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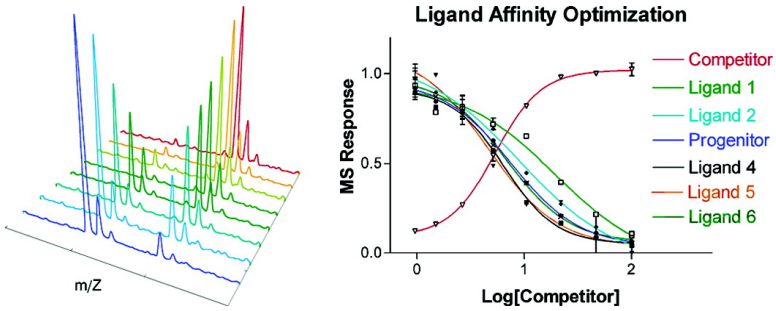

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A General Technique To Rank Protein−Ligand Binding Affinities and Determine Allosteric versus Direct Binding Site Competition in Compound Mixtures

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Abstract: To realize the full potential of combinatorial chemistry-based drug discovery, generic and efficient tools must be developed that apply the strengths of diversity-oriented chemical synthesis to the identification and optimization of lead compounds for disease-associated protein targets. We report an affinity selection-mass spectrometry (AS-MS) method for protein−ligand affinity ranking and the classification of ligands by binding site. The method incorporates the following steps: (1) an affinity selection stage, where protein-binding compounds are selected from pools of ligands in the presence of varying concentrations of a competitor ligand, (2) a first chromatography stage to separate unbound ligands from protein−ligand complexes, and (3) a second chromatography stage to dissociate the ligands from the complexes for identification and quantification by MS. The ability of the competitor ligand to displace a target-bound library member, as measured by MS, reveals the binding site classification and affinity ranking of the mixture components. The technique requires no radiolabel incorporation or direct biochemical assay, no modification or immobilization of the compounds or target protein, and all reaction components, including any buffers or cofactors required for protein stability, are free in solution. We demonstrate the method for several compounds of wide structural variety against representatives of the most important protein classes in contemporary drug discovery, including novel ATP-competitive and allosteric inhibitors of the Akt-1 (PKB) and Zap-70 kinases, and previously undisclosed antagonists of the M_2 muscarinic acetylcholine receptor, a G-protein coupled receptor (GPCR). The theoretical basis of the technique is analyzed mathematically, allowing quantitative estimation of binding affinities and, in the case of allosteric interaction, absolute determination of binding cooperativity. The method is readily applicable to high-throughput screening hit triage, combinatorial library-based affinity optimization, and developing structure−activity relationships among multiple ligands to a given receptor.

Introduction

To realize the full potential of combinatorial chemistry-based drug discovery, generic and efficient tools must be developed that apply the strengths of diversity-oriented chemical synthesis to the identification and optimization of lead compounds for disease-associated protein targets. Ideally, such tools would require no chemical modification, such as isotope labeling, fluorescence tagging, or immobilization on a solid substrate, of either the compounds or their biomolecule target. However, general techniques to directly assess binding mechanisms and evaluate ligand affinities in a multiplexed format are currently lacking, despite advances in chemical synthesis that have enabled considerable sophistication in the construction of diverse compound libraries to probe protein function.[1,2] Furthermore, genome and proteome analyses are rapidly increasing the number of human and bacterial proteins identified as potential targets for small molecule therapy of human disease, creating a critical need for methods of evaluating protein−ligand binding that are applicable to emerging drug targets for which functional assays are unavailable.[3]

Affinity selection-mass spectrometry (AS-MS) techniques hold particular promise for the achievement of these goals. AS-MS techniques uniquely and directly identify protein-bound components from complex mixtures by virtue of their molecular weights or collision-induced fragmentation patterns, making it possible to simultaneously evaluate multiple ligands from compound libraries.[4−17] Mixture components can be self-encoded by their molecular weight and do not require tagging

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with radioisotopes, fluorophores, or other moieties. The exquisite sensitivity of modern MS technology enables AS-MS experiments to be performed using very low (ng) amounts of a purified biomolecular receptor. Additionally, potential ligands can bind to all protein surfaces and not just the "active site", facilitating the study of ligands that act through allosteric binding and other mechanisms, even in the absence of secondary activity assays. The protein target and library components can be kept in solution, and any cofactors, metal ions, buffering reagents, or detergents necessary for proper protein folding and stability can be included in the experiment as well. In contrast to cellular or biochemical assays, AS-MS techniques report only compounds that bind directly to the target of interest, thus precluding false positives that arise from off-target activity or interactions with substrates or other reagents.

We describe here a multidimensional chromatography−mass spectrometry method for ranking the affinity of multiple ligands for a protein receptor while simultaneously demonstrating whether the ligands bind the same site as a competitor ligand or bind at an allosteric binding site. In instances where two ligands bind at different sites, the method yields their absolute binding affinity and a quantitative assessment of the degree of allosteric cooperativity between them. The method, which is generic with respect to protein class and requires only micrograms of a typical purified protein per analysis, is demonstrated with known and previously undisclosed ligands to targets from protein families of current interest in drug discovery, including the Zap-70[18] and Akt-1 (PKBα)[19] kinases and the muscarinic acetylcholine receptor M_2, a G-protein-coupled receptor (GPCR).

Results and Discussion

(A) Theoretical and Experimental Description of the Method. The AS-MS hardware configuration used in this study has been described previously for the discovery of small molecule ligands from mass-encoded combinatorial libraries.[20] Briefly, samples containing a mixture of the protein target and ligands are first incubated to equilibrate binding interactions and then injected into a rapid (<20 s) size-exclusion chromatography (SEC) stage that separates the resulting protein−ligand complexes from unbound components. This time-resolved separation eliminates false positive effects arising from nonspecific interactions with high dissociation rates.[8] The protein−ligand complex-containing band, identified by the protein's native UV absorbance, elutes in the void volume of the SEC column, while nonbinding library members are retained. A valving mechanism then captures and transfers the protein−ligand complex to a reverse-phase chromatography (RPC)-MS system where ligand dissociation from the protein is effected by high temperature (60 °C) and acidic (pH < 2) RPC conditions. The dissociated ligands are subsequently eluted in a high-resolution, electrospray-time-of-flight mass spectrometer for identification and quantification. The rapid SEC step moderates the loss of ligands with slow-to-intermediate dissociation rates; hence, the ligand recoveries measured by MS reflect the equilibrium concentrations of protein−ligand complexes present in a given sample.

The ability of a known competitor ligand to displace a target-bound library member, as measured by AS-MS, reveals the binding site classification and affinity ranking of mixture components. Specifically, equilibrium affinity selection experiments are performed with samples containing a constant concentration of the ligand(s) of interest and serially increasing concentrations of a competitor ligand. The AS-MS recoveries of the ligands and the competitor from such experiments reflect the equilibrium concentrations of each protein−ligand complex, thereby yielding information about the equilibrium dissociation constant (K_d) of each reaction component. The competitor used in these experiments may be either a known ligand for the target of interest, a representative chosen from multiple ligand classes discovered through (e.g.) a high-throughput screening campaign, or the progenitor of a series of structural analogues synthesized for affinity ranking.

Figure 1A shows the ligand recovery from a simulated AS-MS binding displacement experiment for direct competition between a titrant ligand S_1 and a second ligand S_2 for the single-site receptor E (Scheme 1).

Scheme 1

$$E{\cdot}S_2 \overset{K_{d2}}{\rightleftharpoons} E + S_1 \overset{K_{d1}}{\rightleftharpoons} E{\cdot}S_1$$
$$+ S_2$$

Increasing concentrations of titrant S_1 in the absence of ligand S_2 yield increasing concentrations of the receptor−ligand complex $E{\cdot}S_1$, ultimately saturating the receptor at the total protein concentration $[E]_0$, here 5.0 μM. The steepness of the hyperbolic binding curve depends on the equilibrium dissociation constant K_{d1}, here modeled as 2.0 μM. This saturation binding curve in the absence of a competitive ligand can be fit to eq 1 to yield K_{d1} and $[E]_0$.

$$K_{d1} = \frac{([E]_0 - [E{\cdot}S_1])([S_1]_0 - [E{\cdot}S_1])}{[E{\cdot}S_1]} \qquad (1)$$

In the presence of 2.0 μM directly competitive ligand S_2 with $K_{d2} = 0.5$ μM, titration by S_1 yields a shallower binding curve for $E{\cdot}S_1$, and the concentration of protein−ligand complex $E{\cdot}S_2$ decreases as the receptor is saturated by S_1. Importantly, the ratio of $E{\cdot}S_1$ to $E{\cdot}S_2$ increases linearly with total titrant concentration $[S_1]_0$ when the receptor is the limiting reagent (i.e., when the titrant and ligand must compete for the same receptor site). This linear relationship indicates mutually exclusive competitive binding (most simply explained by orthosteric interaction with the receptor) and is described[21] by

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(21) The derivation is provided in the Supporting Information.

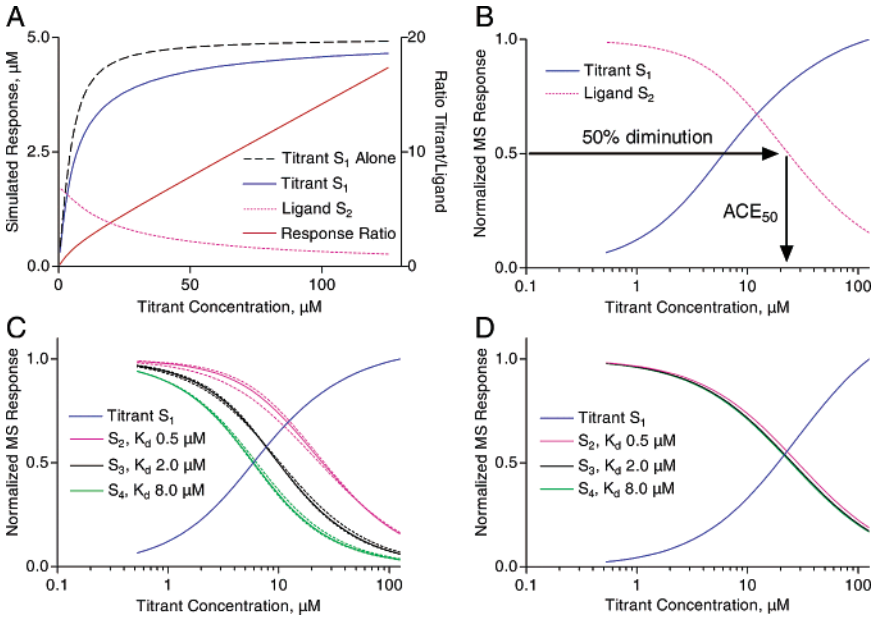


Figure 1. Simulated protein−titrant and protein−ligand complex concentrations from an AS-MS competition experiment. (A) The ratio of protein−titrant to protein−ligand complex concentrations increases linearly with titrant concentration if the ligand and titrant are directly competitive. (B) Data in (A) plotted with a logarithmic titrant concentration axis, illustrating the definition of ACE_{50} as used in the text. (C) A simulated ACE_{50} experiment for a mixture of three ligands of different K_d under receptor-excess conditions: Solid lines show recovery for the given ligand at 1 μM total concentration, and dashed boundary lines indicate recovery where that ligand is present at 0.33 or 3.0 μM. (D) Same as (C) except receptor concentration is limiting (2 μM) with respect to total ligand concentration (15 μM).

the following equation:

$$\frac{[E{\cdot}S_1]}{[E{\cdot}S_2]} = \frac{[S_1]_0 K_{d2}}{[S_2]_0 K_{d1}} \qquad (2)$$

Therefore, a plot of the ratio of ligand responses for $E{\cdot}S_1$ to $E{\cdot}S_2$ versus total titrant concentration $[S_1]_0$ will yield a straight line for directly competitive ligands where $[S_1]_0 > [E]_0$. If the MS response calibration factors of the titrant and ligand are known, then the slope of this line, $K_{d2}(K_{d1}[S_2]_0)^{-1}$, yields the ratio of ligand and titrant affinities.

This linear relationship holds even if the compounds' MS sensitivities differ in magnitude. If the MS responses of the ligand and titrant vary linearly with the concentration injected, then $E{\cdot}S_1$ response = calibration factor$_1$·$[E{\cdot}S_1]$. Substituting this expression (and the corresponding one for $E{\cdot}S_2$) into eq 2 and solving for the ratio of the responses (as opposed to the ratio of concentrations) introduces a linear term (the ratio of the calibration factors) into the right-hand side of eq 2, as shown below in eq 3. Regardless of the difference in magnitude, if the calibration factors are linear terms, then the ratio plot will be linear for directly competitive ligands.

$$\frac{E{\cdot}S_1 \text{ response}}{E{\cdot}S_2 \text{ response}} = \frac{\text{calfactor}_1[S_1]_0 K_{d2}}{\text{calfactor}_2[S_2]_0 K_{d1}} \qquad (3)$$

Importantly, although it is possible to determine the ligands' MS calibration factors (for example, by injecting standards of known concentration and plotting the MS response versus concentration), it is not necessary to know the ligands' MS calibration factors to determine the direct versus allosteric competition mechanism or to perform affinity ranking (vide infra).

For easier visualization and more straightforward interpretation, the MS response data in a titration series can be normalized by dividing the raw MS signal for each data point by the maximum signal observed within the series. For the ligands that are subject to competition, the maximum signal typically occurs where the titrant concentration is lowest. Plotting the normalized protein−ligand complex concentrations from the simulation in Figure 1A against a logarithmic competitor concentration axis yields sigmoidal curves for the increase of $[E{\cdot}S_1]$ and the diminution of $[E{\cdot}S_2]$ (Figure 1B), where the top of each curve is 1.0. The total competitor concentration $[S_1]_0$ at which each protein−ligand complex concentration is reduced to one-half its value in the absence of the competitive ligand is defined as the affinity competition experiment 50% inhibitory concentration (ACE_{50} value) and is dependent upon the K_d of the ligand and other experimental parameters. The ACE_{50} value, which describes the concentration of the competitor required to compete out 50% of the ligand of interest, is the converse of the ordinary definition of a biochemical or biophysical IC_{50}, which describes the concentration of the ligand of interest required to compete out 50% of a known compound, for example, a radioligand. In contrast to a conventional IC_{50} value, a higher ACE_{50} value indicates a higher-affinity ligand: Greater competitor concentration is required to displace the compound of interest from the binding site.

Figure 1C shows a simulated binding displacement experiment for a mixture of three ligands of varying K_d. This figure demonstrates how the ACE_{50} method may be used to simultaneously affinity-rank multiple compounds. In this simulation, the total concentration of all pool components ($[S_2]_0 = [S_3]_0 = [S_4]_0 = 1.0$ μM) is comparable to the total receptor concentration (5.0 μM). Under these conditions, individual library components bind independently to the excess receptor and compete primarily with the titrant. The ACE_{50} values are relatively insensitive to ligand concentration: If one of the ligands is changed to 0.33 or 3.0 μM, representing a 9-fold variation in the concentration

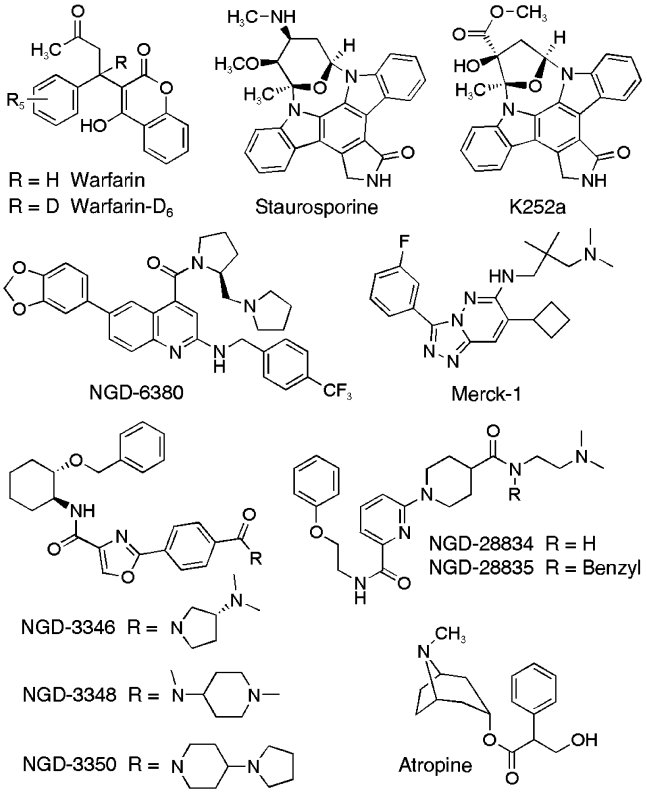


Figure 2. Structures of select compounds used in this study.

of that ligand, the results are virtually unchanged (Figure 1C, dashed boundary lines). This feature of the method is critical when directly evaluating synthetic mixtures where ligand concentration may vary due to differences in synthetic yield or solubility. The consequence of a higher pool concentration than receptor concentration is demonstrated in Figure 1D. In the extreme case shown in this simulation, three ligands of different K_d values yield nearly equivalent ACE_{50} values when the total ligand concentration of each of the three library components is 5 μM and the receptor concentration is 2 μM. This result highlights the importance of maintaining the sum of the library components' concentrations below that of the receptor.

In some respects, the ACE_{50} experiment resembles a radioligand displacement experiment but is far more versatile. No radiolabel is necessary, as all measurements are performed by MS analysis of stable isotopes. Also, no background subtraction of nonspecific ligand binding is required. Furthermore, individual ACE_{50} values are obtained from compound mixtures, which is not feasible for a typical radioligand quench experiment. The ACE_{50} value is independent of MS signal intensity; therefore, normalized MS responses yield readily interpretable ranking information. No MS response calibration is necessary to simultaneously determine ACE_{50} values for compounds that may have different electrospray ionization efficiencies; therefore, no pure calibration standards are needed, allowing unpurified mixtures to be used without a precise knowledge of the components' concentrations.

(B) Experimental Validation of the Method. As a first experimental validation of the ACE_{50} method, an AS-MS binding displacement experiment was conducted using the human serum albumin (HSA) ligand warfarin in competition with stable isotope-labeled warfarin-D_6 (their chemical structures

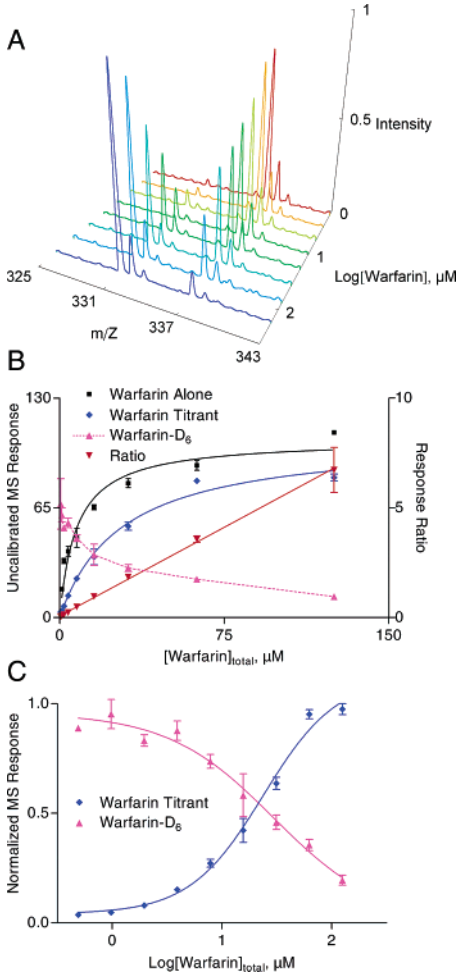


Figure 3. AS-MS-measured HSA−ligand concentrations from a binding displacement experiment between warfarin and warfarin-D_6. (A) A plot of the mass spectra summed under the RPC extracted ion chromatogram for warfarin (m/z = 331, $[M + Na]^+$) and coeluting warfarin-D_6 (m/z = 337, $[M + Na]^+$) with increasing warfarin concentration. (B) Plot of the ligand recoveries and the ratio thereof from the experiment in (A). The linear ratio versus titrant concentration relationship confirms direct binding competition. (C) Ligand recoveries from the experiment in (A) plotted with a logarithmic titrant concentration axis show 50% diminution of warfarin-D_6 signal at 29.6 μM titrant concentration. Data points are the mean ± one standard deviation of duplicate experiments.

are shown in Figure 2). This is an absolute example of orthosteric competition: The labeled and unlabeled compounds are expected to bind to the same receptor site with identical affinities and identical electrospray mass spectrometry response factors, yet be distinguishable by molecular weight.

Figure 3A shows the AS-MS results obtained by titrating a binding reaction between 5.0 μM HSA and 20 μM warfarin-D_6 with increasing concentrations of warfarin. The MS response of the deuterated ligand diminishes as the concentration (and MS response) of the unlabeled titrant increases. Plotting the ligands' MS responses from this displacement experiment versus titrant concentration reveals saturation binding by warfarin as binding by its deuterated counterpart is abated (Figure 3B). The results of an equivalent titration of HSA by warfarin in the absence of warfarin-D_6 are also shown. Nonlinear regression[22] of this saturation binding curve according to eq 1 yields a K_d

(22) We have found that the evaluation of protein−ligand K_d values by saturation binding titration coupled with AS-MS is a general technique for affinity quantification. This will be the subject of a future report.

of 5.5 ± 0.9 μM for warfarin binding to HSA, which is consistent with values reported in the literature for this protein−ligand interaction.[23] The titration curves in the presence and absence of competitor both asymptotically approach the same maximum signal. However, as expected, the binding curve is shallower in the presence of the competitor. The ratio of the uncalibrated MS recoveries versus titrant concentration is linear, indicating direct binding competition, with a slope of 0.054 ± 0.02 μM^{-1} ($r^2 = 0.986$). Given equivalent K_d values for the labeled and unlabeled ligands and equivalent MS responses factors, eq 2 yields an expected value of $1/[S_2]_0 = 0.050\ \mu M^{-1}$ for the slope in this experiment, in agreement with the measured value. This result confirms that the ACE_{50} method can identify directly competitive ligands by virtue of their recovery ratio plots.

In addition to competitive binding mode information, quantitative protein−ligand affinity estimates are also attainable using the ACE_{50} results. The K_d of the ligand of interest can be calculated from its measured ACE_{50} value if the receptor and ligand concentrations and the K_d of the competitor are known. Although the equations describing the relationship between the ACE_{50} and K_d are unwieldy in print, they are readily solvable by numerical methods using commercially available mathematical software programs and are provided, along with their derivations, in the Supporting Information.[24,25] These functions can also be used for ACE_{50} experiments in mixtures if the total ligand concentration is below the receptor concentration. Using the K_d value of 5.5 μM for warfarin measured separately by AS-MS saturation binding analysis, and an HSA concentration of 5.0 μM, the ACE_{50} value of 29.6 μM (95% c.i. 21.4−41.0 μM) from the experiment shown in Figure 3C yields a K_d of 4.9 μM for warfarin-D_6 (95% c.i. 3.1−8.2 μM). This K_d value is consistent with that measured for the unlabeled compound by independent titration and indicates that quantitative K_d estimates can be obtained using the measured ACE_{50} values.

To further validate the ACE_{50} method, orthosteric binding competition experiments between the well-known general kinase inhibitor staurosporine and its structural congener K252a were conducted with the emerging immunosuppression target Zap-70. Titration of 2.0 μM basal Zap-70 by staurosporine in the presence of 20 μM K252a yields the binding curves shown in Figure 4A. As expected, a plot of the ratio of uncalibrated MS responses versus titrant concentration gives a straight line, confirming direct binding competition between these two structurally similar ligands.

ACE_{50} experiments with the Zap-70 ligand NGD-6380 show that the method can also be applied to the study of novel ligands in competition with structurally dissimilar compounds. NGD-6380 was discovered through AS-MS screening of mass-encoded combinatorial libraries against the basal form of Zap-70, and it inhibits activated Zap-70 with a biochemical IC_{50} of 80 nM.[26] Titration of 2.0 μM Zap-70 by staurosporine in the presence of 20 μM NGD-6380 yields the binding curves shown in Figure 4B. The linear response ratio versus titrant concentration indicates direct binding competition between NGD-6380 and staurosporine. By analogy to the staurosporine-K252a result, this outcome suggests that NGD-6380 inhibits Zap-70 activity by overlapping occupation of the ATP binding site. Independent biochemical experiments at varying ATP concentrations confirm that both staurosporine and NGD-6380 are competitive with ATP (data not shown).

(C) Evaluation of Allosteric Ligands by the ACE_{50} Method. Like the Zap-70 ligand NGD-6380, the Akt-1 kinase ligand NGD-28835 was also discovered through AS-MS screening of mass-encoded combinatorial libraries against the basal form of its target.[27] However, NGD-28835 exhibits allosteric binding to its kinase target with respect to staurosporine. An ACE_{50} competition experiment using NGD-28835 as the titrant against 5.0 μM basal Akt-1 in the presence of 1.25 μM staurosporine yields the binding curves shown in Figure 4C. Diminution of staurosporine response with increasing titrant concentration indicates competitive binding. However, the ratio of the ligand recoveries rises to a plateau with increasing titrant concentration rather than increasing linearly as would be expected for orthosteric competition. Considerable nonlinearity of the response ratio is observed at high or low (shown) competitor concentration, and regardless of which ligand is used as the titrant. These results suggest NGD-28835 and staurosporine bind separate sites on Akt-1, with negative cooperativity between the sites.

The results of the Akt-1 titration described above can be explained by the ternary complex model[28] of allosteric binding (Scheme 2). In this model, ligands S_1 and S_2 bind distinct sites on receptor E with dissociation constants K_{d1} and K_{d2}, respectively. However, if both ligands bind simultaneously to the receptor, they affect each other's binding constant by a factor α. For example, S_1 binds to E with dissociation constant K_{d1}, but it also binds to the binary complex $E{\cdot}S_2$ to form ternary complex $E{\cdot}S_1{\cdot}S_2$ with dissociation constant $\alpha{\cdot}K_{d1}$. Where $\alpha > 1$, allosteric interaction by one of the ligands increases the dissociation constant of the other, resulting in negative cooperativity. Where $\alpha < 1$, positive cooperativity results, and if $\alpha = 1$, binding by one ligand has no affect on the binding of the other.[29]

The AS-MS configuration described here cannot separate the binary protein−ligand complexes from allosterically bound ternary complexes; all protein−ligand complexes coelute from the SEC stage. The measured recovery of a particular ligand therefore represents the sum of the protein−ligand complexes containing that ligand. For example, S_1 recovery correlates with the summed concentrations of the complexes $E{\cdot}S_1$ and $E{\cdot}S_1{\cdot}S_2$. Figure 4D shows the simulated AS-MS recovery of two allosteric ligands where S_1 with $K_{d1} = 2.0\ \mu$M is titrated into a mixture of receptor E at 5.0 μM concentration plus S_2 with $K_{d2} = 0.5\ \mu$M at 2.0 μM concentration. For cooperativity

(23) The K_d for HSA binding to racemic Warfarin has been reported from 3 to 6 μM by various techniques, including frontal analysis and equilibrium dialysis, and is temperature and pH dependent. See: Loun, B.; Hage, D. S. *Anal. Chem.* **1994**, *66*, 3814−3822.

(24) Examples are provided in the Supporting Information for *Mathematica* (Wolfram Research Inc., 100 Trade Center Dr., Champaign, IL 61820).

(25) The Cheng−Prusoff equation relating the affinity constant and 50% inhibitory concentration cannot be applied here as the ligand concentrations are comparable in magnitude to their affinity constants. See: Cheng, Y.; Prusoff, W. H. *Biochem. Pharmacol.* **1973**, *22*, 3099−3108.

(26) Delphia Assay: Braunwalder, A. F.; Yarwood, D. R.; Sills, M. A.; Lipson, K. E. *Anal. Biochem.* **1996**, *238*, 159−164.

(27) Scott, M. P.; Makara, G.; Nan, Y.; Mansoor, F.; Takonda, P.; Liu, B.; Hou, Y.; Whitehurst, C.; Falb, D.; Siddiqui, A.; Alaoui-Ismaili, M. H. *Identification of Novel and Selective Akt-1 Inhibitors Using Affinity-based Screening of Both Basal and Activated Forms of Akt-1*. Presented at the American Association for Cancer Research Meeting, Boston, MA, November 17, 2003.

(28) For a recent review, see: Christopoulos, A. *Nat. Rev. Drug Discovery* **2002**, *1*, 198−210.

(29) Ehlert, F. L. *Mol. Pharmacol.* **1998**, *33*, 187−194.

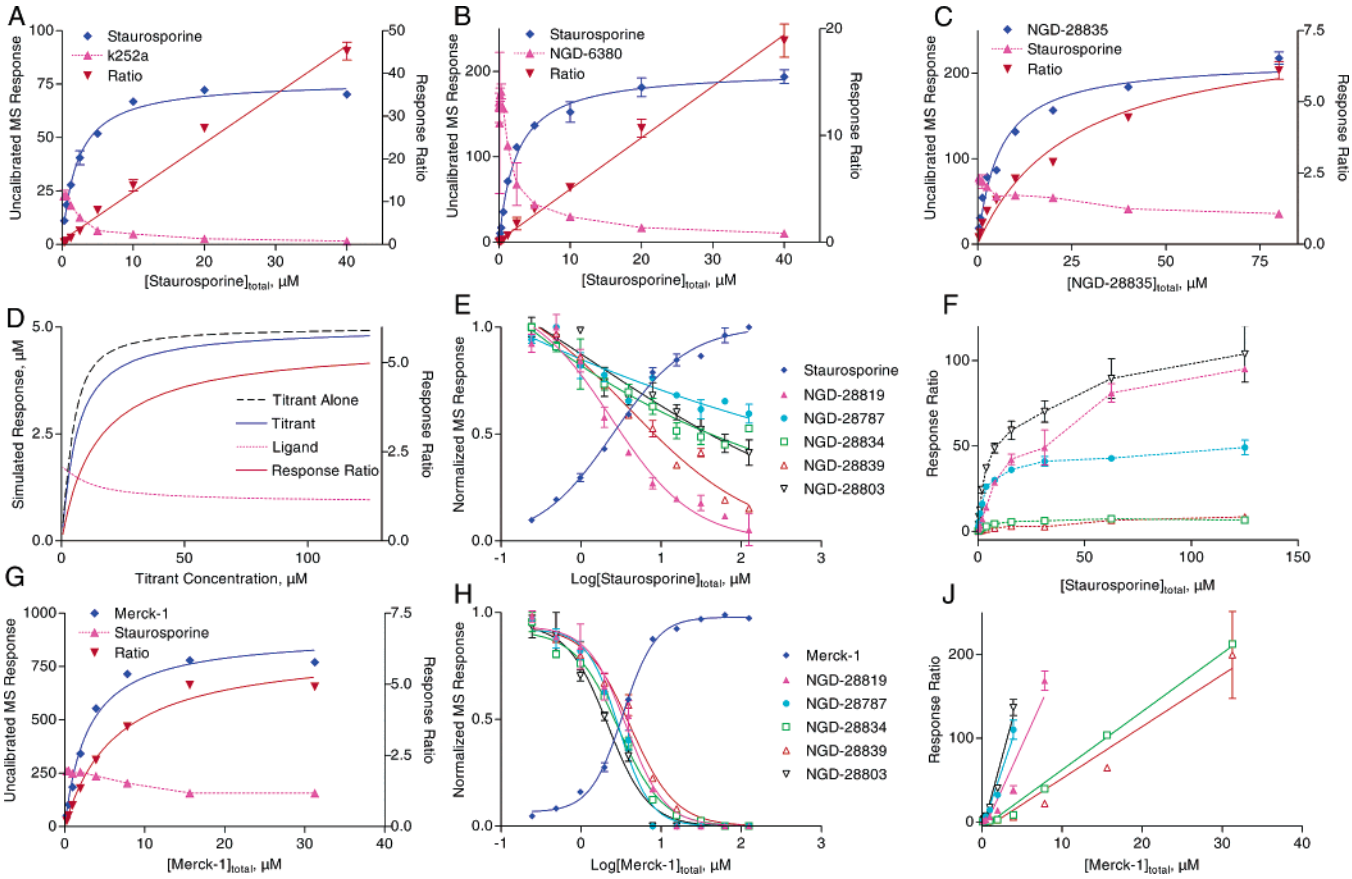
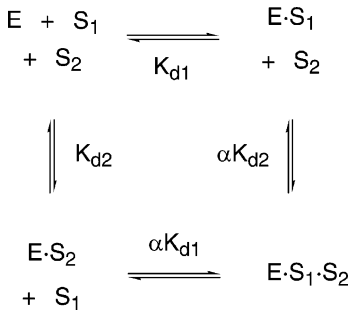


Figure 4. Evaluation of direct and allosteric competition between kinase ligands by the ACE_{50} method. Data points are the mean ± one standard deviation of duplicate experiments. (A) AS-MS-measured Zap-70−ligand complex concentrations versus increasing titrant concentration for titration of K252a and (B) NGD-6380 by staurosporine. The linear ratio versus titrant concentration relationship indicates direct binding competition between staurosporine and both K252a and NGD-6380. (C) AS-MS-measured Akt-1−ligand complex concentrations and the ratio thereof for a titration of staurosporine by NGD-28835. The asymptotically bounded ratio plot indicates allosteric binding competition. (D) Simulated AS-MS recoveries of an allosteric-competitive titrant and ligand and the ratio thereof with increasing titrant concentration. (E) AS-MS-measured protein−ligand complex concentrations and (F) the ratio thereof from an ACE_{50} experiment between staurosporine and a mixture of ligands discovered by screening of mass-encoded combinatorial libraries. The hyperbolic ratio plots indicate allosteric binding. (G) AS-MS-measured Akt-1−ligand complex concentrations and the ratio thereof for a titration of staurosporine by allosteric ligand Merck-1. (H) AS-MS-measured protein−ligand complex concentrations and (J) the ratio thereof from an ACE_{50} experiment between Merck-1 and the mixture of ligands in (E) and (F). In contrast to the results with staurosporine, direct binding competition is observed between the pool members and Merck-1. Note the ratio plots are not corrected for MS response calibration.

Scheme 2



factor $\alpha = 10$, negative cooperativity between the two sites causes the recovery of S_2 to diminish with increasing titrant concentration. However, its recovery does not diminish to zero as was the case for direct binding competition; rather, the ligand's recovery simply decreases to a plateau because its receptor concentration remains constant (S_2's binding site is not occupied by titrant S_1), while K_{d2} is increased by the factor α. This has an important and measurable influence on the ratio of the recoveries of the two ligands: Rather than linearly increasing with titrant concentration under receptor-limiting conditions, the ratio is hyperbolically curved per eq 4:

$$\frac{[E{\cdot}S_1] + [E{\cdot}S_1{\cdot}S_2]}{[E{\cdot}S_2] + [E{\cdot}S_1{\cdot}S_2]} = \frac{[S_1]_0([S_2]_0 + \alpha K_{d2})}{[S_2]_0([S_1]_0 + \alpha K_{d1})} \qquad (4)$$

Also, the ratio is asymptotically bounded at the value expressed in eq 5:

$$\lim_{[S_1]_0 \to \infty} \frac{[S_1]_0([S_2]_0 + \alpha K_{d2})}{[S_2]_0([S_1]_0 + \alpha K_{d1})} = 1 + \frac{\alpha K_{d2}}{[S_2]_0} \qquad (5)$$

Equation 6 describes the case where the negative cooperativity is large. Here, the interactions are indistinguishable from mutually exclusive competitive binding, and the right-hand side of eq 3 reduces to that of eq 2:

$$\lim_{\alpha \to \infty} \frac{[S_1]_0([S_2]_0 + \alpha K_{d2})}{[S_2]_0([S_1]_0 + \alpha K_{d1})} = \frac{[S_1]_0 K_{d2}}{[S_2]_0 K_{d1}} \qquad (6)$$

The response ratio data from an ACE_{50} titration against an allosteric-competitive ligand can be fit to eq 4 to yield the K_d of the titrant and cooperativity factor α given the K_d of the

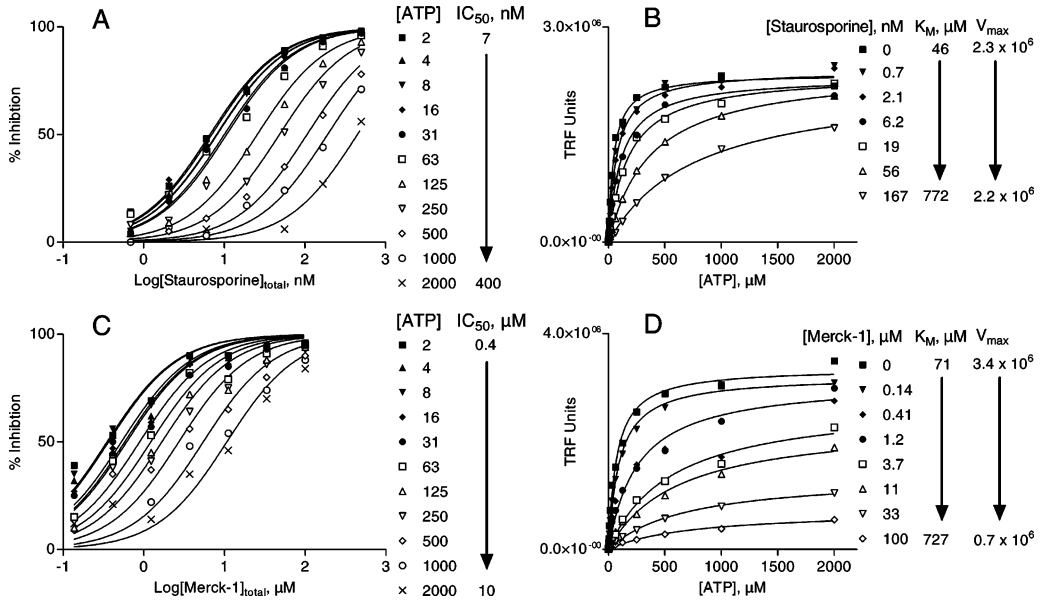


Figure 5. Inhibition of Akt-1 biochemical activity by staurosporine and Merck-1 with increasing ATP concentrations. (A) and (B), staurosporine results, indicating competitive binding between staurosporine and ATP. (C) and (D), results for Merck-1, indicating mixed, noncompetitive binding between Merck-1 and ATP. Staurosporine is plotted versus a nanomolar concentration axis, and Merck-1 is plotted versus a micromolar axis.

ligand, the total ligand concentration $[S_2]_0$, the total receptor concentration $[E]_0$, and relative MS response calibration factors for the titrant and ligand. Nonlinear regression analysis of the Akt-1 ACE_{50} response ratio data from Figure 4C yields a cooperativity factor $\alpha = 8.3 \pm 0.7$ and K_d value of 3.0 ± 0.3 μM for NGD-28835.[30] This K_d value is in good agreement with that of 3.3 ± 1.3 μM measured by an independent titration experiment against the basal kinase.

Allosteric binding competition can also be evaluated in a multiplexed fashion. Figure 4E,F shows an ACE_{50} competition experiment using staurosporine as the titrant against a mixture of Akt-1 ligands.[31] Saturation binding by the titrant staurosporine does not quantitatively displace these ligands, and the response ratio curves vs titrant concentration are asymptotically bounded. These results indicate that the pool components all bind allosterically with respect to the ATP/staurosporine binding site. Similarly, an ACE_{50} experiment between staurosporine and the recently reported[32] Akt-1 ligand Merck-1 also shows allosteric binding competition between these two ligands (Figure 4G). However, titration of the Akt-1 ligand pool by Merck-1 indicates direct binding competition between each component and Merck-1 (Figure 4H−J). These results suggest the Merck compound and the pool components, all of which are directly competitive with it, bind at a site on Akt-1 distinct from the ATP/staurosporine binding site.

To independently evaluate this conclusion, inhibition of Akt-1 kinase activity by Merck-1 and staurosporine was examined at varying ATP concentrations, as shown in Figure 5. Staurosporine binds the ATP-binding site, and, consistent with this implication, increasing ATP concentrations increase the measured IC_{50} for the nanomolar inhibitor staurosporine by greater than 50-fold. The increase in staurosporine IC_{50} with increasing ATP concentration is primarily due to an increase in ATP K_m. The effect of staurosporine on V_{max} for this reaction is modest, even at high ATP concentration. These results confirm staurosporine is a direct competitor of ATP.

Although the IC_{50} of Merck-1 is only micromolar, its IC_{50} increases by a smaller factor over the same ATP concentration range as the nanomolar IC_{50} of staurosporine. The increase in the IC_{50} of Merck-1 with increasing ATP concentration is due to a 5-fold decrease in V_{max} coupled with a smaller increase in K_m than is observed for staurosporine. These results suggest that Merck-1 is a mixed, noncompetitive inhibitor of Akt-1, with a biochemical mechanism of action that includes both ATP displacement (increased ATP K_m) and slowing of Akt-1 kinase activity (decreased V_{max}). This mechanism is most consistent with Merck-1 binding at a site topographically distinct from the ATP/staurosporine-binding pocket of Akt-1. However, although the biochemical assay results presented are typical of a "mixed" inhibitor, they are not unequivocal. Only when evaluated collectively with the ACE_{50} results, which show that Merck-1 and staurosporine simultaneously bind Akt-1, is it clear that at least two classes of inhibitor (ATP-orthosteric and ATP-allosteric) are possible. Furthermore, the ACE_{50} results demonstrate that all of the compounds in the test pool bind Akt-1 allosterically with respect to staurosporine, and therefore bind allosterically with respect to ATP. Akt-1 is a multidomain protein that is activated after binding of its pleckstrin homology (PH) domain to its endogenous target. The data we acquired may suggest the binding of the Merck and NeoGenesis ligands involve the PH domain and effect a biological response through this mechanism, rather than through traditional binding to the kinase active site. Indeed, these ligands fail to bind with high affinity to the kinase domain alone of Akt-1 (data not shown).

(D) Affinity Ranking in Mixtures. In addition to illuminating details of the Akt-1 binding mechanism of the mixture components, the ACE_{50} titration experiment with Merck-1 also yields an affinity ranking for these compounds. NGD-28839 has the highest ACE_{50} value, indicating it is the highest affinity ligand

(30) Independent LC-MS experiments indicate Staurosporine and NGD-28835 have nearly identical MS response factors. In this case, the ratio data are used as-is to estimate the cooperativity factor.
(31) NGD-28834 was used in the pool to avoid mass overlap by its analogue NGD-28835 with another pool component.
(32) International Patent No. WO 02/083139, 2002.

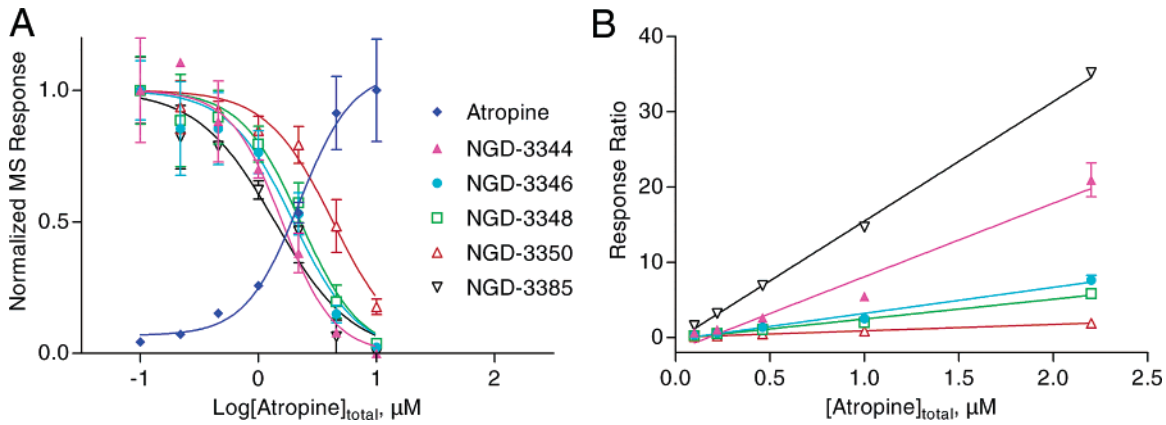


Figure 6. Affinity ranking and determination of the mode of binding competition versus atropine for a pool of ligands to the muscarinic acetylcholine receptor M_2. (A) A log-axis plot of the ACE_{50} experiment results indicates NGD-3350 has higher affinity to M_2 than its congeners NGD-3348 and NGD-3346 and the other pool components. (B) A linear titrant to ligand response ratio plot indicates direct competition between atropine and the pool components. Note the ratio plots are not corrected for MS response calibration.

in this test mixture, as it requires the highest concentration of titrant Merck-1 for displacement. NGD-28839 shows the best biochemical activity of these mixture components, yielding 44 ± 8% inhibition of Akt-1 kinase activity at 50 μM concentration.[26] The ACE_{50} value for NGD-28839 in Figure 4H is 4.1 μM (95% c.i. 3.4−5.0 μM), corresponding to a K_d of 3.5 ± 0.7 μM (95% c.i. 1.5−10.2 μM) given a K_d of 0.3 ± 0.1 for Merck-1.[33] As NGD-28839 binds in an allosteric manner with respect to ATP, its biochemical activity is a function of both the affinity and the cooperativity factor α. Although the affinity and the cooperativity factor of an allosteric ligand are not necessarily coupled, the other ligands in this mixture show weaker inhibition of Akt-1 activity than NGD-28839, correlating with their lower affinities.

Affinity ranking and affinity optimization in compound mixtures using the ACE_{50} method are further demonstrated in Figure 6 using a small library of ligands to the muscarinic acetylcholine receptor[34] M_2, a GPCR. This ligand pool includes chemotype representatives of several compounds discovered through AS-MS-based high-throughout screening of mass-encoded libraries, as well as some structural analogues of NGD-3346. The known M_2 ligand atropine was used as the titrant against 2.0 μM M_2 in the presence of 0.5 μM per component compound pool. The response ratio plots are linear, indicating that all of the ligands examined are directly competitive with atropine.[35] Consistent with this result, independent biochemical assays show that all of the ligands tested, like atropine, are antagonists of M_2. The ACE_{50} curves indicate clear differences in affinity, with NGD-3350 exhibiting a higher affinity than its structural congeners NGD-3348 and NGD-3346. Independent biochemical activity measurements confirm this result: NGD-3350 exhibits an IC_{50} of 1.6 μM in a cell-based cAMP assay[36] and an IC_{50} of 9.6 μM in a tissue-based assay[37,38] for M_2 antagonism. While the remaining compounds all exhibit weaker M_2 antagonist activity in the cAMP assay, only NGD-3350 shows significant activity in tissue. Independent AS-MS saturation binding experiments with the individual M_2 ligands yield the same rank-order of affinities as revealed by the ACE_{50} experiment: K_d values of 0.7, 2.1, 2.9, and 6.2 μM were measured for NGD-3350, NGD-3348, NGD-3346, and NGD-3344, respectively. These results highlight the utility of the ACE_{50} method for simultaneously rank-ordering compounds by affinity, particularly for mixtures of structural analogues synthesized by combinatorial chemistry techniques. The method is especially valuable for identifying those compounds with improved affinity relative to a progenitor, for example, the improved affinity of NGD-3350 relative to its parent NGD-3346. Through multiple iterations of combinatorial analogue synthesis and ACE_{50} analysis, protein−ligand structure activity relationships can be established.

Conclusions

The results presented here show that the ACE_{50} method is a general technique for evaluating the affinity and binding cooperativity of ligand−ligand interactions by directly competitive or allosteric competitive binding. The technique is effective for mixtures of chemical compounds of unique molecular weight, and the number of compounds that can be simultaneously studied is only restricted by the upper limit of the receptor concentration. The only other limitation is the ligands' electrospray ionization sensitivity. While small molecule, drug-like compounds typically ionize well by positive ion electrospray (hence the popularity of the technique in pharmaceutical research and development), many biological cofactors such as ATP do not ionize by electrospray. Therefore, a small molecule surrogate with affinity for a cofactor-binding site, such as staurosporine for the ATP-binding site, is currently necessary for specific binding site assignment by the ACE_{50} method. We are currently exploring AS-MS methods that are not subject to this limitation for binding site assignment relative to MS-insensitive ligands.

As shown in these examples, the technique enables the triage of multiple hits arising from high-throughput screening according to binding site and target-specific binding affinity and facilitates combinatorial library-based structural optimization of these hits to high-affinity lead compounds. The technique can

(33) Determined by an independent AS-MS titration experiment.
(34) Caufield, M. P.; Birdsall, N. J. M. *Pharmacol. Rev.* **1998**, *50*, 279−290.
(35) At high titrant response and low ligand response, the ratio is prone to error, as small changes in the measured ligand response (a small number relative to the titrant response) have a large effect on the ratio. Note that the ratio plot is shown to approximately 50% depletion of ligand response.
(36) CAMP-Screen Chemoluminescent Immunoassay System, Applied Biosystems, 850 Lincoln Centre Dr., Foster City, CA 94404.
(37) Lambrecht, G.; Feifel, R.; Wagner-Roder, M.; Strohmann, C.; Zilch, H.; Tacke, R.; Wailbroeck, M.; Christophe, J.; Boddeke, H.; Mutschler, E. *Eur. J. Pharmacol.* **1989**, *168*, 71−78.
(38) Lundblad, L. K. A.; Persson, C. G. A. *Br. J. Pharmacol.* **1988**, *93*, 909−917.

be applied to protein−ligand interactions with a range of affinities, as demonstrated with ligands varying in affinity from nanomolar to micromolar (M_2 ligands with K_d values from 0.7 to 6.2 μM and Zap-70 ligands with K_d values from staurosporine at 0.01 μM to NGD-6380 at 0.1 μM). By simultaneously classifying ligands of dissimilar structure by binding site, the technique holds promise for developing structure−activity relationships and understanding protein−ligand interactions in multidomain or multisubunit targets, even in the absence of a high-resolution protein−ligand structure. It is noteworthy that this method can suggest the binding site of ligands to the inactive form of a receptor (e.g., the basal form of a kinase), which is a challenging task using traditional biochemical assays. The ACE_{50} method holds particular promise as a unique tool for the study of allosteric ligands, facilitating the advancement of compounds with improved target specificity engendered by binding at sites distinct from those conserved within protein families.[39]

Experimental Section

Materials. Atropine, k252a, and staurosporine were purchased from Calbiochem (San Diego, CA). Warfarin was purchased from Sigma-Aldrich (St. Louis, MO). Commercially available reagents were used as received. Warfarin-D_6 was synthesized according to the literature method for the parent compound,[40] except Benzaldehyde-D_6 (99% atom-D, Cambridge Isotope Labs, Andover, MA) was used to prepare the benzylidene−acetone reagent.[41] Other compounds used in this study were prepared by the NeoGenesis Medicinal Chemistry Group and demonstrated to be of >95% purity by reverse phase chromatography with MS, diode array UV, and evaporative light-scattering detection. Human serum albumin (defatted HSA) and β-lactoglobulin were purchased from Sigma-Aldrich (St. Louis, MO) and were used as received. The other protein targets, Akt-1, M_2, and Zap-70, were prepared according to literature precedence by the NeoGenesis Protein Chemistry Group and were purified to apparent homogeneity by SDS-PAGE according to standard procedures.

Sample Preparation. The following procedure for an ACE_{50} competition experiment between warfarin and warfarin-D_6 is representative: 1 μL DMSO aliquots of a serially diluted stock solution of warfarin (10, 5, 2.5, ..., 0.078 mM) are combined with 1 μL aliquots of 1.6 mM warfarin-D_6. These 2 μL samples are dissolved in 38 μL of PBS (50 mM, pH 7.5 sodium phosphate buffer containing 150 mM NaCl). The resulting solutions are mixed by repeated pipetting and are clarified by centrifugation at 10 000*g* for 10 min. To 1.1 μL aliquots of the resulting supernatants is added 1.1 μL of 10 μM defatted HSA in PBS. Each 2.2 μL experimental sample thus contains 11 pmol (0.7 μg) of protein at 5.0 μM concentration in PBS plus 20 μM Warfarin-D_6, 2.5% DMSO, and varying concentrations (125, 62.5, ..., 0.98 μM) of the competitor warfarin. Samples are incubated at room temperature for 60 min and then chilled to 4 °C prior to AS-MS analysis of 2.0 μL injections.

AS-MS Data Acquisition. The AS-MS hardware configuration used in this study has been described previously (ref 20). Briefly, SEC is performed at 4 °C using phosphate-buffered or TRIS-buffered saline, typically 50 mM pH 7.5 phosphate buffer containing 150 mM NaCl. SEC columns are prepared in-house by proprietary methods. Operationally comparable columns are available from Regis Technologies, Inc. (Morton Grove, IL).[42] An Agilent (Palo Alto, CA) isocratic pump (G1310A) fitted with an Agilent online degasser (G1322A) is used for eluant delivery at 300 μL/min. Using this configuration, the SEC retention times are reproducible to better than ±1 s for a 15−20 s chromatography run. With reproducible SEC chromatography and moderate to slow dissociation rates, the slight variation in retention time from sample-to-sample is not a significant source of error in this method.

The eluant from the SEC column is passed through a UV detector (Agilent G1314A using a G1313 micro flow cell) where the band containing the protein−ligand complex is identified by its native UV absorbance at 230 nm. After a pause to allow the band to leave the first detector and enter a valving arrangement, the protein−ligand complex peak is automatically transferred to an RPC column (Higgins Targa-C_{18}, Higgins Analytical Inc., Mountain View, CA). Ligands are dissociated from the complex and trapped at the head of the RPC column, where they are desalted and eluted into the mass spectrometer using a gradient of 0−95% acetonitrile (0.1% formic acid) in water (0.1% formic acid) over 5 min using an Agilent capillary binary pump (G1376A) for eluant delivery at 20 μL/min. To promote dissociation of ligands from the complex, the RPC column is maintained at 60 °C using an Agilent G1316A column compartment. In this study, MS analysis was performed using a Waters LCT high-resolution time-of-flight mass spectrometer (Manchester, U.K.) with positive mode ionization occurring from a standard nebulized ESI source with the capillary at 3.5 kV, a desolvation temperature of 180 °C, a source temperature of 100 °C, and 30 V "cone" and 3 V extraction lens settings.

Control experiments were conducted for each compound mixture to confirm that any unbound ligand is trapped by the stationary phase and only protein-bound ligand is eluted for analysis (i.e., no chromatographic breakthrough is occurring). Specifically, independent AS-MS experiments were conducted using a protein (β-lactoglobulin) that has no affinity for the ligands of interest, yet elutes at the same SEC retention time as the target protein (under these conditions, all proteins elute in the void volume). The absence of any ligand MS signal when β-lactoglobulin is the target protein indicates no chromatographic breakthrough is occurring.

Data Analysis. For each AS-MS experiment representing a single data point in an ACE_{50} titration, the areas underlying the extracted ion chromatograms (XICs) for the singly protonated, doubly protonated, and monosodiated ($[M + H]^+$, $[M + 2H]^{2+}$, $[M + Na]^+$) species are summed. The resulting raw response for each data point is then normalized for the entire titration curve by dividing each ligand's raw response by its highest response in the titration experiment (typically a data point where the titrant concentration is lowest). The titration data are then fit to a variable slope sigmoidal dose−response using GraphPad Prism (version 3.02 for Windows, GraphPad Software, San Diego, CA, www.graphpad.com) with a maximum normalized value of 1.0. The titrant concentration at which the fit curve passes through 0.5 represents the ACE_{50} value. Because the concentration of competitor necessary to reduce the ligand response to one-half its value in the absence of competitor defines the affinity ranking, normalized responses are a more readily interpretable way to represent the data than raw responses. No calibration curves are necessary to determine the affinities of the mixture components.

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Supporting Information Available: Derivation of equations shown in the text and functions relating ACE_{50} values to K_d. This material is available free of charge via the Internet at http://pubs.acs.org.

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