Frontal Affinity Chromatography with MS Detection of EphB2 Tyrosine Kinase Receptor. 1. Comparison with Conventional ELISA

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FAC-MS offers a convenient method for measuring the relative binding strengths of ligands in a mixture and enables a rapid ranking and identification of ligands in the mixture as potential hits against immobilized targets. Using immobilized EphB2 receptor tyrosine kinase as the target and known kinase inhibitors, the results of FAC-MS screening (% shift) have been shown to correlate with the binding constant, K_d, and with IC_{50} results from the more traditional ELISA assay. Therefore, since FAC-MS can accommodate a wide variety of target proteins, its applications could play a broad role in drug discovery not only at the hit discovery stage but also during the subsequent more rigorous screening at the hit-to-lead and lead optimization stages.

Introduction

Traditionally, the pharmaceutical industry has relied on time-consuming assays of very large historical compound libraries for the identification of small molecule hits against therapeutic targets. During the past decade, advances in high throughput screening (HTS) technologies, combinatorial chemistry, and robotics have dramatically transformed the hit identification process. However, despite the fact that HTS screening of very large compound libraries does indeed provide hits (albeit at a much lower rate than expected), there are numerous difficulties associated with HTS assay development that are beginning to get noticed.[1] Furthermore, the majority of these HTS assays involve methods of detection, such as fluorescence, radioactivity, or absorbance that while very useful only provide indirect evidence of binding. This is because the detection of the binding event is not based on a distinct feature of one of the binding components. Therefore, a screening system that is a measure of direct binding, even at a moderate throughput, would be advantageous. Moreover, due to the large number of nonenzyme targets (ion channels, GPCRs, etc.) entering the drug discovery arena, assay technologies that do not rely on enzymatic activity, and, as such, are adaptable to "any target" screening, are promising. There are only a few technologies such as NMR and a variety of affinity and capture methods that provide binding data and species identification.[2] Frontal affinity chromatography with MS detection, termed FAC-MS, is another method that has been shown to be of value in this regard with the screening of mixtures and determination of binding constants (K_d).[3−6] The Biacore technology has also proven very useful in the determination of the kinetic constants k_{on} and k_{off} and of K_d and has recently been used in the kinetic screening of antibodies.[7] Without the use of MS, however, Biacore is unable to identify the various molecular species involved in binding.

A challenge with any new technology is demonstrating its utility and advantage in the face of already well-established methods and procedures. Therefore, we present a comparison of the results of a FAC-MS analysis with ELISA using the erythropoietin-producing hepatocellular (Eph) receptor tyrosine kinase (RTK) family member, EphB2. Overexpression and/or overactivation of RTKs has been implicated in a number of cancers and the identification of small molecule inhibitors for RTKs is currently a viable strategy for therapeutic intervention.[8,9] The RTK member, EphB2, is overexpressed in a number of cancers[10] such as colorectal cancer[11] and has been proposed as important in angiogenesis.[12] In addition to demonstrating the utility of FAC-MS we were ultimately interested in identifying small molecule inhibitors for EphB2. Furthermore, an EphB2 ELISA assay was previously developed that could independently verify the FAC-MS results.

Results

FAC-MS. The principles behind FAC-MS have been published elsewhere but briefly the technique is based on the continuous infusion of small molecules over a protein target immobilized onto a solid support column with MS dectection.[3,4] In contrast to traditional "capture and release" affinity chromatography methods, FAC-MS is based on an ongoing equilibrium between ligands flowing through the column containing the immobilized protein target. As ligands flow through the column they bind to the target with differing affinities. As a result, individual ligands are retained in the column causing an increase in their "breakthrough volume", that 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 mass spectrometry and corresponds directly to the time that the front (breakthrough time) is observed to pass through the column. As such, FAC-MS offers a very convenient way of measuring the relative binding strengths of ligands in a mixture and enables a rapid

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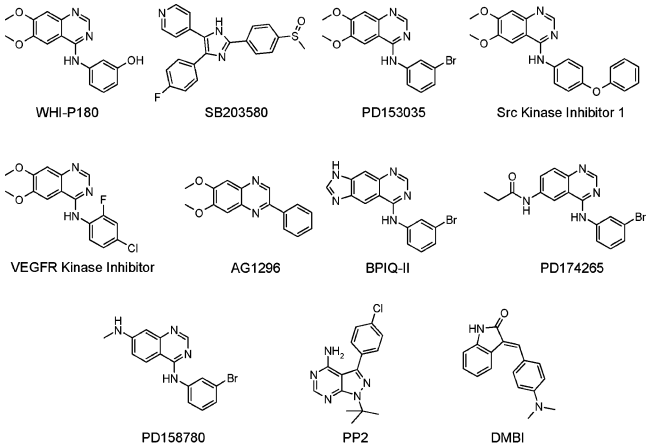

Figure 1. Known kinase inhibitors.

ranking of these ligands and the identification of potential hits against an immobilized target.

His-tagged EphB2 kinase domain was immobilized onto streptavidin-coated controlled-pore glass (CPG) beads by treatment with a biotinylated anti-His monoclonal antibody and packed into FAC-MS columns. To determine whether an immobilized protein is active by FAC-MS, an "indicator" and "void marker" are required. In this case, the indicator identified was the CDK2 inhibitor, WHI-P180[13] , which has an IC_{50} of 1.2 μM for EphB2. It was also determined that WHI-P180 has low nonspecific binding in the FAC-MS column and gave a relatively high stable MS signal. The void marker used was αMan(1→3)[αMan(1→6)]βManO-octyl[3] (M3), which gives the same elution front whether the target protein is present on the column or not.

We evaluated EphB2 by FAC-MS using a number of known kinase inhibitors shown in Figure 1. These kinase inhibitors represent inhibitors of a variety of kinase receptors such as p38 MAP kinase (SB203580[14]), EGFR (PD153035,[15] BPIQ-II,[16] PD174265,[17] PD158780[18]), Src family kinases (Src kinase inhibitor 1,[19] PP2[20]), VEGF family kinases (VEGF receptor tyrosine kinase inhibitor[21]), and PDGF kinase (AG1296,[22] DMBI[23]), respectively.

The crystal structure of WHI-P180 complexed in the ATP binding-site of CDK2 has been solved.[24] Thus, we assumed the binding of WHI-P180 to EphB2 to be ATP competitive as well but carried out kinetic assays to confirm this mechanism of action. In an autophosphorylation assay we varied the ATP and WHI-P180 concentrations, and the double reciprocal plot (Figure 2) shows that WHI-P180 is a competitive inhibitor of ATP. Our goal was to use the FAC-MS system using WHI-P180 as an indicator to profile a set of kinase inhibitors, expected to bind in the ATP binding-site of EphB2. If the kinase inhibitors have affinity for EphB2 at the ATP binding site, they will compete with the WHI-P180 indicator for binding and cause WHI-P180 to breakthrough earlier than it would in the absence of the inhibitor. This reduction in the breakthrough time of the indicator in the presence of an inhibitor is defined as a "shift". An example of this shift is shown in Figure 3, where BPIQ-II caused a reduction in the breakthrough time of WHI-P180 from 24 to 19 min.

We profiled the kinase inhibitors (Figure 1) by FAC-MS, using standard concentrations of WHI-P180 (1 μM),

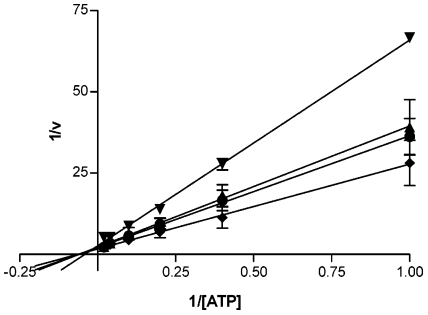

Figure 2. Double reciprocal plot of kinetic data of EphB2 protein kinase autophosphorylation activity at different concentrations of WHI-P180. The ATP concentrations in the reaction mixtures varied from 1 to 50 μM and WHI-P180 concentrations are 0 (♦), 0.25 (●), 0.5 (▲), and 1 μM (▼). *v* is nanomoles phosphate/min.

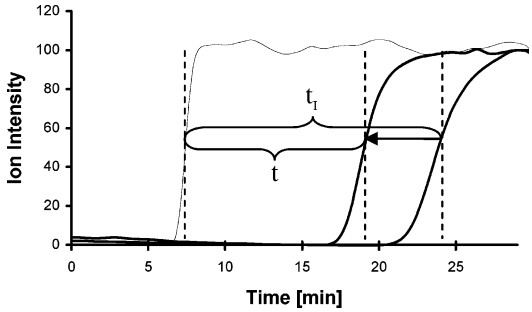

Figure 3. FAC-MS shift for WHI-P180 breakthrough time by BPIQ-II. On a column containing immobilized EphB2, the breakthrough time of WHI-P180 (1 μM, thick line) was reduced from 24.4 min to 19.8 min in the presence of BPIQ-II (5 μM). The thin line corresponds to the void marker, M3. All ion intensities were normalized to 100% of the maximum. The breakthrough time difference (measured at the inflection points, vertical dashed lines) between WHI-P180 and M3 is shown as t_I and the breakthrough time difference in the presence of the kinase inhibitor, BPIQ-II is *t*.

M3 (1 μM), and kinase inhibitor (5 μM), monitoring only WHI-P180 and M3. As exemplified in Figure 3, we monitored the breakthrough time for WHI-P180 alone, then in the presence of the inhibitors. To compare the reductions in breakthrough times for WHI-P180 in the presence of the inhibitors, the % shift is quantified from eq 1.

$$\% \text{ shift} = (t_I - t)/(t_I - t_{NSB}) \times 100\% \qquad (1)$$

where *t* is the breakthrough time difference (measured at the inflection point of the sigmoidal front) between WHI-P180 and M3 in the presence of the tyrosine kinase inhibitors, t_{NSB} is the nonspecific binding breakthrough time difference in the absence of immobilized EphB2 (and is a constant for the indicator used), and t_I is the breakthrough time difference in the absence of the kinase inhibitors (results given in Table 1). In this manner the FAC-MS % shifts can be used to rank the binding affinity of compounds for further analysis and ultimately further biological evaluation. In other words, the greater the % shifts, the greater the degree of competition for the indicator.

In addition to ranking compounds by their shifts of an indicator, an advantage of FAC-MS is the relatively straightforward determination of K_d. There are two ways to determine K_d via FAC-MS, the direct, or

Table 1. FAC-MS % Shift, IC_{50}, and K_d Values for the Kinase Inhibitors

compound	FAC-MS % shift[a]	K_d (μM)[b]	ELISA IC_{50} (μM)[c]
WHI−P180	−	2.4 + 0.2[d]	1.2 ± 0.4
SB203580	36	6.8 + 0.25[d]	7.8 ± 0.7
PD153035	28	8.1	10.2 ± 0.6
Src kinase inhibitor	11	58.4	86 ± 16
VEGFR kinase inhibitor	34	6.2	8.5 ± 0.3
AG1296	12	nd[e]	110 ± 20
BPIQ-II	19	15.2	22 ± 2.4
PD174265	6	nd	180 ± 15
PD158780	16	21.9	34 ± 3.1
PP2	14	36.5	52 ± 5.6
DMBI	17	19.5	27.5 ± 4.2

[a] % Shift = $(t_I - t)/(t_I - t_{NSB}) \times 100\%$. [b] Determined by the direct (or staircase) method, see Figure 4.[3] [c] Average of three measurements ± SD. [d] WHI-P180 and SB203580 were determined twice. [e] Not determined.

staircase, method and the indirect method.[3,4] The staircase method involves correlating the measured breakthrough volume of a ligand with its infusion concentration, whereas the indirect method relies on measuring an indicator as the probe for the state of equilibrium of the column with the ligand of interest. Using the staircase method, K_d values were determined for WHI-P180 (Figure 4) and a number of the kinase inhibitors with the results given in Table 1.

ELISA IC_{50}. We utilized a standard ELISA assay format to determine IC_{50} values for the set of kinase inhibitors for EphB2 (Table 1). Poly(Glu, Tyr) was the immobilized substrate, and horseradish peroxidase conjugate and *o*-phenylenediamine were the chromogenic substrates. DMSO (1%) was well tolerated by both EphB2 and the chromogenic substrate, allowing sufficient solubility of the kinase inhibitors in the assay. Although generally in ELISA, fluorescent or chemiluminescent substrates give increased assay sensitivity and much higher signals than colorimetric substrates, we were able to generate inhibition data for this series of kinase inhibitors in a reproducible manner. WHI-P180 was found to be the most potent inhibitor of EphB2 with an IC_{50} of 1.2 μM. The remaining kinase inhibitors were determined to have IC_{50}'s ranging from 7.8 to 180 μM (Table 1).

Discussion

A key component of FAC-MS is the immobilization of the target protein onto solid supports, and there are a number of ways in which this can be achieved. The most common method is to use the streptavidin/biotin complex ($K_d = 10^{-15}$ M) in which the protein is biotinylated and then coupled to a streptavidin-coated support. The streptavidin/biotin immobilization technique has been successfully used with FAC-MS for a number of different proteins such as β-galactosidase, sorbitol dehydrogenase, and the ligand binding domain of estrogen receptor β.[4] In our case, biotinylation of His-tagged EphB2 and treatment with streptavidin-coated CPG beads was unsuccessful in generating FAC-MS active columns. However, taking advantage of the His-tag on our EphB2 construct and coupling with a biotinylated anti-His antibody, we immobilized EphB2 to the CPG beads through a streptavidin/biotin-His/anti-His antibody system. The stability of the immobilized streptavidin/biotin-His/anti-His antibody EphB2 system on these FAC-MS columns is evident as the breakthrough time for WHI-P180 determined before and after each analysis was reproducible (1−2%) on the same column (data not shown). Subsequently, we also generated FAC-MS active columns by immobilizing EphB2 on beads via a direct covalent attachment to carboxylated CPG beads. In other FAC-MS applications, the direct covalent immobilization of polyclonal antibodies to sepharose beads also has been successful.[25,26] These results illustrate that FAC-MS could utilize any protein target, assuming that immobilization does not affect the activity of the protein (enzyme activity, access to binding pockets etc.).

Since the FAC-MS % shift results of the kinase inhibitors stem from competitive binding with WHI-P180, they should be closely correlated with K_d values, and as shown in Figure 5 (left panel), there is a good correlation (data from Table 1). We were, however, also interested in determining if these % shifts correlated with functional IC_{50} activity values determined from the more traditional ELISA assay. As shown in Figure 5 (right panel) again using the data from Table 1, the FAC-MS % shift, indeed, does correlate with the ELISA IC_{50} data. This correlation is not surprising since a low percent shift corresponds to competition of WHI-P180 with a low affinity kinase inhibitor. As the kinase inhibitor affinity for EphB2 gets closer to the WHI-P180 affinity, the percent shift becomes significant and, as such, the potential inhibitory activity of the kinase inhibitor increases. Similar correlations have been observed in FAC-MS studies with IC_{50} values for two other immobilized proteins. Specifically, using immobilized sorbitol dehydrogenase, the FAC-MS breakthrough times (measured as breakthrough volumes) of a series of closely related compounds were shown to correlate with their IC_{50} values.[4] Also, using an immobilized polyclonal antibody acting as an epidermal growth factor receptor mimic in a FAC-MS column, the breakthrough times of six naturally occurring compounds correlated with ELISA IC_{50} data.[25] Therefore all these correlations with IC_{50} suggest that FAC-MS can be used in practical applications with a variety of immobilized targets.

Moreover, this simple dependence of a FAC-MS % shift of the indicator on strongly binding ligands can be used with confidence as a reliable method in the screening of compound libraries. The % shift of the indicator correlates with compound affinity, which is a valuable asset when analyzing mixtures of compounds. For example, a mixture of all the kinase inhibitors in Table 1 (except SB203580), at 5 μM each, generated a substantial 68% shift of WHI-P180. Analysis of the individual inhibitors (deconvolution) of this mixture ultimately gave the results in Table 1. This illustrates the utility of FAC-MS for screening compound mixtures that upon further deconvolution will reveal the compound(s) responsible for binding.

All the kinase inhibitors tested gave IC_{50} values higher than WHI-P180. However, the theory of FAC-MS[3,4] also describes other effects in terms of discrimination between strong and weak binders. If there are only ligands present that have weaker K_d values than that of the indicator, the breakthrough time of the indicator will shift and retain its sigmoidal shape, as was the case

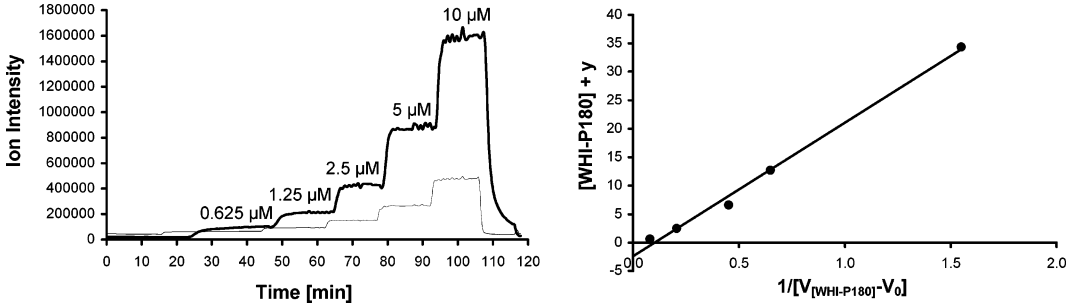

Figure 4. Staircase FAC-MS chromatogram and K_d determination for WHI-P180. Left panel. The thin line corresponds to the void marker, M3, and the thick line to WHI-P180. The concentration ranges injected consecutively for M3 and WHI-P180 were 0.625, 1.25, 2.5, 5, and 10 μM. Right panel. A plot of the successive increasing WHI-P180 concentrations, [WHI-P180] + y versus their reciprocal breakthrough volumes $1/(V_{[WHI-P180]} - V_0)$. This utilizes the staircase equation where y is a concentration correction term[4] and generates a straight line with the slope equal to the column capacity B_t and the y-intercept equal to $-K_d$ (2.4 μM for WHI-P180).

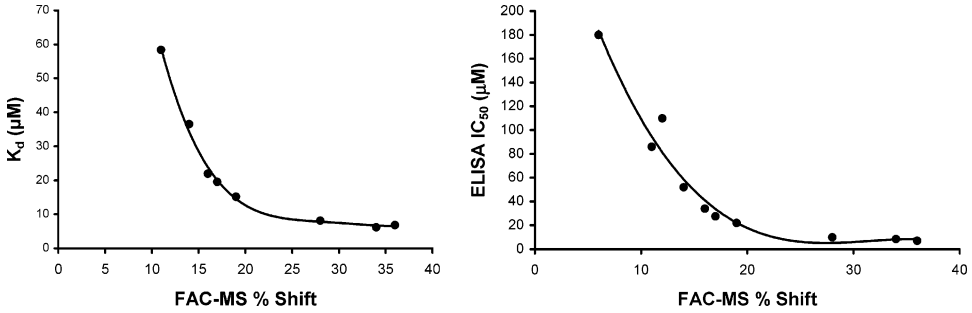

Figure 5. Correlation of the kinase inhibitors FAC-MS % shift with K_d (left panel) and ELISA IC_{50} (right panel). Data taken from Table 1.

for WHI-P180. If, however, there is a ligand present that has a stronger K_d than the indicator, a transient overconcentration is experienced which will manifest in the FAC-MS chromatogram as a characteristic "roll-up". Physically, this occurs because the indicator propagates through the column at a higher linear velocity than the stronger ligand. As the column equilibrates with the stronger ligand, some of the indicator gets displaced from the column, adding to the infusion concentration. We have seen this overconcentration using two of the kinase inhibitors in Table 1, SB203580 and WHI-P180. In this case, however, we used SB203580 as the indicator and monitored both compounds. This mode of indicator operation serves as the fastest route to the identification of mixtures containing "quality" ligands. The appearance of a rollup in the chromatogram indicates that in a tested mixture there exists at least one compound with a higher affinity than the indicator. As shown in Figure 6, WHI-P180 (K_d = 2.4 μM), which is the stronger binder, is causing a roll-up of SB203580 (K_d = 6.8 μM), the weaker binder.

All of the kinase inhibitors generated a shift indicating, as expected, that they are competing with WHI-P180 for binding and thus ATP-competitive. Since we have determined experimentally that WHI-P180 is an ATP-competitive inhibitor for EphB2, the shifts caused by the other kinase inhibitors suggest that they are ATP-competitive as well. It must be noted that, as with any competition assay, FAC-MS can distinguish whether a compound is ATP competitive without revealing the exact binding site. More extensive kinetic experiments or crystallography would need to be performed to establish if binding is occurring at the catalytic or allosteric site.

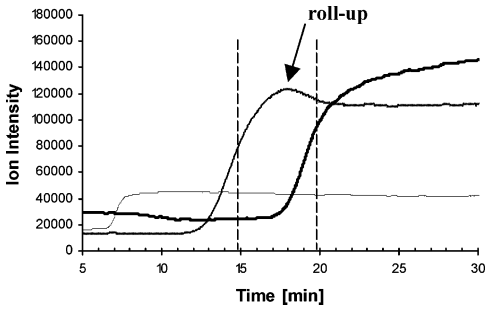

Figure 6. Roll-up of SB203580 (1 μM, K_d = 6.8 μM) caused by an equimolar concentration of WHI-P180 (1 μM, K_d = 2.4 μM). The thin line corresponds to the void marker, M3. The thick line with breakthrough time of 14.7 min and showing a roll-up (arrow) corresponds to SB203580, and the thickest line with breakthrough time 19.8 min corresponds to WHI-P180.

We assayed the set of kinase inhibitors here (Figure 1) with the expectation that they would generate structure−activity relationship data that would reveal ways of designing potent and selective EphB2 inhibitors. Consequently, this SAR was expected to reveal features of the ATP binding pocket that differentiate EphB2 from other kinases. Interestingly, the binding affinities and IC_{50} data of these kinase inhibitors revealed an initial SAR that when used together with structural information allowed us to "categorize" the EphB2 binding site and identify structural requirements desired for compound binding. For example, the most active compounds were SB203580, a potent inhibitor of p38 MAP kinase, and the VEGFR kinase inhibitor, whereas PD174265, a potent EGFR kinase inhibitor, was the weakest, suggesting the EphB2 ATP binding pocket may be structurally more similar to p38 than EGFR. Also, surprisingly, the Src kinase inhibitor was only moder-

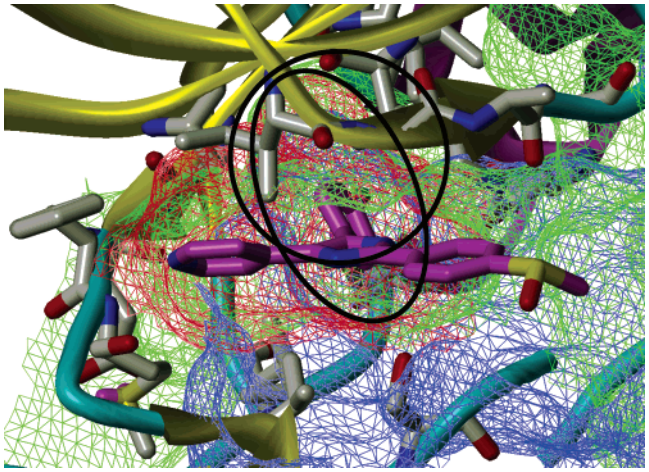

Figure 7. SB203580 docked in the ATP binding site of EphB2 showing overlap of Connolly channels for EphB2 (red), P38 (blue), and EGFR (green) active sites, respectively. The 4-flourophenyl group of SB203580 sits in the hydrophobic pocket of EphB2 whose elliptical shape and orientation are more like P38 than the more spherical pocket of EGFR.

ately active considering the higher degree of homology between the catalytic domains of Eph receptors and the Src family tyrosine kinases.[27] Comparisons of the X-ray crystal structures of p38 in complex with SB203580,[28] with the crystal structure of EGFR complexed with the 4-anilinoquinazoline, erlotinib,[29] and with the structure of EphB2 complexed with AMP-PNP[24] revealed the reason for the pattern in the inhibitor activity profile. Analysis of the superposition of the three structures followed by docking experiments (Figure 7) reveals that the hydrophobic pocket[30] of EphB2 is oriented almost perpendicular to the plane occupied by the adenine group of ATP in the ATP binding-site and can accommodate the 4-flourophenyl group of SB203580. The topology of the EphB2 hydrophobic binding pocket has more similarity to that of p38 than of EGFR.

Docking experiments of SB203580 in EGFR showed that the fit and orientation of the 4-flourophenyl group in EGFR is not optimum. This initial SAR together with structure analysis and further docking exercises will form the basis for the search of EphB2 inhibitors using an approach combining virtual screening and FAC-MS screening to be submitted in a subsequent manuscript.

Conclusion

We have demonstrated that the FAC-MS indicator % shift correlates well with two significant measurements in drug discovery, the IC_{50} value, and K_d constant of a compound for the EphB2 receptor tyrosine kinase. This is of great significance in that FAC-MS can be used as a moderate high-throughput assay tool for profiling compound mixtures in a hit discovery setting without need for deconvolution of every mixture. Only those mixtures that shift the indicator by a significant value contain compounds of interest and need deconvolution. Furthermore, we have demonstrated (data not shown) that the correlation between % shift and IC_{50} or K_d is predictive. Therefore, for a given target and using a small set of compounds to determine a K_d and IC_{50} correlation, these curves can then be used to predict IC_{50} values for other compounds. Moreover, we anticipate that this correlation with FAC-MS % shift and IC_{50} will also translate to many other protein targets, in particular those for which traditional assays have not been established or have been problematic. Since FAC-MS can accommodate a wide variety of target proteins, its applications could play a broad role in drug discovery not only at the hit discovery stage but also during the subsequent more rigorous screening at the hit-to-lead and lead optimization stages.

Experimental Section

Reagents. All kinase inhibitors (WHI-P180, SB203580, PD153035, Src kinase inhibitor, VEGFR kinase inhibitor, AG1296, BPIQ-II, PD174265, PD158780, PP2, and DMBI) and M3 were purchased from Calbiochem (San Diego, CA). Anti-His tag, clone 4D11, biotin conjugate monoclonal antibodies were purchased from Upstate Inc. (Charlottesville, VA). CPG glass beads (20 μm, 500 Å pore size) coated with streptavidin were purchased from CPG (now Millipore, Bedford, MA). $\gamma[^{32}P]$-ATP (6000 Ci/mmol) was purchased from PerkinElmer Life Science (Woodbridge, Ontario, Canada). SDS-polyacrylamide gels (4−20%) were obtained by BioRad (Mississauga, Ontario, Canada). Recombinant His-tagged EphB2 (kinase domain) was generously donated by MDS Proteomics (Toronto, Ontario, Canada). Poly(Glu, Tyr) 4:1, the protein tyrosine kinase assay kit, all solvents, and other reagents were obtained from Sigma-Aldrich (Oakville, Ontario, Canada).

FAC-MS. A solution of biotinylated anti-His tag monoclonal antibodies (100 μL, 100 μg) was incubated with streptavidin-coated CPG (20 μM) beads in 1X PBS buffer overnight at 4 °C. Afterward, 200 μg of EphB2 in 1X PBS with 10 μM $MgCl_2$ and 20 μM $MnCl_2$ was added and incubated for 24 h at 4 °C. After loading immobilized EphB2, the FAC-MS capillary columns (250 μm i.d. × 2.5 cm) were washed with 50 μL (at 200 μL/h) of 1X PBS buffer followed by 50 μL of the running buffer (20 mM NH_4OAc containing 1% DMSO). To block any remaining streptavidin sites, the column was infused with D-biotin (40 μM) and washed again with running buffer. The analyte solution contained WHI-P180 (1 μM) as the indicator 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 (Concord, Ontario, Canada) and syringe pumps (Harvard Biosciences, Holliston, MA) and was allowed to equilibrate with the running buffer until the WHI-P180 $[M + H]^+$ signal was stable, and then data were acquired. After 1 min, the system was switched to the analyte solution and data collection continued until the WHI-P180 signal had maximized for at least 10 min. The column was washed with running buffer until the WHI-P180 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 WHI-P180 and M3.

Competition Assay. Autophosphorylation assays were performed in which three WHI-P180 concentrations (0.25, 0.5, and 1 μM) were tested at six ATP concentrations (1, 2.5, 5, 10, 25, and 50 μM) in kinase assay buffer (20 mM HEPES pH 7.4, 1 mM $MnCl_2$, 1 mM dithiothreitol, 100 μM sodium vanadate, 2 mM $MgCl_2$, and 10 μM ATP (1 μCi $\gamma[^{32}P]$ATP) and 1 μM EphB2. WHI-P180 was dissolved in DMSO with the final DMSO concentration in the assay at 5%. Controls lacking inhibitor contained an equal concentration of DMSO. The reactions were initiated with the addition of ATP, incubated at 25 °C for 10 min. The reactions were terminated by the addition of SDS sample buffer, containing 9 mM EDTA, pH 8.0. Samples were applied without heating to 4−20% (w/v) Tris-glycine SDS-polyacrylamide gels. The gels were visualized by using a phosphoimager (BioRad) and analyzed with Quantity One software (BioRad 4.2.1). The data were plotted on a double reciprocal plot.

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, Rochester, NY) and incubated overnight at 4 °C. After being washed three times with 250 μL/well of coating buffer, the wells were 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 five 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) was prepared. All kinase inhibitors were prepared as 10X working solutions in 10% DMSO. ATP (12.5 μM, 80 μL) 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 five 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 incubated for 60 min at 25 °C. After incubation, the plates were washed six times with 250 μL of wash buffer, 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 SpectraMax Plus microplate reader (Molecular Devices, Sunnyvale, CA) at 492 nm, and IC_{50} values were calculated using Excel.

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