Magnetic Microbead Affinity Selection Screening: Development, Application, and Evaluation

By

MICHAEL RUSH

B.S. Philosophy, University of Idaho, 2008
B.S. Chemistry, University of Idaho, 2008

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Advisory Committee:
Professor Richard B van Breemen, Chair
Dejan Nikolic,
Brian Murphy
Jimmy Orjala
Gerd Prehna, The Center for Structural Biology, Research Resources Center and the Department of Microbiology and Immunology

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MDR

Contributions of Authors

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Michael Rush and Elizabeth Walker worked together in formulating and developing the MagMASS assay. Gerd Prehna developed expressed and purified the protein and guidance during the project. Tristesse Burton extracted the botanical natural products. Richard van Breemen advised during the project.

Chapter 3: Rush, Michael D., *et al*. "Magnetic Microbead Affinity Selection Screening (MagMASS) of Botanical Extracts for Inhibitors of 15-Lipoxygenase." *Journal of Natural Products* 79.11 (2016): 2898-2902.

Michael Rush and Elizabeth Walker formulated and developed the assay. Michael continued the assay through the analytics and structure elucidation. Tristesse Burton extracted the botanical natural products. Richard van Breemen advised during the project.

Chapter 4. Michael Rush developed the assay and pooled Dr. Murphy's actinomycetes library. Gerd Prehna expressed, purified, and refolded the protein for analysis. Michael developed the IT server infrastructure and metabolomics workflow for the assay. Using the selected wells, Michael did the enzymatic inhibition work to confirm hits. Richard van Breemen advised on the project.

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LIST OF ABBREVIATIONS

MagMASS – Magnetic Microbead Affinity Selection Screening

FDA – Food and Drug Administration

UV-Vis – Ultraviolet-Visible light

MS – Mass Spectrometry

MSMS – Tandem Mass Spectrometry

NMR- Nuclear Magnetic Resonance

pH – Negative log of the Hydrogen Concentration

COX-1/2 – Cyclooxygenase-1 / 2

IC_{50} –Concentration Required for 50% Inhibition

LCMS – Liquid Chromatography Mass Spectrometry

PUF – Pulsed Ultrafiltration

PUFMS – Pulsed Ultrafiltration Mass Spectrometry

Gen – Generation

MMP-2 – Matrix Metalloproteinase-2

VDR – Vitamin D Receptor

RXRα – Retinoid X Receptor α

MBP – Maltose Binding Protein

NRF2 – Nuclear Factor Erythroid 2 – Related Factor

LBD – Ligand Binding Domain

DNA – Deoxyribonucleic Acid

HPLC – High Pressure Liquid Chromatography

UHPLC – Ultrahigh Pressure Liquid Chromatography

LIST OF ABBREVIATIONS (continued)

NHS – *N*-hydroxysuccimide

SRC-1 – Steroid Receptor Coactivator-1

LB – Lysogeny Broth

NTA – Nitrotriacetic Acid

TEV – Tobacco Etch Virus Nuclear-Inclusion-a Endopeptidase

SDS-PAGE – Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

LOD – Limit of Detection

SRM – Selected Reaction Monitoring

K_d - Dissociation Constant

NIH – National Institutes of Health

NCCIH – National Center for Complementary and Integrative Health

15-LOX – 15-Lipoxygenase

UIC – University of Illinois at Chicago

IT-ToF – Ion Trap Time of Flight

GP - Glycogen Phosphorylase

AMPK - AMP Activated Protein Kinase

SGLT - Sodium Glucose Cotransporter

SPE – Solid Phase Extraction

CD - Circular Dichroism

CE – Collision Energy

BPC – Base Peak Chromatogram

MTP – Microtiter Plate

SUMMARY

The contemporary approach to natural product drug discovery incorporates bioassay guided fractionation. The work described here is an effort to incorporate mass spectrometry earlier in the natural product drug discovery to alleviate many of the concerns of bioassay guided fractionation. Magnetic Microbead Affinity Selection Screening (MagMASS) targeting retinoid X receptor-α (RXR□) was developed as an alternative to bioassay guided fractionation. This work focuses primarily on the assay development using standards and testing the effects of different bead chemistries, immobilization, and matrices.

Next, MagMASS was applied to screening botanical extracts for ligands to 15-lipoxygenase (15-LOX) in place of bioassay guided fractionation. With the assay optimized, MagMASS was then used as prototype for a natural product drug discovery system. The workflow was developed and produced a hit for the protein target as a proof of concept.

Previously, MagMASS had a small scope of natural product drug discovery; it was limited to a small number of natural product extracts or combinatorial libraries. Here, approximately 900 extracts were screened for activity to fructose-1,6-bisphosphatase. With the expanded natural products, a new workflow was developed to process and visualize the data. This work provided a more complete exploration of MagMASS as a possible drug discovery platform.

MagMASS was then evaluated as a drug discovery platform. With some significant upfront costs, MagMASS can provide a shortcut in natural product drug

discovery in the hands of a skilled researcher. While not a universal walk up technique, it has specific and niche purposes where this technique shines.

CHAPTER 1: CHALLENGES OF NATURAL PRODUCTS AS SOURCES FOR DRUG DISCOVERY

Natural products as sources for drug leads

In the never-ending search for new drug leads, combinatorial libraries and natural products are important sources. Specifically, natural products provide approximately 35-40% of all approved drugs from the FDA[1] in recent years. Compounds made through various biochemical pathways from many different biological organisms provide a significant source of new drug leads in modern pharmaceutical research. Effectively utilizing these resources to find drug leads is a critical and ongoing challenge of natural products research. Unlike combinatorial libraries – which tend to be well curated, single compound, and in equal concentrations – natural product libraries are far more complicated. In general, each natural product extract has multiple compounds of varying concentrations leading to challenges in correlating positive responses in biologic assays for detecting drug leads and identifying the compounds that produce the biological response. This is a monumental task.

Bioassay Guided Fractionation

Bioassay guided fractionation is a contemporary method for processing these complicated natural products to eventually yield the compounds of interest[2]. A standard

bioassay guided fractionation procedure is as follows: first, extract the metabolites of the natural product using any of variety of techniques, most commonly a solid-liquid extraction using an organic phase. Evaporate the extract and reconstitute the natural products in a solvent that is amendable to later biologic testing. Second, test this extract in a biological test for activity. This test drives the drug discovery process regarding what compounds will later be identified. These biological tests range from cellular cytotoxicity for antitumor compounds or antibacterial agents to targeting specific enzymes or proteins. The variety of applications for which bioassay guided fractionation may be used is too extensive to cite here.

Following a positive biologic activity response, the natural product extract is then subjected to fractionation using a wide range of chromatographic methods – liquid chromatography, thin layer chromatography, or affinity chromatography. This fractionation is generally done through a manual or automatic detection protocol. Manual collection is often an operator collecting the eluent from a column or a plate. Automatic fractionation can be carried out with a fraction collector. Many commercially available fraction collectors are small devices in-line with the separation technique. Further, this separation can be guided by a chemical phenomenon. Commonly, UV-Vis detectors are in line with the separation techniques. These can guide the fractionation by allowing each peak to be collected manually or automatically. However, not all substituents are either chromophorically active or have enough concentration to be detected using UV-Vis; this detection method depends on the concentration and the chemical nature of the absorbing compounds. Sometimes there is an insufficient concentration for a response in the detector. Alternatively, collection can be done based

on time. All peaks are collected and each well corresponds to a window of time from the eluent. While this method groups similar compounds based upon the separation technique, it will ensure that all compounds from the eluent is captured.

Once fractionation is complete, each fraction is then reanalyzed in the same bioassay used previously for the complete extract. Ideally, the activity from the initial bioassay will be localized to a few subfractions. Focusing on the active subfractions, the subsequent work is highly dependent upon the complexity of the original natural products. In many cases, these subfractions are still complex, requiring multiple iterations of fractionation followed by bioassays until a relatively pure fraction remains which shows activity in the bioassay. From this point, the relatively pure fraction can be identified using standard structure elucidation techniques such as NMR combined with high resolution mass spectrometry.

Challenges of Bioassay Guided Fractionation

Bioassay guided fractionation is a proven and thorough process to isolate bioactive compounds from mixtures of natural products. However, there are many challenges in this technique. First, it is labor-intensive and requires the diligence of a skilled laboratory worker for success. Second, the bioassay that is critical for directing the fractionation is usually a bottleneck in the process.

Bioassay guided fractionation can be directed using variety of biological tests – for example, testing the effects on cells or enzymatic inhibition – but there are drawbacks to each of these types of assays. For cellular response bioassays, the cost

lies with developing the cells for treatment with the fractions. Many times, cell growth may require days to weeks. Couple the long growing time with the need for multiple iterations of the bioassay for a given extract, it is clear that bioassay guided fractionation can take weeks to complete and become expensive. In addition, each cycle of cell culture may introduce variability in terms of viability and reproducibility.

In contrast, enzymatic bioassays are faster and cheaper than cellular bioassays. Nevertheless, the iterative nature of the bioassay guided fractionation multiplies the cost of the protein and the bioassay. Although an enzymatic bioassay might involve a cheaper workflow and provide less information, an expensive protein can still amount to a significant cost during the fractionation.

Second, the availability of the starting materials may also be problematic. Many times, once a fraction has been processes to sufficient purity for testing and characterization, the amount of remaining material is only of trace levels. Bioassay guided fractionation is an inherently a dilute-then-reconcentrate technique. The final remaining active fraction may be of such low concentration, it may have activity in a bioassay, but not enough material for proper structure elucidation. Unfortunately, this outcome is difficult to predict and may require a second round of harvesting, extraction and fractionation to obtain still greater amounts of material even after the final product of the initial bioassay guided fractionation has been evaluated.

Third, the problem of dereplication of natural products is a constant challenge. Dereplication is important because many of the natural product sources may have similar metabolic pathways. Therefore, it is important to avoid expending effort rediscovering a previously reported compound. Depending upon the bioassay

component, there may not be a way to incorporate a dereplication technique until after the bioassay fractionation is completed and a previously reported compound is isolated.

Searching for a solution to these challenges, our lab sought to find a shortcut to bioassay guided fractionation. If the end goal of bioassay guided fractionation is to identify a structure of an active compound, then incorporating a mass spectrometer into an assay could quickly advance us to the goal.

Development of Mass Spectrometry Assays to Replace Bioassay Guided Fractionation

Pulsed Ultrafiltration: Gen 1

The first iteration of this mass spectrometry-based assay was published by the van Breemen laboratory in1997.[3] In this approach called pulsed ultrafiltration mass spectrometry (PUFMS), a flow-through chamber of 1000 µL is equipped with a stir bar and a protein, which is trap by an ultrafiltration membrane on the exit side of the chamber as depicted in Figure 1. Then, a set amount of a combinatorial library – or a pulse of ligands – is injected into the chamber to interact with the trapped protein. This preserves the native protein ligand interactions. Ligands in the pulse bind to the protein while non-ligands are washed to waste from a continuous flow of buffer through the chamber. Then, the protein ligand complex is disrupted by an external factor: the addition of methanol, change in pH, change in temperature, etc. The small molecule ligands are then released and exit the chamber through the ultrafiltration membrane to a mass spectrometer, where each ligand is characterized by molecular weight. Using

the mass spectrometric characterization of the ligands paired with knowledge of the library, it is possible to identify the ligands in a library.

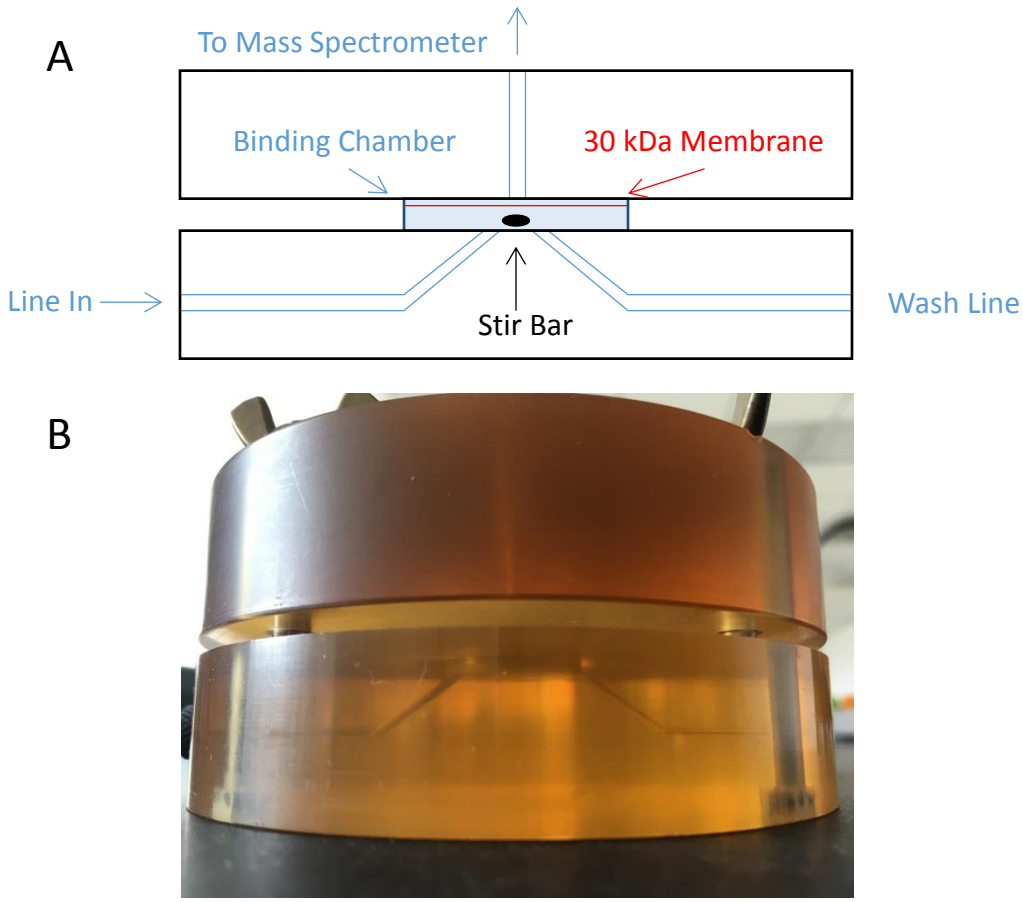


Figure 1 - Pulsed Ultrafiltration Gen 1 described schematically and a photo of the system. A shows the general layout. The protein is introduced to the 1000 µL binding chamber with the stir bar and is trapped by the 30 kDa ultrafiltration membrane. Ligands or libraries are introduced and eventually eluted through the membrane to the mass spectrometer. B is a picture of the PUFMS cell on the bench top. The dimensions are approximately the size of a hockey puck.

In this first report on PUFMS, the protein of interest was adenosine deaminase. This enzyme is a drug target because a number of adenosine analogs used for anticancer or antiviral purposes contain an adenosine motif which is cleaved by this enzyme, thereby lowering the efficacy of those drugs.[4] An inhibitor of adenosine deaminase might therefore prolong the half-life of adenosine analogs, and developing a screening tool to identify such compounds would be valuable.

A library of equimolar adenosine analogs - plus the substrate adenosine - was injected into the chamber with trapped adenosine deaminase. All 20 substrates were easily detected using an electrospray single quadrupole mass spectrometer as they eluted from the ultrafiltration chamber. This method showed an effective way to identify compounds that bound to the protein, and it was even possible to measure affinity constants as the ligands dissociated from the protein and into the mass spectrometer. This method has a problem with throughput – only one pulse can be injected into the chamber at one time and must be washed before the next injection. As a drug discovery method, the technique is feasible, but the throughput ultimately limits this generation of PUFMS.

This PUFMS chamber was used again to screen a small chemical library for ligands of dihydrofolate reductase[5]. An equimolar library of 22 compounds, six with known affinity for dihydrofolate reductase, was injected into the protein trapping chamber containing enzyme. This work added complexity to PUFMS because the library contained known ligands as well as compounds that should not bind. However, there was an issue with nonspecific binding of compounds from the library. While two

ligands were detected, managing the effect of the nonspecific binding signals was recognized as a possible source of interference. Normally using methanol to elute compounds from the protein would also elute compounds nonspecifically binding to the chamber introducing noise into the mass spectrometer. The solution to this nonspecific binding problem was to elute the ligands from the protein using a low pH mobile phase – of all the elution techniques investigated, low pH elution yielded the lowest noise from nonspecific binders.

The value of this work arises from the eventual goal using PUFMS to analyze complex natural product extracts. One of the continuing struggles is to minimize the signal of nonspecific binders. In this work, there were only 16 compounds with no affinity to the protein. However, in the case of natural product extracts, that number could be hundreds to possibly thousands of compounds.

As a test of the methodology, a COX-2 PUFMS assay was developed.[6] COX-1 and COX-2 are important drug targets as nonsteroidal anti-inflammatory agents bind to these targets. In a blind study, bacterial broth, DMSO or plant extracts were spiked with known ligands to COX-2 to evaluate if PUFMS can effectively handle complicated matrices and isolate ligands with IC_{50} values ranging from 100 µM to 10 nM and then identify them using mass spectrometry. Human recombinant COX-2 was trapped in the pulsed ultrafiltration chamber using a 30 kDa filter. The samples were eluted from the chamber using acidified methanol solvent and trapped on a C_{18} cartridge. Then using this C_{18} cartridge as a column, all bound compounds were eluted and identified using liquid chromatography with negative mode electrospray mass spectrometry. Each supplied sample had been spiked with two to three ligands at routine screening

concentrations, and all spiked compounds in this blind study were identified as hits. Most importantly, the complicated matrices of the bacterial fermentation froth or the plant extract had no effect on the signal of the spiked ligands.

The importance of this work is critical to the theoretical possibility of a mass spectrometric drug discovery tool; this work showed that PUFMS could be used to isolate and characterize ligands to macromolecular targets such as proteins in extremely complicated matrices. Further, using mass spectrometric detection provides data for characterization and perhaps even identification of hits without a long and arduous fractionation procedure.

Pulsed Ultrafiltration: Gen 2

Evolving from the chamber, pulsed ultrafiltration as a mass spectrometry protein ligand binding assay began to utilize ultrafiltration spin cartridges, which facilitated parallel separations and higher throughput. Like Gen 1 PUFMS, these ultrafiltration cartridges could be equipped with membranes of a variety of molecular weight cutoffs: 10 kDa, 30 kDa, 50 kDa, and 100 kDa. They also fit within a 1.5 mL centrifuge tube for more rapid separation of protein-bound ligands from non-ligands. Instead of having a single chamber with protein trapped, a number of centrifuge tubes can be used in parallel limited only by the amount of rotor space available to spin these cartridges. These membranes are filtered by spinning these tubes at high speeds to separate nonbinding compounds from proteins trapped above the membrane. Nonbinding compounds are removed during the centrifugation step while ligands bound to the protein are trapped on the protein above the membrane. A subsequent step interrupting the protein ligand

interactions and a further spin will push the previously bound ligands through the membrane leaving the protein above the membrane. Upon this second spin, the eluent is collected and prepared for LC-MS analysis. This general procedure is described in Figure 2.

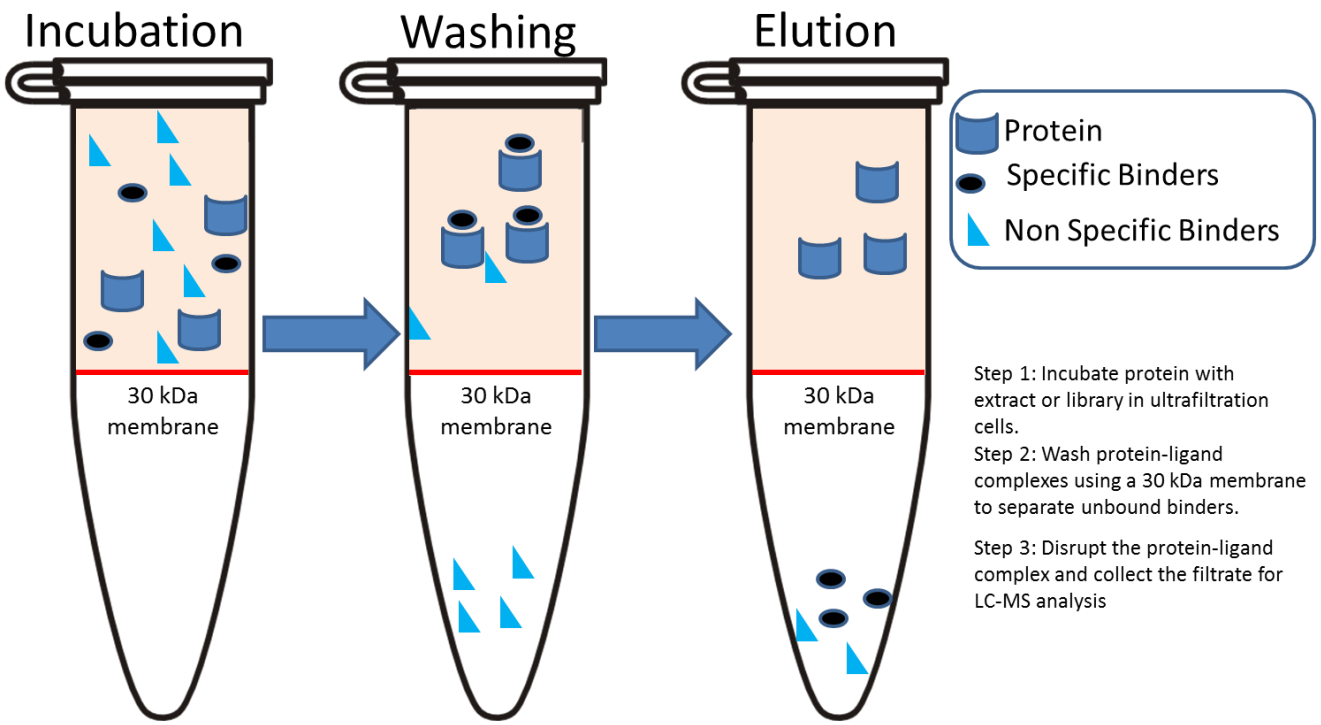


Figure 2 - Pulsed Ultrafiltration Gen 2. The general procedure involves incubating the protein above a 30 kDa membrane then washing away all non-binders. Then the protein ligand complex is disrupted and the eluent from the membrane is collected for LCMS analysis

Liu *et al*. 2001[7] first demonstrated this second generation of PUFMS when screening red clover (*Trifolium pratense*) extracts for estrogenic compounds. Red clover is a commonly used in botanical dietary supplements by women seeking menopausal relief from menopausal symptoms such as hot flashes. Although cell based Ishikawa assays had been used to determine that red clover extracts were estrogenic, but the specific estrogenic compounds were uncertain. PUFMS was used as a screening assay to replace bioassay guided fractionation in identifying these active compounds.

Using a 30kDa membrane, the extract was incubated with the estrogen receptor-β. After washing and spinning to remove non-binders, the protein ligand complex was disrupted using methanol. Another spin and the eluent were recovered and analyzed using LC-MS. Isoflavones were identified as responsible for the estrogenic activity in the Ishikawa assay as follows: genistein, daidzein, biochannin A, and formononetin. The significance of this work begins to show the strength of PUFMS. In previous studies, simple combinatorial libraries of known compounds or standards spiked into different matrices were screened using PUFMS. This was the first application of PUFMS to the identification of ligands to a macromolecular receptor (here estrogen receptor-β) in a complex botanical extract. Although the throughput of PUFMS was enhanced by using ultrafiltration spin cartridges, Pulsed Ultrafiltration Gen 2 lost the capability of measuring affinity constants. Based on signal intensities in the mass spectrometer, it was possible to compare relative binding affinities of ligand but not measure them.

Applications of Pulsed Ultrafiltration Gen 1 and 2

Following these milestones in the invention and development of mass spectrometry-based screening, new applications of Pulsed Ultrafiltration Gen 1 and Gen 2 have expanded their utility in a variety of different directions. Gen 1 was used to study human serum albumin and the binding of warfarin[8], further refined targeting carbonic anhydrase for purposes at Eli Lilly,[9] the identification of estrogenic compounds from chaste-berry,[10] identifying electrophilic quinoids in dietary supplement for safety concerns,[11] finding xenobiotic electrophilic metabolites binding hepatic liver microsomes,[12] developing a technique to monitor metabolic stability of drugs using liver microsomes,[13] and the effects of cranberry extracts on *E. coli* and COX-2.[14] The single chamber version of PUFMS, while effective, was limited in throughput. Moving to the centrifuge spin membrane approach dramatically enhanced throughput but eliminated the ability to measure affinity constants.

Identifying ligands binding to COX-2 from ginger[15] and other natural products,[16] RXRα,[17] investigating the role of small molecules in β-amyloid protein aggregation,[18] developing a competitive binding assay to compare the estrogenic compounds in hops and red clover,[19] screening for quinone reductase-2 natural products,[20] are many examples of the application of PUFMS from the van Breemen research group. The use of PUFMS for natural product ligand discovery continues to grow, especially in regard to Chinese medicine. For example, discovering xanthine oxidase inhibitors from chrysanthemum,[21] characterizing tyrosinase inhibitors in mulberry leaves,[22]development of a *Mycobacterium tuberculosis* ligand-binding assay,[23] characterizing inhibitors of α-glucosidase from hawthorn leaf flavinoids[24] and from *Rhizoma Coptidis* and *Radix Rehmanniae,*[25] identifying aromatase inhibitors from *Corydalis yanhusuo,*[26] and

identifying MMP-2 inhibitors from *Carthamus tinctorius* L.,[27] are some of the numerous examples of the application of Pulsed Ultrafiltration Gen 2. A recent review from H. Wei, *et al*. 2016[28] described 38 additional applications of Pulsed Ultrafiltration Gen 2 for identifying natural product binders to a variety of targets. It further details 16 applications using pulsed ultrafiltration for purification of biological samples to monitor concentrations of specific drugs. The applications of the three generations of PUFMS are described in Table 1.

Table I - APPLICATIONS OF THE THREE GENERATIONS OF PULSED ULTRAFILTRATION.

GEN 2 HAD THE MOST WIDESPREAD USE OUT OF ALL THREE; THERE WAS A FORAY INTO GEN 3, BUT PUFMS LIMITATIONS PREVENTED FURTHER DEVELOPMENT

Pulsed Ultrafiltration Gen 1
Warfarin and Serum Albumin[8]
Carbonic Anhydrase[9]
Estrogenic Compounds of Chaste Berry[10]
Electrophilic Quinoids in Natural Products[11]
Metabolic Stability with Liver Microsomes[12]
Cranberry Extracts on *E.coli* and COX-2[14]

Pulsed Ultrafiltration Gen 2
Ginger and COX- 2[15]
COX -2 and Natural Products[6]
RXRα[17]
β-Amyloid Protein Aggregation[18]
Competitive Binding of Estrogenic Compounds of Hops and Red Clover[29]
Quinone Reductase-2 and Natural Products[20]
Xanthine Oxidase inhibitors from Chrysanthenum[21]
Tyrosinase Inhibitors from Mulberry Leaves[22]
Mycobacterium Tuberculosis Ligand Binding[23]
α-Glucosidase from Hawthron Leaf Flavinoids[24]
α-Glucosidase from *Rhizoma coptidis* and *Radix rehmanniae*[25]
Aromatase Inhibitors from *Corydalis yanhusuo*[26]
MMP-2 Inhibitors from Carthamus tinctorius[27]
Carbonic Anhydrase[30]

Pulsed Ultrafiltration Gen 3
VDR[31]
Carbonic Anhydride[30]

Limitations of Pulsed Ultrafiltration

Pulsed Ultrafiltration Gen: 3

Pulsed Ultrafiltration Gen 2 operates through the use of ultrafiltration cartridges and centrifugation. However, centrifuge rotors have limited capacity, and normal laboratory sized centrifuges can hold approximately 28 centrifuge spin tubes at a time. With the need of a control experiment to correct for nonspecific binding, throughput is cut in half. Although higher compared to the Pulsed Ultrafiltration Gen 1, the bottleneck in throughput becomes availability of spaces in the rotor. Upgrading to a 96-well plate to improve the throughput of this assay was investigated by J White of the van Breemen laboratory in his dissertation.[31]

White used the vitamin D receptor as the macromolecular target for further development of PUFMS. A drug target, the vitamin D receptor has a role in inflammation, osteoporosis, certain cancers, and autoimmune diseases.[32] To develop proof of principle, PUFMS for ligands to the vitamin D receptor was developed first using the Gen 2 approach and then in 96-well plate format (Pulsed Ultrafiltration Gen 3). AM6-36, a synthetic compound that binds to vitamin D receptor and cholecalciferol, a known binder to vitamin D receptor, were used as positive controls, while 8-prenylnaringinin, a non-binder to vitamin D receptor, served as a negative control. Using these compounds, a Gen 2 ultrafiltration assay was validated using centrifuge tubes fitted with 30 kDa membranes.

Next, the method was transferred to a 96-well plate format to enhance throughput by almost 100-fold. Microtiter plates (96-well) containing a 10 kDa

membrane were obtained from Millipore (Billerica MA). Centrifugal separation of bound from free ligands was achieved by using a microtiter plate rotor.

Unfortunately, the 96-well plate separation was unsatisfactory. Moving to a smaller molecular weight cutoff (10 kDa but not 30 kDa membranes were available from commercial sources) drastically increased the time required for ultrafiltration separations. Another limitation was slower centrifugation of the 96-well plate rotor compared with the ultrafiltration centrifugation tube rotor. These two simultaneous effects, the smaller cutoff membrane and the slower centrifugation, multiplied the time required to separate ligand-protein complexes from the unbound small molecules. Whereas each centrifugation for Pulsed Ultrafiltration Gen 2 required approximately 5-10 minutes, 96-well plates required several hours of centrifugation. Instead of a 100-fold increase, at best, a tenfold increase was gained from the move to a 96 well plate in Gen 3 compared to an equal number of ultrafiltration centrifuge tubes in the Gen 2 system.

One of these concerns is limited by the availability of the membranes on the plates. For general commercial purposes, the 96-well plates available for ultrafiltration are limited to 10 kDa filters. The problem with rotor speed is a limitation with the ultrafiltration microtiter plate design. While there are faster centrifuges than that used by White to speed up the filtration process, the membranes in these ultrafiltration plates are quite fragile. Working closely with Millipore scientists, this problem seemed to be unresolvable. Any faster rotation beyond the recommended spins velocities could rupture the ultrafiltration membrane and produce unreliable data.

Concurrent External Development of Pulsed Ultrafiltration Gen 3

Working concurrently on pulsed ultrafiltration was a research team at Abbott.[30] They reported that pulsed ultrafiltration was being used in their high-throughput screening efforts,[33,34] and there was an effort to evolve this technique into a 96-well format for the introduction of automation. Using carbonic anhydrase as the target protein, the goal was to screen libraries containing upwards of 150,000 compounds. During their survey of three affinity screening techniques, they separately developed pulsed ultrafiltration Gen 3. Spinning the plates during washing steps revealed a separate problem from this iteration of Gen 3. The Abbott researchers found variable remaining amounts of liquid above the ultrafiltration membrane. The Abbott team found that Gen 2 screening worked for carbonic anhydrase, but Gen 3 did not.

During the centrifugation of the 96-well plate, the force is not evenly spread across the plate. Although all wells in the microtiter plate have the same rotational frequency in a centrifuge, but speed of each well depends on its distance from the axis of rotation. Newton's second law for a particle in uniform circular motion describes that the particles experience different centrifugal forces based on their distances from the axis. Each well is not necessary in the same axis of rotation – the center of the plate is the center and the wells will radiate outwards to the edges of the plate. Each well will have different force acting on the liquid being forced through the membrane, ultimately creating variable amounts of filtration.

This variable amount of liquid filtered through the membranes is problematic for the development of a method for high throughput automation. In this particular case, there would be irreproducible filtration of each well leading to irreproducible data. This

issue can be overcome by increasing the time for filtration (longer spinning times). This issue was not reported by White, who used very long spin times.

Alternatives to Pulsed Ultrafiltration

With the problem of slow centrifugation limited by the integrity of the membrane and the long spin times required to filter all the wells completely, it seemed as if the constraints of pulsed ultrafiltration might prevent it from becoming a high throughput screening technique. Prior to the development of Pulsed Ultrafiltration Gen 3, Choi in the van Breemen laboratory had developed a different screening assay for ligands binding to estrogen receptor.[35] As an alternative to ultrafiltration, the estrogen receptors in this mass spectrometry screening assay were captured using magnetic microbeads.

Beads functionalized with either aldehyde or carboxylic acid groups were purchased from Bioclone (San Diego, CA). Using procedures detailed from the manufacturer, Choi covalently immobilized estrogen receptors to the magnetic beads. In general, the procedures of the magnetic bead MS-based assay resemble those of pulsed ultrafiltration. First, the bead-tethered protein receptor is incubated with the natural products or combinatorial library. The beads are washed with buffer to removed unbound compounds while preventing the loss of the protein with a magnet. Finally, the bound ligands are released for LCMS analysis using a denaturing solution while the beads are retained using a magnet. Figure 3 provides a general overview to Choi's assay.

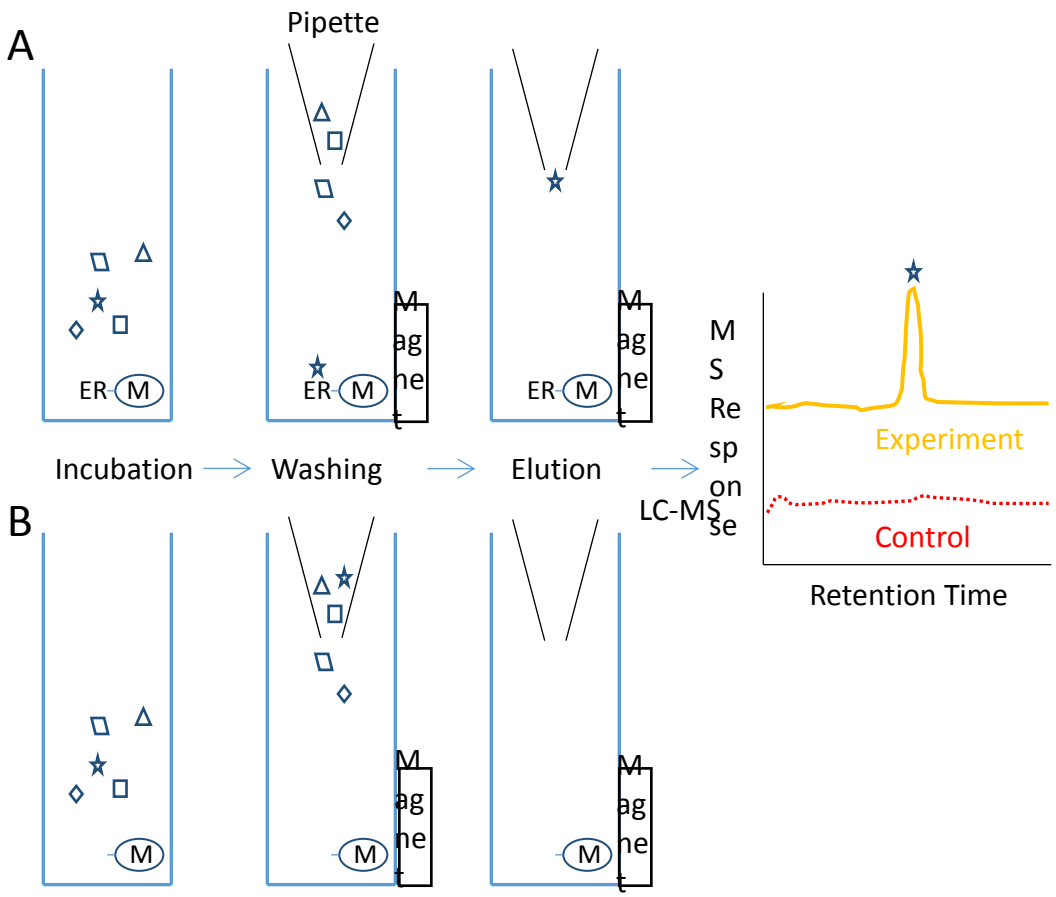


Figure 3 - Choi *et al*'s schema for a magnetic bead alternative to PUFMS. This assay follows the similar steps of PUFMS: incubation, washing, and elution then analysis on LCMS.

In this work, Choi optimized the conditions for the immobilization of estrogen receptors for two bead chemistries and determined that while covalent immobilization produced active beads, noncovalent affinity immobilization of biotinylated estrogen receptor β proved to be more effective in the screening assay. Using magnetic beads with the streptavidin functional group, the protein could be captured noncovalently. This doubled the effective response of the internal standards when detected by mass spectrometry compared to the covalent immobilizations. Choi speculated that covalent immobilization might partially block the protein active site or produce tertiary protein changes.

Using either method worked, albeit the noncovalent magnetic tethering provided better response of the standards. Red cover and hops extracts were screened using these protein-tethered magnetic beads. From the red clover extract, for example, daidzein, genistein, formononetin, and biochannin A were identified using negative electrospray LCMS. This finding was consistent with Liu's development of a Pulsed Ultrafiltration Gen 2 assay screening these extracts.[36]

This work developing an alternative to pulsed ultrafiltration as a natural product screening and combinatorial chemistry screening tool made possible MS-based automated high-throughput screening of complex mixtures such as botanical extracts. Although the limitations of pulsed ultrafiltration prevented it from evolving as a high-throughput drug discovery system, this magnetic bead approach overcame many of those limitations. There are no membrane integrity concerns using magnetic beads. There is no centrifugation or variability using these magnetic beads from well-to-well. In this dissertation, this technique, now called Magnetic Microbead Affinity Selection Screening (MagMASS) was developed further as a high-throughput drug discovery

technique. Previous iterations of this style of natural product drug discovery also included a bioassay prior to the PUFMS. The end goal of MagMASS is to develop a screening technique that can identify potential compounds without a prior bioassay. First, MagMASS was explored for the screening of small molecules for ligand to the anticancer and anti-inflammation target, retinoid X receptor α.

CHAPTER 2: DEVELOPMENT OF MAGNETIC MICROBEAD AFFINITY SELECTION SCREENING ASSAY FOR RETINOID X RECEPTOR

Adapted from Rush, M.D., Walker, E.M., Prehna, G., Burton, T., van Breemen, R.B. Development of a magnetic microbead affinity selection screen (MagMASS) using mass spectrometry for ligands to the retinoid X receptor-α. *J. Am. Soc. Mass Spectrom.* (2017) 28: 479. doi:10.1007/s13361-016-1564-0 with permission.

Introduction

Approximately 50% of the cancer drugs used in the last 50 years have been inspired by natural products.[37] The main sources of discovery of these therapeutic natural product compounds have been ethno medicine and biological screening.[38,39] While these sources have historically been valuable for drug discovery, modern methods using reverse pharmacology drug discovery techniques (also known as target-based drug discovery) have been underutilizing natural products.

Instead of the slow process of testing for changes in a living cell or organism in response to a compound, knowledge of the disease-relevant receptor-ligand interactions allow for interrogation of a specific pathway. For example, a recombinant protein can be exposed to a single compound or mixture of compounds and any receptor-ligand interactions can be detected by using fluorescence, enzymatic product

formation, thermal stability change, or another method. After a hit is detected, the individual compound must be identified, isolated and retested for activity, and eventually developed as a drug lead. However, mixtures of natural products such as botanical extracts are rarely used in high-throughput screening primarily because of the extra expertise and time required to identify active constituents.[39]

As with any reverse pharmacology drug discovery screen, biological relevance depends upon the choice of target. In this investigation, the retinoid X receptor-α (RXRα) was used as the target protein because it is an important nuclear receptor in the cancer protein network, is a known target for multiple chemotherapy agents, and unlike most other nuclear receptors, few ligands are known.[40,41] The endogenous ligand for RXRα is 9-*cis* retinoic acid, a derivative of vitamin A.[42] A knock-out mouse of RXRα develops a phenotype similar to vitamin A deficient mice with characteristic developmental morphology, differentiation, and cellular growth.[43] One major mechanism of cell death linking RXRα activity with cancer is through its inhibition of NF-E2 P45-related factor 2 (Nrf2).[44] Treatment with an RXRα ligand can modulate the genes regulated by Nrf2, including critical cytoprotective genes implicated in cancer. Several RXRα ligands, such as bexarotene, have received FDA approval to treat lymphoma,[40,45] but these compounds have serious toxic side effects. Therefore, less toxic RXRα ligands are needed for therapeutic use.

RXRα has several structural requirements for biological activity and use in high-throughput screening. After binding a ligand, RXRα monomers change conformation and dimerize to form an active confirmation that can bind to DNA.[46,47] Because RXRα

dimerizes with one-third of all nuclear receptors including itself, the challenge in targeting RXRα is obtaining specificity.[48,49] The ligand binding domain (LBD) of RXRα is relatively independent, both structurally and functionally, and has been used instead of the full-length construct to study many structural and functional aspects of RXRα.[50,51] Both full-length RXRα and its LBD were considered in this paper.

To increase the throughput of reverse pharmacology natural product mixture screening, we developed Magnetic Microbead Affinity Selection Screening (MagMASS), in which the target of interest is tethered to magnetic beads instead of being free in solution[35]. To separate ligands from unbound compounds, the receptor-bound fraction is retained in a well of a microtiter plate using a magnet while the unbound fraction is removed (Figure 4). Magnetic beads are often used for affinity isolation of proteins from complex mixtures,[52] but they are less often used to isolate small molecule ligands to a target for discovery.[53]

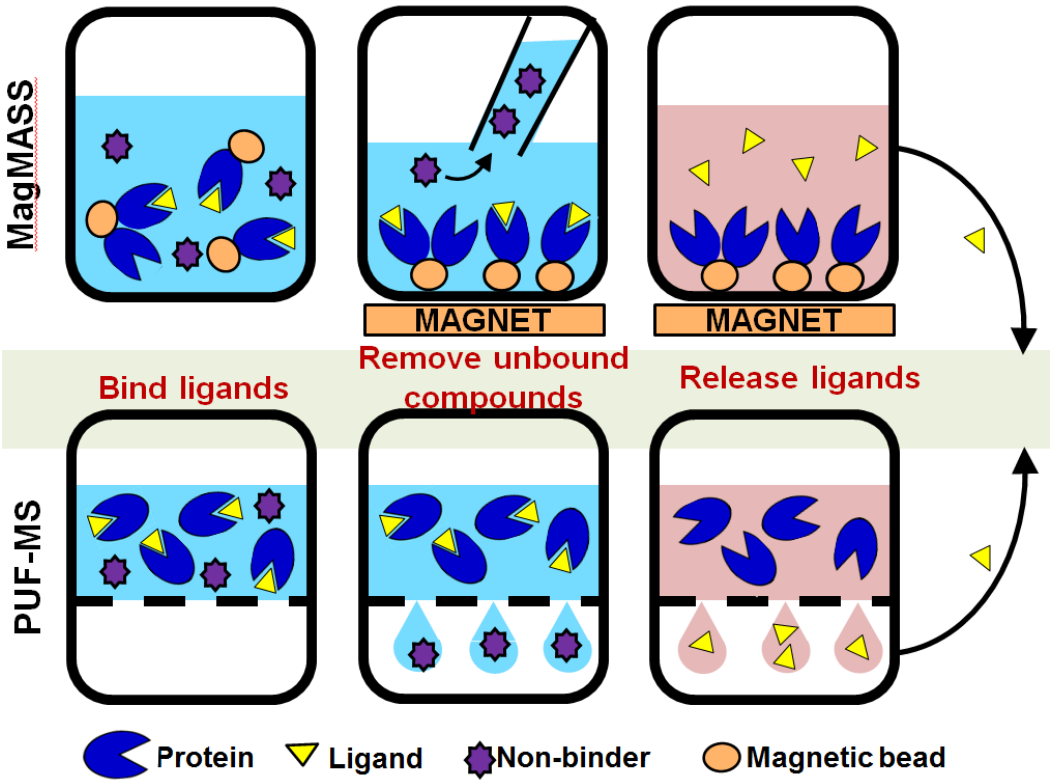


Figure 4 Comparison of PUFMS and MagMASS. During affinity selection screening, ligands (yellow triangles) but no other low molecular weight compounds (purple stars) bind to a macromolecular target (RXRα, blue). In PUFMS, the ligand–receptor complexes are separated in solution from nonbinding compounds by filtration through an ultrafiltration membrane (grey dashed line). Ligands are released by denaturing the receptor and recovered in the ultrafiltrate for LC-MS analysis. **(b)** During MagMASS, an external magnetic force is applied to secure the magnetic beads (orange ovals) containing immobilized receptor and affinity bound ligand while the unbound compounds are washed away. Ligands are released using organic solvent and/or a pH change and separated from the magnetic beads for UHPLC-MS analysis. Reprinted with permission from Rush, M.D., Walker, E.M., Prehna, G. *et al. J. Am. Soc. Mass Spectrom.* (2017) 28: 479. doi:10.1007/s13361-016-1564-0. Copyright 2017 ACS.

With the goal of increasing the throughput of affinity mass spectrometric-based screening, a new MagMASS assay was developed using RXRα as the target protein, which enabled direct comparison with a previously developed PUFMS RXRα assay. Although both full-length and the ligand binding domain of RXRα were active in the PUF-MS assays, only the full-length form of RXRα was active after covalent immobilization for MagMASS. Both mass spectrometry based screening methods were effective at affinity isolation of RXRα ligands from complicated matrices such as botanical extracts. The throughput of both approaches benefited equally from the substitution of UHPLC for HPLC during MS analyses and from the application of metabolomics software for automated UHPLC-MS data analysis. However, MagMASS provided several distinct advantages over PUFMS, including six-fold faster separation of bound ligand from unbound.

Experimental Information

Chemicals and Reagents

Ketoconazole, LG100268, 9-cis-retinoic acid, and 13-cis-retinoic acid were purchased from Sigma-Aldrich (St. Louis MO, USA). Polypropylene 2-mL conical bottom 96-well plates were purchased from Fisher Scientific (Hanover Park, IL). Amicon Ultra Centrifugal Filters 10 K and 30 K were purchased from Millipore (Billerica, MA, USA). Pierce *N*-hydroxy-succinamide (NHS)-activated magnetic beads were purchased from Thermo Scientific (Rockford, IL, USA), and amylose-functionalized magnetic beads

were purchased from New England Biolabs (Ipswich, MA, USA). NanoOrange kits (Life Technologies, Hanover Park, IL) were used to measure protein concentrations. Deionized water was prepared using a PureLab Option-Q purification system (Elga, Woodridge, IL).

A 20-compound equimolar mixture (10 μM each in methanol) of non-RXRα ligands was prepared from an in-house library and was tested for purity using a Shimadzu (Kyoto, Japan) ion trap-time-of-flight high resolution mass spectrometer: PA452, peroxicam, indomethacin, thiamine HCl, custom steroid II-39, custom steroid II-17-1, melatonin, oxomate, acetaminophen, spirobrassinin, yangonin, harmaline, flavone, N-methylserotonin, custom carbolene, isoliquiretigenin, tolbutamide, eridictual, formononetin, and naringenin. The compounds were selected to simulate the chemical diversity of both combinatorial libraries and natural product libraries without introducing the matrix associated with a botanical extract. Aerial plant parts (leaf, stem and inflorescence) of *Proserpinaca palustris* L. (mermaid weed) and *Stenaria nigricans* (Lam.) Terrell (diamond flowers) were obtained from the Chicago Botanic Garden (Chicago, IL) where they were taxonomically identified. Voucher specimens (herbarium voucher numbers 13706 and 18630) were deposited in the Chicago Botanical Garden Herbarium. Extracts were prepared via cold percolation in methanol and dried by rotary evaporation. The extracts were reconstituted at 40 mg/mL in methanol.

RXRα, RXRα ligand binding domain (LBD), and maltose binding protein (MBP).

The plasmid for recombinant RXRα was generously provided by Prof. Matthew Redinbo (University of North Carolina, Chapel Hill, NC). Protein was expressed and purified as described previously. Briefly, both MBP (*N*-terminus, 43 kDa) and the SRC-1 co-activator peptide (*C*-terminus, 2 kDa) are present on the protein construct and required for enhanced stability and solubility of full-length RXRα, totaling a 96 kDa protein.[54] The RXRα construct was transformed into BL21(DE3) cells for over-expression and grown at 37 °C in LB media until reaching A600 > 0.6, upon which isopropyl β-D-1-thiogalactopyranoside was added to a final concentration of 1 mM. The temperature was reduced to 20 °C, and the cells were grown for an additional 20 h. After expression, cells were harvested by centrifugation and lysed by using an Emulsiflex C5 (Avestin, Ottowa, ON). The lysate was cleared by centrifugation at 30,000×*g*, and the soluble material was passed over NTA-Sepharose beads (Life Technologies, Grand Island, NY). The NTA-Sepharose beads were washed with 50 column volumes of 50 mM Tris (2-carboxyethyl)phosphine (pH 7.5) containing 500 mM NaCl and 25 mM imidazole, and the protein was eluted from the column in this buffer with the imidazole concentration increased to 500 mM.

Half of the purified protein was exchanged into 25 mM Tris (pH 7.5) 250 mM NaCl, 20% glycerol, and 0.5 mM TCEP (tris(2-carboxyethyl)phosphine) by overnight dialysis or by gel-filtration using an SD75 column (GE Healthcare, Pittsburgh, PA) to produce pure MBP-RXRα with a final concentration of 15 μM. The remaining starting material was digested with a 1:50 ratio (mol/mol) of TEV protease overnight at 4 °C while being dialyzed into the low imidazole buffer to remove RXRα. After TEV protease digestion, the His6-MBP protein was re-purified using NTA-Sepharose beads and again

dialyzed into the same buffer as MBP-RXRα. Purified MBP alone was used as a control for full-length MBP-RXRα screening experiments. An SDS-PAGE gel was used to confirm the molecular weight and purity of MBP-RXRα and MBP, which showed that MBP was present as a single band of 43 kDa and that MBP-RXRα appeared at 96 kDa and showed some minimal degradation at lower molecular weight bands (Figure 5).

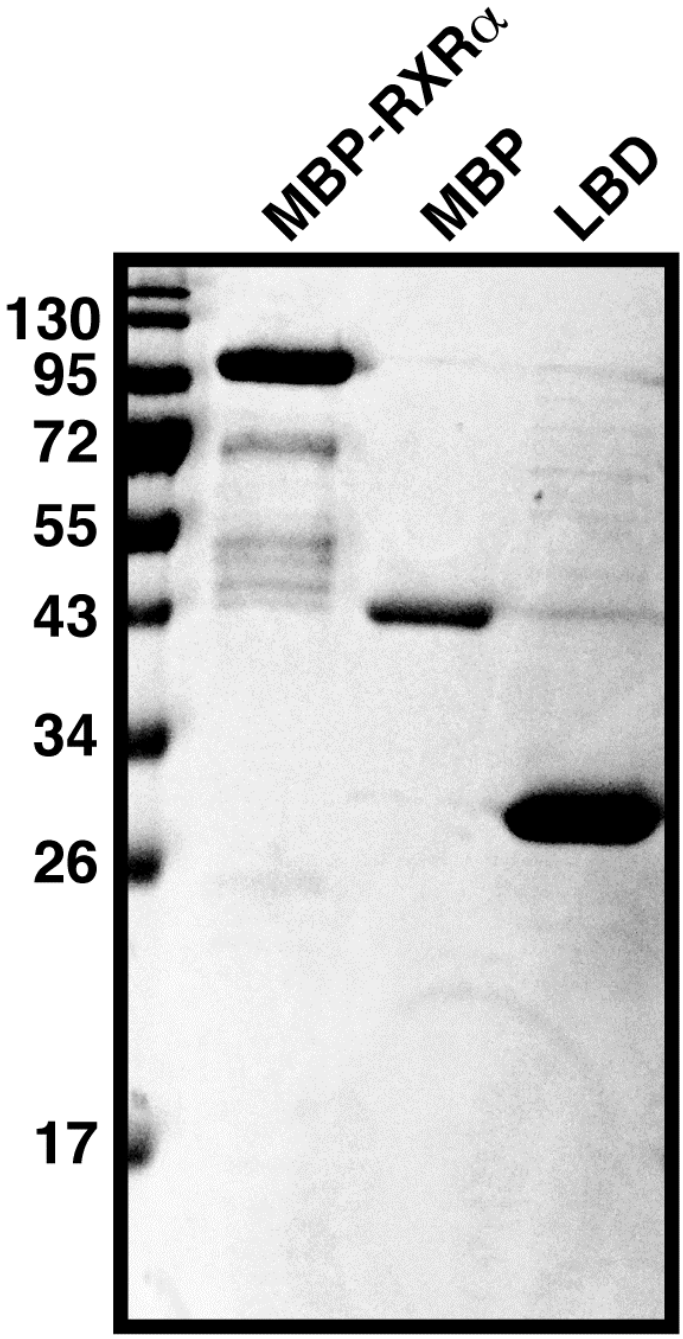


Figure 5. Purification of RXRα protein constructs. A 15% SDS-PAGE 24 gel stained with Coomassie blue shows the relative purity of MBP-RXR (96 kDa), MBP 25 (43 kDa), and the RXRα ligand binding domain (LBD) (27 kDa). The far left lane contains 26 molecular weight standards labeled in kDa. Reprinted with permission from Rush, M.D., Walker, E.M., Prehna, G. *et al*. *J. Am. Soc. Mass Spectrom*. (2017) 28: 479. doi:10.1007/s13361-016-1564-0. Copyright 2017 ACS.

The LBD of RXRα (expressed in *E. coli*), corresponding to amino acids 223 – 463 and an apparent molecular weight of 27 kDa, was purchased from Active Motif (Carlsbad, CA) at 6.17 mg/mL in 50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM dithiothreitol, and 50% glycerol. SDS-PAGE was used to confirm the molecular weight and purity of RXRα LBD, which showed only minimal degradation and minor dimerization (Figure 5). Denatured LBD was prepared by heating at 90 °C for 10 min and was used as a negative control for RXRα LBD experiments.

Immobilization of proteins on magnetic beads.

N-Hydroxy-succinimide (NHS)-activated magnetic beads (20 µL per well, 10 mg/mL beads, approximate binding capacity 20-50 µg protein/mg of bead) were used to immobilize MBP-RXRα, control MBP, LBD, or denatured LBD. A 60-lb magnetic plate consisting of 12 neodymium rare earth magnets (N45 2 × 1 × 1/8" NdFeB) (CMS Magnetics, Garland, TX) was used to retain the beads while storage buffer was removed. The beads were washed with 1 mM HCl, and the beads were resuspended in 300 µL 50 mM borate buffered to pH 7.5. Protein was immobilized by incubating 100 pmol protein with the beads at room temperature for 1 h with gentle shaking every 5 min. Proteins remaining in the supernatant were saved for quantification using a NanoOrange kit. Beads were washed twice with 0.1 M glycine buffer (pH 2.0) and washed once with water. Unreacted NHS sites were saturated by incubating with excess 3 M ethanolamine at pH 9.0 for 1 h. The beads were washed once with water

and resuspended in 300 μL TBS buffer containing 50 mM Tris-buffered saline and 500 mM NaCl for the binding assays.

For immobilization on amylose magnetic beads, the beads were washed with water and resuspended in TBS buffer containing 50 mM Tris-buffered saline and 0.5 M NaCl. Beads (20 μL, 10 mg/mL) were incubated with the target protein, RXRα-MBP or MBP (100 pmol), and a ligand (10 pmol). The amylose magnetic beads will bind with the MBP, and the ligand will bind with the RXRα. The incubation solution was removed from the amylose beads, and unbound protein was quantified using the NanoOrange kit.

RXRα assays.

All screening experiments contained the same incubation buffer (0.2 M NaCl and 20 mM Tris-HCl adjusted to pH 7.4) at the same incubation volume (300 μL), and identical amounts of ligand(s) (33 μM each) and protein (0.3 μM) – a 100 fold difference between ligand and protein moles. Experiments testing the matrix effects of botanical extracts contained *P. palustris* extract or *S. nigricans* extract at 133 μg/mL. Experiments evaluating possible interference from combinatorial libraries screened 20-compound mixtures (10 μM each in methanol).

PUFMS was carried out as described previously[5] with minor alterations. Briefly, the ultrafiltration membrane (10 kDa cut-off for RXRα LBD or 30 kDa cut-off for MBP-RXRα and MBP) was pre-washed with 150 μL binding buffer by centrifugation at 13,000×*g* for 10 min at 4 °C. Protein and ligand mixtures were incubated in the dark for 1 h at room temperature. Then, the unbound fraction was removed by centrifugation at

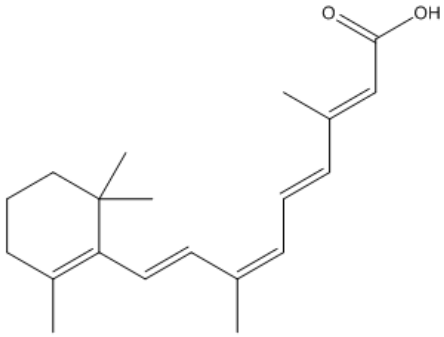
13,000×*g* for 10 min at 4 °C. Protein was washed three times with 300 μL portions of 30 mM ammonium acetate (pH 7.5). Ligand mixtures were eluted with two washes of 100 μL 90% methanol in water. The released ligands were evaporated to dryness and then reconstituted in 50 μL 80% aqueous methanol containing 100 nM ketoconazole internal standard immediately prior to LC–MS analysis.

MagMASS was carried out in 2-mL 96-well plates with conical bottoms. Beads (20 μL containing 100 pmol protein) and ligand were added to 279 μL incubation buffer, and incubated at 4 °C for 1 h. Beads were resuspended every 15 min using a multichannel pipette. Beads were retained on the magnetic plate while the unbound fraction was removed by washing 3 times with 900 μL aliquots of binding buffer and one last wash with water. Ligands were eluted by treating the beads with 100 μL methanol containing 50 nM ketoconazole (internal standard for UHPLC-MS) and transferred to clean wells. After evaporation to dryness, each ligand sample was reconstituted in 50 μL of 80% methanol.

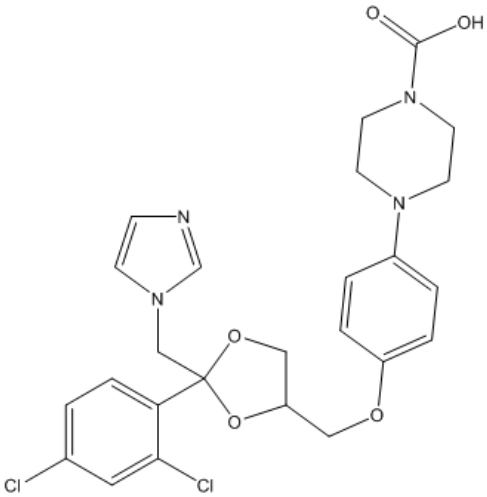
Quantification of proteins was performed using NanoOrange Protein Quantitation Kits as follows. Standard protein samples of MBP-RXRα, MBP, and LBD were prepared by dilution with NanoOrange diluent (2 μM sodium azide) to 20 μg/mL, 15 μg/mL, 10 μg/mL, 5 μg/mL, 2 μg/mL, and 0 μg/mL. The supernatants remaining after protein immobilization on magnetic beads were normalized to the background fluorescence due to the respective buffers. Regression equations were calculated from the protein dilutions normalized to fluorescence from diluent alone. Using this limit of detection and the theoretical amount of protein present in solution if no protein was bound to beads

after the immobilization step, the LOD of percent unbound was calculated for each protein in each buffer.

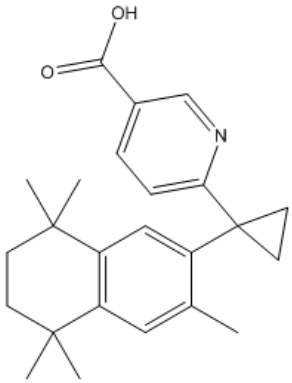
Ligand analyses were carried out using a Shimadzu LCMS-8040 mass spectrometer equipped with electrospray and a Shimadzu Nexera UHPLC system (Kyoto, Japan). Separations were carried out at room temperature using an in-line filter and a Waters Xterra C_{18} HPLC column (2.1 mm × 50 mm, 3 μm) or a BEH UHPLC column (2.1 mm × 50 mm, 1.7 μm). The mobile phase consisted of a 3-min linear gradient from 30% to 100% methanol in 0.1% formic acid in water at a flow rate of 0.6 mL/min. Each library compound, internal standard (ketoconazole), and ligand were measured using UHPLC-MS/MS with collision-induced dissociation and selected reaction monitoring (SRM) using optimized electrospray parameters (nebulizing gas flow 3 L/min, desolvation line 250 °C, heat block 400 °C, drying gas flow 15 L/min), collision energies and SRM transitions. A total of 50-transitions were monitored using a 2 ms dwell time for library screening or a 13 ms dwell time for quantitative analyses of ligands. The SRM transitions for quantitation included *m/z* 364 to 294 for LG100268, *m/z* 301 to 123 for 9-cis retinoic acid, *m/z* 301 to 81 for 13-cis retinoic acid, and *m/z* 531 to 244 for ketoconazole (see chemical structures in Figure 6). All the compounds were analyzed in a single method using the fast polarity switching (5 ms) of the Shimadzu LCMS-8040. The software used for collecting and viewing data was Shimadzu Lab Solutions Version 5.65.



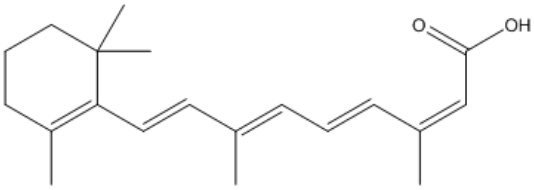
9-*cis*-Retinoic acid
SRM *m/z* 301 to 81



Ketoconazole
SRM *m/z* 531 to 244



LG100268
SRM *m/z* 364 to 294



13-*cis*-Retinoic acid
SRM *m/z* 301 to 123

Figure 6 Chemical structures and SRM MS/MS transitions of LG100268, 9-cis-retinoic acid, 13-cis-retinoic acid, and ketoconazole**.** Used for quantitation during MagMASS method development and validation, these SRM transitions were the most abundant among those detected for each compound during product ion tandem mass spectrometry.

Standards were prepared over the range 10 nM to 1000 nM containing ketoconazole at 100 nM as an internal standard. The upper limit of detection corresponded to 100% retention of a positive control ligand by the target protein. Specific binding was determined based on an increase in peak area in the UHPLC–MS chromatogram of a ligand relative to the corresponding negative control incubation with denatured protein. Statistics on the MagMASS and PUF-MS dataset were performed in Excel for Mac version 14.5.1. A one-way paired t-test was performed on the data for each experimental condition with replicates over four days.

Summary

Results and Discussion

To identify ligands of a target protein most efficiently, the MagMASS technique was optimized and validated at every step, from protein immobilization to the evaluation of matrix effects. Two magnetic bead functional group chemistries were evaluated for immobilizing the target protein: covalent tethering by NHS and immobilization by amylose-MBP affinity. A 10-fold excess of theoretical bead capacity was used to ensure efficient protein immobilization, and untethered protein remaining in solution after immobilization was measured. Both NHS and amylose magnetic beads retained MBP-RXRα or MBP alone with nearly 100% efficiency (Table 2). To verify the functionality of immobilized RXRα compared with solution-phase protein, affinity capture of the ligand LG100268 was compared using the established PUFMS approach[5] and MagMASS

using NHS beads (covalent attachment) or amylose-MBP beads (non-covalent immobilization). Using identical amounts of protein and ligand, LG100268 was detected as a specific ligand for RXRα using all three approaches with MBP as a negative control (Figure 7). Relative to the internal standard (ketoconazole), PUFMS showed the strongest signal for the affinity recovery of LG100268 (79% recovery; $p < 0.05$, $N = 6$) followed by NHS immobilization (33% recovery; $p < 0.001$, $N = 9$) and then amylose (21% recovery; $p < 1 \times 10\text{-}4$, $N = 8$)

TABLE II - PROTEIN IMMOBILIZATION EFFICIENCY ON MAGNETIC MICROBEADS. A NANOORANGE PROTEIN KIT WAS USED TO DETERMINE THE CONCENTRATIONS OF MBP-RXRA, RXRA-LBD, AND MBP AFTER IMMOBILIZATION ON NHS MAGNETIC BEADS OR AMYLOSE BEADS. IMMOBILIZATION EFFICNEY WAS CALCULATED FROM EXPERIMENTAL PROTEIN CONCENTRATIONS SUBTRACTED FROM CONCENTRATION LEVELS IN BUFFER ALONE. VALUES REPRESENT THE MEAN OF FOUR EXPERIMENTAL REPLICATES. REPRINTED WITH PERMISSION FROM RUSH, M.D., WALKER, E.M., PREHNA, G. *ET AL. J. AM. SOC. MASS SPECTROM*. (2017) 28: 479. DOI:10.1007/S13361-016-1564-0. COPYRIGHT 2017 ACS.

Immobilization type	Protein	Binding Efficiency (+/- standard error)
	MBP-RXRα	98.2 ± 2.1 %
NHS beads	MBP	95.4 ± 1.7 %
	LBD	>99.5 %
Amylose beads	MBP-RXRα	>99.5 %
	MBP	>99.5 %

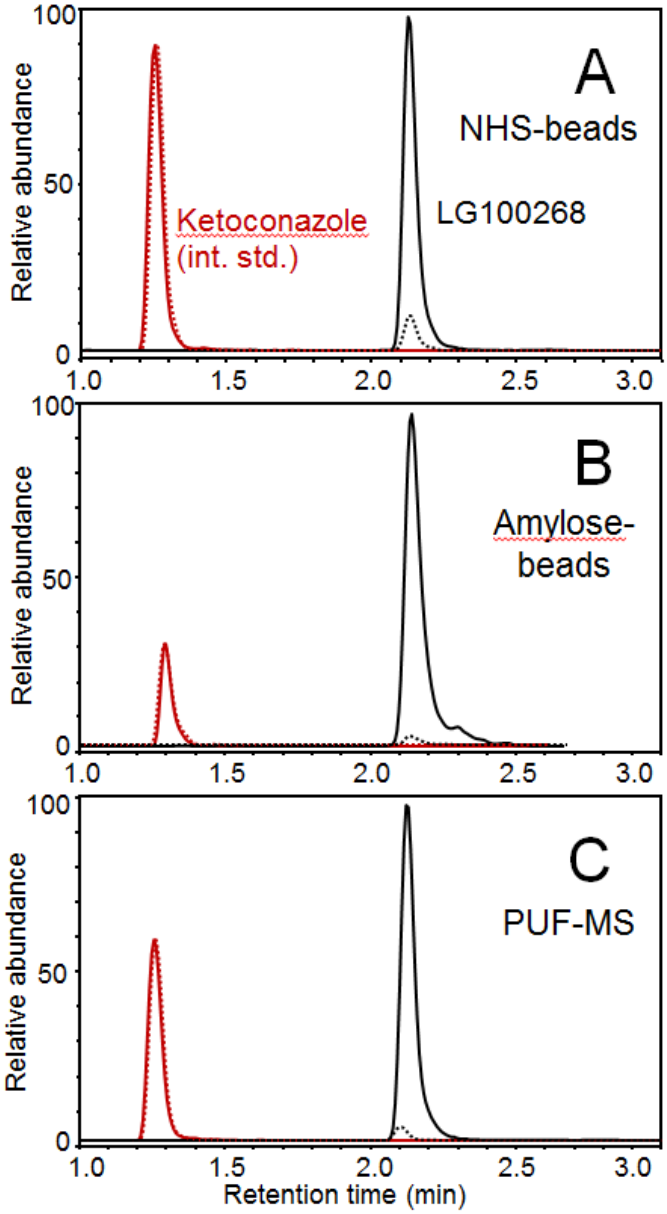


Figure 7 Comparison of MagMASS and Pulsed Ultrafiltration LC-MS (PUFMS) to test ligands for binding to MBP-RXRα. The UHPLC-MS/MS SRM chromatograms show the RXRα ligand LG100268 (SRM transition *m/z* 364 to 294, positive ion electrospray) retained by MBP-RXRα (solid line) compared with the MBP negative control (dashed line). Each incubation contained 100 pmol protein and 100 nM LG100268. The chromatograms were normalized to the internal standard, ketoconazole (100 nM; positive ion electrospray SRM transition *m/z* 531 to 244). (a) MagMASS using MBP-RXRα immobilized on NHS beads; (b) MagMASS using amylose beads to retain the MBP-containing protein; and (c) PUF LC-MS using the MBPRXRα protein in solution. Note that MBP is maltose binding protein (43 kDa) and that MBP-RXRα (96 kDa) is a stable construct of RXRα containing MPB on the *N*-terminus and SRC-1 co-activator peptide (2

kDa) on the *C*-terminus. Reprinted with permission from Rush, M.D., Walker, E.M., Prehna, G. *et al*. *J. Am. Soc. Mass Spectrom*. (2017) 28: 479. doi:10.1007/s13361-016-1564-0. Copyright 2017 ACS.

The covalent NHS mechanism of protein immobilization employs an *N*-succinamide functional group on the magnetic bead, which reacts with primary amines mostly on lysines and arginines of proteins. Although LG100268 was retained in high abundance during MagMASS using NHS-immobilized MBP-RXRα, the immobilization process (priming the beads, immobilizing the protein, and deactivating the unreacted sites on the beads) required ~4 h to complete. Furthermore, immobilization through random lysine and arginine residues, especially through multipoint attachment to the support, can change the functionality of the protein. Although immobilization of receptors and enzymes in some orientations can block access of ligands to the active site, selective protein immobilization can retain activity while enhancing stability such as resistance to denaturation.[53]

Proteins are often expressed with an MBP tag to stabilize the protein during recombinant expression, including the full-length RXRα construct used in this investigation. By leaving MBP on the RXRα target protein, no time had to be expended removing the tag and then repurifying RXRα. To demonstrate the feasibility of MagMASS for natural product discovery, MBP-RXRα immobilized on NHS-magnetic beads was incubated with the known ligand LG100268 spiked into a botanical extract. The UHPLC-MS/MS chromatograms in Figure 8 show that the botanical extract matrix did not interfere with MagMASS affinity extraction and detection of LG100268 as a ligand for immobilized RXRα. Similarly, LG100268 was detected with strong abundance using MagMASS screening of a small 20-compound library or no matrix at all (Figure 8). Table 3 shows additional MagMASS screening data for the selective detection of LG100268 in a botanical matrix extracted from *S. nigricans*. In each case, the ligand

LG100268 was identified with high confidence ($p < 0.006$) compared with controls carried out using denatured protein even in the presence of complicated matrices.

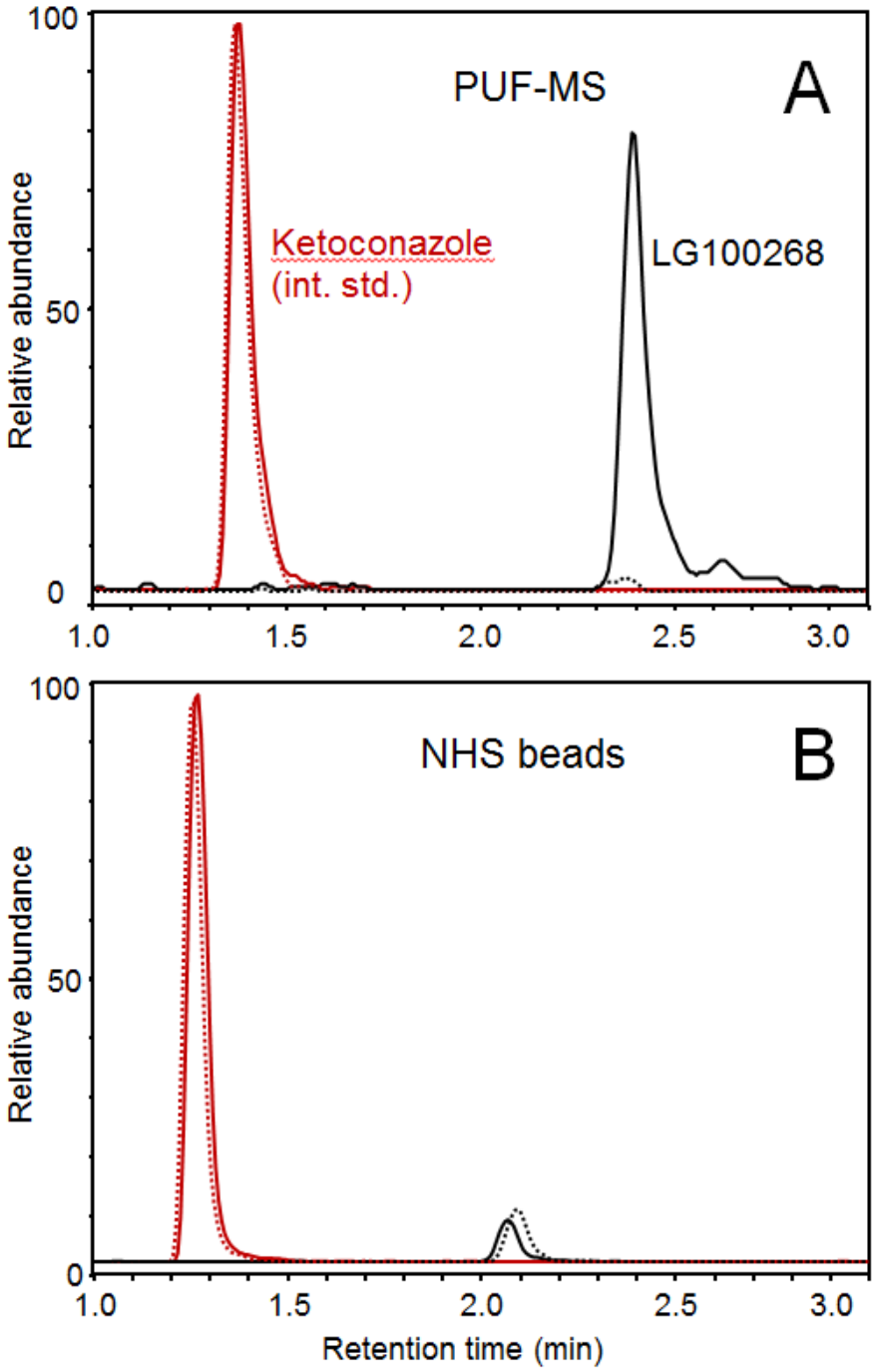


Figure 8 Comparison of PUFMS to NHS immobilization of the RXRα-LBD. While enrichment of LG100268 was observed using PUFMS, it was not observed when covalently immobilized using NHS beads. Reprinted with permission from Rush, M.D., Walker, E.M., Prehna, G. *et al. J. Am. Soc. Mass*

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The effects of matrix as well as substrate on MagMASS screening were investigated using not only MBP-RXRα immobilized on NHS-magnetic beads but also MBP-RXRα immobilized on amylose-magnetic beads (Table 3). Both immobilization methods produced active RXRα that efficiently bound LG100268 or the endogenous ligand 9-cis-retinoic acid in the presence of botanical extract matrix. This is indicated by the large enrichment factors in Table 3, which are calculated as the ratio of peak areas of specifically bound ligand to non-specifically bound ligand (noise). Because 9-cis-retinoic acid (K_d 15.7 nM[55]) has lower affinity for RXRα than does LG100268 (Kd 3 nM[40]), the probability of detecting 9-cis-retinoic acid was lower in the MagMASS screens (Table 3). Although 9-cis-retinoic acid could be detected with significant enrichment factors regardless of matrix when using MBP-RXRα immobilized on NHS-magnetic beads, 9-cis-retinoic acid was not always detected when assayed alone or with the 20-compound library using amylose-magnetic beads. This might have been caused by non-specific binding of 9-cis-retinoic acid to amylose that was blocked in the presence of the botanical extract. As a negative control, note that the non-ligand of RXRα, 13-cis-retinoic acid[55] (an isomer of 9-cis-retinoic acid) was not detected as a ligand of RXRα during MagMASS screening regardless of the matrix or the form of immobilized protein (Table 3).

TABLE III IMMOBILIZATION METHODS OF MAGMASS FOR ALL PROTEINS INVESTIGATED HAVE AN EXTREMELY HIGH BINDING EFFICIENCY. ALMOST ALL THE PROTEIN FOR IMMOBILIZATION IS AVAILABLE IN THE WELL FOR LIGAND BINDING.

RXRα Construct[a]	Matrix[b]	LG100268[c] Enrichment ± standard error[e]	9-cis-Retinoic acid[d]	13-cis-Retinoic acid
MBP-RXRα NHS	Buffer only	17.2 ± 13.9 (N = 9) $P < 0.01$[f]	6.7 ± 1.7 (N = 8) $P < 0.01$	1.1 ± 0.1 (N = 4) $P > 0.2$
"	Compound library	42.4 ± 90.1 (N = 8) $P < 0.01$	5.6 ± 1.9 (N = 8) $P < 0.01$	0.9 ± 0.1 (N = 5) $P > 0.2$
"	*P. palustris* extract	298.6 ± 459.7 (N = 7) $P < 0.01$	24.3 ± 7.2 (N = 6) $P < 0.01$	N/A (N = 4) $P > 0.2$
"	*S. nigricans* extract	72.5 ± 49.8 (N = 4) $P < 0.01$	12.0 ± 4.6 (N = 3) $P < 0.05$	N/A (N = 4) $P > 0.2$
MBP-RXRα amylose	Buffer only	8.6 ± 2.0 (N = 4) $P < 0.01$	2.8 ± 0.9 (N = 8) $P = 0.22$	0.9 ± 0.1 (N = 5) $P > 0.2$
"	Compound library	10.5 ± 4.2 (N = 7) $P < 0.01$	1.9 ± 0.4 (N = 8) $P = 0.06$	1.6 ± 0.3 (N = 4) $P > 0.2$
"	*P. palustris* extract	56.8 ± 34.3 (N = 9) $P < 0.01$	9.5 ± 2.2 (N = 9) $P < 0.01$	N/A (N = 5) $P > 0.2$
"	*S. nigricans* extract	59.0 ± 47.0 (N = 4) $P < 0.01$	6.1 ± 2.2 (N = 4) $P < 0.05$	N/A (N = 5) $P > 0.2$

[a]MBP-RXRα was immobilized using either covalent attachment of amino groups via NHS on the magnetic beads or through non-covalent interaction between MPB and amylose beads.

[b]Possible interference of ligand binding to MBP-RXRα was investigated using different matrices ranging from simple buffer to complex botanical extracts.

[c]A positive control, LG100268, was used as a high affinity synthetic ligand of RXRα (Kd 3 nM[40])

[d]9-cis-Retinoic was tested as an endogenous ligand for RXRα (Kd 15.7 nM[55]), while isomeric 13-cis retinoic was used as a non-binding negative control.[55]

[e]The enrichment factor (peak area compound bound to RXRα/peak area compound bound to denatured protein) was averaged over all replicates. One-way paired t-test was used to evaluate the difference between results obtained using active RXRα and denatured protein.

Like other affinity selection mass spectrometric screening approaches, MagMASS may be used to rank ligands with respect to affinity for a receptor. Under all test conditions, the highest affinity ligand LG100268 produced the highest enrichment factor as shown in Table 3. The ligand with the next highest affinity for RXRα, 9-cis-retinoic acid, showed the next highest enrichment factors during MagMASS, and the non-ligand 13-cis-retinoic acid produced the lowest enrichment factors. Note that enrichment factors obtained using PUF LC-MS have been used to rank human and equine estrogens based on their relative affinities for the estrogen receptors.[56] Although enrichment factors provide relative binding data that may be used to rank ligands in order of affinity, they do not provide affinity constants.

The protein construct used for magnetic bead immobilization was crucial for maintenance of protein integrity. This was particularly evident in the case of the ligand binding domain (LBD) of RXRα. Although active in solution during PUF-MS screening assays, RXRα-LBD completely lost the ability to bind ligands such as LG100268 after immobilization on NHS-magnetic beads but not when immobilized on amylose beads (Figure 8). It is hypothesized that truncated proteins such as RXRα-LBD have fewer sites available for NHS tethering to occur, and this increases the likelihood of covalent immobilization through amino acids side chains at or near the active site. This might block the active site or cause tertiary structural changes of the protein at the active site, of which might lower the affinity of the receptor for ligands.

As a solution to the problem of maintaining activity upon immobilization of RXRα, we expressed MBP with RXRα (which had been used during protein expression and purification) and immobilized the protein on amylose magnetic beads. Because MBP

binds amylose, amylose-functionalized beads may be used to immobilize MBP-RXRα without affecting the conformation or sterically hindering ligand access to the active site (Figure 6). As illustrated by these examples, optimization of protein construct and type of functionalized magnetic bead need to be experimentally determined for each protein target during the development of new MagMASS assays.

Protein consumption during MagMASS screening is minimal, requiring only 100 pmol per well. MBP-RXRα was stable for at least 24 hours after immobilization with NHS beads. Immobilization on NHS beads requires approximately 4 h, but this step may be eliminated when using amylose magnetic beads. MagMASS is a fast, automatable assay requiring 1.5 hours to prepare each 96-well plate. UHPLC separation is the rate limiting step. In comparison, PUFMS has not yet been automated and requires 9 h to process each 96-well plate.

Conclusion

The diversity of natural products in botanical and microbial extracts is currently not being leveraged adequately by the pharmaceutical industry largely because these mixtures are slower to screen than discreet compounds in combinatorial libraries. To address this issue, MagMASS offers a new approach for high-throughput natural product screening that is fast, automatable, requires minimal protein and extract, and yields reproducible screening results. Compared with our previous PUFMS approach, MagMASS is over 100-fold faster due to a combination of using UHPLC in place of HPLC, faster sample processing using magnetic beads in place of ultrafiltration, and

using 96-well plates. Nevertheless, PUF-MS still has advantages such as the convenience of skipping the immobilization process and higher ligand recovery.

The versatility of MagMASS extends beyond screening for ligands of the active sites of receptors and enzymes. For example, functional assays may be carried out using enzymes immobilized using our MagMASS approach in which the incubation mixture is analyzed for a reaction product. MagMASS does not require displacing or binding to an active site, as all sites are available for binding ligands. Consequently, it is important to note that MagMASS may be used to identify allosteric ligands in addition to those binding to the active site of the immobilized protein. Binding of ligands to allosteric sites may be differentiated from binding at the active site by demonstrating binding or displacement in the presence of a high affinity active site ligand.

The RXRα MagMASS assay research of this dissertation focused on optimizing and develop the various parts of the assay. With this work complete, the MagMASS assay was then applied to natural product drug discovery with the target of 15-lipoxygenase.

Acknowledgements

The authors thank Matthew Redinbo for providing the RXRα plasmid. This work was supported by grants R01 AT007659 and T32 AT007533 from the NIH National Center for Complementary and Integrative Health. The authors acknowledge the Chicago Botanic Garden for donating all plant material used during the development of this assay. All plant extractions were performed with support from NIH grants

P50AT000155-13S1 and P50AT000155 from the Office of Dietary Supplements and the National Center for Complementary and Integrative Health.

CHAPTER 3: APPLICATION OF MAGNETIC MICROBEAD AFFINITY SELECTION SCREENING TO NATURAL PRODUCT DRUG DISCOVERY

Adapted from Rush, M.D., Walker, E.M., Burton, T., van Breemen, R.B. Magnetic microbead affinity selection screening (MagMASS) of botanical extracts for inhibitors of 15-lipoxygenase. *J. Nat. Prod.* (2016) 79(11): 2898-2902 with permission. Copyright 2017 ACS.

Introduction

Natural products have always been a rich source of drug leads.[57] Unfortunately, screening natural products for drug discovery has been replaced largely by high-throughput screening of combinatorial libraries of discrete compounds due to concerns about the complexity of natural products.[58] Despite the speed of combinatorial synthesis and high-throughput screening, the chemical diversity and biological relevance of the compounds covered by this approach cannot compete with those of natural products.[57] Pulsed Ultrafiltration-mass spectrometry (PUFMS)[59] and magnetic microbead affinity selection screening (MagMASS)[29] were invented to overcome the limitations of traditional natural product drug discovery. Both PUFMS and MagMASS utilize affinity selection to separate ligands bound to protein targets from nonbinding compounds contained in complex mixtures such as botanical extracts and then utilize the speed and selectivity of mass spectrometry to characterize and help identify the ligands. Although PUFMS has been effective in finding ligands for a wide variety of targets, the

ultrafiltration separation step is inherently slower and more challenging to carry out in multi-well format.

Pursuing higher-throughput screening applications, reported here is a faster and more efficient MagMASS approach and its application to an anti-inflammation target, 15-lipoxygenase (15-LOX). During inflammation, the enzymatic products of 15-LOX display both pro-inflammatory and anti-inflammatory properties.[60] 15-LOX oxidizes free and esterified fatty acids, such as its main substrates, arachidonic acid and linoleic acid, to form a wide range of bioactive products including 13-HODE, 12-HETEs, lipoxins, and leukeotrienes.[61] These products contribute to a variety of inflammatory disorders, including atherosclerosis.[60,62] The MagMASS screening approach involves tethering the protein target to magnetic microbeads, incubating with an extract or other mixture containing potential ligands, using magnetism to separate the ligand−protein/bead complexes from unbound compounds remaining in solution, and then releasing the bound ligands for analysis using mass spectrometry (Figure 9). Compared with conventional high-throughput screening that utilizes fluorescent or absorption readouts, MagMASS does not suffer from interference from samples containing fluorophores or chromophores, which are common in natural product samples.

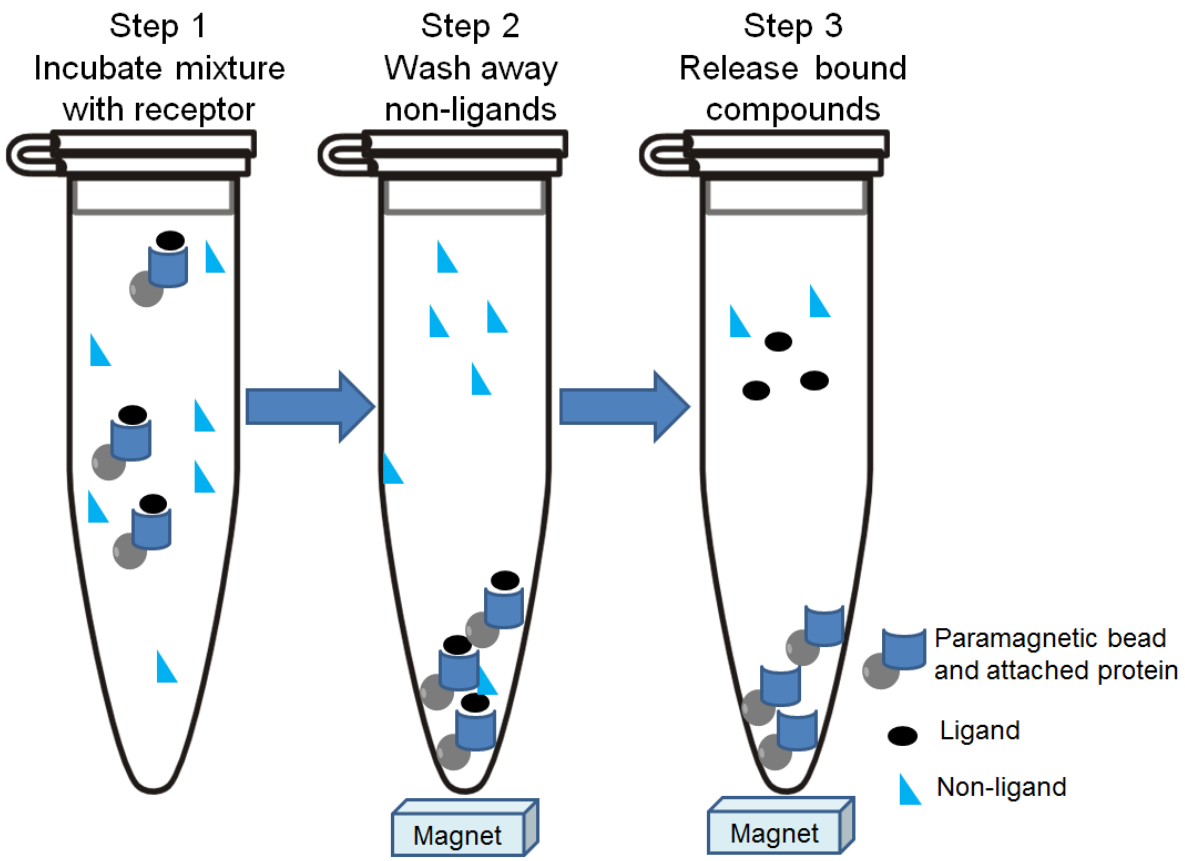


Figure 9 - Scheme for Magnetic Microbead Affinity Selection Screening (MagMASS). Step 1: Target protein (15-LOX) bound to paramagnetic microbeads is incubated with a mixture of compounds such as a botanical extract. Step 2: Protein-ligand complexes are held at the bottom of the incubation well by a magnetic field while unbound compounds are washed away, typically using a pipette. Step 3: Ligands are released by denaturing the receptor with organic solvent and then removed for UHPLC-MS analysis. Reprinted with permission from Rush, Michael D., *et al*. *J. Nat. Prod.* (2016) 79(11): 2898-2902. Copyright 2017 ACS.

Experimental Information

Test Materials

Except as noted, all biochemicals and standard phytochemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). 15-Lipoxygenase soybean P1 (15-LOX) was purchased from Cayman Chemicals (Ann Arbor, MI, USA). All solvents were LC-MS grade and were purchased from Thermo Fisher (Waltham, MA, USA). Ketoconazole was used as an internal standard. The activity of 15-LOX was determined according to the supplier's instructions. Briefly, linoleic acid (0.02%) was incubated with 15-LOX tethered to magnetic beads (as described below) in 50 mM sodium borate buffer (pH 7.5), and the absorbance was monitored at 234 nm. Immobilized 15-LOX was denatured by heating at 90 °C for 20 min and used as a control for nonspecific binding.

Immobilization of 15-LOX on Magnetic Beads

NHS derivatized magnetic microbeads were purchased from Life Technologies (Grand Island, NY, USA) or AvanBio (Parsippany, NJ, USA). Magnetic microbeads (300 μL) were added to a 1.5 mL centrifuge tube or a well (V-bottom; 1.3 mL capacity) of a 96-well microtiter plate, drawn to the bottom using a magnetic plate consisting of 12 neodymium rare earth magnets (N45 2 × 1 × 1/8 in. NdFeB) from CMS Magnetics (Garland, TX, USA), and washed with 300 μL of 1 M HCl. The supernatant was removed, and 300 μL of 50 mM sodium borate buffer (pH 7.5) was added to each tube

or well. After mixing, 50 pmol of 15-lipoxygenase soybean P1 (15-LOX) in 300 μL of sodium borate buffer (50 mM, pH 7.5) was added to each tube or well, and the mixture was shaken every 5 min during a 60 min incubation at room temperature. The beads containing immobilized protein were drawn magnetically to the bottom of the well, the supernatant was removed, and 300 μL of 0.1 M glycine (pH 2.0) was added to the protein-beads. This glycine treatment step was repeated. The protein beads were drawn to the bottom of the wells, and the supernatant containing unreacted glycine was removed. The beads were then washed with 300 μL of water. To quench any remaining reactive sites on the beads, the protein-beads were drawn magnetically to the bottom of the tube, the supernatant was removed, and 300 μL of 3 M ethanolamine (pH 9.0) was added. After 2 h with shaking every 5 min, the supernatant was removed, and 300 μL of 50 mM sodium borate (pH 7.5) containing 0.05% (m/v) sodium azide was added as storage buffer.

Botanical Extracts

All botanicals were obtained from the Chicago Botanic Garden (Chicago, IL, USA), where they were taxonomically identified. Voucher specimens were deposited in the Chicago Botanic Garden Herbarium. Wild-collected from Kansas, Illinois, Missouri, Colorado, Wisconsin, and South Dakota, the botanical specimens included *Geranium caespitosum* E. James (Geraniaceae) [Herbarium voucher 15556], *Proserpinaca palustris* L. (Halorgaceae) [Herbarium voucher 13706], *Dasistoma macrophylla* (Nutt.) Raf. (Scrophulariaceae) [Herbarium voucher 13844], *Lithospermum canescens* (Michx.)

L (Boraginaceae) [Herbarium voucher 14549], *Oenothera macrocarpa* Nutt. (Onagraceae) [Herbarium voucher 14836*], Ruellia humilis* Nutt. (Acanthaceae) [Herbarium voucher 14856], *Desmanthus illinoensis* (Michx.) MacMill. ex B.L. Rob. & Fernald (Fabaceae) [Herbarium voucher 14939], *Silphium perfoliatum* L. (Asteraceae) [Herbarium voucher 14965], *Chamaecrista fasciculata* (Michx.) Greene (Fabaceae) [Herbarium voucher 14964], *Ludwigia alternifolia* L. (Onagraceae) [Herbarium voucher 15005], *Stenaria nigricans* (Lam.) Terrell (Rubiaceae) [Herbarium voucher 18630], *Symphyotrichum oblongifolium* (Nutt.) G.L. Nesom (Asteraceae) [Herbarium voucher 15001], *Echinocystis lobata* (Michx.) Torr. & A. Gray (Cucurbitaceae) [Herbarium voucher 15023], and *Verbena stricta* Vent. (Verbenaceae) [Herbarium voucher 15031]. These species were selected for screening for ligands of 15-LOX because they have biological data regarding anti-inflammatory activity and have been used historically by Native Americans or are native to the region and have not previously been studied.[63–72] Inflorescence tissue (200−400 g dry weight) of each botanical was dried and ground. Methanol extracts of 30 g portions of ground tissue were prepared using cold percolation (1:20, w/v) in separatory funnels. Each extract was evaporated to dryness under vacuum and reconstituted in dimethyl sulfoxide to a stock solution of 40 mg/mL

MagMASS Protocol

For assay validation, luteolin (IC_{50} 5 μM),[62],= a known inhibitor of 15-LOX, was used as a positive control, while the nonligand ebselen was used as a negative control. A 25 μL aliquot of 15-LOX immobilized on magnetic beads was incubated with one or more

standards (100 pmol each) at 23 °C for 1 h with shaking every 5 min. For screening, each botanical extract stock solution was diluted to 10 mg/mL using DMSO, and 1 µL (10 µg) of the extract was incubated with a 25 µL aliquot of immobilized 15-LOX for 1 h at 23 °C. After incubation, the magnetic beads were drawn to the bottom of the well using the magnet, and the supernatant was removed using a pipet (Figure 9). The beads were rinsed to remove unbound compounds by washing with 300 µL of 30 mM ammonium acetate. Washing was repeated twice with buffer and once using water. To release bound ligands remaining after incubation and washing, immobilized 15-LOX was treated with 100 µL of 90% aqueous methanol. The beads were drawn magnetically to the bottom of the well, and the supernatant was transferred to a new tube or well (Figure 9). The supernatant was evaporated *in vacuo* and reconstituted in 100 µL of 50% water/acetonitrile containing 1 ng/L ketoconazole as internal standard for UHPLC-MS or UHPLC-MS/MS analysis. The total time to complete the assay from incubation to drying of the wells was. 3.5 h. Control incubations were carried out in parallel to each experiment and were identical except that the active, immobilized 15-LOX was replaced with denatured 15-LOX.

Mass Spectrometry

After release from 15-LOX, ligands were characterized using UHPLC-MS/MS on a Shimadzu (Kyoto, Japan) Nexera UHPLC system interfaced with a Shimadzu electrospray ion trap-time-of-flight (IT-ToF) hybrid high-performance mass spectrometer. UHPLC separations were carried out using a Waters (Milford, MA, USA)

Acquity C_{18} CSH column (1.7 μm, 2.1 × 150 mm) with a 12 min linear gradient from 5% to 95% methanol containing 0.1% formic acid. The injection volume was 1 μL. High resolution (resolving power 10 000) accurate mass positive-ion and negative-ion mass spectra were recorded using polarity switching over the mass range *m/z* 100 to 1000. Mass spectra were recorded whenever the ion trap collection reached 10 000 000 counts. Natural product hits were identified by coelution with authentic standards during UHPLC-MS/MS on a Shimadzu LCMS-8040 triple quadrupole mass spectrometer and Nexera UHPLC system equipped with a Waters Cortecs C_{18} column (2.7 μm, 2.1 × 50 mm). Compounds were eluted using a 4-min linear gradient from 5% to 75% methanol containing 0.1% aqueous formic acid. Collision-induced dissociation and selected reaction monitoring were optimized for each ligand.

Data Management

A positive hit in the assay was determined by comparing the UHPLC-MS chromatogram of an extract after MagMASS incubation with intact 15-LOX to that obtained after incubation with the denatured protein. An increase in area of a chromatographic peak for the experimental chromatogram relative to the denatured protein control constituted a potential hit (Figure 10). The free metabolomics software, XCMS online,[73] was repurposed to automate the comparison of the experimental and control UHPLCMS chromatograms generated for each sample. This online software automatically aligned the experimental and control chromatograms and indicated which peaks of particular *m/z* values were enriched. The spectra were aligned and normalized to the ketoconazole internal standard. To characterize and dereplicate 15-LOX ligands contained in botanical extracts, elemental compositions were determined based on the high-resolution IT-ToF mass spectra of the hits. The elemental compositions were searched against natural product and mass spectrometry databases including Reaxys, Metlin, SciFinder, NAPRALERT, and Massbank. After predicting the chemical structures of hits through this dereplication process that included high-resolution MS/MS as well as elemental composition determination, the identities of known natural products were confirmed by comparison with authentic compounds during UHPLC-MS/MS as described above.

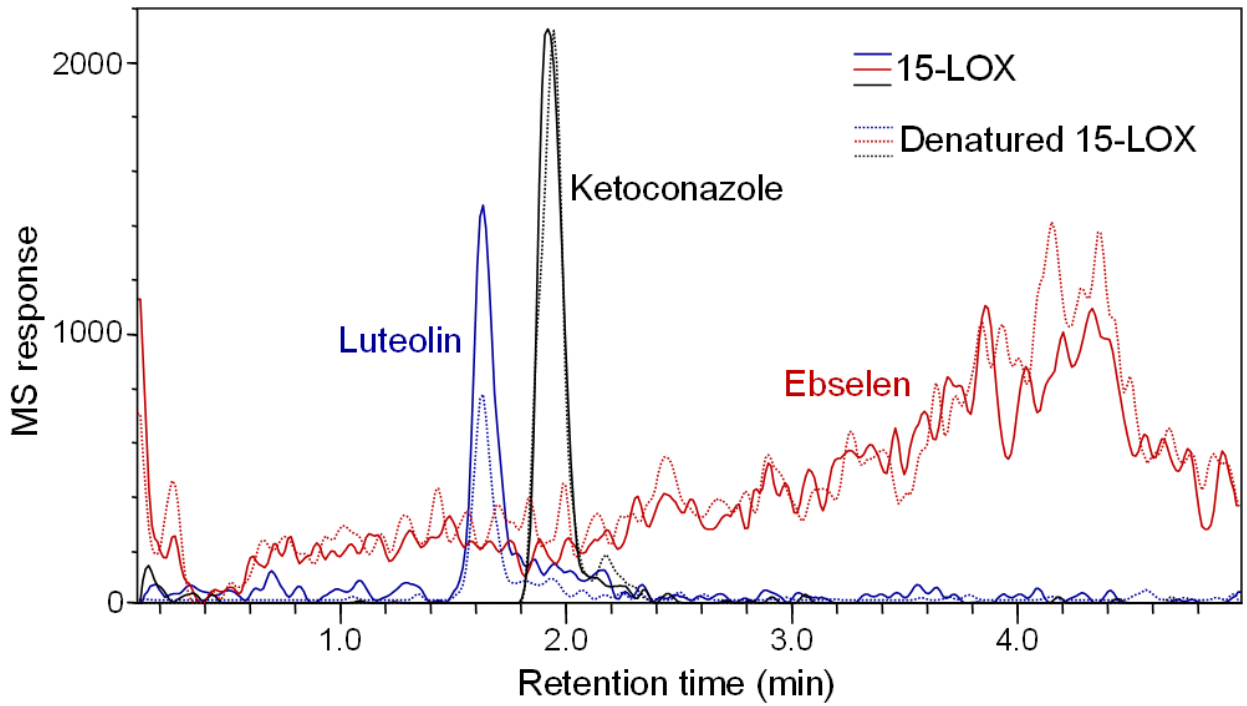


Figure 10 MagMASS UHPLC-MS chromatograms following incubation of 15-LOX (solid lines) or denatured 15-LOX (dashed lines) with the known ligand luteolin and the non-ligand ebselen. Only the positive control, luteolin, showed peak enhancement that is indicative of specific binding to 15-LOX. Ketoconazole was added to each sample as an internal standard immediately before UHPLC-MS analysis. Reprinted with permission from Rush, Michael D., *et al*. *J. Nat. Prod.* (2016) 79(11): 2898-2902. Copyright 2017 ACS.

Results and Discussion

The specific activity of 15-LOX before immobilization on magnetic microbeads was 33 000 units/mg. After immobilization, 15-LOX remained active at 20 000 units/mg, while denatured immobilized 15-LOX had no measurable activity. Covalent enzyme immobilization will invariably cause some loss of activity, perhaps due to steric hindrance of the active site, restriction of enzyme flexibility, and/or changes in tertiary structure. MagMASS can accommodate this problem by using different bead chemistries for immobilization as needed. In this case, enzymatic activity remained sufficiently high as to enable the assay to be successful. As proof of concept, the 15-LOX MagMASS approach was tested using an equimolar mixture of the ligand, luteolin, and the nonligand, ebselen. Comparing the UHPLC-MS chromatograms for the experiment (active 15-LOX) with the control (denatured 15-LOX), there was peak enrichment for luteolin in the experimental chromatogram as expected but not for the non-ligand ebselen (Figure 10). This experiment was performed in triplicate during three replicates of both the experiment and protein control, showing a significant enrichment of the luteolin peak compared to the control.

Next, extracts of 14 North American prairie plants were screened for ligands to 15-LOX. Analyses of each pair of chromatograms (active 15-LOX for the experiment and denatured 15-LOX for the matched control) were carried out using the metabolomics software program XCMS. Only one hit from the series of botanical extracts was observed during MagMASS analysis, and this corresponded to the methanol extract of *Proserpinaca palustris* L. (Haloragaceae; mermaid weed) (Figure 11). For the peak eluting at 8.3 min during UHPLC-MS/MS, there was enrichment in the

experimental chromatogram relative to the control with a significance of $p < 0.05$, as indicated by the XCMS statistical analysis (Figure 11). Investigation of the data confirmed that the enriched peak corresponded to a natural product with a high-resolution accurate mass of *m/z* 449.108 (+) and *m/z* 447.095 (−), which corresponded to a neutral elemental composition of $C_{21}H_{20}O_{11}$ (≤5 ppm). Based on high-resolution accurate mass measurement and dereplication of the hit using NAPRALERT (UIC College of Pharmacy, Chicago, Illinois, USA), SciFinder (Chemical Abstracts Service, Columbus, Ohio, United States), Massbank (National Bioscience Database Center, Tokyo, Japan), Metlin (Scripps Center for Metabolomics, San Diego, California, USA), and Reaxys (Elsevier, Amsterdam, The Netherlands), possible structures were determined to be either luteolin 7-*O*-glucoside (cymaroside) or else quercitrin (quercetin 3-*O*-rhamnoside) (Figure 12).

Product-ion tandem mass spectra and retention times of the unknown hit and the two standards were similar, but the relative abundances of the product ions suggested that the hit was quercitrin (Table 4). To confirm that the hit was quercitrin, both quercitrin and luteolin 7-*O*-glucoside were screened against 15-LOX using MagMASS. Only quercitrin showed specific binding to 15-LOX (Figure 13), confirming the hit in *P. palustris* extract. A literature search confirmed that quercitrin inhibits 15-LOX with an IC50 value of 79.8 μM.[74] Because quercitrin is a known ligand of 15-LOX, additional experiments to determine the site of quercitrin binding, such as displacement by previously characterized active site ligands,[6] were not carried out.

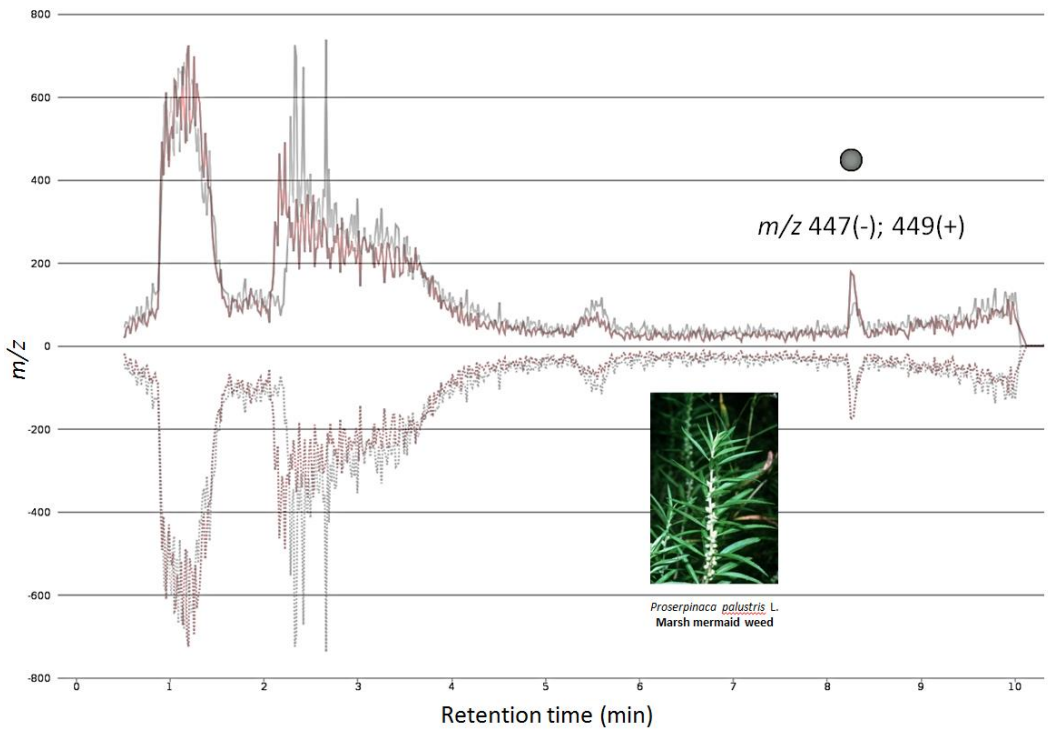
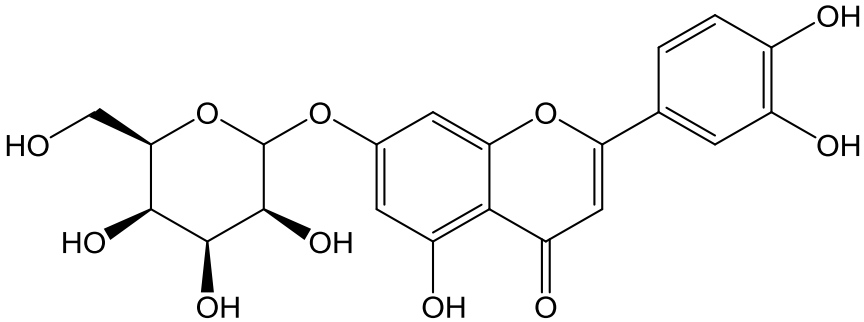
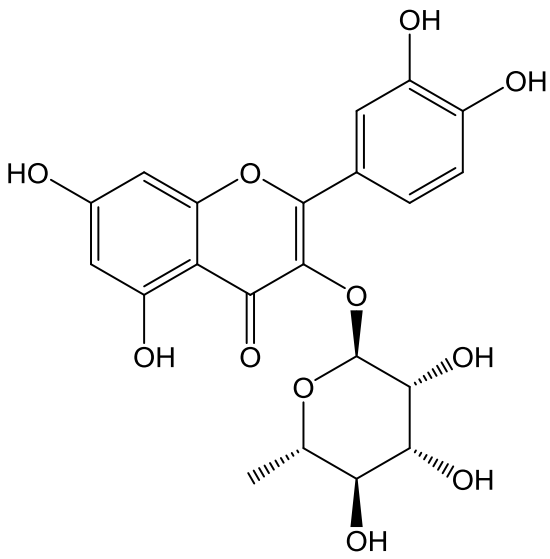


Figure 11 XCMS online metabolomics analysis overlay for the screening of mermaid weed extract for ligands of 15-LOX. MagMASS screening against active 15-LOX is shown in red and denatured 15-LOX MagMASS as a negative control is shown in grey. Negative ion electrospray UHPLC-MS chromatograms are shown on the top and the corresponding positive ion chromatograms are displayed on the bottom. A hit representing a ligand with MW 448 (*m/z* 449, $[M+H]^+$; *m/z* 447, $[M-H]^-$) was detected at a retention time of 8.2 min. Reprinted with permission from Rush, Michael D., *et al*. *J. Nat. Prod.* (2016) 79(11): 2898-2902. Copyright 2017 ACS.



7-Luteolin-*O*-glycoside



Quercitrin

Figure 12 Two possible structures of the hit observed during MagMASS testing of mermaid weed for ligands of 15-LOX with an accurate mass of 448.106 (ΔM <5 ppm $C_{20}H_{21}O_{11}$:), dereplication indicated two probable natural products isomers, 7-luteolin-*O*-glycoside or quercitrin. Reprinted with permission from Rush, Michael D., *et al. J. Nat. Prod.* (2016) 79(11): 2898-2902. Copyright 2017 ACS.

TABLE IV HIGH RESOLUTION ELECTROSPRAY MS/MS ANALYSES OF THE XCMS SCREENING HIT OF *PROSERPINACA PALUSTRIS* L. (MERMAID WEED) AND COMPARISON WITH NATURAL PRODUCT STANDARDS 7-LUTEOLIN-O-GLYCOSIDE AND QUERCITRIN. REPRINTED WITH PERMISSION FROM RUSH, MICHAEL D., *ET AL*. *J. NAT.L PROD.* (2016) 79(11): 2898-2902. COPYRIGHT 2017 ACS.

MagMASS 15-LOX hit	Retention time (min)	Precursor ion (*m/z*)	Collision energy (eV)	MS/MS product ions (relative abundance)
Positive ion	1.88	449.108	-30	303.05 (100), 287.06 (28)
Negative ion	1.88	447.095	30	301.03 (100)

7-Luteolin-*O*-glycoside	Retention time (min)	Precursor ion (*m/z*)	Collision energy (eV)	MS/MS product ions (relative abundance)
Positive ion	1.81	449.108	-35	287.06 (100)
Negative ion	1.81	447.095	29	285.05 (100)

Quercitrin	Retention time (min)	Precursor ion (*m/z*)	Collision energy (eV)	MS/MS product ions (relative abundance)
Positive ion	1.88	449.108	-15	303.05 (100),
Negative ion	1.88	447.095	29	301.03 (100)

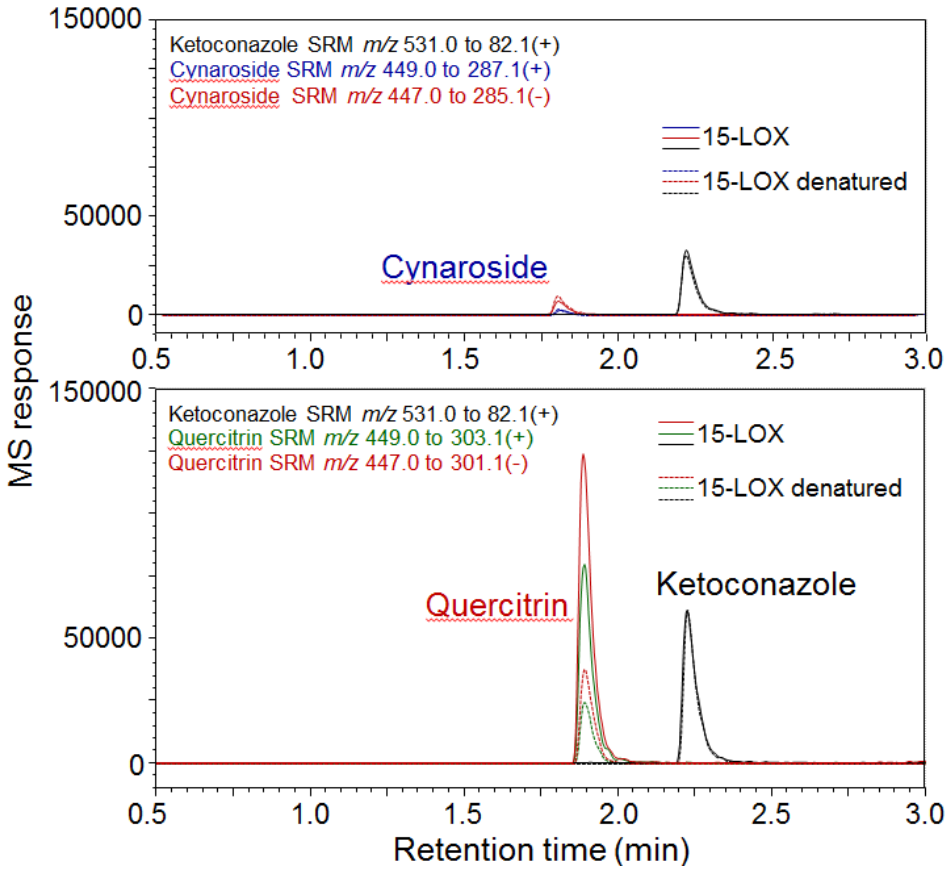


Figure 13 MagMASS screening of the standards of 7-luteolin-*O*-glycoside and quercitrin confirmed the binding of only quercitrin to 15-LOX based on significant quercitrin but not 7-luteolin-*O*-glycoside peak enhancement. Reprinted with permission from Rush, Michael D., *et al. J. Nat. Prod.* (2016) 79(11): 2898-2902. Copyright 2017 ACS.

Given the complexity of extracts of botanicals and microbial fermentations, many laboratories have ceased screening the molecular diversity of natural products and instead test discrete compounds contained in combinatorial libraries. As demonstrated here, MagMASS may be used to screen mixtures of compounds including natural products in botanical extracts faster than was possible previously. Furthermore, assays based on MagMASS are beginning to gain popularity since first having been reported by our group in 2008 for estrogen receptor screening.[29,75] Wilson *et al*.[76] recently used kynurenine 3-monooxygenase immobilized on magnetic microbeads for MS-based screening of a commercial combinatorial library. Instead of exploiting the selectivity of mass spectrometry to screen combinatorial library mixtures or natural product extracts as in our approach, this group screened discrete compounds in series. Also, instead of covalently attaching the enzyme as in our assays, Wilson *et al*.[76] covalently attached an antibody to the magnetic beads first and then used it to capture kynurenine 3-monooxygenase.

The innovations reported here for MagMASS are significant in that they speed up screening complex mixtures of natural products and make this approach a more appealing alternative to bioassay-guided fractionation. The application of XCMS software originally developed for metabolomics to the analysis of MagMASS data is a significant advance over manual data inspection.[29] This approach to data analysis alone enhances throughput by orders of magnitude and enables the process to be automated. When a hit is observed during MagMASS, mass spectrometric data are used to identify the compound through the process of dereplication. If the structure of the compound is novel, then the LC-MS retention time facilitates its chromatographic isolation for

additional structure elucidation. Shortening the iterative process used in bioassay-guided fractionation to just one-step in MagMASS significantly enhances the drug discovery process. Another innovation reported here is the use of UHPLC-MS instead of HPLC-MS, which shortened chromatographic separation and enhanced throughput 5-fold over previous applications of MagMASS. As a proof of concept, a new MagMASS method was developed and demonstrated for the discovery of natural product inhibitors of 15-LOX from botanical extracts. Incorporating the metabolomics program XCMS online for automated data analysis increases throughput an estimated from 10-fold to 100-fold depending upon the sample complexity.

These 15-LOX assays demonstrate proof of concept that MagMASS is compatible with the complexities of natural products. The next step for the MagMASS assay is to explore the application in a much larger and broader drug discovery experiment.

Acknowledgements

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CHAPTER 4: MAGNETIC MICROBEAD AFFINITY SELECTION SCREENING AS A HIGH-THROUGHPUT DRUG DISCOVERY PLATFORM

Fructose-1,6-bisphosphatase as a protein target

Obesity is an issue at the forefront of public health. The Center for Disease Control estimates that 16.9% of youth and 34.9% of adults are obese.[77] The estimated medical cost of obesity is ~$147 Billion in the United States; and the medical costs for an obese person were $1,429 per capita higher than for a non-obese person in 2008.[78] Low carbohydrate diets are the first line of defense against obesity, and although effective, many individuals have difficulty adhering to these diets.[79] Unabated high caloric diets lead to obesity, metabolic syndrome, diabetes mellitus, and worse. Pharmaceutical companies are developing synthetic medications targeting particular biochemistries that lower blood glucose levels and lessen the impact of sugar on the body. However, there is vast potential for the discovery of natural products and botanical dietary supplements that can serve as alternatives to help in the fight against excessive carbohydrate related obesity.

There are many possible pharmacologically important targets in the metabolic pathways of glucose and fructose. Many small molecules have been designed by the pharmaceutical industry to lower free blood glucose levels, and these compounds have a wide range of targets[80] including glucokinase, fructose-1,6-bisphophatase, glycogen phosphorylase, sodium glucose cotransporter, and AMP-activated protein kinase. Glucokinase activators are losing their promise as a target. Recent studies show that glucokinase activators cause higher hepatic triglyceride levels and episodes of

hypoglycemia in human studies.[81] Glycogen phosphoralyse (GP) inhibitors have a selectivity issue; these small molecules are unlikely to be able to selectively inhibit hepatic GP and not muscle GP.[82] AMP activated protein kinase (AMPK) small molecule activators show promise as a target as many natural products and drugs activate this enzyme, however there are concerns over liabilities of this target as the possible side effects are unknown.[83]

The development of sodium glucose cotransporters has a history of using natural products as a drug lead, and phlorizin, isolated from apple tree bark, is an example. This compound has been used extensively in diabetes research since the 1900s. In the 1970s, phlorizin's mechanism of action was determined to be inhibition of sodium glucose cotransporters (SGLT). It inhibits both SGLTI and SGLTII but has poor gastrointestinal absorption. Analogs of this natural product have been discovered that circumvent these problems.[84] Two small molecules, dapaglifozin and canagliflozin (Faxiga and Invokana, respectively) are FDA approved drugs that resulted from of using phlorizin as a natural product drug lead. However, these drugs have concerns of increased cardiovascular risk.[85]

Fructose-1,6-bisphosphatase (F16BPase) is another target for obesity and metabolic syndrome that has been investigated by the pharmaceutical industry but with only limited success. Fructose-1,6-bisphosphatase should be a viable anti-obesity target since it is solely a gluconeogenic enzyme and is not involved in any other pathway.[86] As indicated in Figure 15, the only function of fructose-1,6-bisphosphatase is to convert fructose-1,6-diphosphate to fructose-6-diphosphate, which is an essential step in the formation of glucose in humans. This limited role for fructose-1,6-bisphosphatase

enhances its value as a therapeutic target, because inhibiting this enzyme should have few deleterious effects on other pathways. Note that current inhibitors of many other targets of gluconeogenesis are bioactive in a variety of pathways resulting in a wide array of side effects.[87]

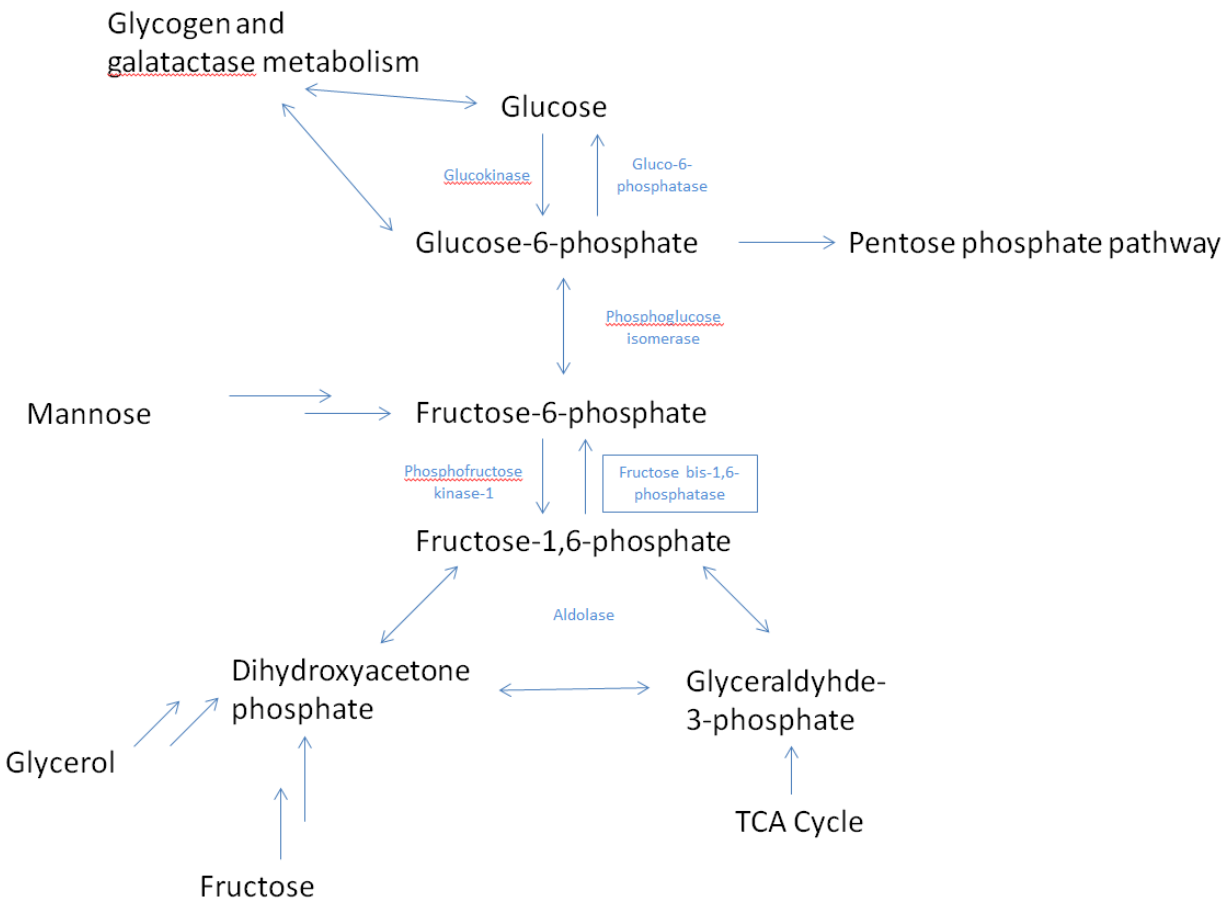

Figure 14 F16BPase plays a critical role in gluconeogenesis and its role is solely in this pathway.[88] This is advantageous for reducing interactions caused by promiscuous ligands.

There are two main binding sites on fructose-1,6-bisphosphatase – the fructose binding site and the AMP cofactor binding. There has been little success in designing compounds that act as competitive inhibitors to the active site for fructose-1,6-diphosphate.[89] However, allosteric inhibitors have been discovered that bind to the subunit interfaces of fructose-1,6-bisphosphatase.[90–92] These compounds show some activity, but more work can be done in finding pharmacologically active compounds targeting fructose-1,6-bisphosphatase. Compounds that have been chosen for possible clinical trials are all AMP mimics. These compounds bind to the site on fructose-1,6-bisphosphatase where the cofactor, AMP, would normally bind. By denying access to AMP – a cofactor that would accept a phosphate group from fructose-1,6-diphosphate – these compounds can inhibit the enzyme as a noncompetitive inhibitor. However, these inhibitors have problems with potency; they tend to be promiscuous with any enzyme that can bind AMP and there is a greater chance of side effects. Since a second generation inhibitor of fructose-1,6-bisphosphatase from Metabasis Therapeutics, MB07803,[93] has shown improvement by reaching the primary endpoint of decreased free blood glucose at day 28,[94] targeting fructose-1,6-phophatase to lower free blood glucose is a promising target.[82] However, there are still no FDA-approved medications targeting fructose-1,6-bisphosphatase.

MagMASS is a unique tool that can be used to find ligands for fructose-1,6-bisphosphatase. For unknown libraries or botanical/microbial extracts, using a high-resolution mass spectrometer as the detector provides distinct advantages for rapidly characterizing and identifying active compounds. If mass spectrometry alone were insufficient, the LC conditions and compound retention time would be known, which

would facilitate compound isolation for further structural determination by NMR, etc. Another advantage of MagMASS compared to traditional high throughput screens is the possibility of finding allosteric ligands. Conventional competition-based assays only measure binding to a single active site and ignore the possibility of allosteric binding. During MagMASS, allosteric ligands can bind and still be captured for detection. Known fructose-1,6-bisphosphatase inhibitors tend to be AMP analogs that interact with a cofactor binding site;[87] but there are other and possibly more important binding sites on fructose-1,6-bisphosphatase with different modes of action. Using MagMASS as a screening technique can yield compounds that not only might bind to the AMP site but alternatively to novel inhibitory sites. Therefore, MagMASS theoretically enables the discovery of compounds with novel mechanisms of action.

Natural products have always been a rich source of drug leads.[57] Unfortunately, screening natural products for discovery has declined as there were concerns of the technical limitations of using natural product extracts libraries in high-throughput competition screens and then isolating the active compounds.[58] Available to this project at the University of Illinois at Chicago is a wide range of natural products, extracts, and fractions for screening. These resources include natural products, extracts, and fractions from marine bacteria, cyanobacteria, and botanicals. There is significant need to identify new compounds that bind to fructose-1,6-bisphosphatase, and testing a wide range of natural products might provide new lead compounds representing hitherto unknown scaffolds with novel binding mechanisms. Further, these new ligands would be natural products offering their own advantages. Instead of serving as a stand-alone therapeutic agent to treat or prevent obesity (or used as a lead compound for anti-

obesity drug development), novel inhibitors of fructose-1,6-bisphosphatase might be discovered within existing natural products. These supplements could then be standardized to these active natural products and used for the maintenance of optimum weight.

Experimental Information

Chemicals and Reagents

Fructose bisphosphatase inhibitor and MB05032 were purchased from Caymen Chemicals (Ann Arbor, MI, USA). Ketoconazole, cymaroside, and all buffer salts were purchased from Sigma-Aldrich (St. Louis, MO, USA). All reagents and solvents were purchased from Thermo Fisher (Waltham, MA, USA) or VWR (Radnor, PA, USA). AvanBio (Parsippany, NJ, USA) provided all magnetic beads for testing. A 60-lb magnetic plate consisting of 12 neodymium rare earth magnets (N45 2 × 1 × 1/8" NdFeB) (CMS Magnetics, Garland, TX) was used to in the assay.

Actinomycetes Library

The marine and freshwater actinomycetes extracts were generously donated from the laboratory of Dr. Brian T Murphy. In general, each actinomycetes strain was fermented in 1 L for approximately seven days and extracted with Amberlite XAD resin. The resin was then extracted using acetone, concentrated, and partitioned between ethyl acetate and water. The ethyl acetate extract was separated using a step gradient into four fractions over a silica gel solid phase extraction (SPE) cartridge. After testing the

fractions in our assay, subfractions from two strains were repooled for use in MagMASS assays at a final concentration of 1 mg/mL.

Fructose-1,6-bisphosphatase

Human fructose-1,6-bisphosphatase cloned with a *C*-terminal 6His-tag was obtained from GeneCopoeia (Rockville, MD) in the expression vector pFL-B31cl (Catalog number EX-C0133-B31). BL21(DE3) cells were transformed with the expression vector and grown to an OD >0.6 at 600 nm and 37 °C. The temperature was reduced to 20°C with the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and the cells were grown overnight. Cells were harvested by centrifugation, resuspended in column buffer (50 mM Tris pH 7.5, 50 mM sodium acetate pH 7.5, 500 mM NaCl, 25 mM imidazole, 5 mM $MgCl_2$), and lysed using Emulsiflex-C5 (Avestin). The lysate was cleared by centrifugation at 30,000x*g*, and then passed over a nickel NTA gravity column (Pierce, Rockford, IL) which was washed with 50 column volumes of chilled column buffer.

Although fructose-1,6-bisphosphatase was eluted and dialyzed as described previously,[95] the protein exhibited heavy precipitation after dialysis. To refold fructose-1,6-bisphosphatase, the precipitated material was resuspended in 50 mL of 50 mM potassium phosphate pH 7.5 and 6 M guanidine HCl for a final protein concentration of ~0.5 mg/mL. The denatured protein was dialyzed at 4 °C for 2 hr against 1 L of refolding buffer (25 mM Tris pH 7.5, 50 mM sodium acetate pH 7.5, 250 mM NaCl, 2.5 mM $MgCl_2$, 7.5% glycerol, and 2 mM β-mercaptoethanol). The refolding buffer was changed two additional times after 2 hr and overnight incubations. After refolding, no visible precipitation was observed, and fructose-1,6-bisphosphatase was concentrated to 1

mg/mL. To verify refolded protein, the secondary structure of refolded fructose-1,6-bisphosphatase was measured by circular dichroism. The protein was diluted to ~0.03 mg/mL in 20 mM potassium phosphate pH 7.5 and 100 mM NaF, and data were recorded using a JASCO J-815 spectrometer and a cuvette with a 0.2 cm path length. The purified refolded material and CD spectra are shown in Figure 16.

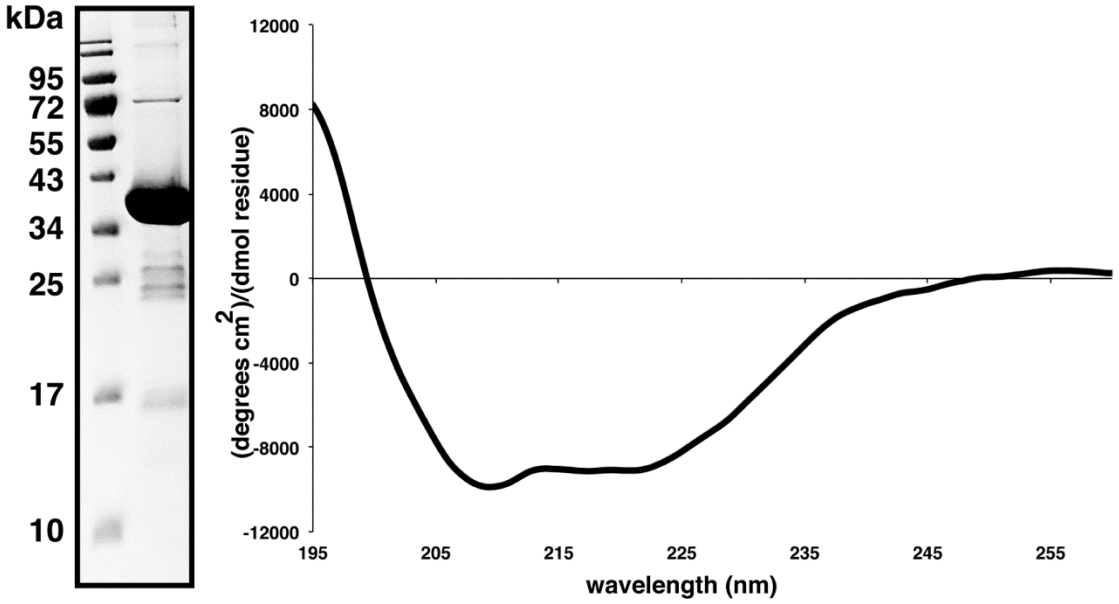

Figure 15 Recombinant Fructose-1,6-biphosphatase. Control data for purification and refolding of human fructose-1,6-biphosphatase. (Left) SDS-PAGE gel of purified and refolded F16BPase visualized by Coomassie stain. (Right) Circular dichroism spectrum of refolded F16BPase.

The activity of the enzyme was assessed by monitoring the conversion of NADP to NADPH at 340 nm using a Shimadzu UV2401PL UV-Vis recording spectrophotometer (Kyoto, Japan). 25 mM HEPES at pH 7.5 containing 2 mM $MgCl_2$, 1 mM DTT, 2 mM ammonium sulfate, 0.2 mM NADP, 0.05 mM EDTA, 20 units / mL glucose 6-phosphate dehydrogenase, 20 units/mL phosphoglucose isomerase, 100 µg/mL bovine serum albumin, and 10 nM fructose-1,6-bisphosphatase was made and aliquoted into 1 mL plastic cuvette. Fructose-6-bisphosphate was added to a final concentration of 100 µM and the reaction was continuously monitored at 340 nm. The activity was calculated to be 19 units / mg. The E.coli expressed fructose-1,6-bishphosphatase has lower activity than protein expressions from yeast[96] or collection from rat liver cells[97] or spinach leaves[98]. The refolding of the protein restored some activity – this can be offset by using higher concentrations of the enzyme during the MagMASS protocols.

MagMASS Protocols

In developing the MagMASS assay for fructose-1,6-bisphosphatase, selecting the appropriate bead chemistry is an important step. Because the His tag was removed during preparation, covalent immobilization was required. AvanBio provided five types of magnetic beads with different function chemistries: NHS, Sulfo-NHS, maleimide, amine, -COOH, and Hydrophilic-COOH. NHS, Sulfo NHS, –COOH, and Hydrophilic –COOH magnetic beads bond with primary amines to form amide linkages. Amine magnetic beads bond with primary amines or carboxyl-containing proteins. Maleimide magnetic beads react with thiols on the proteins. All procedures were scaled down from the manufacturer’s suggested protocols for smaller scale bead immobilization. Figure 17 describes the immobilization mechanisms.

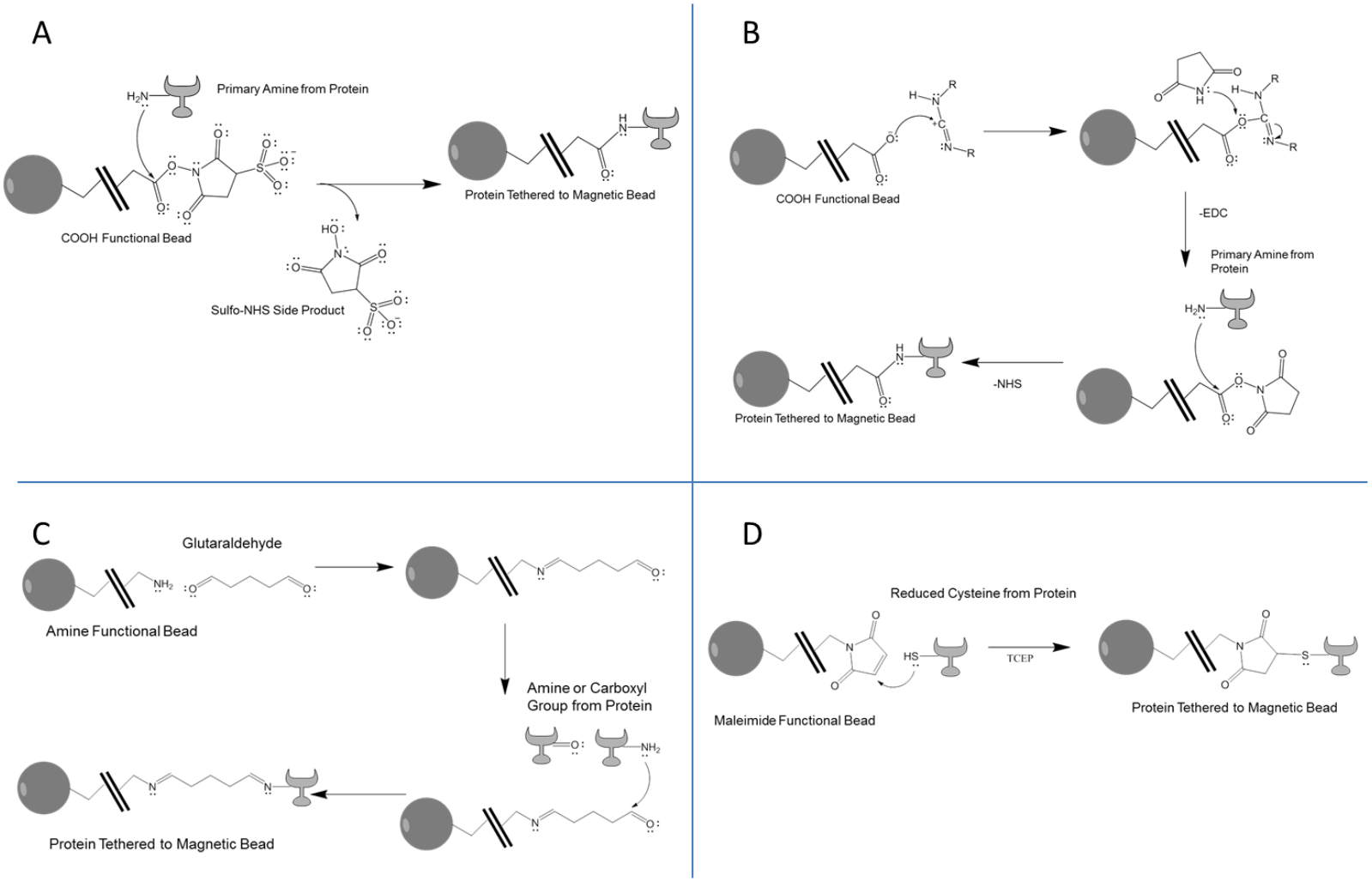

Figure 16 - Mechanism of immobilization for the four types of functional bead chemistries. A is the mechanism for NHS and Sulfo-NHS functional beads. A primary amine from the protein reacts with the NHS function group replacing the NHS with a covalent bond to form the covalent bonded protein-bead. B describes the mechanism for COOH and HCOOH functional beads. The COOH beads are activated using EDC. The NHS then replaces the EDC. A primary amine from the protein covalently forms the protein-bead bond. C is the

mechanism for Amine function beads. The Amine function beads are activated using glutaraldehyde. A carboxyl or an amine from the protein will replace the carboxyl on the glutaraldehyde chain. D is the mechanism for the Maleimide functional beads. The Maleimide beads are mixed with reduced cysteines made by TCEP. A sulfur bridge forms between the protein and the maleimide.

Protein immobilization on the NHS and Sulfo-NHS beads used identical procedures. To remove the storage buffer, the beads were retained using a magnetic plate while being washed twice with 500 µL PBS x1. Fructose-1,6-bisphosphatase was resuspended in 500 µL 150 mM NaCl, 0.01% Tween-20 and 50 mM MES pH 7.0, and then incubated for 2 hr with the NHS or Sulfo-NHS beads at ratios 30 µg protein / mg of beads. Then, the beads were retained using the magnet while the solution was replaced with a quenching buffer of 500 µL of 150 mM NaCl and 100 mM Tris pH 7.0, which deactivated any remaining reactive sites on the beads. The quenching reaction proceeded for 2 hr. The beads were drawn down again using a magnet, washed twice with the 500 µL quenching buffer, and then resuspended in 300 µL of 150 mM NaCl and 100 mM Tris pH 7.0 with 0.1% sodium azide.

Immobilizations of fructose-1,6-bisphosphatase on COOH and HCOOH magnetic beads were carried out using procedures identical to each other. After removing the storage buffer (as described above), the beads were washed 3 times using 500 µL of 50 mM MES at pH 7.0. The beads were resuspended in the coupling buffer of 150 mM NaCl, 0.01% Tween-20, 50 mM MES pH 7.0, and molar excesses of EDC and NHS. The protein was added and incubated for 2 hr. Then, the beads were incubated for 2 hr with a blocking buffer 500µL of 150 mM NaCl and 50 mM Tris pH 7.0. The beads were washed twice and resuspended in 300 µL blocking buffer containing 0.1% sodium azide.

For immobilization of fructose-1,6-bisphosphatase on the Amine magnetic beads, the storage buffer was removed and washed three times using 500 µL coupling buffer

10 mM pyridine, pH 6.0. The beads were resuspended in 500 µL a 5% glutaraldehyde solution containing 10 mM pyridine, pH 6.0, and incubated for three hours. After washing 3 times in the coupling buffer, the beads were resuspended in the coupling buffer, fructose-1,6-bisphosphatase was added and the mixture was incubated for 24 hr. Next, the quenching solution (1.0 M glycine, pH 8.0) was added and incubated for 24 hr. While being retained by a magnet, the beads were washed 3 times using 0.01 M Tris, 150 mM NaCl, 0.1 % BSA (w/v), 1 mM EDTA, and 0.1% sodium azide.

For the immobilization of fructose-1,6-bisphosphatase on Maleimide magnetic beads, the storage buffer was removed, and the beads were washed 3 times with 100 mM Tris, pH 7.2. The beads were resuspended in the coupling solution (100 mM Tris, pH 7.2, 5 mM TCEP) and incubated for 24 hr. Next, the coupling solution was replaced by a blocking solution consisting of 100 mM Tris, pH 7.2, and100 µg/mL L-cysteine, and incubated for 24 hr. The beads were washed using 100 mM Tris pH 7.2 twice and resuspended in the same buffer. Table 5 shows an abridged immobilization protocol for each bead chemistry.

TABLE V - ABRIDGED PROCEDURES FOR THE VARIOUS BEAD CHEMISTRIES. NHS/SULFO NHS AND COOH/HYDROPHILIC COOH BEADS REQUIRED THE LEAST AMOUNT OF STEPS AND THE SHORTEST TIME REQUIRED FOR IMMOBILIZATION. AMINE AND MALEIMIDE IMMOBILIZATIONS REQUIRED MULTIPLE 12 OR MORE HOUR INCUBATIONS TO COMPLETE IMMOBILIZATIONS.

NHS/Sulfo-NHS	COOH/Hydrophilic COOH	Amine	Maleimide
1. Remove Storage Buffer	1. Remove Storage Buffer	1. Remove Storage Buffer	1. Remove Storage Buffer
2. Wash 3x with Incubation Buffer	2. Wash 3x with Coupling Buffer	2. Wash 3x with Glutaraldehyde Solution	2. Wash 3x with Tris Buffer
3. Resuspended with Incubation Buffer	3. Resuspend with Coupling Buffer	3. Resuspended with Glutaraldehyde Solution	3. Resuspended with Tris and TCEP Buffer
4. Add Protein	4. Add Protein	4. Incubate 3 hours	4. Add Protein
5. Incubate for 2 Hours	5. Incubate for 2 Hours	5. Add Protein	5. Incubate 12 Hours
6. Replace with Quenching Buffer	6. Replace with Quenching Buffer	6. Incubate 24 Hours	6. Replace with L-cysteine Solution
7. Incubate for 2 Hours	7. Incubate for 2 Hours	7. Replace with Quenching Solution	7. Incubate 12 Hours
8. Wash 3x with Quenching Buffer	8. Wash 3x with Quenching Buffer	8. Incubate 24 Hours	8. Wash 3x with Tris Buffer
9. Resuspended in Quenching Buffer for use	9. Resuspended in Quenching Buffer for use	9. Wash 3x with Tris Buffer	10. Resuspended in Tris Buffer for use
		10. Resuspended in Tris Buffer for use	

Control beads were prepared using identical procedures for each bead chemistry except for the omission of fructose-1,6-bisphosphatase. For the evaluation of each immobilization procedure and bead chemistry, incubations were carried out in microtiter plates using immobilized fructose-1,6-bisphosphatase or beads prepared without the target protein and the following controls: no ligand, 100 µM fructose bisphosphatase inhibitor, 100 µM MB05032, and 100 µM cymaroside. Each bead chemistry was compared with respect to background noise, positive control peak enrichment, and lack of negative control enrichment. The general assay procedure was as follows: beads were incubated for 1 hour with100 µM controls or without ligands. Then, the beads were washed once with resuspension buffer and then twice with 50 mM ammonium acetate, pH 7.0. The protein-ligand complex was disrupted using 150 µL of methanol. The supernatants were pipetted to a new 96-well plate and evaporated to dryness using a vacuum centrifuge. The compounds in each well were reconstituted in 100 µL of water/acetonitrile (50:50, v/v) containing100 nM ketoconazole as an internal standard and measured using LC-MS/MS as described below.

Based on these preliminary experiments, Hydrophilic COOH magnetic beads were selected for subsequent use and optimization. The optimized procedure for fructose-1,6-bisphosphatase immobilization was as follows: the storage buffer was removed from 500 µL of HCOOH magnetic beads (20 mg/mL), which were then washed 3 times with 1 mL of 50 mM MES, pH 7.0. The beads were resuspended in 1 mL of the MES buffer, and 5 mg each of EDC and NHS were added. After 15 min incubation, the beads were resuspended in 0.5 mL of 150 mM NaCl, 0.01% Tween-20, and 50 mM

MES, pH 7.0, and then 1 mL of 1 mg/mL fructose-1,6-bisphosphatase was added. After incubating at 4 C for 2 hours, the beads were washed and then resuspended in 1 mL of the blocking buffer: 150 mM NaCl, 50 mM MES, 0.1% sodium azide (w/v) and 50 mM Tris pH 7.0. After 2-hour incubation, the beads were washed twice with the MES buffer and resuspended in 1.2 mL of the blocking buffer.

After immobilization, the fructose-1,6-bisphosphatase beads were used in a MagMASS assay of marine natural products. In a deep well microtiter plate, 300 µL of 150 mM NaCl, 50 mM MES, 0.1% sodium azide (w/v) and 50 mM Tris pH 7.0 were added to each well. A 10 µL aliquot of the protein tethered beads was added to each well along with a 2.5 µL aliquot of the actinomycetes library and incubated for 1 hour. The last column on each microtiter plate was consisted of controls that were prepared identically except as follows: two wells contained no ligands and two wells contained 2.5 µL 100 µM fructose-1,6-bisphosphatase inhibitor, 2.5 µL 100 µM MB05032, and 2.5 µL 100 µM cymaroside. The beads were drawn down and washed twice with 150 mM NaCl, 50 mM MES, 0.1% sodium azide (w/v), and 50 mM Tris pH 7.0. Then the beads were washed twice using water. 150 µL of methanol was added to disrupt the protein ligand complex. After 30 minutes of denaturing, the supernatant was collected into a clean microtiter plate and evaporated to dryness using vacuum centrifuge (about 3 hours). In parallel, a duplicate negative control plate was processed that was identical except that no F16BPase beads were added. The plates were stored at -20 °C freezer until LCMS analysis.

Immediately before analysis, the contents of each well were reconstituted in 100 µL of 1% aqueous formic acid/acetonitrile (50:50; v/v) containing 100 nM ketoconazole

as an internal standard. After a minimum of 30 minutes, each plate was placed into an auto sampler for LCMS analysis. Each plate was analyzed simultaneously with the corresponding negative control plate. Each actinomycetes sample well was evaluated in duplicate on different days.

Mass Spectrometry

LC-MS/MS analyses were carried out using a Shimadzu LCMS-8040 triple quadrupole mass spectrometer and Nexera UHPLC system equipped with electrospray ionization and a Waters Cortecs C_{18} column (2.7 µm, 2.1 × 50 mm). Compounds were eluted using 0.25 minute divert to waste to remove salts, then a 2.0-min linear gradient from 25% to 90% acetonitrile containing 0.1% aqueous formic acid at a flow rate of 0.5 mL/minute. Between analyses, the column was re-equilibrated at 25% 0.5 for 1 minute.

Collision-induced dissociation and selected reaction monitoring (SRM) were optimized for each compound. For MB05032, the SRM transitions were *m/z* 303.10(+) to*m/z* 285.05(+) and *m/z* 151.05(+). The Q1 pre bias was -15V, the collision energies were -25 eV and -38 eV, and the Q3 pre bias values were -27 V and -25 V. For fructose bisphosphatase inhibitor, the SRM transitions were *m/z* 376.90 (+) to *m/z* 168.10(+) using positive ion electrospray and *m/z* 374.90 (-) to *m/z* 145.0(-) and *m/z* 208.90(-) in negative ion mode. The Q1 prebias was -19, 17, and 17 V, respectively. The collision energies were -30, 28, and 21 eV, and the Q3 pre bias values were -30, 28, and 23 eV, respectively. Ketoconazole and cymaroside were measured as described in Chapter 3.

High resolution mass spectrometric analyses of marine natural products were carried out using a Shimadzu LCMS-IT-ToF mass spectrometer equipped with a

Shimadzu Alliance XR HPLC system and a Waters Cortecs C_{18} column (2.7 µm, 2.1 × 50 mm). The HPLC mobile phase was identical to that used above for the Shimadzu LCMS-8040 triple quadrupole analyses. High resolution mass spectra were acquired from *m/z* 150 to 1000 with polarity switching (200 ms per spectrum) with a total cycle time of 0.6 seconds. Ion accumulation in the ion trap was 10 ms and the base peak chromatogram was set to 70%. Two microtiter plates (MagMASS experiment and control) were loaded into the auto sampler microtiter tray at a time. Each microtiter plate was covered with a thin plastic cover to prevent solvent evaporation.

XCMS Infrastructure

To develop an offline metabolomics infrastructure, a small business server was purchased from Newegg (Industry, CA, USA). The server consisted of two Intel Xeon E5-2670 processors for 16 total cores. With 128 GB of memory and a terabyte hard drive, XCMS Plus (La Jolla, CA, USA) was installed for the offline processing of the experiments using VMWare to run the virtual machines (Palo Alto, CA, USA).

Each experiment consisted of positive ion and negative ion data processing in XCMS as determined by the .mzxml files. During the feature detection, the maximal error threshold for consecutive scans was set to 5 ppm. The min/max peak widths were set to 3 and 60 secs. The retention time correction window set to 0.5 *m/z*. The width of overlapping *m/z* windows for density chromatograms was set to 0.025. For XCMS to align the experiment and control chromatogram peaks the threshold of minimum fractions to align was set to 0.5 – the *m/z* value had to be detected in at least one of

each experiment and control experiments. The allowable retention time deviations were set to 5. The statistical analysis was set to use an unpaired parametric t-test or Welch t-test. The threshold of enrichment and p value was set to 1.5 and 0.05 for XCMS Plus to exclude any other *m/z* values for analysis.

Although annotation and identification were available through external database sources, such as metlin or mzmine, this function was not utilized. In the complete version of XCMS Plus, the accessible libraries are greater and include some actinobacteria species. For this demo version of XCMS Plus, these databases were unavailable, so this assistance in annotating and possibly identifying structures was not available. This option may not be useful for structural determination of compounds from Actinomycetes because the number of entries for these natural products in the database on XCMS online is limited.

Tableau Data Visualization

XCMS Plus is designed for the analysis of data files one by one. Therefore, processing 1000 experiments in this manner became a bottleneck. To overcome this bottleneck, all the processes XCMS Plus data were exported into spreadsheets and visualized using Tableau (Seattle, WA, USA), which is designed for business intelligence and analytics.

Inhibition Assay

Based on the data visualization, MagMASS wells enriched in ligands for fructose-1,6-bisphosphtase ligands were selected for follow up experiments using afunctional assay. This confirmatory assay was designed to determine if the wells testing positive for ligands using MagMASS contained compounds that inhibited the activity offructose-1,6-bisphosphtase. The functional assay carried out using a previously established protocol.[99] malachite green microtiter kits were purchased from Caymen Chemicals (Ann Arbor, MI, USA).Each well contained a final volume of 150 µL. In each well, 50 mM MES pH 9.0, 2 mM $MgCl_2$, 0.2 µg fructose-1,6-bisphosphatase. Each extract was diluted and added to have a final concentration of 0.1 mg/mL, 1 µg / mL, and 10 ng/mL. The plate was incubated for 30 minutes at 37 C and lightly shaken. Fructose-1,6-bisphosphate was added to a final concentration of 0.1 mM. The reaction proceeded for 20 minutes at 37 C and was terminated by adding 5 µL Malachite Green Acidic Reagent. The plate was incubated for 10 minutes at room temperature and 15 µL of the Malachite Green Blue Solution was added. After gentle mixing for 20 minutes at room temperature, the plate was read at 630 nm. The standard curve for the plate was created following the procedures from the kit. Fructose-1,6-Bisphosphatase Inhibitor and MB05032 were used as positive controls. Instead of the extracts at three different concentrations, the inhibitors were added at a final concentration of approximate IC_{50} values, 156 µM and 20 nM respectively.

Results

Selecting Bead Chemistry

Five different bead chemistries were tested for the greatest compatibility with fructose-1,6-bisphosphatase. The experiments were done in pairs – immobilizing protein and immobilization without protein via the manufacturer's instructions. The enriched peak areas of the two positive controls and the negative controls were the metrics to analyze which bead chemistry would be used in the larger screening project.

During the analysis, the nanomolar positive control was not detected in the LCMS analysis. MB05032 was not detected in any protein-tethered bead wells. This could be a result of the inhibitor acting as a nonreversible inhibitor. The mechanism of action of MB05032 is described as an AMP mimic[100]. MagMASS requires that the inhibitors to be released from the protein. For an unknown reason, MB05032 was not detected post MagMASS assay. Therefore, the micromolar inhibitor, fructose bisphosphatase inhibitor, became the metric of peak enrichment for evaluating the magnetic bead chemistries. The ranking of the various bead chemistries for immobilizing active fructose-1,6-bisphosphatase was as follows: HCOOH > NHS > COOH =Sulfo-NHS > amine = maleimide. For all functional bead chemistries, the negative control, cymaroside, was not detected. Figure 18 shows a representative chromatogram comparing the detection of FBPI of each functional bead experiment to its control.

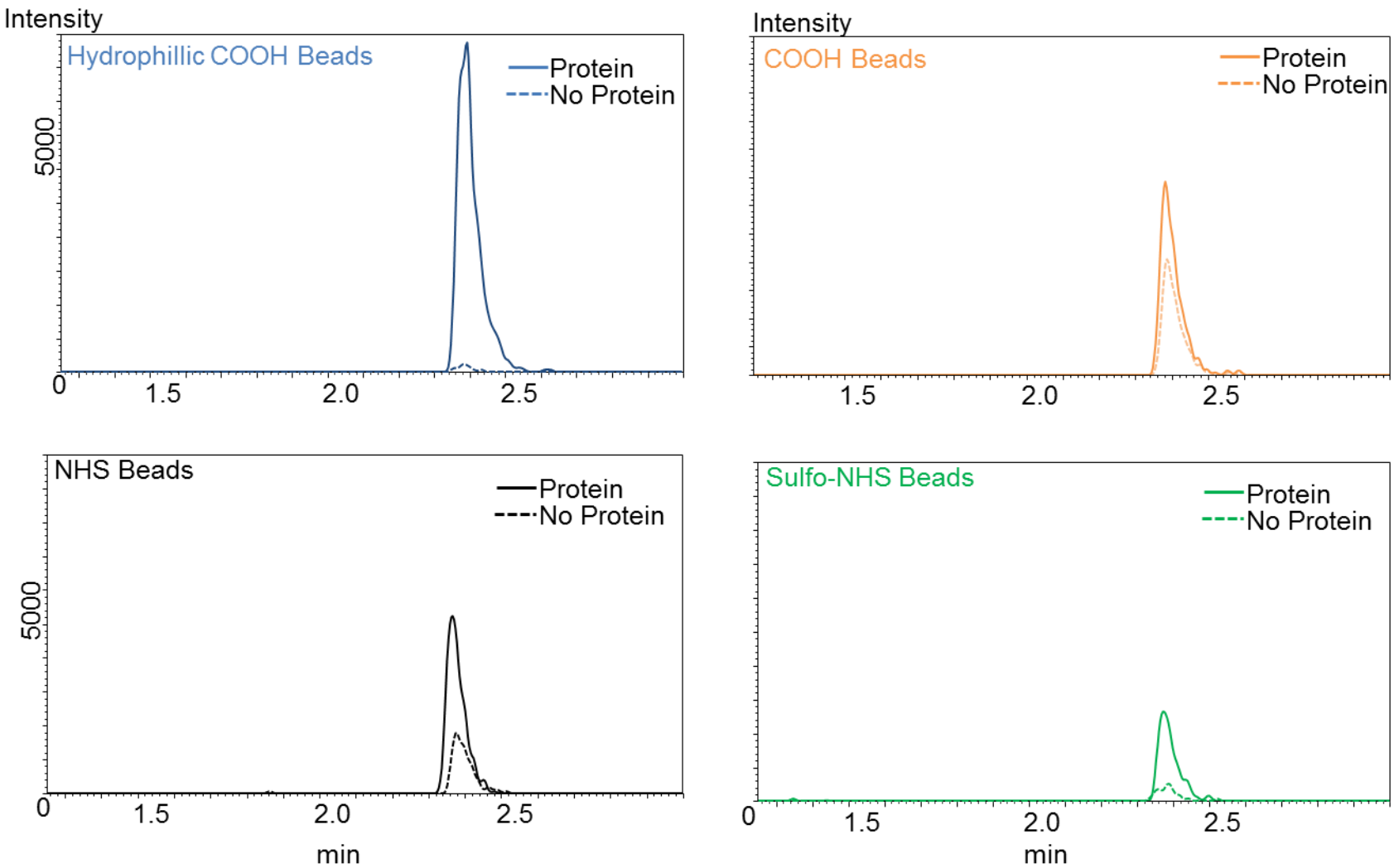

Figure 17 - The effect of different bead chemistry on the enrichment of FBPI. Hydrophilic COOH beads provided the best enrichment and intensity of the positive control FBPI. COOH beads enrichment is low, NHS beads enrichment is satisfactory, but the intensity is low. Sulfo-NHS beads had the satisfactory enrichment, but the lowest

intensity. Amine and maleimide beads are not shown from the concern of irreproducibility in bead recovery. Comparing the bead chemistries, Hydrophilic COOH beads are chosen for further use – out of all the bead chemistries, Hydrophilic COOH beads have the highest intensity and greatest enrichment.

The longer immobilization procedures using amine and maleimide functionalized beads produced the least active immobilized fructose-1,6-bisphosphatase. The length of these procedures, 4 hours for NHS/Sulfo-NHS and COOH/Hydrophilic COOH vs. 51 hours for Amine vs. 24 hours for maleimide beads might have contributed to enzyme degradation. Although not investigated further, adding protease inhibitors or using sterilized buffers and reagents might have enhanced the activity of immobilized fructose-1,6-bisphosphatase, especially for the amine and maleimide bead chemistries.

During all steps of the MagMASS assay, maintaining maximal bead recovery using a magnetic plate is critical. At the onset of the immobilization procedure when the beads are aliquoted and the storage buffer is removed, there is a slight loss of magnetic beads. This is visible as an orange-brown instead of clear supernatant. After this initial small loss of beads, most bead chemistries showed nearly complete bead recovery. For two functional bead chemistries, amine and maleimide, bead recovery was low during the longer 24-hour immobilization procedure. When magnetically drawing down the beads for washing or during the addition of the blocking buffer, a visible amount of beads would adhere to the sides of the plastic wells. Physically removing the beads from the walls with a scraper was ineffective and also possibly introduced contamination. As each immobilization batch is eventually compared to others, variable bead recovery introduces error into the analysis. Out of the remaining functional bead chemistries, HCOOH functional bead chemistries were chosen based on the high enrichment of the positive control.

Data Processing, Analytics, and Hit Selection

After MagMASS of approximately 1000 marine microbial fermentation extracts for ligands to fructose-1,6-bisphosphatase using HCOOH beads, the high resolution LC-MS chromatograms were analyzed in just a few days using the repurposed metabolomics software XCMS Plus with the virtual machine server architecture. MagMASS experiments which were run in duplicate were averaged and compared with the no protein experimental controls. Using the set parameters, 601 either produced no significant features or failed in processing. Figure 19 is a visual representation of the frequency of each issue.

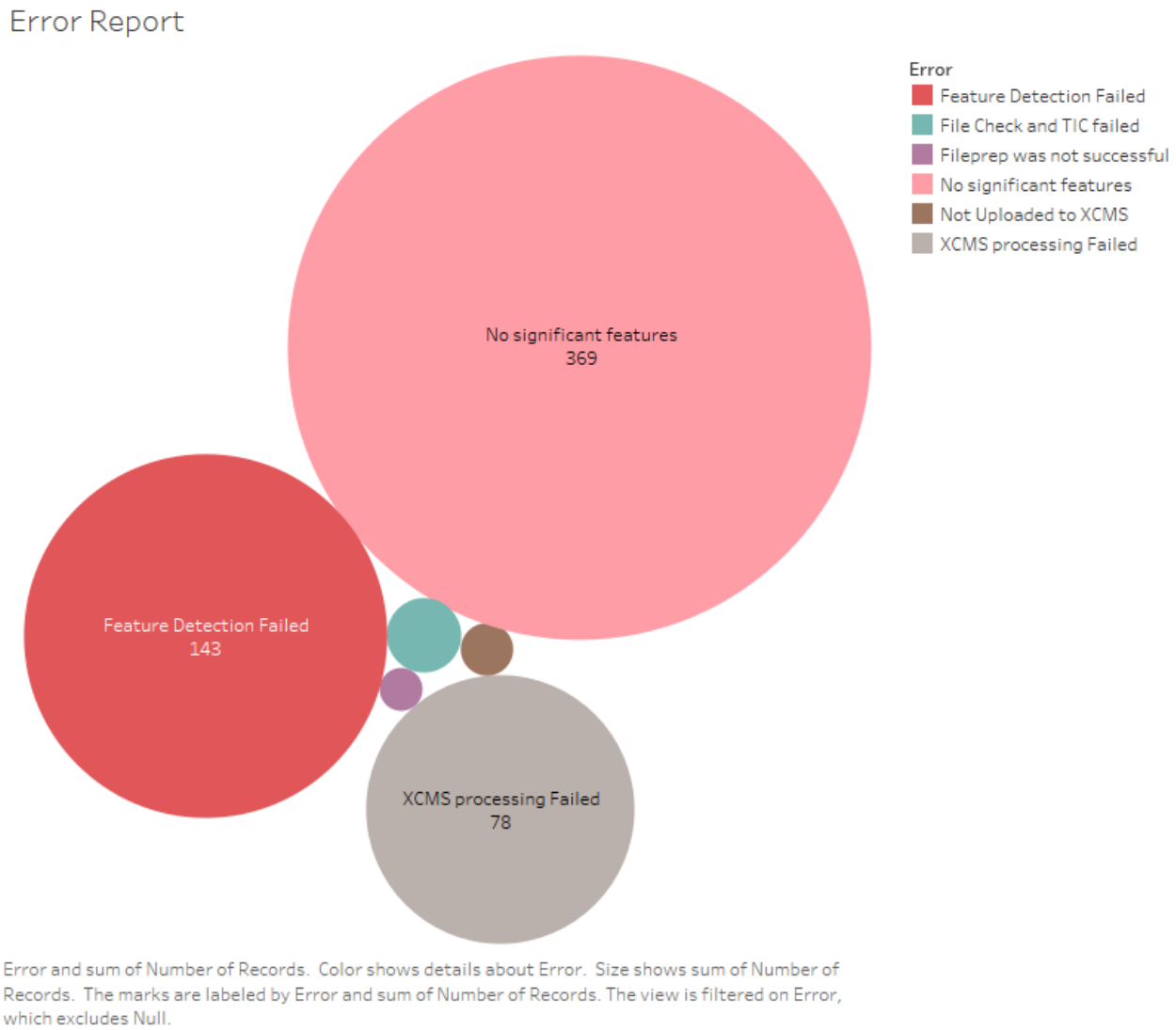

Figure 18 Visual breakdown of the errors encountered during data processing. Out of the 1000 experiments processed, approximately 30% failed in processing and approximately 40% did not have any detected features with an enrichment of at least 1.5 with a p value of 0.05. The size of the circle represents the frequency of the encountered error.

Among the remaining wells, there were approximately 1700 LC-MS peaks determined to be significant hits based on XCMS Plus data processing. XCMS Plus also generated computer-reconstructed ion chromatograms for each hit. Manual examination for each significant hit would be time consuming, a new workflow prioritizing the hits for further investigation is required. Figure 20 shows a plot of enrichment factor versus significance for all 1700 features.

In our previous analyses of MagMASS data, the numbers of analyses and resulting hits were smaller, so that all hits all with low significance and high enrichment could be investigated.. Features with high enrichment and higher p values or features with low enrichment but high p value should receive high priority, while those with low enrichment and low p value should be the last set of significant features for follow up analysis.

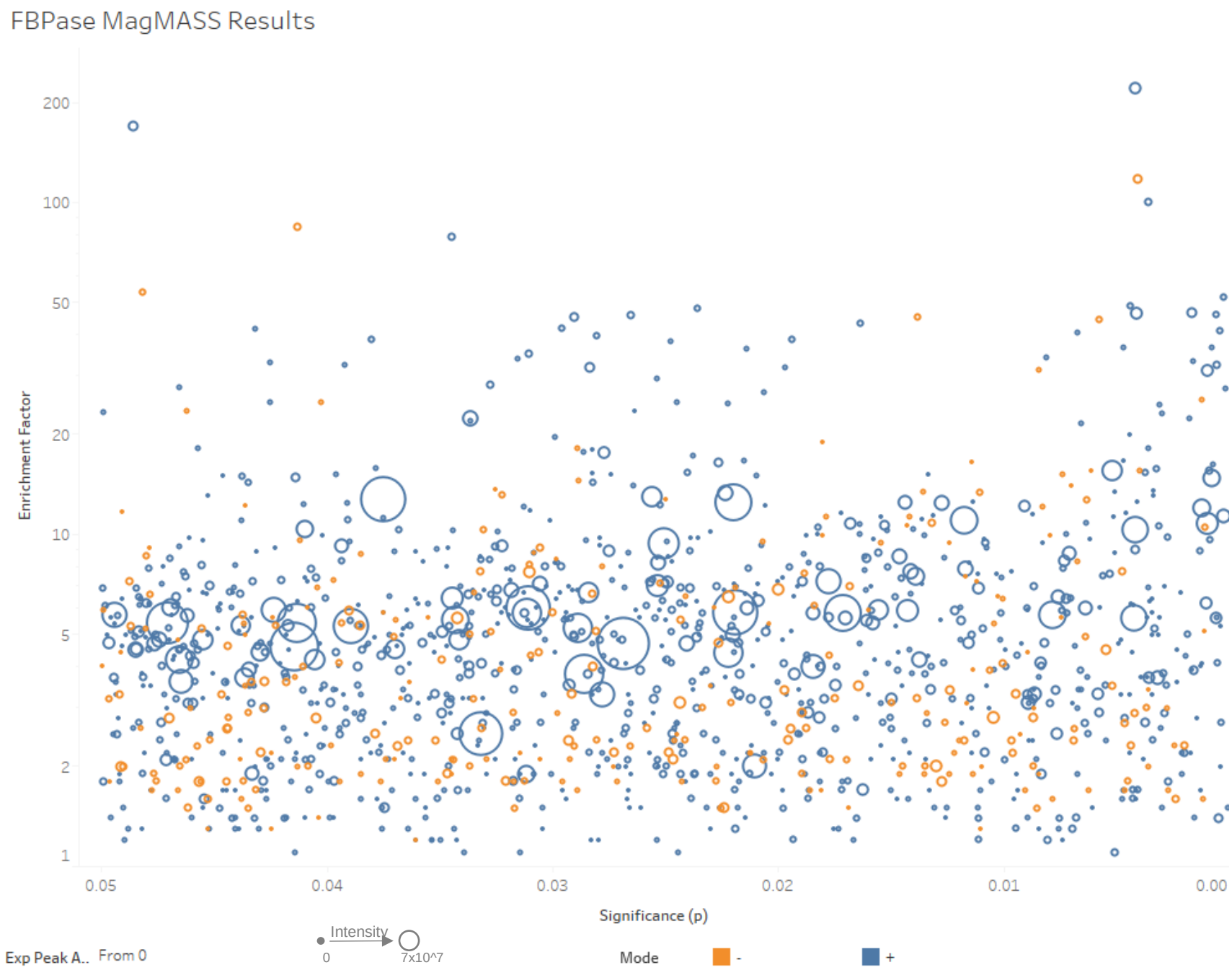
FBPase MagMASS Results
Enrichment Factor
200
100
50
20
10
5
2
1
0.05
0.04
0.03
0.02
0.01
0.00
Significance (p)
Intensity
0
7x10^7
Exp Peak A..
From 0
Mode
-
+

Figure 19 Over 1000 MagMASS experiments were processed through XCMS Plus, and 1700 significant features were identified. Plotted as enrichment factor versus significance, each circle corresponds to a different significant feature, and the size of the circle corresponds to the peak area of the feature, and the color denotes positive ion (blue) or negative ion (orange) electrospray mass spectrometry. The Previous iteration of MagMASS workflow would select features for future analysis by a juxtaposition of the lowest p value to the highest enrichment factor. In effect, all results landing in the top right quadrant of the plot of all data points.

During manual data inspection, qualitative decisions can be made about the intensity of each feature relative to background noise, peak shape, etc. These decisions on the quality of the feature are challenging to incorporate in an automated decision tree, but are necessary for prioritizing 1700 features. The lack of these quality decisions means a number of features are background noise. Criteria are required to remove potential noise signals needs to be incorporated in the analysis to parse out the significant features. Most features that are noise tend to have the lowest intensities out of the features. This problem is easily displayed if the feature list is parsed to include only the lowest intensity features. For example, Figure 21 shows positive ion electrospray MagMASS features from Figure 20 that have a maximum peak area 50 000 intensity. From this subset of low peak area features, an example feature of *m/z* 384.80(+) (from pooled plate 3 well G7) was selected for having the ideal characteristics of high enrichment and a low p value. Inspection of the computer-reconstructed mass chromatogram of the ion of *m/z* 384.80(+) for this feature (Figure 22) shows that it is a false hit containing only noise. The quality of selecting features for follow up requires eliminating these noise features. Therefore, instrument noise needs to be added to the automated decision tree for hit identification and prioritization.

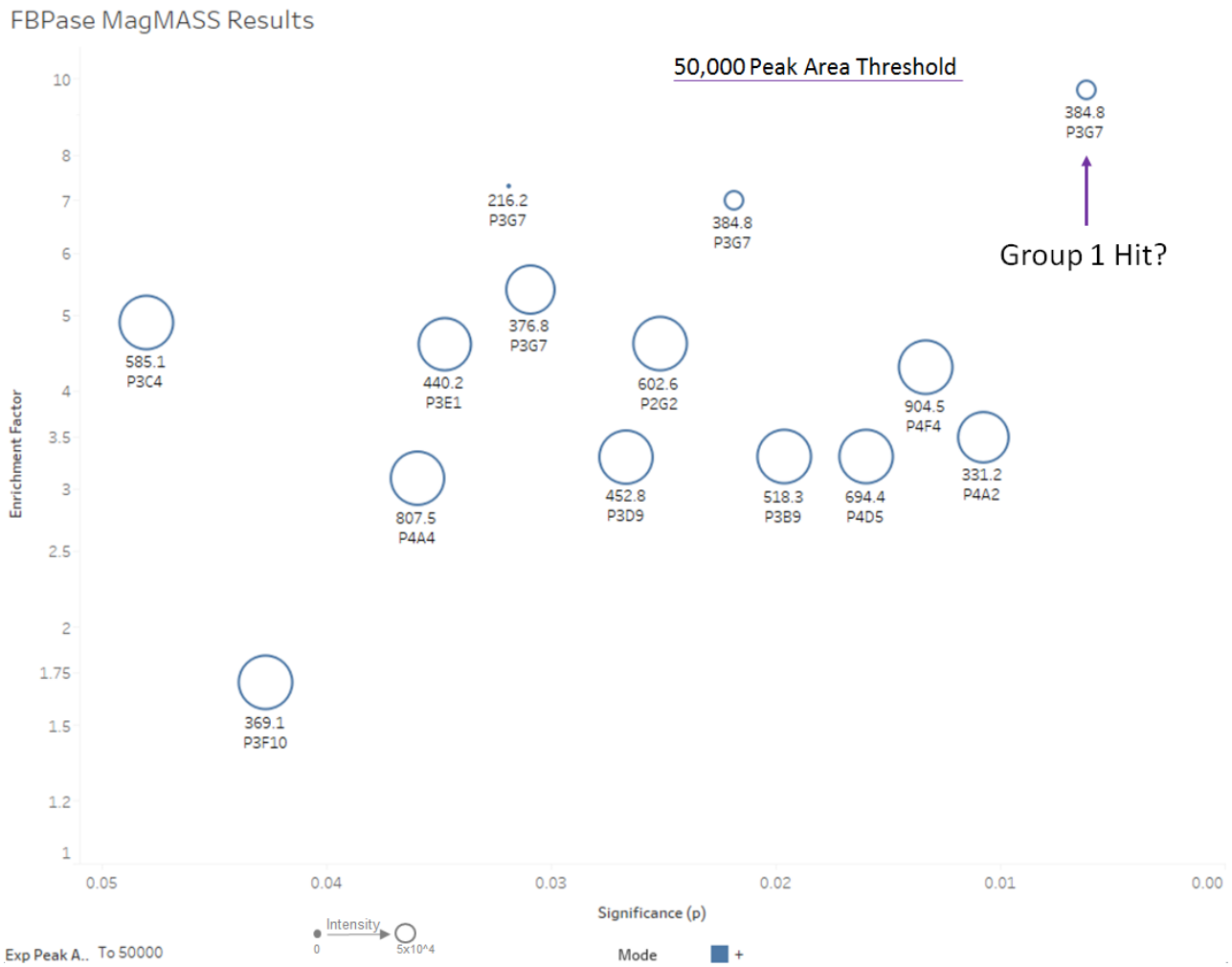

Figure 20 Fructose-1,6-biphosphatase MagMASS positive ion electrospray features plotted as enrichment factor versus significance with a maximal peak area of 50 000 intensity. The size of each circle corresponds to the peak area of the feature. One feature is highlighted for additional investigation.

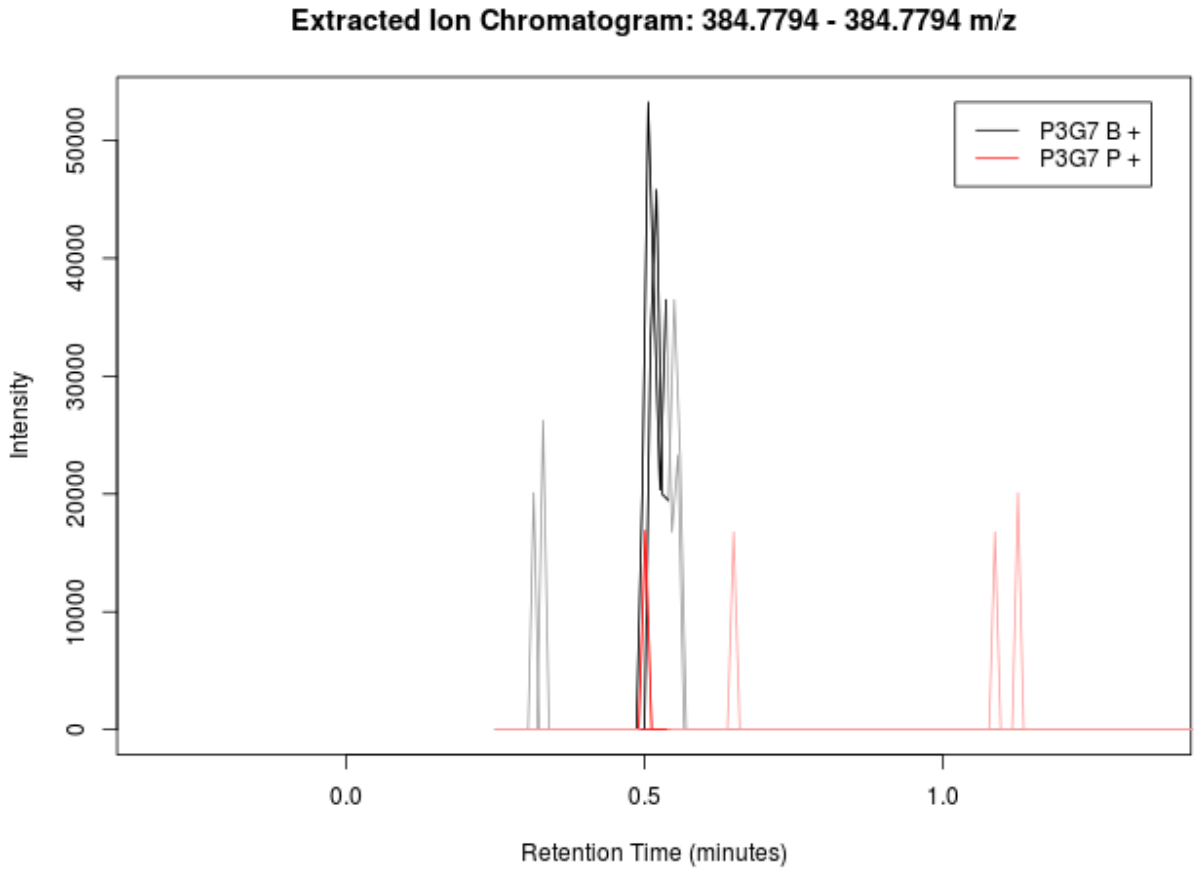

Figure 21 Extracted ion chromatogram selected from features in Figure 21. This feature, while having high enrichment and low p value, is most likely noise. Even if the feature was not noise, the abundance is too low to have any biological effect in a functional assay. Features with low chance for follow up success can be eliminated by having a minimum peak area threshold.

A minimum threshold for MagMASS peak area was required as a metric for automated feature evaluation. This metric provides two benefits. First, many noise signals are eliminated, and there is a higher likelihood of identifying compounds found in the extract. Secondly, MagMASS fundamentally works by identifying compounds that bind to the protein. There is no expectation or estimation of biological activity. Therefore, MagMASS ultimately requires a bioassay to confirm features into hits. While MagMASS eliminates a lot of the problems of bioassay guided fractionation, it does not eliminate the role of the bioassays. While there may be low intensity compounds from these actinomycetes library that high enrichment factors and significance, their absolute concentration may be too low for any biological activity in a bioassay. This may produce false negative results from the bioassay. Along the same lines, if this workflow is to produce direction in finding compounds that not only bind, but also are possible to collect metabolites from these actinomycetes from later growths, focusing on high peak area features is advantageous. If there is a choice to divide resources – money and time for follow up investigation – focusing on metabolites of greater abundance improves the odds of follow up purification and isolation. While these compounds may be binders and have biological activity, any follow up work is exceedingly difficult if they extracted in trace amounts. For greatest success for narrowing down the wells for further investigation, this minimum area threshold can be set to find more abundant metabolites for biological activity. If the features are parsed down with a minimum peak area threshold to 2 000 000 intensity, the number of features becomes much more manageable (Figure 23).

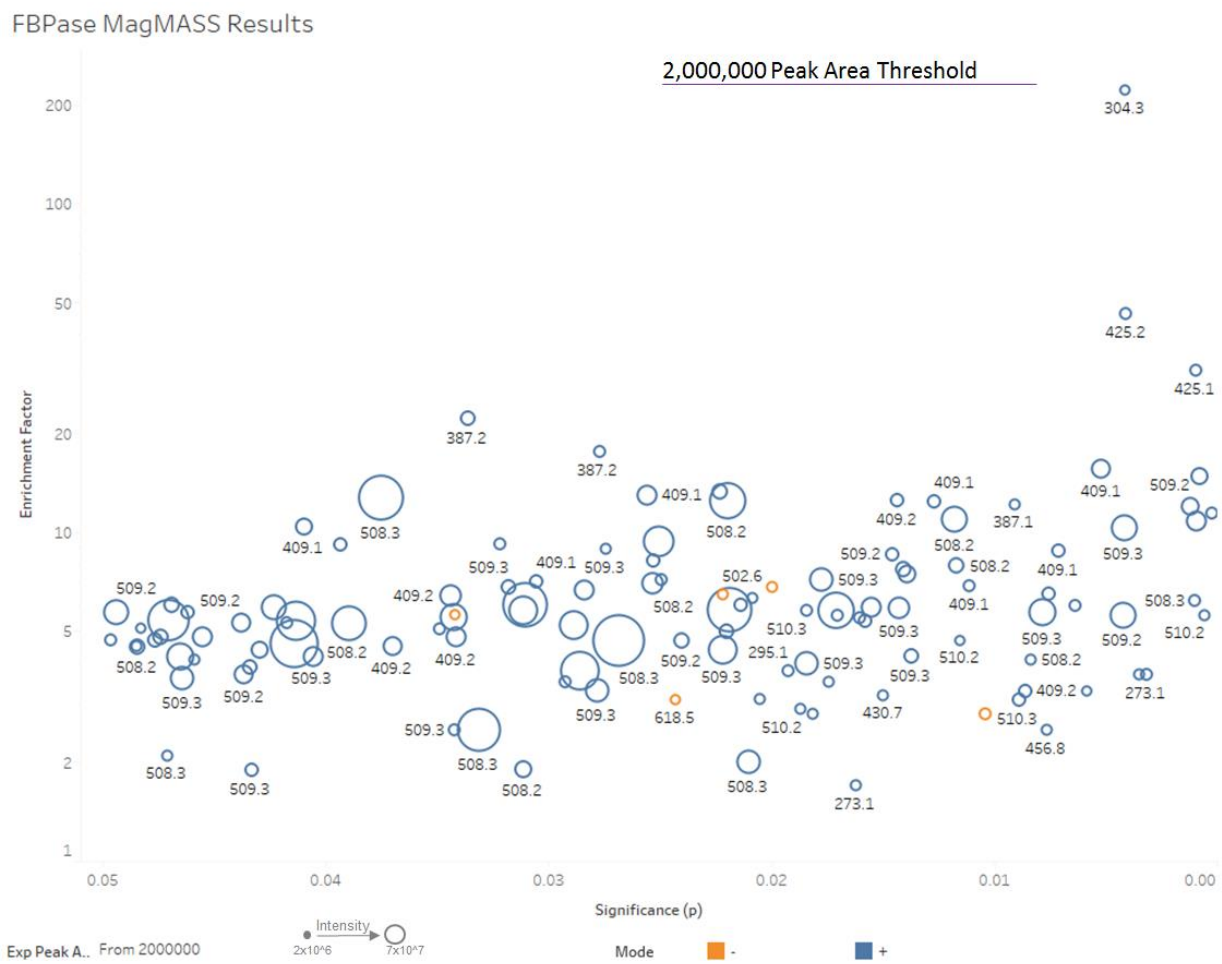

Figure 22 Fructose-1,6-bisphosphatase MagMASS features plotted as enrichment factor versus significance with a minimal peak area of 2 000 000 intensity. The size of the feature corresponds to the peak area of the feature. This additional parsing puts reduces the features to a manageable amount for further investigation.

After reducing the number of features using a minimal MagMASS peak intensity, many of the remaining features were isomeric, as indicated by identical high resolution *m/z* values. This redundancy might be due, in part, to similar types of compounds occurring in different actinomycetes cultures. Therefore, the significant features shown in Figure 24 were replotted after grouping by similar *m/z* values. By eliminating redundant hits, the total number of active wells to be retested in functional bioassays was again reduced. In particular, the wells corresponding to the most abundant ions of each *m/z* value were selected for functional assay confirmation. These selected wells are summarized in Table 6.

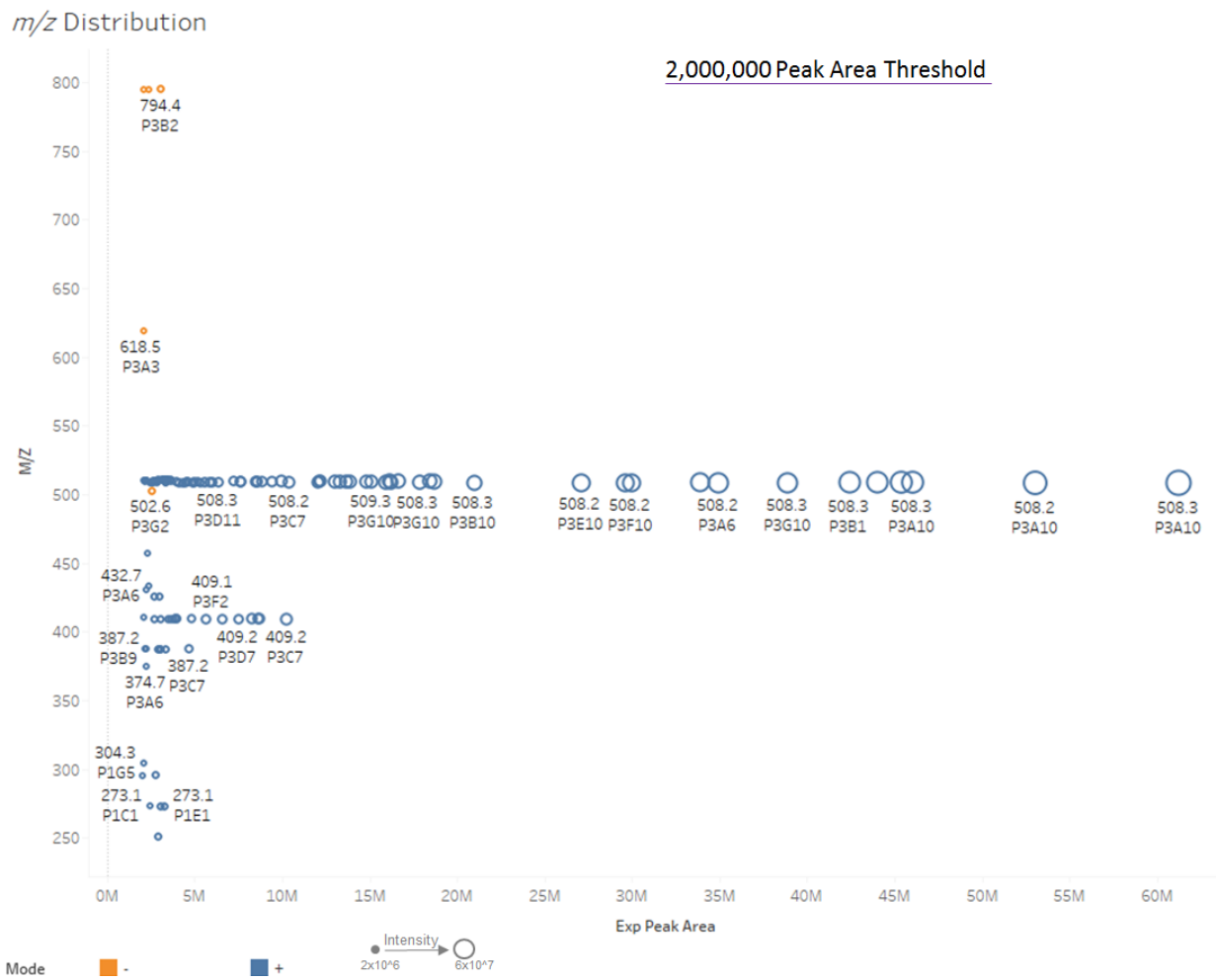

Figure 23 Fructose-1,6-bisphosphatase MagMASS features from Figure 23 (enrichment >1.5, significance $p < 0.05$, and peak area ≥ 2 000 000) were re-plotted according to *m/z* value versus peak area. An enrichment of 1.5 has been a minimum enrichment for selecting a hit using PUFMS. A significance of 0.05 allows more confidence that this feature is reproducible. A minimum peak area of 2 000 000 removes many of the undesirable problems of either trace metabolites or noise.

TABLE VI SELECTED SIGNIFICANT FEATURES AND THEIR CORRESPONDING WELLS FOR FOLLOW-UP OF BIOLOGICAL ACTIVITY.

m/z	Corresponding Well
251.1(+)	P1C1
273.1(+)	P1E1
304.3(+)	P1G5
374.7(+) / 432.9(+)	P3A6
387.2(+) / 409.1(+)	P3C7
409.1(+)	P3D7
425.1(+)	P3H5
430.7(+)	P3D2
508.3(+)	P3A10
509.3(+)	P3C11
618.5 (-)	P3A3
794.4 (-)	P3B2

Functional Assay

Figure 25 shows the response from the fructose-1,6-bisphosphatase inhibition assay in duplicate. After reading the absorbance at 630 nm, each well had the background subtracted. The absorbance values for each well on the plate is normalized to the vehicle control well and indicated potential inhibition of fructose-1,6-bisphosphatase. Wells P1C1 were selected because of the possibility that the feature detected through XCMS might have been the same compound *m/z 251.1* and *m/z 273.1* corresponding to $[M+H]^+$ and $[M+Na]^+$ respectively. These wells showed inhibition, although low levels. Well P3A10 shows significant inhibition at the intermediate and high concentrations of the extract. This feature is a possible candidate for follow up investigation. Well P3C7 had two features that are detected at *m/z* 387.2 and *m/z* 409.1 possibly correspond to $[M+H]^+$ and $[M+Na]^+$ adducts. In addition, well P3D7 also showed a similar feature of *m/z* 409.1. The lack of signal at *m/z* 387.2 in well P3D7 suggests these may not be adducts. It is important to note that these *m/z* values are averaged masses of the extracted chromatograms. Both of these wells show weak inhibition – these might be candidates for further follow up investigation. All other wells showed little reproducible inhibition.

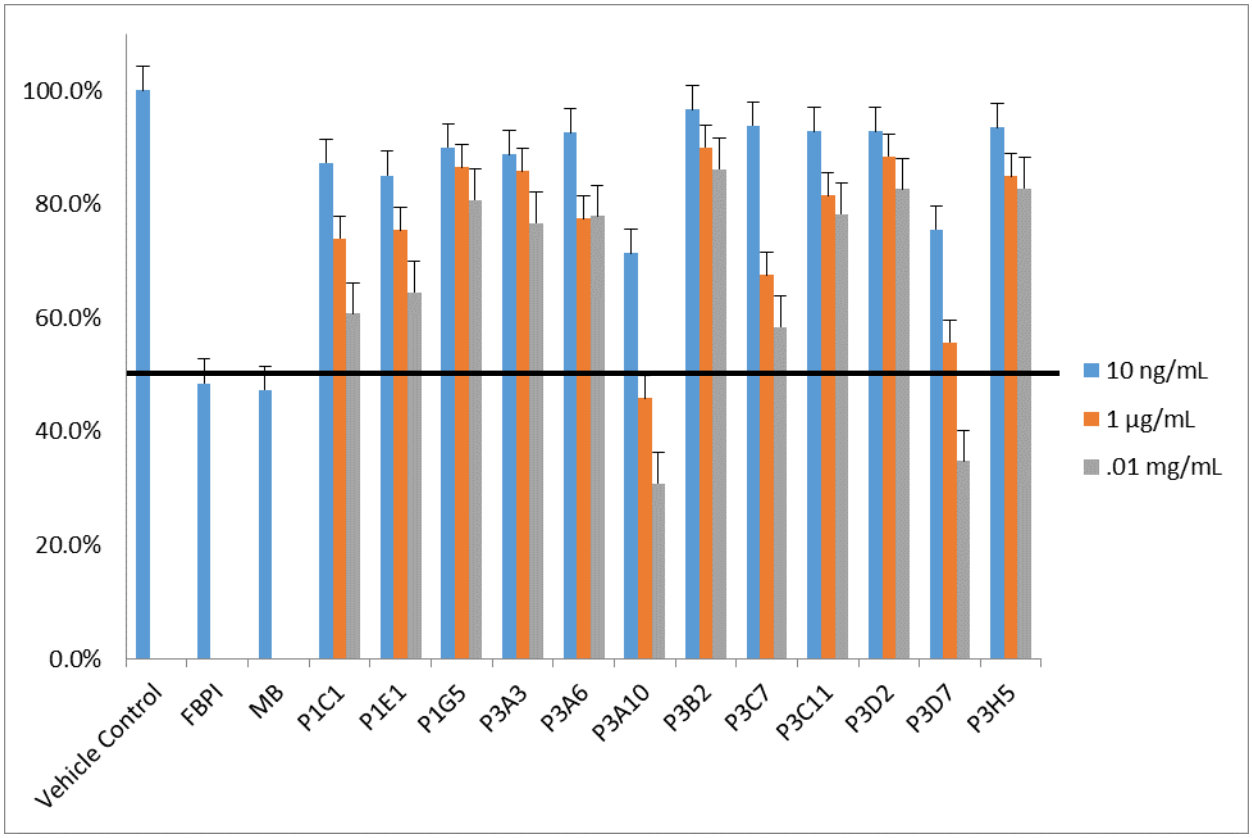

Figure 24 Enzymatic Inhibition of Fructose-1,6-bisphosphatase by Selected Wells. The Malachite green assay was normalized to the vehicle controls. The positive controls FBPI and MB were added at their IC50 concentrations. Each selected well was tested at three different concentrations: low, intermediate, and high. Well P3A10 and Well P3D7 are possible candidates for follow up investigation. Wells P1C1, P1E1, and P3C7 are also possible candidates.

Hot Wells and Possible Structures

Table 7 shows the accurate mass for each of the significant features. The most promising feature, *m/z* 508.253 from P3A10 is indicated. Within an error of 5 ppm, there are 10 possible elemental compositions. There are two compounds of *m/z* 508)+) previously reported from actinomycetes strains: Levantilide A[101] or a dimer of an unreported structure[102]. Levantilide A can be excluded since the exact mass would be 508.376 (+) – the mass error for this possible structure is large enough that it could not be the detected compound of *m/z* 508.253 (+). Tanvir *et al*[102] isolated a novel actinomycetes compound that produces a $[2M+H]^+$ at *m/z* 508.2. Their compound 3 had a $[M+H]^+$ of *m/z* 254.2, but was not detected for the mass spectra for well P3A10. Unfortunately, Tanvir *et al*[102] did not elucidate a structure, but a possible formula of $C_{16}H_{31}NO_2$ was reported. While a possible lead, more work is needed to confirm the structure. There are no published entries for the ions of *m/z* 387.178(+) from P3C7, *m/z* 387.170(+) and *m/z* 409.160(+) from P3D7, *m/z* 251.142(+) from P1E1, *m/z* 251.144 and *m/z* 273.126(+) from P1C1. A future project from this work can be the structure elucidation of these particular compounds. Figure 26 is a series of chromatograms of the extracts from the library with extracted ion chromatograms of the ions of interested from each well.

TABLE VII- HIGH RESOLUTION ACCURATE MASS MEASUREMENTS FOR SIGNIFICANT FEATURES WITH ENZYMATIC INHIBITION. THE POOLED WELLS ARE DECODED BACK TO THE STRAINS FOUND IN DR. MURPHY'S ACTINOMYCETES LIBRARY.

m/z	Accurate Mass	Corresponding Pooled Wells	Strains
251.1(+)	251.142 (+)	P1C1	D059/B004
273.1(+)	273.126 (+)	P1E1	A044/A045
387.2(+) / 409.1(+)	387.170 (+) / 409.160 (+)	P3C7	J260/K084
409.1(+)	387.178 (+) / 409.161 (+)	P3D7	K124/K127
508.3(+)	508.253 (+)	P3A10	J202/J203

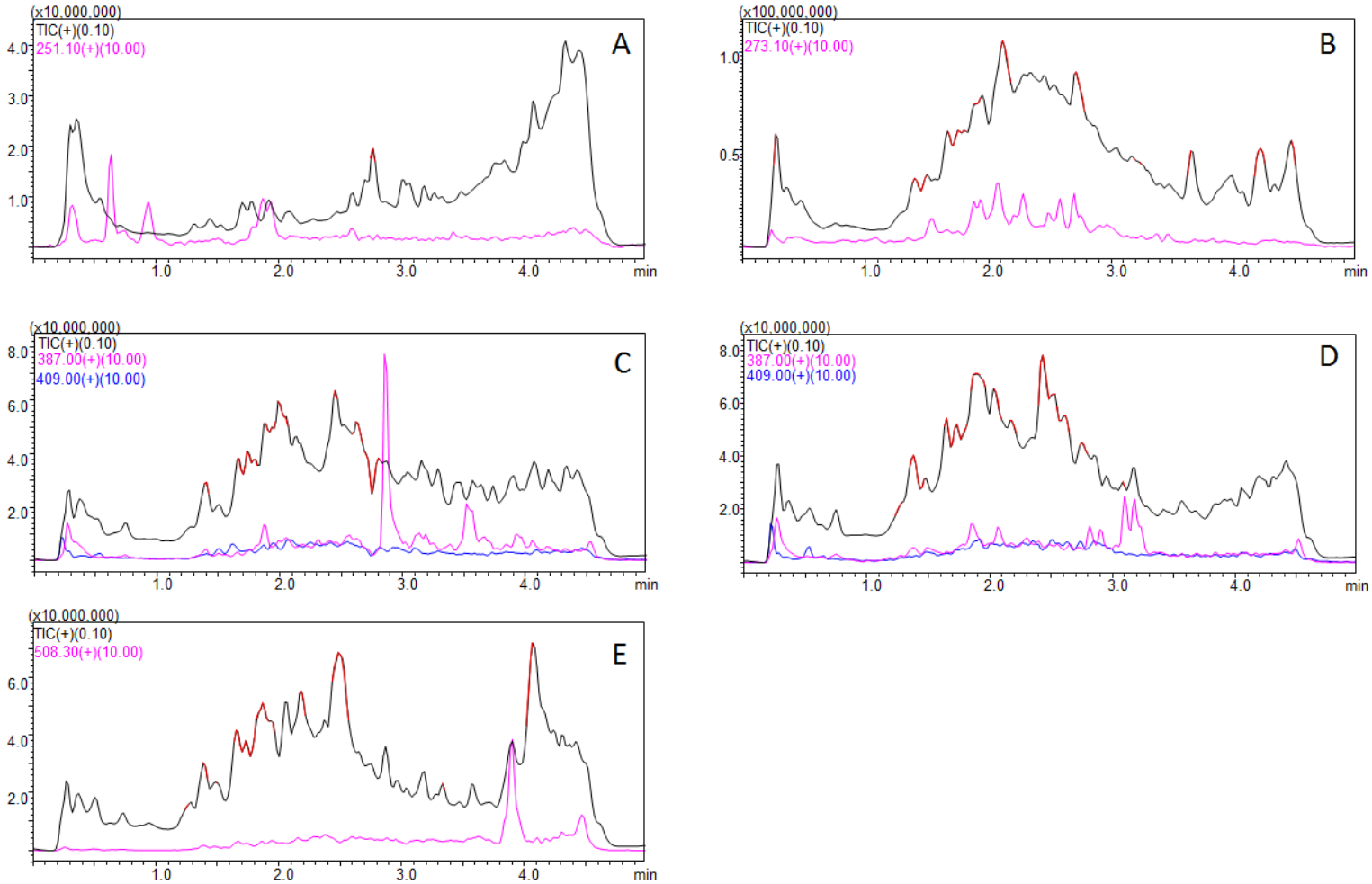

Figure 25 Extracted ion chromatograms for each extract and their ion of interest. These were collected on Shimadzu 8060 using a C18 50 x 2.1 1.6 µ column using a gradient of 0.1% formic acid in water and acetonitrile starting at 5% acetonitrile going to 90% over 5 minutes. The mass spectrometer was collecting a Q3 scan in positive mode with a range of 200 to 600 *m/z* with a scanning rate of 400 units / sec. These experiments confirms if the ions of interested as identified by the inhibition assay are found in the library well. A is the well P1C1 with an XIC for ion 251.1 (+). B is the well P1E1 with an XIC for ion 273.1 (+). C is the well P3C7 with an XIC for ion 387/409 (+). D

is the well P3D7 with an XIC for ion 387/409 (+). E is the well P3A10 with an XIC for ion 508.3 (+). For many of these extracted ion chromatograms, there could be a few possible compounds correlating to a few peaks, but for C and E there are single major peaks relating to each of the ions of interested for follow up structure identification and isolation.

Conclusions

Through this work, MagMASS has been developed to evaluate libraries on a large scale. Previous iterations has been limited in terms of scope; this application to fructose-1,6-bisphosphatase is an high through put natural product drug discovery platform. The assay was developed, the metabolomics and data analytics guide to particular features, and the inhibition provides confirmation for follow up analysis. Compared to previous iterations, the enumeration of the decision making and data analysis is the strongest addition to the history of this screening.

Acknowledgements

I would like to thank Shimadzu Scientific Instruments and Shimadzu Corporation for providing the UHPLC-MS/MS systems used during this investigation. I would like to thank the Brian Murphy for donating the actinomycetes libraries for analysis. I would also like to thank Gerd Prehna for his work on protein expression and purification. This project was funded by the National Center for Complementary and Integrative Health grants R01 AT007659 and F31 AT009039-01.

CHAPTER 5: MAGNETIC MICROBEAD AFFINITY SELECTION SCREENING EVALUATION

Bead Chemistries

Vendor Variations

The quality and the functional chemistries of the beads have turned out to be a critical aspect for success. The RXRα assay used amylose beads from New England Biolabs and ThermoFisher brand, Pierce, for the NHS beads. During the analysis, a graduate of the van Breemen research group founded a magnetic bead company – AvanBio - and was interested in our magnetic bead screening techniques. The 15-LOX assay tested NHS beads from Thermo and AvanBio. The physical properties of the three different vendors could be tested for differences between the beads.

There are two physical properties of the magnetic beads that became important issue during MagMASS development. First was the response of the beads when applied to the magnet. During the steps of drawing the beads to the bottom of the wells to remove storage solutions and buffers, each vendor's beads responded differently. When a suspension of the beads was placed on the rare earth magnet, ThermoFisher Pierce NHS beads would slowly collect at the bottom of the well. In addition, there would be a noticeable amount of bead loss that did not respond to the magnet. New England Biolabs collected faster and there was less loss. AvanBio beads collected the fastest and had minimal bead loss. The effects of the bead loss are normalized since each well was compared to another well with beads from the vendor, but the effects cannot be ignored. Bead loss means there are less active sites for the protein to

immobilize. Less immobilized protein means less sites for ligands to bind and, thus, loss of sensitivity. This effect is most likely minimal; however, using the most compatible methods increases likelihood of success.

The second physical property at issue was the speed of collecting the beads when applying a magnetic field. Drawing down the beads in a faster manner is important for the overall throughput of the assay. In each assay, there are generally twenty steps requiring the beads to be collected. If the collection can be completed in a few seconds instead of a minute, the throughput increases during the assay. The AvanBio and New England Biolabs beads could be recovered twice as fast as the Thermo beads. The overall time for bead collection using AvanBio beads was approximately 15-30 sec depending on the amount of beads in the well.

Functional Bead Chemistries

Initially, the MagMASS assay was developed using fewer bead chemistries. Choi *et al.*[35] used aldehyde and carboxylic acids functional bead chemistries for covalent immobilization. Subsequent covalent immobilizations utilized with MagMASS: NHS, Sulfo-NHS, Amine, Maleimide, and HCOOH. They are not interchangeable; many target the same functional groups on an enzyme, but the immobilization reactions conditions are different. The conditions for the covalent bond formation can damage the integrity of protein targets. During the assay development stage for a novel target, it is critical to select the appropriate bead chemistry. As part of any future MagMASS assay developments, a screening for selecting the most responsive functional chemistry should be implemented.

For *Choi et al.*[35] and the RXRα MagMASS assay[103] noncovalent immobilization techniques has several advantages. First, there is no chemical immobilization step. During covalent immobilization, it is possible that the bead protein bond formation can disrupt the protein; either by blocking important binding sites or changing tertiary structure changing binding pockets. Possibly, the immobilization step may denature the protein. Non-covalent beads do not have this problem. Second, covalent immobilization steps can require significant amounts of time ranging from 5 hours to 48 hours and might involve extra steps to activate the beads prior to immobilization. In contrast, beads for non-covalent immobilization are typically ready for immediate use, thereby requiring less preparation and immobilization reaction time. Lastly, beads for non-covalent immobilization are technically reusable, although none of the MagMASS assays described in this dissertation use recycled magnetic beads.

There is a drawback to noncovalent immobilization using magnetic beads: the beads require an affinity tag or target group on the receptor to be immobilized. These groups tend to be expression tags that are used during protein purification. Many times, these tags are cleaved off the proteins before use, but they need not be removed when using with these immobilization chemistries.

Both types of immobilizations worked throughout the MagMASS assay developments. Choi *et al.* and the RXRα approaches both showed gains in sensitivity using noncovalent immobilization, but covalent immobilization is also effective.

Advantages of MagMASS

Target Independent

One of the advantages of MagMASS is the versatility of this technique. As of May 2017, MagMASS has been used to analyze estrogen receptors α and β ligand binding domains[20], retinoid X receptor α both the full length and ligand binding domain[103], 15-lipoxygenase[104], and progesterone receptor. This technique can target a wide range of proteins; from nuclear receptors to enzymes. As a target based drug discovery system, MagMASS is target independent.

Low Amounts of Natural Product Sources

Bioassay guided fractionation has a problem of scale and consumption. Bioassay guided fractionation starts with an analytical exploratory step for biological activity. If positive biological data is found, yields of the subfractions become vanishingly small. These yields may be too low to continue further bioassays. A follow up fractionation can require copious amounts of starting materials for higher yields of subfractions. This may introduce even more complicated challenges: the same strain does not produce the original metabolites; the starting material may not be available, intellectual property concerns over collecting greater materials, and more.

An advantage of MagMASS is the amount of natural products required for each well for analysis. Only trace amounts of natural products are required for MagMASS initial screening, and then more for the follow up functional assay. The overall

consumption is much less than bioassay guided fractionation. Each well required 10 µg of botanical or microbial extract per experiment and control wells (run in duplicate).

Disadvantages of MagMASS

Cost

MagMASS is not an inexpensive assay. There are many unique significant costs. Approximately 250 mg of beads were required for the screening of the actinomycetes pooled library, at a cost of the beads of approximately $200 (in 2017). This repeated cost can add up for each analysis. A greater cost is instrumentation time for the mass spectrometry. From developing the assay with positive controls to the screening the library, approximately 150 LC-MS hours to screen an actinomycetes extract library of 900 strains and 3600 fractions. At a cost of $25/hour for instrument time, this amounted to 0.24 USD per sample for instrument time.

Although the costs of high-throughput screening of combinatorial libraries can be as low as $1 per well, this approach must be combined with bioassay-guided fractionation, which adds substantially to the expenses. Even in situations with exceedingly low labor costs – say those of a graduate student – the labor intensive process of bioassay guided fractionation cost a minimum of several months of human effort.

Complexity Involving Metabolomics

XCMS plus software has a few variables and options that need to be selected based on the type of instrumentation and the accuracy of the instrumentation using during metabolomics data collection. Optimizing these parameters is required to streamline the data analysis process. In this dissertation,, a tight error range for feature detection was selected. This is narrower than the defaults processing methods. This caused the piecing together of the extracted ion chromatograms to create addition significant figures when the normal instrumentation drift would be wider than the narrow range. In Figure 24 of Chapter 4, the top three most abundant ions (all of *m/z* 508.3 (+)) were obtained from well P3A10. The instrumentation drift probably was wide enough during the analysis of that well to be outside of the processing error window. Most likely, the same compound is being identified as three significant features.

Other metabolomics software packages are available, and there will be a learning curve for each. Another metabolomics package – MzMine – was evaluated. This open source metabolomics package did similar processing as XCMS, however there was a critical step missing from the workflow. The analytics for selecting significant hits was not readily incorporated into the processing. While not problematic when analyzing a small number of experiments, rapid sifting of the data for significant features is required for the larger number of experiments in this dissertation.

Robustness

There are many sources of errors and problems with reproducibility associated with MagMASS. Most times, the response from comparing the experimental wells to controls was just noise due to in part nonspecific binders. MagMASS requires focus and skill in ensuring there is as minimal variation and contamination from well to well.

Useful in the hands of a researcher with significant assay experience, MagMASS requires at least 6 months of experience before that researcher can obtain positive and consistent results. Nevertheless, this assay holds promise as a means to discover natural products with potential pharmacological activity.

Future Direction

Automation

One solution to the problem of robustness with MagMASS is to incorporate automation into the liquid handling. Automated liquid handling eliminates the uniformity issues that can occur using manual pipetting. In this work, MagMASS progressed from experiments using individual wells to microtiter plates. Moving to multichannel pipettes improved consistency from well-to-well. Automating the process would further improve the analysis. This avenue was investigated with many automated liquid handling vendors.

MagMASS could be easily brought into these automated systems. According to manufacturers, the magnet would not interfere with normal functions. In addition, the software for handing plates and moving them on and off the magnet would not be a problem, a simple change in the default commands. It would require a specific command for a plate to be placed on the magnet at a specific location to a nondefault

height. The biggest limitation with MagMASS and automation is that these liquid handling devices have several decks and the magnetic plate would sit in one of these decks. To accommodate the rest of the trays – tips, waste, buffer, organic solvent, clean MTPs – an automated liquid handling system would need to have enough decks. A system with six to ten decks would be enough for a MagMASS assay to be incorporated. The more decks, a greater amount of the MagMASS assay can be automated in a single workflow. A system with fewer decks requires more start and stop automation to change the decks. A system with four or fewer decks would be insufficient for MagMASS screening. Although expensive, using these robotic systems could drastically increase the workflow and quality of the MagMASS data. The immobilization procedures could also be automated.

Software Improvements

In previous MagMASS research, XCMS online had been used for the data processing. Each data file was exported from the Shimadzu file type into a .mzxml file. This file type is a generic format that is compatible with XCMS online. However, XCMS online has a job limit per user throttle to ensure wide access. Furthermore, uploading files can be slow and processing can take extended times depending on the server usage. If uploading files took on average sixty minute to upload and complete, total computation time for this dissertation would have been approximately 1 000 hours. The servers do not allow batch uploading, so uploading one file at a time for data analysis would have been impractical at best. After creating a variety of accounts hidden behind VPNs to have multiple jobs being analyzed at a time to bypass the throttling, I received a cease-

and-desist letter from XCMS online. As a solution to this dilemma, XCMS online provided an offline demo of their licensed software for offline software usage. I developed the offline server infrastructure to queue and process my 4000 files automatically. Instead of 1 000 hours, total software analysis was completed in 5 days.

To run these data analytics on the scale of higher throughput natural product discovery, this type of server architecture is mandatory. Using free and accessible programs and software is possible, but processing these files requires more computing power than the average desktop to complete quickly. In this work, the metabolomics software was repurposed for this alternative approach. When troubleshooting a problem with the XCMS online programmers, Dr. Paul Denton of XCMS was surprised I had so many jobs for the software. It was clear in the design of the windows and the number of clicks that the workflow was not designed for evaluating 1000 jobs at a time. For future development of this MagMASS platform, one direction that should be pursued is finding or amending metabolomics software with the explicit focus of processing an enormous amount of jobs. If the XCMS Plus software license were purchased, these alterations would be included in the purchase.

Conclusions

MagMASS is a technique that can supplement or possibly replace bioassay guided fractionation of natural products. MagMASS offers an immense short cut by eliminating the need for reiterative fractionation typically used today. Briefly, MagMASS can quickly identify exactly the biologically active peak in a chromatogram that should be isolated for structural determination while providing its molecular weight and elemental

composition. In the current iteration, MagMASS might not be cost effective as an alternative to high-throughput screening of combinatorial libraries. However, for natural products, MagMASS is a far cheaper and faster alternative to bioassay guided fractionation.

This dissertation describes the evolution of MagMASS from the basics of Pulsed Ultrafiltration Gen 1 to a semi-automated system suitable for screening 900 microbial extracts for ligands to a pharmacological receptor. MagMASS has evolved from a single experimental system into a high-throughput natural product drug discovery system. The addition of fully automated liquid handling and metabolomics software designed for natural product screening will be the next iteration of MagMASS as it continues to evolve into an efficient approach for natural product drug discovery.

APPENDICES

APPENDIX A

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Author: Michael D. Rush

Publication: Journal of The American Society for Mass Spectrometry

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Author: Michael D. Rush, Elisabeth M. Walker, Tristesse Burton, et al

Publication: Journal of Natural Products

Publisher: American Chemical Society

Date: Nov 1, 2016

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VITA

Mass Spectrometrist 2012 – Present

University of Illinois at Chicago (UIC) Department of Medicinal Chemistry and Pharmacognosy,

Chicago, IL

Mentor: Richard B. van Breemen Ph.D.

- Developing new bioaffinity screening technology by tethering protein to magnetic beads, known as MagMASS – magnetic microbead affinity selection screening - for use in conjunction with mass spectrometers for rapid identification of novel ligands as potential drug leads for a variety of different protein targets – RXRα, 15 LOX, COX2 ERα/β, and Progesterone receptor.
- Awarded an F31 NIH/NCCAM F31 Predoctoral Fellow Ruth L. Kirschtein Individual Predoctoral National Research Service Award to apply MagMASS to novel topic – F16BPase to identify novel ligands found in marine and cyanobacteria extracts using high-resolution tandem mass spectrometry on Shimadzu IT-TOF. Screened approximately 900 actinomycetes and fungal extracts through MagMASS. Created server infrastructure for data processing, confirmed hits through functional assays, and identified novel ligands through structure elucidation
- Planned and conducted six different methods for extracting oil from whole frozen krill following specifications and requirements in an international patent law suit. This

work involved understanding and integrating a variety of legal and scientific concerns to develop krill oil for use in legal argumentation. Proceeded to test the extracted krill oil for omega-3-fatty acid content in GLP and analytical practice on Shimadzu 8040

- Investigated the role of ammonium mobile phase additives commonly used in the literature for quantitative measurements of phosphatidylcholines. Optimized best ionization parameters using a Shimadzu 8060 Triple Quad for best front end settings for greatest ionization of this class of compounds. Also, determined that commonly used mobile phase additives suppress the ionization of phosphatidylcholines and developed a method and LC conditions for best quantitation of this class of phospholipids.
- Analyzed extremely high molecular weight caramel class IV polymers for Pepsi Co. Chemical and structural differences were analyzed between the three generations of caramel solids. High molecular weight polymers were isolated and analyzed on Shimadzu 8050 Triple Quadrupole analyzing their MS ionization patterns and PDA fingerprints. Solids were further separated and analyzed on Bruker AutoFlex MALDI-TOF. Enzymatic cleavage and chemical hydrolysis were utilized to create caramel polymer fragments for analysis.
- Interpreted MS^2 spectra of long chain procyanidins purified from Planta Analytica were analyzed using a Bruker AutoFlex MALDI-TOF-TOF. Created an in depth guide to determining structural information for long chain procyanidin polymers as well as identify novel fragmentation pathways. In addition to the MS developments, screening procyanidins for COX-2 activity and identified IC_{50}'s for the three

procyanidins with the greatest activity. Assisted in the development of pairing MS^2 techniques with ion mobility for further specificity for 15 to 30 oligomeric chains.

- Developed effective chromatography techniques using a variety of stationary phases and techniques ranging from LC: normal, reverse, HILIC, ion exchange, size exclusion, supercritical fluid and other chromatographic methods: solid phase extraction, micro SPE, liquid-liquid extraction, solid-liquid extraction, TLC, bioaffinity, paper chromatography, and more.
- Trained, operated, and maintained 11 mass spectrometers; including Shimadzu 8040 Triple Quad, Shimadzu 8050 Triple Quad, Shimadzu 8060 Triple Quad, Shimadzu IT-TOF, Thermo FT-ICR, Thermo Orbitrap, Agilent Triple Quad, Agilent Q-TOF, SciEx Q-Trap 6500, SciEx Q-Trap 5500, and Bruker MALDI-TOF mass spectrometers and LC systems. Other instruments include the Shimadzu UV-VIS, Shimadzu PDA, and, BioTeK Synergy G2 Plate Reader.
- Assumed the responsibilities of the laboratory manager and chemical safety officer for the Richard van Breemen lab group consisting of over a dozen graduate students and postdocs. Duties include ordering, organizing, maintaining the laboratory, identifying and rectifying safety concerns, and instructing new students on proper techniques for analytical and LCMS work.
- Spent 2 four-month rotations learning advanced techniques and finer aspects in synthetic medicinal chemistry and cell culture laboratories.

Organics Analyst Oct 2011 – Jan 2012

Energy Laboratories, Casper, Wyoming

- Prepared soil and water samples for BTEX and other volatile organic compound testing. After preparing the samples, analysis was conducted with GC/MS, Quadrapole and Ion Trap MS. Job responsibilities included operating and maintaining GC/MS, preparing samples according to EPA methods, and sample management, Good Laboratory Management requirements, including organization and disposal.

Student Lab Researcher May 2007 – Aug 2007

Idaho National Laboratories, Idaho Falls, Idaho

- Operated a Nitrogen physisorption instrument (Quantachrome 1-C) Quadrupole mass spectrometer. Worked with continuous-flow experimental setups equipped with gas manifolds and electronic mass flow controllers. Other duties included researching and analyzing literature and producing transmission electron microscopy images for particle size distribution.

Student Lab Researcher May 2005 – Aug 2005

Idaho National Laboratories, Idaho Falls, Idaho

- Maintained a laboratory set-up testing different rare earth catalysts in a sulfuric acid - hydrogen production system with a GCMS to detect sulfur decomposition products. Responsibilities included preparing the samples, loading the reactors, ending the reaction, cleaning and maintain the experimental set up.
-

Teaching Experience

PCAT Test Prep Instructor 2014

University of Illinois at Chicago (UIC) College of Pharmacy, Chicago IL

- Prepared lessons and taught 12 students enrolled in the Urban Health and Outreach PCAT preparation course over the course of 6 weeks totaling 24 hours of instruction

Teaching Assistant , 2012 – 2014

Pham 331: Medicinal Chemistry I, Pham 332: Medicinal Chemistry II

University of Illinois at Chicago (UIC) College of Pharmacy, IL

- Supported the education of hundreds of first year pharmacy students. Assisted in preparation and administration of lectures, tests, and recitation sessions. Actively participated in working with students during office hours and grading

Secondary School Educator and Teacher Trainer 2008 – 2010

U.S. Peace Corps, Kondoa Girls High School, Kondoa Dodoma, Tanzania

- Received 140 hours of technical training, including educational pedagogy and practice, and development theory as well as an internship involving teaching and shadowing Peace Corps Volunteers
- Taught advanced placement equivalent chemistry and math to 700 students. Co-authored a manuscript for chemistry teacher training. Maintained laboratory and conducted student experiments. Served as advisor for self-empowerment club.

Conducted training for other Peace Corps in teaching techniques and food preservation methods.

- Achieved a 40% increase in the students' scores on college-qualifying exams.
- Totaled 2533 teaching hours in classroom and recitation hours.
- Led JICA funded country wide teacher training workshops over four days for approximately 100 experienced teachers on introducing the use of local materials for use in student scientific laboratories and in class exercises.

Teaching Assistant 2008

University of Idaho, Moscow, ID

- Assisted in the education approximately 60 undergraduate philosophy students. Taught one lecture, ran weekly recitation sections, and graded exams.

Student Tutor 2007 –2008

University of Idaho, Moscow,

- Provided approximately 150 hours of group and individual tutoring for approximately 25 undergraduates in chemistry and philosophy courses

Education

PhD in Medicinal Chemistry 2012 – Present

University of Illinois at Chicago (UIC) College of Pharmacy, Department of Medicinal Chemistry and Pharmacognosy, Chicago, IL

- Relevant Coursework: Medicinal Chemistry, Natural Products, Advanced Organic Chemistry, Spectroscopy in Medicinal Chemistry, Elucidation of Natural Products,

Chemical Separations, Biostatistics, Cancer Biology and Therapeutics, Philosophy of Ethics.

Bachelors of Science in Chemistry – Cum Laude 2004 – 2008

University of Idaho, Moscow, ID

- Relevant Coursework: Inorganic Chemistry, Physical Chemistry, Quantitative Analysis, Laboratory Statistics, Organic Chemistry, Drug Design

Bachelors of Science in Philosophy – Cum Laude 2004-2008

University of Idaho, Moscow, Idaho

- Relevant Coursework: Ethics, Philosophy of Science, Philosophy of Language, Ecological Jurisprudence, Philosophy of Law, Symbolic Logic.

Professional Publications

EA Rue, **MD Rush**, RB van Breemen. Procyanidins: a comprehensive review encompassing structure elucidation via mass spectrometry. Phytochemistry Reviews. 10.1007/s11101-017-9507-3

MD Rush, RB van Breemen. Role of Ammonium Salts in the Ionization and Fragmentation of Phosphatidylcholines Found in Krill Oil. Rapid Communications in Mass Spectrometry. doi: 10.1002/rcm.7788. Cover art for the journal.

MD Rush, EM Walker, G Prehna, T Jones, and RB van Breemen. Development of a magnetic microbead affinity selection screen (MagMASS) using mass spectrometry for ligands to the retinoid X receptor-α. Journal of the American Society of Mass Spectrometry. J Am Soc Mass Spectrom. 2016 Dec 13. DOI: 10.1007/s13361-016-1564-0. Cover art for the journal.

MD Rush, EM Walker, RB van Breemen. Magnetic Microbead Affinity Selection Screening (MagMASS) of Botanical Extracts for Inhibitors of 15-Lipoxygenase. Journal of Natural Products, 2016, 79(11) 2989-2902.

MD Rush, P Kowalski, J Glinksi, RB van Breemen. Identifying long chain procyanidins via MALDI-TOF-TOF spectra interpretation. Journal of Natural Products (under review)

Aron Walker, **Michael Rush**, *Shika na Mikono,* September 2010, Peace Corps Tanzania.

Daniel M. Ginosar, Harry W. Rollins, Lucia M. Petkovic, Kyle C. Burch, **Michael Rush**. High-Temperature Sulfuric Acid Decomposition Over Complex Metal Oxide Catalysts. International journal of Hydrogen Energy 34 (2009) 4065 – 4073.

Oral Presentations

MD Rush, Richard B van Breemen, Structure Elucidation of Procyanidin Oligomers by MALDI-TOF-TOF. Phytochemical Society of North America, Davis CA 2016

MD Rush, Richard B van Breemen, Role of Ammonium Salts in the Ionization and Fragmentation of Phosphatidylcholines Found in Krill Oil. Chicago Area Mass Spectrometry Discussion Groups Mass Spec Day Chicago IL 2016

Professional Organizations

American Associated for the Advancement of Science, 2012 – present

American Chemical Society, 2012 – present

American Society of Mass Spectrometry, 2013 – present

American Society of Pharmacognosy, 2014 – present

Chicago Mass Spectrometry Discussion Group, 2012 – present

American Phytochemical Society 2016 – present

College of Pharmacy Involvement

Medicinal Chemistry and Pharmacognosy Graduate Student Council, 2013 - present

Member of Diversity Strategic Thinking and Planning Committee, 2015 - present

Awards

- W.E. van Doren Scholar - 2017

- F31 National Institutes of Health National Center for Complementary and Integrative Health F31 Predoctoral Fellow Ruth L. Kirschtein Individual Predoctoral National Research Service Award - 2016
- Charles Bell Award for Medicinal Chemistry -2016
- University of Idaho's Alumni Award of Excellence - 2008

Volunteer Work

- O'Hare Legal Team Interpreter Volunteer, Chicago 2017
- Homework Helper, Howard Washington Library, Chicago 2013 – 2015
- US Peace Corps Volunteer, Kondoa, Dodoma, Tanzania 2008 - 2010