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Magnetic Microbead Affinity Selection Screening (MagMass) of Botanical Extracts for Inhibitors of 15-Lipoxygenase

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Abstract

To expedite the identification of active natural products in complex mixtures such as botanical extracts, a Magnetic Microbead Affinity Selection Screening (MagMASS) procedure was developed. This technique utilizes target proteins immobilized on magnetic beads for rapid bioaffinity isolation of ligands from complex mixtures. A MagMASS method was developed and validated for 15-lipoxygenase. As a proof of concept, several North American prairie plants used medicinally by Native Americans were extracted with MeOH and screened. A hit from an extract of *Proserpinaca palustris*, also known as mermaid weed, was flagged for further characterization using high-resolution tandem mass spectrometry, dereplication, and identification using XCMS online. Through the application of high-resolution product ion tandem mass spectrometry, comparison with natural product databases and confirmation using standards, the hit was identified as quercitrin, which is a known inhibitor of 15-lipoxygenase. The overall workflow of MagMASS is faster and more amendable to automation than alternative methods designed for screening botanical extracts or complex mixtures of combinatorial libraries.

Natural products have always been a rich source of drug leads.¹ Unfortunately, screening natural products for drug discovery has been replaced largely by high-throughput screening of combinatorial libraries of discreet compounds due to concerns about the complexity of natural products.² Conventional natural product drug discovery involves the use of bioassay-guided fractionation³ followed by structure determination of the isolated, active compound. 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.¹

Pulsed ultrafiltration-mass spectrometry (PUF-MS)⁴ and magnetic microbead affinity selection screening (MagMASS)⁵ were invented to overcome the limitations of traditional natural product drug discovery. Both PUF-MS and MagMASS utilize affinity selection to separate ligands bound to protein targets from non-binding compounds contained in complex mixtures such as botanical extracts and then utilize the speed and selectivity of

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mass spectrometry to characterize and help identify the ligands. Although PUF-MS has been effective in finding ligands for a wide variety of target proteins including adenosine deaminase,⁶ carbonic anhydrase,⁷ dihydrofolate reductase,⁸ cyclooxygenase-2,⁹ estrogen receptors- α and β ,¹⁰ and retinoid X receptor,¹¹ 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.

During inflammation, the enzymatic products of 15-lipoxygenase (15-LOX) display both proinflammatory and anti-inflammatory properties.¹² 15-LOX oxidizes free and esterified fatty acids, such as its main substrates, arachidonic and linoleic acid, to form a wide range of bioactive products including 13-HODE, 12-HETEs, lipoxins, and leukotenes.¹³ These products contribute to a variety of inflammatory disorders, including atherosclerosis.^{12,14}

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 1). 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. Whereas conventional competition-based assays only measure binding to a single active site of a target protein, MagMASS also enables the discovery of ligands to allosteric sites of the target. Therefore, MagMASS enables the discovery of compounds with potentially novel modes of binding.

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 measureable 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 chemistry for immobilization as needed. In this particular 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 non-ligand, 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 2). 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 MeOH extract of *Proserpinaca palustris* L. (Haloragaceae; (mermaid weed) (Figure 3). 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 3). 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 (cynaroside) or else quercitrin (quercetin 3-*O*-rhamnoside) (Figure 4). 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 1). 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 5) confirming the hit in *P. palustris* extract. A literature search confirmed that quercitrin inhibits 15-LOX with an IC_{50} value of 79.8 μ M.¹⁵ 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,⁹ were not carried out.

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.^{5,16}

Wilson, et al.¹⁷ 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.¹⁷ 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 expedite the process of screening complex mixtures of natural products and make this approach a more appealing alternative to bioassay-guided fractionation. In particular, the application of XCMS software originally developed for metabolomics to the analysis of MagMASS data is a significant advance over manual data inspection.⁵ 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 five-fold over previous applications of MagMASS. Not only did UHPLC-MS and XCMS data processing accelerate MagMASS compared with LC-MS and manual data inspection, the separation of ligand-receptor complexes from unbound compounds was over ten-fold faster during MagMASS compared to PUF-MS. This difference was particularly evident when processing 96-well plates, because the fragile ultrafiltration membrane available with 96-well plates necessitated slower separations than normal for PUF-MS (data not shown).

By upgrading our previous magnetic bead assay⁵ to 96-well format, streamlining data analysis, and implementing UHPLC separations, MagMASS is now faster than ever before and suitable for automation. 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. Compared to our previous PUF-MS and magnetic bead assays, improvements reported here using MagMASS include utilizing 96-well plate format, which increases throughput by four-fold, the implementation of UHPLC, which increases throughput by at least six-fold, and incorporating the metabolomics program XCMS online for automated data analysis, which increases throughput from ten-fold to a thousand-fold depending upon the sample complexity. Another improvement compared with PUF-MS alone is the separation of ligand-receptor complexes from unbound compounds using magnetic force instead of centrifugation, which increases the throughput by another four-fold.

EXPERIMENTAL SECTION

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 2x1x1/8" NdFeB) from CMS Magnetics (Garland, TX, USA), and washed with 300 μ L of 1 M HCl. The supernatant was removed, and 300 μ L of 50 mM sodium borate buffer (pH 7.5) were added to each tube or well. After mixing, 50 pmol of 15-lipoxygenase soybean P1 (15-LOX) in 300 μ L sodium borate buffer (50 mM, pH 7.5) were 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 removed, and 300 μ L of 0.1 M glycine pH 2.0 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 μ L 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 μ L of 3 M ethanolamine (pH 9.0) were added. After 2 h with shaking every 5 min, the supernatant was removed, and 300 μ L of 50 mM sodium borate (pH 7.5) containing 0.05 % (m/v) sodium azide were 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. (Haloragaceae) [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 to 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 previously.^{18–27}

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.

MassMASS Protocol

For assay validation, luteolin (IC₅₀ 5 μ M)¹⁴, a known inhibitor of 15-LOX, was used as a positive control, while the non-ligand ebselen was used as a negative control. A 25 μ L 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 μ L (10 μ g) of the extract was incubated with a 25 μ L 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 pipette (Figure 1). The beads were rinsed to remove unbound compounds by washing with 300 μ L 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 μ L 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 1). The supernatant was evaporated in vacuo and reconstituted in 100 μ L 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 ca. 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 C₁₈ CSH column (1.7 μ m, 2.1 $\times$ 150 mm) with a 12-min linear gradient from 5% to 95% methanol containing 0.1% formic acid. The injection volume was 1 μ L. 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 co-elution 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 C₁₈ column (2.7 μ m, 2.1 $\times$ 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 (SRM) 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 the area of a chromatographic peak for the experimental chromatogram relative to the denatured protein control constituted a potential hit (Figure 2). The free metabolomics software, XCMS online,²⁸ was repurposed to

automate the comparison of the experimental and control UHPLC-MS 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 describe above.

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Figure 1. 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. Step 3: Ligands are released by denaturing the receptor with organic solvent and then removed for UHPLC-MS analysis. Although use of an Eppendorf tube is shown, assays were also carried out using 96-well microtiter plates.

Figure 2.

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.

Figure 3.

XCMS online metabolomics analysis overlay for the screening of *P. palustris* 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.

Figure 4.

Two possible structures of the hit observed during MagMASS testing of *P. palustris* for ligands of 15-LOX in Figure 3. With an elemental composition of $C_{21}H_{20}O_{11}$, based on positive ion and negative ion accurate mass measurements of m/z 449.108 ($C_{21}H_{21}O_{11}$) and m/z 447.095 ($C_{21}H_{19}O_{11}$), respectively ($M \pm 5$ ppm), dereplication indicated two probable natural products isomers, luteolin 7-*O*-glucoside or else quercitrin.

Figure 5.

MagMASS screening of the standards of luteolin 7-*O*-glucoside and quercitrin confirmed the binding of only quercitrin to 15-LOX based on significant peak enhancement by quercitrin but not luteolin 7-*O*-glucoside.

Table 1

High-resolution Electrospray Product-Ion Tandem Mass Spectrometric Analysis of the XCMS Screening Hit of *Proserpinaca palustris* (Mermaid Weed) and Comparison with Natural Product Standards Luteolin 7-*O*-glucoside and Quercitrin.

MagMASS 15-LOX hit	retention time (min)	precursor ion	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)

luteolin 7- <i>O</i> -glucoside	retention time (min)	precursor ion	collision energy (eV)	MS/MS product ions (relative abundance)
positive ion	1.81	449.108	−35	303.06 (100)
negative ion	1.81	447.095	29	301.03 (100)

quercitrin	retention time (min)	precursor ion	collision energy (eV)	MS/MS product ions (relative abundance)
positive ion	1.88	449.108	−15	303.05 (100), 287.06 (10)
negative ion	1.88	447.095	29	287.05 (100)