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Global Kinase Screening. Applications of Frontal Affinity Chromatography Coupled to Mass Spectrometry in Drug Discovery

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Utilizing frontal affinity chromatography with mass spectrometry detection (FAC-MS), we have identified novel applications in the discovery of small-molecule hits to protein targets that are difficult if not impossible to accomplish using traditional assays. We demonstrate for the first time an ability to distinguish between competitive ligands for the ATP and substrate sites of protein kinase C independently in the same experiment and show that ATP competitive ligands using a functionally inactive receptor tyrosine kinase can be identified. This ability of FAC-MS to simultaneously monitor binding at the ATP and substrate binding sites, as well as measure ligand binding to both active and inactive kinases, suggests that FAC-MS can be used as a "global kinase binding assay".

The combination of frontal affinity chromatography coupled with mass spectrometry detection (FAC-MS), established in 1998 by Schreimer and Hindsgaul,[1] is a tool that has been utilized in the drug discovery process and successfully applied to a wide range of biological targets.[2−8] The details of FAC-MS have been described elsewhere,[1,3,7] but basically it takes advantage of the ongoing equilibrium between ligands flowing through a capillary column containing an immobilized protein target. As ligands flow through the column and bind with the target, individual ligands are retained in the column on the basis of their affinity for the target. This causes an increase in each ligand's specific "breakthrough volume", which is the effluent volume passing through the column that allows the output ligand concentration to equal the input ligand concentration. The breakthrough volume, characterized as a sigmoidal front, can readily be detected by MS and corresponds directly to the time that the front is observed to pass through the column. The use of a void marker (a compound that has no affinity for the immobilized protein target and gives the same elution front whether the target protein is present in the column or not) and MS detection allows for multiple ligands to be evaluated simultaneously in a direct readout fashion (Q1 scan). Therefore, depending on the ligand's affinity for the target, FAC-MS offers a very convenient way of measuring the relative binding strengths of ligands and enables rapid ranking with ligand identification. This method can be utilized in an initial screen, and depending on the amount of immobilized protein on the column, relatively high throughput (up to 10 000 compounds/day with one FAC-MS system) can be reached with no special requirements. The concept of using an "indicator" for monitoring real-time competition with ligands in screening applications has increased the capability of FAC-MS.[3,7,8] An indicator is a compound detectable by MS that binds to a specific site on the immobilized protein target with a known affinity in a specific column infusion time. In screening cases using an indicator only, the indicator and void marker are monitored by MS. Although there is no set affinity requirement for an indicator to an immobilized target, for practical reasons the affinity should be in the 0.5−50 μM range. Therefore, these two FAC-MS screening possibilities—with and without an indicator—give FAC-MS extra flexibility in screening assays.

It has been shown in the development of FAC-MS that frontal affinity chromatography theory[9−13] was also applicable to the corresponding microcapillary columns utilized in FAC-MS. Specifically, the relationship between V (the breakthrough volume of ligand A), V_0 (the breakthrough volume of the void marker), [A] (the ligand concentration), B_t (the total amount of immobilized active protein) and K_d (the binding constant) is given by the following equation.[7]

$$V - V_0 = B_t/([A] + K_d)$$

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Hence, for an immobilized protein, a series of ligand concentrations are infused through a FAC-MS column sequentially starting with the lowest concentration of the ligand, A. For each concentration, the breakthrough volume ($V - V_0$) is determined by monitoring the ligand and void marker by MS and a plot of the ligand concentration versus the reciprocal of $V - V_0$ generates a line with the y intercept equal to $-K_d$ and the slope equal to B_t.[7]

All of the FAC-MS applications to date have focused on screening of a known specific site on a protein target. Protein targets, however, often have multiple sites for potential inhibition, but these multiple sites are not specifically addressed using traditional high-throughput screening (HTS) methodologies. Such screens are based on assays that are limited to measuring the functional effects of small-molecule interactions without revealing the site of inhibition. Traditional functional HTS assays also require the protein to be in its active conformation to be screened. This requirement limits the quality of potential ligands discovered, as the functional assays are unable to distinguish between ligands that interact with the inactive form of the target or at allosteric binding sites. As many proteins are allosterically regulated or exist in equilibrium between active and inactive conformations, there is increasing interest in discovering inhibitors that target these specific protein conformations and therefore possess greater levels of specificity for the target of interest.[14]

The search for kinase inhibitors is an example of the potential limitations inherent in the use of traditional functional assays. Protein kinases catalyze the transfer of a phosphate from ATP to a substrate protein. Overactivation of kinase activity has been implicated in a number of proliferative disorders, such as cancer. Consequently, inhibition of kinase activity is currently a very attractive avenue in drug discovery.[15] Several strategies have emerged for keeping kinase activity at bay: (1) inhibition by blocking the ATP binding site,[16] (2) inhibition by blocking the substrate binding site,[17] (3) inhibition by binding at an allosteric site, a mechanism recently discovered for how BIRB 746[18] inhibits the p38 MAP kinase, or (4) inhibition by transforming the kinase into an inactive form upon ligand binding resembling its autoinhibited state, as seen in the binding of Gleevec to Bcr-Abl.[19] The majority of protein kinase inhibitors on the market or in advanced clinical trials are ATP competitive.[20] Although many are proving efficacious, the potential toxicity of these ATP-competitive ligands still looms because of the estimated 500 additional human protein kinases that could be potential pharmacodynamic targets of these drugs.[21] In fact, it has been reported that, for a variety of kinase inhibitors tested against a large panel of protein kinases, many were found to be so nonspecific that conclusions drawn from their use were rendered meaningless.[22] Despite this, the search for ATP-competitive kinase inhibitors is still being aggressively pursued.

Since protein kinases target specific protein substrates, it has been suggested that compounds that target the substrate binding site, instead of the ATP binding site, may provide drugs with improved kinase selectivity and potentially less toxicity.[12] It has also been suggested that, in the search for selectivity, the inactive forms of kinases are attractive targets for drug design.[9] As mentioned, Gleevec provides a prototype of this model; this drug binds unphosphorylated Abl and locks the kinase in an inactive conformation, likely as the result of an induced fit effect.[14] With the methods currently available, however, it is not possible, in an initial screen, to distinguish between the binding sites using traditional radioactive, luminescent, or antibody-based kinase assays.[23] With these assays, once a ligand has been identified, additional competition studies are required to elucidate whether compounds are ATP competitive or substrate competitive. Since these methods rely on kinase activity readout and thus an active kinase, it is also not possible to screen against an inactive kinase. In this study, we extend the applications of FAC-MS to show that (1) both the ATP binding site and the substrate binding site of a kinase target can be assayed in a simultaneous, yet independent fashion in one single experiment (dual binding site assay) and (2) an inactive kinase can be assayed.

EXPERIMENTAL METHODS

Materials. All kinase inhibitors (WHI-P180, SB203580, PD153035, VEGFR kinase inhibitor), αMan(1→3)[αMan(1→6)]βManO-octyl (M3), chelerythrine chloride, human recombinant protein kinase Cα (PKCα), and PKC inhibitor peptide 19−36 were obtained from Calbiochem. Anti-His tag, clone 4D11, and biotin conjugate monoclonal antibodies were purchased from Upstate. Streptavidin-coated CPG glass beads (20 μm, 500-Å pore size) were purchased from CPG (now Millipore). Recombinant His-tagged EphB2 (kinase domain) and mutant (Y604/610F) murine EphB2 were generously donated by MDS Proteomics. Poly(Glu, Tyr) 4:1, protein tyrosine kinase assay kit, solvents, and other reagents were obtained from Sigma-Aldrich.

FAC-MS. Mutant (Y604/610F) murine EphB2 and PKCα were biotinylated using biotin-XX-sulfosuccinimidyl ester (Molecular Probes), purified by gel filtration, and then dialyzed against 1X PBS (pH 7.4). The biotinylated proteins (100 μL, ~200 μg) were incubated with streptavidin-coated CPG beads in 1× PBS buffer overnight at 4 °C. A solution of biotinylated anti-His tag monoclonal antibodies (100 μL ~100 μg) was incubated with streptavidin-coated CPG (20 μM) beads in 1× PBS buffer overnight at 4 °C. Afterward, 200 μg of recombinant His-tagged EphB2 (kinase domain) in 1× PBS with 10 μM $MgCl_2$ and 20 μM $MnCl_2$ was added and the resultant mixture incubated for 24 h at 4 °C. After loading immobilized protein, the FAC-MS capillary columns (250 μm i.d. × 2.5 cm) were washed with 50 μL (at 200 μL/h) of 1× PBS buffer followed by 50 μL of the running buffer (20 mM NH_4-OAc containing 1% DMSO). To block any remaining streptavidin sites, the columns were infused with D-biotin (40 μM) followed by washing with the running buffer. Analyte solutions were prepared to contain the indicator (1 μM), the ligand (5 μM), or

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both, and M3 (1 μM) as the void marker in 20 mM NH_4OAc containing 1% DMSO. The makeup buffer was 90% methanol containing 0.1% acetic acid in water. The flow rates used were 800 μL/h for the makeup buffer and 100 μL/h for the FAC-MS columns. The column was connected to an AB/Sciex API 3000 triple-quadrupole mass spectrometer (Sciex) and syringe pumps (Harvard Biosciences) and was allowed to equilibrate with the running buffer only (no indicator) until the background level of the ion $[M + H]^+$ signal corresponding to the indicator was stable, and then data were acquired. After 1 min, the system was then switched to the analyte solution (now containing the indicator with or without test ligands) and data collection continued until the indicator signal had maximized for at least 10 min. The column was washed with running buffer until the indicator signal had reduced to its background level to regenerate the column. The data were analyzed using a customized Excel macro to determine the breakthrough times of indicators, ligands, and M3, which uses the principle of taking the first derivative to calculate the point of inflection of the breakthrough front, which is independent of the shape of the front (sigmoidal or asymmetric).

ELISA. A standard ELISA assay was performed according to instructions as supplied with the kit. The plates were coated by the addition of 100 μL of 10 μg/mL poly(Glu, Tyr) in coating buffer (0.1 M sodium carbonate, 0.9% NaCl, pH 9.6) to 96-well MaxiSorp plates (Nalge Nunc International) and incubated overnight at 4 °C. The plate wells were washed 3 times with 250 μL/well coating buffer and then blocked with 200 μL of 1% BSA in coating buffer for 45 min at 25 °C. Just prior to the addition of ATP, plates were washed 5 times with coating buffer. ATP (12.5 μM) in protein tyrosine kinase (PTK) assay buffer (50 mM HEPES pH 7.5, 100 mM NaCl, 20 mM $MgCl_2$, 10 mM $MnCl_2$, 0.2 mM Na_2VO_4, 1 mM DTT) and 0.1−10 μL of the kinase inhibitor (100 μM, 10% DMSO), plus appropriate PTK assay buffer, were added to each well. The assay was initiated with EphB2 (120 ng/well) with incubation at 25 °C for 60 min. For the negative and positive controls, PTK assay buffer was used instead of protein or kinase inhibitor, respectively. The kinase incubation was stopped by aspirating the well contents and washing 5 times with 250 μL of wash buffer (10 mM phosphate buffer, pH 7.4, 2.7 mM KCl, 137 mM NaCl, 0.02% Tween 20). After washing, 100 μL of a 1:40 000 dilution of anti-phosphotyrosine peroxidase conjugate in wash buffer with 1% BSA was added and the resultant mixture incubated for 60 min at 25 °C. After incubation, the plates were washed 6 times with 250 μL of wash buffer and 100 μL of freshly prepared *o*-phenylenediamine substrate solution was added, and the plates were incubated for 7 min in the dark at room temperature. The reaction was stopped by the addition of 100 μL of 2.5 N H_2SO_4, plates were read in a SpectroMax Plus microplate reader (Molecular Devices) at 492 nm, and IC_{50} values were calculated using Excel.

RESULTS

Simultaneous ATP and Substrate Site FAC-MS Kinase Assay (Dual Binding Site Assay). To showcase the dual binding site FAC-MS kinase assay method, we chose PKCα as the target protein since there were known and commercially available substrate site ligands that could potentially act as indicators. We anticipated that we would find ATP binding site ligands that could act as indicators from commercially available known ATP site kinase inhibitors. As mentioned, an indicator is a compound that binds to a specific site on the immobilized protein target and will saturate the available binding sites in a specific column infusion time. If there are any competing ligands present that also bind to the same site as the indicator, then they will compete with the indicator for binding and decrease its breakthrough time shifting the indicator "front" to the left.

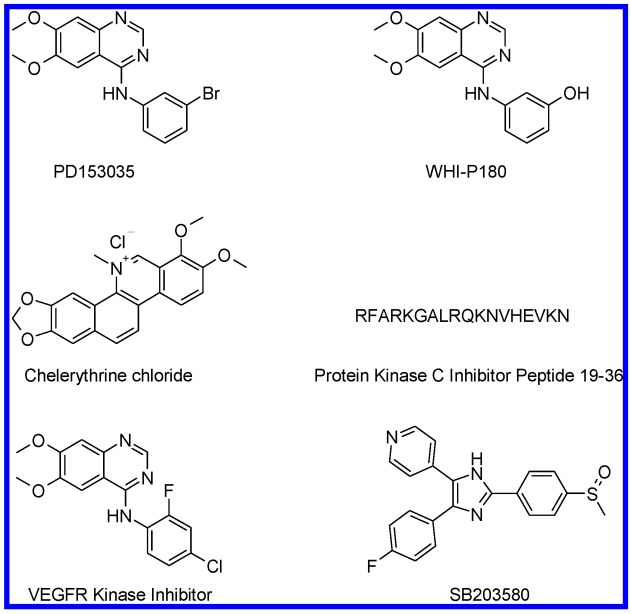

Figure 1. PKC and other protein kinase inhibitors.

Specifically, the ligands for the PKCα kinase substrate site were the PKC peptide inhibitor peptide 19−36, (IC_{50} = 147 nM)[24,25] and the small molecule, chelerythrine chloride,[26,27,28] which is a PKCα substrate site competitive inhibitor (IC_{50} = 660 nM) that is noncompetitive with respect to ATP (Figure 1). The ligands for the ATP binding site were identified to be PD153035 and WHI-P180 (Figure 1).

After a protein of interest has been immobilized in the FAC-MS column, its binding activity must be confirmed. This is accomplished by infusing a ligand with a known affinity for the immobilized protein target. If the immobilized protein is indeed binding the ligand, the ligand's breakthrough time will be delayed compared to that of a void marker, which has no affinity for the immobilized protein. The nonspecific binding of the ligands to the FAC-MS system (capillary lines, columns, streptavidin beads, etc.) is accounted for by analysis in the absence of the target protein. Hence, after PKCα immobilization, confirmation of FAC-MS activity was obtained (shown in Figure 2a) by observing significant binding (delayed breakthrough time of 30 min compared to the void marker, M3 at 7 min) of the substrate binding site ligand, chelerythrine chloride. In addition, we determined K_d for chelerythrine chloride to be 698 nM against PKCα (Figure 3), which is comparable to the literature IC_{50} value of 660 nM. With respect to the ATP binding site, we identified the EGFR

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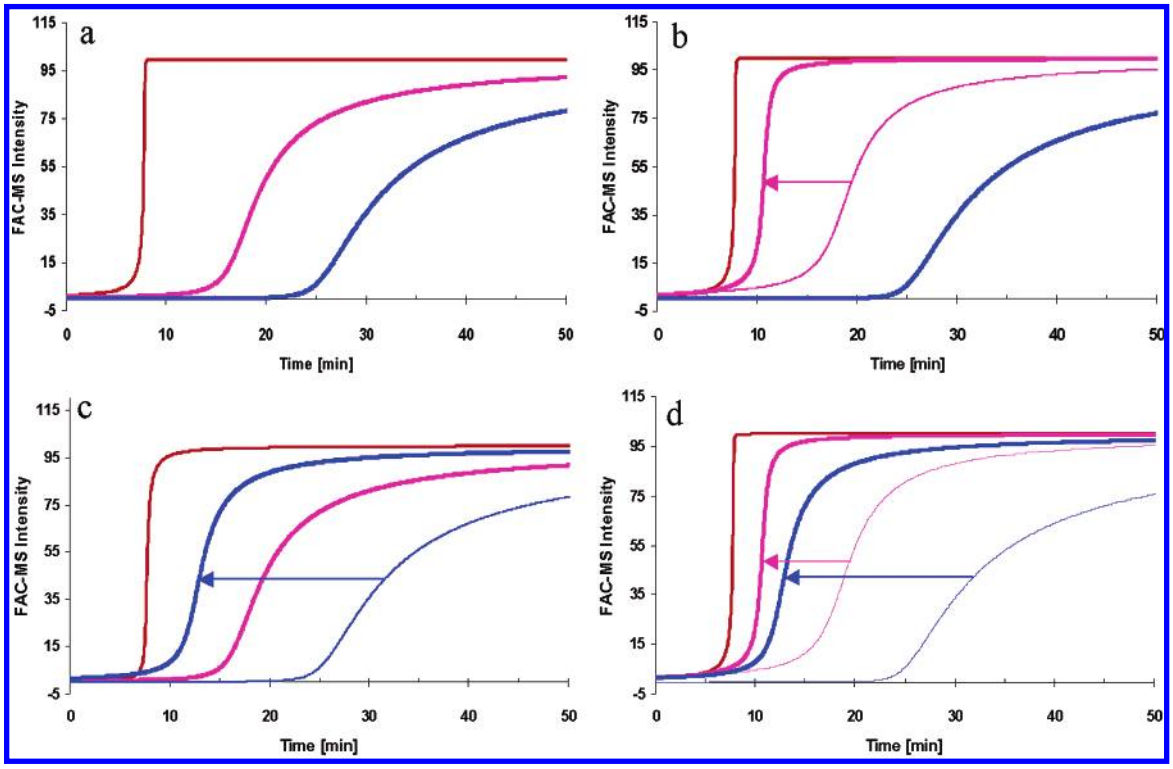

Figure 2. FAC-MS chromatograms of dual indicators for PKCα immobilized to CPG beads through the streptavidin/biotin complex. (a) In the chromatograms, the red lines correspond to the void marker, M3; blue lines correspond to chelerythrine chloride (1 μM), the substrate site indicator; and magenta lines correspond to PD153035 (1 μM), the ATP site indicator. Arrows indicate respective shifts. FAC-MS intensities smoothed and normalized for clarity. (b) Effect of adding WHI-P180 (5 μM) to the infusate. Only PD152025 (1 μM), the ATP site indicator, shifted to the left from 19 to 10 min. (c) Effect of adding protein kinase C inhibitor peptide 19−36 (5 μM) to the infusate. Only chelerythrine chloride (1 μM), the substrate site indicator, shifted to the left from 30 to 12 min. (d) Effect of adding both WHI-P180 (5 μM) and protein kinase C inhibitor peptide 19−36 (5 μM) to the infusate. Both indicators shifted to the same extent as when they were analyzed separately.

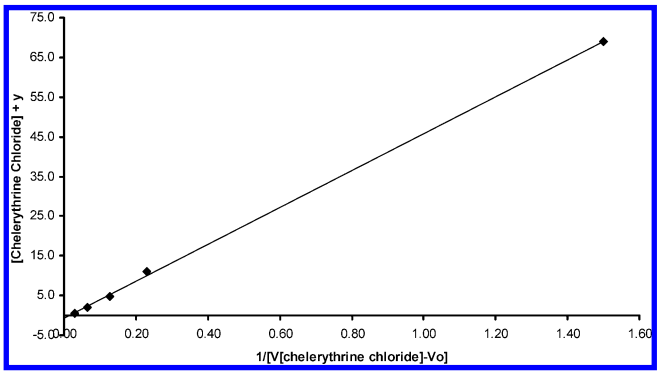

Figure 3. K_d determination for chelerythrine chloride. A plot of successive increasing [chelerythrine chloride] + y versus the reciprocal breakthrough volumes $1/(V_{[\text{chelerythrine chloride}]} - V_0)$. This utilizes the staircase equation, where y is concentration correction term[2] and generates a straight line with the slope equal to the amount of immobilized protein B_t (46 pmol for this PKCα column) and the y intercept equal to $-K_d$ (698 nM for chelerythrine chloride in this case).

kinase inhibitor, PD153035,[29] that gave a delayed breakthrough time of 18 min compared to void marker M3 (also in Figure 2a), again indicating activity with a measured K_d of 7.6 μM. Neither compound affected each other's breakthrough time, regardless of the concentration used. In addition, both compounds also gave good MS signals, had a high dynamic range, and had low nonspecific binding. Therefore, chelerythrine chloride and PD153035 were able to act simultaneously and independently as dual indicators for their two respective binding sites of the catalytic domain of PKCα.

With indicators for the two binding sites for PKCα now established, we wished to identify additional competitive ligands for the two sites from our kinase inhibitor collection. When WHI-P180[30] was added to the infusate, only the ATP binding site indicator (PD153035) shifted to the left from 19 to 10 min as shown in Figure 2b, indicating that WHI-P180 is an ATP-competitive ligand for PKCα (K_d = 14.5 μM). WHI-P180 had no effect at the substrate binding site of PKCα since the breakthrough time of chelerythrine chloride remained unchanged. An analogous situation occurred for the substrate binding site, shown in Figure 2c, when PKC inhibitor peptide 19−36 (a PKCα substrate site inhibitor) was added and only chelerythrine chloride shifted to the left from 30 to 12 min but the breakthrough time of PD153035 remained unchanged. The PKC inhibitor peptide 19−36 only affected the substrate site indicator. These results were further confirmed when by mixing all ligands together, identical results were obtained (Figure 2d) with both chelerythrine chloride and PD153035 shifting to the left.

Regarding indicators for the ATP binding site, we could not use the known group of aminoalkyl bisindolylmaleimide[31] inhibi-

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tors for PKC as indicators for FAC-MS because of their very high nonspecific binding interactions tested with neutral proteins (BSA) and the FAC-MS system. Bisindolylmaleimide II, however, as expected, generated a substantial shift to the left (>70%) of the PD153035 indicator, showing that it was a potent binder of PKCα.

Inactive Kinase FAC-MS Assay. Nature has designed very sophisticated regulatory processes to regulate kinase activity in cells.[32] Kinases themselves use a variety of autoinhibitory mechanisms to maintain inactive configurations in quiescent cells.[33] The problem is that once these mechanisms are disrupted, disregulated kinase activation can occur leading to disease. Most kinase inhibitors aim at inhibiting this aberrant kinase activity and, thus, target active kinases. It has been established, however, that even in well-regulated systems there is equilibrium between active and inactive kinases.[11]

There is also the case regarding the mechanism of action of Gleevec. The crystal structure of Gleevec bound to its primary target, Abl, reveals that upon binding it stabilizes or, rather, traps Abl in an inactive conformation.[14] In this way, Gleevec abrogates the aberrant kinase activity of the constitutively activated Bcr-Abl fusion protein. Thus, targeting a unique inactive conformation of a kinase, in addition to the current targeted active kinases, may provide a new means for kinase inhibition. It has been suggested that this avenue may yield compounds with improved selectivity profiles due to enhancement of structural and topological differences arising from the differences in activation or conformational states.[9] A major question, however, is how do you find inhibitors that favor one state over the other and take advantage of these differences when current screening methods that rely on a kinase activity readout require active kinases? Crystal structures clearly provide valuable information and insight with respect to binding but cannot be used effectively in a screening exercise. Here again we show the utility of FAC-MS as a unique assay tool by screening for binders of an inactive form of the EphB2 receptor tyrosine kinase.

The erythropoietin-producing hepatocellular (Eph) receptor family and several other receptor tyrosine kinases have invariant tyrosine residues in their juxtamembrane regions.[34] Autophosphorylation of these tyrosines provides a docking site for many downstream signaling proteins but also plays a crucial autoinhibitory function and has been shown to directly stimulate kinase catalytic activity.[35] It has been shown with EphB2 that mutation of the two juxtamembrane tyrosines 604 and 610 to phenylalanines[36] (Y604/610F) generated an inactive kinase. It is believed that without autophosphorylation of these tyrosines, this (Y604/610F) mutant of EphB2 remains in a pseudoautoinhibited state.

To demonstrate the capability of FAC-MS to identify binders of an inactive kinase, we evaluated both the active and inactive forms of EphB2 under similar conditions. It should be expected that the conformations of the ATP binding pockets of both the active and inactive EphB2 proteins are similar and it is the (Y604/610F) juxtamembrane mutations that render the kinase inactive.[31] By analyzing both active and inactive forms, the FAC-MS results for the active EphB2 act as a reference and provide confidence that any observed binding for the inactive (Y604/610F) EphB2 is real. To confirm that the (Y604/610F) EphB2 mutant was inactive, we first measured the ability of both active and (Y604/610F) inactive EphB2 kinases to phosphorylate a peptide substrate in an ELISA format at two different protein concentrations, 120 and 240 ng/well, respectively. As shown in Figure 4, the activity of the active/wild-type EphB2 doubled as the concentration doubled, whereas as expected, the (Y604/610F) EphB2 mutant showed no activity at either concentration. For FAC-MS, active EphB2 (His tagged) was immobilized first by treatment with biotinylated anti-His tag monoclonal antibodies and then immobilized onto the streptavidin-coated CPG beads through the streptavidin/biotin complex.

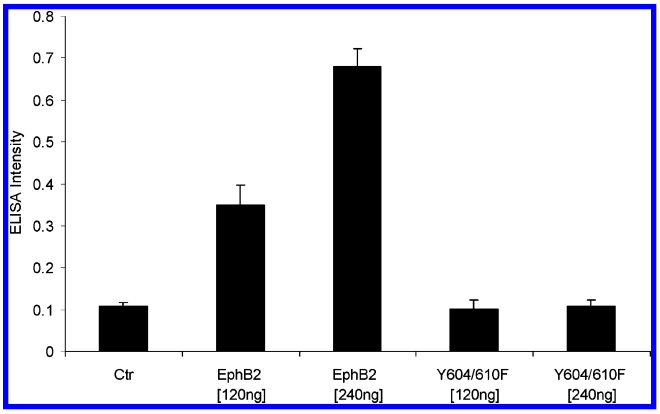

Figure 4. ELISA comparison of EphB2 (active) with Y604/610F EphB2 mutant (inactive) evaluated at 120 and 240 ng/well protein. The results are an average of three experiments.

To validate the ability to screen the inactive (Y604/610F) EphB2 mutant kinase, we first needed to show binding to active EphB2 since the assumption was that the ATP binding site was similar in both proteins. Previously we had identified a series of known commercially available kinase inhibitors as binders to active EphB2.[8] We chose four of these inhibitors (VEGF kinase inhibitor, $K_d = 6.2\ \mu M$; WHI-P180, $K_d = 2.4\ \mu M$; SB203580, $K_d = 6.8\ \mu M$; PD153035, $K_d = 8.1\ \mu M$; see Figure 1) and passed them as a mixture over active EphB2 generating the FAC-MS chromatogram shown in Figure 5a. Although all the compounds are competing for the same (ATP) site, each compound will also be competing with each other, and as a result, their respective fronts are reduced compared to if they were run individually (data not shown). The point, however, is that these four known inhibitors are still displaying binding since their breakthrough times were greater than that of the nonbinding void marker, M3, and can be used as control compounds for evaluating FAC-MS screening of the (Y604/610F) inactive EphB2 mutant.

The inactive (Y604/610F) EphB2 mutant was immobilized in a slightly different fashion to the beads since it was GST-tagged (as opposed to the His-tagged active EphB2). It was first biotinylated and then attached to the beads through the streptavidin/biotin complex. When the same four kinase inhibitors were screened against the (Y604/610F) inactive EphB2 kinase, essentially the same results and rank order were obtained (Figure 5b). Although the methods of immobilization were different for the two EphB2 kinases, we found that the nonspecific binding interactions were minimal and similar. Subtle differences between

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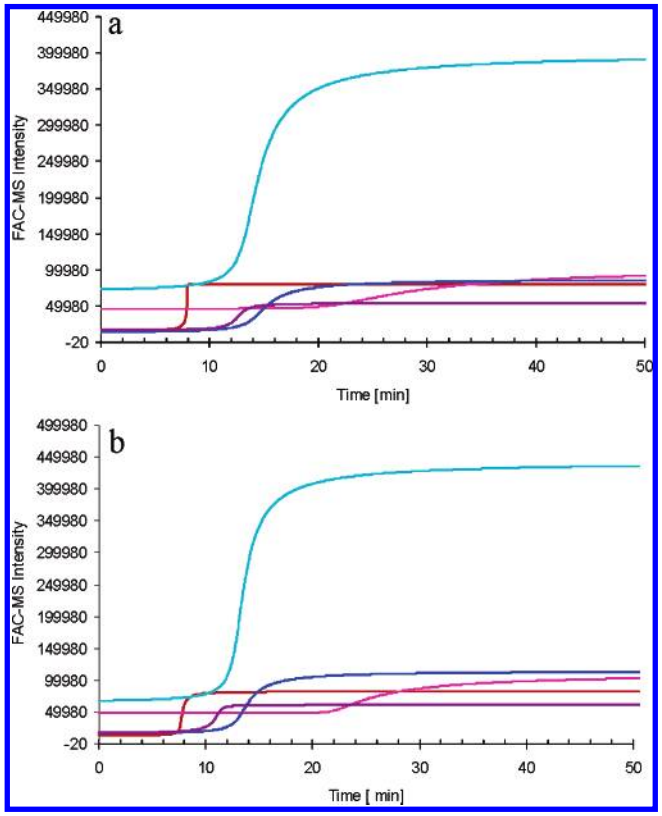

Figure 5. FAC-MS chromatograms showing the effect of four known kinase inhibitors evaluated against (a) active EphB2 and (b) inactive Y604/611F EphB2 mutant. In the FAC-MS chromatograms, red lines correspond to the void marker, M3 (1 μM), blue lines correspond to SB203580 (5 μM), magenta lines correspond to PD153035 (5 μM), purple lines correspond to WHI-P180 (5 μM), and turquoise lines correspond to the VEGF kinase inhibitor (5 μM). Original FAC-MS intensities preserved but smoothed for clarity.

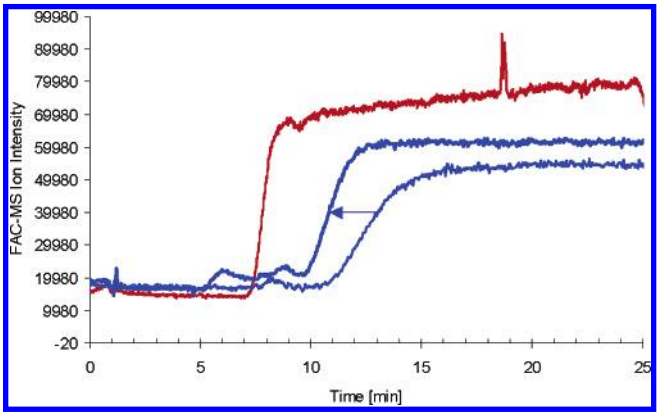

Figure 6. Original FAC-MS chromatograms showing the effect of a mixture of nine compounds on the indicator, WHI-P180, using immobilized inactive Y604/611F EphB2 mutant. Red line corresponds to the void marker, M3 (1 μM), thin blue line corresponds to WHI-P180 (1 μM), and thick blue line corresponds to WHI-P180 (1 μM) plus the mixture of nine compounds each at 5 μM. Arrow shows a shift indicating the presence of at least one compound competing with WHI-P180 for this inactive kinase.

the two sets of chromatograms are present and expected due to the different immobilization methods and differing amounts of protein on the columns. Nonetheless, to our knowledge, this shows for the first time an ability to assay a functionally inactive kinase to identify binding ligands.

To further elaborate and show that FAC-MS can screen for ligands against this inactive kinase, we also screened a mixture of nine ligands that also had been previously shown to be active against active EphB2 using WHI-P180 as the indicator. As shown in Figure 6, this mixture shifted WHI-P180 to the left, demonstrating that at least one compound in this mixture was competing with WHIP-180 for binding to this inactive (Y604/610F) EphB2 kinase. Although Figure 6 is an example of a FAC-MS chromatogram typically seen in a screen,[8] the important difference is that this screen was carried out with a functionally inactive (or dead) kinase. The conclusion from this inactive (Y604/610F) EphB2 kinase FAC-MS study is that even though the structure of the inactive EphB2 has been solved,[31] until now it would have been difficult to use this inactive variant of EphB2 directly in a traditional functional assay or screen.

DISCUSSION

We have introduced novel applications of a screening method (FAC-MS) that allows the discovery of small-molecule hits to protein targets that could be problematic to accomplish using traditional screening assays. By specifically targeting multiple sites on protein targets and allowing the screening of proteins in their inactive form, FAC-MS screening allows a potentially higher level of target specificity to be achieved and a corresponding ability to reduce the potential toxic effects of the selected compounds. Although we chose to emphasize its application as a "global kinase" screen, the principles demonstrate that FAC-MS screening can be applied to most kinds of protein classes that possess multiple binding sites, whether their function is known (and assayable) or not. Since current standard kinase assays can only monitor one specific binding site at a time and no information is derived that indicates to which site the compounds are actually binding, this dual-site FAC-MS capability represents an advancement regarding kinase assays.

We chose PKCα as the model target to illustrate the dual binding site FAC-MS assay since there were well-known substrate binding site ligands (chelerythrine and PKC peptide inhibitor peptide 19−36) that are noncompetitive with respect to ATP. Since we are using immobilized PKCα, it is possible that chelerythrine is binding not at the substrate site but at some other binding site arising from changes to the protein upon immobilization. Since, however, these substrate binding site ligands, in our FAC-MS system, are conforming to their published observations, this indicates that our immobilized PKCα is behaving as expected toward these ligands. Although not yet shown in the literature, it is possible that, with a conventional kinase assay, two different fluorophore-tagged compounds for the two binding sites (as in PKCα) could potentially be used as long as there was minimal readout overlap.

Screening functional proteins is traditionally done using ELISA-type assays that measure the enzymatic reaction through fluorescent labeling detection methods. Recent advances have adopted the use of MS detection[37] to these general assay procedures, eliminating the need for the often-problematic fluorescent detection methods. The benefits of MS detection (e.g., highly quantitative data and sensitive measurements) and these FAC-MS screen-

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ing methods take this concept to the "next level" by applying MS detection to a binding assay that does not rely on the presence of a functional assay with the demonstrated ability here to screen an inactive form of EphB2 receptor tyrosine kinase. This application could easily be extended to other protein classes where there is equilibrium between active and inactive states of the target. By specifically targeting the inactive conformation, a higher level of specificity could be achieved that may reduce potential side effects. To our knowledge, this is the first time that such a unique screening capability has been described and may open new avenues of drug discovery especially with regard to kinases.

CONCLUSION

FAC-MS provides a versatile hit discovery and development methodology that can be applied in areas where traditional screening assays are not applicable such as determining binding site information and screening with inactive conformations of proteins. The use of FAC-MS to assay multiple binding sites on protein targets and against inactive forms of targets can potentially increase the chances of finding selective compounds for a particular target and potentially reduce these selective compound's toxicity.

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