Affinity Selection Mass Spectrometry (ASMS) High-Throughput Screening of the GLP1 Receptor

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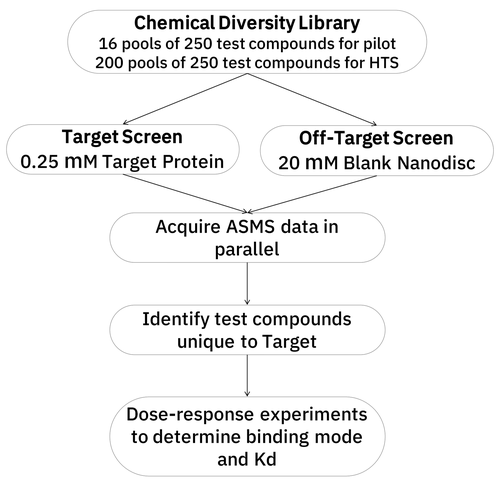
Abstract

High-throughput screening of membrane-bound proteins can be particularly challenging, because hydrophobic test compounds bind non-specifically to the artificial membranes and detergent micelles typically used to solubilize and stabilize target constructs. In this study, we describe a successful high-throughput screen against the GLP1 receptor (GLP1R), an important target in the treatment of type 2 diabetes and obesity. The NativeMP™ platform was used to prepare the target construct, which consisted of the full-length GLP1R protein expressed in a copolymer-based nanodisc. A customized ASMS workflow was developed to enable compounds to be screened simultaneously against the GLP1R nanodisc (target screening) and a copolymer-matched blank nanodisc (off-target screening). Customized hit selection criteria were implemented using Aston ASMS data analysis software, and test compounds that displayed higher affinity for GLP1R than for the blank nanodisc were selected as hits. This screening configuration enabled test compounds that non-selectively bind the nanodisc construct to be efficiently eliminated. A successful pilot project (4K compounds) identified 5 unique stoichiometric binders, while a subsequent diversity screen (50K compounds) identified 75 hits (0.15% hit rate), of which 34 were confirmed in secondary assays. This screening approach can be applied to a wide range of membrane-bound protein targets, including GPCRs and ion channels.

Advantages of ASMS

Minimal assay development required	Ideal platform for selectivity screening
High throughput	100K compounds screened in pools of 320 in 38 hours
Low protein / RNA usage	Primary screening (100K compounds in pools of 320) requires less than 1mg of a 50 kD target protein.
Solution-phase binding with native target	No need for target tagging or surface immobilization
Ability to work with large targets (including protein/protein and protein/oligonucleotide complexes)	Large targets can be challenging for SPR or interferometry
Hits are immediately available for confirmation and follow up	Unlike DEL screening, the identity of hits are known, and hit compounds can be available as powders
Signal level is quantitatively correlated to target occupancy	Subtle changes in binding affinity can be detected in selectivity screening

HTS Project Workflow



Screening and Data Analysis Parameters

ASMS Screening Conditions	
Sample volume	10 μL
GLP1 Target Conc	0.25 μM
Blank Nanodisc Conc	20 μM
Test Compound Conc	1 μM each
Pooling Density	250

Hit Criteria Applied for Aston Software	
Peak Area	Min 5x background
Blank Subtraction	Min 4x peak area for target vs DMSO
Cross-well filter	Max 3
Score	Min 60
Off-target filter	Min 2x peak area target vs off-target

GLP1R – Type 2 Diabetes Target

Target for Type 2 Diabetes and Obesity Treatment
GLP1R drugs manage blood sugar levels and promote weight loss

Enhanced Metabolic Health
GLP1R drugs can improve metabolic parameters beyond glucose and weight management, potentially benefiting patients with metabolic syndrome and associated conditions.

Drug Discovery at Momentum Bio and Cube Biotech
Discovery and development of compounds targeting GLP1R, from hit to lead

Cardiovascular Protection
Some GLP1R therapies reduce the risk of heart attack and stroke, which is valuable for patients with cardiovascular disease

Oral Availability
Small molecules can be taken orally, eliminating the need for injections and making treatment for diseases like type 2 diabetes and obesity more convenient.

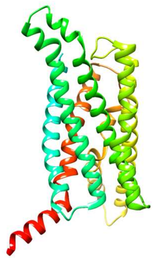
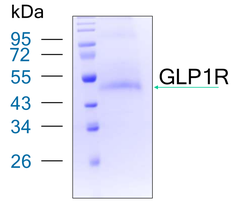


Figure 1: A relaxed-state 3D conformation of the GLP1 receptor (GLP1R) shown in cartoon representation (PDB: 8JIS; 2.46Å)

NativeMP™ Platform
Stabilization of GLP1R
- Full-length
- First ever stabilized wild-type
- Retention of native host cell lipid environment
- Function and stability preserved
- Available in mg amounts
- Compatible with assay development at body temperature

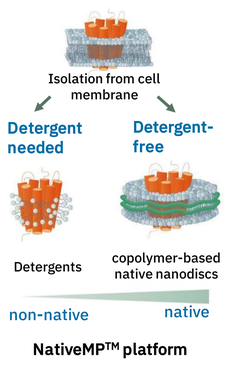
Sample Purity

SDS-PAGE



Unique characteristics of Lipid-Membrane Protein Particles

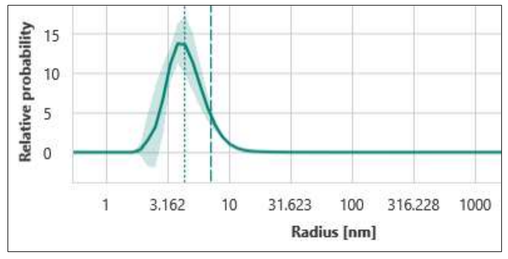
Detergent-stabilized Membrane Protein
- Non-lipid environment
- Function and stability compromised
- Restricted assay temperatures
- High-risk project with long timeline
- Not compatible with full-length wild-type GPCRs



NativeMP™ platform

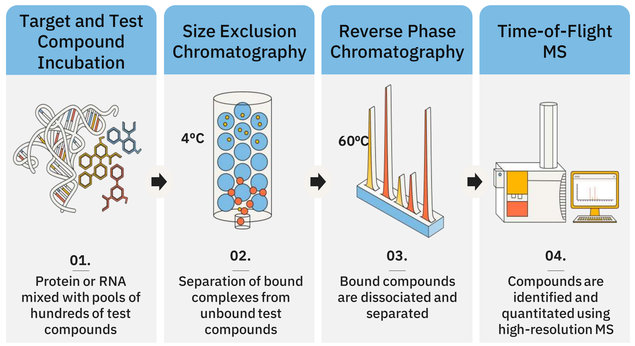
Lipid Membrane Protein Particle in Synthetic Polymers
- native host cell lipid environment
- Function and stability preserved
- Assays can be performed at body temperature
- Lower risk and faster project timeline
- **Enables analysis of full-length wild-type GPCRs**

Homogenous sample without aggregation

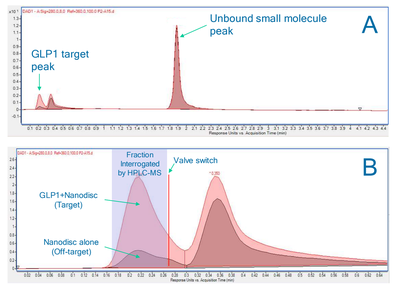


DLS- analysis

ALIS-ASMS Workflow

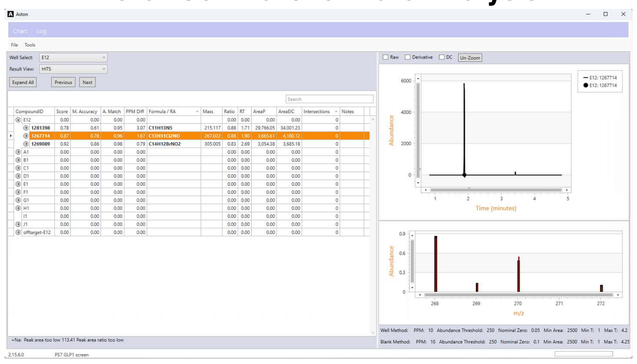


SEC-UV Chromatograms



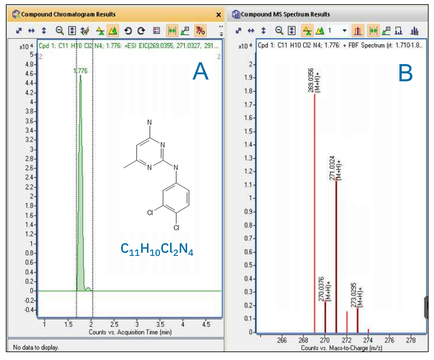
Overlaid SEC-UV chromatograms of GLP1R target protein (pink) and blank nanodisc (gray). Panel A shows clear separation between the target (eluting at 0.21 minutes) and unbound small molecules (eluting upon a 70% acetonitrile wash at 1.9 minutes). The shaded portion of the zoomed chromatogram in Panel B (between 0.17 and 0.27 minutes) represents the fraction of interest, which is diverted to the reversed phase column via a switching valve so that ligands can be dissociated (60˚C, pH 1.0, high organic) and detected by ToF-MS.

Aston Software for Data Analysis



Example screenshot of custom Aston ASMS Software for data analysis. Each test compound is interrogated in the target well in parallel with DMSO-only controls, matched off-target injections (in this case, blank nanodisc), and non-target wells. Primary hits must pass user-selected criteria for MS signal quality, and signal must be unique to the the target well. Graphical displays of each compound's Extracted Ion Chromatogram and Mass Spectrum, as well as each injection's SEC-UV trace, are displayed to enable rapid manual QC of results as needed

Hit Confirmation Assay



Example results from a singleton hit confirmation assay using Agilent Mass Hunter Software. The Extracted Ion Chromatogram (Panel A) and Mass Spectrum (Panel B) of this test compound indicate that the structure has been detected and quantified. ASMS signal intensity is defined as the area under the curve for the compound chromatogram peak (shaded in green in Panel A) and corresponds to the concentration of the test compound. Test compound concentration is proportional to target occupancy, which is in turn proportional to the affinity of the ligand for the target.

Results

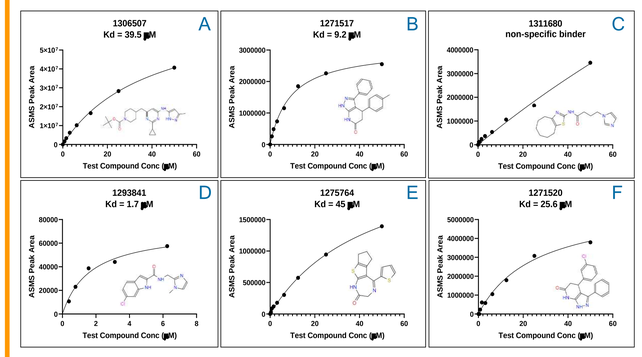
Initial testing was performed using a set of 4,000 test compounds (16 pools *x* 250 compounds) selected at random from the Momentum diversity library (300,000 compounds). Assays were run using a range of GLP1R and blank nanodisc concentrations to optimize parameters. Ultimately, a target concentration of 0.25 uM and an off-target (blank nanodisc) concentration of 20 uM were selected, resulting in 6 verified hits (0.15% hit rate). These conditions were then used to perform a primary ASMS screen with 49,546 test compounds (Momentum PS7 sub-library). This library consists of 199 mass-coded pools of 250 test compounds each, assorted such that each test compound in a given pool has a mass difference ≥ 0.2 g/mol from all pool-mates. The primary screen was completed in under 24 hours and identified 75 hits (0.15% hit rate), which were further interrogated in a confirmation assay. Primary hits were run as singletons against the target and blank nanodisc, resulting in 34 confirmed hits for an overall confirmation rate of 45%. Confirmed hits were subjected to 8-point dose-response experiments using serial dilutions starting at 50 uM and including DMSO-only and no-target controls. Many test compounds exhibited saturating dose-response curves, indicating specific and stoichiometric binding to the target protein.

Target Selectivity of Hits

Cpd ID	Molecular Formula	Target ASMS Peak Area	Off-target ASMS Peak Area	Ratio: Target/Off-target
1271517	C19 H17 N3 O	119486	ND	NA
1271520	C18 H14 Cl N3 O	81644	ND	NA
1272547	C19 H23 N3 O	37321	ND	NA
1275201	C19 H31 N5	555608	ND	NA
1275764	C14 H12 N2 O S2	18017	ND	NA
1275958	C16 H21 N3 O3 S	7797	ND	NA
1290789	C20 H19 N3 O3	87402	ND	NA
1291652	C21 H30 F N3 O	133181	ND	NA
1292203	C21 H27 N5	85755	ND	NA
1293841	C14 H13 Cl N4 O	32335	ND	NA
1299453	C24 H29 N3 O2	96432	ND	NA
1301285	C23 H24 N4 O4	21947	ND	NA
1305682	C21 H25 N5 O3	116767	ND	NA
1306039	C26 H28 F N3 O2	401944	ND	NA
1306554	C22 H27 N5 O S	44319	ND	NA
1310492	C18 H30 N2 O3 S	47378	ND	NA
1310653	C19 H25 N3 O2	128147	ND	NA
1311222	C19 H21 Cl N2 O2	24869	ND	NA
1311531	C16 H20 F N3 O S	44724	ND	NA
1311680	C16 H22 N4 O S	228963	ND	NA
1312044	C22 H23 N3 O2	276813	ND	NA
1314345	C18 H21 N7 S	224715	ND	NA
1314986	C19 H17 F N4 O	16347	ND	NA
1315345	C22 H27 N5	49095	ND	NA
1306217	C27 H27 F N4 O	639956	28490	22.5
1291709	C17 H28 N6 O	621132	63855	9.7
1274598	C19 H24 F3 N5 O S	76527	10939	7.0
1293685	C22 H24 F N O3	80234	14928	5.4
1306507	C22 H32 N6 O2	894676	167319	5.3
1272721	C16 H15 F N4 O S	340691	93393	3.6
1290335	C17 H18 Cl N5	65722	21021	3.1
1309913	C14 H16 F2 N2 O3	26181	8574	3.1
1277620	C18 H20 N6 O S	51366	17329	3.0
1298002	C22 H23 N5 O	72438	27082	2.7

Hit confirmation assays were run against both 0.25 uM GLP1R target protein and 20 uM blank nanodisc. The peak area for each injection is shown in the table to the left. Of 34 confirmed hits, 24 bound only the target protein, with no detectable binding to the blank nanodisc. The remaining 10 confirmed hits bound both the target protein and blank nanodisc to some extent, suggesting weak non-specific binding to the nanodisc lipids in addition to specific target interactions. A target peak area of at least 2X the off-target peak area was required for hit selection.

Affinity Determination



Eight-point serial dose-response experiments were run on the 34 confirmed hits to determine the binding affinity of each test compound. A saturating binding curve indicates specific and stoichiometric binding to the target, from which the binding affinity can be determined. A linear dose-response curve (as seen in Panel C) indicates super-stoichiometric binding, likely due to non-specific interactions between the target and test compound; this compound represents a true binder but is unlikely to be a useful lead compound. Kd values for the 5 specific binders ranged from 1.7 uM to 45 uM. Interestingly, two test compounds (shown in Panels B and F) share over 97% structural homology but exhibit a three-fold difference in Kd values, providing early insight into the structure-activity relationship.

Conclusions

Membrane-bound proteins pose unique challenges for binding assays due to extensive non-specific binding between poorly soluble test compounds and lipids required to stabilize the target protein. We developed a rapid, robust and automated binding assay that overcomes these challenges to target a novel GLP1R-nanodisc construct. The use of the nanodisc system minimizes the lipid load used in the assay, while the off-target screening approach effectively eliminates non-specific binders from the hit pool, greatly streamlining downstream interrogation and increasing the percentage of compounds useful for a drug discovery campaign.

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