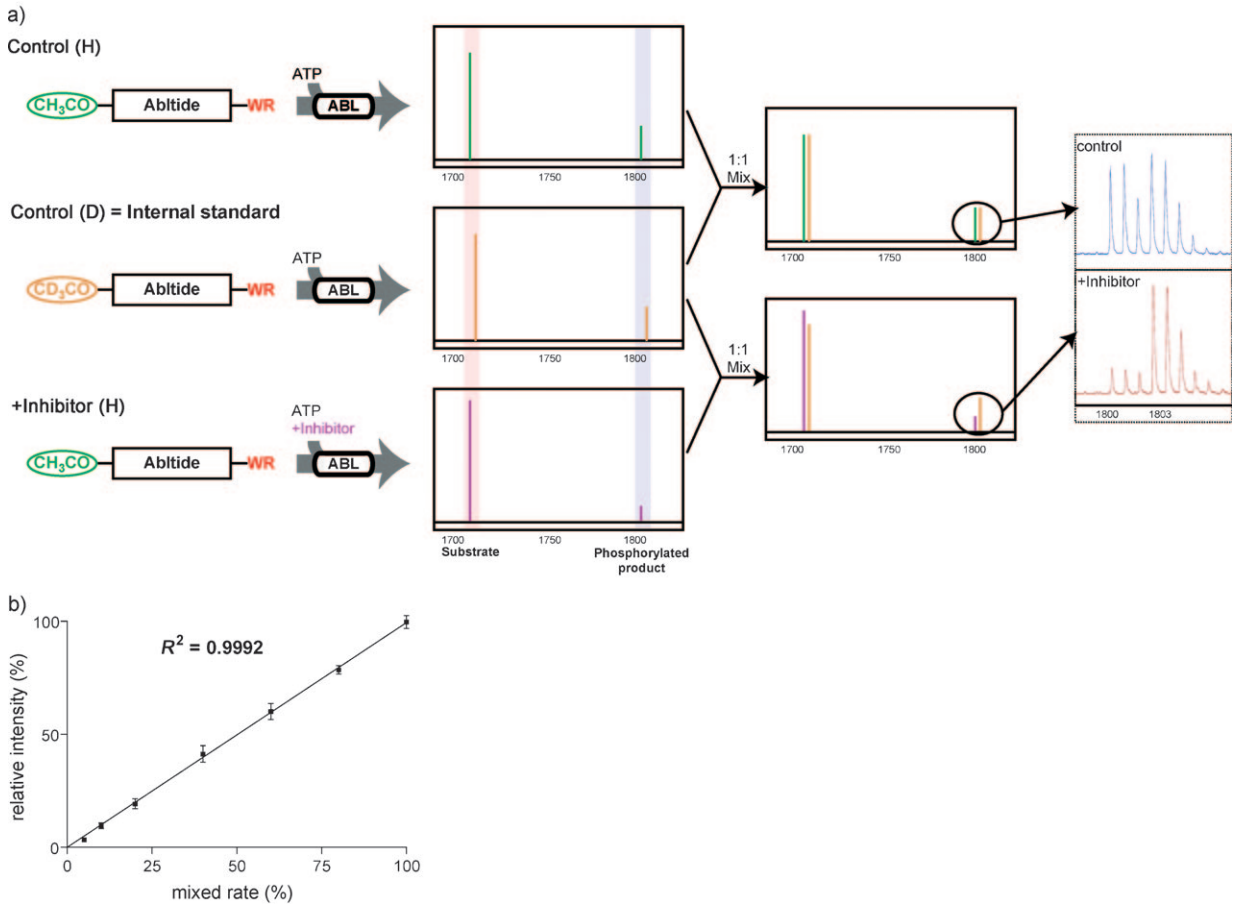

Figure 2. MS-based quantitative kinase assay by using doubly probed abltides. a) General protocol for the direct monitoring of phosphorylation by c-Abl kinase and its application to kinase inhibitor assay. b) Standard curve plotted by measuring various ratio of phosphorylated bltid–WR(H) and the heavy-isomer phosphorylated abltide–WR(D).

screening of compounds that can differentiate an inactive kinase conformation from its active conformer.

Application to cellular kinase assay: We illustrate that this protocol can be applied to the quantitative cellular kinase assay with mouse B16 melanoma cells under the following conditions and by using the following procedure: Cell extracts (final concentration of total protein = 200 μg mL^{-1}) were mixed with 15 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris–HCl) buffer (50 μL, pH 7.5) containing 200 μM abltide–WR(H), 500 μM ATP, 0.01 % Tween20, 2 mM dithiothreitol (DTT), 5 mM $MgCl_2$, and 1 mM sodium orthovanadate. The mixture was incubated at 25 °C for 17 h. After quenching of the enzymes by the addition of 2,6-dihydroxybenzoic acid (DHB) in trifluoroacetic acid (50 μL), the mixture was spiked with an internal standard, namely, phosphorylated abltide–WR(D) (the final concentration was adjusted to be 13.3 μM), and subjected directly to MALDI-TOF mass-spectrometric analysis. Abltide–WR(H) was readily phosphorylated by cellular tyrosine kinase(s) in the presence of supplemented ATP (500 μM; Figure 6b), whereas no detectable product was synthesized from intracellular ATP (Figure 6a). It was demonstrated that the effects of 10 μM imatinib (Figure 6c), 1 μM staurosporine (Figure 6d), and 10 μM gefitinib (ZD-1839, a potential inhibitor against tyrosine epidermal growth factor receptor (EGFR-like kinase);[3,20] Figure 6e) on this modification by cellular kinase(s) can be quantitatively determined. Comparing the relative inhibitory effects by these three inhibitors on cell-extract kinases and recombinant c-Abl kinase (1 μg mL^{-1}), as indicated in Figure 6f, it was revealed that mouse B16 melanoma cells express various tyrosine kinases that show high affinity with abltide–WR(H), namely, Abl-like kinases, in addition to c-Abl. Interestingly, abltide–WR(H) was a good substrate for EGFR-like kinase despite an optimal peptide substrate for Abl kinase.[16] This outcome means that these two important classes of tyrosine kinases, that is, Abl-like and EGFR-like kinases, were independently inhibited by imatinib and gefitinib. This exciting result means that the MALDI-TOF MS-based quantitative kinase assay of disease-related culture-cell extracts by means of a new class of peptide substrate labeled by double probes would greatly improve the throughput for profiling the activity of small-molecular kinase inhibitors and predicting their mode-of-action in living cells and animal models.

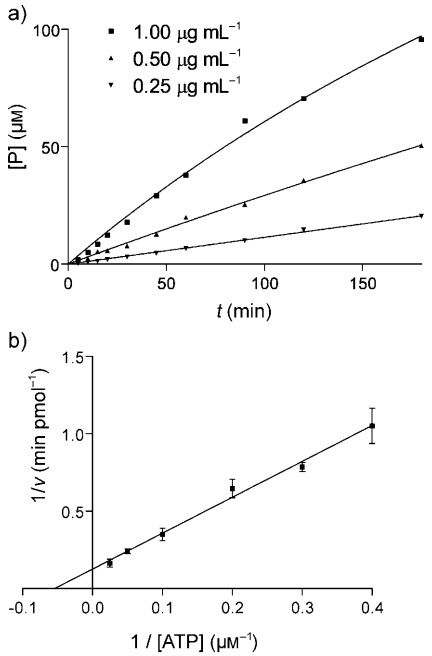

Figure 3. Kinetic analysis of recombinant c-Abl kinase by MALDI-TOF MS. a) Time-course assay that used three different concentration of abltide–WR(H); [P] shows the concentration of phosphorylated Abltide–WR(H) estimated by means of the standard curve. b) Lineweaver–Burk plot; the error bar indicates the standard deviation in triplicate experiments.

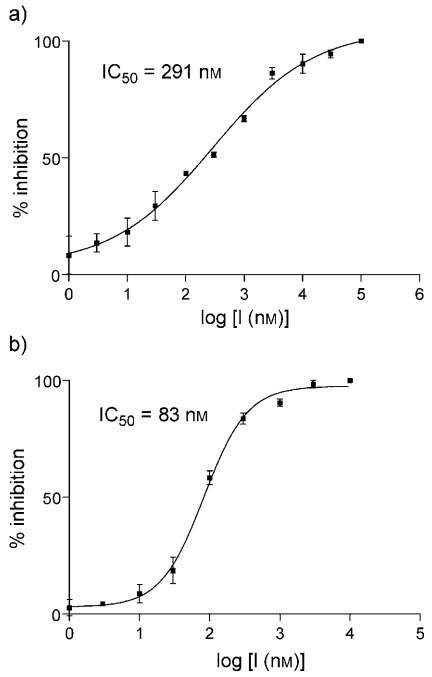

Figure 4. Inhibition assay in the presence of a) imatinib and b) staurosporine.

We demonstrated preliminarily that a cocktail of seven synthetic substrates becomes a nice tool for profiling multiple kinases of human cancer cells, that is, the K562 cell line. It seemed that K562 cells express at least four kinases that

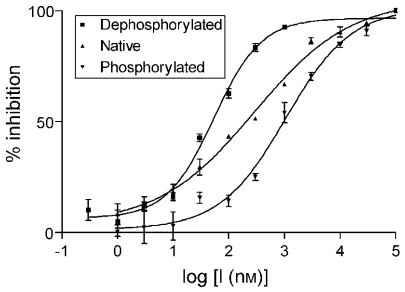

Figure 5. Differentiation of the conformations of recombinant c-Abl kinases by imatinib.

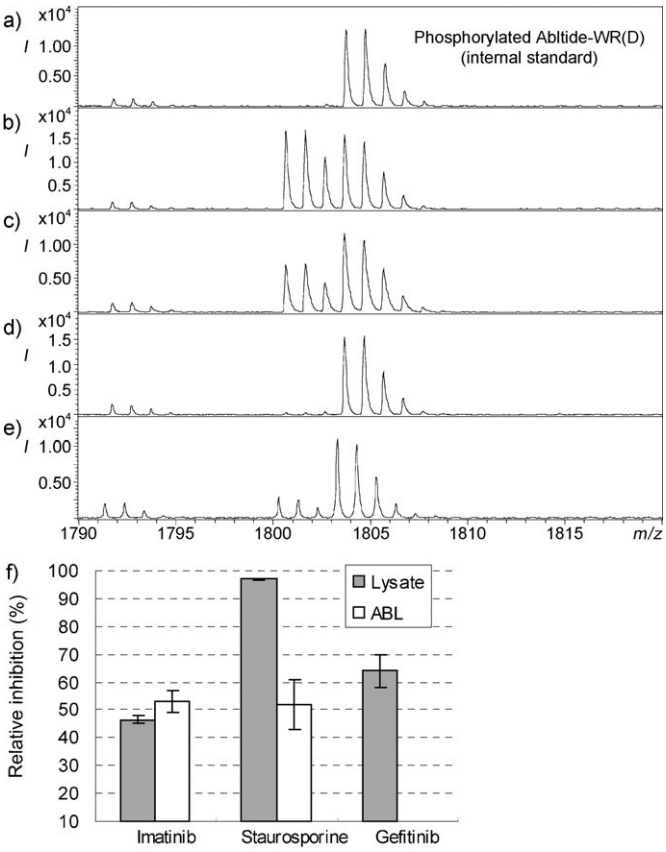

Figure 6. Direct assay of cellular kinase activity and the feasibility in inhibitor profiling by using mouse B16 melanoma cells. a) Abltide–WR(H)+cell lysate, b) abltide–WR(H)+cell lysate+ATP, c) abltide–WR(H)+cell lysate+imatinib+ATP, d) abltide–WR(H)+cell lysate+staurosporine+ATP, e) abltide–WR(H)+cell lysate+gefitinib+ATP, and f) the effects of three inhibitors on whole kinase activity of B16 cell lysate and recombinant c-Abl. I=intensity.

modify the CaMKII substrate, erktide, abltide, and srctide among these peptides (Figure 7). Next, we examined the effects of three inhibitors (i.e., staurosporine, imatinib, and gefinitib) on phosphorylation by K562 cell extracts by using CaMKII substrate–WR(H)/(D), erktide–WR(H)/(D), abltide–WR(H)/(D), and srctide–WR(H)/(D), respectively (Figure 8). The results summarized in Figure 9 should allow us to predict the key kinases expressed in K562 cells and the suited drug candidates, concurrently.

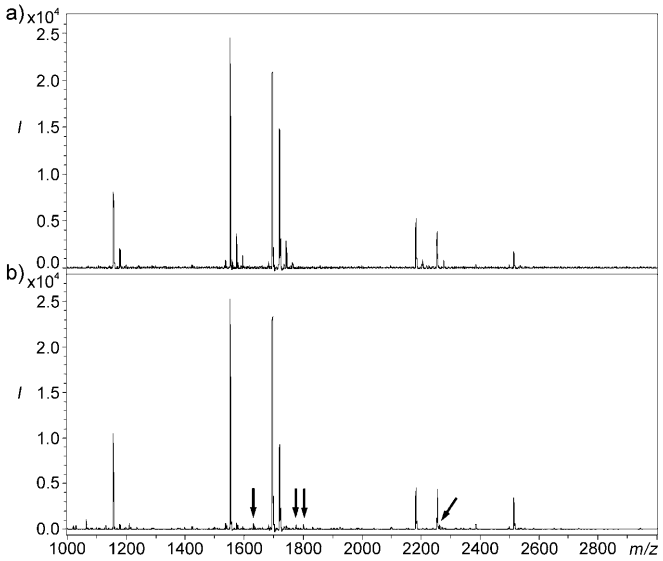

Figure 7. Profiling of whole-kinase activity of human cancer K562 cell lysate in the a) absence and b) presence of seven synthetic substrates. It was revealed that K562 exhibited four kinase activity to modify CaMKII substrate, erktide, abltide, and srctide among these peptides. I = intensity.

Conclusion

In conclusion, we have established a novel and general method for the MALDI-TOF MS-based quantitative characterization of human protein kinases by means of novel synthetic peptides bearing both N-terminal stable isotope tags and C-terminal MS-sensitivity enhancement reagents. Our approach has allowed the rapid and precise kinetic analysis of recombinant c-Abl kinases in vitro and direct monitoring of cellular kinase activity by using mouse B16 melanoma cells and human cancer K652 cells. The advantage of this strategy is evident because the combined use of stable isotopes and sensitivity-enhancement reagents allows high-throughput and quantitative kinase/inhibitor profiling of not only tyrosine kinases but general serine/threonine kinases. We believe that this radioactive isotope-free quantitative assay will contribute to the discovery of new-generation kinase inhibitors that exhibit highly selective inhibitory effects based on desired "specific" mechanisms. It should be emphasized that a highly sensitive MS-based quantitative kinase assay that uses a doubly probed peptide set will greatly accelerate the investigation of the relationship between the expression levels of cellular kinases and inhibitor sensitivity for any specific kinase. Our findings should provide some insight into the activity-based abundance of specific protein kinases within the protein kinase superfamily and relative to other cellular proteins.[21]

Experimental Section

General: Recombinant human Abl kinase was purchased from Carna Biosciences Inc. (Kobe, Japan). Adenosine 5′-triphosphate disodium salt (ATP) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Imatinib was extracted from Gleevec tablet (Novartis, Basel, Switzerland). Staurosporine was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). HPLC was conducted on a HITACHI L-7100 HPLC system equipped with a Inertsil ODS column (20×250 mm, GL Sicence Inc., Tokyo, Japan) and HITACHI L-7420 UV/vis detector. Dishes (10-cm) were obtained from BD Falcon. Minimum Essential Midium Eagle, phosphate buffered saline (PBS), penicillin/streptomycin, L-glutamin, and Trypan blue were purchased from Invitrogen. A cell scraper was obtained from SUMILON. Mouse B16 melanoma cells were purchased from the Health Science Research Resources Bank. Human K562 cells were obtained from the Riken Cell Bank (Tsukuba, Japan).

All mass measurements were performed by using an Ultraflex TOF/TOF mass spectrometer (Bruker Daltonics GmbsH, Bremen, Germany) equipped with a reflector and controlled by the Flexcontrol 1.2 software package. Ions generated by a pulsed UV laser beam (nitrogen laser, λ = 337 nm) were accelerated to a kinetic energy of 23.5 kV. External calibration of the MALDI mass spectra was carried out by means of singly charged monoisotopic peaks of a mixture of human angiotensin II (m/z 1046.542), bombesin (m/z 1619.823), ACTH (m/z 2465.199), and somatostain 28 (m/z 3147.472). To achieve mass accuracy better than 60 ppm, internal calibration was carried out by doping the matrix solution with a mixture of the calibration peptides. Calibration of these mass spectra was performed automatically by utilizing a customized macro command of the XMASS 5.1.2 NT software package. The macro command was used for the calibration of the monoisotopic singly charged peaks of the above mentioned peptides. All of the data processing and calculations were performed by Microsoft Excel and Graphpad Prism.

Synthesis of peptide–WR(H) and peptide–WR(D): The syntheses of all the peptides and peptide–WR moieties were carried out on a TentaGel S RAM resin by using an automated peptide synthesizer (Advanced ChemTech, APX396). For example, abltide–WR(H)/(D) was carried out on this resin (0.24 mmol g^{-1}, 83 mg, 0.02 mmol) and one cycle on the automated peptide synthesizer was as follows: The resin was mixed and stirred with piperidine/DMF (20:80, v/v) for 5 min, and this process was repeated once more over 15 min to complete the N-deprotection. After filtration, the resin was washed with *N*-methylmorpholine (NMP) and DMF and added to a solution of an Fmoc-protected amino acid (5 equiv, 0.10 mmol), 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU; 5 equiv, 0.10 mmol), *N*-hydroxybenzotriazole monohydrate (HOBt; 4.3 equiv, 0.086 mmol), and diisopropylethylamine (DIEA; 7 equiv, 0.14 mmol) in NMP/DMF (1:1, 18 mL). The reaction mixture was stirred for 1 h at ambient temperature, and this coupling was conducted twice. The reaction mixture was filtered, the residual resin was washed with NMP and DMF, and the Fmoc group was removed as described above. This process was repeated to complete the synthesis of the target peptides. In the final step, the normal acetyl group or deuterated acetyl group was incorporated into the N-terminal glutamate (Glu) residue by treatment with $(CH_3CO)_2O$ or $(CD_3CO)_2O$ (95 μL, 1.00 mmol) for 30 min at room temperature in the presence of DIEA (45 μL, 0.27 mmol) and HOBt (4 mg, 0.03 mmol) in DMF (2 mL). Finally, the resin was washed with NMP and CH_2Cl_2, and dried in vacuo. The protected peptide on the resin was treated with 95% trifluoroacetic acid (TFA) containing 2.5% of triisopropylsilane for 1.5 h at room temperature. The mixture was filtered and the resin was washed with TFA. The yellow solution was evaporated and the residual syrup was precipitated from ice-cold diethyl ether. After centrifugation at 15000 rpm for 15 min, the supernatant was removed and then the crude solid was subjected to purification by reverse-phase HPLC under a linear A–B gradient (eluent A: 0.1% TFA in water, eluent B: 0.1% TFA in acetonitrile) at a flow rate of 10 mL min^{-1}. The fraction containing the product was lyophilized to afford the pure peptide derivative as a colorless amorphous solid.

Abltide–(H): HRMS: m/z: calcd for $C_{66}H_{104}N_{16}O_{16}$: 1377.7894; found: 1377.7893; abltide–WR(H): HRMS: m/z: calcd for $C_{83}H_{123}N_{22}O_{18}$: 1718.9620; found: 1718.9644; abltide–WR(D): HRMS: m/z: calcd for $C_{83}H_{123}D_3N_{22}O_{18}$: 1721.9809; found: 1721.9786. The results of the analysis of the amino acids were in good agreement with the theoretical data.

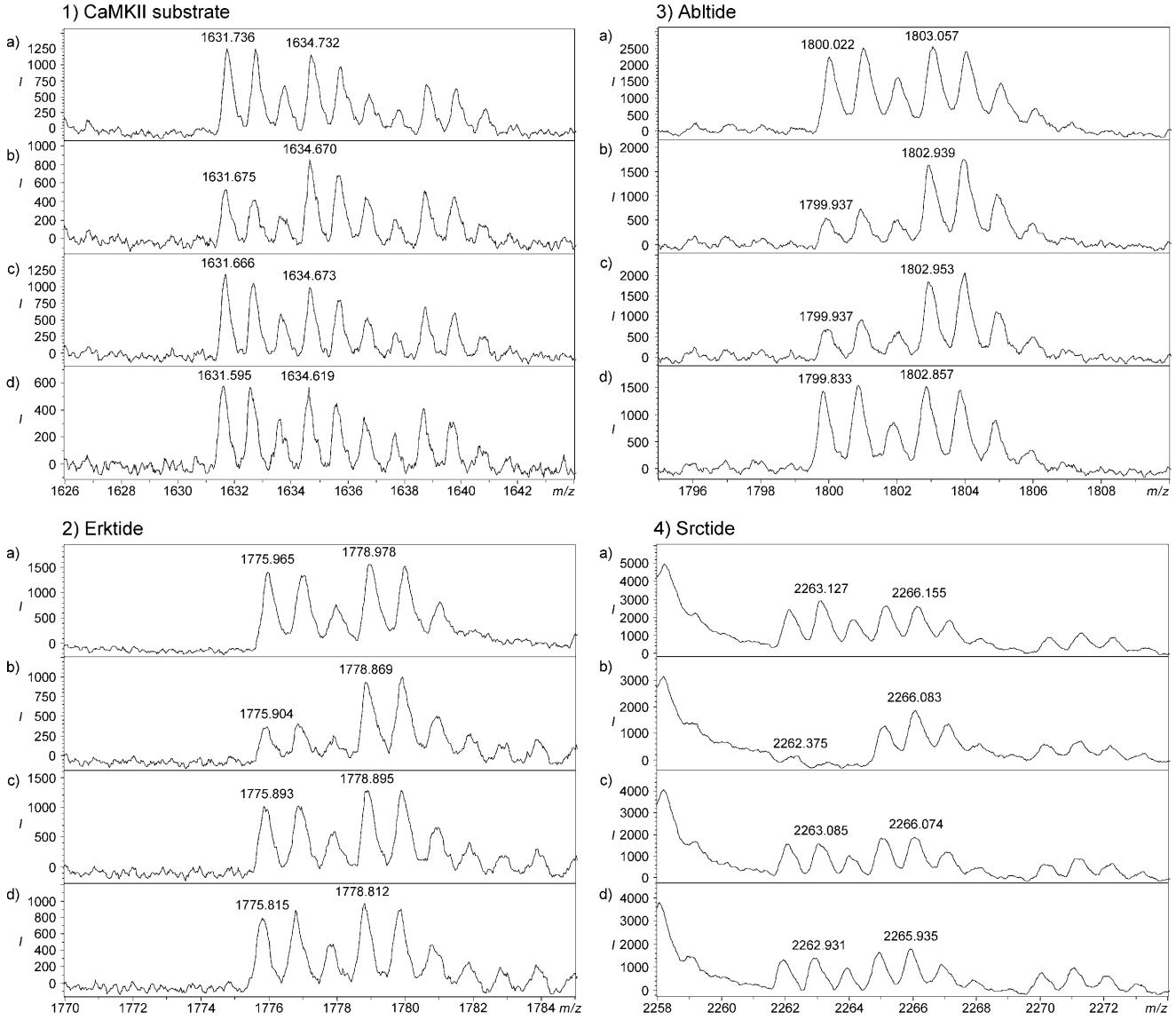

Figure 8. Inhibitory effects by staurosporine, imatinib, and gefitinib on K562 cell kinases in the presence of four synthetic substrates. Fully phosphorylated peptides with the stable-isotope tag were employed as an internal standard. I = intensity.

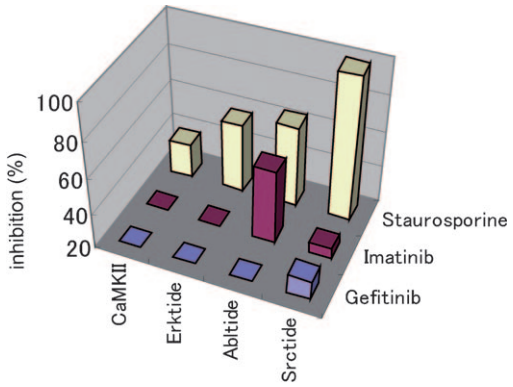

Figure 9. Summary of the multiple kinase assay by using human cancer K562 cell lysate.

In a similar manner, peptide–WR(H)/(D) of kemptide, CaMKII substrate, erktide, srctide, neurogranin, and CKI substrate were synthesized. Kemptide–(H): HRMS: m/z: calcd for $C_{34}H_{64}N_{14}O_9$: 813.5059; found: 813.5079; kemptide–WR(H): HRMS: m/z: calcd for $C_{51}H_{86}N_{20}O_{11}$: 1155.6863; found: 1155.6891; kemptide–WR(D): HRMS: m/z: calcd for $C_{51}H_{83}D_3N_{20}O_{11}$: 1158.7051; found: 1158.7075; CaMKII–(H): HRMS: m/z: calcd for $C_{55}H_{84}N_{16}O_{15}$: 1209.6380; found: 1209.6387; CaMKII–WR(H): HRMS: m/z: calcd for $C_{72}H_{106}N_{22}O_{17}$: 1551.8184; found: 1551.8220; CaMKII–WR(D): HRMS: m/z: calcd for $C_{72}H_{103}D_3N_{22}O_{17}$: 1554.8373; found :1554.8397; erktide–(H): HRMS: m/z: calcd for $C_{60}H_{96}N_{20}O_{16}$: 1353.7391; found 1353.7427; erktide–WR(H): HRMS: m/z: calcd for $C_{77}H_{118}N_{26}O_18$: 1695.9195; found 1695.9227; erktide–WR(D): HRMS: m/z: calcd for $C_{77}H_{115}D_3N_{26}O_{18}$: 1698.9384; found 1698.9376; srctide–(H): HRMS: m/z: calcd for $C_{83}H_{130}N_{20}O_{27}$: 1839.9492; found 1839.9520; srctide–WR(H): HRMS: m/z: calcd for $C_{100}H_{152}N_{26}O_{29}$: 2182.1297; found 2182.1325; srctide–WR(D): HRMS: m/z: calcd for $C_{100}H_{149}D_3N_{26}O_{29}$: 2185.1485; found 2185.1502; neurogranin-(H): HRMS: m/z: calcd for $C_{83}H_{142}N_{30}O_{20}S$: 1912.0815; found 1912.0839. neurogranin–WR(H): HRMS: m/z: calcd for $C_{100}H_{164}N_{36}O_{22}S$: 2254.2620; found 2254.2653; neurogranin–WR(D): HRMS: m/z: calcd for

$C_{100}H_{161}D_3N_{36}O_{22}S$: 2257.2808; found 2257.2831; CKI–(H): HRMS: *m/z*: calcd for $C_{87}H_{142}N_{28}O_{35}S$: 2171.9991; found 2171.9991; CKI–WR(H): HRMS: *m/z*: calcd for $C_{104}H_{164}N_{34}O_{37}S$: 2514.1795; found 2514.1816; CKI–WR(D): HRMS: *m/z*: calcd for $C_{104}H_{161}D_3N_{34}O_{37}S$: 2517.1984; found 2517.1961. The results of the analysis of the amino acids were also in good agreement with the theoretical data.

Phosphorylated abltide–WR(H)/(D) and the standard curve: Phosphorylated abltide–WR was synthesized enzymatically in 15 mM Tris–HCl buffer (100 μL, pH 7.5) containing Tween20 (0.01%), DTT (2 mM), and $MgCl_2$ (5 mM) in the presence of abltide–WR(H) or abltide–WR(D) (200 μM), ATP (600 μM), and c-Abl (10 μg mL^{-1}). The mixture was incubated for one day at 25°C. After completing the conversion into the fully phosphorylated form, as judged by MALDI-TOF MS, the reaction was stopped by the addition of DHB (200 μL, 10 mg mL^{-1} in 33% acetonitrile containing 0.1% TFA). The phosphorylated abltide–WR(H) and abltide–WR(D) were mixed in the ratios of 1:1, 1:0.8, 1:0.6, 1:0.4, 1:0.2, and 1:0.1 (v/v), respectively. The solution (2 μL) was moved onto polished stainless steel sample plate, allowed to dry to induce crystallization, and analyzed by MALDI-TOF mass-spectrometric analysis in positive-ion reflectron mode. A total of 300 laser shots were applied for each spectrum. The standard curve was constructed by plotting the ratio of MS intensity of phosphorylated abltide–WR(H) and abltide–WR(D). The ratio was calculated by Equation (1) to the correct MS intensity of the deuterated product because the apparent intensity of phosphorylated abltide–WR(D) at *m/z* 1803 involves the signal intensity ($I_H = 18.73\%$) from the isotope of abltide–WR(H).

$$\text{Ratio}\ (\%) = I_H/(I_D - I_H \times 0.1873) \times 100 \quad (1)$$

where I_H and I_D are the peak intensities from phosphorylated abltide–WR(H) and abltide–WR(D) at *m/z* 1800 and 1803, respectively. The solution of phosphorylated abltide–WR(D) in DHB was stored at −20°C as an internal standard stock solution.

Time-course assay for the phosphorylation of abltide–WR(H) by c-Abl kinase: The reactions by c-Abl (1 μg mL^{-1}, 0.50 μg mL^{-1}, 0.25 μg mL^{-1}) were conducted in 15 mM Tris–HCl buffer (100 μL, pH 7.5) containing Tween20 (0.01%), DTT (2 mM), and $MgCl_2$ (5 mM) in the presence of abltide–WR(H) (200 μM) and ATP (200 μM). The mixture was incubated at 25°C and an aliquot of solution (10 μL) was collected at 5, 10, 15, 20, 30, 45, 60, 90, 120, and 180 min. After quenching by the addition of a solution of DHB (20 μL), all the test solutions were mixed with an internal standard, that is, phosphorylated abltide–WR(D) (4.33 μM for 0.25 μg mL^{-1} of c-Abl, 8.33 μM for 0.5 μg mL^{-1} of c-Abl, and 33.3 μM for 1.00 μg mL^{-1} of c-Abl, respectively) in a ratio of 1:1 (v/v), and subjected to analysis by using MALDI-TOF MS as described above.

The quantization was performed on the basis of the ratio of the MS intensity of phosphorylated abltide–WR(H) and the internal standard. The concentration of the phosphorylated peptide was calculated by applying Equation (2).

$$P_H = P_D \times I_H/(I_D - I_H \times 0.1873) \quad (2)$$

where P_H is the concentration of phospholyrated abltide–WR(H) in the quenched sample, P_D is the concentration of the phosphorylated abltide–WR(D) (internal standard), and I_H and I_D are the peak intensities of phosphorylated abltide–WR(H) and abltide–WR(D), respectively.

The kinetic constants of recombinant c-Abl against ATP: All the experiments were carried out in 15 mM Tris–HCl buffer (25 μL, pH 7.5) in the presence of ATP (40, 20, 10, 5, 3.33, 2.5, and 1.25 μM), bltide–WR(H) (200 μM), and c-Abl (0.50 μg mL^{-1}). The mixture was incubated at 25°C for 30 min and the reactions were terminated by the addition of a solution of DHB (50 μL). The concentration of the internal standard was adjusted to 2.17 μM when 40 and 20 μM of ATP were employed, whereas other experiments were carried out in the presence of 0.67 μM of the internal standard. The MALDI-TOF MS analyses ($n = 3$) were performed and kinetic constants were calculated by fitting the data to the Michaelis–Menten equation using Prism software.

The kinetic constants of recombinant c-Abl against abltide–WR(H): All the experiments were carried out in 15 mM Tris–HCl buffer (25 μL, pH 7.5) in the presence of ATP (100 μM), abltide–WR(H) (100, 75, 50, 25, 10, 5, 3.3, 2.5 μM), and c-Abl (0.50 μg mL^{-1}). The mixture was incubated at 25°C for 30 min and the reactions were terminated by the addition of a solution of DHB (50 μL). Each sample was mixed with 2.16 μM of internal standard in a ratio of 1:1 (v/v) and analyzed by MALDI-TOF MS as described. The MALDI-TOF MS analysis ($n = 3$) was performed and kinetic constants were calculated by fitting the data to the Michaelis–Menten equation using Prism software.

Determination of IC_{50} values of imatinib and staurosporine: The inhibition assays were conducted in 15 mM Tris–HCl buffer (25 μL, pH 7.5) in the presence of ATP (20 μM), abltide–WR(H) (200 μM), c-Abl (0.50 μg mL^{-1}), and inhibitors at 25°C for 30 min. The concentration of the inhibitors was fixed at 0–100 μM (imatinib) or 0–10 μM (staurosporine). The reactions were terminated after 30 min at 25°C by the addition of a solution of DHB (50 μL). Each solution was mixed with 1.33 μM of the internal standard in a ratio of 1:1 (v/v) and subjected to MALDI-TOF MS as described above. The reaction rate was estimated by applying Equation (2) and the data were converted into percent inhibition relative to the reaction rate detected without inhibitor. The assays were repeated three times and averaged. The data processing and calculation of IC_{50} values were performed by using Microsoft Excel and Graphpad Prism.

Dephosphorylation of recombinant c-Abl (preparation of the inactive form c-Abl): Dephosphorylated recombinant c-Abl was obtained by treating recombinant c-Abl (15 μg mL^{-1} at final concentration) with *Yersinia enterocolitica* phoshatase (6 U μg^{-1} of recombinant c-Abl) in 15 mM Tris–HCl buffer (100 μL, pH 7.5) containing Tween20 (0.01%), DTT (2 mM), and $MgCl_2$ (5 mM) for 30 min at 25°C. 200 μL of Na_3VO_4 (7.5 mM in buffer) was added and the reaction mixture was immediately used for the inhibition assay using imatinib.

Phosphorylation of recombinant c-Abl (preparation of the active form c-Abl): Phosphorylation of recombinant c-Abl was carried out in 15 mM Tris–HCl buffer (pH 7.5) containing Tween20 (0.01%), DTT (2 mM), and $MgCl_2$ (5 mM) in the presence of ATP (600 μM), Src (0.3 μg mL^{-1}), and recombinant c-Abl (15 μg mL^{-1}) at 25°C for 30 min. This reaction mixture was diluted with buffer and used for the determination of the IC_{50} value of imatinib.

Cell culture and cellular kinase assay using mouse B16 melanoma cells: Mouse B16 melanoma cells were grown in 5.0% CO_2, water-saturated atmosphere at 37°C in Minimum Essential Medium Eagle supplemented with fetal calf serum (FCS, 10%), penicillin (50 units mL^{-1}), streptomycin (0.05 mg mL^{-1}), and L-glutamine (2 mM). The number of cells was determined by staining with Trypan blue. B16 melanoma cells grown in 10-cm dishes were rinsed with cold PBS (free of Ca^{2+} and Mg^{2+} ions) three times. Cold PBS containing 10 mM ethylenediaminetetraacetic acid (EDTA) was added to the dishes and the cells were collected with a cell scraper. The solution of PBS containing B16 cells was centrifuged at 4°C, and the supernatants were removed to give the cell preparation as a pellet (1.8×10^7 cells). Buffer (200 μL; Tris–HCl (15 mM), DTT (2 mM), $MgCl_2$ (5 mM), and Tween20 (0.01%), protease inhibitor) was added into the cell pellet and sonicated. The samples were centrifuged after sonication at 5000 *g* at 4°C for 15 min, and the resulting supernatant was used as a whole-cell lysate sample (total protein: 10 mg mL^{-1}) for the subsequent phosphorylation assay.

Profiling of the inhibitors by using cell lysate was carried out in 15 mM Tris–HCl buffer (50 μL, pH 7.5) containing Tween20 (0.01%), DTT (2 mM), and $MgCl_2$ (5 mM) in the presence of ATP (500 μM), abltide–WR(H) (200 μM), sodium orthovanadate (1 mM), inhibitors (imatinib (10 μM), stauroseporine (1 μM), and gefitinib (10 μM), respectively), and cell lysate (total protein: 200 μg mL^{-1}). The mixture was incubated at 25°C for 17 h. The reaction was terminated by the addition of a solution of DHB (50 μL). Each sample solution was mixed with the internal standard (13.3 μM) in a ratio of 1:1 (v/v) and analyzed by MALDI-TOF MS as described for the kinase assay in vitro.

Preparation of human K562 cell extracts and multiple kinases assay: K562 cells were cultured at 37°C and 5% CO_2 in HamF12 nutrient mix-

ture containing 10% FBS (Fetal Bovine Serum). Approximately 10^7 cells in culture medium (10 mL) were washed twice with ice-cold PBS and then lysed in buffer (1 mL) containing Tris–HCl (50 mM, H 7.5), NaCl (100 mM), $MgCl_2$ (5 mM), Triton-100 (1%), glycerol (10%), Na_3VO_4 (1 mM), and protease inhibitor cocktail (2%). After incubation on ice for 15 min, the lysis mixture was centrifuged and the extract supernatant was transferred to fresh tubes and used immediately. The cell extract (5 μL) was diluted fivefold with assay buffer containing 15 mM Tris–HCl (pH 7.5), $MgCl_2$ (10 mM), DTT (1 mM), ATP (200 μM), and a synthetic peptide substrate (40 μM). The mixture was incubated at 25 °C for 2 h and the reaction was terminated by the addition of a solution of DHB (50 μL). Each sample solution was mixed with the internal standard and analyzed by MALDI-TOF MS as described for the B16 cells assay.

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