Analysis of the Structure and Function of Endocannabinoid-Hydrolyzing Enzymes Using Biophysical and Nanomedical Techniques

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Dedication

This work would not have been possible without the love and support of my family and friends. Specifically, my loving partner Emily Sechny, my mother Mary Rice, and my father Richard Johnson. Thank you for encouraging me to follow my dreams.
Acknowledgements

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Abstract of Dissertation

Presented here is a systematic structural, and functional study of endocannabinoid metabolizing enzymes, including: a potential biomarker for breast cancer and the development of a novel nanoplatform for studying these membrane proteins. Investigating the impact of phospholipid bilayers on the structure and function of membrane proteins is an essential precursor to developing drugs that target these dynamic systems. The membrane-associated serine hydrolase, monoacylglycerol lipase (MGL), and the membrane-bound serine hydrolase, fatty acid amide hydrolase (FAAH), are well-recognized therapeutic targets that regulate endocannabinoid signaling. In particular, overexpression of MGL in certain tumor cells elevates the levels of pro-tumorigenic signaling lipids, and as such, MGL regulates a fatty acid network that promotes pathogenesis in some cancers. Reported here is the application of phospholipid bilayer nanodiscs that mimic the native cell membrane environment to evaluate the effects of membrane systems on the catalytic properties, and conformational dynamics, of human MGL (hMGL) and rat FAAH (rFAAH). Specifically, hMGL’s kinetic properties (apparent maximum velocity \([V_{\text{MAX}}]\) and substrate affinity \([K_M]\)) were enhanced in the presence of anionic and charge-neutral phospholipid bilayer nanodiscs. In order to examine further the effects of modulating the activity of hMGL, a novel nano-medicinal agent (derived from a dynamic class of nano-materials that can be applied to \textit{in vitro}, \textit{in vivo}, and clinical applications) was synthesized, and characterized. Nanoparticles exist in a physical state between that of bulk material and a single molecule. In this transitional state, surface properties and quantum-mechanical dynamics can be “tuned” simply by altering particle size and shape. The ‘fLPA-SPIO@AuNS’ particle design proposed here presents a multilamellar nanoplatform for imaging, drug delivery, and therapeutic applications that, in this case, target the endocannabinoid system. Another novel imaging motif, proposed here involves probing
the mechanism of hMGL inhibition by 5-(4-hydroxyphenyl)-pentanesulfonyl fluoride (AM3506) using biochemical and mass spectrometric (MS) approaches. After hMGL was treated with AM3506, the conversion of sulfonyle serine (Ser\textsubscript{122}) to dehydroalanine via a β-elimination mechanism was observed, and confirmed by tandem MS analysis. Targeting the resultant dehydroalanine hMGL with thiophenol resulted in the conversion of Ser\textsubscript{122} to S-phenyl-cysteine (addition of 92 Da), which demonstrates a selective approach for serine hydrolase modification at the catalytic serine. This modification confers a new function to in this case- hMGL without genetic manipulation. The results of this work contribute to the understanding of key regulatory pathway involved in breast cancer progression (as well as other disease states) and provides evidence of the feasibility of the development of a novel, pharmacologic intervention toward the diagnosis and treatment of metastatic disease.
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<td>(-)MSP</td>
<td>A his-tag cleaved variant of membrane scaffold protein 1D1</td>
</tr>
<tr>
<td>2-AG</td>
<td>2-arachidonoylglycerol</td>
</tr>
<tr>
<td>βME</td>
<td>Beta-mercaptoethanol</td>
</tr>
<tr>
<td>ΔTM-rFAAH</td>
<td>Transmembrane deleted, or truncated rat fatty acid amide hydrolase</td>
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<tr>
<td>AA</td>
<td>Arachidonic acid</td>
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<tr>
<td>AAMCA</td>
<td>N-arachidonoyl 7-amino-4-methylcoumarin amide</td>
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<td>α-β Hydrolase Domain Containing Enzyme 6/12</td>
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<td>Activity based protein profiling</td>
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<td>AEA</td>
<td>Anandamide</td>
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<td>AHMMMCE</td>
<td>Arachidonoyl 7-hydroxy-6-methoxy-4-methylcoumarin ester</td>
</tr>
<tr>
<td>AMC</td>
<td>7-amino-4-methylcoumarin</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
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<tr>
<td>APTES</td>
<td>(3-Aminopropyl)triethoxysilane</td>
</tr>
<tr>
<td>AuNP</td>
<td>Gold nanoparticle</td>
</tr>
<tr>
<td>BSA</td>
<td>Fatty acid-free bovine serum albumin</td>
</tr>
<tr>
<td>CAN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>CB</td>
<td>Cannabinoid</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(3-Cholamidopropyl)dimethylammonio]-1-Propanesulfonate</td>
</tr>
<tr>
<td>Click chemistry</td>
<td>Azide-alkyne Huisgen cycloaddition</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>CYMAL</td>
<td>6-cyclohexyl-1-hexyl-β-D-maltopyranoside</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
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<td>-------------</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DAGL</td>
<td>Diacylglycerol lipase</td>
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<tr>
<td>DCC</td>
<td>N, N’-Dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DCU</td>
<td>N,N'-dicyclohexylurea</td>
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<tr>
<td>DDM</td>
<td>N-dodecyl-beta-D-maltoside</td>
</tr>
<tr>
<td>DI</td>
<td>Deionized</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EA</td>
<td>Ethanolamine</td>
</tr>
<tr>
<td>ECB</td>
<td>Endocannabinoid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>EPR</td>
<td>Enhanced permeability and retention effect</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
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<td>FAAH</td>
<td>Fatty acid amide hydrolase</td>
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<tr>
<td>fLPA-SPIO@AuNS</td>
<td>Lipoic acid propargyl amide-functionalized superparamagnetic iron oxide core, gold nanoshell</td>
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<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
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<tr>
<td>GBMSDG</td>
<td>Greater Boston Mass Spectrometry Discussion Group</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein coupled receptor</td>
</tr>
<tr>
<td>h/rFAAH</td>
<td>Humanized rat fatty acid amide hydrolase</td>
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<tr>
<td>H121S hMGL</td>
<td>Histidine 121 to serine single mutant recombinant human monoacylglycerol lipase</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
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<tr>
<td>HMMC</td>
<td>7-hydroxy-6-methoxy-4-methylcoumarin</td>
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<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
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<tr>
<td>HX MS</td>
<td>Hydrogen deuterium exchange mass spectrometry</td>
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<tr>
<td>IGERT</td>
<td>Integrative Graduate Education and Research Traineeship</td>
</tr>
<tr>
<td>IMA</td>
<td>Imidazole</td>
</tr>
<tr>
<td>IMAC</td>
<td>Immobilized metal affinity chromatography</td>
</tr>
<tr>
<td>ImageJ</td>
<td>Image processing and analysis in java software</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>Kan</td>
<td>Kanamycin A</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Broth</td>
</tr>
<tr>
<td>LC-TOF MS</td>
<td>Liquid chromatography time-of-flight mass spectrometry</td>
</tr>
<tr>
<td>LDAO</td>
<td>Lauryldimethylamine-oxide</td>
</tr>
<tr>
<td>LPA</td>
<td>Dihydrolipoic acid, or lipoic propargyl amide</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix-assisted laser desorption ionization-time of flight</td>
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<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<td>Matrix</td>
<td>α-cyano-4-hydroxycinnamic acid</td>
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<td>MD</td>
<td>Molecular dynamics</td>
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<td>MGL</td>
<td>Monoacylglycerol lipase</td>
</tr>
<tr>
<td>MNPs</td>
<td>Magnetic nanoparticles</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MSP</td>
<td>Membrane scaffold protein</td>
</tr>
<tr>
<td>n.d.</td>
<td>Not determined</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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</tr>
<tr>
<td>NAPE</td>
<td>N-arachidonoyl phosphatidyl-ethanolamine</td>
</tr>
<tr>
<td>NCI</td>
<td>National Cancer Institute</td>
</tr>
<tr>
<td>ND</td>
<td>Nanodisc</td>
</tr>
<tr>
<td>ND-CPX</td>
<td>Nanodisc protein complex</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institute of Health</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NP</td>
<td>Nanoparticle</td>
</tr>
<tr>
<td>NSF</td>
<td>National Science Foundation</td>
</tr>
<tr>
<td>OD&lt;sub&gt;600&lt;/sub&gt;</td>
<td>absorbance (optical density) of a sample measured at a wavelength of 600 nm</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein data bank</td>
</tr>
<tr>
<td>PDI</td>
<td>Polydispersity index</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidyl-ethanolamine</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethanesulfonfluoride</td>
</tr>
<tr>
<td>POPC</td>
<td>1-palmitoyl-2-oleoylphosphatidylcholine</td>
</tr>
<tr>
<td>POPG</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol</td>
</tr>
<tr>
<td>RARE</td>
<td>Rapid acquisition with relaxation enhancement, aka. rapid spin echo</td>
</tr>
<tr>
<td>rFAAH</td>
<td>Rat fatty acid amide hydrolase</td>
</tr>
<tr>
<td>rFAAH I250H</td>
<td>Isoleucine to histidine single mutant rat fatty acid amide hydrolase</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature, approximately 22 °C</td>
</tr>
<tr>
<td>SC-SPION</td>
<td>Silica coated superparamagnetic iron oxide nanoparticle</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SEC</td>
<td>Size exclusion chromatography</td>
</tr>
<tr>
<td>SH-PEG/mPEG2000-SH</td>
<td>O-[2-(3-Mercaptopropionylamino)ethyl]-O’-methylpolyethylene glycol 2,000</td>
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<td>SMPL</td>
<td>Solubilized membrane protein library</td>
</tr>
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<td>Sol-hMGL</td>
<td>L169S, L176S double mutant human monoacylglycerol lipase</td>
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<td>SPIO(N)</td>
<td>Superparamagnetic iron oxide (nanoparticle)</td>
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<tr>
<td>SPIO-NH₂</td>
<td>Amine-functionalized, silica coated, superparamagnetic iron oxide nanoparticles</td>
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<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
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<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
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<tr>
<td>TeOS</td>
<td>Tetraethyl orthosilicate</td>
</tr>
<tr>
<td>TEV Protease</td>
<td>Tobacco etch virus protease</td>
</tr>
<tr>
<td>THPC</td>
<td>Tetrakis(hydroxymethyl)phosphonium chloride</td>
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<tr>
<td>TM</td>
<td>Transmembrane domain</td>
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<tr>
<td>TRP</td>
<td>Transient receptor potential</td>
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<tr>
<td>UPLC</td>
<td>Ultra high pressure liquid chromatography</td>
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<tr>
<td>WT hMGL</td>
<td>Wild type recombinant human monoacylglycerol lipase</td>
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Introduction

According to the National Cancer Institute, more than 12% of female newborns in the United States will be diagnosed with breast cancer within their lifetime; those with aggressive forms of the disease, will have less than a 25% chance of surviving 5 years post-diagnosis. Without pharmacological intervention, breast cancer will remain a significant global health issue for the foreseeable future. Currently, a major hurdle in the breast cancer drug discovery initiative is the identification of highly selective imaging and therapeutic agents for the diagnosis and treatment of the disease.

The regulation of endocannabinoid signaling has profound effects on the progression of aggressive/metastatic breast and prostate cancer cell growth, migration, and invasion. Monoacylglycerol lipase (MGL), a serine hydrolase that regulates endocannabinoid signaling, has a typical serine-histidine-aspartate catalytic triad belongs to the α/β hydrolase family, and is the major enzyme responsible for the hydrolysis of 2-arachidonoylglycerol (2-AG); which is an endocannabinoid that is synthesized and localized in the membrane bilayer. Increased tissue 2-AG levels, consequent to pharmacological or genetic MGL ablation, are associated with preclinical therapeutic benefit against pain, inflammation, neurodegenerative disorders, psychological stressors, nausea/emesis, and most notably cancer pathogenesis. Overexpression of MGL, and the resultant over-hydrolysis of 2-AG, elevates the level of protumorigenic signaling lipids in cancer cells. As such, the development of highly selective imaging and therapeutic agents for examining functional MGL presents a key step in advancing our understanding of the complete metabolic role this potential biomarker for breast cancer plays.
The serine esterase/amidase fatty acid amide hydrolase (FAAH) similarly regulates a bioactive lipidome by catabolizing cannabinoid and non-cannabinoid compounds,\textsuperscript{16} including its primary substrate anandamide (AEA),\textsuperscript{17} and 2-AG.\textsuperscript{18} FAAH also regulates signaling through the transient receptor potential (TRP) ion channels.\textsuperscript{19} The crystal structures of rat FAAH (rFAAH),\textsuperscript{20} truncated rFAAH (ΔTM-rFAAH),\textsuperscript{20,21} and a humanized rFAAH construct (h/rFAAH)\textsuperscript{22} are known, but the structural dynamics, and oligomeric states of this enzyme have yet to be elucidated.

Unlike FAAH, which is anchored by a transmembrane spanning domain,\textsuperscript{20} MGL transiently associates with cell membranes.\textsuperscript{23} To gain a comprehensive understanding of the mechanism of action of these enzymes, it is vital that we first unravel the impact of this protein-membrane interaction. Like most lipases, MGL appears to exhibit interfacial activation and undergoes a transition from a “solution” to a “membrane-associated” conformation, a process that involves attachment of part of its lid domain to the phospholipid bilayer.\textsuperscript{24} As a prerequisite for developing effective small molecule drugs that target MGL and FAAH, it is essential to probe these conformational and spatial dynamics. Presented here is the determination the kinetics of MGL hydrolysis in a nanodisc biological membrane mimetic, and the further characterization of the nanodisc model with use of high resolution imaging techniques, measurement of the structural dynamics of MGL and rFAAH in nanodiscs using hydrogen-deuterium exchange mass spectrometry (HX-MS), use a novel “Michael probe” system as a proof-of-concept for a novel imaging agent which specifically labels active human MGL, and the development of a silica-coated, super-paramagnetic, iron oxide core/gold shell, theranostic nanoplatform that could be used to target these proteins and treat metastatic disease.
Chapter 1

Characterization of the Nanodisc Model Using High-resolution Techniques
Introduction

Nanodiscs (NDs), originally described and synthesized by Stephen Sligar et al.\textsuperscript{25,26} at the University of Illinois at Urbana-Champaign, are phospholipid bilayers constrained by two amphipathic membrane scaffold proteins (MSPs). NDs are highly stable in aqueous solution and are compatible with analytical techniques that are typically limited to soluble proteins (see review by Malhotra and Alder\textsuperscript{27}). Unlike micelles, bicelles, and liposomes, which traditionally have been used as \textit{in vitro} models of biological membranes,\textsuperscript{28} NDs do not suffer from aggregation, geometric distortion, or heterogeneity. Nanodiscs are formed through a self-assembly process whereby detergent-stabilized (usually sodium cholate) phospholipids and detergent-stabilized membrane proteins are combined with MSP in specific stoichiometric ratios. Detergent is subsequently removed, catalyzing self-assembly of nanodisc-membrane protein complexes (ND-CPX) through dialysis or the use hydrophobic-adsorbent beads. ND-CPX formation is then traditionally confirmed using fast protein liquid chromatography (FPLC) equipped with a size-exclusion analytical column and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). This system has been used to aid the study many different membrane proteins, such as GPCRs\textsuperscript{25,29,30}, pore proteins\textsuperscript{31,32}, enzymes\textsuperscript{24,33-35}, and multimeric complexes\textsuperscript{36,37}. This represents an invaluable tool for studying these proteins, which make up 20-30\% of the proteome for any given organism,\textsuperscript{38} especially considering that membrane proteins make up less than 1\% of the crystal structures in the protein data bank (PDB).\textsuperscript{27}

In the current study, we examined the identity and purity of MSP1D1 using FPLC-SEC and liquid chromatography time-of-flight mass spectrometry (LC-TOF MS) before forming ND-MGL complexes. The ND-MGL was analyzed using immobilized metal affinity chromatography (IMAC) co-purification, FPLC-SEC, and SDS-PAGE to confirm complex formation. Nuclear
magnetic resonance (NMR) spectroscopy was also used to confirm the interaction between NDs and MGL. Densitometric analysis was performed to analyze the stoichiometry of the ND-MGL complex using our synthetic scheme (ratio of MSP/lipid/MGL/detergent), and dynamic light scattering (DLS) was used to study the association kinetics of ND-MGL formation. Finally, high resolution electron microscopy was performed to image ND-MGL.
Results and Discussion

Confirming the identity and purity of MSP1D1

Before proceeding with nanodisc self-assembly, the identity and purity of MSP1D1 was confirmed using SDS-PAGE, FPLC, and LC-TOF-MS (Fig. 1). The multimeric state of the protein is important to measure before initiating ND-CPX self-assembly, since MSP readily forms oligomers. Monomeric MSP1D1 forms NDs more quickly than older, more aggregated protein (data not shown). Dimers will disaggregate in the presence of cholate in the ND buffer, but higher order oligomers will not and therefore cannot be used to form NDs. SEC for freshly purified MSP1D1 shows MSP-monomer as the predominant peak, but significant amounts of MSP-dimer and trivial amounts of higher order oligomers are also present in the sample (Fig. 1a). SDS-PAGE analysis shows only a single band for purified MSP1D1, indicating that MSP multimers disaggregate in SDS (Fig. 1a inset). LC-TOF electrospray ionization (ESI)-MS reveals two predominant masses for MSP1D1, 24,660.6 Da, and 24,716.0 Da (Fig. 1b). The first is the expected protein mass (± 1 Da) as interpreted from the protein’s amino acid sequence. The second peak is only present when IMAC purification is carried out using Talon® affinity resin, not Clontech His60 Ni Superflow™ resin, and most likely represents the addition of a divalent metal-ion to the 6-His tag, although we did not rule out other possible (+56 Da) chemical modifications such as addition of a butyl group.

Nanodisc assembly and complex co-purification

Since MGL associates with membranes, but is not tethered to the membrane like many “membrane-bound” proteins, a co-purification experiment was performed to give additional proof of complex formation beyond SDS-PAGE analysis of SEC fractions. ND-MGL was isolated using (Scheme 1). To demonstrate complex formation, untagged MSP1D1 “(-)MSP”
was used for nanodisc complex formation with 6-His tagged, wild-type human MGL (WT hMGL). (-)MSP does not bind to Talon® cobalt affinity resin, unlike MSP1D1, TEV protease, and WT hMGL, and (-)MSP clearly separates from uncleaved MSP1D1 on an SDS-PAGE gel (Fig. 2). Anti-his western blotting showed a clear signal for WT hMGL and no signal for (-)MSP (data not shown). The pooled and concentrated elution, following nanodisc formation, showed bands for both WT hMGL, and (-)MSP (Fig. 2). The (-)MSP could only be observed in complex with MGL. This pooled and concentrated elution contained ND-MGL, ND, and WT hMGL (which presumably disassociated during SEC), as evidenced by size exclusion chromatography, and silver stained SDS-PAGE (data not shown), providing direct evidence of complex formation. In order to select for a population of ND-MGL, rather than ND and WT hMGL in the disassociated state, an excess of ND should be self-assembled to ensure the ND-MGL conformation in solution.

NMR analysis of ND-MGL association

MGL shows a distinct pattern of low-field “downfield” resonances (12-18 ppm) in proton NMR. This pattern, or “fingerprint,” represents a hydrogen bond network that changes when the enzyme is in an “open” active, or a “shielded” inactive 3-dimentional conformation. By altering the pH of the aqueous buffer, this conformation can be shifted from open to closed or left in a dynamic state where the MGL population readily switches between open and shielded (Scheme 2). To determine if the addition of NDs would change the conformation of MGL in solution, suggesting interaction/complex formation, NDs were titrated into the NMR tube with a mixed population of MGL (pH 8). Addition of nanodiscs elicited two changes in the downfield resonance pattern. First, a clear shift from the open to the shielded conformation was observed, and second, a significant decrease in overall downfield signal was evident (Fig. 3). This result
suggests that MGL in the open conformation was interacting with the NDs. The resultant complex would be too large to measure by our method for solution-probe proton NMR due to its slow rate of tumbling\textsuperscript{39}. Therefore, addition of NDs may have served to sequester active protein, leaving only inactive and uncomplexed MGL available for detection in the NMR tube. This experiment adds to the preponderance of evidence that suggests NDs and MGL form complexes in solution.

**Densitometry analysis of the stoichiometry for ND-MGL preparation**

To determine the stoichiometry of the ND-MGL complex, which was designed to have an excess of NDs to drive the associated state, densitometry analysis was performed. A ratio of 1 ND-MGL per 6 NDs formed was found for this preparation (Fig. 4a). The MSP1D1 standard curve had an $R^2$ value of 0.987 (Fig. 4b), and the MGL standard curve had an $R^2$ value of 0.996 (Fig. 4c). This stoichiometry is sufficient for future analysis of this sample, and this densitometry-based methodology should be used to analyze future ND complexes.

**Dynamic Light Scattering analysis of ND-MGL association kinetics**

DLS measures particle size based on motion in solution (based on a Brownian motion assumption) and has been widely used to study protein aggregation\textsuperscript{40-46}. We used this technique to analyze the association kinetics for ND-MGL complex formation. When NDs were added to a solution containing WT hMGL, at first widely variant Z-averages (intensity based harmonic mean particle size) were calculated (Fig. 5). This variance from the mean particle size decreased steadily over 15 min until a metastable state was achieved (Fig. 5). This suggests that the ND-MGL complex forms rapidly through a dynamic process of association and disassociation. The time to achieve this equilibrium is essential to consider before proceeding to analyze ND-MGL samples.
Imaging ND-MGL using transmission electron microscopy

The fine structure physical of ND-MGL samples was imaged using TEM (Fig. 6). This representative micrograph shows two, well-formed, POPC/POPG (3/2) ND-MGL complexes. Due to the destructive nature of TEM sample preparation, and the need for a complete vacuum in the sample chamber, many NDs were destroyed during this procedure; and as such, misfolded protein aggregates were visible in the imaging field. For this experiment, negative staining with the electron dense uranyl acetate was used to create contrast in the image. The stain accumulates/concentrates along the edges of the NDs creating dark circles, and the stain deposits on any proteins in complex with the NDs. Therefore, the dark spots within the 10 nm circles designated by red arrows, are presumed to be MGL in complex with NDs.
Conclusions

In this study, high-resolution imaging techniques were used to analyze the ND membrane model. After confirming the fidelity of the membrane scaffold protein, we provided evidence that NDs will form complexes with MGL using a co-purification protocol. These data offer evidence that hMGL associates with nanodiscs to form an ND-MGL complex with a mean Stokes diameter slightly greater than either the enzyme or the unassociated nanodiscs.

To characterize further this interaction, a ND-MGL mixture was analyzed using in-gel densitometry. The stoichiometric ratio of this mixture can be altered by modulating the amount of MGL in the self-assembly mixture, but a synthetic scheme leading to an excess of blank NDs was preferred in this situation to drive the ND-MGL associated state, since MGL is not a membrane bound, or integral membrane protein and can disassociate and reassociate with biological membranes readily. Dynamic light scattering was then used to determine the length of time required for complex formation between blank NDs and detergent-free WT hMGL in solution. The time required to reach a dynamic equilibrium is essential to consider if using this method for complex formation. We have provided evidence that 15 min is sufficient for this equilibrium to be reached at room temperature, but 30 min will be used in the future to ensure the steady state has been reached.

Complexation was also analyzed using NMR spectroscopy. Sol-hMGL gives distinct NMR spectra, depending on whether it is in the open (active), shielded (catalytically inactive) form, or a mixture of the two conformation(s). ND-hMGL complexes are too large to measure by solution probe NMR as their “tumbling time” is too slow, but hMGL that has yet to form a complex can be detected. We showed that when NDs are titrated into a solution of sol-hMGL, the NMR-detectable enzyme population is completely in the closed form at a stoichiometric ratio
of ND::MGL 1::32. This may be due to a high-affinity interaction between open hMGL and the nanodisc. Indeed it has been postulated that hMGL will only disassociate from the membrane through transition from the open-conformation, membrane-associated complex to the membrane-dissociated, closed conformation\textsuperscript{47}. 
Materials and Methods

Materials

SDS-PAGE supplies, SM-2 biobeads, and Bio Spin columns were purchased from Bio-Rad (Hercules, CA). Culture media, standard laboratory chemicals, and buffers were purchased from Fisher Chemical (Pittsburgh, PA) and Sigma Chemical Co. (St. Louis, MO). The plasmid expressing MSP1D1 was purchased from Add Gene (Cambridge, MA). POPC and POPG were purchased from Avanti Polar Lipids (Alabaster, AL) as stock solutions in chloroform. 1,2-Deuterium oxide (>99%) was purchased from Cambridge Isotope Laboratories (Andover, MA).

Methods

Expression and purification of hMGL

Expression and purification of hMGL followed the following procedure. A single E. coli BL21 (DE3) colony, containing pET45His6hMGL plasmid, was used to inoculate 20 mL of Luria broth medium containing ampicillin (100 µg/mL) and was grown overnight at 37°C with shaking (~250 rpm). The following day, 10 mL of the overnight culture was used to inoculate 1 L of fresh Luria broth-AMP and allowed to grow until the culture reached an OD600 of 0.6. Protein expression was induced with isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and the temperature lowered to 33°C. Following a 4 hr. induction, the cells were collected by centrifugation at 5000 x g for 10 min at 4°C and stored at –80°C. For purification, the frozen pellet was thawed on ice, re-suspended in lysis buffer (50 mM Tris, 100 mM NaCl, 1% Triton X-100, pH=8.0) and sonicated on ice. The cell lysate was then centrifuged at 30,000g for 30 min at 4°C. The supernatant was added to 0.5 mL (bed volume) of Cobalt-NTA resin (Clonetech) pre-equilibrated with lysis buffer and mixed for 1 hr. at 4°C. The suspension was transferred to a gravity-flow column and washed with 20 mL lysis buffer, then
with 10 mL of lysis buffer containing 10 mM imidazole. Finally, hMGL was eluted with 3 mL of lysis buffer containing 250 mM imidazole and the purity was checked with SDS-PAGE.

**Expression and purification of MSP1D1**

MSP1D1 growth and expression was carried out according to the protocol published by Bayburt *et al.* In brief, a single colony of *E. coli* BL21 Gold (DE3) expressing the pET28a plasmid with the MSP1D1 gene was grown in 30 mL LB-kan until an OD$_{600}$ of 0.5-1 was achieved (~4-5 h.), then the inoculum was kept at 4 °C overnight. 1 L of 37 °C TB-kan was then inoculated with 20 mL of the overnight culture. The cells were induced with 1 mM IPTG at OD$_{600}$ of 2.5 to 3.0 (~4-5 h.). 1 hr. post-induction, the temperature was reduced to 28 °C. Approximately 3.5-4 h post-induction, cells were collected by centrifugation at 8 k x g for 15 min. and cell pellets were stored at -80 °C.

For purification, 2 g cell pellets were lysed by sonication in 20 mL of lysis buffer (20 mM NaH$_2$PO$_4$ with 1% Triton X-100, and 1 mM fresh PMSF, pH 7.4). The sonicate was centrifuged at 30 k x g for 30 min at 4 °C. The supernatant was then added to Clontech His60 Ni Superflow™ resin and purified using the manufacturer’s instructions. In brief, equilibrated beads (0.5-1 mL bed volume) were incubated on a rotator with the MSP1D1 supernatant for 1 hr. at 4 °C, then transferred to a gravity flow column where they were washed with 10-20 bed volumes of lysis buffer, then 10-20 bed volumes of ND buffer (20 mM Tris/HCl, pH 7.4, containing 100 mM NaCl, 0.01% NaN$_3$ and 50 mM cholate), then 10-20 bed volumes of ND buffer without cholate, the 10 bed volumes of ND buffer without cholate, with 30 mM imidazole. MSP1D1 was eluted in fractions (1 bed volume each) of ND buffer without cholate, with 300 mM imidazole. The fractions were pooled and dialyzed overnight against 1,000 X v/v ND buffer without cholate. The dialyzed protein was then concentrated using 10 kDa cutoff Amicon® Ultra.
centrifugal filter units, to 100 µL and stored at 4 °C for immediate use, or at -80 °C for long-term storage. Purity was checked with SDS-PAGE, and concentration measured by Pierce® 660 nm protein assay (Thermo Scientific) before use.

**LC-TOF analysis of MSP1D1**

A Waters LCT-Premier mass spectrometer was used to determine the intact mass of MSP1D1. The instrument was calibrated with 500 fmol/mL horse myoglobin (Sigma-Aldrich) before and after each run. Instrument conditions were as follows: source temperature, 80°C; desolvation, 175°C; capillary voltage, 3200 V; and cone voltage, 35 V. Dialyzed, detergent-free MSP1D1 samples in ND buffer without cholate (200 pmol) were injected onto a self-packed POROS 20 R2 2 mm x 20 mm trap column which traps the protein but allows buffer salts through. Samples were manually desalted with an appropriate (>5 x the volume of the sample) volume of 0.1% formic acid in H₂O. The desalted protein was then eluted from the column with a 15-75% gradient of acetonitrile containing 0.05% TFA at flow rate 50 µL/min in 4 min.

**ND-MGL co-purification**

Tabaco etch virus (TEV) protease was used to remove the 6-his tag from MSP1D1 using the manufacturer’s (Sigma-Aldrich) protocol. In brief, MSP1D1 in ND buffer without cholate was diluted to 1-2 mg/mL in dialysis buffer (25 mM Tris-HCl, pH 8.0 containing 200 mM NaCl). TEV protease was added at a ratio of 1:100 (w/w) TEV/MSP1D1. This mixture was dialyzed against 4 L of dialysis buffer at 4 °C overnight. MSP1D1 and TEV protease were removed by IMAC using Talon® resin according to the manufacturer’s protocol, and (-)MSP was spin-concentrated as described previously. Purity of (-)MSP was measured by SDS-PAGE and concentration was determined by absorption at λ 280 using the molar absorptivity constant 18,200. ND assembly was performed with a (-)MSP/POPC/POPG/WT hMGL/cholate/Triton X-
100 ratio of 1/47/31/0.31/156/0.5%. The mixture was incubated for 1 h on ice, and cholate detergent was removed during a gentle 10 hr. rotation with SM-2 biobeads at 4°C. IMAC purification was then performed using Talon® resin according to the manufacturer’s protocol to isolate WT hMGL and ND-MGL. Pooled eluent was purified by size-exclusion chromatography with an Amersham-Pharmacia AKTA FPLC Protein Purifier System (GE Healthcare Life Sciences, Pittsburgh, PA) on a Superdex 200 10/300 column eluted with 20 mM Tris–HCl, pH 7.4, containing 100 mM NaCl and 0.5 mM EDTA at 0.5 mL/min. Column eluate absorbance was monitored at 280 nm, and SDS-PAGE was used to evaluate the purity of the fractions.

**ND self-assembly and isolation**

Purified MSP1D1 in ND buffer was added to a solubilized mixture of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC)- 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) (3:2 molar ratio) with sodium cholate, and the final molar ratio was adjusted to 1:78:200 (MSP1D1:phospholipid:sodium cholate). NDs were formed and purified as described above. FPLC fractions containing purified NDs were collected and concentrated for experimental use. ND concentration was calculated from the absorbance at 280 nm and the MS1D1 molar extinction coefficient 21,000, accounting for two MSP1D1 molecules per disc.

**Proton NMR analysis**

L169S, L176S double hMGL mutant (sol-hMGL) was expressed and purified as hMGL (above) and used for detergent-free solution NMR study. NMR analysis was performed according to the protocol developed by Karageorgos *et al.* In brief, Enzyme samples for NMR were prepared in a solution of 93% H₂O–7% D₂O (v/v) containing 50 mM Tris, 100 mM NaCl, pH 8.0. sol-hMGL protein concentration was 200 mM and NDs in the same buffer were titrated in at a molar ratio of ND/sol-hMGL of 1/64 or 1/32.
All 1D $^1$H NMR spectra were obtained at 37 °C on a 700-MHz Bruker AVANCE II NMR spectrometer equipped with a 5 mm triple resonance probe. NMR spectra for low-field exchangeable protons were recorded using the 1331 pulse sequence with the excitation maximum at 16 ppm to minimize water excitation at 4.7 ppm. Acquisition was performed with a 90° flip angle, recycle delay 0.6 s, and 32 K data points for 4 K scans. Data were processed and analyzed using the Topspin 2.1 software package (Bruker). Exponential weighting resulting in a 30 Hz line broadening was applied. The proton chemical shift values were directly referenced to 4,4-dimethyl-4-silapentane-1-sulfonic acid at 0 ppm.

**Densitometry analysis**

Nanodisc samples, and controls (MSP and MGL at known concentrations) were separated by SDS-PAGE and stained with Comassie. Gel images were acquired using an Alpha Innotech FluorChem SP MultiImage™ light cabinet. Images were processed using Image Processing and Analysis in Java (ImageJ) software freely available here: http://imagej.nih.gov/ij/ from the National Institute of Health (NIH).

**Dynamic Light Scattering**

DLS experiments were carried out on a Malvern Zetasizer Nano-S (Malvern Inc., UK) instrument. Samples containing ND and detergent-free WT hMGL in ND buffer were added in a 2/1 (ND/MGL) stoichiometric ratio in the DLS cuvette at 25 °C. Readings were recorded every 15 sec for 15 min and Z-average (the intensity-based harmonic mean particle size) values were calculated using the Malvern software, version 6.

**TEM imaging of nanodiscs**

A single drop of nanodisc-containing solution was added to a carbon-coated, copper, TEM square-grid (Agar Scientific). This drop was allowed to dry for 1 min, and then excess
buffer was wicked away using Whatman #4 filter paper. The sample was negatively stained with a 1.5% uranyl acetate or phosphotungstic acid solution in DI water. The grid was then washed 3 times with DI water and dried completely before imaging using a JEOL JEM-1010 general purpose transmission electron microscope, with digital image acquisition operated at 75 kV.
Scheme 1: Workflow for co-purification of ND-MGL.

a) Purification and concentration of untagged MSP1D1. [inset: in red, the residues cleaved by TEV protease, in black, the sequence of (-)MSP1D1]. b) Formation of ND-MGL using (-)MSP1D1 and the subsequent co-purification of the complex using IMAC for the 6-His tag on WT hMGL and SEC.
Scheme 2: Determination of MGL conformation by proton NMR spectroscopy.
(Scheme provided by Sergiy Tyukhtenko) MGL exists in solution in two defined states, open (active) and shielded (closed) which have distinct downfield resonance patterns. The conformation of MGL can be controlled by changing the pH of the aqueous buffer.
Fig. 1. Identity and purity of MSP1D1.
(a) FPLC chromatogram for MSP1D1 showing multimeric state. [Inset: pure MSP1D1 SDS-PAGE image] (b) LC-TOF ESI-MS chromatogram showing intact mass of MSP1D1 [Inset: signal to noise ratio was 67]
**Fig. 2. SDS-PAGE analysis of ND-MGL formation.**

4 µL of prestained SDS-PAGE standards #161-0318 ladder were added to lanes 1 and 15. Lane 2: MSP1D1, Lane 3: MSP1D1 and TEV protease, Lane 4: wash, Lane 5: his tag cleaved MSP1D1, Lane 6: concentration of his tag cleaved MSP1D1, Lane 7: ND formation mixture, Lane 8: unbound fraction, Lane 9: pooled elution, Lane 10: concentration of pooled elution, Lane 11: WT hMGL, Lane 12: Talon® resin from MSP cleavage protocol, Lane 13: Talon® resin from complex isolation wash, Lane 14: Talon® resin from complex isolation.
Fig. 3. Downfield resonances of ND-MGL complex formation using proton NMR. 
Lane 1: Downfield resonance “fingerprint” for “open” conformation MGL. Lane 2: Fingerprint for MGL at pH 8.0 showing a mixture of open and “shielded” conformations. Lane 3: Fingerprint for MGL with ND added at a stoichiometric ratio of 1 to 64. Lane 4: Fingerprint for MGL with ND added at a stoichiometric ratio of 1 to 32.
Fig. 4. Densitometry Analysis of ND-MGL Complex.
a) SDS-PAGE gel showing 4 µL of prestained SDS-PAGE standards #161-0318 ladder, empty NDs, ND-MGLs, and MSP/MGL standards. b) Standard curve for MSP1D1. c) Standard curve for WT hMGL. d) ImageJ densitomograms for the corresponding cyan squares in panel a.
Fig. 5. ND-MGL Association Kinetics as Measured by DLS.
Complex association is highly dynamic, but a metastable (low variance from the linear regression trend line of Z-average vs. time) state is achieved within 15 minutes. Inset: normalized trend line representing dynamic instability reaching metastability over time.
Fig. 6. TEM Image of ND-MGL.
Negative stained (1.5% uranyl acetate solution) image of ND-MGL. Red arrows show ND-MGL. Inset: 100 nm scale bar.
Chapter 2

Membrane Phospholipid Bilayer as a Determinant of Monoacylglycerol Lipase Kinetic Profile and Conformational Repertoire$^{24}$
Introduction

A member of the α/β serine-hydrolase superfamily, monoacylglycerol lipase (MGL) is a lipid hydrolase featuring a characteristic serine (Ser\textsuperscript{122})-histidine (His\textsuperscript{269})-aspartic acid (Asp\textsuperscript{339}) catalytic triad.\textsuperscript{50} MGL is primarily responsible for deactivating 2-arachidonoylglycerol (2-AG), the predominant endocannabinoid signaling lipid in the central nervous system, which is synthesized on-demand from membrane phospholipid precursors.\textsuperscript{23,51,52} The hydrolysis of 2-AG by MGL also links the endocannabinoid and eicosanoid signaling systems by generating arachidonic acid precursor for eicosanoid biosynthesis.\textsuperscript{50,52} Since 2-AG acts as a full agonist capable of activating both principal 7-transmembrane cannabinoid receptors, designated CB1R and CB2R, MGL represents a major control point for 2-AG-mediated cannabinergic transmission that influences myriad (patho)physiological processes from psychobehavioral status to energy metabolism.\textsuperscript{53,54} Increased tissue 2-AG levels consequent to pharmacological or genetic MGL ablation are associated with preclinical therapeutic benefit against pain,\textsuperscript{8,9} inflammation,\textsuperscript{10,11} neurodegenerative disorders,\textsuperscript{12} psychological stressors,\textsuperscript{13} nausea/emesis,\textsuperscript{14} and cancer pathogenesis.\textsuperscript{2,15} Although protracted MGL inhibition invites functional CB1R desensitization in rodents,\textsuperscript{55} such salutary results in preclinical disease models have helped validate MGL as a drug target, focusing interest on the design and application of temporally-tuned human MGL (hMGL) inhibitors as medicines capable of elevating 2-AG tone and, indirectly, CB1R transmission to therapeutic levels with less risk of inciting the adverse events observed with systemic application of direct CB1R agonists.\textsuperscript{56,57}

Lipases are acyl hydrolases that cleave long-chain triacylglycerols at the boundary between aqueous and lipid-substrate phases, and their biocatalysis is activated at the interface. The interfacial potentiation of lipolysis has been attributed to factors such as increased local
substrate concentration in close proximity to the enzyme and optimized orientation of triacylglycerol scissile ester bonds.\textsuperscript{58} Many lipases feature a lid domain that regulates substrate access to the binding pocket/active site,\textsuperscript{59} and crystallographic data have supported inference that lipase structural changes upon association between the enzyme’s lid region and the boundary of a lipid matrix also contribute to interfacial lipase activation. Atomic-level analysis of \textit{Rhizomucor miehei} lipase, for example, suggested that this enzyme’s activation-associated conformational change reflects a hinge-type motion involving displacement of its lid domain so as to enhance the hydrophobic area for both enzyme interaction at the lipid interface and substrate binding.\textsuperscript{60} The mechanism of other lipases appears to involve more complex motions of multiple enzyme helices upon association with triacylglycerol substrate.\textsuperscript{61} Several studies have demonstrated that 2-AG hydrolytic activity is found at varying proportions between membrane and soluble tissue subfractions, depending upon cell/tissue type. In mouse brain, 2-AG hydrolase activity is primarily (\textasciitilde 90\%) membrane-associated,\textsuperscript{23} whereas in rat macrophages and gastrointestinal tract MGL activity is enriched in the cytosol.\textsuperscript{62,63} The enzymatic properties of cytosolic and membrane-associated MGL differ as well: in rat gastrointestinal tissue, the latter is less sensitive to pharmacological inhibition.\textsuperscript{63} These collective data have invited the hypothesis that, \textit{in situ}, MGL interacts reversibly with cell membranes, allowing the enzyme to extract 2-AG substrate from membrane-associated pools and into its hydrophobic substrate-binding pocket containing the catalytic triad, thereby facilitating substrate engagement.\textsuperscript{50}

Recent X-ray analyses of \textit{apo} and liganded hMGL variants have suggested a mechanistic rationale for this hypothesis.\textsuperscript{47,64,65} Reminiscent of many other lipid hydrolases, the (h)MGL active site is gated by a flexible lid domain positioned to shield the entrance to the enzyme’s substrate-binding pocket and thereby regulate substrate access to the catalytic center. A
comparison of the crystal structures of apo-hMGL and hMGL in complex with a reversible inhibitor has supported the view that the hMGL lid domain also participates in anchoring the enzyme reversibly to the cell membrane during the catalytic cycle and in structural rearrangements upon inhibitor binding, eliciting a shift from an “open” apo-enzyme to a “closed,” ligand-bound form in which the active site is shielded.\textsuperscript{47,64} In contrast to this purported mechanism, a crystal structure of hMGL covalently bound to a serine-reactive carbamylating agent displayed an open-- not closed-- conformation.\textsuperscript{65} Thus, although static representations of unique states of apo- and liganded-hMGL variants are available at atomic resolution, ambiguities remain concerning the structural and functional ramifications of hMGL-membrane interaction.

In the current study, we have chosen to examine this issue by using purified recombinant hMGL and phospholipid bilayer nanodiscs. A nanodisc is a discoidal form of high density lipoprotein composed of a nanometer-sized phospholipid bilayer surrounded by two α-helical membrane scaffold proteins (MSPs).\textsuperscript{66} Nanodiscs represent a unique, water-soluble biomimetic system for studying membrane-associated proteins, for monodisperse nanodiscs do not suffer from the propensity for aggregation, geometric distortion, and heterogeneity characteristic of other structured lipid platforms such as micelles, bicelles, and liposomes.\textsuperscript{67,68} In conjunction with a nanodisc-hMGL reconstitution system, we have used hydrogen exchange mass spectrometry (HX MS) and molecular dynamics (MD) simulation as conformational-analysis methods to gain insight into membrane influence on hMGL catalysis, structure, and ligand engagement. Peptide-level HX MS involves experimental quantification of protein amide-hydrogen exchange with heavier deuterium isotope in D\textsubscript{2}O media, the extent and kinetics of deuteration in peptide fragments generated through digestion of the intact protein reflective of the regional solvent accessibility of the protein as a proxy for protein conformational state and dynamics.\textsuperscript{69} MD
simulation is a computational approach toward modeling protein structure, dynamics, and interactions. Our data constitute evidence indicating that hMGL interaction with the nanodisc phospholipid bilayer involves a transition from a solution to a membrane-associated conformation, as evidenced primarily by structural changes in the major helical component (helix α4) of the hMGL lid domain and neighboring regions. These conformational changes may help stabilize an open hMGL conformation at the membrane-water interface, facilitate hMGL-ligand (substrate, inhibitor) interaction, and enhance catalysis.
Results and Discussion

Phospholipid bilayer nanodisc characterization

Nanodiscs are formed through a controlled self-assembly process from a defined molar ratio of phospholipid micelles in detergent and a stabilizing MSP to yield monodisperse, nanometer-size discoidal phospholipid bilayers encircled by the MSP, whose length defines the diameter of the disc. For this study, purified scaffold protein MSP1D1 was mixed with micelles composed of sodium cholate and either 1-palmitoyl-2-oleoyl-"sn"-glycero-3-phosphocholine (POPC) only or a 3:2 molar ratio of POPC and 1-palmitoyl-2-oleoyl-"sn"-glycero-3-phosphoglycerol (POPG). From an initial MSP1D1:phospholipid:sodium cholate molar ratio of 1:78:200, gradual detergent removal was used to effect component assembly. The resulting nanodiscs, as isolated by fast protein liquid chromatography (FPLC) on a calibrated size-exclusion column, evidenced the expected Stokes hydrodynamic diameter of ~10 nm (Fig. S1a, red) and by SDS-PAGE contained MSP1D1 as the sole protein component (Fig. S1b, red), substantiating the purity and integrity of our nanodisc preparation.

Phospholipid bilayer nanodiscs enhance hMGL catalytic activity and substrate affinity

Diacylphosphoglycerides such as POPC and POPG are not MGL substrates, and the charged head groups of bilayer phospholipids typically endow the surface of biological membranes with a net negative charge. These considerations, along with MGL’s membrane association observed in various tissues, led us to examine initially whether anionic phospholipid bilayer nanodiscs containing a 3:2 POPC:POPG molar ratio might influence the kinetic properties of hMGL, the recombinant enzyme expressed and purified as previously detailed by this laboratory.
As compared to hMGL in aqueous Tris buffer, the presence of POPC/POPG nanodiscs increased by ~3-fold the enzyme’s rate of hydrolysis (V\textsubscript{max}) of the fluorogenic reporter substrate, arachidonoyl 7-hydroxy-6-methoxy-4-methylcoumarin ester (AHMMCE),\textsuperscript{74,75} and enhanced by ~2.5-fold the enzyme’s affinity for this substrate (K\textsubscript{m}) (Table 1). The POPC/POPG nanodiscs elicited a similar enhancement of hMGL kinetic properties for hydrolysis of the enzyme’s natural endocannabinoid substrate, 2-AG (Table 1). Nanodisc enhancement of hMGL substrate turnover and affinity was independent of phospholipid bilayer charge, since charge-neutral POPC-bilayer nanodiscs increased hMGL V\textsubscript{max} and decreased its K\textsubscript{m} to extents comparable to those of anionic POPC/POPG nanodiscs (Table 1). Although the nonionic surfactant Triton X-100 can serve as a hydrophobic phase that helps solubilize both lipases and their triacylglycerol substrates to elicit apparent lipase activation,\textsuperscript{76} we found that Triton X-100 at a final concentration of 0.05 mM (i.e., ~ 2-fold its critical micelle concentration\textsuperscript{76}) did not affect hMGL substrate affinity and increased hMGL substrate turnover by ~1.5-fold relative to the enzyme in buffer alone (Table 1). Thus, Triton X-100 micelles affected hMGL’s V\textsubscript{max} only modestly as compared to the enhanced effect of either negatively-charged or charge-neutral phospholipid bilayer nanodiscs on both hMGL V\textsubscript{max} and K\textsubscript{m}.

The coincident enhancement of hMGL activity and substrate affinity induced by phospholipid-bilayer nanodiscs suggests that these biomembrane mimetics facilitate hMGL interaction with 2-AG or AHMMCE in a manner distinct from merely serving as a solubilization depot for hydrophobic substrate, since quantitatively parallel effects in hMGL kinetic properties were not induced by detergent micelles. Since both anionic and charge-neutral nanodiscs enhanced hMGL activity and substrate affinity (Table 1), it is tempting to hypothesize that hydrophobic interactions between the structured nanodisc phospholipid bilayer and hMGL may
form and establish an interfacial microenvironment that enhances hMGL kinetic properties 
underpinning by facilitating 2-AG/AHMMCE diffusion from the membrane into the enzyme’s 
open, hydrophobic substrate-binding pocket.

**hMGL interacts with phospholipid bilayer nanodiscs**

Our hypothesis that a hydrophobic association between hMGL and phospholipid bilayer 
nanodiscs enhances the enzyme’s kinetic properties is congruent with the concept that physical 
interaction between the hydrophobic lid region of lipases with an α/β hydrolase fold and their 
lipid-phase substrates helps govern lipase catalysis by influencing substrate supply/accessibility, 
orientation, and partitioning from the lipid phase to the enzyme’s substrate-binding pocket. The 
paradigm for interfacial effects on lipase activity extends to conformational rearrangement 
of the enzyme’s lid domain as a mechanism for gating substrate access to the active site. These 
concepts led us to probe experimentally whether hMGL associates with the nanodiscs 
themselves. For this purpose, we analyzed the POPC/POPG nanodisc and hMGL preparations 
themseles and an hMGL-nanodisc mixture by size-exclusion FPLC. The results demonstrate 
that two distinct nanodisc-containing populations with similar, but differentiable, Stokes 
hydrodynamic diameters are resolvable from hMGL itself (Fig. S1a, green), the hMGL-nanodisc 
mixture (Fig. S1a, black) evidencing a mean Stokes diameter greater than the nanodiscs alone 
(Fig. S1a, red). Only the hMGL-nanodisc mixture contained both MSP1D1 and hMGL protein 
(Fig. S1b). These data offer provisional evidence that hMGL associates with nanodiscs to form 
hMGL-nanodisc complexes having a mean Stokes diameter greater than either the enzyme or the 
nanodiscs.
hMGL interaction with nanodiscs modifies enzyme regional conformation

We next investigated experimentally whether phospholipid bilayer nanodiscs influence hMGL conformation in the two regions of α/β lipid hydrolases that are most critical for substrate interaction and turnover: the lid and the substrate-binding pocket/active site domains. For this purpose, we used peptide-level HX MS to compare the kinetics with which these hMGL regions exchange amide hydrogens for heavier deuterium isotope (i.e., the degree of solvent accessibility) when the intact, functional enzyme is incubated in D₂O medium in the presence or absence of POPC/POPG nanodiscs. Deuterium uptake into hMGL was monitored over incubation periods of 10 sec to 4 h, after which times pepsin hydrolysates were generated and analyzed by MS to determine the extent of hMGL deuteration in peptides within the enzyme’s lid domain and substrate-binding pocket. At a given pH and temperature, deuteration rate is modulated by protein conformational properties: rapid deuterium exchange is characteristic of more disordered, solvent-exposed protein regions. Conversely, slower, limited exchange indicates a more compact, solvent-shielded protein state. The difference between deuterium incorporation into hMGL peptides in the absence or presence of phospholipid-bilayer nanodiscs serves as proxy for nanodisc structural impact on the enzyme. Since peptide-level HX MS readout is based on the masses of peptides assignable to the protein under study (here, hMGL), the presence of MSP1D1 in some hMGL samples does not interfere with HX MS analysis of the enzyme itself.

Supplementary Table S1 lists the common peptic peptides identified through electrospray ionization-MS/MS (ESI-MS/MS) from three independent hMGL preparations for enzyme in the absence or presence of nanodiscs, along with the maximum possible deuterium incorporation for each respective peptide. This peptide subfamily includes peptic peptides representing the hMGL
lid and substrate binding pocket/active site domains that are the focus of the current study.
Specifically, residues 101-137 have been localized to the bottom of the substrate-binding pocket and encompass the catalytic-triad Ser. Residues 158-191 span what has been considered apo-hMGL’s lid domain, with residues 166-178 encompassing helix α4, which is flanked by residues 158-165 (leading from sheet β6 into helix α4) and residues 179-191 (loop conjoining helices α4 and α5). Residues 217-223 encompass helix α6 which, along with residues 224-241, are in the vicinity of the active site.

The peptide-level HX MS results for the hMGL lid domain and substrate-binding pocket/active-site region are plotted in Fig. 1a, and the peptides considered are highlighted in the hMGL structure representation (PDB ID: 3JW8) in Fig. 1b. The limited, gradual deuteration of peptides 101-125 and 126-137 indicates that the distal region of the substrate binding pocket containing catalytic Ser maintains only modest solvent exposure whether nanodiscs are present or not and is likely a stable structural element of the enzyme without significant breathing/unfolding motions. Peptide 224-241 was likewise shielded from solvent, but appeared somewhat dynamic, since it acquired significant deuterium over time to become labeled to 46% of maximum by 10 min, regardless of the presence of nanodiscs. Deuterium uptake into hMGL lid-domain peptides 158-166, 166-178, and 182-191 was extremely rapid, indicative of high solvent exposure. Within the lid domain, however, deuteration of peptide 166-178 constituting helix α4 was markedly suppressed before 10 min in the presence of nanodiscs and increased thereafter to parallel the peptide’s deuterium uptake in the absence of nanodiscs. Yet the deuterium exchange in hMGL peptides 158-166 and 182-191 flanking helix α4 was unaffected by the nanodiscs. A nanodisc-induced suppression of deuterium uptake into peptide 217-223 (i.e., helix α6) was also evident with an even more protracted approach to maximal deuteration.
than was observed for the influence of nanodiscs on lid-domain helix α4. Especially as compared to hMGL helices α4 and α6, other hMGL peptic peptides identified but not considered in Fig. 1 generally evidenced modest deuterium uptake that remained unaffected by the phospholipid bilayer nanodiscs (data not shown). This latter observation is congruent with the ordered, shielded nature of the core of eukaryotic α/β-hydrolases across species, a characteristic shared by apo-hMGL, as evident in a refined homology model of the enzyme and the finding that wild-type apo-hMGL and unliganded variants crystalize as compact globular proteins.

The nanodisc-induced suppression of deuterium uptake by hMGL helix-α4 peptide 166-178 along with the nanodisc’s enhancement of hMGL reaction velocity and substrate affinity suggest that this region of the enzyme’s lid domain associates with the nanodisc phospholipid bilayer, the interaction serving both to shield this lid region from solvent and facilitate access of lipid substrate to the lid-gated ligand-binding pocket. By analogy with other lipases, it has been speculated that, in situ, hMGL substrate diffuses into the enzyme’s binding site from a biomembrane pool by virtue of hMGL-membrane association. To the authors’ best knowledge, the present study provides the first experimental evidence supporting such a mechanism for hMGL. As observed for hMGL helix α4 peptide 166-178, nanodiscs acutely suppressed deuteration of helix α6 peptide 217-223 which is localized near the active site, suggesting that hMGL interaction with the nanodisc phospholipid bilayer hinders the solvent accessibility of this enzyme region also. Nanodisc shielding of the hMGL active-site region may also be appreciated from the less dynamic and more protected nature of peptide 224-241 (as indicated by its attenuated deuterium uptake) in the presence of nanodiscs. Collectively, these data indicate that hMGL association with phospholipid bilayer nanodiscs influences hMGL conformation within the enzyme’s lid domain and the vicinity of its active site.
Covalent hMGL inhibitor modulates enzyme conformation in the presence of nanodiscs

Potent MGL inhibitors share intrinsic lipophilicity with both the hMGL endocannabinoid substrate, 2-AG, and the reporter substrate, AHMMCE.\textsuperscript{9,10,15,50,52,53,56,57} In light of our demonstration that phospholipid bilayer nanodiscs enhance hMGL kinetic properties and induce enzyme lid-domain and active-site conformational changes consonant with hMGL-nanodisc association, we used HX MS to determine the potential influence of a small-molecule inhibitor on these hMGL regions in the presence of POPC/POPG nanodiscs. For this purpose, we selected AM6580 (Fig. 2a) as being representative of the principal class of covalent hMGL inhibitors, agents with the potential to carbamylate the enzyme’s catalytic serine.\textsuperscript{50,57} We previously demonstrated that AM6580 inhibits hMGL with nanomolar potency\textsuperscript{75} and now provide evidence from matrix-assisted laser desorption ionization-time of flight (MALDI-TOF/TOF) MS substantiating that AM6580’s fluorenyl piprazine moiety covalently modifies hMGL catalytic Ser\textsuperscript{129}, resulting in the expected hMGL mass increase of 277 Da (Fig. S2).

In the presence of phospholipid bilayer nanodiscs, AM6580 did not influence deuterium uptake into the hMGL lid domain (peptides 158-166, 166-178, and 182-191) (Fig. 2b). In contrast, carbamylation of hMGL Ser\textsuperscript{129} by AM6580 significantly reduced deuterium uptake in the vicinity of the enzyme’s active site [i.e., peptides 217-223 (helix-α6), 224-240, and 242-256 (from sheet β7 to helix α7 and including Asp\textsuperscript{246} in the catalytic triad)].\textsuperscript{64,65} Interaction between the AM6580-derived fluorenyl moiety and hydrophobic residues in carbamylated hMGL may be responsible for shielding helix-α6 peptide 217-223, as suggested by docking the fluorenyl piprazine moiety from AM6580 into the active site of the hMGL structure representation PDB ID: 3JWE\textsuperscript{65} (Fig. 2c). The fluorenyl piprazine group covalently attached to hMGL Ser\textsuperscript{129} may also render the enzyme’s active-site region more rigid and less dynamic. Notably, in the presence
of nanodiscs, deuterium uptake by the helix-α4 lid peptide (residues 166-178) was the same whether hMGL was inhibited or not (Fig. 2b), suggesting that the inhibitor enters the enzyme’s substrate-binding pocket from the nanodisc phospholipid bilayer via a membrane-associated lid, the inhibited enzyme remaining associated with the nanodisc in an open conformation.

**MD simulations predict hMGL interaction topology with nanodiscs**

As a computational approach for characterizing further the conformational impact of hMGL membrane interaction, we next performed MD simulations with POPC/POPG nanodiscs and hMGL modeled from the X-ray structure of wild-type hMGL (PDB ID: 3JW8) in an open conformation. Following 10 ns of MD simulation, significant components of the hMGL lid region were found to interact with the bilayer. Lid-domain helix α4 penetrated into the nanodisc phospholipid bilayer, whereas helix α6 in the vicinity of the lid evidenced a less intimate membrane association, whether the enzyme was in its unliganded apo form (Fig. 3a), or carbamylated by AM6580 inhibitor (Fig. 3b).

Superposition based on Cα positions of the starting hMGL model with hMGL after 10 ns of MD simulations revealed that the majority of Cα atoms could be superimposed with very little difference, whereas helices α4 and α6 displayed large variations after 10 ns simulations (Fig. 4). Helix α4 rotated 31° counter-clockwise in the same plane away from the hMGL active-site opening, resulting in the helix α4 ends being separated by a distance of 5.5Å. Helix α4 rotation was accompanied by simultaneous motion of the loop region connecting helices α4 and α5 (residues 177-192). In particular, residues Leu179 and Ile182 shifted and were present as different rotamers, substantially altering the hMGL active-site conformation. Helix α6 has rotated 22° counter-clockwise in the same plane away from the active site. The net result of these
collective conformational changes is an increased opening of the hMGL substrate-binding pocket and accessibility of the active site.
Conclusions

Crystallographic analyses of various apo and liganded hMGL variants have furnished atomic-level structural detail on a few static conformational states of an enzyme that has garnered intense interest as a therapeutic target. Despite documentation that MGL exists in situ as a membrane-associated enzyme and observations that lipase association with supramolecular lipid assemblies activates these enzymes, direct experimental evidence regarding the potential influence of membrane association on hMGL molecular properties is lacking. Our biochemical, HX MS, and computational analyses of the impact of a well-recognized biomembrane mimetic, the phospholipid bilayer nanodisc, on hMGL kinetic properties and conformation constitute the first detailed study to address this subject. The collective data provide evidence that a subdomain within the hMGL lid associates intimately with the membrane phospholipid bilayer through hydrophobic interaction, creating an interfacial microenvironment that enhances the enzyme’s kinetic properties. Our HX MS and MD simulation data indicate that the process of hMGL membrane association involves hMGL lid-domain helix α4 and, more distally, helix α6 and is accompanied by dynamic regional alterations in the conformation of these helices and in the enzyme’s active-site region that would help stabilize an open hMGL conformation at the lipid/water boundary receptive to substrate/inhibitor partitioning from the membrane bilayer and into the enzyme’s substrate-binding pocket. The shielding of select hMGL regions in proximity to the active site upon Ser carbamylation by AM6580 demonstrates directly that considerable conformational plasticity is associated with regions of the enzyme proximal to the active-site in response to covalent inhibitors. The emergence of covalent enzyme inhibitors as potential drug candidates for various diseases and the identification of serine-reactive carbamylating agents as the lead chemical class of hMGL
inhibitors\textsuperscript{50,57} make this observation particularly relevant to the design and targeting of hMGL inhibitors as potential medications. More generally, the present study demonstrates the suitability of peptide-level HX MS combined with nanodisc technology for investigating the structure-function correlates of enzyme-membrane interaction.
Materials and Methods

Materials

SDS-PAGE supplies, SM-2 bio beads, and Bio Spin columns were purchased from Bio-Rad (Hercules, CA). MS-grade trypsin (Trypsin Gold) was from Promega (Madison, WI). AM6580 and AHMMCE were synthesized at the Center for Drug Discovery, Northeastern University (Boston, MA) by standard routes. HPLC grade acetonitrile, ethylenediaminetetraacetic acid (EDTA 99%) and 85% phosphoric acid were purchased from Fisher Scientific (Pittsburgh, PA). Arachidonic acid was from Nu-Check Prep (Elysian, MN), and 2-AG was a generous gift from the National Institute on Drug Abuse (Bethesda, MD). Fatty acid-free bovine serum albumin, magnesium chloride tetrahydrate, and Trizma base were purchased from Sigma-Aldrich (St. Louis, MO). The plasmid expressing MSP1D1 was from Add Gene (Cambridge, MA). POPC and POPG were purchased from Avanti Polar Lipids (Alabaster, AL) as stock solutions in chloroform. 1,2-Deuterium oxide (>99%) was purchased from Cambridge Isotope Laboratories (Andover, MA).

Methods

Purification of recombinant wild-type hMGL

Recombinant hexa-His-tagged, wild-type human hMGL was expressed in *E. coli* and purified by cobalt affinity chromatography, as previously detailed.\textsuperscript{74} Chromatographic fractions were pooled and dialyzed against 50 mM Tris-HCl, pH 8.0, containing 100 mM NaCl, and protein concentration was estimated by absorbance at 280 nm. Purity was monitored by SDS-PAGE.
**MSP1D1 purification**

MSP1D1 was expressed and purified as described. The protein was isolated by cobalt affinity chromatography, and purity was monitored by SDS-PAGE. Fractions containing MSP1D1 were pooled and dialyzed against 20 mM Tris-HCl, pH 7.4, containing 100 mM NaCl, 0.5 mM EDTA, and 0.01% NaN₃. Protein concentration was estimated by absorbance at 280 nm.

**Nanodisc self-assembly and isolation**

Purified MSP1D1 in 20 mM Tris-HCl, pH 7.4, containing 100 mM NaCl, 0.5 mM EDTA, and 0.01% NaN₃ was added to a solubilized mixture of either POPC/sodium cholate or POPC-POPG (3:2 molar ratio)/sodium cholate, and the final molar ratio was adjusted to 1:78:200 (MSP1D1: phospholipid: sodium cholate). The mixture was incubated for 1 h over ice, and cholate detergent was removed during a gentle 10-h rotation with SM-2 bio beads at 4 °C. Nanodiscs were purified by size-exclusion chromatography with an Amersham-Pharmacia ÄKTA FPLC Protein Purifier System (GE Healthcare Life Sciences, Pittsburgh, PA) on a Superdex 200 10/300 column eluted with 20 mM Tris-HCl, pH 7.4, containing 100 mM NaCl and 0.5 mM EDTA at 0.5 mL/min. Column eluate absorbance was monitored at 280 nm, and SDS-PAGE was used to evaluate the purity of the nanodisc-containing fractions. FPLC fractions containing purified nanodiscs were collected and concentrated for experimental use. Nanodisc concentration was calculated from the absorbance at 280 nm and the MS1D1 molar extinction coefficient, accounting for 2 MSP1D1 molecules per disc.

**FPLC analysis of nanodiscs incubated with hMGL**

Purified, detergent-free hMGL was incubated with purified nanodiscs for 30 min at room temperature at an hMGL:nanodisc molar ratio of 1:2. Samples of hMGL alone and the hMGL-nanodisc co-incubation were analyzed by FPLC as detailed above for nanodiscs.
Determination of hMGL kinetic properties

hMGL kinetic constants were determined by measuring the hydrolysis of either natural substrate (2-AG) or fluorogenic reporter substrate (AHMMCE) with methods adapted from our prior work. In brief, 2-AG at varying concentrations (10 to 400 μM) was incubated at 37 °C with either 2.9 nM hMGL alone or 2.9 nM hMGL with nanodiscs at an MGL:nanodisc molar ratio of 1:2 in TME buffer (25 mM Tris base, 5 mM MgCl₂, and 1 mM EDTA, pH 7.4) in a total reaction volume of 300 μL. Reaction samples (50 μL) were taken immediately at the start of the incubation and after 4 min, diluted 1:2 by volume with acetonitrile, and centrifuged at 20,000 x g for 5 min at 4°C. A 20-μL aliquot of each supernatant was subjected to reverse-phase HPLC on an Agilent Zorbax XDB-C18 column (4.6 mm x 150 mm, 3.5 μm) (Agilent Technologies, Santa Clara, CA). Mobile phase A was 100% acetonitrile, and mobile phase B consisted of 8.5% aqueous phosphoric acid/acetonitrile (60:40, v/v) with the following gradient at a flow rate of 1 mL/min: 100% mobile phase B for 2 min, 5% mobile phase B for 5 min, 100% mobile phase B for 1 min followed by a 5-min injection delay. In an 8-min run, 2-AG was eluted at 4.2 min, and arachidonic acid at 5.0 min, allowing the reaction to be followed by either substrate utilization or product formation.

A fluorogenic hMGL assay based upon the conversion of AHMMCE reporter substrate to coumarin fluorophore was conducted as described. Reaction samples at room temperature included various concentrations of AHMMCE incubated with either 2.9 nM hMGL alone, 2.9 nM hMGL with 0.05 mM Triton X-100, or 2.9 nM hMGL with 5.8 nM nanodiscs in 50 mM Tris-HCl buffer, pH 7.4, at a reaction volume of 200 μL. Fluorescence readings at 360 nm/460 nm (λ_excitation/λ_emission) were taken every 15 min for up to 2 h, and relative fluorescence units were converted to the amount of coumarin fluorophore formed based upon a coumarin standard curve.
Michaelis-Menten kinetic parameters were derived with Prism software (GraphPad, San Diego, CA). Apparent $K_m$ and $V_{max}$ values are the means ± SD for triplicate determinations across three independent enzyme preparations. Statistical significance of group-mean differences was evaluated by a two-sample independent $t$-test, the significance level set at $p \leq 0.05$.

**Peptide-based HX MS analysis**

Continuous labeling hydrogen-deuterium exchange experiments were initiated by diluting 10-fold 12 μL of an hMGL-nanodisc mixture (5 μM hMGL and 10 μM nanodiscs) into 99% deuterium oxide buffer (50 mM Tris-HCl containing 100 mM NaCl, pH 7.6) at room temperature. At selected times ranging from 10 sec to 4 h after the introduction of D$_2$O, samples of the exchange reaction were taken and immediately quenched by acidifying to pH 2.5 with formic acid and placed on ice to limit back-exchange. After quenching, nanodiscs were rapidly disassembled with the addition of ice-cold sodium cholate in a 25:1 molar ratio of sodium cholate:nanodisc phospholipid. Porcine pepsin (1.2 μL of a 10 mg/mL stock solution) was added to digest the sample during a 5-min incubation on ice. In the last minute of digestion, ZrO$_2$ beads were added to the digestion mixture to facilitate phospholipid removal. The sample was filtered through a prechilled, 0.45-μm cellulose acetate membrane by centrifugation at 18,000 x g and 4 °C for 1 min at to trap both the pepsin and ZrO$_2$ beads. The flow-through was injected without delay into a precolumn trap (Waters VanGuard C18, 2.1 mm × 5 mm, 1.7 μm) and desalted with 0.05% formic acid in water for 5 min. The trap was placed in-line with a second identical pre-column directly connected to the analytical column (Waters Xbridge C18, 1.0 mm × 100 mm, 1.7 μm). HX data were acquired on a Waters nanoAcquity UPLC with HDX Technology. Peptides originating from hMGL pepsinolysis were identified from triplicate analyses of undeuterated control enzyme samples using Waters ProteinLynx Global Server 2.4.
All reported peptide-based HX MS data were derived from triplicate hMGL preparations, each analyzed in triplicate. The error of peptide HX MS measurements was ± 0.50, as determined by replicate analyses of peptide standard and prior HX MS data from this experimental setup.86,87

**MALDI-TOF/TOF MS analysis of hMGL covalent modification by AM6580**

Purified hMGL was incubated with AM6580 (molar ratio 1:5, enzyme:inhibitor) at room temperature for 1 h, at which time the enzyme was verified by direct biochemical assay to be inhibited (above). The incubation was terminated by desalting using a Bio-Spin 6 column and 25 mM ammonium bicarbonate buffer, pH 8.0, containing 0.05% CYMAL. The desalted enzyme sample was digested overnight with 200 ng Trypsin Gold. Equal volumes of the tryptic digest and α-cyano-4-hydroxycinnamic acid matrix (5 mg/mL in aqueous 50% acetonitrile-0.1% trifluoroacetic acid) (0.5 μL each) were co-crystallized. MS characterization of hMGL covalent modification by AM6580 were acquired on a 4800 MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Framingham, MA) fitted with a 200-Hz solid-state ultraviolet laser (wavelength 355 nm) from samples spotted on Opti-TOF 384-well plate inserts.
Computational methods

*MD simulation setup.* A 128-lipid, racemic POPG bilayer neutralized with 128 Na\(^+\) counter ions and hydrated with 3527 water molecules was used as the basis of a POPC/POPG bilayer.\(^{51}\) All water molecules and ions were removed. Since the experimental nanodisc contained POPC and L-POPG in a 3:2 molar ratio, all D-POPG and 11 L-POPG lipid molecules were replaced with POPC, and 3 L-POPG lipid molecules were removed. This resulted in a phospholipid bilayer containing 75 POPC and 50 L-POPG lipid molecules, which was solvated with 4270 water molecules and neutralized with 50 Na\(^+\) counter ions. The system was fully minimized with steepest descent, and 40 ns of unrestrained MD were then performed to produce a fully-solvated, fully-equilibrated POPC/POPG bilayer (simulation procedure below). The potential energy reached a stable plateau after 3 ns. After 10 ns, the area of the xy plane per lipid fluctuated around 69.7 Å\(^2\), a value within the experimental range for POPC (63.0-68.3 Å\(^2\)) and probable range for POPG (64-70 Å\(^2\)).\(^{88,89}\) Two simulation systems were established: one with unliganded, wild-type hMGL (PDB ID: 3JW8),\(^{47}\) the other with hMGL covalently carbamylated by AM6580. The carbamylating moiety of AM6580 was docked to hMGL (PDB ID: 3JWE)\(^{47}\) using Glide at the XP level,\(^{90}\) and a covalent bond was made between this moiety and the catalytic Ser\(^{122}\) (i.e., Ser\(^{129}\) for the 6-His-tagged hMGL). To enable MD simulation of a covalently-bound ligand, a custom residue was defined as Ser\(^{122}\) carbamylated by AM6580 and was parameterized.\(^{91}\)

*MD simulation procedure.* Unmodified and AM6580-carbamylated hMGL were placed with the enzyme’s lid domain partially embedded in the POPC/POPG bilayer, as given by the Orientations of Proteins in Membranes database.\(^{92,93}\) Any lipid or water molecules clashing with the enzyme were removed. The system was made electrically neutral and minimized with
steepest descents to relax unfavorable intermolecular contacts. To stabilize the lipid environment during production dynamics, equilibration MD was performed for 2 ns, with all heavy atoms in the protein positionally restrained with a force constant of 1000 kJ mol$^{-1}$ nm$^{-2}$. Unconstrained production MD was performed for 10 ns on each system. All MD simulations were performed in the NPT (isothermal–isobaric) ensemble with periodic boundary conditions. A temperature of 300 K was maintained with time constant 0.1 ps by an extension of the Berendsen thermostat to which a properly constructed random force was added.$^{94}$ Semi-isotropic pressure coupling was used, with the reference pressure set at 1.0 bar and time constant at 5 ps. Coulomb and short-range neighbor list cut-offs were both set to 0.9 nm, and Lennard-Jones cut-offs were set to 1.2 nm. The electrostatic interactions were computed using the Particle-Mesh Ewald method with an interpolation order of 4 and a maximum grid spacing of 0.12 nm.$^{95,96}$ A time-step of 2 fs was used, and pair lists were updated every 10 steps. The LINCS algorithm$^{97}$ was employed to preserve bond lengths. The simple point charge water model$^{98}$ was used in all simulations. The simulations were carried out with the GROMACS program, version 4.5.5, using the GROMOS96 53a6 force field.$^{88,99,100}$
Tables

Table 1. Kinetic parameters for hydrolysis of AHMMCE and 2-AG by hMGL in the presence and absence of detergent or phospholipid bilayer nanodiscs.

<table>
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<th>Incubation Conditions</th>
<th>Substrate</th>
<th></th>
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<tr>
<td></td>
<td>AHMMCE</td>
<td>2-AG</td>
</tr>
<tr>
<td></td>
<td>$K_m$ (μM)</td>
<td>$V_{max}$ (μM/min/mg)</td>
</tr>
<tr>
<td>Buffer</td>
<td>18.3 ± 2.8</td>
<td>191.4 ± 10.5</td>
</tr>
<tr>
<td>Buffer + Triton X-100</td>
<td>18.3 ± 4</td>
<td>295 ± 37.2</td>
</tr>
<tr>
<td>Buffer + POPC/POPG nanodiscs</td>
<td>7.5 ±1.8</td>
<td>565.4 ± 52</td>
</tr>
<tr>
<td>Buffer + POPC nanodiscs</td>
<td>7.9 ± 2</td>
<td>590 ± 45</td>
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For AHMMCE substrate, buffer was 50 mM Tris-HCl, pH 7.4. For 2-AG substrate, TME buffer (25 mM Tris base, 5 mM MgCl$_2$, and 1 mM EDTA, pH 7.4) was used. Apparent $K_m$ and $V_{max}$ values are the means ± SD for triplicate determinations across three independent enzyme preparations. Statistical significance of group-mean differences was evaluated by a two-sample independent $t$-test, the significance level set at $p \leq 0.05$. n.d., not determined.
Fig. 1. Localization of conformational changes in hMGL induced by phospholipid bilayer nanodiscs.

(a) Deuterium incorporation curves derived from hydrogen-exchange mass spectra for the hMGL peptic peptides designated by their amino-acid residue numbers. Relative deuterium uptake (Da) is plotted vs. time of hMGL incubation in D$_2$O in the absence (blue lines, □) or presence (red lines, ◆) of POPC/POPG bilayer nanodiscs. The maximum amount of deuterium incorporation possible for each respective peptide is designated on the y-axis of each kinetic plot. Data were obtained through peptide-based HX MS analysis of hMGL. The error of peptide HX MS measurements with this experimental setup was ± 0.50 Da as determined by replicate analysis of peptide standards in prior HX MS work with this instrumentation.

(b) The hMGL peptides for which deuterium uptake curves are presented in panel (a), above, are highlighted in the hMGL structure representation derived from (PDB ID: 3JW8).
Fig. 2. Localization of conformational changes in hMGL induced through active-site Ser carbamylation by the covalent inhibitor, AM6580.

a) Deuterium incorporation curves derived from hydrogen-exchange mass spectra for the hMGL peptic peptides designated by their amino-acid residue numbers. Relative deuterium uptake (Da) is plotted vs. time of hMGL incubation in D$_2$O in the presence of POPC/POPG phospholipid bilayer nanodiscs without (red lines, ◆) or with (green lines, ▼) AM6580. The maximum amount of deuterium incorporation possible for each respective peptide is designated on the y-axis of each kinetic plot. Data were obtained through peptide-based HX MS analysis of hMGL. The error of peptide HX MS mesurements with this experimental setup was ± 0.50 Da as determined by replicate analysis of peptide standards in prior HX MS work with this instrumentation. $^{49,50}$

b) Structure of AM6580.

c) Schematic depicting the docking of the AM6580-derived fluorenyl piprazine group covalently attached to hMGL active-site Ser$^{122}$ (i.e., Ser$^{129}$ for the 6-His-tagged hMGL) in a portion of the hMGL structure representation derived from (PDB ID: 3JW8). Helix-α6 peptide 217-223 (orange) is shielded by the carbamylating group modification at Ser$^{122}$. 
Fig. 3. MD simulation of hMGL interaction with membrane phospholipid bilayer. Snapshots of hMGL (structure derived from PDB ID: 3JW8) with a phospholipid bilayer membrane of the same composition as in the experimental nanodiscs (POPC: POPG, 3:2 molar ratio) after 10 sec of MD simulation. The enzyme is depicted: a) unliganded as apo-hMGL and b) occupied with carbamylating inhibitor, AM6580, in the active site. Helix α4 in the lid domain and helix α6 in the vicinity of the active site are depicted in red, and AM6580 is shown in green.
Fig. 4. Cartoon superposition of the X-ray structure of hMGL (cyan, PDB ID: 3JW8) and the model of hMGL obtained after 10 ns of MD simulation at a water/phospholipid interface (gray).

The structures are virtually identical, except for helices α4 and α6 and the loop region from amino-acid residues 177 to 192 connecting helices α4 and α5. The movements of helices α4 and α6 induced by a phospholipid bilayer (POPC:POPG, 3:2 molar ratio) are indicated by red arrows. a) side view of hMGL; b) top view of hMGL rotated 90° from the orientation in panel a, as indicated. The active-site Ser$^{122}$ (i.e., Ser$^{129}$ for the 6-His-tagged hMGL) is depicted in orange.
Supplemental Material

Supplemental Tables

Table S1. Peptic peptides common to hMGL in the absence or presence of nanodiscs. Peptides were identified by ESI-MS/MS and are shown with their respective sequence number, length, amino-acid sequence, z, m/z, and maximum possible deuterium incorporation.

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<th>End</th>
<th>Length</th>
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<th>m/z</th>
<th>Max Deuterium Incorporation</th>
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Supplementary Figures

(a) Analysis of hMGL (green line), POPC/POPG phospholipid bilayer nanodiscs (red line), and hMGL-nanodisc mixture (black line) by a) size-exclusion fast protein liquid chromatography and b) SDS-PAGE. The calibration scale for the size-exclusion column is shown and was derived from proteins of known Stokes diameter: thyroglobulin (bovine thyroid) (17 nm), ferritin (horse spleen) (12.2 nm), catalase (bovine liver) (10.4 nm), albumin (bovine serum) (7.1 nm).
Fig. S2. Demonstration that AM6580 covalently inhibits hMGL by carbamylation of the enzyme’s Ser129 nucleophile.

a) MALDI-TOF/TOF MS mapping of a tryptic digest of purified recombinant hMGL. b) Tryptic hMGL peptide that contains the hMGL nucleophile Ser129. c) New hMGL tryptic peptide after incubation with AM6580, showing a mass increase of 277 Da. d) Illustration of the carbamylation reaction between hMGL Ser129 of hMGL and AM6580.
Chapter 3

Expression and Purification of Full-length rFAAH and Subsequent Incorporation within the Nanodisc Model
Introduction

Serine hydrolases such as fatty acid amide hydrolase (FAAH) comprise the largest, and structurally and functionally -- most diverse class of enzymes within the human proteome.\textsuperscript{101} These enzymes actuate the catabolism of amide, ester, and thioester bonds in biosignaling molecules, proteins, and xenobiotics. This process is mechanistically dependent upon a catalytic triad, Ser\textsuperscript{241}, Ser\textsuperscript{217}, and Lys\textsuperscript{142}, for FAAH (PDB: 1MT5)\textsuperscript{20} and proceeds through the formation of a covalent, acyl-enzyme complex intermediate that is subsequently hydrolyzed by a water/hydroxide-induced saponification process that regenerates the catalytic serine. FAAH is the only characterized mammalian amidase in the serine hydrolase family of enzymes,\textsuperscript{102} and it is extremely hydrophobic. FAAH has three domains that interact with biological membranes, the NH\textsubscript{2}-terminal transmembrane domain (residues 9-29), the α18 domain (residues 410-426), and the α19 “lid” domain (residues 429-438).\textsuperscript{20} Despite the wealth of structural, chemical, and biochemical information available for this enzyme, the only molecular dynamic studies to date for this enzyme have been in silico simulations (most recently reviewed by Palermo \textit{et al}.).\textsuperscript{102} Due to the unique nature of this enzyme’s structure, with a comprehensive molecular dynamic analysis in a biological membrane, it should be possible to design inhibitors with exceptional selectivity for FAAH. Such inhibitors could target either the orthosteric, cytoplasmic channel and/or the hydrophobic enzyme-acyl chain pocket through the membrane in a manner consistent with the mechanism by which FAAH accesses its primary substrate, anandamide (AEA).\textsuperscript{20}

FAAH rapidly degrades AEA, forming arachidonic acid (AA) and ethanolamine (EA).\textsuperscript{103} AEA is a partial-to-full agonist at CB\textsubscript{1} (assay-dependent) and a weak partial agonist at CB\textsubscript{2}.\textsuperscript{104} It is synthesized from N-arachidonoyl phosphatidyl-ethanolamine (NAPE) when dephosphorylated by phospholipase-D.\textsuperscript{105} NAPE is synthesized from dietary lecithin and phosphatidyl-
ethanolamine (PE) via enzymatic n-acyltransferase activity whereby AA is bioconjugated to PE.\textsuperscript{106} Inhibition of FAAH leads to increased levels of AEA, which has been shown to be therapeutic for treating pain and inflammation.\textsuperscript{107-109} Increased expression levels of FAAH have also been found in breast,\textsuperscript{110} prostate,\textsuperscript{111,112} and lung carcinomas,\textsuperscript{113} as well as in lymphocytic leukemia,\textsuperscript{114} making FAAH inhibition an essential target for therapeutic development.

Presented here is the expression and purification of full-length rFAAH. Traditional purification schemes did not yield pure protein due to the hydrophobic nature of FAAH and the significant portion of histidine-rich proteins present in the raw \textit{E. coli} lysate. These factors lead to either insufficient binding to the IMAC resin in the presence of imidazole or an abundance of nonspecific protein binding. To circumvent these limitations, a fractionation-purification protocol was used to remove soluble and insoluble proteins (leaving only solubilized membrane proteins or “membrane fraction”) before purification. This membrane fraction was used to purify rFAAH for expression in the nanodisc model and in parallel, to generate a solubilized membrane protein library (SMPL).\textsuperscript{115} ND-rFAAH was also purified from the SMPL, although this method had a low yield. This represents the first time FAAH has been incorporated into the ND system.

Protein dynamics for purified rFAAH and ND-rFAAH were then studied using hydrogen-deuterium exchange mass spectrometry (HX MS). Peptide-level HX MS examines solvent accessibility over time by measuring changes in peptide mass after exposure to D\textsubscript{2}O, which will exchange deuterium with the hydrogen atoms on the “backbone” amide nitrogens of proteins.\textsuperscript{69} This study represents the first, non-simulated, molecular dynamic study for FAAH both in solution and in an intact membrane.
Results and Discussion

Purification of full length rFAAH

The *E. coli* sonicate showed activity in the fluorescence assay\(^{116}\) comparable to that seen in a control lysate, and no activity was lost during membrane solubilization (Fig. 1). The pelleted insoluble fraction retained some ability to breakdown the substrate (greater than the blank), but significantly less than the solubilized membrane fraction (Fig. 1 blue). These results suggest that rFAAH completely partitions into the membrane fraction and can be solubilized using n-dodecyl-beta-D-maltoside (DDM) (1%) in aqueous buffer. This solubilized membrane fraction was used to purify C-terminal 6-his tagged full-length rFAAH by IMAC. SDS-PAGE analysis of the purification showed that despite a high-capacity resin, only a small portion of the rFAAH bound to the resin (Fig. 2). However, little loss was observed when washing the resin either with buffer B (20 mM Na\(_2\)PO\(_4\) pH = 7.8 with 500 mM NaCl and 1% DDM), or buffer B that contained 50 mM imidazole (IMA) (Fig. 2). To isolate pure rFAAH, FPLC-SEC was used (Fig. 3a). Fractions 7 through 17 (mL) were analyzed by SDS-PAGE, and fraction 10 appeared to have the highest purity (Fig. 3b). This fraction was tested for activity using the fluorogenic substrate assay at increasing sample concentrations. This fraction showed activity which increased in a concentration dependent manor (Fig. 3c). Unfortunately, the activity level measured here was low as compared to the sonicate and membrane fraction. This decrease was attributable to significant dilution and not a loss in protein abundance or intrinsic activity, although some protein was undoubtedly lost in the SEC column. This small-scale experiment demonstrated a superior method for purifying full length rFAAH for incorporation into the ND model.
rFAAH incorporation into the nanodisc biological membrane model

To express full-length rFAAH in the ND model, two methods were used: an adapted version of the SMPL technique, and in parallel, the Sligar et al. method of combined self-assembly. Using the Sligar technique, purified rFAAH was incubated with MSP1D1, POPC and POPG, and disc self-assembly was catalyzed by detergent removal with hydrophobic-adsorbent beads. FPLC-SEC was used to isolate the ND-rFAAH population, and a clear peak was visible at ~12 mL (Fig. 4a). Coomassie stained SDS-PAGE analysis of the pooled fractions 9 through 13 (mL) inclusive showed clear bands for MSP1D1 and rFAAH (Fig. 5) indicating the formation of ND-rFAAH.

In the SMPL technique, MSP1D1 was added to the membrane fraction with POPC and POPG, to augment the native lipid population, and detergent removal beads were used to catalyze ND formation. FPLC-SEC was used to separate the population of unincorporated proteins and aggregates from the SMPL, and a clear ND peak population was observed between fractions 9 and 13 (mL) inclusive (Fig. 4b). Coomassie stained SDS-PAGE analysis of the pooled fractions 9 through 13 (mL) inclusive showed clear bands for MSP1D1 and rFAAH as well as several other bands (Fig. 5) indicating the formation of ND-rFAAH and the SMPL.

Either technique would be viable for ND-rFAAH preparation, but in this experiment, purifying rFAAH from the detergent-solubilized membrane fraction before Sligar synthesis gave a higher yield, and a more pure population of ND-rFAAH (data not shown). When the additional step of removing the 6-his tag from MSP1D1 that is necessary for purifying ND-rFAAH from the SMPL is considered, the data suggest that the traditional method should be used to prepare ND-rFAAH in the future.
**In-solution dynamics of rFAAH**

Peptide-level HX MS was used to measure the dynamics for rFAAH in solution over a 4-hour time period. No deuterium uptake was measured in the peptic peptide (residues 10 – 23 LSGVSGVCLACSL) from the transmembrane (TM) domain (residues 9 – 29, TLGVSVLGLACSLLAASVAVL) or in the active site peptic peptides (residues 138 – 148, PVSLKECFSTK, 208 – 230, WKSSKPGGSSGEGALIGSGGS, and 227 – 242 SGGSPLGLGLTDGGSI) (Fig. 6, Fig. 7). This indicates that the TM domain is shielded from the solvent when FAAH is in solution. This could be due to interaction between the protein and detergent micelles, or through dimerization in solution. Unfortunately, the proposed dimerization sites (residues 299 – 314, CEHLFTLDPTVPPSSL and Trp$^{445}$) were not covered by our peptic digest (Fig. 6, Fig S2). Similarly, the membrane binding domain α18 (residues 410 – 426, LPSWFKRLSLPSWFKRLSL) and the lid domain α19 (residues 429 – 438, LAAFLNSMRP) were also not covered (Fig. 6, Fig S2).
Conclusions

In this study, catalytically active full length rFAAH was successfully expressed, purified, and incorporated into NDs. This represents the first experiment where a mammalian amidase, serine hydrolase was studied using this model. A fractionation purification protocol was necessary to first separate the solubilizable membrane proteins from other proteins in the E. coli lysate. Following this critical step, direct IMAC purification was achieved, as well as formation of a SMPL. From the SMPL, ND-rFAAH was successfully isolated. Additionally, traditional methods were employed to isolate ND-rFAAH. These protocols for isolating the membrane-protein pool, and for disc formation, are generalizable to other hydrophobic mammalian recombinant proteins expressed in E. coli. For the purification of rFAAH, the FPLC-SEC purification step, while giving some information on the oligomeric state of rFAAH in solution, should not be necessary to isolate protein of acceptable purity for ND formation in the future.

Preliminary analysis of the in-solution protein dynamics for rFAAH reviled areas of high exchange, and zero to low exchange over the four hour time period used. Notably, the lack of exchange in the active site domain, and the transmembrane domain support previous findings that these hydrophobic regions interact directly with the membrane, and are not accessible to the cytosol. The shielding from exchange in the TM domain in particular suggests that in solution rFAAH may exist as a dimer imbedded within detergent micelles. This makes rFAAH an excellent target for analysis in the ND membrane model, which is compatible with peptide-level HX MS analysis.
Materials and Methods

Materials

SDS-PAGE supplies, SM-2 biobeads, and Bio Spin columns were purchased from Bio-Rad ( Hercules, CA). Culture media, standard laboratory chemicals, and buffers were purchased from Fisher Chemical (Pittsburgh, PA) and Sigma Chemical Co. (St. Louis, MO). The plasmid expressing MSP1D1 was purchased from Add Gene ( Cambridge, MA). POPC and POPG were purchased from Avanti Polar Lipids (Alabaster, AL) as stock solutions in chloroform. 1,2-Deuterium oxide (>99%) was purchased from Cambridge Isotope Laboratories (Andover, MA).

Methods

Expression and Purification of rFAAH

Full length rFAAH was purified from _E. coli_ (BL21-CodonPlus (DE3)-RIPL) that was transformed with the pTrcHis2-TOPO vector containing full length, wild type rFAAH. A 1L cell pellet (~4-5 g) was suspend in 40 mL of Buffer A (20 mM Na$_2$PO$_4$, 100 mM NaCl, pH = 7.8) and rotated for 15 min. Cells were lysed cells by probe sonication (55 sec pulse, on 1 sec, off 5 sec), spun at 5,000 x g for 20 min, and the pellet was discarded. The supernatant was centrifuged at 100,000 x g for 1hr and the supernatant was discarded. The cell pellet was suspended in buffer A (40 mL) and spun at 100,000 x g for 1hr. This wash step was then repeated 2 more times. The resulting cell pellet was suspended in 40 mL of Buffer B (20 mM Na$_2$PO$_4$, 500 mM NaCl, 1% DDM, pH = 7.8) and spun at 100,000 x g for 1hr. The supernatant (“membrane fraction”) was added to 1.0 mL (bed volume) of His60 Ni Superflow™ resin (Clonetech) pre-equilibrated with buffer B and mixed at 4°C for 1 hr. The suspension was transferred to a gravity-flow column and the resin was washed with 10 mL of Buffer B, then 10 mL of Buffer B with 20 mM imidazole, followed by 10 mL of Buffer B with 50 mM imidazole. rFAAH was eluted with 5 to 6 mL of
Buffer B with 200 mM imidazole. Size exclusion chromatography was performed as described above using a Superdex 200 10/300 GL column and Gel Filtration Buffer (10 mM Tris/HCl, 150 mM NaCl, 0.015% LDAO, pH = 8.0). Purified rFAAH samples were stored at -80°C in Gel Filtration Buffer with 10% Glycerol.

**Assay of rFAAH activity using AAMCA**

A fluorometric assay was used to evaluate the catalytic activity of rFAAH. This was monitored by the hydrolysis of the fluorogenic substrate: N-arachidonoyl,7-amino-4-methylcoumarin amide (AAMCA) to form the fluorescent product 7-amino-4-methylcoumarin (AMC). DMSO (10 µL), rFAAH samples, and AAMCA (final concentration 20 μM) were added to a total volume of 200 µL in assay buffer (50 mM Tris-HCl, pH 9.0) in a 96-well plate. The reaction was allowed to proceed for 10 hr at 25°C. Fluorescence readings were taken every 15 minutes at 360 nm/460 nm (λ_{excitation}/λ_{emission}) on a BioTek Synergy HT Microplate Reader (BioTek Instruments, Winooski, VT).

**Solubilized membrane protein library (SMPL) method for rFAAH nanodisc incorporation**

Purified MSP1D1 in ND buffer (20 mM Tris/HCl, pH 7.4, containing 100 mM NaCl, and 0.01% NaN₃) was added to a the rFAAH membrane fraction described above. A solubilized mixture of POPC-POPG (3:2 molar ratio)/sodium cholate was also added, and the final molar ratio was adjusted to 1:0.5:5.5:2.3:20 (MSP1D1:total membrane protein:phospholipid:native lipid:sodium cholate). The mixture was incubated for 1 h on ice, and cholate detergent was removed during a gentle 10 hr rotation with SM-2 biobeads at 4°C. This self-assembly mixture was added to a 1.0 mL (bed volume) of His60 Ni Superflow™ resin (Clonetech) pre-equilibrated with ND buffer and mixed at 4°C for 1 hr. The suspension was transferred to a gravity-flow column and the resin was washed with 10 mL of ND buffer, then 10 mL of ND buffer with 20
mM imidazole, then 10 mL of ND buffer with 50 mM imidazole. ND-rFAAH was then eluted with 5 to 6 mL of ND buffer with 200 mM imidazole. Size exclusion chromatography was performed as described above, using a Superdex 200 10/300 GL column in ND buffer with 0.5 mM EDTA.

**Coself-assembly of purified rFAAH**

Purified MSP1D1 in ND buffer and rFAAH in buffer B was added to a solubilized mixture of POPC-POPG (3:2 molar ratio)/sodium cholate, and the final molar ratio was adjusted to 1:0.5:75:200 (MSP1D1:phospholipid:sodium cholate). The mixture was incubated for 1 h on ice, and cholate detergent was removed during a gentle 10 hr rotation with SM-2 biobeads at 4°C. Nanodiscs were purified by size-exclusion chromatography with an Amersham-Pharmacia ÄKTA FPLC Protein Purifier System (GE Healthcare Life Sciences, Pittsburgh, PA) on a Superdex 200 10/300 column eluted with 20 mM Tris–HCl, pH 7.4, containing 100 mM NaCl at 0.5 mL/min. Column eluate absorbance was monitored at 280 nm, and SDS-PAGE was used to evaluate the purity of the nanodisc-containing fractions.

**Peptide-level HX MS analysis**

Continuous labeling hydrogen-deuterium exchange experiments were initiated by diluting 10-fold 12 µL of a rFAAH–nanodisc mixture (5 µM rFAAH and 10 µM nanodiscs) into 99% D₂O buffer (50 mM Tris/HCl containing 100 mM NaCl, pH 7.6) at room temperature. At selected times ranging from 10 s to 4 h after the introduction of D₂O, samples of the exchange reaction were taken and immediately quenched by acidifying to pH 2.5 with formic acid on ice to limit back exchange, and nanodiscs were rapidly disassembled with the addition of ice-cold sodium cholate in a 25:1 molar ratio of sodium cholate to nanodisc phospholipid. Porcine pepsin (1.2 µL of a 10 mg/mL stock solution) was added to digest the sample during a 5 min incubation
on ice. In the last minute of digestion, ZrO$_2$ beads were added to the digestion mixture to facilitate phospholipid removal. The sample was filtered through a prechilled, 0.45 µm cellulose acetate membrane by centrifugation at 18,000 x g and 4°C for 1 min to trap both the pepsin and ZrO$_2$ beads. The flow through was injected without delay into a precolumn trap (Waters VanGuard C18, 2.1 mm 3 5 mm, 1.7 µm) and desalted with 0.05% formic acid in water for 5 min. The trap was placed in line with a second identical precolumn directly connected to the analytical column (Waters BEH C18, 1.0 mm 3 100 mm, 1.7 lm). HX data were acquired on a Waters nanoAcquity UPLC with HDX Technology. Peptides originating from rFAAH pepsinolysis were identified from triplicate analyses of undeuterated control enzyme samples, with use of Waters Protein-Lynx Global Server 2.4.
Fig. 1. rFAAH membrane fraction activity tracking using the fluorogenic substrate AAMCA.
Relative fluorescent units vs. time for: in **black** circles, autohydrolysis of the fluorogenic substrate. In **blue**, the insoluble portion of the membrane fraction. In **green**, the solubilized membrane fraction. In **black** triangles, the sonicate without cellular debris. In **red**, control WT rFAAH lysate (~6 mg/mL total protein) isolated from rat brain homogenate (n=6).
Fig. 2. Coomassie stained SDS-PAGE analysis of rFAAH purification from solubilized membrane fraction.
4 µL of prestained SDS-PAGE standards #161-0373 ladder were run between lanes 2 and 3. Lane 1: membrane fraction before IMAC. Lane 2: membrane fraction after IMAC. Lane 3: unbound fraction. Lane 4: Wash with buffer B. Lane 5: wash with buffer B containing 50 mM imidazole. Lane 6: purified rFAAH. Lane 7: IMAC resin. rFAAH was successfully purified from the membrane fraction and no irreversible binding to the Ni^{2+} IMAC resin was observed.
Fig. 3. FPLC-SEC separation for purified rFAAH. 
a) FPLC-SEC chromatogram for purified rFAAH. b) Coomassie stained SDS-PAGE for 
fractons 7 through 17. c) Activity analysis for varying volumes (µL) of fraction 10 using the 
fluorogenic substrate AAMCA.
Fig. 4. FPLC-SEC separation for purified ND-rFAAH.

a) Chromatogram for ND-rFAAH isolated using the Sligar method. b) Chromatogram for SMPL separation.
Fig. 5. Coomassie stained SDS-PAGE for ND-rFAAH.
Lane 1: “L” 4 µL of prestained SDS-PAGE standards #161-0373 ladder. Lane 2: “P” ND-rFAAH isolated using the Sligar method. Lane 3: “DS” SMPL including ND-rFAAH
Fig. 6. Uptake plots for rFAAH in solution.
Fig. 7. In-solution dynamics for rFAAH (PDB: 1mt5)\textsuperscript{20}. Residues 10 – 23 in the TM domain are indicated in black. These showed no exchange over 4 hours indicating that this hydrophobic domain is shielded from the solvent, perhaps through dimerization. The protein is oriented in a top-down view, such that the membrane bilayer would be parallel with the page, under/behind the protein as represented. The blue arrows indicate residues 229 – 314, the suspected oligomerization domain.
Supplementary Data

Coverage of peptic peptides for rFAAH and MSP1D1 nanodiscs

Peptic peptide coverage for MSP1D1 in samples of ND-rFAAH was 93.7% (Fig. S1). Coverage of the peptic peptides for undeuterated control samples of rFAAH in solution was 77.9% (data not shown), but only 52.1% coverage was achieved for the deuterium exchange rFAAH in solution sample (Fig. S2). Coverage of the peptic peptides for rFAAH in ND-rFAAH, was only 27.4% (data not shown). Digestion conditions need to be optimized in order to get greater coverage for rFAAH, both in solution, and in the ND system. Additionally, IMAC purification of ND-rFAAH using untagged MSP1D1 may lead to detecting a greater percent coverage for this protein since it would decrease the relative signal intensity coming from MSP1D1.
Supplementary Figures

Fig. S1. Coverage map for MSP1D1 in ND-rFAAH samples.
Fig. S2. Coverage map for rFAAH in solution.
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Chapter 4

Mechanistic Analysis of Human Monoacylglycerol Lipase Inhibition by the Sulfonyl Fluoride-based Inhibitor AM3506
Introduction

The endocannabinoid system comprises receptor proteins (CB1 and CB2), signaling lipids (endocannabinoids -- ECBs), catabolic and anabolic enzymes, transport proteins, and allosteric receptor modulators. The CB1 receptor is the most abundant G-protein coupled receptor (GPCR) in the central nervous system (CNS)\(^{117}\). CB1 is also found, albeit at lower concentrations, in various peripheral tissues. The CB2 receptor is expressed primarily in cells of the immune system, but is also present in the brain, liver, pancreas, and bone\(^{118}\). The two receptors have relatively low sequence homology (about 44% overall -- 68% in the transmembrane “TM” domain\(^{119}\)), and receptor-selective agonists and antagonists have been developed. Both CB1 and CB2 primarily signal through the Ga\(i/o\) pathway (inhibiting adenylate cyclase),\(^{120}\) but signaling also occurs via mitogen-activated protein (MAP) kinase\(^{121}\), and β-arrestin\(^{122}\), as well as G\(\beta\gamma\) regulation of calcium and potassium flux\(^{123}\). Cannabinoid signaling is retrograde in nature. Signaling compounds are synthesized “on-demand” in the postsynaptic neuron, and then signal at presynaptic neurons, thus decreasing neurotransmission from the presynaptic neuron onto the postsynaptic cell (feedback)\(^{124}\). CB receptors also demonstrate biased agonism (a.k.a. functional selectivity), a ligand-dependent selectivity for specific signal transduction pathways\(^{125}\), possibly due to various ligand-bound structural conformations, homo and hetero-multimeric state of the receptor, allosterism, and/or test system-dependent effects. This makes the regulation of the cannabinoid system both complex and fascinating through the perspective of investigating structure-function relationships.

The endocannabinoid metabolome consists of eicosanoid lipids (oxidized 20-carbon fatty acids) in their acid (e.g. arachidonic acid “AA”), ethanolamide (e.g. arachidonoyl ethanolamide - - anandamide “AEA”), monoacil-glycerol derivatized (e.g. 2-arachidonoyl glycerol “2-AG”),
ether (e.g. arachidonoyl glycerol-ether), amino acid conjugated (e.g. arachidonoyl-serine), or hormone-conjugated (e.g. arachidonoyl-dopamine) form. AEA and 2-AG are the primary signaling ligands. AEA is a partial-to-full agonist at CB1 (assay-dependent), and a weak partial agonist at CB2; it is synthesized from phospholipase-D cleavage of N-arachidonoyl phosphatidyl-ethanolamine (NAPE). NAPE is synthesized from dietary lecithin and phosphatidyl-ethanolamine (PE), via enzymatic “N-acyltransferase” activity, by conjugating AA to PE. AEA is degraded rapidly by fatty acid amide hydrolase (FAAH) forming AA and ethanolamine. 2-AG is a full agonist at both CB1 and CB2 receptors, and is synthesized from diacylglycerol (DAG) by DAG lipase (DAGL), which is located in the postsynaptic membrane; 2-AG is catabolized (inactivated) by MGL to form AA and glycerol.

The endocannabinoid system plays a major role in regulating several disease states, including: cancer, stroke, Multiple Sclerosis, Parkinson’s, Huntington’s and Alzheimer’s diseases, amyotrophic lateral sclerosis, epilepsy, schizophrenia, anxiety/depression, insomnia, nausea, drug and alcohol addiction, cardiovascular disease, glaucoma, gastrointestinal/liver disease, arthritis, and osteoporosis to name a few. Regulation of the ECB system directly inhibits the growth, migration, and invasion of cancer cells. These anti-proliferative and anti-metastatic effects make the cannabinoid system an ideal target for drug discovery efforts. Various cannabinoids, including cannabidiol, AEA, 2-AG, and ECB transport inhibitors, also induce apoptotic cell death. Elevated ECB levels due to MGL and/or FAAH inhibition have marked anti-nociceptive, anti-allodynic, and anti-inflammatory effects as well. MGL inhibition specifically limits AA proinflammatory and tumorigenic eicosanoid signaling. As such, MGL inhibitors have a lot of promise for the treatment of inflammatory disorders, and cancer.
Serine hydrolases such as MGL and FAAH comprise the largest, and structurally, and functionally most diverse class of enzymes within the human proteome. These enzymes actuate the catabolism of amide, ester, and thioester bonds in biosignaling molecules, proteins, and xenobiotics. This process is mechanistically dependent upon a catalytic triad (Ser^{122}, His^{269}, and Asp^{239} for MGL, Ser^{241}, Ser^{217}, and Lys^{142} for FAAH^{50}) and proceeds through the formation of a covalent, acyl-enzyme complex intermediate that is subsequently hydrolyzed by a water/hydroxide-induced saponification process that regenerates the catalytic serine. MGL, like other characterized metabolic serine hydrolases, such as acetylcholinesterase (AChE) has the classic α/β-hydrolase fold and the GXSXG consensus motif^{3}. MGL hydrolyses various fatty acid glycerol’s, including 2-AG (the primary signaling lipid of the endocannabinoid metabolome) and regulates a fatty acid network, that can promote cancer cell growth, migration, and invasion.

MGL inhibition has been observed in the presence of hexadecylsulphonyl fluoride (AM374) and phenylmethylsulphonyl fluoride (PMSF)^{130}. Brain Serine Hydrolase activity, as measured by competitive activity based protein profiling (ABPP), also demonstrates that MGL, α-β Hydrolase Domain Containing Enzyme 6 (ABHD6), and FAAH, are completely inhibited by 5-(4-hydroxyphenyl) pentanesulfonyl fluoride (AM3506)^{131}. Direct investigation of serine residues in sulfonylated-enzymes is a challenge, since the sulfonyl group can be rapidly eliminated in the form of a sulfonate anion^{132}. Use of Matrix Assisted Laser Desorption Ionization, Time of Flight, Tandem Mass Spectrometry (MALDI-TOF MS/MS) is valuable for examining the mechanism of inhibition of these enzymes by sulphonyl fluorides. In the present study, we characterized AM3506-inhibited serine hydrolases using a comprehensive mass spectrometric analysis.
Results

Covalent inhibition of hMGL, H121S hMGL, and rFAAH by AM3506

AM3506 reacts stoichiometrically with hMGL to generate an inactive, monosulfonyl enzyme product. Using sensitive, fluorescence-based assays, AM3506 inhibits hMGL in the nanomolar range with an IC50 of 236 nM (Fig. 1). Maximal hMGL, H121S hMGL (data not shown), ΔTM-rFAAH, and MBP-hFAAH inhibition by AM3506, at a fixed substrate concentration of 20 µM AHMMCE or AAMCA, is obtained at a compound to enzyme molar ratio of 10:1.

Intact mass analysis of hMGL inhibited by AM3506

Following the determination that AM3506 inhibits hMGL, LC-TOF mass spectrometry was used to confirm the attachment of the covalent ligand to hMGL. The spectrum of inhibitor-free sample shows two prominent peaks with average masses of 34,123.6 Da (calculated 34,123.2 Da) and 34,179.4 (+56 Da addition that represents hMGL with one divalent cation equivalent, from the affinity resin in purification) (Fig. 2a). The average intact mass of AM3506-treated hMGL is 225.2 Da greater than that of the unmodified enzyme, a result consistent with enzyme sulfonylation by AM3506 and the consequent calculated mass increase of 226.2 Da (Fig. 2b). These data suggest that AM3506 interacts stoichiometrically with hMGL, producing the monosulfonyl enzyme. Samples of hMGL incubated with AM3506 at pH 12.0 show a new peak with an average mass of 34,161.4 Da (Fig. 2c). This new peak is 18 Da less massive than the expected 34,179.4 Da peak. This modification is characteristic of a β-elimination reaction following the sulfonylation of the catalytic serine (Scheme 1).
MALDI-TOF analysis of hMGL and H121S hMGL inhibited by AM3506

To pinpoint the specific sulfonylation site, AM3506-inactivated hMGL samples were subjected to overnight, in-solution trypsin digestion and the tryptic peptides were analyzed by MALDI-TOF MS. A comparison of the spectra of the tryptic digest from native and AM3506-treated hMGL reveal (in the latter) a new peptide at \( m/z \) 5,233.11 with mass 18 Da less than the peptide containing the catalytic serine at \( m/z \) 5,251.10 (DYPGLPVFLGHSMGGAIALTAERPGHFAGMVLISSLVLAPESATTFTKVALK -- position 117-172) (Fig. 3a). The 18 Da decrease was provisionally attributed to a \( \beta \)-elimination reaction, which led to the conversion of sulfonyl serine to dehydroalanine. Intermediate peptides attributable to sulfonylation of the catalytic serine (+226.2 mass increase) were not observed by MALDI-TOF analysis. Because there are three serine residues in the tryptic fragment, tandem MS was performed on both the modified and unmodified ions to determine the exact serine residue modified by AM3506. The MS/MS spectra of the \( m/z \) 5251.10 and \( m/z \) 5,233.11 ions derived from trypsin digests of untreated, and AM3506-treated hMGL, are shown (Fig. 3b). MS/MS spectral optimization was complicated by the high molecular weights involved, as well as the low intensity of the peptides in question. However, Ser\(^{122}\) is unambiguously detectable, as the residue that was converted to dehydroalanine.

It can be hypothesized that an alkaline microenvironment, provided by the Ser\(^{122}\)-adjacent His\(^{121}\), is essential for this \( \beta \)-elimination reaction. To examine this, the mutant H121S hMGL was expressed and purified. This mutant retained catalytic activity comparable to that of the wild type and was inhibited by AM3506 (Fig. 4a). H121S hMGL was treated with AM3506 at a compound to enzyme molar ratio of 10:1, and then subjected to overnight trypsin digestion. The MS spectra of the tryptic digest from untreated H121S hMGL and AM3506-treated H121S hMGL are
virtually identical; the 18 Da lighter peak is absent in the latter case (Fig. 4b). This experiment demonstrates that His$^{121}$ plays an essential role in the β-elimination reaction.

**MALDI-TOF MS analysis of the Michael addition reaction**

If the β-elimination reaction converted Ser$^{122}$ to a dehydroalanine residue, activated thiols would act as Michael donors, creating specific hMGL thio-conjugates. In this proof-of-concept experiment, tryptic digests of hMGL, and H121S-hMGL, pretreated with AM3506 were incubated with either thiophenol or beta-mercaptoethanol (βME). Each sample was then subjected to MALDI-TOF analysis. A comparison of the spectra from the tryptic digest of either untreated and thiophenol-treated hMGL revealed that the latter contains a new, modified peptide with a 92 Da increase in mass (m/z 5,342.8 Da) (Fig. 5a) that is consistent with the addition of thiophenol to the dehydroalanine via the Michael addition reaction. Also, the spectra of βME-treated samples revealed a new peptide with a 60 Da increase in mass (m/z 5,310.74 Da) that is consistent with the addition of βME to dehydroalanine via Michael addition (Fig. 5b). The successful conjugate addition confirms the formation of a dehydroalanine residue in hMGL, treated with AM3506, and this transmuted residue’s availability for nucleophilic addition.
Conclusions

We have characterized the molecular mechanism of hMGL’s inhibition by AM3506 through a comprehensive mass spectrometric (MS) analysis. The reaction of AM3506 with both MGL and FAAH exhibited a “one–to-one” stoichiometry. We showed that AM3506-based inhibition of MGL occurs through sulfonylation of the catalytic serine (Ser$^{122}$), followed by a His$^{121}$ mediated β-elimination reaction that resulted in desulfonylation of the inactivated enzyme and the subsequent formation of a dehydroalanine$^{122}$ residue. To confirm the formation of dehydroalanine, a Michael acceptor, we performed conjugate addition (Michael reaction) using the activated thiols, thiophenol and betamercaptoethanol as nucleophiles. A comparison of the spectra of the tryptic digest from untreated and thiol-treated hMGL confirmed the addition of these thiols to dehydroalanine$^{122}$. Additionally, intact hMGL pretreated with AM3506, was incubated with thiophenol after adjusting the pH to 12.0 to catalyze dehydroalanine formation, and the intact mass was analyzed by LC-MS. The spectrum showed a peak with an average mass of 34215.2 Da (+91.6 Da), a mass consistent with the formation of S-phenyl cysteine and the consequent calculated mass increase of 92.1 Da (Supplementary Data, Fig S1)

To provide evidence that His121 is essential in directing the desulfonylation via β-elimination mechanism, we expressed and purified mutant H121S hMGL and treated it with AM3506. We did not observe any new peptides attributable to the β-elimination mechanism. Similarly, we did not observe any peptides attributable to β-elimination in AM3506-treated fatty acid amide hydrolase (FAAH), a serine hydrolase enzyme that has no histidine preceding the catalytic serine (Supplementary Data, Fig. S2, Fig. S3, and Fig. S4). We attempted to analyze a rFAAH I250H mutant in the attempt to induce β-elimination, but unfortunately this mutant was catalytically silent (data not shown). LC-MS/MS analysis of this mutant showed no difference in
the 221-251 peptide upon incubation with AM3506 (Supplementary Data, Fig S5a). In the rFAAH sample that was treated with AM3506, no β-elimination occurred because the mutant did not interact with AM3506 (Supplementary Data, Fig S5b).

In general, desulfonylation of a sulfonyl enzyme can be achieved by three different mechanisms: (1) direct displacement of the sulfonate anion by a nucleophile\textsuperscript{132} 2) an intramolecular cyclization reaction that leads to an oxazoline ring\textsuperscript{133}, or 3) β-elimination to form dehydroalanine\textsuperscript{134}. FAAH clearly undergoes the first process since the catalytic serine residue is regenerated on desulfonylation. Intramolecular cyclization requires complete departure from the ridged structure of the peptide. While this is possible during trypsin digestion, in no case was the presence of an oxazoline ring detected. The β-elimination mechanism has only been observed in intact proteins for p-toluenesulfonyl chymotrypsin in 0.1 N NaOH (pH ≥13.0). Our discovery that the path of desulfonylation in hMGL occurs via β-elimination during overnight trypsin digestion (pH= 8.0) due to a specific histidine residue outside of the catalytic triad presents a selective approach for serine hydrolase modification at the catalytic residue that confers new function without genetic manipulation. We believe that H\textsuperscript{121} creates a microenvironment surrounding the catalytic Ser\textsuperscript{122} in which the pH increases; and as a result, elimination is favored over substitution\textsuperscript{21}. Also, we show that increasing the pH to 12.0 causes the intact monosulfonylated hMGL to undergo β-elimination quickly (in less than 1hr), which results in the formation of dehydroalanine.

Nucleophilic additions to the α,β-unsaturated dehydroalanine residue by different nucleophiles demonstrates a novel, selective approach for serine hydrolase modification. Fluorogenic probes can now be designed using this paradigm to target catalytically active serine
hydrolases. The ability to confer new functions on MGL specifically can enable us to study this potential cancer-cell biomarker, and provide a greater understanding of its function within a global, context.
Materials and Methods

Materials

Culture media, Bio-Spin™ P-6 columns and SDS-PAGE supplies were purchased from Bio-Rad (Hercules, CA). MS-grade trypsin (Trypsin Gold) was from Promega (Madison, WI). AM3506 and AHMMCE were synthesized at the Center for Drug Discovery, Northeastern University.

Methods

Expression and purification of hMGL and H121S hMGL

Expression and purification of hMGL and the histidine-to-serine single mutant human MGL (H121S hMGL) followed a similar procedure as reported by Zvonok et al. A single E. coli BL21 (DE3) colony, containing either pET45His6hMGL or pET45His6H121ShMGL plasmid, was used to inoculate 20 mL of Luria broth (LB) medium containing ampicillin (100µg/mL) and was grown overnight at 37°C with shaking (~250 rpm). The following day, 10 mL of the overnight culture was used to inoculate 1 L of fresh Luria broth-ampicillin and allowed to grow until the culture reached an OD₆₀₀ of 0.6. Protein expression was induced with isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and the temperature lowered to 33°C. Following a 4 hr. induction, the cells were collected by centrifugation at 5000 x g for 10 min at 4°C and stored at ~80°C. For purification, the frozen pellet was thawed on ice, re-suspended in lysis buffer (50 mM Tris, 100 mM NaCl, 1% Triton X-100, pH=8.0) and sonicated on ice. The cell lysate was then centrifuged at 30,000g for 30 min at 4°C. The supernatant was added to 0.5 mL (bed volume) of Cobalt-NTA resin (Clonetech) pre-equilibrated with lysis buffer and mixed for 1 hr. at 4°C. The suspension was transferred to a gravity-flow column and washed with 20 mL lysis buffer, then with 10 mL of lysis buffer
containing 10 mM imidazole. Finally, hMGL was eluted with 3 mL of lysis buffer containing 250 mM imidazole in 0.5 mL fractions, and the purity was checked with SDS-PAGE.

**Interaction of AM3506 with hMGL and H121S hMGL**

A solution containing purified hMGL or H121S hMGL (50μl of 5 μM) in lysis buffer was incubated with AM3506 (in DMSO) at a compound-to-enzyme molar ratio of 10:1 at room temperature for 1 hr. In control samples, DMSO was added to the enzyme solution instead of AM3506. An aliquot of each mixture was used to check activity after 1 hr. using the fluorescent assay method (described below). The reaction was terminated by desalting with a Bio-Spin 6 column in 25 mM ammonium bicarbonate buffer containing 0.05% CYMAL (pH 8.0). The sample was digested with 200 ng of Trypsin Gold (Promega, Madison, WI) overnight at 37°C.

**Assay of hMGL and H121S hMGL inhibition using AHMMCE**

An in-house fluorometric assay\textsuperscript{135} was used to evaluate the hMGL and H121S hMGL inhibition by AM3506, after incubation with AM3506 for 1 hr. at room temperature. MGL activity was monitored by the hydrolysis of the fluorogenic substrate: 7-hydroxy-6-methoxy-4-methylcoumarin ester (AHMMCE) to form the fluorescent product 7-hydroxy-6-methoxy-4-methylcoumarin (HMMC). hMGL (30 ng) and AHMMCE (final concentration 20 μM) were added to a total volume of 200 μL in assay buffer (50 mM Tris-HCl, pH 7.4) in a 96-well plate. The reaction was allowed to proceed for 3 hr. at 25°C. Fluorescence readings were taken every 15 minutes at 360 nm/460 nm (\(\lambda_{\text{excitation}}/\lambda_{\text{emission}}\)) on a BioTek Synergy HT Microplate Reader (BioTek Instruments, Winooski, VT).
Intact mass analysis of AM3506-treated hMGL

A Waters LCT-Premier mass spectrometer was used to determine the intact mass of AM3506-treated hMGL samples. The instrument was calibrated with 500 fmol/mL horse myoglobin (Sigma-Aldrich) before and after each run. Instrument conditions were as follows: source temperature, 80°C; desolvation, 175°C; capillary voltage, 3200 V; and cone voltage, 35 V. Dialyzed, detergent-free hMGL in buffer A (50 mM Tris, 100 mM NaCl, pH 8.0) were incubated with AM3506 at a compound to protein molar ratio of 10:1. After 1 hr. of incubation at pH 8.0, samples (200 pmol) were injected onto a self-packed POROS 20 R2 2 mm x 20 mm trap column which traps the protein but allows buffer salts through. Samples were manually desalted with an appropriate (>5 x the volume of the sample) volume of 0.1% formic acid in H2O. The desalted protein was then eluted from the column with a 15-75% gradient of acetonitrile containing 0.05% TFA at flow rate 50 µL/min in 4 min. The intact mass of AM3506-treated hMGL was also determined after adjusting the pH of buffer A to 12.0.

MALDI-TOF MS analysis of tryptic digests (AM3506 hMGL and H121S hMGL)

MS data were obtained using a 4800 MALDI TOF/TOF instrument (Applied Biosystem, Framingham, MA) equipped with a 200 Hz solid-state ultraviolet laser (wavelength 355 nm). Samples were crystallized on a 384-well MALDI plate (Opti-TOF™ 384 Well Insert, Applied Biosystems) by spotting 0.5 µL tryptic digest and 0.5 µL α-cyano-4-hydroxycinnamic acid matrix (5 mg/mL in 50% water/50% acetonitrile/0.1% trifluoroacetic acid). The digested samples were diluted 5-fold and 10-fold with α-cyano-4-hydroxycinnamic acid matrix solution and 1 µL of the diluted samples were spotted for analysis. External calibration of the spectra was carried out by using a mixture of 6 calibrants with masses (MH+) of 904.468, 1296.685, 1570.677, 2093.086, 2465.198, and 3657.929 corresponding to des-Arg1-bradykinin, angiotensin I, glu-
fibrino peptide, ACTH (fragment 1–17), ACTH (fragment 18–39), and ACTH (fragment 7–38) respectively. For data analysis, the monoisotopic peaks were compared with the theoretical molecular weights corresponding to the expected tryptic peptides. MS-digest (University of California, San Francisco, CA) was used to calculate the theoretical molecular weights of the expected peptides after digestion. The maximum error limit was set to 80 parts per million (ppm).

**Additions of thiols to the tryptic digest samples of hMGL and H121S hMGL**

Tryptic digest samples of hMGL and H121S hMGL (40 μL, 120 μM) pretreated with AM3506 were incubated with either thiophenol, or beta-mercaptoethanol (βME) at a thiol to MGL molar ratio of 2:1. The incubation was performed at 37°C for 30 min. For the thiophenol sample, the pH was adjusted to 8.0 and for βME sample, the pH was adjusted to 12.0. Samples were directly spotted on a 384-well MALDI plate (Opti-TOF™ 384 Well Insert, Applied Biosystems) and crystallized by adding α-cyano-4-hydroxycinnamic acid matrix as described above.

**Addition of thiophenol to sulphonylated intact hMGL**

Samples of hMGL (50 μL, 120μM) pretreated with AM3506 were incubated with thiophenol at a thiophenol to MGL molar ratio of 2:1. The incubation was performed at room temperature for 1 hr. at pH 8.0. After incubation, samples (200 pmol) were injected onto a self-packed POROS 20 R2 2 mm x 20 mm trap column. Samples were manually desalted with 0.1% formic acid in H2O. The desalted protein was then eluted from the column with a 15-75% gradient of acetonitrile containing 0.05% TFA at flow rate 50 μL/min in 4 min.
Scheme 1: Proposed mechanism of hMGL inactivation by AM3506.
The path of desulfonylation outlined above is consistent with the MS data.
Fig. 1. Inhibition curves for serine hydrolases using AM3506.
a) MBP-hFAAH hydrolysis of AAMCA substrate. b) ΔTM-rFAAH hydrolysis of AAMCA substrate. c) hMGL hydrolysis of AHMMCE substrate. d) Structure of AM3506.
Fig. 2. LC-TOF ESI-MS analysis of hMGL enzyme masses.

a) Intact mass of unmodified hMGL at pH 8.0. b) Intact mass of AM3506-treated hMGL at pH 8.0. c) Intact mass of AM3506-treated hMGL, incubated at pH 12.0 for 1 hr. at RT.
Fig. 3. MS/MS analysis of hMGL inactivation by AM3506.

a) MALDI-TOF MS mapping of the tryptic digest for purified hMGL. Top: a unique tryptic peptide (m/z 5233.1) in AM3506-treated hMGL (18 Da lighter when compared to the respective unmodified peptide). Bottom: high-resolution spectra of the precursor ion for the unmodified peptide (m/z 5251.6) that contains the catalytic serine.

b) MALDI-TOF MS/MS analysis identifying Ser\textsubscript{122} as the modified residue. Top: unmodified peptide. Bottom: AM3506-treated peptide.
Fig. 4. H121S hMGL inhibition by AM3506.
a) The activity of H121S hMGL as a function of time before and after treatment with AM3506.
b) MALDI-TOF MS analysis of AM3506-inhibited H121S hMGL. Top: mass spectra of the tryptic peptide containing the catalytic serine. Bottom: The peptide remains unmodified following AM3506 treatment.
Fig. 5. MALDI TOF MS analysis of the conjugate addition of thiophenol and β-mercaptoethanol (βME) to AM3506-treated hMGL.

a) hMGL treated with AM3506 only. b) hMGL treated with AM3506 followed by the addition of thiophenol. c) hMGL treated with AM3506 followed by the addition of βME.
Supplementary Data

Expression and purification of ΔTM rFAAH

The transmembrane-deleted rat FAAH (ΔTM rFAAH) was expressed in E. coli cells and purified using a procedure adapted from Patricelli et al21. Briefly, 1L of LB medium was inoculated with 10 mL of the overnight culture. Cultures were then induced with IPTG (1mM) at an OD₆₀₀ of 0.6-0.8 for 4 h and pelleted at 5000 rpm for 10 min. For purification, the cells (3g) were thawed on ice, resuspended in lysis buffer (50 mM Tris, pH 8.0 containing 100 mM NaCl, and 1% Triton X-100) and lysozyme was added to a final concentration of 1 mg/mL. The lysate was then sonicated and centrifuged at 10,000 x g for 35 min at 4 °C. The supernatant was added to 0.5 mL (bed volume) of pre-equilibrated cobalt-NTA beads and mixed for 1 hr. at 4 °C. The suspension was transferred to a gravity-flow column and washed with 20 mL lysis buffer, then with 10 mL of lysis buffer containing 10 mM imidazole, and then to 10 mL of lysis buffer containing 30 mM imidazole. ΔTM rFAAH was eluted with 4 mL of lysis buffer containing 250 mM imidazole. The elution was transferred directly to a heparin column pre-equilibrated with imidazole elution buffer. The heparin column was washed with 20 mL of imidazole elution buffer, 20 mL of wash buffer 1 (20 mM HEPES (pH 7.8), 1 mM EDTA, 10% glycerol and 0.5% CHAPS) with 150 mM NaCl, and 20 mL of wash buffer 1 with 300 mM NaCl. ΔTM FAAH was eluted with 5 mL of buffer 1 with 700 mM NaCl. The elution fractions were desalted and concentrated using Millipore Ultra centrifugal filter tubes (10K cut off membrane). The desalted samples were then loaded into Sephacryl S-200 high resolution gel filtration columns equilibrated in (20 mM HEPES (pH 7.8), 150 mM NaCl, 1mM EDTA, and 0.1% LDAO) at a flow rate of 0.5 mL/min. Eluted fractions were concentrated and analyzed by SDS-PAGE to confirm purity.
Interaction of AM3506 with ΔTM rFAAH

A solution (50 µL, 4 µM) containing purified ΔTM FAAH in lysis buffer was incubated with AM3506 at a compound:enzyme molar ratio of 2:1. The incubation was conducted at room temperature for 1 hr. Control samples (DMSO was added to the enzyme solution instead of the test compound) were prepared in parallel. An aliquot of each mixture was used to evaluate activity after 1 hr. using the fluorescent assay method as described previously. The incubations were terminated by desalting with a Bio-Spin 6 column in 25mM ammonium bicarbonate buffer containing 0.05% CYMAL (pH 8.0), and the samples were subjected to digestion with 200 ng of Trypsin Gold (Promega, Madison, WI) overnight at 37 °C.

Assay of ΔTM FAAH inhibition using AAMCE

The ΔTM FAAH inhibition by AM3506 was checked after incubation with AM3506 for 1 hr. at room temperature. ΔTM FAAH activity is monitored by the hydrolysis of the fluorogenic substrate N-arachidonoyl, 7-amino-4-methylcoumarin amide (AAMCA) to form the fluorescent product, 7-amino-4-methylcoumarin (AMC). Briefly, the assay was performed in a 96-well format in 50 mM HEPES, 1 mM EDTA, 0.1% BSA, pH 7.4 buffer with ΔTM FAAH alone and ΔTM FAAH that has been incubated with AM3506. AAMCA was then added to a final concentration of 10 µM in a total volume of 200 µl. The incubation was continued for 4 hr., during which fluorescence readings at 360 nm/460 nm (λexcitation/λemission) were taken on a BioTek Synergy HT Microplate Reader (BioTek Instruments, Winooski, VT) every 20 min.

LC/MS ESIMS Intact mass analysis of AM3506-treated ΔTM rFAAH

Waters LCT Premier mass spectrometer was used to determine the intact mass of AM3506-treated ΔTM FAAH samples. Instrument conditions were as follows: source
temperature, 80 °C; desolvation, 175°C; capillary voltage, 3200 V; and cone voltage, 35 V. Dialyzed, detergent free ΔTM FAAH solutions were incubated with AM3506 at a compound:protein molar ratio of 2:1. After 1 hour of incubation, samples (200 pmol of protein) were injected onto a 2mm x 20mm trap column containing self-packed POROS 20 R2 which traps the protein but allows buffer salts through. Samples were manually desalted with an appropriate (>5x the volume of the sample) volume of 0.1% formic acid in H2O. The desalted protein was then eluted from the column with 15-75% acetonitrile containing 0.05% TFA over 4 min. All samples were calibrated with infusing 500 fmol/mL myoglobin at 10ul/min at the end of each run.

**MS/MS Analysis of rFAAH I250H Tryptic Peptides**

LC-MS/MS analysis of rFAAH I250H tryptic peptides was performed with a Waters Synapt G2 mass spectrometer coupled to a Waters nanoACQUITY UPLC System. Separation of the peptides was carried out on a Waters ACQUITY UPLC HSS T3 column (1.0 x 50 mm, packed with 1.8 µM silica particles). The sample, 100 pmol of rFAAH I250H in 100 mM NH₄CO₃ and 0.05% CYMAL-6 detergent, was diluted up to 50 µL in water and injected onto the column. The sample was desalted on Waters ACQUITY UPLC BEH C18 VanGuard Pre-column (packed with 1.7 µM BEH particles) over 3 min with 95% water and 5% acetonitrile at 60 µL/min. Mobile phase A (water and 0.1% formic acid) and B (acetonitrile and 0.1% formic acid) were applied as a gradient (8% B to 40% B over 6 min, then 40% B for 1 min, then 40% B to 85% B over 30 seconds, then 85% for 1 min, then 85% B to 5% B over 30 seconds, then 5% B for 3 min) over 12 min at a flow rate of 60 µL/min. MS scans were acquired from m/z 50 to 1990. Peptides were identified using Waters software (Protein Lynx Global Server 2.5).
Assignment of Trypsin Digested Peptides from rFAAH I250H

Acquired spectra were searched by Protein Lynx Global Server 2.5 using the sequence of rFAAH I250H. Peptides were further filtered using Waters software DynamX. Peptides could not have an error greater than 20 ppm, must contain a minimum of two consecutive amino acid fragments and at least 0.2 products/aa. Peptides belonging to rFAAH I250H that were treated with inhibitor were searched with a side chain dehydration variable modifier on.
Supplementary Figures

**Fig. S1**: LC-ESI MS analysis of hMGL enzyme masses at pH 8.0.

a) The intact mass of unmodified hMGL.  
b) intact mass of AM3506-treated hMGL.  
c) Intact mass analysis of AM3506-pretreated hMGL that was incubated with thiophenol after adjusting the pH to 12 to catalyze dehydroalanine formation. The analysis of C shows the covalent attachment of AM3506; an addition of 226.1 Da to the total mass of hMGL that is consistent with enzyme sulfonylation. There is a peak with an average mass of 34,215.2 Da (+91.6 Da), a mass consistent with the direct replacement of sulfonyl group with thiophenol. (* indicates Co^{++}-6-His adduct formed during purification)
Fig. S2. Coomassie-stained SDS-PAGE gel for purified, hexa-histidine-tagged ΔTM rFAAH
Fig. S3. ΔTM rFAAH inhibition by AM3506.
a) The activity of ΔTM rFAAH as a function of time before and after treatment with AM3506. b) LC-ESI MS analysis of intact masses of unmodified (top) and AM3506-treated ΔTM rFAAH (bottom). The analysis shows the covalent attachment of AM3506; an addition of 226 Da to the total mass of ΔTM rFAAH consistent with enzyme sulfonylation.
Fig. S4: MS analysis of ΔTM rFAAH inhibition by AM3506.
a) MALDI-MS fingerprint of a tryptic digest of purified ΔTM rFAAH only. b) Tryptic digest of purified ΔTM rFAAH that was treated with AM3506. The analysis shows no difference between the two preparations; we did not observe any peptides with (-18 Da or +226 Da) mass changes in the AM3506-treated sample. The unmodified precursor ions at m/z 2670.2 corresponds to the tryptic peptide SPGGSSGEGALIGSGSPGLGLGTGDSIR (position 221-251) which contains the catalytic serine (Ser249) are circled.
Fig. S5: LC-ESI MS analysis ΔTM rFAAH I250H

a) MS and MS/MS spectra of the 221-251 tryptic peptide in the +3 charge state for rFAAH I250H mutant.
b) MS and MS/MS spectra of the 221-251 tryptic peptide in the +3 charge state for rFAAH I250H in the presence of the AM3506 inhibitor.
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Chapter 5

Synthesis and Characterization of a Novel, Theranostic, Click-Enabled Nanoplatform
Introduction

Nanomedical agents, such as gold nanoparticles (AuNPs), comprise a dynamic class of materials with \textit{in vitro}/\textit{in vivo} and clinical (ClinicalTrials.gov Identifier: NCT01436123, and NCT01270139) applications. Nanoparticles exist in a physical state between that of bulk material and a single molecule. In this transitional state, surface, and quantum-mechanical properties can be “tuned” simply by altering particle size and shape. Nanoparticles can be synthesized to present a unique combination of magnetic, chemical, and optical properties, which can be optimized for biomedical applications. Using click chemistry, MGL and FAAH inhibitors can be conjugated to these therapeutic, and diagnostic imaging “nanoplatforms,” which can then be utilized for hyper-thermal ablation therapy, and MRI contrast enhancement in real time. Coupling endocannabinoid-hydrolyzing enzyme inhibiting medications to this platform in this way would expand the field from palliative, to direct antineoplastic treatment with these agents.

Herein we synthesize and characterize a multi-lamellar nanoplatform based on the particles synthesized by Melancon \textit{et al.} for imaging, drug delivery, and therapeutic applications. These “fLPA-SPIO@AuNS” particles are comprised of a superparamagnetic iron oxide SPIO core (Scheme 1a), which was chosen to enable deep tissue magnetic hyperthermia, MRI contrast enhancement, and the magnetic targeting/trapping of these particles. Localized hyperthermia (or thermal ablation therapy) can be used to kill cancer cells by coagulating intracellular proteins (inducing apoptosis) and destroying nearby blood vessels, while leaving healthy, non-tumor cells intact. SPIO nanoparticles will produce localized heating when exposed to an alternating current magnetic field, which can be produced in an MRI instrument. The mechanism by which hyperthermia (temperature: 42–45 °C) and hyperthermic
Ablation therapy (temperature: >50 °C) proceeds is through subsequent increased immunologic response (increased levels of peripheral blood CD4+ T cells, and serum IL-2). These particles also act as T1 and T2, contrast enhancing imaging agents in the MRI, which allows for better imaging of tumor borders, and allows for real time tracking of treatment efficacy. These particles can also be directed to, and or confined within, tumor sites, with use of an externally applied magnetic field. When the particles enter the field, the tumor cells are unable to clear them, leading to highly precise, targeted treatments.

The dielectric SiO2 that makes up the second layer of the fLPA-SPIO@AuNS (Scheme 1a) enhances the surface plasmon resonance (SPR) of the gold shell that was designed for biocompatibility, facile bioconjugation, and both photothermal and imaging capabilities. SPR in gold nanoshells is the vibrational oscillation of surface electrons, stimulated by monochromatic light. In smaller particles, light in the blue-green spectrum is absorbed, while red light is reflected. As particle size increases, however, SPR absorption redshifts to longer wavelengths, eventually landing in the infrared (IR) spectrum of 700 to 1,000 nm. The excitation wavelength can be further tuned by varying the thickness of the gold shell. This SPR phenomena leads to localized heating, which can be used to hyper thermally ablate solid tumors. Infrared light can penetrate tissue to a depth of 1-2 cm, making this therapy useful for the treatment of superficial, solid tumors.

Gold nanoshells can be functionalized with dihydrolipoic acid (LPA), since the dithiol readily adsorbs onto gold surfaces. This can be used to facilitate conjugation of targeting, therapeutic, or imaging agents to the fLPA-SPIO@AuNS platform (Scheme 1b). Therefore, a modified LPA, the product of the conjugation of lipoic acid and propargyl amine (using N, N’-Dicyclohexylcarbodiimide (DCC) as the dehydration agent) can be used to present a functional
alkyne moiety on the surface of these particles for azide-alkyne Huisgen cycloaddition\textsuperscript{168} (‘click’ addition) of azido-functionalized entities. In this case, conjugation of a novel, azido-functionalized MGL inhibitor could be used to target metastatic breast cancer cells.\textsuperscript{2} In this case, the fLPA-SPIO@AuNS MGL inhibitor would act as both a targeting ligand, and an anti-metastatic agent. Presented here is the complete synthesis, monitored using transmission electron microscopy (TEM), dynamic light scattering spectroscopy (DLS), and nuclear magnetic resonance imaging (MRI), of fLPA-SPIO@AuNS particles.
Results and Discussion

TEM imaging to monitor particle synthesis

Hydrophobic superparamagnetic iron oxide nanoparticles (SPIONs) (core particles – Scheme 1) were synthesized using a co-precipitation method. TEM images of these particles showed them to be disperse, and ranging in size from around 5 to 15 nm, with an average diameter of 10 nm (Fig. 1). These particles were of sufficient stability to store at 4 °C for future use in nanoplatform development. The core particles were highly attracted to a neodymium magnet, and would disperse in cyclohexane when the magnet was removed. Upon lyophilization, the particles did not appear to oxidize (no change in color) and the NP powder could be resuspended easily in cyclohexane.

The hydrophobic SPIONs were then coated in SiO₂ using the Stöber process and a monolayer of SiO-NH₂ was grown on the surface of these particles to facilitate seeding with 2 nm gold nanoparticles. TEM micrographs showed a clear core-shell morphology (Fig. 2top). Particles ranged in size from 35 to 45 nm and had an average diameter of 40 nm (Fig. 2). After adding THPC stabilized 2 nm gold nanoparticles to the now hydrophilic population of particles in solution, seeding was clearly evident on the surface of the particles (Fig. 2bottom).

Dynamic light scattering analysis particle synthesis

For the hydrophilic nanoparticles, dynamic light scattering (DLS), a nondestructive analysis technique, was used to determine the hydrodynamic size of these particles. In DLS imaging, a coherent, monochromatic light source (of a large wavelength relative to the particle size to be measured) is used to measure particle motion in solution. This motion (beam crossing) is then filtered through an autocorrelation function which is subsequently deconvoluted to solve for the particle’s diffusion coefficient, which is a function of particle-in-solution hydrodynamic
size. The intensity weighted chromatograms from the silica coated SPIONs (SC-SPOINs) showed a hydrodynamic size of 135.8 nm with a polydispersity index (PDI) of 0.005 (Fig. 3a). This differs greatly from the physical size of these particles as measured by TEM, and suggests these particles have a layer of water that attaches to the particles’ surface and moves with them in aqueous solution. This interpretation is consistent with the hydrophilicity of the surfaces of these particles. After seeding, the particle hydrodynamic size was measured 136.6 nm with a PDI of 0.005 (Fig. 3b). As such, no significant change in particle hydrodynamic size, or polydispersity was detectible by DLS after particle seeding.

Following fLPA-SPIO@AuNS formation with various surfactant ratios, and solvents, a distinct change in hydrodynamic size was evident. For fLPA-SPIO@AuNSs formed with a 2/1 ratio of LPA to SH-PEG in water, a 93.9 nm hydrodynamic size was measured with a PDI of 0.030 (Fig. 4). For fLPA-SPIO@AuNSs formed with a 1/1 ratio of LPA to SH-PEG in water, a 111.2 nm hydrodynamic size was measured with a PDI of 0.005 (Fig. 4). For fLPA-SPIO@AuNSs formed with a 1/1 ratio of LPA to SH-PEG in 50% DMSO(aq), a 138.8 nm hydrodynamic size was measured with a PDI of 0.005 (Fig. 4). Many factors can contribute to the size of gold nanoshells, but in this case the surfactant LPA appears to be the determining factor. It is known that surfactants can stabilize nanoparticles, but they also mould the size and shape of the particles. From these results, it appears that LPA has higher solubility in DMSO than on the surface of SPIO@AuNS particles. As such, the more LPA available to react with the particles, the smaller the particles hydrodynamic size will be. Another interpretation of these results could be that the hydrophilicity of the surface changes, but the particle size is unaffected. This interpretation does not reconcile the visible color change in the fLPA-SPIO@AuNS
solutions, a clear sign of differing particle sizes and differing optical properties of these populations.

**T2 contrast enhancement as measured by MRI**

SC-SPIONs of varying concentrations from 1 nM to 1 mM were analyzed for T2 contrast enhancement against agar phantoms using nuclear magnetic resonance imaging (MRI) (Scheme 2). Slices were analyzed for T2 enhancement with variable contrast (Fig. 5a). T2 values decreased in a dose-dependent manor as expected for SPIONs, becoming significant in the µM concentration range (Fig. 5b). Changes in T1 relaxivity were not significant, but became pronounced for the 1 mM concentration (data not shown). Imaged slices of samples containing µM concentrations of SC-SPIONs showed clinically relevant contrast enhancement (Fig. 5c), similar to that of Feridex®, an FDA approved contrast agent. Relevant concentrations were likely much lower, perhaps in the high nM range, since the concentration estimations used here assumed no loss of iron in the synthesis of SC-SPOINs. From the TEM images, it would appear that small and large iron oxide particles were not incorporated in the SC-SPIONs, so some loss would be expected. Further study is required to determine the intracellular concentration of fLPA-SPIO@AuNS particles necessary to image small neoplasms with MRI, but this study has provided a proof of concept for this technique.
Conclusions

In this study, a novel nanomedical agent was synthesized and characterized. This fLPA-SPIO@AuNS nanoplatform could be coupled to a targeting moiety, such as an MGL or FAAH inhibitor, in order to target this diagnostic imaging, and therapeutic agent to aggressive breast cancer cells. The synthesis of the core SPIO particles was characterized by TEM. As predicted, hydrophobic, magnetic nanoparticles with an average diameter of 10 nm were synthesized. These particles were then coated with silica, and functionalized with a monolayer of NH₂-silica. SC-SPIONs with an average physical diameter of 40 nm, and an average hydrodynamic diameter of ~140 nm were then seeded with 2 nm gold to provide nucleation sites for gold shell growth. This seeding did not change the hydrodynamic diameter of the particles, and this process was monitored by TEM and DLS. Subsequent growth of the gold shell was regulated by surfactant LPA ratio and availability for binding. A greater amount of LPA lead to thinner shell formation. Additionally, these particles acted as T2 contrast enhancing agents in the MRI. Development of the fLPA-SPIO@AuNS platform opens the door to a plethora of targeting, therapeutic, and diagnostic imaging agents that could be bioconjugated via click chemistry to this nanomedical agent. Additionally, by adjusting the size of this platform, the optical and chemical properties of this drug can be tuned for specific applications. Due to the size of these particles, even in the absence of additional targeting agents, bioaccumulation in tumor cells would be expected due to the enhanced permeability and retention (EPR) effect for nanoparticles under 200 nm in physical and hydrodynamic size. This makes the fLPA-SPIO@AuNS platform an attractive launchpad for future drug discovery and development.
Materials and Methods

Materials

Standard laboratory chemicals and buffers were purchased from Fisher Chemical (Pittsburgh, PA) and Sigma Chemical Co. (St. Louis, MO).

Methods

SPIO Synthesis via modified co-precipitation method

5 mL of 0.1 M FeCl$_2$ was mixed with 30 mL of 0.1 M FeCl$_3$ in a round bottomed flask equipped with a temperature probe, and stirred for 20 min in a chemical hood, at which point 100 mg of oleic acid was added. The solution was heated to 50°C, and 3 mL of 5 M NH$_4$OH was added drop wise. Heating was continued until a temperature of 80°C was reached; then this temperature was maintained for 30 min. The clear, pale, yellow-green solution turned brown-black, indicating the formation of iron oxide nanoparticles. The sample was then cooled to room temperature, and MNPs were isolated using a small, neodymium magnet. Magnetic particles were then suspended in cyclohexane.

Silica coating of SPIO nanoparticles

In a 20 mL scintillation vial, 60 μL of concentrated, aqueous ammonia, 0.53 g of Igepal CO-520, 150 μL of cyclohexane solution containing 2 mg/mL Fe$_3$O$_4$ NPs, and 10 mL of cyclohexane were vigorously stirred for 30 mins. To this, 0.5 mL of a cyclohexane solution containing 0.056 g/mL of freshly prepared, tetraethyl orthosilicate precursor was added. The solution was then stirred overnight (20 h) at room temperature, and 1 mL of absolute ethanol was then added to precipitate the particles from the micro emulsion. This solution was centrifuged at 11,000 rpm for 30 min. The supernatant was discarded; the pellet was washed at least 2 times with absolute ethanol, then dispersed in 80% ethanol (in DI water), and stored at 4°C. After one
day, fresh (3-Aminopropyl)triethoxysilane (APTES) was added to the silica solution. This solution was stirred for 2 hr., and then heated to boiling temperature where it was held for 4 hours (additional 80% ethanol was added, as needed, to maintain volume). Five cycles of centrifugation and re-dispersion in pure ethanol were performed to remove any residual reactants.

**THPC-stabilized gold solution synthesis**

To a flask containing 45 mL of water, 0.5 mL of freshly prepared 1 M NaOH and 1 mL of Tetrakis(hydroxymethyl)phosphonium chloride (THPC) (12 µL of 80% THPC in water) were added. The mixture was stirred for 5 min and then 10 mL of 5 mM HAuCl₄ was added. A color change from yellow to dark brown indicated the formation of THPC-gold.

**Particle Seeding**

To a 50 mL volume of THPC-stabilized gold, 500 µL of the amino-silica coated particles was added; the mixture was stirred, and after 1 hour, the solution was centrifuged and the pellet redispersed in DI water. The “seeded” solution was centrifuged a second time, then redispersed in 40 mL of water.

**Synthesis of LPA**

(R)-Lipoic acid (3 g, 4.85 mmol) was dissolved in dry dichloromethane (50 mL) followed by the addition of propargyl amine (933 µL, 4.85 mmol). The solution was cooled to 0°C in an ice bath, and N,N'-dicyclohexylcarbodiimide (DCC) (27 g, 14.55 mmol) was added in one portion. After 3 hours, an additional 10 g DCC was added. The reaction was stirred overnight and warmed to room temperature, filtered through paper to remove DCU, and concentrated. The crude product was re-dissolved in dichloromethane and filtered (3x) to completely remove DCU, purified on a silica gel column, and eluted with 50% ethyl acetate/petroleum ether. 1H NMR
(399 MHz, CDCl3) δ 5.67 (s, 1H), 4.05 (dd, J = 5.1, 2.5 Hz, 2H), 3.57 (dt, J = 12.7, 6.4 Hz, 1H), 3.22 – 3.06 (m, 2H), 2.46 (dt, J = 12.2, 6.5 Hz, 1H), 2.25 – 2.17 (m, 4H), 1.91 (dt, J = 19.8, 6.9 Hz, 2H), 1.57 – 1.38 (m, 4H). 13C NMR (100 MHz, CDCl3) δ 172.52, 79.78, 71.90, 56.62, 40.49, 38.72, 36.37, 34.84, 29.42, 29.10, 25.42.

**Gold Shell Growth**

To 40 mL of water, 500 µL (20 mg/mL) of K₂CO₃ aqueous solution was added; the solution was stirred for 10 min, then 600 µL of 1% HAuCl₄ solution was added, and it was stirred again for 30 min. To this, 2 mL (5 mg/mL) of “seeded” particles were then added with 10 µL of 2.5 g/mL aqueous NH₂OH, and the solution was gently rocked for 1 min. Then, 100 µL of 10 mg/mL mPEG2000-SH, and 5 µL of 100 mg/mL LPA in DMSO were added before shaking for 24 hr.

**Dynamic Light Scattering**

The manufacturer’s protocol (Malvern Instruments) was followed and results were recorded as intensity-weighted scans.

**MRI contrast enhancement**

Flame-sealed glass tubes containing water with various concentrations of SC-SPIONs were suspended in a 1% (w/v) agarose gel solution, which was used as a tissue phantom following solidification. SC-SPIONs were diluted in water logarithmically, in a concentration range of 1 nM to 1 mM. T2 was determined on a Bruker 7T MRI machine, by varying TE and keeping TR constant with a standard gradient eco sequence, Multi-Slice-Multi-Eco. T1 was determined by keeping TE constant and varying TR with a RARE sequence.
Scheme 1: fLPA-SPIO@AuNS Synthetic Scheme.

a) 1) 10 nm SPIO particles are coated with silica, 2) the silica is functionalized with a monolayer of para-amino silica, 3) 2 nm gold nanoparticles are seeded to provide nucleation sites for shell growth, 4) gold is reduced to grow a shell on the SPIO-core, silica coated nanoparticles. b) Dihydrolipoic acid propargyl amide is absorbed on the gold surface and click chemistry is used to attach, in this case, an MGL inhibitor.
Scheme 2: Agar phantoms for MRI contrast enhancement.
Sample solutions were prepared in H₂O and placed in flame-sealed NMR tubes at varying concentrations. These tubes were then embedded in a 1 % (w/v) agarose gel solution and slices were analyzed for MRI contrast enhancement.
Fig. 1. TEM micrographs of oleic acid stabilized Fe₃O₄ (SPIO) particles. Representative images showing hydrophobic SPIO core particles with an average diameter of 10 nm. White lines show physical particle size as measured by the image processing software. [Insets: image scale and magnification]
Fig. 2. TEM micrographs of SiO2-NH2 coated Fe3O4 (SPIO) particles (Core-Shell) and “seeded” particles.
Representative images showing a particle size (average diameter) of 40 nm. Black arrows designate 2 nm gold nanoparticles beginning to adhere to the surface of the particles to provide a nucleation site for gold shell growth. White lines show physical particle size as measured by the image processing software. The lower images show completely seeded particles. [Insets: image scale and magnification]
Fig. 3. Dynamic light scattering analysis of SPIO-NH₂ and seeded nanoparticles.

a) The measured hydrodynamic diameter for SPIO-NH₂ particles was 135.8 nm with a polydispersity index of 0.005. 
b) The measured hydrodynamic diameter for AuNP seeded SPIO-NH₂ particles was 136.6 nm with a polydispersity index of 0.005. AuNP seeding does not affect hydrodynamic diameter for this population of particles.
Surfactant ratio and abundance determines the hydrodynamic size of fLPA-SPIO@AuNS particles during gold shell growth/functionalization. In black, a 2/1 ratio of LPA to SH-PEG in water produced the smallest particles and represents the most surfactant. This sample showed had the highest PDI. In red, a 1/1 ratio of LPA to SH-PEG in water produced intermediate particles. In blue, a 1/1 ratio of LPA to SH-PEG in 50% DMSO produced the largest particles. It is possible that LPA has higher solubility in DMSO than on the surface of SPIO@AuNS particles.
Fig. 5. Magnetic resonance images of SC-SPIONs.
a) A representative slice image of SC-SPIONs with varying contrast. b) Signal intensity vs. time for SC-SPIONs of varying concentration (1 nM – 1 mM) showing a dose-dependent enhancement of T2 relaxation (contrast enhancement) c) Mean T2 vs SC-SPION concentration. T2 darkening below 100 msec is considered clinically relevant.
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Chapter 6

Conclusions
Conclusions

This work has contributed significantly to the understanding of a key regulatory pathway involved in breast cancer progression (as well as other disease states). First, the characterization of the nanodisc model through the use of NMR spectroscopy demonstrated that hMGL associates with nanodiscs. This finding was confirmed through the use of copurification of (-)MSP ND in complex with 6-his tagged MGL. This complex is formed with a stoichiometry of 1 ND-MGL for every 6 discs assembled. This population of excess empty discs drives MGL to remain in complex, rather than dissociating from the membrane. Additionally, determination of the length of time required for complex formation between blank NDs and detergent-free WT hMGL in solution provided us with yet another method for incorporating MGL in NDs, which allowed us to examine the impact of phospholipid bilayers on MGL without the confounding data free MGL may have provided.

Next, we used biochemical, HX MS, and computational approaches to measure the impact of a phospholipid bilayer on the kinetic properties, and conformational dynamics of MGL, successfully providing the first detailed study of this interaction. We demonstrated that an area within MGL’s lid domain associates through hydrophobic interactions with the membrane, and this association enhances the enzyme’s catabolic kinetics. Using HX MS and MD simulation we determined that for hMGL, the lid-domain helix α4, and the distal helix α6 associated with the membrane directly, stabilizing an open (active) conformation. Upon inhibition (carbamylation) with AM6580, several regions of the active site of hMGL showed less deuterium exchange. The dynamic shift in response to this covalent inhibitor provided data that can lead to the design of novel hMGL carbamylating agents. More generally, this work demonstrated the suitability of peptide-level HX MS for the analysis of membrane associated
protein dynamics, and the nanodisc membrane model, for investigating the structure-function relationship of this interaction.

Following the successful analysis of MGL in the ND model, catalytically active full length rFAAH was successfully expressed, purified, and incorporated into NDs. This represents the first experiment where a mammalian amidase, serine hydrolase was studied using this model. The protocols used for isolating the membrane-protein pool, and for ND-rFAAH formation, are generalizable to other hydrophobic mammalian recombinant proteins expressed in E. coli. The subsequent analysis of the in-solution protein dynamics for rFAAH reviled areas of high exchange, and zero to low exchange over the four hour time period used. Notably, the lack of exchange in the active site domain, and the transmembrane domain support previous findings that these hydrophobic regions interact directly with the membrane, and are not accessible to the cytosol. Additionally this suggested that in solution, rFAAH may exist as a dimer imbedded within detergent micelles. This makes rFAAH an excellent target for further analysis in the higher fidelity ND membrane model.

Next, we characterized AM3506-inhibited hMGL and ΔTM-rFAAH via comprehensive mass spectrometric (MS) analysis. We showed that AM3506-based inhibition of MGL occurs through sulfonylation of the catalytic serine (Ser122), followed by a His121 mediated β-elimination reaction that resulted in desulfonylation of the inactivated enzyme and the subsequent formation of a dehydroalanine122 residue. To confirm the formation of dehydroalanine, a Michael acceptor, we performed conjugate addition (Michael reaction) using the activated thiols, thiophenol and betamercaptoethanol as nucleophiles. A comparison of the spectra of the tryptic digest from untreated and thiol-treated hMGL confirmed the addition of these thiols to dehydroalanine122. Additionally, intact hMGL pretreated with AM3506, incubated with thiophenol showed a peak
with an average mass of 34215.2 Da (+91.6 Da), a mass consistent with the formation of S-phenyl cysteine and the consequent calculated mass increase of 92.1 Da.

To provide evidence that His121 is essential in directing the desulfonylation, we expressed and purified mutant H121S hMGL and treated it with AM3506. We did not observe any new peptides attributable to the β-elimination mechanism. Similarly, we did not observe any peptides attributable to β-elimination in AM3506-treated ΔTM-rFAAH which lacks any histidine residues adjacent to the catalytic serine. We analyze a ΔTM-rFAAH I250H single mutant in the attempt to induce β-elimination, but the mutant was catalytically silent.

Our discovery that the path of desulfonylation in AM3506-treated hMGL occurred via β-elimination due to a specific histidine residue outside of the catalytic triad presents a selective approach for the modification of serine hydrolases to confer new function without genetic manipulation. Subsequent nucleophilic additions to α,β-unsaturated dehydroalanine-serine hydrolases represents a novel, selective approach for labeling these proteins which are potential biomarkers for certain cancers.

Following this, another approach for targeting and treating aggressive cancers was developed. The fLPA-SPIO@AuNS nanoplatfrom, which could be coupled to a targeting moiety such as an MGL or FAAH inhibitor, in order to deliver this agent to aggressive breast cancer cells. This nanoplatfrom acted as a T2 contrast enhancing agent for MRI, and could be used for hyperthermal ablation therapy. Development of this platform opened the door to a plethora of targeting, therapeutic, and diagnostic imaging agents that could be bioconjugated via click chemistry to this nanomedical agent. Additionally, by adjusting the size of this platform, the optical and chemical properties can be tuned for specific applications. Due to the size of these
particles, even in the absence of additional targeting agents, bioaccumulation in tumor cells would be expected due to the enhanced permeability and retention (EPR) effect\textsuperscript{172} for nanoparticles under 200 nm in physical and hydrodynamic size. This makes the fLPA-SPIO@AuNS platform an attractive launchpad for future drug discovery and development.

The results of this work contribute significantly to the understanding of endocannabinoid-hydrolyzing enzymes, which modulate key regulatory pathways involved in breast cancer progression (as well as other disease states). This work has additionally opened the door for the further development of a novel, nanomedical, pharmacologic intervention for the diagnosis and treatment of metastatic disease.
References


Saario, S. M., Savinainen, J. R., Laitinen, J. T., Jarvinen, T. & Niemi, R. Monoglyceride lipase-like enzymatic activity is responsible for hydrolysis of 2-arachidonoylglycerol in


Appendix

Awards

For work presented here, the author has received the following awards:

1. University Academic Excellence Award
2. NSF/NCI (IGERT) Nanomedicine Fellowship
3. American Foundation for Pharmaceutical Education Fellowship (Twice)
4. Provost’s Certificate of Excellence in Research Award
5. GBMSDG Annual Advances in Separation Science and Mass Spectrometry Symposium First Place Award
6. John L. Neumeyer Research Achievement Award

Thank you to all those who have contributed to this work, and to those agencies that have recognized its significance with the commendations above.