High-Throughput Screening (HTS) of Potential Lead Compounds for Target Proteins with No False Identifications Using LC/MS

By

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Submitted to the graduate degree program in Chemistry and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Abstract

Developing effective high throughput screening (HTS) methods is of paramount importance in the early stage of drug discovery. When a protein binding event can be detected via spectroscopy, then absorbance, fluorescence or chemiluminescence based assays can be used to screen for tens of thousands of compounds per day. However, this is not feasible for many protein targets. To screen for drug candidates for target proteins where standard light based assays are not viable, we developed a high throughput screening method using LC-MS. Our assay is label free, which allows for rapid assay development and eliminates the risk of label interfering with the drug-protein complex. The main challenge of MS based screening methods is the high rate of false positives; our MS based screening method identifies binding partners for a target protein with no detection of false positives. In this method, ligands are mixed with the immobilized target protein and the non-binding analytes are detected, binding lignads are identified by comparing with a control where no protein was added. The tightest binders completely disappear from the ligand sea after the protein is added, therefore, these high affinity ligands are readily detected as peaks that are absent in the MS data. We then extended our study to eliminate not only the false positives but also the false negatives under high throughput conditions. The assay, which is similar to a ligand fishing experiment, mitigates false positives by selectively identifying positive hits when a ligand at the binding site of interest is displaced. The reporter molecule ionizes well, eliminating false negatives caused by non-ionizing compounds. Finally, we further extended our study to develop MS-based HTS assay using minute amount of target proteins to identify weak affinity ligands while still mitigating false positives and negatives. In summary, this dissertation addresses the major challenges in the field of MS-based HTS to identify binders for target

proteins with no false positives or negatives. These improvements meet the current requirements of the drug discovery field better than any existing MS-based method.

Acknowledgements

First, I would like to thank my advisor Dr. Heather Desaire for her wonderful support and guidance. Your encouragement pushed me to improve myself in both research and class work and I cannot thank you enough for all you have done for me during my graduate carrier. Also, I would like to thank the Chemistry faculty of the University of Kansas, especially my graduate committee for their suggestions and help. I thank all the past and present lab members of the Desaire group for being such wonderful colleagues. I appreciate all the help provided by Dr. Eden Go, Dr. Zhikai Zhu, Jude Lakbub, Kenneth Avocetien, and Joshua Shipman with my research. Thank you to all the friends, especially Manjula Wijesinghe and the Sri Lankan community in Lawrence for your support both in personal and professional level. Finally, I would like to thank my family for their unwavering love and support through graduate school. I wouldn't have achieved any of this without you.

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Chapter 1: Mass spectrometry based high throughput screening methods for discovering new ligands for target proteins.

1.1 Introduction

Finding an inhibitor for a target protein of interest by screening millions of compounds has a high statistical probability. Currently, there is an enormous increase in the number of therapeutic targets and the number of massive compound libraries being created from combinatorial and parallel synthesis techniques. Therefore, high throughput screening (HTS) is an increasingly popular technique in the early drug screening process, since it drastically decreases the time needed to find a lead compound for a particular target out of millions of potential compounds. However, there are several important factors that have to be considered in developing an assay for HTS: relevance, effectiveness, speed, robustness, accuracy, and reproducibility.[1] Relevance is the validation of the screen with a standard or a known compound that has proven successful in animal and human model systems. An effective assay minimizes the number of compounds identified by the screen and avoids the need for counter screens. Normally, speed is necessary to reduce screening time and to test compounds with diverse chemistry or functionality. However, a faster assay is often achieved at the expense of accuracy, which can result in lower productivity. Additionally, a HTS assay should be pertinent to different classes of chemical compounds. This is known as the robustness of an assay. Accuracy and reproducibility measure the lack of false positives and negatives without the need to re-assaying several times for discovery of a hit with excellent efficacy.

Moreover, an effective assay is faster because different compounds are assayed in the same run/batch, instead of in repeated runs. Therefore, developing an assay that optimizes all these factors is vital and not trivial.

The most commonly used HTS assays use florescence and radiometric detection ,[2] owing to the fast detection, simplicity, and miniaturizing ability of these techniques. However, developing an HTS assay for a particular therapeutic target can be time-consuming or impossible, as there is a need to synthesize and modify new compounds or tagged compounds to the target or in library compounds in order to obtain a sensitive florescence or radiometric signal upon ligand binding.[3] Moreover, use of a tagged compound on the target could alter the binding of a ligand, resulting in false positives or false negatives. Surface plasmon resonance (SPR) and nuclear magnetic resonance (NMR) are alternative spectroscopic techniques that are label free, with no alteration of ligand binding to the target protein. However, both SPR and NMR are not suitable for HTS due to the intense effort required for these techniques. Mass spectrometry (MS) based assays are, on the other hand, label free, have fast detection, are able to disclose structural information, and are highly selective and sensitive. In addition, MS can simultaneously differentiate between the multiple components of a complex reaction mixture. These advantages make MS a more appropriate candidate for developing an assay to screen ligands for target proteins without the time-consuming development of procedures of spectroscopic techniques. Several MS based techniques have been developed over the decades, in combination with or without multidimensional chromatography, to screen for ligands for target proteins.

The application of mass spectrometry to high throughput ligand screening has developed over the last 25 years. The advances in MS-based HTS methods originated with new developments in instrumentation and techniques. The purpose of this chapter is to discuss the principles, applications, advantages and disadvantages of MS-based techniques in early drug discovery. In addition, our aim is to provide information for researchers to develop novel techniques with increased functional benefits or apply the available techniques to new applications.

1.1.1 Different MS based techniques for drug screening

MS-based HTS techniques can be divided into two categories: functional based and affinity based screening. Functional based screenings consist of three major methods. These are MALDI-TOF, SAMDI, and Rapid fire-MS. Affinity based screening can be classified into two major categories based whether the protein is immobilized on a solid support. In this chapter, we categorize each technique based on the principle that the screening method employs.

1.2 Functional based screening – Enzymatic assay/MS

In these methods, an enzymatic reaction is used to screen for compounds that can regulate or inhibit enzyme activity. These techniques can be used to detect the formation of product or the disappearance of substrate during a target enzyme/substrate reaction. The assays tend to be very rapid and are easily automated. The ability to simultaneously monitor different species by MS has made this screening approach an attractive platform for HTS, compared to optical-based assays. The following section

discusses different MS-based platforms' ability, with or without chromatographic separation, to identify ligands that modulate the activity of enzyme targets.

1.2.1 Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS)

The most common non-chromatographic MS-based technique is matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The MALDI-TOF approach provides several advantages:[4] fast analysis, consumption of minute amounts of sample, high sensitivity, and the possibility of automation.[5] Sample analytes are mixed and co-crystallized with an easily volatilized matrix and, after deposition on an appropriate MALDI plate, are irradiated with a laser. The resulting plume produces molecular ions from the sample, which are then detected by TOF-MS. The success of this ionization depends heavily on the choice of the matrix compound used for co-crystallization. The compound must be volatile, low weight, and able to absorb UV more efficiently than the analyte of interest (effectively, matrix molecules should contain highly conjugated π systems). It must also be polar and contain acidic protons that will be transferred to the analyte molecules to produce molecular ions. Apart from proton transfer, the matrix should not directly or indirectly structurally affect the target analyte, which would result in altering its detection by MS.

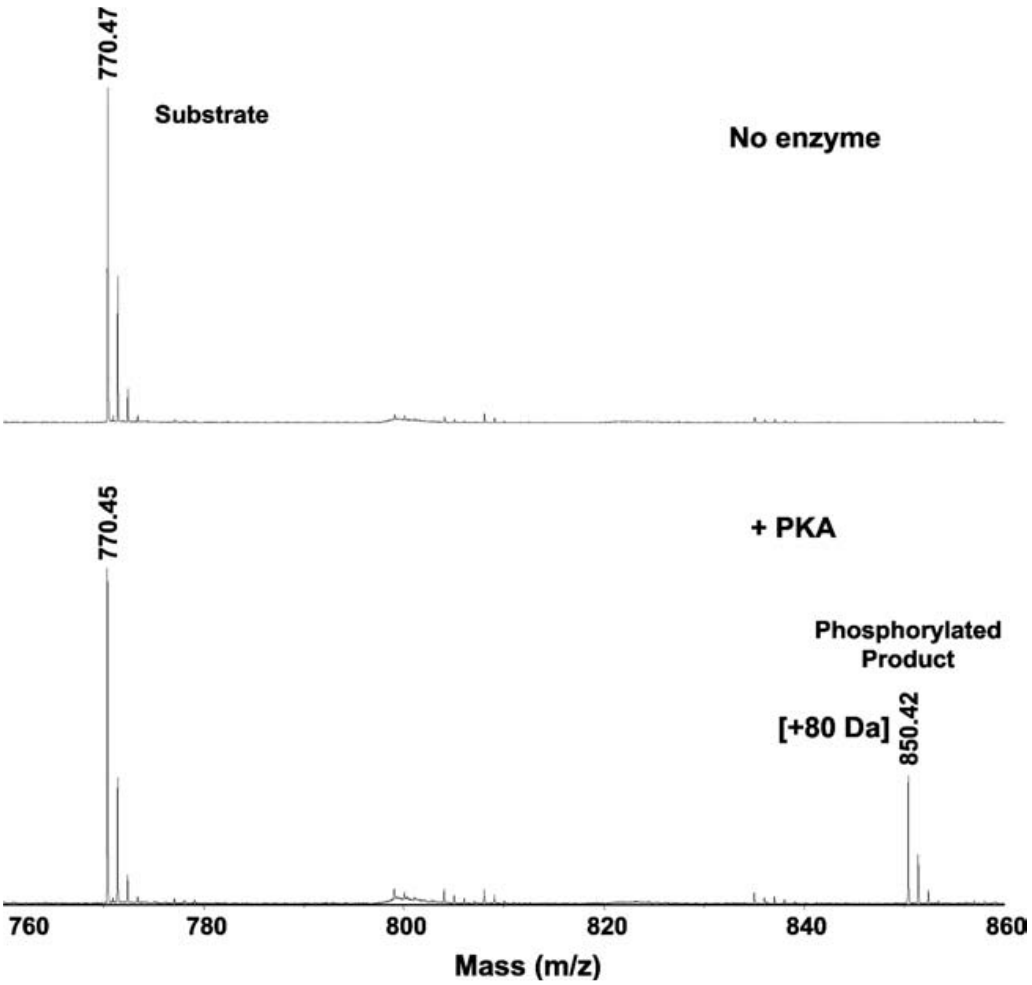

Figure 1: MALDI-TOF MS profiles for the enzyme reaction for cAMP-dependent Protein Kinase, catalytic subunit (PKA). Upper panel: Peptide substrate (kemptide) with buffer, without the enzyme. Lower panel: Peptide substrates incubated with the enzyme for 30 min. This reaction converts a portion of substrate to the expected phosphorylated product.[6]

Monitoring the conversion of substrate to product by MALDI-TOF-MS has been used over the last few decades to identify inhibitors of various target enzymes[4, 6-7] As early as 1998, Hsieh et al.,[4] developed a high throughput bioanalytical method to screen small molecule libraries using only picoliter to nanoliter quantities of samples, allowing the screening of 4000 samples on a single MALDI plate. Three enzyme targets: angiotensin converting enzyme (ACE), N-myristoyltransferase (NMT), and protein

tyrosine phosphatase (PTPase) were used in screening for inhibitors. Three separate substrates of the target enzymes were used to monitor enzyme activity to identify possible inhibitors. The conversion of angiotensin I (*m/z* 1296.98) to angiotensin II (*m/z* 1046.52) for ACE, NMT substrate when combined with myristoyl-CoA (*m/z* 801.35 to *m/z* 1011.59), and PTPase substrate (*m/z* 1702.88 to *m/z* 1622.85) were used as the reaction indicators. MALDI-TOF has been used to identify inhibitors and produce IC_{50} curves for many different classes of target enzymes, including kinases,[8] hydroxylases,[4] oxygenases,[9] histone demethylases, and acetylcholinesterases[7]. Due to its simplicity, fast detection ability and improved screening throughput of compound/protein interactions, MALDI-TOF has gained significant attention in the past decades. Figure 1 shows the MALDI-TOF-MS profiles of the enzyme reaction for cAMP-dependent Protein Kinase, catalytic subunit (PKA). The profiles clearly show the generation of product from the substrate only in the presence of the enzyme PKA.[10] Moreover, MALDI-TOF–MS has been successfully used to monitor and screen enzymatic activities of multiplexed target proteins to identify inhibitors from molecular libraries. With the multiplexing ability of MS, MALDI-TOF becomes a useful technique in drug screening analysis. MALDI-MS has been used to screen small molecules,[11-13] peptides,[14-16] proteins,[17-19] and polymers.[20-22] For example, Figure 2 represents MALDI-TOF-MS profiles for enzymatic activity of acetylcholinesterase, where the acetylcholine substrate is converted to choline, in the presence and absence of the active inhibitor compound. In the presence of an active inhibitor, the catalytic activity of the acetylcholinesterase is reduced.[23]

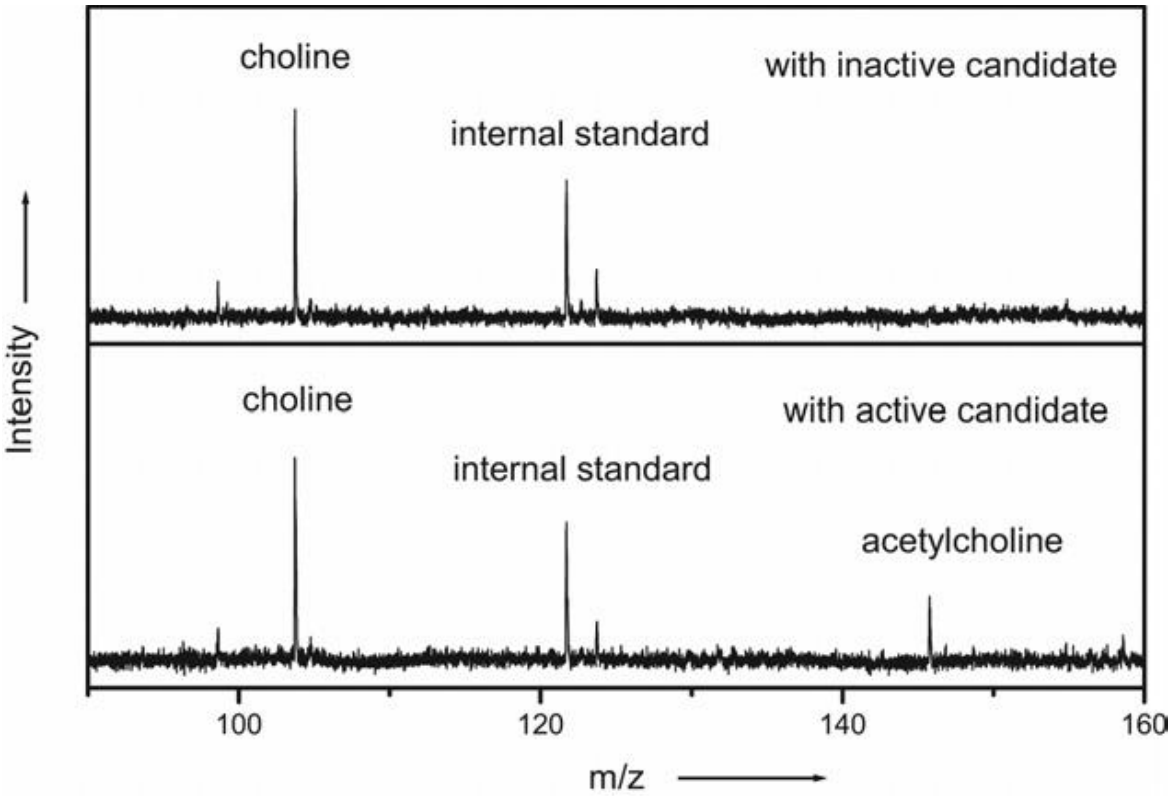

Figure 2: Upper panel: Typical mass spectrum for enzymatic hydrolysis reaction of acetylcholine to choline in the presence of acetylcholinesterase inactive inhibitor. Under these conditions, the reaction would proceed completely. Lower panel: Typical mass spectrum for the same enzymatic hydrolysis reaction in the presence of acetylcholinesterase active inhibitor. Under these conditions, the reaction would be incomplete.[23]

MALDI-TOF suffers from a few notable limitations. One major disadvantage is its poor quantification ability. This occurs due the inhomogeneous sample preparation, i.e. due to the nature of the spotting matrix and sample droplets causing not only poor sample-to-sample reproducibility but also poor laser shot-to-shot reproducibility. This problem can be minimized, to a certain extent, with the use of an internal standard[23-24] optimized measurement protocol[25-26] and sample preparation procedures that improve homogeneity.[27] However, finding or synthesizing an appropriate internal standard for the monitored substrate can be challenging, which is a problem also shared with all other

quantification methods. Though MALDI-TOF-MS based enzymatic methods have an edge over spectroscopic methods due to the ability to identify inhibitors in a label free manner, this same quality can be shown to be a disadvantage. Failure to find screening-compatible enzymatic activity for the target enzyme can decrease MALDI-TOF's usefulness in HTS. Even when an assay is available, MALDI-based techniques are limited by the same requirements as all other MS methods. Mainly, the analyte of interest must be detectable as a gas phase ion. Also, multiple analytes in complex samples compete for ionization. Their differential ionization efficiency may result in an inaccurate observation of their relative abundances.[28] Another major limitation of MALDI-TOF is matrix interference, caused by the ample ionization of matrix molecules, which are more concentrated, compared to the analyte, in the sample. This leads to saturation of the MS detector and suppression of the analyte signal. This problem can be treated by cutting off the m/z range if the m/z of the analyte of interest is higher than that of the matrix ions.

1.2.2 Self-assembled monolayer desorption ionization (SAMDI)-MS

Another new technique, called self-assembled monolayer desorption ionization (SAMDI)-MS, has recently emerged in the field. In this technique, the substrate is immobilized onto a gold-plated surface via maleimide-terminated self-assembled monolayers using thiol groups. Then, the enzyme and the library compounds are incubated with the immobilized substrate. Finally, the plates are rinsed and the monolayer is irradiated with a laser, resulting in cleavage of the thiolate gold bond and efficient desorption and ionization of the chains, which are then analyzed using mass spectrometry. Unlike MALDI-TOF-MS, any back ground interferences from salt or matrix compounds is not a problem in SAMDI-MS, since the plates are rinsed. Figure 3 is the

schematic representation of the SAMDI-MS assay. In this example, the immobilized peptide substrate Ac-GRKAcFGC-NH2 was converted to product in the presence of the enzyme lysine deacetylase 8 (KDAC8) and in the absence of an inhibitor compound.[29] Absence and presence of the product after incubation of small molecule libraries is assessed in order to identify inhibitors. SAMDI has been used to screen inhibitors for many protein targets including methyltransferases,[30] nucleic acid ligases,[31] kinases,[32] proteases,[33] galactosyltransferase,[34] lysine deacetylases,[35] sirtuin deacetylases,[36] and phosphatases.[37] SAMDI-MS has been developed to screen 10^5 compounds/day.[29] It has also been applied to screen enzyme activities in cell lysates[37] in a high throughput manner, representing a potential improvement over other available fluorescence-based HTS methods.[38] Similar to MALDI-MS, poor laser shot-to-shot reproducibility is a drawback, not only for quantification, but also for qualitative analysis of certain compounds that may require a high concentration for detection.

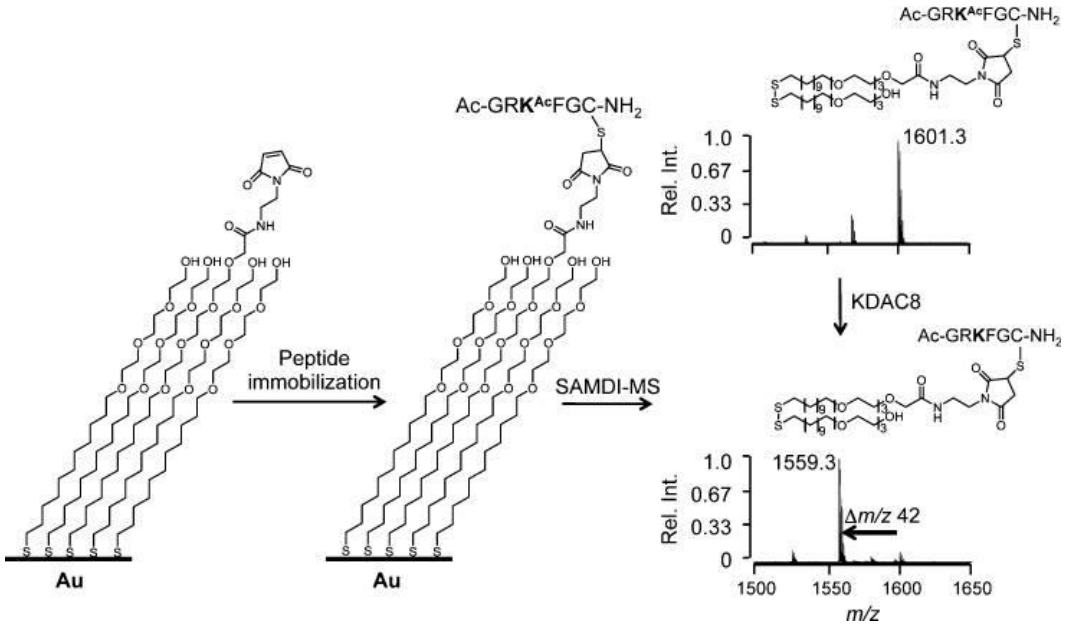

Figure 3: Schematic illustration of a SAMDI assay. Peptide substrates and products are covalently bound by the reaction between maleimide-terminated self-assembled monolayers and the cysteine thiol group. The thiolate gold bond is cleaved upon laser irradiation of the monolayer and the chains are efficiently desorbed and ionized, either as disulfides or alkanethiolates. The starting peptide substrate used in the screen results in the peak at a mass to charge ratio (*m/z*) of 1601.3, and the deacetylated products are shifted by *m/z* 42 to a peak at *m/z* 1559.3.[30]

1.2.3 Rapid Fire™ mass spectrometry

Another technique that uses the conversion of substrate to product is Rapid Fire™ mass spectrometry.[39] In this method, samples are prepared by mixing target protein, substrate, and library of compounds. Next, the samples are quickly run through a small column to separate protein complexes from other solution phase components. Then, the separated solution phase is sprayed onto a MS for the detection. This technique removes any salts, detergents, and buffers through fractionation steps and subsequently supplies purified analytes to the mass spectrometer.[39] Unlike MALDI and SAMDI-MS, the rapid

fire technique does not use laser irradiation on the samples. Since there is no need for a matrix to ionize the analyte of interest, unlike in MALDI and SAMDI-MS, the Rapid Fire technique has been successful in addressing reproducibility and ion suppression problems associated with MALDI-TOF. In addition, Rapid Fire™ still shares important benefits, such as salt tolerance and fast detection, with MALDI-TOF. Rapid Fire™ uses an automated sample collection and purification system that is directly connected to the ESI –MS. The Rapid Fire technique, which has the capability of screening ~300 K compounds per week,[40] has been used to screen for inhibitors of different enzymes, including acetylcholinesterases,[39] decarboxylases,[41] monooxygenase,[42] proteases,[43] kinases,[44] demethylases,[40] and cytochrome p450[45] in a high throughput manner.[46] However, this method requires chromatographic or (in most cases) solid state separation, which can decrease the sensitivity of the assay due to peak broadening, or loss of analytes in the separation cartridge prior to the ionization compared to MALDI-TOF-MS.

1.3 Affinity based screening

Developing assays to screen for binders of target proteins can be carried out using the possible affinity of the target towards small molecules, even if the function or structure of the target is unknown.[10] In this way, protein binders can be distinguished from the remaining unbound compounds. Affinity based screening methods are applicable for wide variety of targets. Therefore, developing target protein-specific assays is not necessary, as opposed to enzymatic assays. In particular, once an assay is developed for one target, it can be applied to screen for ligands of different targets. However, one should be mindful of nonspecific binding and MS detection incompatibility. The former can lead to false

positive identifications of non-specific binders. The latter reduces confidence in the identification of binders that may not be fully ionizable in the MS source.

In these methods, a protein binder is identified by distinguishing between a sea of compounds that remain unbound and the target -bound compounds. The initial step in affinity-based screening methods is the formation of a protein-ligand complex. Then, detection is performed in one of three ways. First, the bound ligand can be directly identified by detection of the protein-ligand complex by MS. Second, the complex can be separated from the sea of compounds, followed by identification of the ligand after dissociation of the complex. Third, the bound ligand can be identified by comparing the unbound compounds with a control sample of library compounds, but without protein present.

Affinity selection methods can be divided into two categories (Figure 4) based upon the treatment of the protein of interest. In the first category, the protein is immobilized on a solid support. This category can be further classified based on whether the binding events occurred online or prior to detection of the ligand involved. Frontal affinity chromatography (FAC)-MS is one of the major techniques that uses affinity/binding events be online with the detection whereas the HAMS method[47] is one of the major techniques that uses binding event before the detection. In the second category, the protein is not immobilized. This category is divided into two screening techniques: direct and indirect screenings. Direct screening involves a homogeneous format where both receptor and ligands are in solution. In the direct screening technique, separation of the receptor-ligand complexes from the nonbinding compounds takes place inside the MS. During indirect screening, other methods of separation are used prior to MS detection. In

fact, only the ligand reaches the MS detector. The execution of these techniques, as well as their advantages and disadvantages, are discussed in the following section.

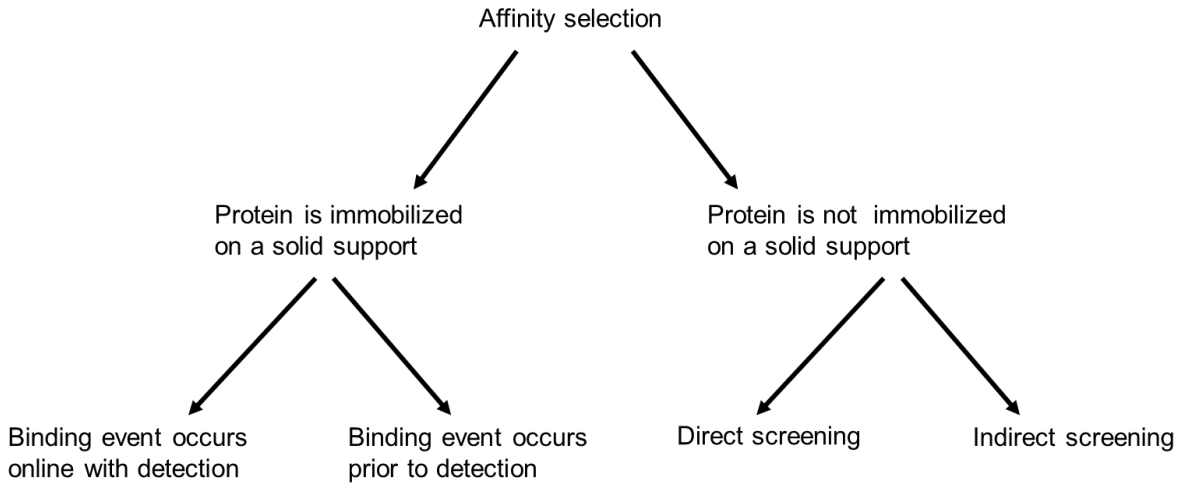

Figure 4: Different categories of MS-based affinity selection methods.

1.4 Techniques with binding event online with the detection

In this category, the target protein is immobilized on a solid support packed inside a column. Continuous infusion of the analytes through the column is a key feature of this technique. Numerous methods belonging to this category use mass spectrometry to identify compounds that bind with a particular target protein. Among those techniques, frontal affinity chromatography can be considered as the most commonly used method, owing to functionality with very low amounts of target protein.

1.4.1 Frontal Affinity Chromatography- Mass Spectrometry (FAC-MS)

Frontal affinity chromatography (FAC)-MS is considered one of the most useful techniques among the MS-based affinity chromatography methods.[48-50] Figure 5 is a

schematic representation of the principles behind FAC-MS.[51] This method is carried out using a continuous flow of small molecules that pass through an immobilized target protein in a column. When small molecules pass by the immobilized target protein, they bind to it based on their affinities and finally reach an equilibrium between being immobilized on the target protein and continuing to travel in the mobile phase. This equilibrium controls their elution from the column. The end point of the experiment is determined when the composition of the effluent is equal to that of the influent. The compounds are detected by mass spectrometry as they exit the column.[48] Since the protein ligand binding affinities vary, the effluent volume that passes through the column varies to match the concentration of the input ligand. This volume is known as the “breakthrough volume” and the higher the affinity of the ligands, the higher the breakthrough volume. Breakthrough volumes of analyte compounds are compared with that of the void marker, a compound that does not have any affinity to the target protein and therefore elutes faster from the column, to estimate the affinity of the analyte compounds.

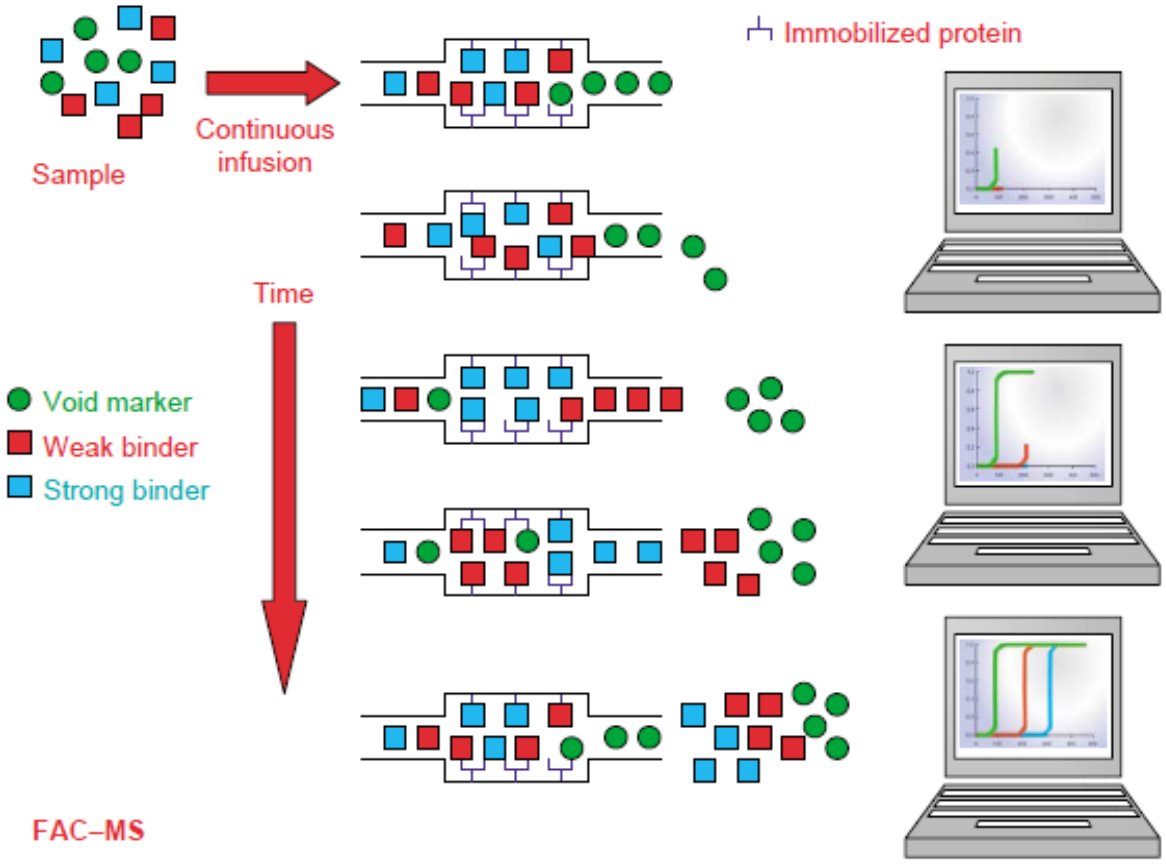

Figure 5: A schematic diagram showing the principle of FAC–MS. Ligands and a void marker are continuously passed through a column containing an immobilized protein.[51]

The roll up method is a form of FAC-MS that uses a known binder of a target protein as an indicator ion. First, the breakthrough curve for the indicator, demonstrated in Figure 5, is obtained. After that, ligand(s) are mixed with the indicator and the breakthrough curves for the indicator are collected. As shown in Figure 6, the presence of high affinity ligands eluting with the indicator will show a high roll up effect, compared to moderate or low affinity ligands.[51]

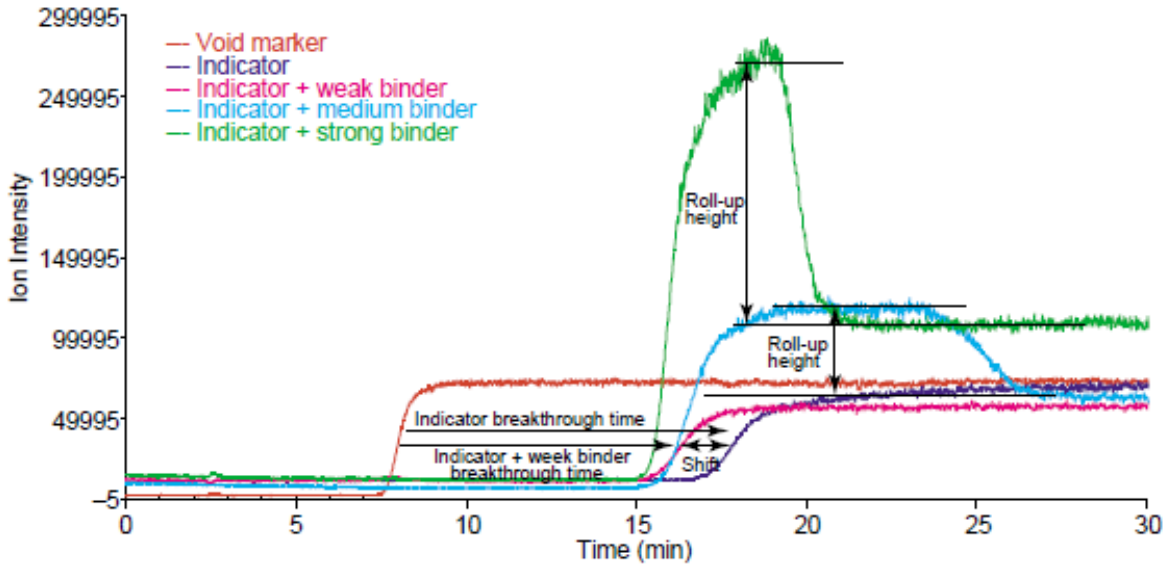

Figure 6: Typical FAC–MS indicator responses (roll-ups and shifts) in screening experiments. Purple trace shows the breakthrough front of the indicator in the absence of any competing ligands. Magenta trace indicates a ligand that is competitive with the indicator, but has a K_d that is weaker than the K_d of the indicator. Such a ligand will generate a shift of the indicator's front to the left. Cyan trace indicates a competitive ligand with a K_d that is similar to, or slightly stronger than that of the indicator. This type of a ligand will produce a roll-up effect that dominates over the shift of the indicator. Stronger competitive binders compared to the indicator will generate significant roll-ups for the indicator as shown in green trace.[51]

There have been many successful attempts to develop HTS methods with FAC-MS using libraries with limited number of compounds. Ng *et al*.[52] developed a HTS method using a large compound library in which they screened 5000 compounds/day to search for inhibitors for N-acetalyglucosamyltransferase. Later, they were able to automate the system and demonstrated[53] the possibility of screening 10,000 compounds/day with human estrogen receptor β (hERβ) as the target protein.

The FAC-MS method has multiple advantages. This technique is used to rank ligands based on their affinity towards the target protein, since low affinity ligands elute first and high affinity ligands elute later. Therefore, it is easy to estimate the relative K_d

values of different ligands[54-55] Use of an indicator ligand in the roll-up method of screening provides the opportunity to discriminate between mixtures containing strong binding ligands from mixtures with weak binding ligands. Moreover, the roll-up effect helps to confirm whether two ligands interact with the same target protein.[49]

Even though FAC-MS possesses many advantages, it has some limitations. The requirement of a void marker, time consuming data analysis, and the need for the construction of breakthrough curves to identify strong binders impedes the use of this method for HTS.[51] False negatives, due to buffer compatibility, ion suppression, and the limitations of mass resolution power for some instruments are also noteworthy limitations.[56] Another major drawback arises from immobilization of the target protein. Buffer conditions used during immobilization may have an effect on the folding of the target protein, influencing the binding of the ligand. In addition, nonspecific binding is possible with the linker system and with the stationary phase.[51] In summary, even though FAC–MS has the limitations mentioned above, it provides valuable information regarding the binding events of a ligand to a target protein than MS-based HTS methods with high false identification rates.

1.5 Direct screening technique

Direct screening can be used to detect both covalent and noncovalent target-ligand complexes. This approach is made possible by the soft and tunable ionization process of electrospray ionization (ESI). Even though covalent complexes can also be formed via a fragment based discovery assembly process,[57-58] detection of noncovalent complexes by

direct screening is more valuable, since it is a better representation of the target-drug interaction.

In direct screening, bound compounds are identified by directly analyzing the protein-bound compounds using ESI-MS. This native MS concept has been used to validate both the inter- and intramolecular interactions of proteins[59-60] to screen for binders [61-62] and to carry out fragment screening.[63] Figure 7(i) shows the anticipated mass spectrum for direct-screening analysis upon binding of a ligand to a target. Figure 7(ii) shows the deconvoluted mass spectra for the binding of an endocellulase mutant, Cel6A D117Acd, with various saccharide molecules. The mass difference between the protein and the complex increases with increasing mass of the saccharide binding molecules.[64] An instance where direct screening was used successfully to screen for peptide binding partners to target proteins was reported by Maaty et.al.[65] They have used hydrogen exchange mass spectrometry to screen binders for calmodulin and ribonuclease S from an Escherichia coli proteome containing over 6000 peptide fragments.[65]

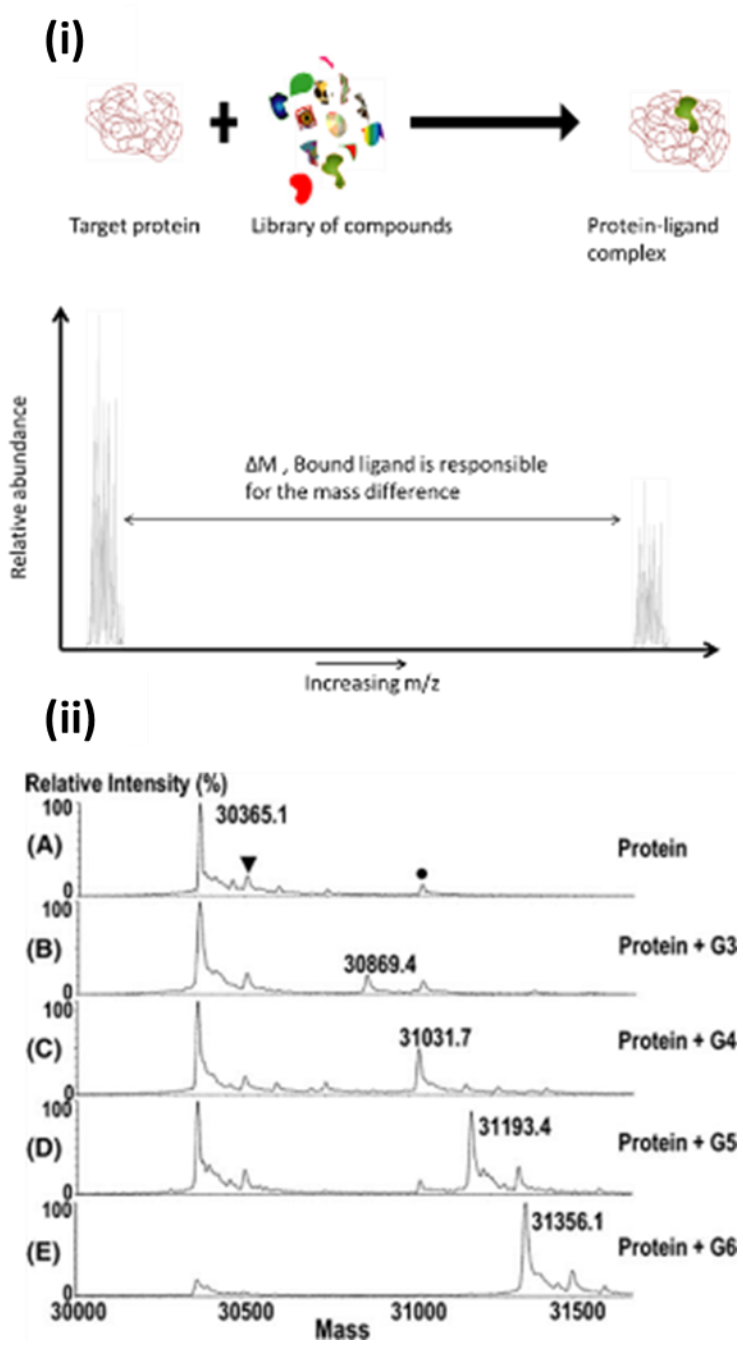

Figure 7: (i) The expected mass spectrum of the direct ASMS method. The complex shows mass shifts upon binding of a ligand. **(ii)** The deconvoluted mass spectra indicating the noncovalent interactions for an endocellulase mutant, Cel6A D117Acd (panel A) with the oligosaccharides cellotriose (G3, panel B), cellotetraose (G4, panel C), cellopentaose (G5, panel D) and cellohexaose (G6, panel E). The ▼ and ● labels indicate protein mutants present at low abundance.[64]

Although very useful, direct screening suffers from several limitations. The mass of the protein-ligand complex is not significantly different from the mass of the protein. Therefore, it can be difficult to deduce the mass of the ligand, even if there is a binding event. As a result, mass spectrometers with high mass accuracy and high resolving powers, such as the Orbitrap, Fourier transform ion cyclotron resonance (FTICR), and quadrupole time of flight (Q-TOF) mass analyzers, are essential for direct screening. In addition, an argument can be made that the structural integrity of the proteins is disturbed when the protein is desolvated and transported into the gas phase. Furthermore, full or partial alteration of the target-bound compound complex resulting from the ESI process cannot be completely eliminated, at least for transient (millimolar to micromolar range) binders, as opposed to tight (nanomolar to picomolar range) binders.[66] Applying this native MS ligand screening technique to a high throughput platform is hindered by certain additional limitations. These limitations include the necessity for highly purified and concentrated protein samples, the need for keeping the target-bound compound complex intact, using a suitable solvent system in ESI-MS, concurrently bringing the complex into gaseous phase by desolvation, the requirement for incubation of a small pool or individual library compounds with the target to mitigate nonspecific bindings,[61] and lastly the time consuming data analysis, where spectrum deconvolution is obligatory for obtaining ligand information, due to the fact that multiple charge states are often observed.

1.6 Indirect screening technique

Another incarnation of the affinity selection method that uses non-immobilized proteins is the indirect screening technique. This method was developed to circumvent certain problems associated with the direct screening method, specifically the need for a

solvent system that is compatible with both ESI-MS and ligand-target complexes, need for incubation of the individual library compounds with the target (instead of a large pool of compounds) to minimize nonspecific bindings, and, finally, time-consuming data analysis. In this method, the target protein is first incubated with library compounds. Then, the unbound compounds are separated from the complexes (target protein receptor-ligands), then the bound compounds are released from the target protein and detected using MS.

For efficient separation of unbound compounds from target-ligand complexes, ultra-filtration membranes, or size exclusion chromatography (SEC) have been used. Figure 8 shows a work flow for a method called “Speed Screen” that has been developed using SEC for indirect screening and for which the authors have demonstrated HTS capability[67-68] A centrifuged 96-well plate assembly of SEC columns with a sample loading plate on the top and pinholes at the bottom of each well to collect effluent from protein-ligand complexes was used for this approach. Therefore, it was possible to screen ~ 60,000 compounds per day.[69] The speed screen technique has been used to identify drug like compounds for multiple target molecules.[69]

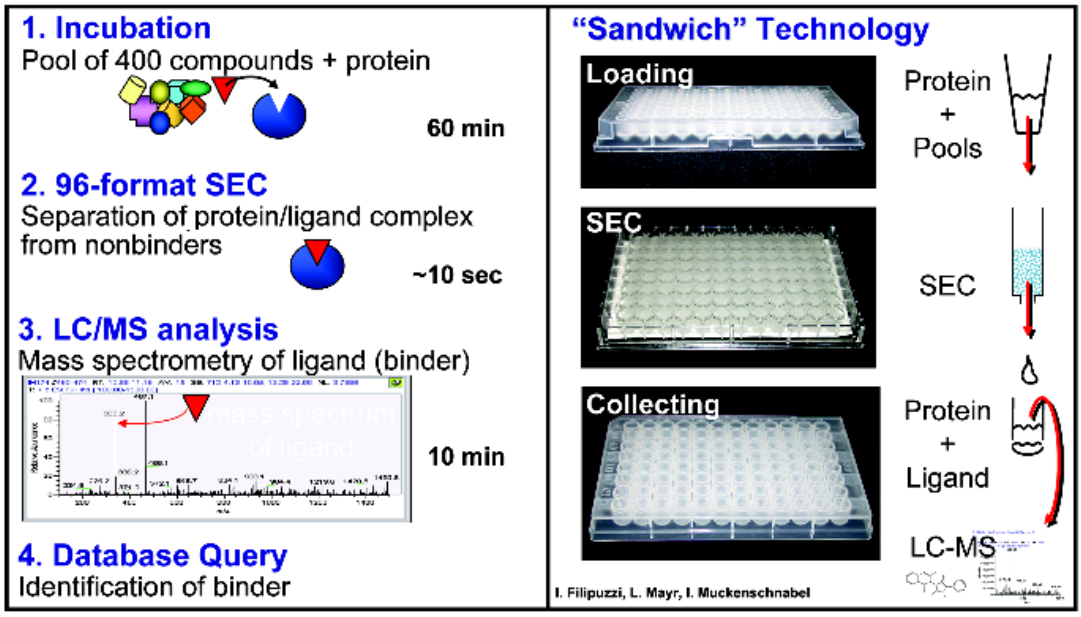

Figure 8: Major steps in a typical Speed Screen technique (Left) and composition of the "Speed Screen sandwich" (Right).[70]

Automated ligand identification system (ALIS) has been developed using the SEC-LC-MS platform for HTS.[71-72] The workflow for ALIS is described in Figure 9.[72] This system is fully automated. In the ALIS process, the target receptor molecule and the mixture of compounds are allowed to equilibrate in 96-well plates. Then the unbound compounds are separated from the complexes by an SEC column, followed by an analytical column. The complexes are monitored by UV detection that is integrated into the system. After detecting the protein (receptor-ligand complex) peak via UV, an automatic valve system directs the complex to a reverse phase chromatography column, where the complex is desalted and allowed to dissociate. The eluted ligands are then detected by MS. ALIS has been used to discover novel inhibitors for protease β-secretase, a target related to Alzheimer's disease[73] and receptor ligands for orthostatic and allosteric muscarinic M2 acetylcholine, G-protein coupled receptors.[74-75] ALIS has

also been used to study the competitive binding profile of multiple target receptor ligands and to evaluate the structure-activity relationship in a single reaction.[76]

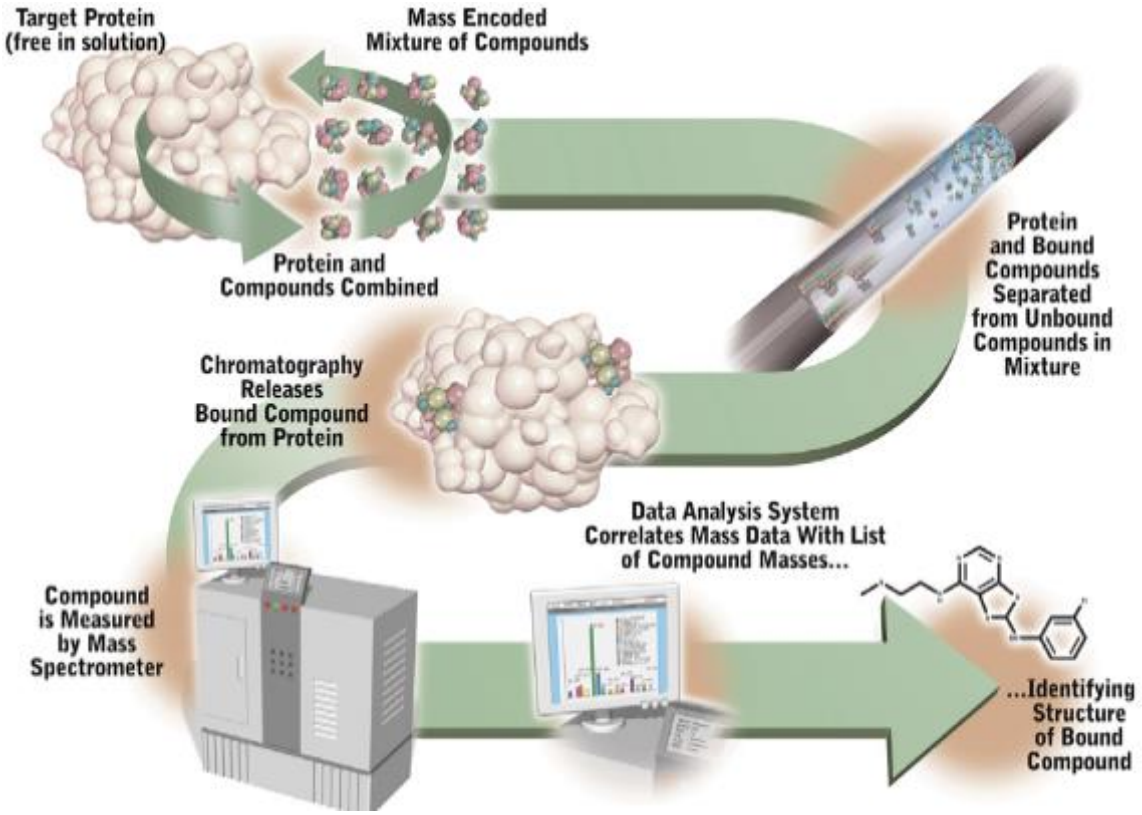

Figure 9: Schematic representation of the ALIS process.[72]

An automated, solution-based indirect screening system that displays considerable overlap with the ALIS method has been developed by O'Connell et al.[77] using commercially available parts. However, accurate mass analysis gives it a substantial edge over ALIS in simplifying hit identifications. Overall, this improved technique is capable of screening $1x10^5$ compounds per day with a low hit rate, which is indicative of more specific binding and a lower number of false positives.[77]

Ultrafiltration membranes have also been used to separate receptor-ligand complexes from nonbinding compounds.[78-80] First, the target receptor is incubated with a

library of compounds. Then the incubation mixture is subjected to centrifugation with molecular cutoff filters to separate the unbound compounds from the complex. The bound compounds are then released by denaturing the protein/ligand complex. Finally, the released compounds are detected using MS. This ultrafiltration-based indirect screening method has been used to study the structure and affinity of the human estrogen receptors[81] and also to identify new inhibitors for MurF, a *Streptococcus pneumoniae* cell wall biosynthesis enzyme.[80]

While it is true that affinity based techniques are more compatible with HTS and automation, compared to functional based screening, overall they suffer from high rates of false positive identification which significantly complicates analysis, though the rate of false positive identification varies among the different techniques. Minimizing or eliminating false positives and improving upon the existing techniques is of paramount importance for the drug development process.

1.7 Techniques with binding event prior to the detection

In this category, proteins are immobilized on a solid support for use in assays, but not packed in a column. Most of the techniques in this category use ligand fishing, where a binder is caught from a ligand sea and identified after release. Other techniques involve the comparison of unbound compounds in binding experiments with control experiments.

1.7.1 High Affinity Mass Spectrometry (HAMS)

HAMS[47] is an MS-based high throughput screening method that is used to selectively detect binding compounds from complex library mixtures without reporting false positives. Figure 6 shows a schematic diagram of the HAMS method. A control

experiment, where no protein is used, generates spectra that are compared to binding experiment spectra, and the absence of certain peaks is indicative of binding compounds, as shown in Figure 10.[47] The authors have demonstrated the applicability of HAMS on three different target proteins: pepsin, carbonic anhydrase, and maltose binding protein. Binders in three affinity ranges (pM, nM and μM) were screened without detecting any other compounds as hits, except the known binders for the respective proteins. A comparison experiment of the HAMS and Affinity selection mass spectrometry (ASMS)[77] methods was carried out with carbonic anhydrase as the target protein, further confirming the lack of false positives in HAMS, whereas a 10% false identification rate was observed for ASMS. However, a HAMS experiment is more time consuming, since it necessitates acquiring two LC-MS spectra for the same set of compounds, specifically binding and control experiments. Similar to all other MS techniques, the HAMS method suffers from false negative identifications due mostly to compounds that are not ionizable (and thus not detectable) by MS.

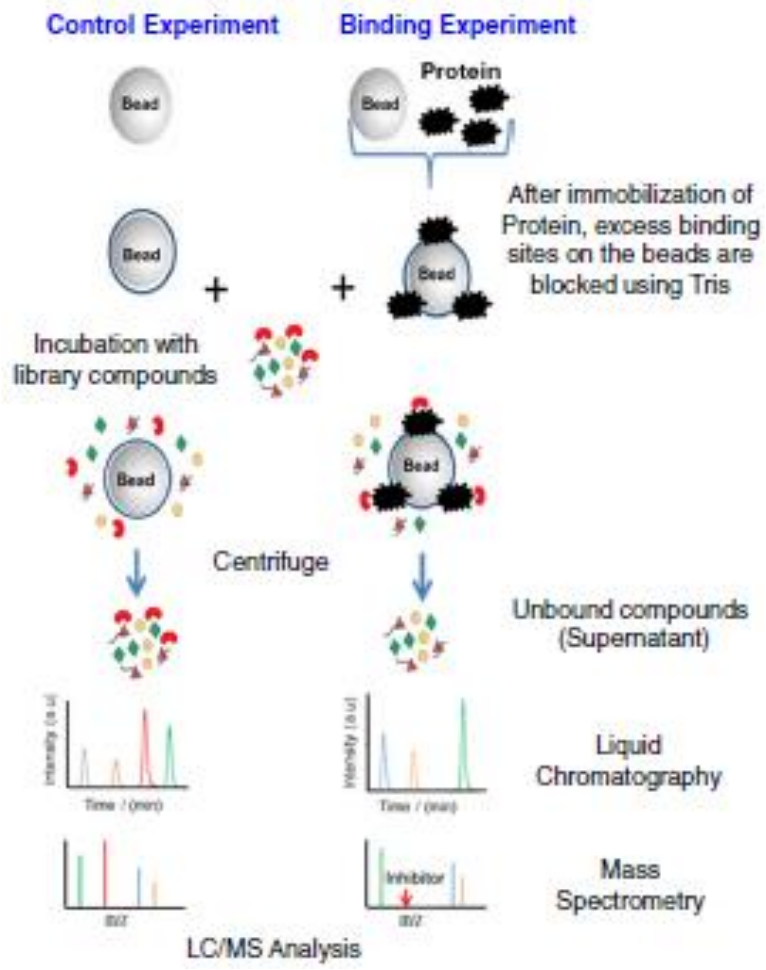

Figure 10: Schematic diagram of the HAMS method. Inhibitors/binders are incubated with the immobilized protein (and, separately, with blocked beads in the control experiment), removed, and analyzed by LC-MS. Strong binders were identified by comparing the spectral data of the control and binding experiments. The binders are present in the data for the control experiment, but absent in the data for the binding experiment.[47]

Our group recently developed an improved version of the HAMS method, which features several advantages over previous methods. The assay can be used to selectively identify positive hits when a ligand at the binding site of interest is displaced, which lowers the false positives. In this method, a reporter compound that ionizes well, rather than the ligand binder which perhaps is a non ionizable compound, is detected. This mitigates the appearance of false negatives. In this method, four hundred

compounds can be assayed in 10 minutes, demonstrating fast detection, and the assay requires low (nanogram) amounts of target protein per compound. In addition, binders with K_d's in the picomolar to micromolar range can be detected by this method. Known binders of three proteins, Pepsin, Maltose Binding Protein (MBP), and Carbonic Anhydrase (CA), in the presence of hundreds of non-binders were used to validate this assay. Furthermore, a novel CA binder, pifithrin-μ, a compound with poor ionization efficiency, which could not have been identified by any other MS-based assay, was also identified by this method.

1.7.2 Additional detection methods

Ligand fishing is a method originally developed to "fish out" any bioactive compounds that bind to a target of interest from sets of natural products.[82] In this method, activated surfaces are used to immobilize bait molecules and can be carried out either online or offline. In offline analysis, the target protein (the bait) is first immobilized on a solid surface, such as resin beads. Then the bait (immobilized protein molecule) is immersed in the ligand seas. After that, the immobilized protein is washed thoroughly to remove any nonspecifically bound compounds. The specifically bound compounds are subsequently released and identified using MS.[82] Offline ligand fishing has mainly been conducted using magnetic beads or agarose beads coated with biomolecules. Figure 11 is a schematic representation of offline ligand fishing of active compounds from plant extract for a target protein. In this process, protein-coated magnetic beads are incubated with the plant extract. After several washing steps, the bound ligand is eluted using an organic buffer, and is then identified using MS.[82] In an alternative format, where the affinity selection is conducted online with the detection step, the immobilized protein is in a

column which ligands seas are flowed through. After that, any excess molecules are washed out, followed by elution of the bound compound, which is finally identified using mass spectrometry. Immobilized enzymes, such as lipases,[83] galactosidases,[84] pectinases,[85] glucosidases,[86] are mostly used to fish out ligands from complex matrixes. Even though the applications are promising in the field of drug screening, false positives due to non-specific interactions can hinder interpretation of results.

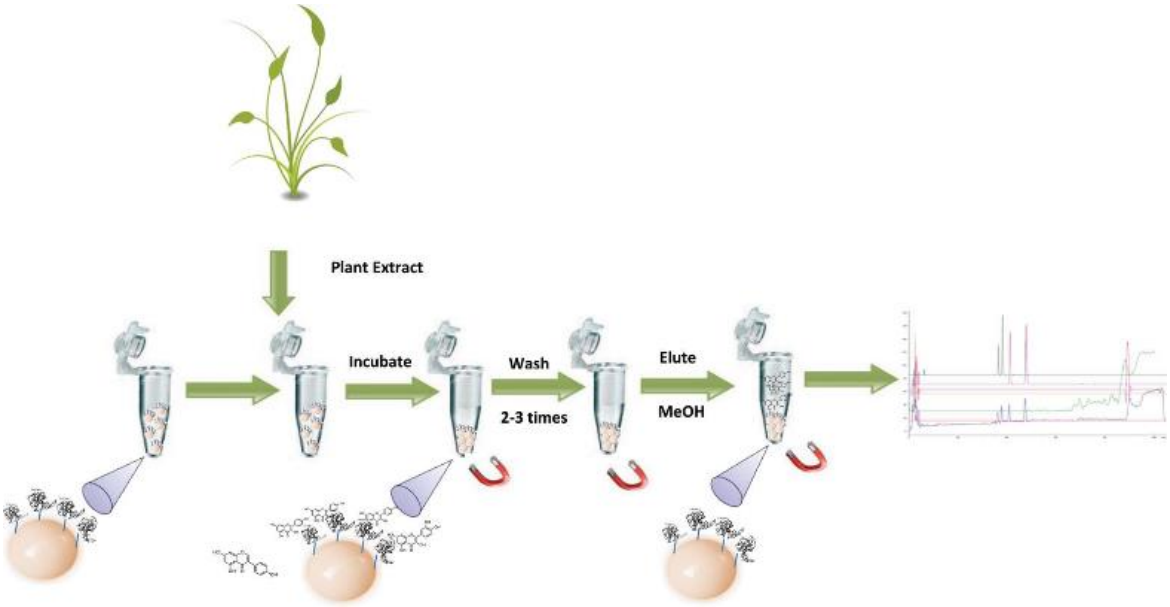

Figure 11: In the ligand fishing approach, protein coated magnetic beads are incubated with a plant extract, washed, and then eluted in an aqueous organic buffer. The elution buffer is then analyzed by HPLC-MS.[82]

High through put screening of drug candidates is a necessity in many drug discovery pipelines. When a Mass spectroscopy (MS) based method is used for screening, false positive identifications become a major challenge. Chapter 2 describes a detection strategy designed to eliminate false positives when screening for the high affinity ligands of target proteins. In this approach, the protein is immobilized on a solid support and incubated with a library of ligands. Then, the non-binders are separated from

the protein-ligand complexes for detection. Binders are identified by comparing MS data with a control consisting of a ligand sea to which no target protein was added. Binders are not detectable by MS due to being removed with the protein, but readily discernable in the control experiment. The assay was demonstrated using three different proteins, Pepsin, Maltose Binding Protein (MBP), and Carbonic Anhydrase (CA), with hundreds of non-inhibitors. No false positive hits were identified in our approach, as opposed to many that were identified in an already reported method. Our findings demonstrate that the approach described here compares favorably to traditional MS based screening methods.

Chapter 3 introduces a new MS-based HTS assay that possess substantial advantages over existing methods. This high throughput assay is designed to identify ligands that are able to inhibit non-enzymatic targets. This method is similar to a ligand fishing experiment, where a reporter molecule is detected instead of the actual binder. Three proteins, Pepsin, Maltose Binding Protein (MBP), and Carbonic Anhydrase (CA), in the presence of hundreds of non-binders, were used to validate this method. This new method addresses many of the challenges that are encountered by current high throughput screening methods. False positives are eliminated by selectively identifying a known weak ligand that is displaced from the binding site by a stronger affinity counterpart. False negatives are mitigated by detecting the ionizable reporter molecule instead of the binding ligand, which may or may not be ionizable. This allows non-ionizable compounds to be consistently assayed using a MS based method. Four hundred compounds can be screened in 10 minutes with this method, using only low (nanogram) quantities of target protein per library compound. In addition, a wide variety of binders, from the picomolar to micromolar range, can be detected. Moreover, a novel

CA binder, pifithrin-μ, which could not have been identified by any other MS-based assay, due to its poor ionization efficiency, was identified using this technique.

Improvements to the method described in Chapter 3 are introduced Chapter 4. We developed an assay that reduces the amount of protein needed to screen for micromolar binders by 50%. This is significantly advantageous for a high throughput assay since the target protein is often the limiting reagent that influences the number of ligands that can be screened at once. Identifying ligands with a Kd in the micromolar range is challenging due to their lower affinity, and this is exacerbated when the available quantity of the target protein is limited. In this study, our previous ligand fishing HTS assays described in Chapter 2, in which 20 μg of protein was used, were re-characterized to identify micromolar binders. An internal standard was used to negate non-reproducible detection of the reporter molecule for identification of potential binders with a reduced amount of target protein, due to the variation of the mass spectrometry ionization source. This novel assay mitigates false positives and false negatives in a similar manner to our previous methods, in addition to lowering the consumption of proteins.

Chapter 5 outlines potential future uses of our method. In this chapter, we briefly discuss the major barriers for automation and increasing the throughput capability by reducing the sample preparation time. Furthermore, the current projects that the Desaire group are conducting are also discussed, specifically focusing on screening for new inhibitors for protein-protein interaction (PPI). This would be the first assay that ever been developed using MS-based high-throughput screening assay to screen for inhibitors for PPI.

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Chapter 2: HAMS: High-Affinity Mass Spectrometry Screening. A high-throughput screening method for identifying the tightest-binding lead compounds for target proteins with no false positive identifications.

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A major challenge in drug discovery is the identification of high affinity lead compounds that bind a particular target protein; these leads are typically identified by high throughput screens. Mass spectrometry has become a detection method of choice in drug screening assays because the target and the ligand need not be modified. Label free assays are advantageous because they can be developed more rapidly than assays requiring labels, and they eliminate the risk of the label interfering with the binding event. However, in commonly used MS based screening methods, detection of false positives is a major challenge. Here, we describe a detection strategy designed to eliminate false positives. In this approach, the protein and the ligands are incubated together, and the non-binders are separated for detection. Hits (protein binders) are not detectable by MS after incubation with the protein, but readily identifiable by MS when the target protein is not present in the incubation media. The assay was demonstrated using three different proteins and hundreds of non-inhibitors; no false positive hits were identified in any experiment. The assay can be tuned to select for ligands of a particular binding affinity by varying the quantity of protein used and the immobilization method. As examples, the method selectively detected inhibitors that have K_i values of 0.2 μM, 50 pM and 700 pM.

These findings demonstrate that the approach described here compares favorably to traditional MS based screening methods.

2.1 Introduction

In recent decades, combinatorial and parallel synthesis methods have been employed to synthesize millions of library compounds that potentially could be used as new therapeutic drugs. One of the vital tasks of drug discovery is fast and effective identification of high affinity ligands from libraries containing these vast numbers of compounds. When a screening method based on fluorescent[1-2] or chemiluminescent[3] read-out is feasible, tens of thousands of compounds can be assayed per day. Many potential drug candidates, however, cannot be probed using these standard assays because some druggable interactions, such as protein binding events, cannot be readily monitored by a change in fluorescence. In these cases, assays of lower throughput based on Nuclear Magnetic Resonance (NMR) or Surface Plasmon Resonance (SPR) are routinely employed.[4]

Mass spectrometry (MS) is increasingly used for High Throughput Screening (HTS) due to its advantages of fast and sensitive detection, high specificity, and the ability to detect compounds without labels. One of the commonly used MS-based screening methods is Affinity Selection Mass Spectrometry (ASMS); this approach probes the affinity of various ligands by combining large batches of library compounds with the protein of interest and using MS to detect the binding of a small sub-set of those ligands to the protein.[5-6] In one incarnation of the method, the direct ASMS method, any protein/ligand complex that forms is detected directly by MS. A lead is identified when

the mass of the protein is shifted to include the mass of the binding ligand. The disadvantage of this approach is that the ESI process may fully or partially distort the protein-ligand complexes; determining appropriate MS parameters for each type of complexes is challenging and time consuming. In addition, the mass of the protein/ligand complex is not substantially different than that of the protein itself, and even when a binding event is detected, it can be difficult to infer the exact mass of the ligand.[7]

In another embodiment of the ASMS method, often referred to as "the indirect approach," the binding ligands are identified after the protein-ligand complex is isolated and the ligands are dissociated. This approach has shown to be highly amenable to automation, with a throughput of ~ $1*10^5$ compounds per day.[8-9] However, it has some limiting disadvantages that we seek to address herein. Namely, the ligands could bind weakly and nonspecifically to the protein, and in the ASMS assay, they would still be detected as protein binders.[8-10] In addition, the method suffers from a higher incidence of false positive hits, which can be introduced during the isolation of the ligands from the protein complex. To alleviate this problem, others have used molecular weight cut off filters,[11] or size exclusion gels[12-14] to isolate the protein complexes prior to ligand dissociation. While these approaches are helpful, non-binding ligands may interact with these separation media as well, also leading to false positives. Additionally, these solutions do not address the problem of detecting numerous nonspecific and weak binders.

Herein we present a new MS-based high throughput screening assay that shares several advantages with the indirect ASMS method described above, but it eliminates several of the key disadvantages. In the approach described here, the protein and ligands are mixed

as in the ASMS method, but the non-binding analytes are detected, instead of the binders. This detection strategy was chosen to reduce the detection of false positives – those compounds that bind weakly or nonspecifically to the protein. Only the tightest binders completely disappear from the ligand sea after protein is added, so those tightest binders are readily detected as peaks that are *absent* in the MS data, when the non-binding ligands are analyzed. The method described herein not only picks out the tightest binding ligands, it can also be used to detect ligands with different binding affinities, by tuning the concentration of protein used in the assay. As an additional benefit of screening in this manner, the amount of protein required, and the amount of each binding ligand, can be substantially reduced compared to the state-of-the-art comparator method.[9] Reduction in the amount of protein required, in particular, is a strong asset in a high throughput screening method; it can substantially reduce assay cost because the quantity of the most expensive reagent, the protein, is reduced.

2.2 Methods:

2.2.1 Reagents

The library compounds used in this study are all FDA-approved drugs. For all the experiments conducted with 176 compounds, the drug molecules are selected from the Prestwick library, which was obtained from the High Throughput Screening Laboratory at the University of Kansas. The library was provided in combined batches of 176 compounds per vial, dissolved in DMSO at a concentration of 14.2 µM per compound. For all the experiments conducted with 352 compounds, the drug molecules were obtained from LOPAC1280-small scale library that was purchased from Sigma Aldrich

(St. Louis, MO) as individual compounds, dissolved in DMSO at a concentration of 10 mM per compound. When combining compounds for high throughput screens from the LOPAC library, any compound larger than 150 Da was considered a candidate, and no effort was made to remove compounds from the library that would likely not ionize by ESI. Pepsin, Aminolink plus coupling resin and disposable plastic columns were acquired from Pierce Biotechnology, Inc (Rockford, IL), while nitrocellulose drop dialysis membranes were purchased from Fisher Scientific (Houston, TX). Maltose binding protein and Carbonic Anhydrase were purchased from My BioSource (San Diego, CA) and Sigmal Aldrich (Milwaukee, WI), respectively.

2.2.2 Binding Experiment on agarose.

Pepsin and Carbonic Anhydrase (CA) immobilization was carried out by adapting a procedure published elsewhere.[15] The following buffers were used for the binding experiment with Pepsin: Citric acid and $NaHPO_4$ (0.1 M, pH 4.5) were used as the coupling buffer; the blocking buffer was Tris HCl (1.0 M, pH 4.5); the incubation buffer was ammonium acetate (0.02 M, pH 4.0); and the wash buffer was a solution of 1 M NaCl in coupling buffer. Similarly, for CA, the coupling buffer was phosphate buffered saline (PBS) (0.1 M, pH 7.4); the blocking buffer was Tris HCl (1.0 M, pH 7.4); the incubation buffer was ammonium acetate (0.02 M, pH 7.4); and the wash buffer was a solution of 1 M NaCl in coupling buffer. Both Pepsin and CA were dissolved in 100 µL of coupling buffer and were dialyzed for 1 hr with the coupling buffer using a 0.025 mm nitrocellulose drop dialysis membrane. A disposable plastic column was filled with coupling resin and washed with 2 mL of coupling buffer. Then, dissolved protein was added (in the amounts described below) followed by addition of 1 M $NaCNBH_4$ prepared in coupling buffer, until

the final concentration of $NaCNBH_4$ is 50 mM. The column was rocked overnight and washed with 10 mL of coupling buffer followed by 5 mL of blocking buffer. Thereafter, 1mL of blocking buffer was added followed by 1M $NaCNBH_4$, until the final concentration is 50 mM and the mixture was rocked for 2 hrs. Next, the column was washed with 10 mL of coupling buffer, 10 mL of wash buffer, and 15 mL of incubation buffer, respectively. Finally, the immobilized protein was transferred to Eppendorf tubes for the incubation with the library compounds.

The quantities of protein used for testing different libraries are as follows: For the limited library, 10 mg of Pepsin was immobilized onto 1000 µL of resin beads. For the experiments with larger libraries, significantly less protein was used. For the 176 compound library, 450 µg of Pepsin was coupled to 250 µL of resin beads. For the 352 compound library, initial experiments used,100 µg of Pepsin coupled to 100 µL of resin beads. Optimization of the amount of protein used for screening was carried out using a 352 compounds library, with 100, 50, 25, 12.5, 6.25 and 3.125 µg of Pepsin in 100 µL of resin beads. For the Carbonic Anhydrase experiments 120, 60, 30, and 6 µg of protein was coupled to 100 µL of resin beads.

Library compounds were diluted from, 14.2 µM to 150 nM in incubation buffer prior to incubation with the immobilized protein. Then, 200 µL of the library mixture was added to 100 µL of immobilized protein mixture to make the final concentration of the library compounds 100 nM. Subsequently, the mixture was rocked for 1hr at room temperature. After incubation, tubes were centrifuged at 3000 × g for 5 minutes; the supernatant was removed and directly used in the LC-MS analysis. All experiments using agarose were repeated and at least 3 times. No changes were detected in the percent of compounds

that ionized in the control experiments (described later) or the identified inhibitors from the binding experiments (described above).

2.2.3 Alternative binding procedure using magnetic beads.

Maltose binding protein (MBP) was immobilized on N-hydroxysuccinimide (NHS)-activated magnetic beads by following the manufacturer's protocol (Pierce™ NHS-Activated Magnetic Beads, Thermo Scientific). Briefly, after washing the magnetic beads per the manufacturer's instructions, 100 µg of maltose binding protein in coupling buffer (PBS, 0.1 M; pH 7.4) was added to 300 µL of magnetic beads and slowly rocked overnight at room temperature. The supernatant was removed and the immobilized protein was washed with 1 mL of coupling buffer. After that, 300 µL of quenching buffer (Tris.HCl, 1.0 M, pH 7.4) was added and slowly rocked for 2 hours. The supernatant was removed and the immobilized protein was washed with 2 mL of ammonium acetate (0.02 M, pH 8.0) incubation buffer. The supernatant was removed again and 50 µL of 352 compounds library mixture and a known inhibitor of maltose binding protein, maltotriose, was added. The final concentration of the library compounds was 100 nM. The mixture was then rocked for 1 hour at room temperature. After incubation, the supernatant was removed and directly used in the LC-MS analysis. It was assayed three times to assure that the results were reproducible. No changes were detected in the percent of compounds that ionized in the control experiment (described next) or the inhibitors identified in the binding experiment (described above).

2.2.4 Control Experiments

Coupling resin (agarose or magnetic beads) without protein, was used for the control experiments. It was treated in the same way as described for its corresponding binding experiment, except no protein was added. The same sets of library compounds, at the same concentrations as described above, were combined with the resin. For each control experiment all conditions were identical to the matched binding experiment, and the LC-MS analysis, described below, was also conducted identically, for both the control experiments and the binding experiments.

2.2.5 Capture and release experiment, an alternative testing method

The 352 compound library and a known strong inhibitor, ethoxzolamide, was incubated with 60 µg of immobilized Carbonic Anhydrase. The concentrations of the library compounds and the incubation conditions were replicated from the binding experiment. After incubation, the supernatant was removed, and the immobilized protein was washed five times with incubation buffer. In each washing step, 1 mL of incubation buffer was added and vortexed for 10 s, followed by centrifugation at 3000 x g for five minutes. After washing was complete, 200 µL of acetonitrile was added to denature the protein, and the sample was kept at 70^{o}C for 10 minutes. The sample was centrifuged at 3000 x g for five minutes and the supernatant was analyzed using LC/MS.

2.2.6 LC/MS Analysis

Liquid chromatography/mass spectrometry analysis was done using an Acquity UPLC system (Waters Corporation, Milford, MA) coupled to an Orbitrap Velos Pro mass

spectrometer. Mobile phase A was 99.9% water with 0.1% formic acid and mobile phase B was 99.9% MeOH with 0.1% formic acid. For each run, 5 µL of the supernatant was injected onto a C_{18} Hypersil Gold column (Particle Size: 5 µm; 1 mm *i.d* X 100 mm, 175 Å, Thermo Electron Corporation, Thermo Fisher Scientific, Pittsburgh, PA) at a flow rate of 50 µL/min. The following multi step gradient was used for the limited library: The column was equilibrated at 98% solvent A for 5 min. Solvent B was linearly increased to 30% in 3 min, followed by a linear increase of solvent B to 60% in 10 min and a final linear increase to 98% in 2 min, where the solvent composition was maintained for an additional 3 min. The multi-step gradient used for the large libraries (176 compounds, and 352 compounds) was as follows: 100% solvent A for 5 min, then a linear increase of B to 10% in 5 min, followed by the linear increase of B to 60% in the next 30 min, followed by another linear increase to 85 % in additional 10 min, followed by a linear increase to 95 % in the final 7 min, where the column was maintained for another 3 min. A short wash and a blank run were carried out to ensure there was no sample carryover between runs. The eluent was diverted to waste for 5 min at the beginning of each run except for the experiments conducted with maltose binding protein, where the time was reduced to 1 min, to prevent salts and DMSO from entering the MS source. The mass spectrometer was operated in the positive ion mode with a 3 kV potential on the ESI needle, and the capillary temperature was set at 250 °C. Full scan MS data were acquired at a mass range of *m/z* 150-1000 using the Orbitrap mass analyzer at a resolution of 30,000 for *m/z* 400.

All LC-MS data were interrogated using the MS analysis software, Apex (Sierra Analytics, Modesto, CA), which identifies compounds based on both mass match and a match of the isotopic distribution. The molecular formulas were input, and the software

extracted chromatograms of each compound from the .raw data files. All analyses were conducted using a 5 ppm mass accuracy threshold and an MS similarity score of 0.05. The spectral data of the compounds that were not identified by the software were manually identified. A mass accuracy of 5 ppm and the correct isotopic cluster pattern, especially the presence of C^{13} isotopic peak, were required for a positive identification during manual analysis. Blank runs for both the binding and control experiments were analyzed, in order to eliminate any false identification of compounds that could arise due to background contaminants.

2.3 Results and Discussion

Figure 1 shows a workflow of the screening method described in this manuscript. Coupling resin is used for both the binding assay and the control experiment. In the binding assay, the protein is immobilized on the resin. The resin, without protein, is used for the control experiment. After immobilization, the remaining active sites on the resin, for both the binding and control experiments, are blocked using Tris. The resins (agarose, or magnetic beads) are then incubated with the library compounds, and the unbound compounds are detected by analyzing the supernatants using LC-MS. The compounds that are absent in the spectrum acquired from the binding experiment compared to that of the control, are considered bound to the protein. It should be noted here that the control experiment is used to determine which compounds are detectable in the assay. Any compound not detectable in the control experiment could be assayed by a different ionization method, such as APCI. However, these compounds are not false positives or false negatives. They are simply “unassayable in this experiment.”

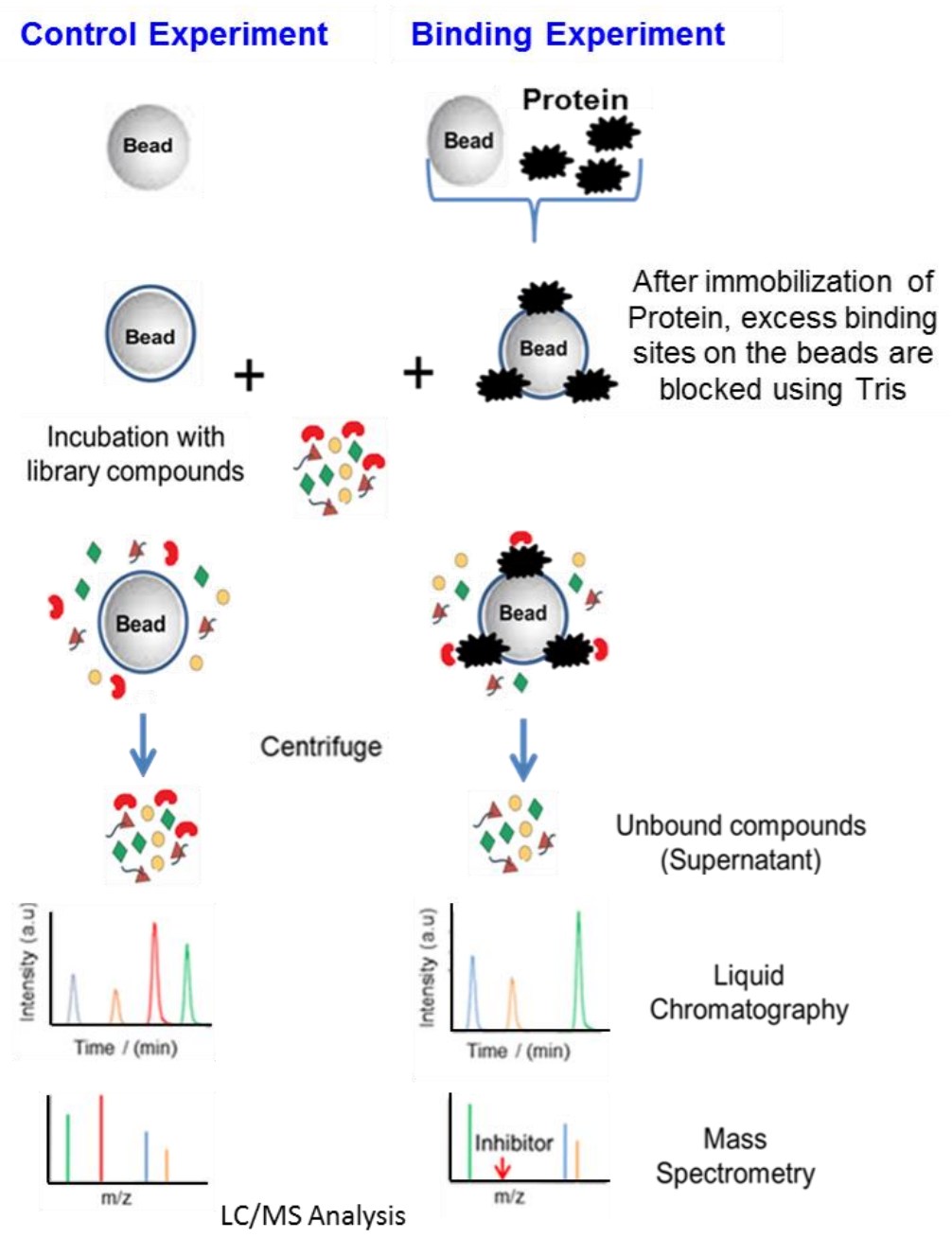

Figure 1: Workflow used herein. The inhibitors/binders were incubated with the protein (and, separately, with blocked beads), removed, and analyzed by LC-MS. Strong binders were identified by comparing the spectral data of the control and binding experiments. The binders are present in the data for the control experiment but absent in the data for the binding experiment.

The effectiveness of the approach was demonstrated in several experiments. First, the feasibility of the method was assessed using immobilized Pepsin and a limited (seven compound) protease inhibitor library, so the results could be compared directly to a previous method upon which this one was loosely based.[15] Next, larger libraries of drug-like candidates were incubated with the protein target, to demonstrate that the method was amenable to larger sets of compounds. Subsequently, the amount of protein was reduced, to demonstrate the method was scalable to very large screens where thousands of compounds could be tested. We demonstrated that the assay could be tuned to only identify inhibitors with the strongest binding constants by optimizing the amount of protein used. Finally, we demonstrated that the assay was transferrable to other protein/inhibitor analyses and to detecting inhibitors with Ki's approaching the micromolar range.

2.3.1 Feasibility study

A well-studied, limited protease inhibitor library[15] was used for proof of concept. Pepsin, immobilized on agarose, was incubated with a small number of protease inhibitors, including one strong Pepsin inhibitor, pepstatin A, and the raw data from this experiment is shown in Figure 2. Overlaid selected ion chromatograms (SICs) of the seven compound inhibitory library incubated with blocked resin beads (from the control experiment) and after incubation with immobilized Pepsin (from the binding experiment) are shown in Figure 2a and 2b respectively.

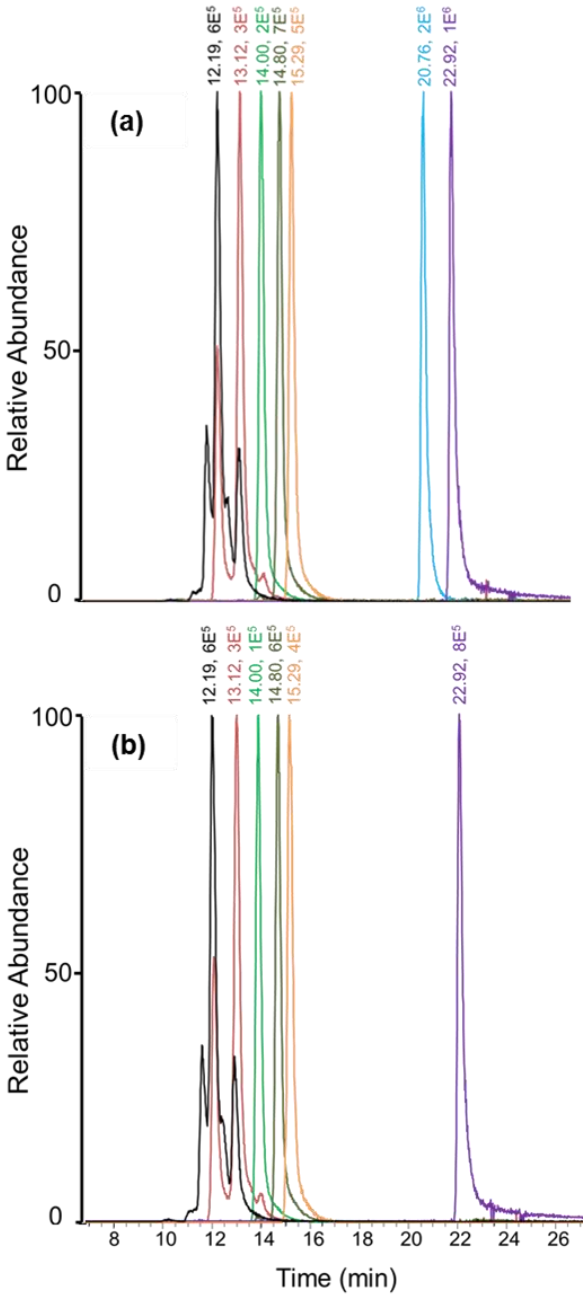

Figure 2: Selected ion chromatograms of known protease inhibitors, from the (a) control and (b) binding experiments. Pepstatin A (blue trace) was not detected in the binding experiment (b) because it is a strong binder compared to other compounds. Seven known protease inhibitors were used in both experiments. They are (in order of increasing retention time): antipain, black; leupeptin (red); bestatin (green); (+)-diisopropyl L tartarate (olive); N-acetyl-3,5-diiodo-Tyr (orange); peptstatin A (blue); cholic acid (purple)

Analysis of data from both the control experiment (Figure 2a) and binding experiment (Figure 2b) shows only one compound, pepstatin A is absent in the binding experiment, indicating it is the single strong-binding inhibitor. This result is significant because pepstatin A is the strongest binder to Pepsin in the group of compounds. In earlier work by Cancilla *et. al*,[15] several compounds were identified as potential inhibitors, including pepstatin A. In the previous experiment, any compound whose MS signal was reduced, compared to the signal obtained prior to incubation with the protein, was identified as a potential inhibitor. Therefore, weak and/or nonspecific binders were detected as possible hits. In contrast to the protocol by Cancilla *et al,* the strongest binders can be exclusively identified if the detection strategy requires that the binder be *completely absent* after incubation with the protein. This change in detection strategy was intended to eliminate false positives and weak binders that are commonly detected in MS-based screening assays. The method was further adapted for high throughput screens by reducing the protein requirements and increasing the number of compounds to be analyzed in one batch, as described next.

2.3.2 Testing large libraries

The number of compounds that were incubated with immobilized Pepsin was increased ~20 fold in order to investigate the method's capacity for screening large numbers of compounds. Figure 3 illustrates the results from screening 176 compounds in one analysis while simultaneously scaling back the protein consumption by more than a factor of 20, from 10 mg to 450 µg. Figure 3a shows a representative TIC from this experiment, demonstrating that the library compounds elute throughout the course of the experiment. Each compound in the library was searched for individually in the dataset

using Apex, as described in the experimental section. Comparison of data from the control experiment and binding experiment shows only one compound, the known inhibitor, is present in the control and absent in the binding experiment. Data in Figures 3b and 3c show the selected ion chromatograms for pepstatin A, demonstrating that it is identifiable as a Pepsin binder. These data clearly demonstrate the method's potential for high specificity, high throughput screens.

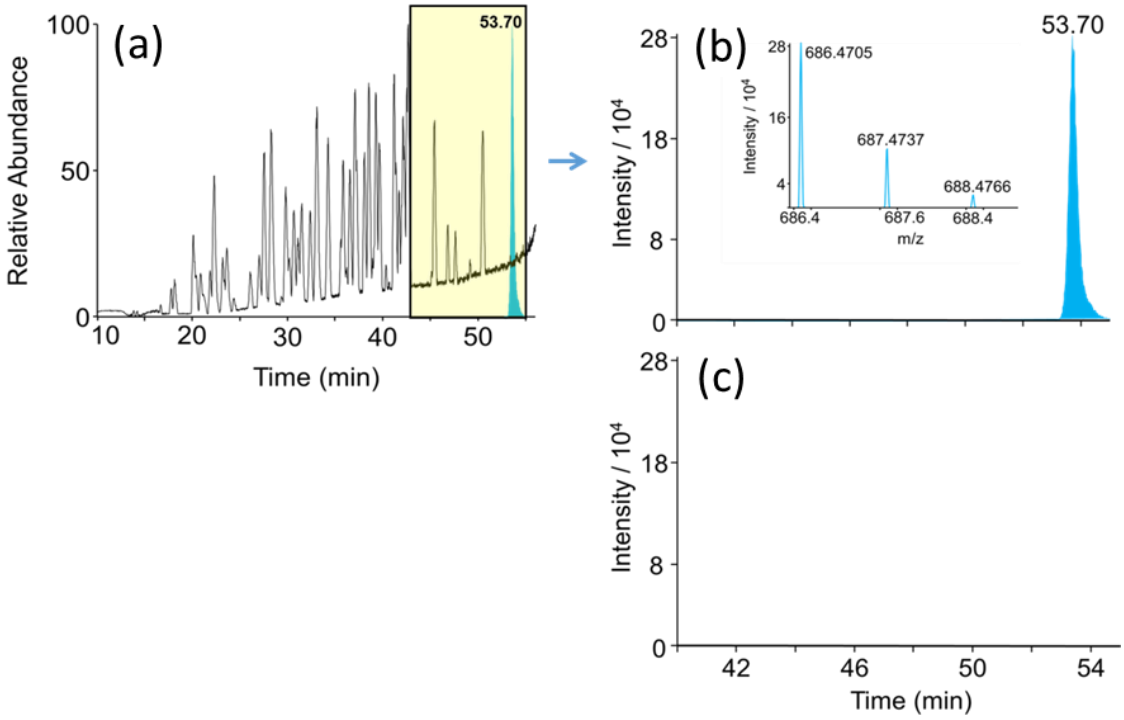

Figure 3: (a) Selected Ion Chromatogram (SIC) of pepstatin A in (a) overlaid on TIC. (b) SIC of pepstatin A in the control experiment, and (c) in the presence of 450 µg of pepsin in the binding experiment. pepstatin A was not detected in the binding experiment because it is a strong binder. A large library of 176 compounds and the known inhibitor (each with 100 nM concentration) were used in all experiments.

In an effort to further test the method, the number of library compounds was doubled again (from 176 to 352), and the amount of Pepsin used for binding experiment was further reduced from 450 µg to 100 µg. Again, only the known inhibitor is absent in comparing the data from the control experiment and the binding experiment; see Figure 4. The number of detectable compounds in the control experiment was 313, or ~88% of the total number of compounds assayed. The compounds that were not detectable in the control experiment (~12%) would need to be assayed by another method. They are not false positives or false negatives. Overall, the results from the 176 compound library and the 352 compound library demonstrate that large numbers of compounds can be analyzed in batches and that the protein necessary for these types of experiments is substantially lower than originally reported in reference 15.

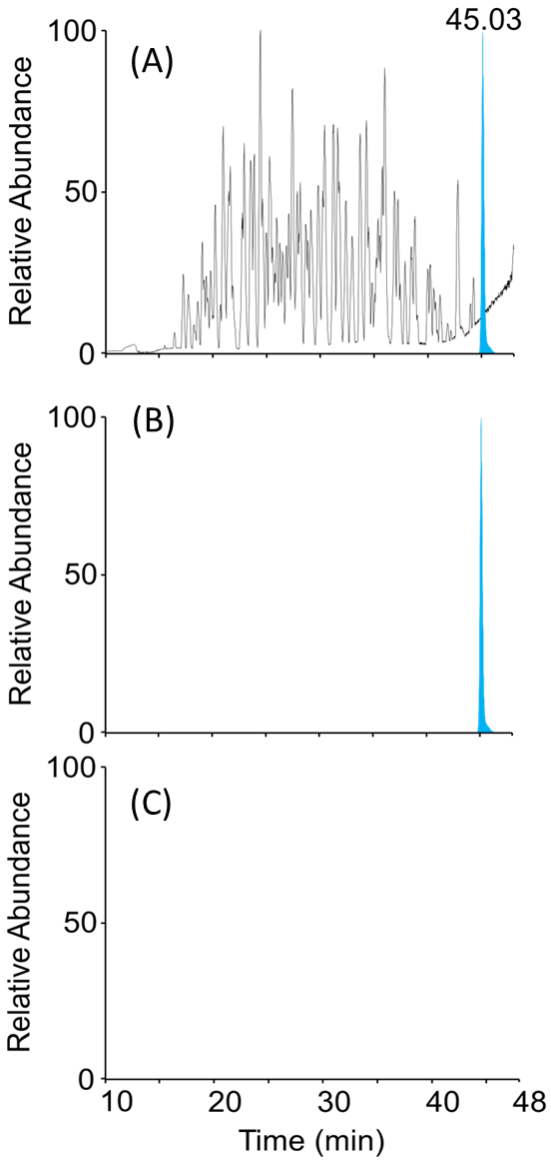

Figure 4: Selected Ion Chromatogram of Pepstatin A in (A) Control experiment overlaid on TIC, (B) in the control experiment, and in the presence of (C) 100 µg of pepsin in the binding experiment. 352 library compounds and known inhibitor (each with 100 nM concentration) were used in all experiments.

2.3.3 Optimization of the protein amount

One important measure of a good of high throughput screening method is the ability to test large numbers of compounds on small quantities of protein. Therefore, we asked the question: What is the minimum quantity of protein needed to detect pepstatin

A, while still screening >350 compounds at a time? Figure 5 illustrates the MS data collected in a number of experiments where the amount of Pepsin was systematically reduced in order to determine the minimum amount of the protein needed for assay to remain successful. (A successful assay is one in which the known, strong inhibitor, peptstatin A, does not appear in the selected ion chromatogram when analyzing the supernatant from the binding experiment.) Several quantities of Pepsin were tested, including 50 µg, 25 µg, 12.5 µg and 6.25 µg. In each case, the pepstatin A was not detected in the supernatant from the binding experiments. However, when the amount of Pepsin was reduced to 3.125 µg, the inhibitor finally appeared in the chromatogram (Figure 4b). These data demonstrate that the high throughput screening campaign could be conducted on a scale that consumed as low as 6 µg (171 pmol) of protein to screen batches of >350 compounds. We expect that this protein quantity is at the low end of what one would want to use in this kind of assay, particularly if weaker-binding ligands are targeted. This inhibitor has a very low K_i, 45 pM, so it is likely to be a very strongly binding inhibitor. Additional experiments, below, indicate that the assay can be done on less than 100 µg of protein per well, even when lower-affinity binders are to be detected. In each of the experiments described above, all the other compounds that are observed in the control experiment, except the inhibitor, were also observed in the binding experiments, demonstrating that zero false positives were identified, which is a key strength of this screening method.

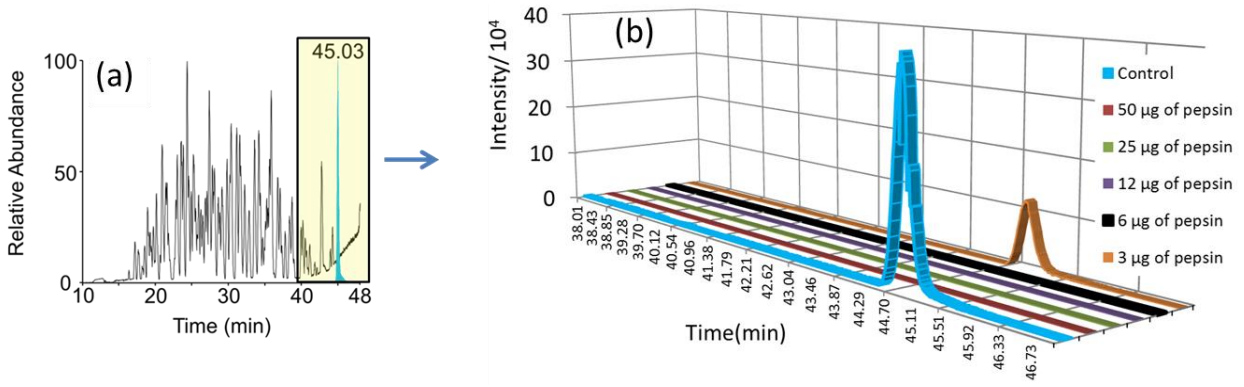

Figure 5: Selected Ion Chromatogram of pepstatin A in (a) Control experiment overlaid on TIC, and in the presence of (b) 0 µg (Control), 50 µg, 25 µg, 12.5 µg, 6 µg, and 3 µg of Pepsin in binding experiments. A library of 352 compounds and the known inhibitor (each with 100 nM concentration) were used in all experiments.

2.3.4 Application to a new protein, Carbonic Anhydrase (CA)

A second protein target, Carbonic Anhydrase (CA) immobilized on agarose beads, along with the 352 compound library described above, was used to further demonstrate the feasibility of this screening method. The library was spiked with two known CA inhibitor standards; ethoxzolamide with K_i of 700 pM ,and sulpiride with K_i of 63 µM. The data in Figure 5a show the TIC for the control experiment, and Figure 5b contains the data from the binding experiment. In each case, the SICs of two known inhibitors, ethoxzolamide (red) and sulpiride (blue) are overlaid in the Figures. Inserts show the mass spectral data for each inhibitor. Approximately 88% (312) of the compounds were detected from the 352 compounds in the control experiment (Figure 6a). All 312 compounds that were observed in the control experiment were also observed in the binding experiment, except ethoxzolamide, the strong inhibitor (Figure 6b). Even though sulpiride is known to be a CA inhibitor, it was not identified as such in this experiment since the K_i is rather high (63

μM). These data demonstrate that only the strongest binders are identified using this approach.

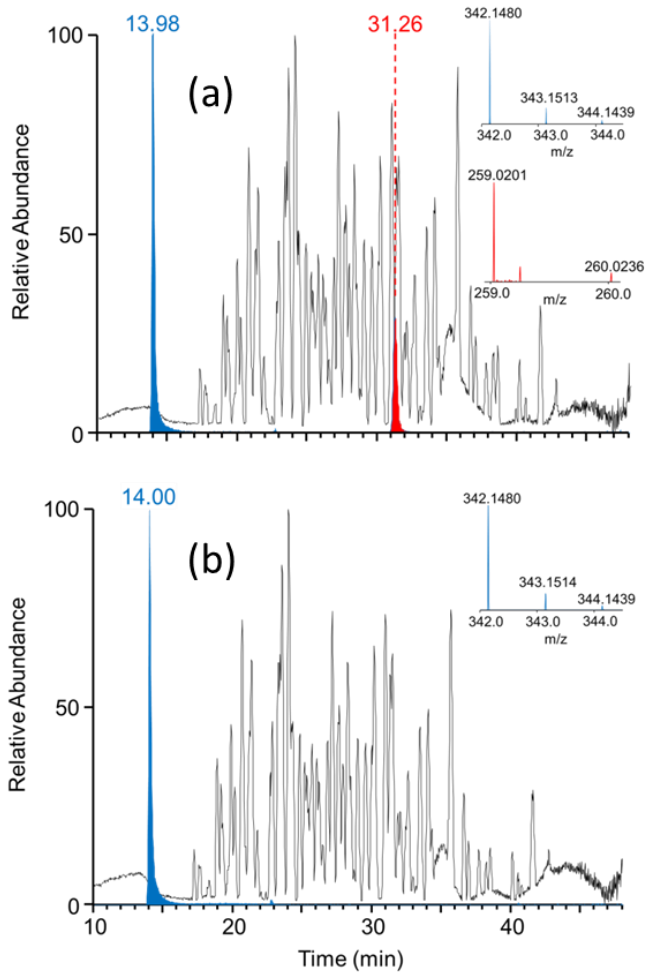

Figure 6: TIC of the (a) control and (b) binding experiments (with 120 μg of CA), including selected ion chromatograms of two known CA inhibitors, ethoxzolamide (red), and sulpiride (blue), overlaid. Ethoxzolamide was not detected in the binding experiment because it is a strong binder. Every other compound detected in the control experiment (a) was also detected in the binding experiment (b), including the weak binder. This experiment shows that zero false positives were detected and that the assay is selective for the strongest binder, when more than one binder is present.

We optimized the assay to determine the minimum amount of protein required to detect ethoxzolamide. These data are presented in Figure 7. When 60 µg of CA was used, ethoxzolamide was not detected. When the amount of CA is reduced in half, significant spectral data were observed for the inhibitor, and even more intense spectral data were observed with the use of 6 µg of CA. These results indicate that approximately 60 µg of the protein is needed to identify this inhibitor, which has a K_i of 700 pM. We demonstrated in Figure 5 that the amount of protein required for this assay is substantially less, if stronger binding inhibitors are to be identified. Specifically, just 6 µg of protein was necessary to test 352 compounds and selectively identify an inhibitor with a K_i of 45 pM. In comparing the data herein for Carbonic Anhydrase (6 pmol protein per compound was required to identify a compound with a K_i of 700 pM) and the previous assay (0.5 pmol of protein per compound was required to identify a compound with a K_i of 45 pM), one can see that the amount of protein required for the assay is roughly proportional to the K_i for the compound to be detected. The strongest binding inhibitors can be detected with very low quantities of protein.

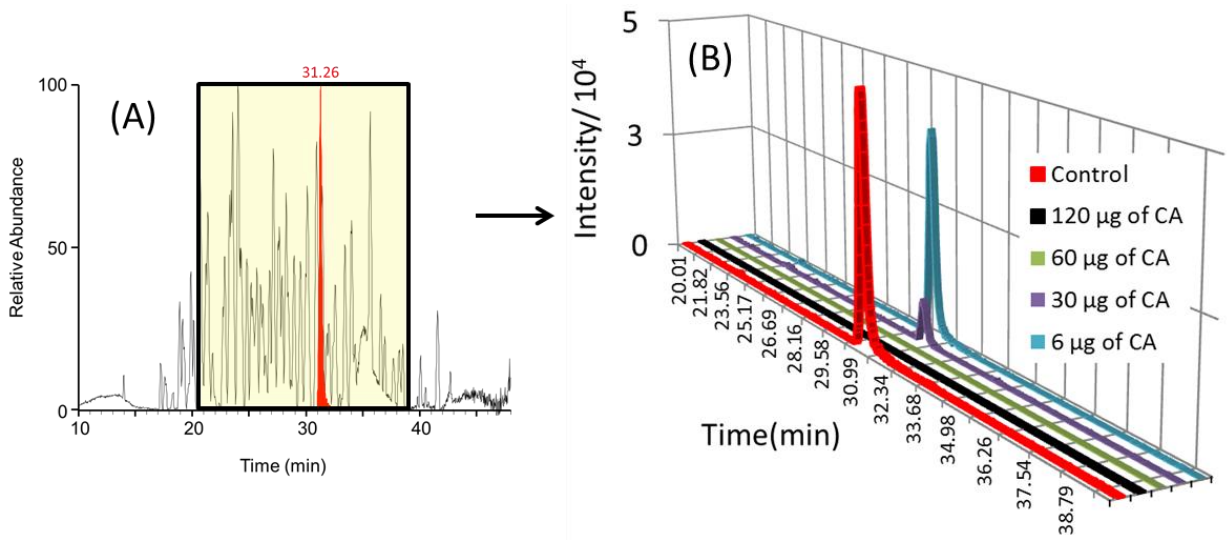

Figure 7: (A) SIC of Ethoxzolamide in (A) the control experiment overlaid on TIC and in the presence of (B) 0 µg (Control), 120 µg, 60 µg, 30 µg, and 6 µg of CA in binding experiments. 352 library compounds and two known CA inhibitors (each with 100 nM concentration) were used in all experiments.

2.3.5 Detecting lower affinity ligands.

To screen for the inhibitors in K_i ~ µM range using the immobilization conditions described above, a large amount of protein would be required. To successfully conduct the HAMS assay with lower affinity ligands, the protein can be immobilized on magnetic beads instead of agarose. This change in immobilization conditions is necessary because the beads can then be removed from the supernatant without centrifugation, so weak protein-ligand complexes are not disturbed while separating the proteins from the ligands

To demonstrate that the HAMS method can identify inhibitors with K_i's approaching the µM range, a screening was conducted using Maltose Binding Protein (MBP), immobilized on magnetic beads, and a 352 compound library, which was spiked with a known MBP inhibitor, maltotriose, with a K_i of ~ 0.2 µM. The data in Figure 8a show the

SIC of maltotriose in the control experiment, where the peak is clearly detectable. The maltotriose peak completely disappears in the binding experiment, as shown in Figure 8b. The data clearly illustrate that maltotriose binds to the protein and can readily be detected as a hit in the HAMS assay. Similar to the previous assays, not one false positive hit was detected. Every compound that appeared in the control experiment was also detected in the binding experiment, except the known inhibitor. These data show that it is fully feasible to screen for inhibitors with K_i approaching = 0.2 µM using the HAMS assay. In this case, less than 7 pmol of protein was needed per compound screened.

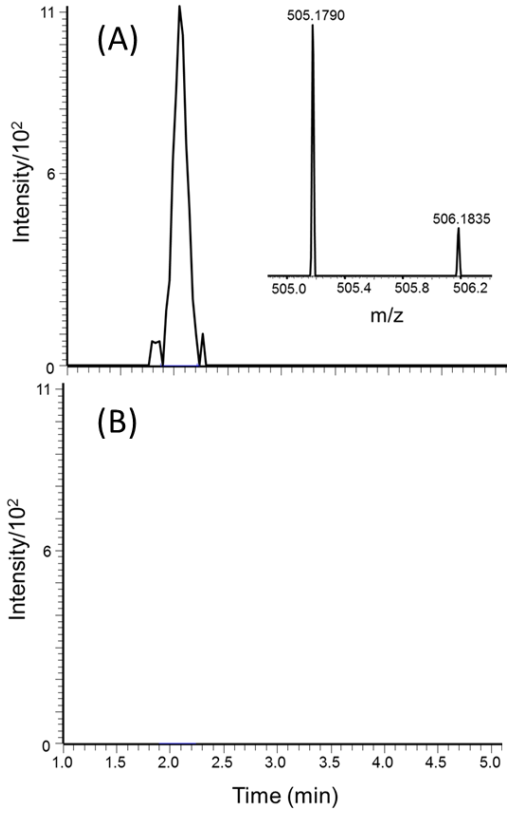

Figure 8: Selected ion chromatograms of known maltose binding protein (MBP) inhibitor: maltotriose, in (A) control and in the presence of (B) 100 µg of MBP in the binding experiments. 352 library compounds and known inhibitor (each with 100 nM concentration) were used in all experiments.

2.3.6 Comparison to alternative testing methods.

One major benefit of the described method is its consistent ability to identify only the strongest inhibitors, substantially eliminating false positive interactions. Other MS-based screening methods have not yet been capable of identifying only the strongest inhibitor present. With this in mind, we asked the question: Did we just get lucky and test a bunch of compounds that would not show up as false positives in other assays either? To answer the question, the library of 352 compounds was used to determine how many false positives would have been identified as Carbonic Anhydrase binders in the ASMS method, a state-of-the-art screening approach.

To screen the compounds using the ASMS method, the 352 potential binders (used in Figure 5) were incubated with CA; the non-binders were removed, and the protein was washed using stringent conditions, as described in the experimental section. The protein was deactivated, and released compounds were analyzed using LC-MS, following the procedure described in reference 9. Using this approach, one could still detect the presence of the strong inhibitor. However, in addition to this compound, 30 other non-inhibiting compounds were also detected. (See Table 1.) This experiment emphasizes the advantage of the newly described protocol over existing methods. Even though the protein was washed thoroughly prior to releasing the bound compounds, almost 10% of the ligands were detected as potential binders. Thus, the traditional high throughput screening approach may leave the investigators with numerous compounds for further investigation, when in fact, only one of them would be desirable to detect in this case, the known inhibitor with K_i in the pM range.

Table 1: The list of compounds that were detected as false positives in the capture and release experiment.

Name	m/z	Found	ppm	RT
Astemizole	459.2555	459.2545	2	29.6
Tamoxifen citrate	372.2322	372.2313	2	46.7
Miconazole	414.9933	414.9927	2	44.33/45.65
Clemizole hydrochloride	326.1419	326.1415	1	35.03
Isoconazole	414.9933	414.9927	2	44.33/45.65
Oxybutynin chloride	358.2377	358.2367	3	38.44
Dicyclomine hydrochloride	310.2741	310.2736	1	42.57
Oxethazaine	468.3221	468.3218	1	44.89
Thioridazine hydrochloride	371.161	371.1599	3	41.99
Vinpocetine	351.2067	351.2065	1	35.03
Cinnarizine	369.2325	369.2319	2	41.51
Pimozide	462.2351	462.2343	2	40.01
Mianserine hydrochloride	265.1699	265.1694	2	32.01
Clomipramine hydrochloride	315.1623	315.1617	2	40.42
Tolnaftate	308.1104	308.1097	2	49.33
Fendiline hydrochloride	316.206	316.2055	2	39.73
Benfluorex hydrochloride	352.1519	352.1513	2	37.58
Clomiphene citrate (Z,E)	406.1932	406.1928	1	45.64
Meclozine dihydrochloride	391.1936	391.1929	2	43.86
Chlorprothixene hydrochloride	316.0921	316.0916	2	40.4
Bepridil hydrochloride	367.2744	367.2736	2	44.1
Dequalinium dichloride	228.1621	228.1625	2	35.78
Doxorubicin hydrochloride	544.1813	544.1818	1	35.66
Propafenone hydrochloride	342.2064	342.2056	2	36.87
Amiodarone hydrochloride	646.031	646.0304	1	48.8
Prenylamine lactate	330.2216	330.2205	3	40.92
Sertaconazole nitrate	437.0043	437.0041	1	45.67
Naftifine hydrochloride	288.1747	288.1743	1	36.48
Sulfadoxine	311.0809	311.0802	2	21.71
Saquinavir mesylate	671.3915	671.3911	1	43.1

Even though the ASMS method suffers from a large number of false positives, it is perhaps best described as complementary to the HAMS method, instead of inferior to it, when all metrics are considered. The ASMS method's two key advantages are its rapid throughput and simple data analysis. The throughput of the HAMS method is about 5,000 compounds per day, whereas the ASMS method can process more than twice as many compounds in the same timeframe. Additionally, the ASMS method, which relies on detecting inhibitors based on the presence of their MS signal, has a simpler data analysis workflow compared to HAMS, which relies on disappearance of the analyte's signal. Therefore, these two methods are quite complementary. A method that could deliver the speed and simplicity of ASMS, along with the complete absence of false positives, like HAMS, would be a remarkable advancement in the field.

2.3.7 Investigation of the undetected compounds

In all the large screening assays we conducted, ~88% of compounds were observed for both the control and binding experiments. In other words, ~12% of the compounds were not detected in the control experiment and therefore, not assayable. They were not false positives or false negatives. They were simply not testable compounds. If these compounds were not detectable due to the assay conditions or due to matrix effects, that would represent a potential weakness that would need to be considered prior to selecting this assay for a large screen. Therefore, we examined the reasons behind their absence in the data sets.

The undetectable compounds from the Carbonic Anhydrase screen were tested individually, to determine if they were undetected due to poor ionization efficiency, or due

to the assay, or due to matrix effects. Each compound was prepared individually and subjected to direct infusion experiments, but none of the compounds were detected in the positive ion mode during these experiments, indicating that all the compounds that possibly can be observed using positive mode ESI were successfully detected in the Carbonic Anhydrase screen. The undetected compounds also were individually tested in negative ion mode, and only 17 were detected. These compounds could potentially be detected if the LC-MS analyses were to be repeated in negative ion mode; however the value of that experiment would have to be balanced against the analysis time required to perform it. In sum, 7% of the compounds did not ionize at all by ESI. Those compounds were, therefore, not detected in the screen. Of the remaining 93% that were ionizable by ESI, 312 out of 329 were detected in one chromatographic run, using only positive ion mode, with no gradient optimization performed. Overall, these experiments demonstrate that the number of detectable compounds is not limited by the assay conditions or matrix effects; rather, it is directly related to the ability of the compounds to ionize by ESI-MS. Every MS-based assay suffers from the fact that it cannot detect compounds that don't ionize. The HAMS assay is unique in that it can identify these compounds as "untestable", and they are not mistakenly characterized as false positives or false negatives.

2.4 Conclusion

Inhibitors were quickly identified for three different proteins by comparing the mass spectra of the library molecules before and after incubation with immobilized protein. In every experiment conducted, all the compounds that were observed in the control experiment were also observed in the binding experiments, except the known inhibitors. This observation indicates that the method described herein is unprecedented for MS-

based assays in that the false positive detection rates are exceedingly low, approaching zero. This method was used to selectively detect inhibitors that have K_i values of 50 pM (with Pepsin), 700 pM (with Carbonic Anhydrase), and the minimum amount of protein needed for these assays was 0.5 and 6 pmol per compound, respectively, was used for screening. These results indicate that one can control the affinity of the ligands detected by controlling the amount of protein used in the assay, a feature which has not been demonstrated by other MS-based screening assays. By using magnetic beads for protein immobilization, inhibitors with Ki's in the micromolar range could also be assayed, using just 7 pmol of protein per compound screened. Finally, the method compares very favorably to the state-of-the-art MS-based assay (ASMS),[9] where the false-positive identification rate was nearly 10% for the ligands assayed herein.

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Chapter 3: A Rapid LC-MS Based High-Throughput Screening Method That Affords No False Positives or False Negatives and Identifies a New Nanomolar Affinity Inhibitor for the Therapeutically Valuable Protein, Carbonic Anhydrase

Developing effective high-throughput screening (HTS) methods is of paramount importance in the early stage of drug discovery. While rugged and robust assays may be easily developed for certain enzymes, HTS assays designed to identify ligands that block a protein binding site are much more challenging to develop. In these protein binding assays, ruling out false positives and false negatives under high-throughput conditions is particularly challenging. To overcome these challenges, we developed an MS-based HTS assay that has several major advantages over existing methods. The assay mitigates false positives by selectively identifying positive hits exclusively when a ligand at the binding site of interest is displaced; it mitigates false negatives by detecting a reporter compound that ionizes well, not by detecting the ligand binder, which may not ionize. This interrogation approach allows non-ionizable compounds to be assayed. The screening is fast (400 compounds in 10 minutes) and requires low (nanogram) amounts of target protein per compound. It can detect binders with K_d's in the picomolar to micromolar range. The method was validated by detecting known binders of three proteins, pepsin, Maltose Binding Protein (MBP), and Carbodic Anhydrase (CA) in the presence of hundreds of non-binders. We also identified a novel CA binder, pifithrin-μ, which could not have been identified by any other MS-based assay because of its poor ionization efficiency. This new method addresses many of the challenges that are currently encountered during high-throughput screening.

3.1 Introduction:

Identifying potent and high affinity ligands for target proteins is a vital first step in drug development. Generally, these molecules are identified during high-throughput screening campaigns, and the most commonly used analysis methods are florescence,[1,2] chemiluminescence,[3] and surface plasmon resonance (SPR).[4] All of these methods suffer from the same limitation: modification of the analytes or the protein is typically necessary for detection. Thus, assay development can be laborious, and the molecular labels themselves can alter the integrity of the binding between ligands and the target protein, leading to false positives and false negatives. Although nuclear magnetic resonance (NMR) is an alternative label free spectroscopy technique,[5,6] it requires substantially more protein, and data analysis is laborious, resulting in very low throughput. As a result of the continued need for fast, label-free detection methods, mass spectrometry (MS) based screening techniques are becoming more common. The key advantages of an MS-based method are the label free nature, high sensitivity, and the ability to differentiate ligands based on the analytes' masses.

Various MS-based techniques have been developed over the past few decades, and these methods can be subdivided into those approaches that either do or do not require a chromatographic separation step as part of the analysis. The most commonly used MS-based methods without chromatography rely on MALDI-TOF-MS.[7,8,9] This MS platform is fast and sensitive, but matrix interferences and poor reproducibility are some shortcomings that can contribute to false positive and false negative identifications.[10,11] Common MS-based HTS methods that incorporate a separation step allow for more compounds to be interrogated at one time; example separation platforms include size-

exclusion chromatography coupled with reverse phase chromatography-MS (SEC-RPC-MS),[12] ultrafiltration-MS,[13] gel filtration-MS,[14] frontal affinity chromatography-MS,[15,16] and affinity capillary electrophoresis-MS.[17] In these methods, binders are generally identified either by direct detection of the protein-ligand complex,[12] or by detection of bound compounds after dissociation of the protein-ligand complex,[14-16,18] or by detection of unbound compounds compared with a control.[18] The most common limitations to these approaches are false positives, due to non-specific binding, false negatives, due to the presence of non-ionizable compounds, the need for large amounts of target protein, and insufficient throughput.[12,14,19,20]

The most commonly used MS-based HTS method is the affinity selection–MS screening method (ASMS). This approach currently has the best balance of strengths and limitations, and it can be used for screening over 1×10^5 compounds per day.[21,22,23,24] While this level of throughput is a clear advantage, false positives, which are due to nonspecific binding of small molecules, introduce a significant disadvantage.[12,14,19] As such, the hits from this type of screen need to be validated with an alternative screening technique in order to completely rule out possible false positives. Additionally, the method is not able to detect hits that do not ionize, so false negatives are also a concern.

Recently, we reported a novel MS-based HTS method, High-Affinity Mass Spectrometry screening (HAMS), which uniquely evades the detection of false positive hits.[25] However, the method requires acquisition of two LC-MS datasets per set of 350 compounds, and this requirement limits throughput. Therefore, further development in MS-based high-throughput screening is needed to overcome the field's current limitations.

Herein, a new approach is developed that fills the existing technology gaps in high-throughput screening. This method avoids false positives and false negatives; it can be used to screen over 10,000 compounds per day, while consuming limited protein quantities. Target proteins are incubated with a known ionizable weak binder (reporter molecule), and the complex is then introduced to a batch of library compounds, while an equimolar amount of the complex, without the library compounds, is used as a control sample. LC-MS is used to detect the reporter molecule. If a stronger binder is present in the library, the signal of the reporter molecule increases compared to the molecule's signal in the control samples. In this way, a binding event is measurable, even if the strong-binding ligand is not detectable by mass spectrometry; in other MS-based assays, non-ionizing compounds result in false negatives. In addition, the assay has very modest protein requirements, consuming ng of protein per compound analyzed. . Finally, the analysis time for the method described herein is a quick 10 minutes per batch of 300-400 compounds, which extrapolates to well over 10,000 compounds per day. Overall, this MS-based HTS method meets the current needs of the drug discovery field better than any existing MS-based method. Furthermore, the assay's value is demonstrated by identifying a new inhibitor for Carbonic Anhydrase, a therapeutically valuable protein.

3.2 Methods:

3.2.1 Reagents

FDA approved drug compounds (1280 compounds) were purchased from Sigma Aldrich (St. Louis, MO). The library was provided as a single compound per vial (LOPAC1280-small scale), dissolved in DMSO at a concentration of 10 mM. Pepsin,

Aminolink Plus coupling resin, and disposable plastic columns were obtained from Pierce Biotechnology, Inc. (Rockford, IL). Pierce™ NHS-Activated Magnetic Beads were purchased from Thermo Scientific (Workford, IL). Maltose Binding Protein and Carbonic Anhydrase were purchased from My BioSource (San Diego, CA) and Sigma Aldrich (Milwaukee, WI), respectively. Nitrocellulose drop dialysis membranes were purchased from Fisher Scientific (Houston, TX).

3.2.2 Protein immobilization onto Aminolink Plus coupling resin

Immobilizations of Carbonic Anhydrase (CA) and pepsin were carried out by adjusting a previously published procedure.[56] Each protein was maintained at its optimal pH. Therefore, for CA the coupling buffer was phosphate buffered saline (PBS) (0.1 M, pH 7.4), the blocking buffer was Tris HCl (1.0 M, pH 7.4), the incubation buffer was ammonium acetate (0.02 M, pH 7.4), and the wash buffer was 1 M NaCl in coupling buffer. Similarly, for pepsin, citric acid and $NaHPO_4$ (0.1 M, pH 4.5) were used as the coupling buffer; the blocking buffer was Tris HCl (1.0 M, pH 4.5), the incubation buffer was ammonium acetate (0.02 M, pH 4.0), and the wash buffer was 1 M NaCl in coupling buffer. All of the pepsin assays were carried out through immobilization of 1 or 2 µg of pepsin with 25 µL of resin beads. Similarly, 10 µg of carbonic anhydrase was coupled to 25 µL of resin beads.

Both CA and pepsin were dissolved in 100 µL of coupling buffer before drop dialysis was conducted. Dialysis of the dissolved proteins with the coupling buffer was performed over 1 h using a 0.025 mm nitrocellulose drop dialysis membrane. Subsequently, the protein was added to a disposable plastic column that was filled with

coupling resin which had been washed with 2 mL of coupling buffer. Then, a solution of 1 M $NaCNBH_4$, in coupling buffer, was added to the resin solution until a final concentration of 50 mM $NaCNBH_4$ was reached. The column and resin solution was rocked overnight, then washed with 10 mL of coupling buffer, followed by 5 mL of blocking buffer. Next, 1mL of blocking buffer was added, and 1M $NaCNBH_4$ was added again until a final concentration of 50 mM $NaCNBH_4$ was reached. The mixture was rocked for 2 h, and the column was washed with 10 mL of coupling buffer, 10 mL of wash buffer, and 15 mL of incubation buffer, in that order. Finally, the immobilized protein was transferred to Eppendorf tubes for incubation with the library compounds immediately.

3.2.3 Preparation of library compounds for binding experiment

To prepare the 100-compound libraries, 2 µL of each compound's stock solution (10 mM) was combined into 100 compound batches at a final concentration of 100 µM. Then, 2 µL of the 100 µM batches (100 compounds each) was diluted with incubation buffer, specific to each protein, to obtain a 25 µM stock solution. When preparing the library of 400 compounds, 2 µL solutions from each of the four libraries of 100 compounds were combined, achieving a final concentration of 25 µM. Then, for both libraries of 100 or 400 compounds, 2 µL of the 25 µM library was diluted to 337.5 nM using the incubation buffer, specific to each protein.

3.2.4 Binding experiment using proteins immobilized on Aminolink coupling resin.

Immobilized pepsin and CA were incubated with 200 µL of 300 nM known weak binders, pepsinothipsongen or methoxzolamide respectively. After incubation, tubes were centrifuged at 3000 × g for 5 minutes; the supernatant was removed, and the immobilized

proteins were washed twice with 500 µL of incubation buffer. Next, 200 µL of the 100 or 400 compound library mixture was added to 25 µL of immobilized protein mixture, bringing the final concentration of the library compounds down to 300 nM. The mixture was rocked for 1 h at room temperature, then centrifuged at 3000 × g for 5 minutes; the supernatant was removed and used directly for LC-MS analysis.

3.2.5 Binding experiment for low-affinity binders.

Maltose binding protein (MBP) was immobilized on N-hydroxysuccinimide (NHS)-activated magnetic beads by following the manufacturer's protocol. Briefly, drop dialysis was conducted on MBP in PBS buffer (0.1 M, pH 7.4). Then, 100 µL of magnetic beads were washed according to the manufacturer's instructions and 20 µg of MBP in coupling buffer (PBS, 0.1 M, pH 7.4) was added. The mixture was slowly rocked overnight at room temperature. Subsequently, the supernatant was removed, and the immobilized proteins were washed with 1 mL of coupling buffer. Then, 300 µL of quenching buffer (Tris HCl, 1.0 M, pH 7.4) was added and the mixture was rocked for 2 hours. The supernatant was removed, and immobilized proteins were washed with 300 µL of washing buffer (1 M NaCl in coupling buffer) followed by 2 mL of ammonium acetate (0.02 M, pH 8.0) incubation buffer. The supernatant was removed again and 50 µL of 50 nM maltose, a known weak binder, was added. The mixture was rocked for an additional two hours at room temperature. After removing the supernatant, 50 µL of the 400 compound library (300 nM) was added, and the mixture was rocked again for one hour. After incubation, the supernatant was removed and used directly in the LC-MS analysis.

3.2.6 Positive and Negative Controls

Positive and negative control samples were prepared, as explained in the *binding experiments* section above, for high and low affinity ligands, except libraries with a known strong binder were used as positive control samples. For each positive control, 401 library compounds were used. Negative control samples contained either buffer only or 400 non-binding compounds. Supernatants for both controls were removed and subjected to LC-MS analysis.

3.2.7 LC/MS Analysis

An Acquity UPLC system (Waters Corporation, Milford, MA) coupled to an Orbitrap Velos Pro Mass Spectrometer was used for liquid chromatography/mass spectrometry analysis. Mobile phase A was 99.9% water with 0.1% formic acid, and Mobile Phase B was 99.9% MeOH with 0.1% formic acid. Five microliters of the supernatant was injected onto a C_{18} Hypersil Gold column (Particle Size: 5 µm; 1 mm *i.d* X 100 mm, 175 Å, Thermo Electron Corporation, Thermo Fisher Scientific, Pittsburgh, PA) at a flow rate of 50 µL/min. The multi-step gradient used for assays, excluding any analyses for MBP, was as follows: 100% solvent A for 1 min, then a linear increase of B to 80% in 4 min, followed by the linear increase of B to 95% in the next 3 min; then B was maintained at 95% for an additional 30 seconds, finally a linear decrease of B to 0% in the next 30 seconds, where the solvent composition was maintained for another 2 min. The following multi-step gradient was used for MBP assays: 100% solvent A for 3 min, then a linear increase of B to 80% in 2 min, followed by the linear increase of B to 95% in the next 3 min, then B was maintained at 95% for an additional 30 seconds, finally, a linear decrease of B to 0 % in

next 30 seconds, where the column was maintained for another 2 min. The eluent was diverted to waste for 7 minutes at the beginning of each run, except for the experiments conducted with MBP, where the divert time was reduced to 1 min. The mass spectrometer was operated in the positive ion mode with a 3 kV potential on the ESI needle, and the capillary temperature was set at 250 °C. Full scan MS data were obtained at a mass range of *m/z* 200-700 using the Orbitrap mass analyzer at a resolution of 30,000 for *m/z* 400.

All peak areas were calculated using the extracted ion chromatograms from the .raw data files for each compound. The extracted ion chromatograms were generated for the monoisotopic peaks of protonated or/and sodiated adducts for every reporter molecule as follows: pepsinostreptin (*m/z* 672.4548), methoxzolamide (*m/z* 237.0110), maltose (*m/z* 343.1235, 365.1054), and chlorothiazide (*m/z* 295.9561). The peak area for each extracted ion chromatogram was calculated using the software (Xcaliber) supported algorithm, Genesis.

3.2.8 Calculation of IC50 of pifithrin-μ

IC50 was determined by plotting the % inhibition versus the log of the concentration of the inhibitor, adapting a well-established method.[26] Experiments were repeated in triplicate. For each trial, 100 µL of 0.125 mg/mL carbonic anhydrase (4.3 µM) in Tris buffer (pH 8.4) was used. First, the protein solution was mixed with 9.9 mL Tris buffer containing different concentrations of inhibitor. The inhibitor concentrations were 0 nM, 1 nM, 5 nM, 10 nM, 20 nM, 40 nM, 60 nM, 100 nM, 130 nM, 200 nM, 350 nM, 500 nM, 1 µM, 2 µM, 4 µM, 6 µM, 8 µM, and 10 µM. These protein-inhibitor mixtures were incubated for 1 hour on a rocking platform at 4° C (on ice). After incubation, 4 mL of

carbonated Tris buffer, which was pre-equilibrated at 4^{o} C (on ice), was added with stirring. The final concentration of the protein in the reaction mixture was 0.03 µM. Simultaneously, the pH of the solution was recorded over time. The time required for the pH of the solution to change from 8.4 to 6.4 was recorded in order to calculate the % inhibition. The entire reaction was carried out at 4^{o}C (on ice) with stirring. The % inhibition was calculated using the following equation: % inhibition = [(normal activity – inhibited activity)] / (normal activity)].

3.3 Result and discussion:

3.3.1 Method Overview

Figure 1 describes the workflow for the assay developed herein. Immobilized protein of interest is first incubated with a compound known to bind to the target protein; hereafter, this compound is referred to as “the weaker binder” or “reporter molecule”, although its binding affinity can be in the micromolar to picomolar range. After incubation, excess weaker binder is removed with the incubation buffer, and the immobilized protein / weaker binder complex is incubated with the library compounds of interest; typically batches of 400 compounds are tested. If the library contains a stronger binder, the high affinity binder competes for the same binding site in the protein with the known weaker binder. The weaker binder is, therefore, displaced from the complex. Hence, the impact of a stronger binding compound being present is that the concentration of the weaker binder in the supernatant increases compared to that of the negative control, when no library compounds are added. Although no strong binders are present in the negative control, a certain degree of dissociation of the weaker binder from the protein occurs, depending on the concentrations of the protein and ligand and the strength of the binding interaction.

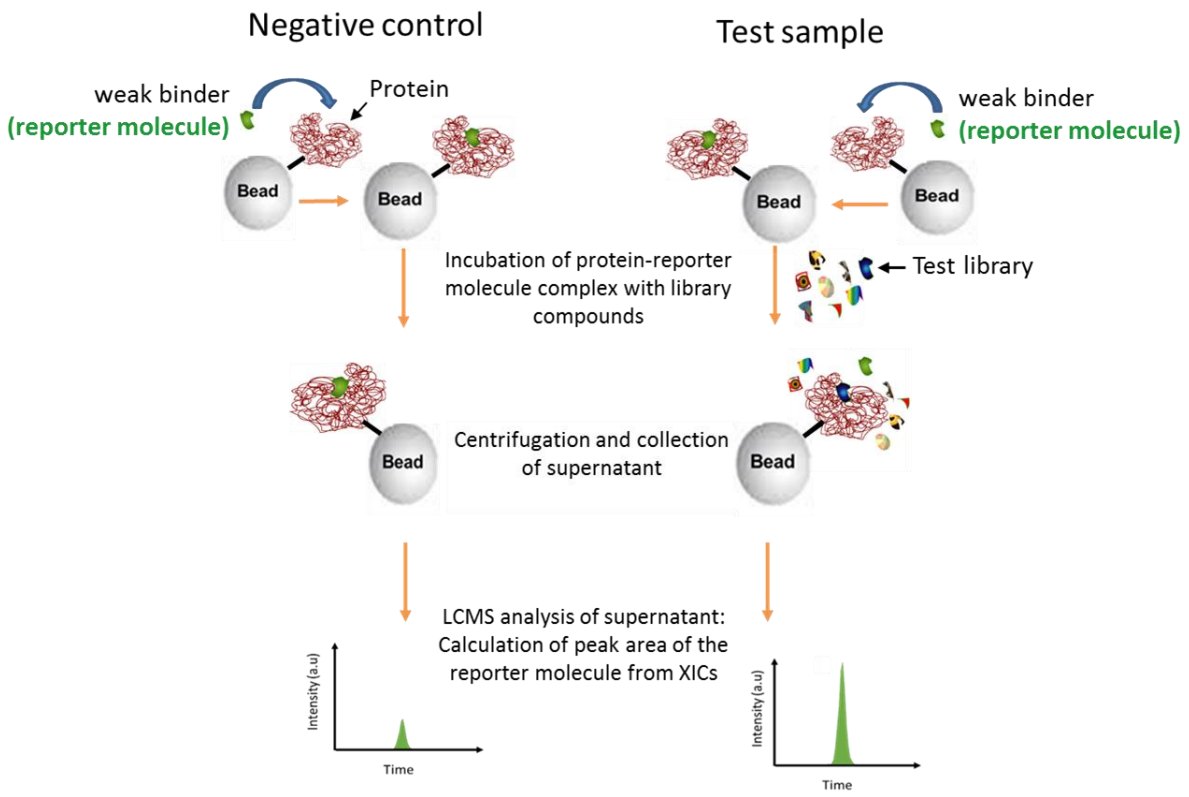

Figure 1: Experimental workflow. The weaker binder (reporter molecule) is incubated with the immobilized protein, and the excess reporter molecule was washed off. The library compounds are added to the test sample. During validation experiments, a known strong binder is spiked into the library, generating a positive control sample. When present, a strong binder replaces the reporter molecule on the binding site of the protein, and the concentration of the reporter molecule increases in the supernatant relative to the negative control. Supernatants for both the negative control and the test samples are analyzed for the presence of reporter molecule, and the peak area of the reporter molecule is used to calculate the Z' factor during validation experiments.

To demonstrate that this workflow provides sufficient discriminatory power between ligand sets that do or do not have a strong binder present, several control experiments were completed on different protein/ligand systems. During these control experiments, a stronger binder is always included in the positive control. Three model proteins (pepsin, Carbonic Anhydrase (CA), and Maltose Binding Protein (MBP)), their known weaker binders, and their known strong binders were used in three separate proof-of-concept experiments. In each case, the peak area of the weaker binder in the positive

control was compared to the signal for the weaker binder in the negative control. These peak areas were used to calculate a Z' factor, which is a statistical measure used to determine the quality of an HTS assay.[27] If the Z' factor is between 0.5 and 1.0, the assay is considered excellent, whereas if the calculated Z' factor is <0.5 and > 0, the method is not considered to be very effective, by most standards.[27] Each experiment is discussed in detail next, starting with the proteins that have known tight-binding inhibitors.

3.3.2 Identification of high affinity binders (pM to nM range)

The assay was first validated using proteins that have known high-affinity ligands. Immobilized pepsin and CA were incubated with their known binding ligands (reporter molecules), pepsinostreptin and methoxzolamide, respectively. For negative controls, the protein/ligand complexes were each incubated with a library of 400 compounds that did not contain any strong binders, while the validation samples (positive controls) were prepared by incubating the protein-reporter molecule complexes with the same library as the negative control and also the protein's known stronger binder. One strong binder (pepstatin A, K_d = 50 pM) was used for pepsin, while two strong binders (Ethoxzolamide, K_d = 750 pM, and Brinzolamide K_d = 3 nM.), were used in two separate experiments for CA. After incubation with the library compounds, the negative controls and the positive controls were each centrifuged, and the supernatants were analyzed using LC-MS. In each case, detection and quantification of the reporter molecule, the weaker binder, was achieved in a 10 minute chromatographic run. Five replicates were completed for each protein.

Figure 2 shows the results of these experiments. For both proteins, a significant increase of the reporter molecules was detected in the supernatant of the positive controls for all five trials compared to the five negative controls. This result indicates that the strong binders in the positive controls displaced the weaker binders in the active sites of the proteins, leading to an increase of the weaker binders in the supernatant of the positive controls. The peak area of pepsin's weaker binder is approximately four times larger in the positive controls than the negative controls (Figure 2a). The peak areas for the weaker binder in both the positive and negative controls are reproducible; and the calculated Z' factor is > 0.6, indicating that the assay is appropriate for high throughput screening of strong binders in the pM range. Figure 2b shows similar results for the two different CA binding experiments. The Z' factors for both experiments are also greater than 0.6, falling into the "excellent" category for HTS assays of strong binders in the nM range. In addition, Figure 2b also shows slight differences in the peak areas of the reporter molecule for the two different strong binders for CA. The variation in peak areas in these two experiments is expected due to the difference between the dissociation constants K_ds of the two binders. These trials required only 1 µg (~30 pmol) of pepsin and 10 µg (~310 pmol) of CA to screen 400 compounds in each library. While the CA assay, which profiled a lower affinity ligand, required more protein, this protein quantity is still significantly lower than the commonly used amount for the screening batches of 400 compounds against CA.[24]

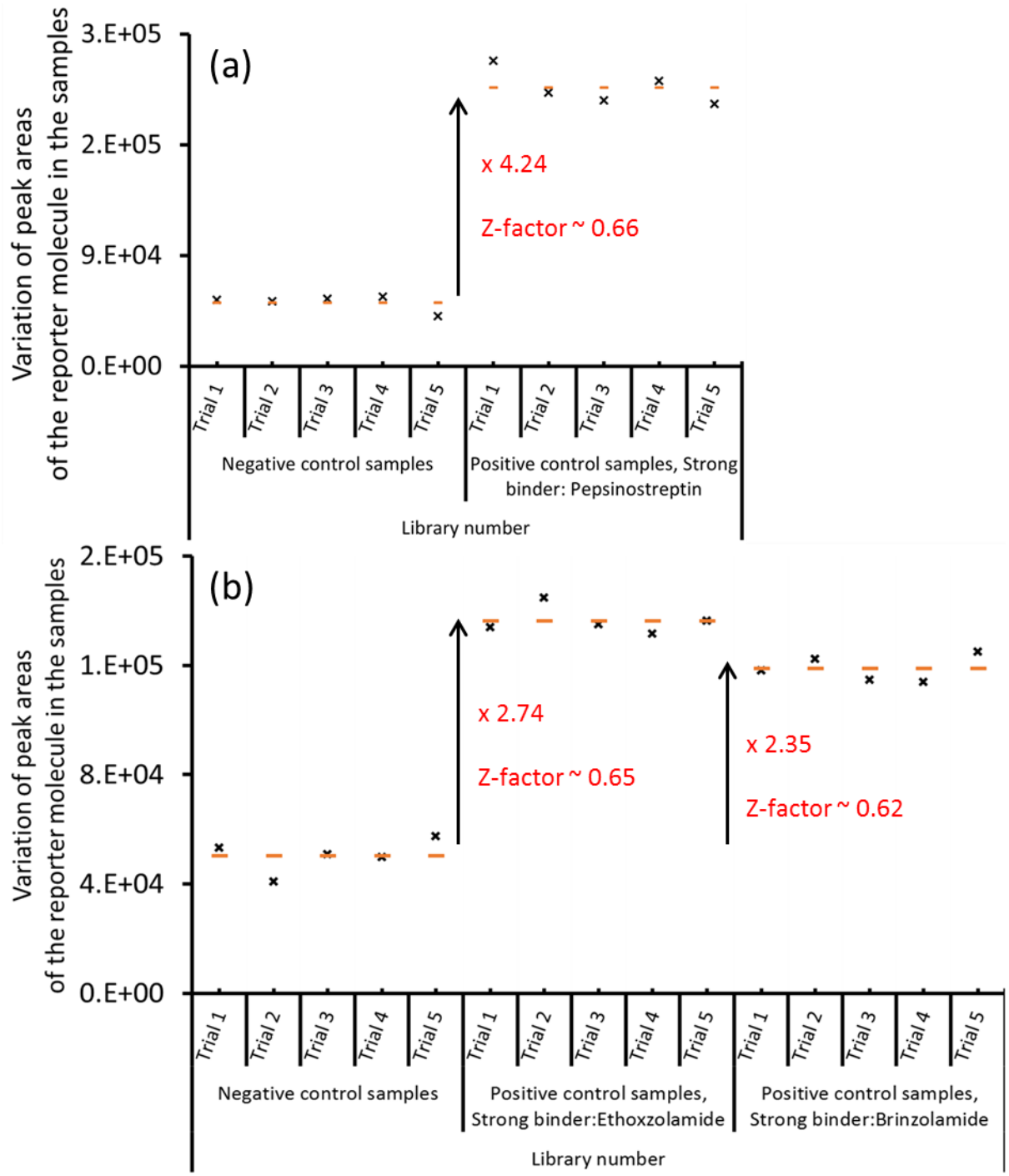

Figure 2: Results of the validation experiments for (A) pepsin and (B) carbonic anhydrase. The calculated Z' factors indicate that the assays have appropriate discriminatory power for a high-throughput screen.

3.3.3 Identification of transient binders (µM range)

After successfully applying the assay to screen for tight-binding ligands, we tested whether the same procedure could be used to identify lower-affinity ligands. In these experiments, maltose binding protein (MBP) was the target; maltose (K_d of ~3 µM) was used as the weaker binder (reporter molecule), while maltotriose (K_d =0.2 µM) was the stronger binder. As before, the stronger binder was spiked into the positive controls, but not the negative controls. No significant difference in the signal of the reporter ion in the positive and negative controls was detectable in these experiments. In considering the potential reasons for the experiment's failure, we suspected that the centrifugation step, which is necessary to separate the immobilized protein from the supernatant, was causing the weakly bound reporter molecule to dissociate from the complex to a significant degree, even when a stronger binder was not present.

To address this problem, magnetic beads were used to immobilize the protein, instead of agarose beads. Since magnetic beads were used, no centrifugation was done, thereby minimizing the dissociation of reporter molecules from immobilized protein due to the centrifugal force.

After making the aforementioned changes, MBP was immobilized on magnetic beads followed by incubation (loading) with the reporter molecule, maltose. Subsequently, the supernatant was removed without centrifugation and the MBP-maltose complex was incubated with library compounds. A library of 400 compounds was used for the negative control, for the positive control, maltoriose, the stronger binder, was spiked into the same library. Finally, the protein was sequestered from the supernatant without centrifugation, and the supernatant was then analyzed for the reporter molecule

using LC-MS. Five trials were conducted for each control. Figure 3 shows the results of the experiments. The increase in the amount of reporter molecules in the supernatant of the positive controls compared to negative controls indicates the experiment was a success. The calculated Z' factor for the experiments was 0.53, showing that the assay can be applied for high-throughput screening of low-affinity binders.

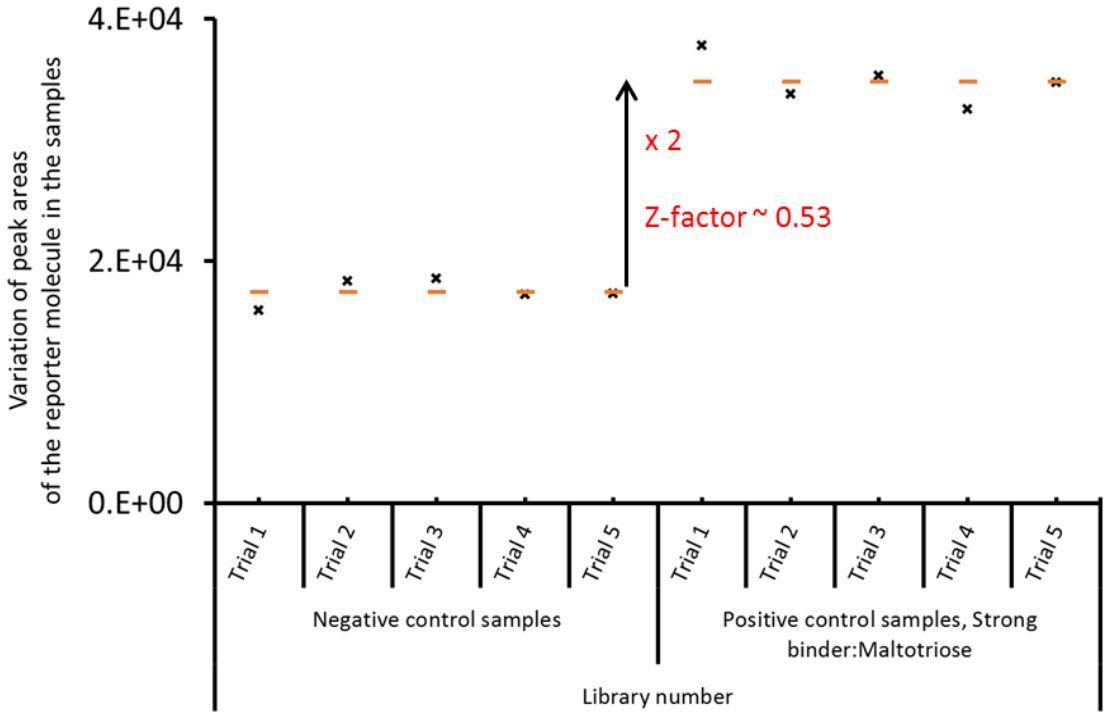

Figure 3: Results of the validation experiment for maltose binding protein. The calculated Z' factor indicates that the assay has appropriate discriminatory power for a high-throughput screen.

Since our assay demonstrated the capability of use in HTS even for transient binders, we validated the assay by using a library of 1200 compounds (same library used in high affinity binders screening) to verify if the known spiked binder could be readily identified. Three sets of 400 compounds each were prepared, and one set was spiked with a known MBP binder, maltotriose (K_d = 0.2 µM). Library B (Figure 4)

contains the compound set with the known binder. The known strong binder was effective at out-competing the reporter molecule (maltose, K_d = 3 µM) that had initially been pre-loaded into the MBP binding site. Figure 4B shows that in three replicate trials, the concentration of the reporter molecule was almost three times that of the concentration of the control sample, where no library compounds competed for binding. The reporter molecule was readily detectable in the supernatant, signaling that a compound in the library was out-competing it for MBP binding. Figure 4A and B also show results for the two other sets of 400 compounds, which have a value close to one. These values, shown in the Y axis, represent a ratio comparing the signal of the reporter ion in the presence and absence of the library compounds. It is clear that only Library B had a strong binder, suggesting the capability of screening enhanced libraries for transient binders was feasible without any false identification. The amount of protein needed was ~ 20 µg to screen for K_d = µM binders in a 400-compound mixture. The K_d was 1000 times larger for the transient binder compared to the K_d of the high affinity binders. However, we were able to develop this assay using only twice the amount of protein that was used for the high affinity assay.

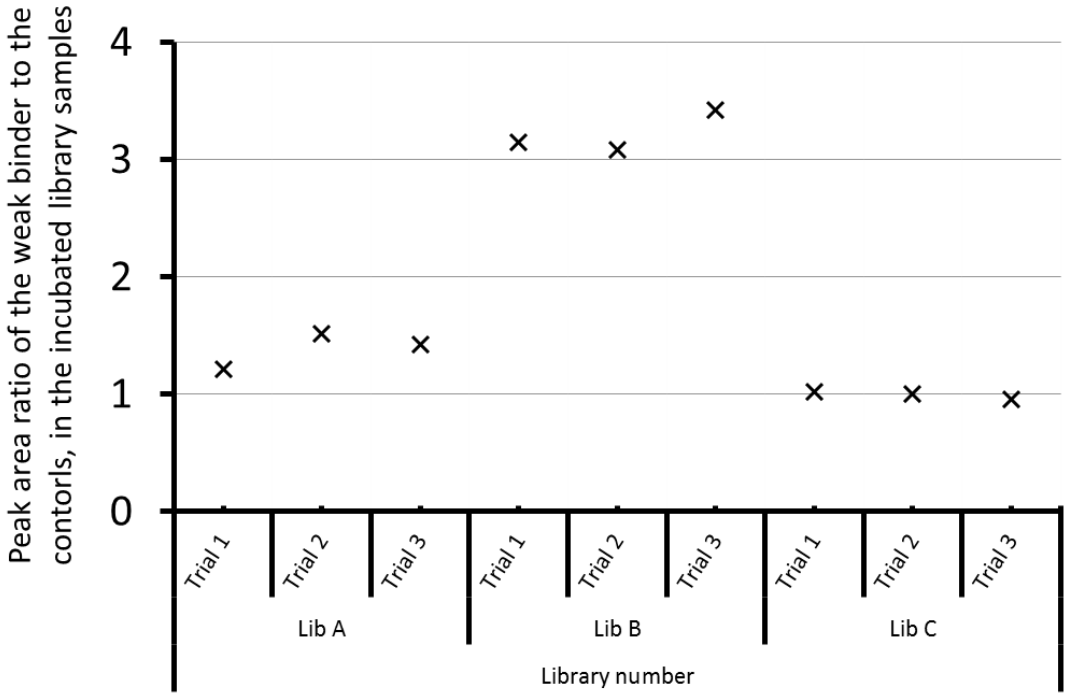

Figure 4: Peak area ratio of the weak binder, maltose, to the controls among all the 400 compound libraries tested. The ratios for Library A and C are close to 1, indicating the absence of possible strong binders. Library B, where the strong binder, maltoriose, was spiked clearly showed a larger peak area**.**

3.3.4 Addressing false negatives

A significant limitation of current MS-based HTS methods is the high rate of false negative identifications due to non-ionizable compounds not being detected in MS-based assays. We carried out an experiment to demonstrate that the method described herein overcomes this limitation. The experiment was conducted using carbonic anhydrase (CA) as the model protein, chlorothiazide as the reporter molecule, and acetazolamide, which is a non-ionizable molecule, as the strong binder. Figure 5 shows a pictoral description of the experiment along with the resulting data. As shown in Figure 5A, acetazolimide is not detectable by ESI-MS when it is analyzed directly. Figure 5B shows the signal for the

reporter molecule, chlorothiazide, when no strong binders are present in the assay; this is the negative control. When acetazolamide is incubated with a CA-chlorothiazide complex, however, a significant increase of the peak area of the reporter molecule, chlorothiazide, is detectable. This increase in signal can be seen by comparing the data in Figure 5B, the negative control, to the data in Figure 5C, when acetazolimide is present. The increase in concentration of the reporter molecule, chlorothiazide, in the supernatant is due to the competitive binding of the non-ionizable strong binder, acetazolamide, to carbonic anhydrase. Hence, the assay can identify the presence of strong binders from a pool of library compounds, irrespective of whether the compounds ionize well or not.

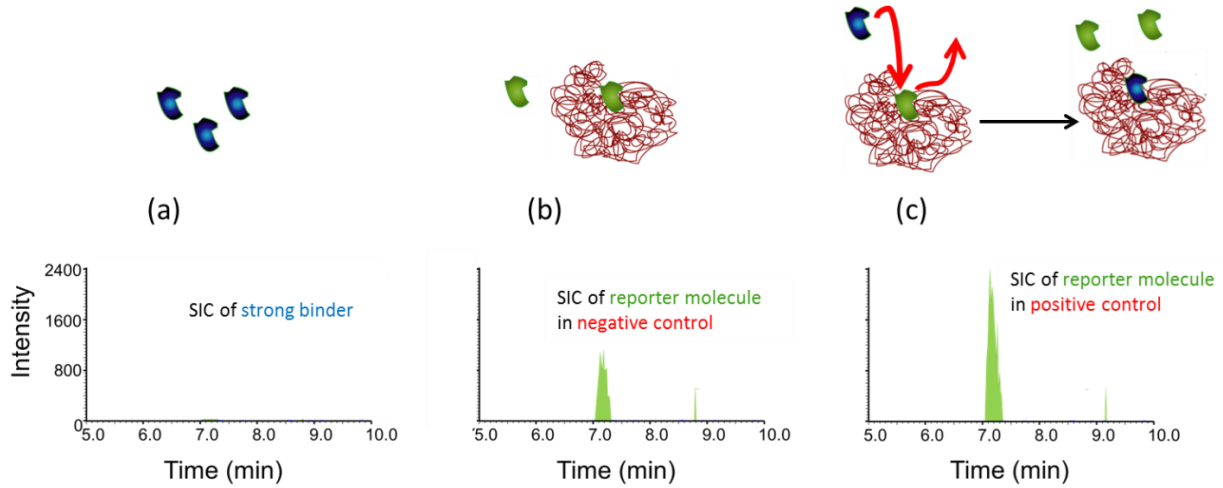

Figure 5: (a) Selected ion chromatogram (SIC) of non-ionizing strong binder, acetazolamide. (b) SIC of the weak-binding reporter molecule, chlorothiazide, in the supernatant of the negative control. (c) SIC of the reporter molecule in the supernatant after the strong binder, acetazoiamid, had been added. This experiment demonstrates that the assay can detect binders that do not ionize. In other MS-based assays, these compounds are either un-assayable or they are false negatives.

3.3.5 Screening for new inhibitors

Because the new assay had demonstrated strong performance metrics during method development, we next tested a well-known target protein against a moderate sized library of 1200 compounds, in order to both verify that a known inhibitor could be readily identified and also to pan for unknown inhibitors. A known CA binder, ethoxzolamide, was spiked into one set of four hundred compounds, and two other compound sets, each containing 400 compounds, were also prepared. These three batches were used to screen for CA binders. The compound set with the known inhibitor, henceforth referred to as Library B, was effective at out-competing the weak inhibitor that had initially been pre-loaded into the CA binding site. The weaker binder was readily detectable in the assay supernatant, signaling that a compound in the library was out-competing it for CA binding. Figure 6A shows that in three replicate trials, the concentration of the weaker binder was almost three times that of the concentration of the control sample, where no library compounds competed for binding. Figure 6A also shows results for the two other sets of four hundred compounds, which are referred to as Library A and Library C. Neither one of these sets had a known CA inhibitor present. With no inhibitor present, each of the data points in the graphs in Figure 6A should have Y coordinates of approximately one. These data points represent a ratio of the reporter ion's signal in the presence and absence of the library compounds. When no strong binder is present, the reporter ion's signal should be the same, regardless of whether or not other compounds are present; therefore, the ratio comparing when compounds are present to when they are absent, should be one if no inhibitor is present. For Library A, all three replicates gave such a value, near 1.0. However, the signal for the reporter ion

was much higher when Library C was added. These results indicate that some compound in Library C is out-competing the reporter molecule for the CA binding site and is therefore inhibiting CA binding. This exciting result suggested that a new CA inhibitor was identified by screening just 1200 well-known compounds against a well-studied target protein.

To further validate the results of the initial screen, and to gain more insight into the new inhibitor that had been identified, Library A, B, and C (Figure 6a) were divided into four batches with 100 compounds each (Figure 6b), and the same experiment was conducted as described above. The known strong binder was spiked in to Library B3 (Figure 6b). Ten of the 100 compound libraries indicated that no strong binding compounds were present; this finding emphasizes the assays power in evading false positives. The only two libraries that contained hits were Library B3, which had been spiked with the known strong binder, ethoxzolamide, and Library C4 (Figure 6b). The high response from Library C4 was consistent with our previous experiment, and it further indicated that a compound in Library C4 was binding to CA and displacing the weaker-binding ligand.

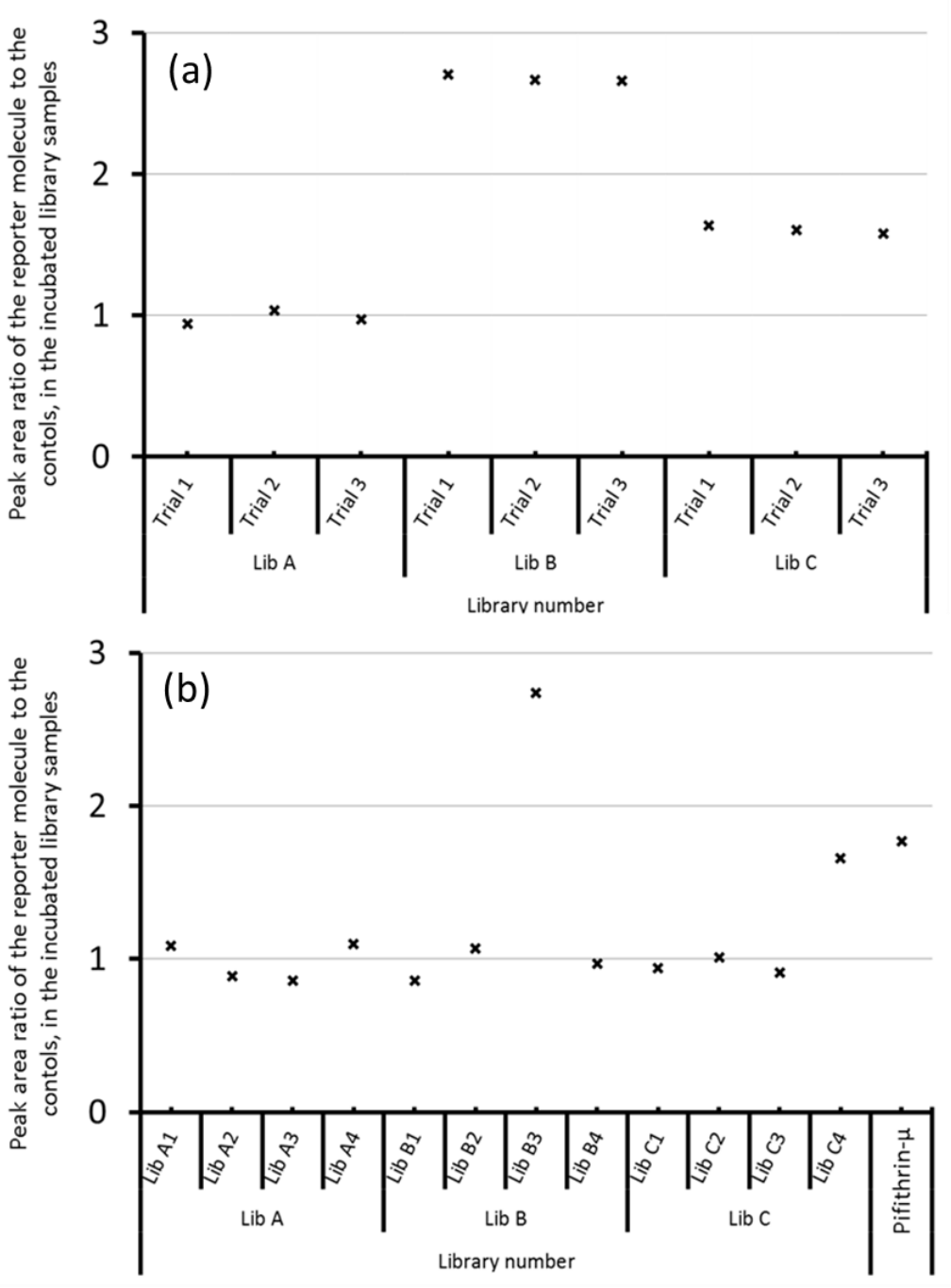

Figure 6: Screening assay for CA binders with methoxzolamide as the reporter molecule. (a) Tests conducted with 400 compound libraries. (b) Tests with 100 compound libraries and with pifithrin-μ. A strong binder, ethoxzolamide, was spiked into Library B (400 compound set) and Library 3B (100 compound set) to verify that a positive response would be readily detectable when a strong binder was present. The positive response for Library C and Library C4 resulted from pifithrin-μ, a CA inhibitor that was newly-identified in this assay.

After we had identified the set of 100 compounds that likely contained a new hit for CA, we attempted to identify the inhibitor based on structural similarity to known CA inhibitors. The chemical structures of the 100 compounds in Library C4 were compared with well-known CA inhibitors. The compound pifithrin-μ was found to have considerable functional group similarities with other CA inhibitors (Figure 7). However, to our knowledge, pifithrin-μ has not been reported as a CA inhibitor. To confirm that pifithrin-μ is responsible for the competitive binding effect, another screen was carried out where methoxzolamide was used as the weak binder and only pifithrin-μ was added. As expected, pifithrin-μ out-competed some of the weak binder that was present, at approximately the same degree as was observed when all 100 compounds in Library C4 were present (Figure 6b). This experiment confirms that pifithrin-μ was responsible for the positive hit in the assay. Pifithrin-μ itself is not ionizable, so it would not be detectable by ESI-MS in either in the positive or negative mode. This property likely explains why it had not previously been identified as a CA inhibitor, even though MS-based HTS assays, screening over 100,000 compounds had been conducted previously using CA as a target protein.[24]

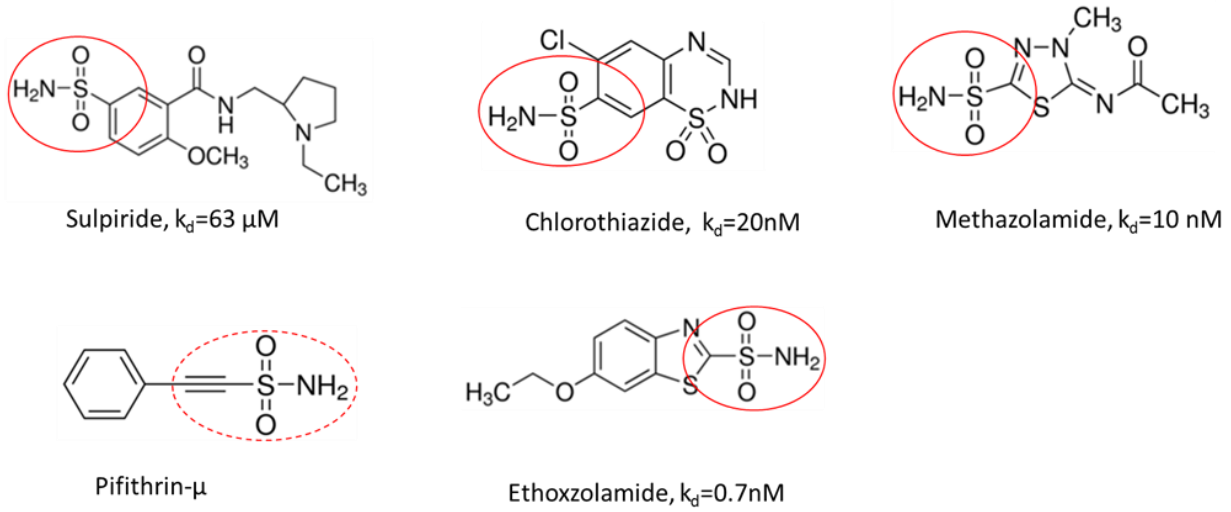

Figure 7: Structures of all the known CA inhibitors and the unknown CA binder pifithrin-µ. Similar functional groups were circled in red in all the structures except dash lines were used on pifithrin-µ.

Is pifithrin-µ a new CA inhibitor, or is it a false positive hit? To answer this question, an inihibition assay was carried out using a standard protocol where the substrate of carbonic anhydrase, carbon dioxide, is transformed to carbonic acid, and this conversion is monitored by a change in pH of a buffered solution. Pifithrin-µ was tested for its inhibitory ability, and it dramatically impacted the activity of carbonic anhydrase, even at low concentrations. The IC_{50} was determined to be ~25 nM (Figure 8). These results confirm that pifithrin-µ is a newly-identified inhibitor of CA. CA inhibitors are highly sought-after for the development of a variety of drug products, treating diseases as broad as cancer to glaucoma.[28,29,30,31] This new lead, therefore, expands the CA structures that can be considered for drug development.

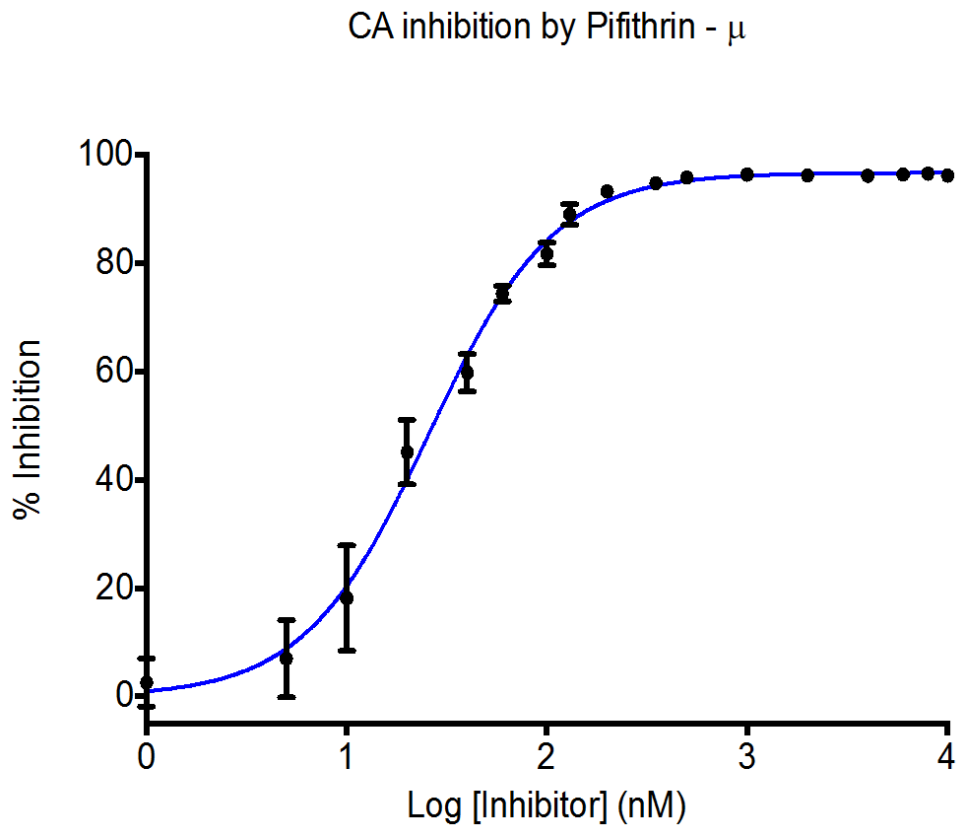

Figure 8: The plot of % inhibition versus the log concentration of the inhibitor. IC50 value (~ 25 nM) for pifithrin-μ compound identified as carbonic anhydrase inhibitor in the LOPAC1280 library.

3.4 Conclusion:

We developed a new MS-based HTS method that has two key advantages over existing methods: First, the assay evades false positives, since compounds that bind nonspecifically do not out-compete the weak binding ligand at the protein's binding site; second, since the method does not detect binding ligands directly, but rather detects when a ligand out-competes a weaker binder, even non-ionizing compounds can be profiled; therefore, false negatives are mitigated as well. The assay is rapid, screening 400 compounds in 10 minutes, and it requires only minute quantities (pmols to nmols) of

target protein. After demonstrating that the assay had appropriate discriminatory power for three different proteins, CA was screened against a library of 1200 compounds and one known strong binder of the targets. We detected the spiked strong binder each time it was present, and none of the 1200 compounds tested gave a false positive response. Furthermore, a new inhibitor, pifithrin-μ, was identified; this is a surprising and exciting result, considering that CA inhibitors are therapeutically valuable drug targets for a number of diseases. This rapid and simple assay can be implemented on a variety of protein targets; binding ligands with a broad range of binding affinities can be detected.

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Chapter 4: High-Performance Ligand Fishing for Target Proteins Using Rapid LCMS: Finding Ligands That Exhibit a K_d in the µM Range Using Limited Amounts of Target Protein with No False Identifications.

Ligand fishing for target proteins is becoming increasingly popular due to its potential to expedite the time of discovery for lead compounds in the drug screening process. Target protein is the most valuable reagent of an assay and, thus, is an important limiting factor that influences the number of ligands that can be screened at once. Ligands with a K_d in the micromolar range are difficult to identify due to their lower affinity, and identification becomes more challenging when there is a limited supply of target protein. In this study, we developed an improved high-throughput screening (HTS) assay that reduces the amount of protein that is needed to screen binders by 50%. In addition, our previous ligand fishing HTS assays, in which d 20 µg of protein was used, were re-characterized to identify micromolar binders in library batches, each containing 400 compounds. The variation of the mass spectrometry ionization source can result in irreproducible detection of the reporter molecule in identification of potential binders with a reduced amount of target protein. Therefore, an internal standard was used to negate any such variation. In addition to lowering the consumption of proteins, this novel assay, similar to our previous methods, mitigates false positives by selectively identifying positive hits during displacement of the ligand in the binding site of interest and false negatives by detecting an ionizable reporter compound

4.1 Introduction:

Mass spectrometry has become the preferred method of ligand fishing for target proteins due to its ability to perform label-free, highly selective, and sensitive high-throughput screening.[1,2,3] With ligand fishing, it can be difficult to discern tightly binding ligands from a library of compounds, most of which will lack the required binding motifs. Searching for low-affinity binders is still beneficial, because the structural information that can be gained from assessing the moieties that bind to the protein is useful for synthesis of new compounds with higher affinities or more druggable properties. Even MS-based techniques suffer from a high rate of false positives associated with nonspecific binding of compounds, which can be misleading in the drug screening process and prolong the discovery pipeline.

Recently, our group developed (as described in the chapter 3) a method for discovering high-affinity ligands for target proteins from ligand seas in a high-throughput manner, allowing us to discover ligands with a K_d in the picomolar to micromolar range without any false positives or false negatives. It is important that these assays be able to function using a low amount of target protein due to the difficulty and expense of obtaining pure material. Our method involves an initial incubation of the target protein with a known weak binder (reporter molecule) and then incubation of the protein-reporter complex with library compounds. If there is a stronger binder in the library, the signal for the reporter molecule in the assayed supernatant increases, compared to controls in which no compounds competes for binding. The signal increases due to displace of the reporter molecule by the strong binder and therefore becomes detectable. This assay identifies strong binders in a specific pool of ligands from batches of ligand seas that have been

tested in our validated HTS assays. The LC-MS method only requires one to quickly scan for known weak-binding reporters in a mixture of up to 400 compounds; thus, screening can routinely be achieved in 10 minutes per set of compounds.

To achieve validation for HTS, it is necessary to evaluate the robustness of an assay by calculating the Z' factor, a statistical parameter that was introduced by Zhang *et al*..[4] It is calculated using a small number of positive and negative control samples. The positive control samples are prepared by spiking in a known strong binder, whereas the negative controls contain every compound in the positive controls except for the known binder. The Z' factor must be higher than 0.5 for an assay to be considered well-validated. A well-validated assay has the potential to yield significantly fewer false positives when millions of samples are screened. A Z' factor of less than 0.5 is considered a marginal assay and generates overlap between the responses of the positive and negative controls, which can lead to false positive identifications.

One difficulty of this assay (chapter 3) is excessive bleeding of reporter molecule into the supernatant when the small amount of protein is reduced further than the optimized amount. This phenomenon is expected; a decreased amount of protein results in more dissociation of the protein-reporter molecule complex. Thus, the number of reporter molecules that are already bound to the target protein before being displaced by a strong binder is small. As a result, the mean difference in the reporter molecule response between positive and negative controls remains close. It is difficult to obtain an acceptable Z' value (> 0.5) if the standard deviation (SD) of the responses (signal fluctuation) is high, as shown by the following equation:

$$\text{Z Factor} = 1 - \frac{(3 \times \text{SD of positive control} + 3 \times \text{SD of negative control})}{|\text{positive control mean} - \text{negative control mean}|}$$

The MS ionization process influences the fluctuations in signal. Internal standards can be used to account for this variation due to the ionization source. Decreasing the SD in the signals of the positive and negative controls by using internal standards is important in the development of a well-validated assay.

By including an internal standard in our current study, we discovered binders with K_d values in the micromolar range using a small amount of target protein. We used an isotopically labeled reporter molecule as an internal standard to validate the assay for HTS and demonstrated its ability to identify a strong binder in a library with much higher reproducibility, allowing us to use only half the initial amount of target protein (10 µg, or ~200 pmol) to fish out a ligand in the micromolar K_d range from a 400 compound library. This approach requires a smaller amount of protein for screening, which is the most valuable reagent in the assay, and includes all of the advantages of the previous method, specifically no false positives or false negatives and fast screening. These properties make it possible to screen for ligands with K_d values in the micromolar range, even for low-abundance target proteins, and reduces the time and effort required for early discovery of drug leads.

4.2 Methods:

4.2.1 Reagents

The library compounds were all FDA-approved drugs, purchased from Sigma Aldrich (St. Louis, MO, USA). The library was provided as a single compound per vial

(LOPAC1280-small scale), dissolved in DMSO at 10 mM. Maltose-binding protein was purchased from My BioSource (San Diego, CA). Nitrocellulose drop dialysis membranes were purchased from Fisher Scientific (Houston, TX). [UL-$^{13}C_{12}$]maltose monohydrate was obtained from Omicron (South Bend, IN).

4.2.2 Binding experiment

Maltose-binding protein (MBP) was immobilized on N-hydroxysuccinimide (NHS)-activated magnetic beads per the manufacturer's protocol (Pierce™ NHS-Activated Magnetic Beads, Thermo Scientific.Workford, IL). Briefly, after the magnetic beads were washed, 10 µg MBP in PBS coupling buffer (0.1 M, pH 7.4) was added to 100 µL of magnetic beads and slowly rocked overnight at room temperature. Drop dialysis was conducted on MBP (in 1.0 M Tris-HCl) before an appropriate aliquot of protein was added to the tube that contained the beads. After the mixture was rocked, the supernatant was removed and the immobilized proteins were washed with 1 mL coupling buffer. Then, 300 µL quenching buffer (Tris-HCl, 1.0 M, pH 7.4) was added and slowly rocked for 2 hours. The supernatant was removed and immobilized proteins were washed with 300 µL washing buffer (1 M NaCl in coupling buffer), followed by the addition of 2 mL ammonium acetate (0.02 M, pH 8.0) incubation buffer. The supernatant was removed again and 50 µL of 50 nM of the known weak binder, maltose, was added; the mixture was then rocked for 2 hours at room temperature. After removal of the supernatant, 50 µL of the 400-compound library (300 nM) was added and rocked for 1 hour. The library compounds were prepared by diluting them from 10 mM to 300 nM in incubation buffer prior to incubation with the immobilized protein. Here, 2 µL was taken from each compound to prepare stock solution of library of 100 compounds with a final concentration of 100 µM.

When preparing the library with 400 compounds, four libraries of 100 compounds were combined by taking 2 µL from each library with a final concentration of 25 µM. Then 2µL of 25 µM compound was diluted to obtain a final concentration of 300 nM in the incubation buffer. After incubation, 20 µL of the supernatant was removed and 2 µL of 550 nM internal standard in incubation buffer, [UL-$^{13}C_{12}$]maltose monohydrate, was spiked and used in the LC-MS analysis.

4.2.2 Positive and negative control experiments

Positive and negative control samples were prepared following the procedure in the *binding experiment* section, except libraries with a known strong binder were spiked to positive control samples. For each positive control, 401 library compounds were used. Negative control samples contained either buffer only or 400 non-binding compounds. Supernatants for both controls were removed and spiked with 2 µL of 550 nM internal standard and used in the LC-MS analysis.

4.2.3 LC/MS Analysis

Liquid chromatography/mass spectrometry analysis was done using an Acquity UPLC system (Waters Corporation, Milford, MA) coupled to an Orbitrap Velos Pro mass spectrometer. Mobile phase A was 99.9% water with 0.1% formic acid and mobile phase B was 99.9% MeOH with 0.1% formic acid. For each run, 5 µL of the supernatant was injected onto a C_{18} Hypersil Gold column (Particle Size: 5 µm; 1 mm *i.d* X 100 mm, 175 Å, Thermo Electron Corporation, Thermo Fisher Scientific, Pittsburgh, PA) at a flow rate of 50 µL/min. The following multi-step gradient was used for MBP assays: 100% solvent A for 3 min, then a linear increase of B to 80% in 2 min, followed by the linear increase of

B to 95% in the next 3 min, followed by maintaining B at 95 % in additional 30 seconds, after that linear decrease of B to 0 % in next 30 seconds where the column was maintained for another 2 min. The eluent was diverted to waste for 1 min at the beginning of each run to prevent salts, DMSO, and compounds that are eluted from the front end of the chromatogram (until the weak binder is eluted) from entering the MS source. The mass spectrometer was operated in the positive ion mode with a 3 kV potential on the ESI needle, and the capillary temperature was set at 250 °C. Full scan MS data were acquired at a mass range of *m/z* 200-700 using the Orbitrap mass analyzer at a resolution of 30,000 for *m/z* 400.

All peak areas were calculated using extracted ion chromatograms from the .raw data files for each compound. Peak areas were taken from the monoisotopic peak of proton or/and sodium adduct for the reporter molecule, maltose (*m/z* 343.1235, 365.1054), and the internal standard, [UL-$^{13}C_{12}$] maltose monohydrate (*m/z* 355.1643, 377.1462). The peak area for each extracted ion chromatogram was calculated using the software (Xcaliber) supported algorithms, (Genesis).

4.3 Results and Discussion:

4.3.1 Method Overview

In this project, we improved on our previously developed assays through the use a significantly smaller amount of target protein for ligand fishing. Figure 1 illustrates a schematic representation of the assay development. The magnetic beads with immobilized target protein were first loaded with the known compound, which is referred to as the weak binder or reporter molecule. After this step, the supernatant was removed and the immobilized-protein reporter molecule complex was incubated with ligand seas, typically batches of 400 compounds. A strong binder (high affinity binder) in an incubated ligand sea then replaced the reporter molecule from the target protein. As a result, the concentration of the reporter molecule in the supernatant increases, compared to negative controls. Then the collected supernatants were spiked with an isotopically-labeled reporter molecule as an internal standard. Next, the reporter molecule and the internal standard were quickly detected by LC-MS. The peak area ratio between the reporter molecule and the internal standard was obtained as an assay signal for each control. The assay was statistically validated by calculating the Z' factor using positive and negative controls.

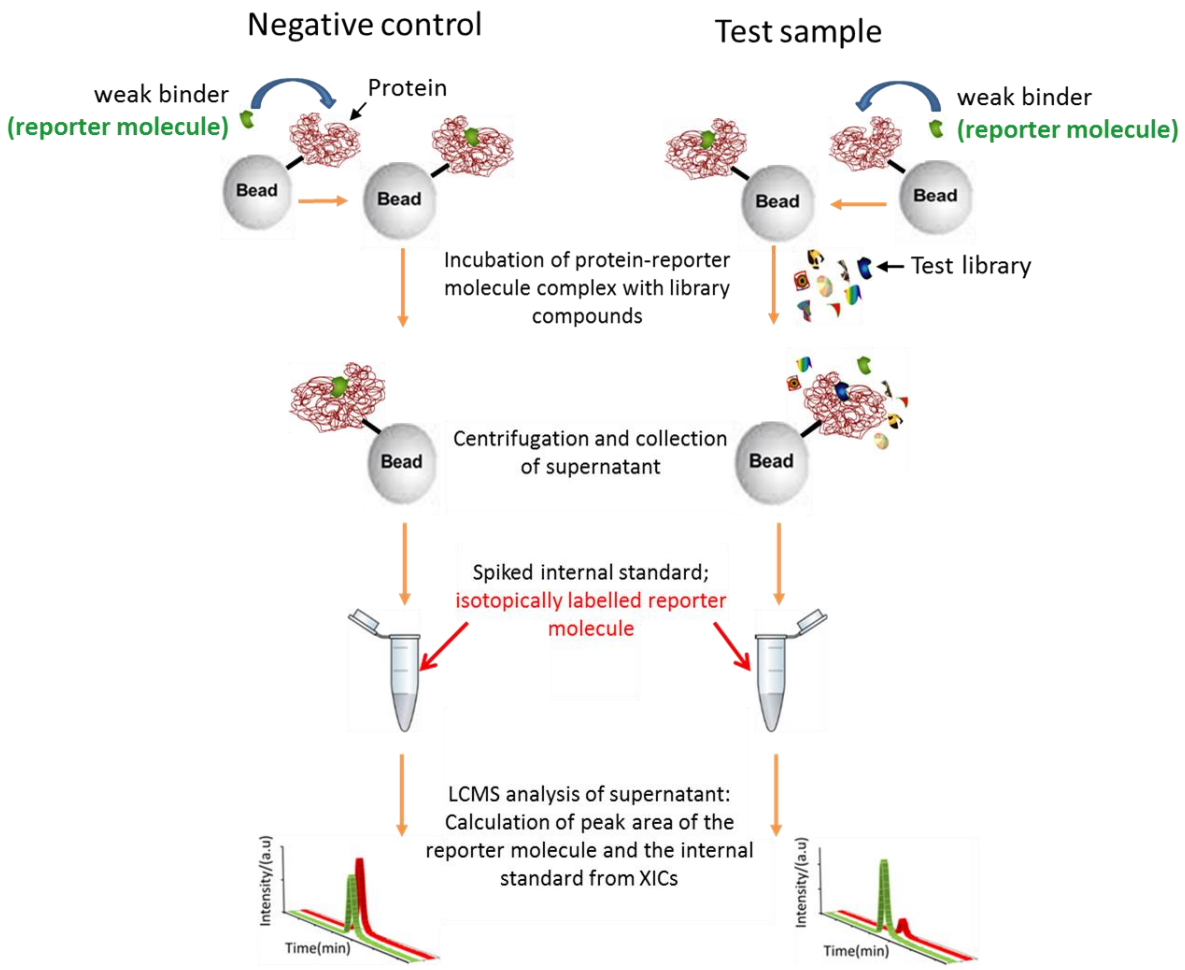

Figure 1: Experimental workflow. The weaker binder (reporter molecule) is incubated with the immobilized protein, and excess reporter molecule removed. The library compounds are added to the test sample. During validation experiments, a known strong binder is spiked into the library, generating a positive control sample. When present, a strong binder replaces the reporter molecule on the binding site of the protein, and the concentration of the reporter molecule increases in the supernatant relative to the negative control. Supernatants are collected and the internal standard (the ^{13}C isotope of the reporter molecule) is spiked into each of the collected supernatants. Analysis consists of confirming the presence of reporter molecule and the internal standard. Peak area ratios of the extracted ion chromatogram between the reporter molecule and the internal standard in both controls are used to calculate the Z factor to validate the assay.

4.3.2 Identification of transient binders with minute amount of protein

To demonstrate that this workflow provides sufficient discriminatory power between ligand sets that do or do not have a strong binder present, several control experiments were completed on MBP/ligand systems. Figure 2 shows a representative comparison of the variation in all controls with (Figure 2a) and without (Figure 2b) internal standard when 10 µg of target protein was used. The calculated Z' factors were 0.65 and ~0.2 in the presence or absence of internal standard, respectively. These values clearly indicate that the internal standard is necessary to validate the assay thoroughly before use for HTS.

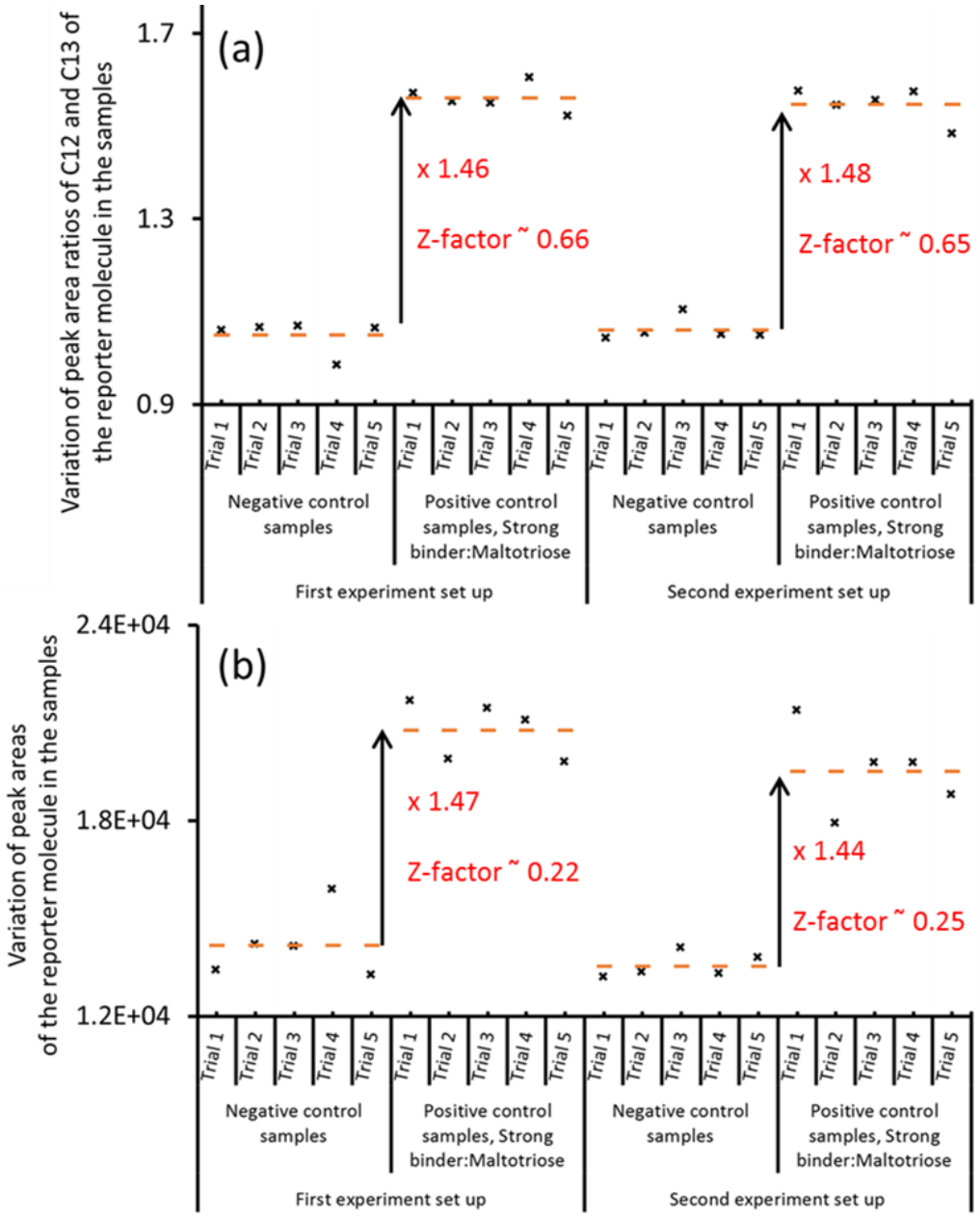

Figure 2: Peak area variation of the weak binder (reporter molecule), maltose (K_d= 3µM, (a) with and (b) without internal standard, ^{13}C-Maltose, in the samples used for Z factor calculations. For each sample, 10 µg of MBP was incubated with 400 non-binding compound, with the exception of positive controls samples, which were spiked with a known strong binder, maltotriose (K_d = 0.2 µM). The orange dash lines represent the mean value of the corresponding trials for each control.

Another important factor to consider is the separation band, which is defined as the difference in the assay signal distribution between the positive control samples and the

negative control samples, as explained in the introduction. The difference between the mean values of the controls decreases as the amount of protein is reduced. The smaller the amount of protein used, the less target protein-reporter molecule complex is available. Therefore, the amount of reporter molecule that can be replaced by a strong binder is smaller and the variation in concentration of the reporter molecule in the supernatant is negligible. When these “low reporter” samples are subjected to LC-MS analysis, the variation in the ion count due to the ESI source becomes more significant, which results in a large SD within the samples. This large SD would not be significant if the separation band attained statistical validation of the HTS, as shown in previous chapter in which 20 µg of MBP was used. To reduce the large SD that is caused by the ionization fluctuation in the ESI source, an internal standard is added to the controls. This allows us to obtain well-validated assays that can be used to investigate much larger libraries that are routinely encountered in real HTS.

4.3.3 Screening of enhanced libraries

To mimic real HTS, three ligand seas, each with 400 compounds, were used and one (library B) was spiked with a strong binder, maltotriose. The calculated peak area ratios of the reporter to internal standard in each library samples were divided by the same ratio of blank controls, in which no library compounds were added to the protein-reporter molecule complex. Figure 3 represents the peak area ratio calculated for each of the samples. Figures 3a and 3b represent screens that were carried out with and without internal standard, respectively. Library B was spiked with a strong binder, maltotriose. The y-axis value of 1 was considered to be the threshold value to identify any ligand sea with a strong binder. Figures 3a and 3b both show high y axis values of more

than 1 for library B, which alone contains the strong binder. However, this conclusion needs to be statistically assessed to test the validity (i.e. is the strong binder in library B, in this case known to be maltotriose, absent from libraries A and C), as shown in Figure 2. The Z' factor was calculated in Figure 2 to validate the assay for use in HTS. Obtaining a larger Z' factor demonstrates the strength of the assay, indicating that there is no overlap between negative and positive responses, even in millions of screens. On the other hand, a lower Z' factor represents a lower robustness for the assay, due to overlap between negative and positive control responses. As shown in Figure 3b, where identical libraries were run in duplicate without internal standard, the SD of the detected amount of reporter was high compared to data from treatment with an internal standard, shown in Figure 3a. In addition, even though library B in trial 1 is distinguishable from libraries A and C (at 99.5% CL), this was not observed in trial 2. In the latter trial, a strong binder could thus be misidentified as a false negative. When an internal standard was used, however, library B in both trial 1 and 2 was distinguishable from libraries A and C (at 99.5% CL). This study shows the feasibility of using a significantly lower amount of protein to fish out very low affinity ligands from ligands seas with the use of internal standards. There are also the additional benefits of no false positives and negatives, the ability to screen 400 compounds in 10 minutes, and site specific binding, all of which are inherent to the assays that we have already developed.

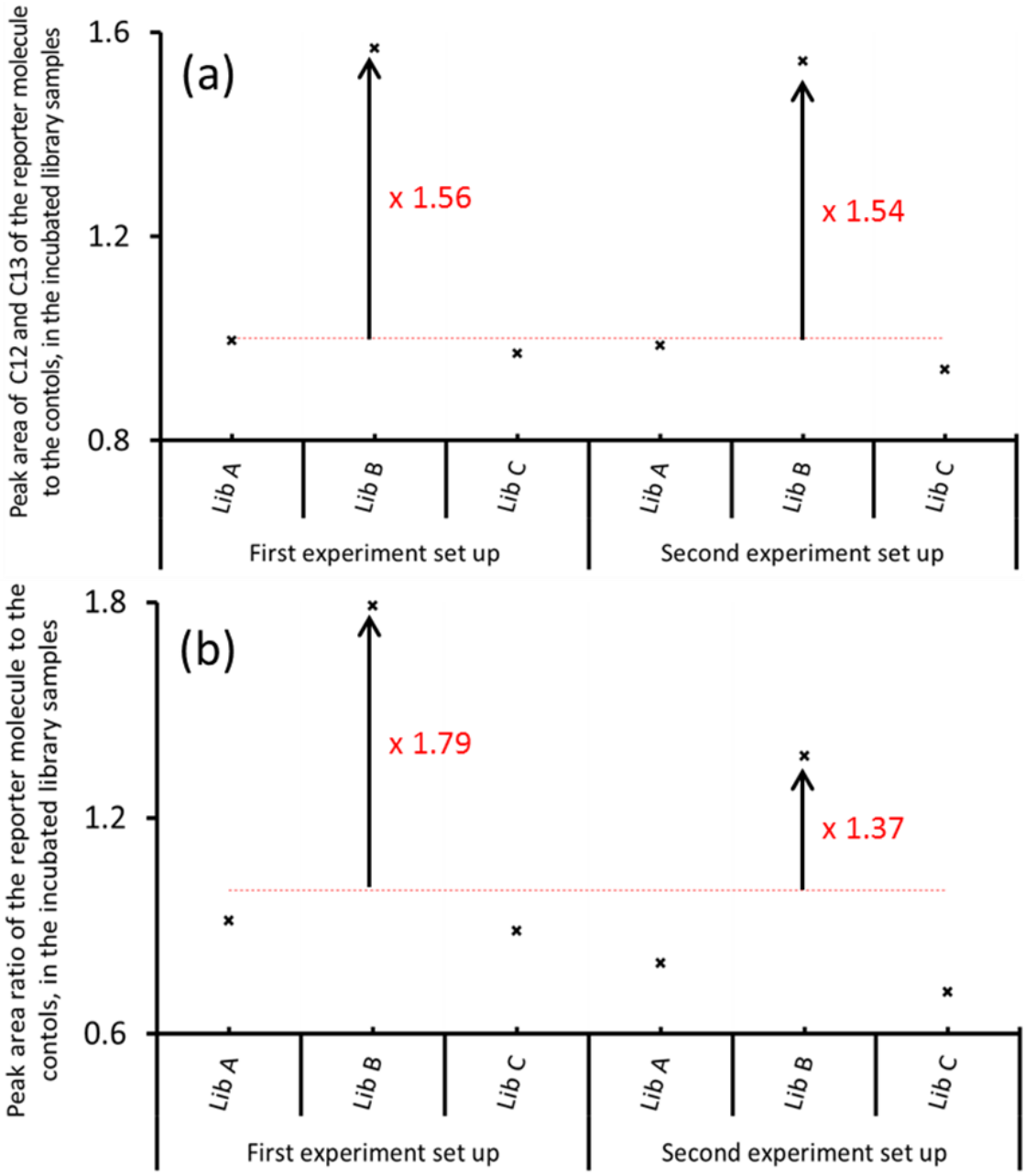

Figure 3: Peak area variation of the weak binder, maltose, to control (a) with and (b) without internal standard, ^{13}C-Maltose, in libraries a, b, and c. For each sample, 10 µg of MBP was used and incubated with libraries containing 400 different non-binding compounds, with the exception of library B, which was spiked with a known strong binder, maltotriose. The red dotted line represents the value of 1, which characterizes the controls. Two different controls were used in each experimental setup.

4.4 Conclusion:

The most valuable reagent in a fishing assay is the target protein. We have used an internal standard to account for ion signal variation during ESI-MS and developed a well validated HTS assay for ligand fishing from ligand seas of binders with K_d's in the μM range. It has improved upon our previously developed assays by decreasing the amount of target protein used. We were able to reduce the amount of protein necessary to assay 400 library compounds by half (20 μg to 10 μg), while still obtaining an excellent Z' factor (0.6) to validate the assay for HTS. Without the internal standard, the reduced amount of protein is only useful for obtaining a marginally validated assay with a Z' factor of 0.2. After using the validated assay to screen various ligand seas, only the ligand sea that was spiked with a strong binder was identified, with no false positives or negatives.

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Chapter 5: Future Directions towards the Automation and Screening of Protein-Protein Interactions

5.1 Dissertation Summary:

This dissertation describes a novel and improved MS-based High Throughput Screening (HTS) method to screen for drug candidates for a given protein target. Chapter one summarizes the execution, advantages, and limitations of the currently available MS based high throughput methods. Chapter two describes the development of an MS-based HTS method to identify the tightest-binding lead compounds for target proteins with no false positive identifications. Three different proteins and hundreds of non-inhibitors were used to demonstrate the validity of the assay; no false positive hits were identified in any experiment. The assay was modified to select for ligands of a particular binding affinity by varying the quantity of protein used (and the immobilization method), as evidenced by the results where inhibitors that have K_i values of 0.2 µM, 50 pM, and 700 pM were selectively detected. The results included in Chapter two demonstrate that this methodology compares favorably to traditional MS based screening methods.

Chapter three describes the modifications applied to the assay explained in Chapter two in order to increase the throughput, in addition to addressing false negative identifications. The method described in Chapter three dramatically increases the pace of library screening from 400 compounds/ hour to 400 compounds/ 10 minutes. Finally, a novel carbonic anhydrase inhibitor, pifithrin-µ (IC_{50} = 25 nM), that would have been a false negative in most MS-based HTS methods, was identified using the modified assay. Chapter four extended the work described in Chapter three by modifying the method to

further reduce the quantity of the protein required for the assay by 50% when screening for weak affinity binders.

5.2 Future Directions:

The most challenging portion of the early drug screening process is the identification of a therapeutically valuable binder to a target protein. As explained in Chapter three, introduction of an ionizable reporter molecule to the target protein mitigates false positives and false negatives, thereby increasing the probability of finding a therapeutically valuable compound, as we discovered in the case of carbonic anhydrase. For our assay, immobilization of the target protein is particularly necessary to separate unbound compounds from the protein-ligand complex. However, immobilization requires several hours and confers numerous disadvantages. These limitations include inactivation of the target protein, incompatibility of the solid support with the immobilization of target protein, alteration of the binding site, and hindrance of possible ligand approaches to the binding site of the target. Furthermore, multiple step experimental approaches and manual error can alter the results. Therefore, an automated experimental set up that does not require target immobilization would be a promising approach for the advancement of our method. If a membrane filter can separate the unbound compounds from the complex, the immobilization step can be omitted from the experimental procedure. However, in our experience, the currently available membrane filters demonstrate poor success in filtering out unbound compounds from the protein-ligand complex. Some common problems of this approach are the high affinity of some small molecules to the membrane material, and spectral overlap of leachable materials from membrane with the reporter molecule. Most of the commercially available

membranes are made of cellulose materials, which acts as hydrophobic surfaces, resulting in the binding of hydrophobic small molecules. If the membranes are made of glass fibers, the separation of unbound compounds from the complex could be a possibility. However, the pore sizes of commercially available glass membranes are not small enough to retain the protein complex. Overcoming this membrane issue is a necessity for the development of an automated ultra-high-throughput screening assay.

The modulation of biological pathways by targeting protein-protein interaction (PPIs) opens up an exciting class of potential drug targets. One of the current projects in the Desaire group is focused on developing an assay to screen for inhibitors of protein-protein interactions. Fluorescence polarization (FP),[1,2,3] fluorescence resonance energy transfer (FRET),[4,5] and surface plasmon resonance (SPR),[6] and enzyme linked immunosorbent assays[7] are some of the currently used common methods for screening PPI inhibitors/modulators. These assays share many limitations, including the requirement for extensive labeling and large amounts of proteins, and show high rates of false positives due to nonspecific signal. Therefore, mass spectrometry can be used as an alternative to traditional methods of screening for PPI inhibitors, due to increased sensitivity, lower sample consumptions, and label-free specificity. However, previously reported mass spectrometry based screening approaches for protein-protein interactions in cell cultures using affinity chromatography and affinity purification pull-down techniques require specific antibodies and purification that is often inefficient.[8,9] Also, to our knowledge, a high throughput screening method to screen for inhibitors for protein-protein interactions is not currently available.[10,11] To develop this novel type of assay, one protein is first immobilized on a solid support and allowed to form a protein-protein complex with

the second protein. Then, the protein complex is incubated with library compounds. The existence of a probable inhibitor in a particular library can be identified by monitoring for the presence of the released protein (from the complex) in the supernatant, compared to the control where no library compounds are added. One of the model protein-protein interactions that we are planning to utilize is the complex formed by p53, a tumor suppressor protein,[12] and the oncoprotein murine double minute 2 (MDM2), an inhibitor of p53 which is analogous to human double minute 2 protein.[13] Even with this PPI approach, a suitable membrane cut off filter could be used when there is a significant size difference between the two interacting proteins. If the necessary improvements are implemented, our methods would be a vital contribution to the field of drug discovery.

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