Rapid rank ordering of binding affinities of pools of test compounds against an RNA target by Affinity Selection Mass Spectrometry (ASMS)

Somayeh Talebzadeh[1], Zane Thistleford[1], Arrin Katz[1], Samuel Hasson[2] and Can Ozbal[1]

[1]Momentum Biotechnologies, 3 Federal St., Suite 300, Billerica, MA 01821; 2Rgenta Therapeutics, 300 Trade Center, Suite 6600, Woburn, MA 01801

Abstract

Small molecules selectivity targeting RNA is a growing area of interest in drug development. Unlike most proteins that have relatively rigid structures and well-defined binding pockets, RNA may adopt multiple confirmations known as the structural ensemble. The biologically active confirmation of an RNA may be a high energy/low probability structure. As a result many of the tools that are commonly used to characterize ligands of protein targets are less effective when applied to RNA due to this inherent flexibility. For example, Surface Plasmon Resonance (SPR) is a powerful tool very commonly used in characterization of protein-ligand interactions. Immobilization of an RNA molecule to a surface or addition of an intercalating dye limits the number of accessible ensembles and may preclude a subset of ligands from being detected. The Affinity Ligand Identification System (ALIS) is a well-established form of affinity selection mass spectrometry (ASMS) for identifying and characterizing small molecule binders of proteins[1]. Because ALIS is a fully solution phase assay that does not require the labelling of the target or ligand the techniques has recently been successfully applied to RNA targets[2]. The ability to interrogate heavily multiplexed pools of test compounds in a single experiment (typically several hundred) makes ASMS an efficient tool for the high-throughput screening of chemical libraries to identify small molecules that bind to an RNA target. Under a typical high-throughput screen over 100K test compounds can be screened in under 36 hours (~1 second per test compound). Because many of the biophysical assays commonly used for protein targets (eg: SPR, DSF) are not appropriate for RNA targets further characterization of ligands is often done by ASMS. Further characterization may include assays such as the determination of the binding affinity or site selectivity through competition assays. However, these assays typically require individual ligands to be interrogated as singletons often at multiple ligand and/or target RNA concentrations greatly reducing the throughput of the approach. Here we describe an approach which enables the rapid and efficient rank ordering of the relative binding affinity of test compounds in support of a structure-activity relationship project for an RNA target. The addition of a well characterized tool compound with known binding affinity to each pool enables the test compounds in the pool to be quickly categorized as tighter or weaker binders than the tool compound.

Introduction

ASMS is a well-established biophysical assay platform where one or more small molecule test compounds are mixed with a target of interest the target/test compound mixture is fractionated by size-exclusion chromatography (SEC) and the fraction containing the target is interrogated by HPLC-MS. Identification of a test compound in the target fraction is indicative of a binding event (Figure 1). Targets interrogated by ASMS can be RNA, proteins and protein/protein or protein/oligonucleotide complexes while typical test compounds consist of traditional drug-like small molecules that are ideally rule-of-five compliant with molecular weights below 480 g/mol. The ability to multiplex hundreds of test compounds into a single injection allows for rapid and efficient identification of ligands at throughputs around 1 second per test compound. However, further characterization of these ligands is much less efficient as binding affinity determination requires analysis of singleton test compounds at multiple concentrations. In this sturdy we describe a technique known as ACE50 where the binding affinities of small pools of test compounds (typically 8-10 per pool) can be rank ordered and benchmarked to a well characterized tool compound[3]. This is especially useful when performing structure-activity relationship studies where groups of structurally similar test compounds can be quickly interrogated and those of most interest can be selected for additional investigation as needed. In this approach a constant amount of a pool of test compounds are incubated with a diminishing amount of target. Eight structurally related compounds from a hit-expansion project were pooled with a well-characterized 9th tool compound. Each test compound was at a final concentration of 1uM and incubated with a range of the target RNA concentrations (8-point serial dilution starting at 10 uM) for 30 minutes prior to ASMS analysis. At 10 uM target RNA there are enough available binding sites for all 9 test compounds to bind. However, as the target RNA concentration is lowered competition between the test compounds is introduced such that weaker binders are displaced by stronger ones until only the tightest binders remain.

ASMS Methods

The ALIS instrumentation consisted of an Agilent 1260 HPLC pump for the size-exclusion chromatography coupled to an Agilent 1290 UHPLC pump for the reversed phase (RP) chromatography with a high-pressure switching valve interfaced to an Agilent 6230B Time-of-Flight Mass Spectrometer. A UV detector between the SEC and RP dimensions was used to monitor the elution of RNA and test compounds. All MS acquisitions were done in positive ion acquisition mode. Data analysis was performed with a combination of Agilent Mass Hunter and proprietary custom software.

SEC conditions: Buffer A: 700 mM Ammonium Acetate
Buffer B: 70% Acetonitrile
Column: 50 x 2.1 mm Polyhydroxyethyl A, 3 um 60 Å porosity (PolyLC)

RP conditions: Buffer A: Water + 0.1% Formic Acid
Buffer B: 90% Acetonitrile + 0.1% Formic Acid
Column: 50 x 2.1 mm Kinetex 2.6 um C18 100 Å (Phenomenex)

ALIS-ASMS Workflow

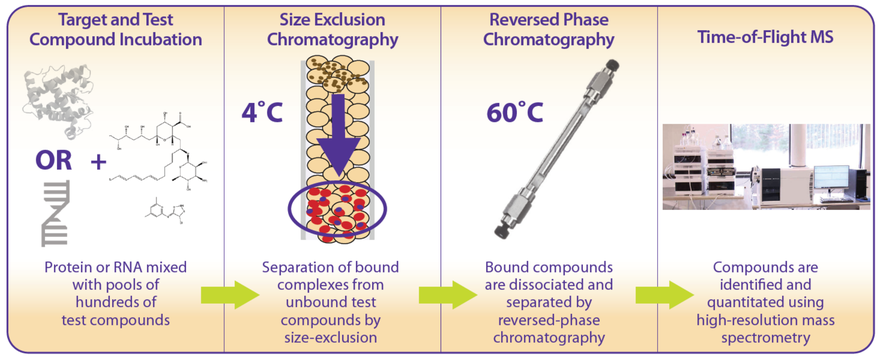


Figure 1: Schematic of the Affinity Ligand Identification System (ALIS). A mixture of one or more small molecule test compounds and a target are fractionated by size-exclusion chromatography. The target fraction is selectively interrogated by HPLC-MS to identify potential ligands

SEC-UV Chromatogram at 280 nm

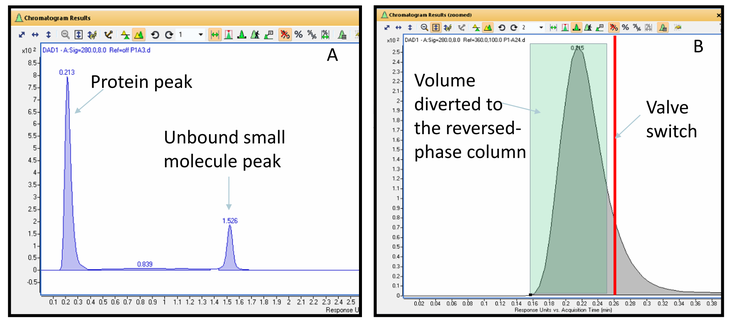


Figure 2: (A) UV chromatogram of the SEC column elute. The early eluting peak at 0.21 min contains the target consisting of molecules that are too large to fit through the pores of the SEC column packing material while the later eluting peak at 1.53 min are small molecules trapped in the SEC column pores and subsequently washed out with 70% acetonitrile. **(B)** Closeup of the protein peak in panel A. A 50 µL high pressure switching valve is used to divert the green shaded portion from the SEC dimension to the HPCL dimension of the system. Any test compounds identified by MS in this region are inferred to be ligands to the target

Isotope Pattern Matching for Hit Identification

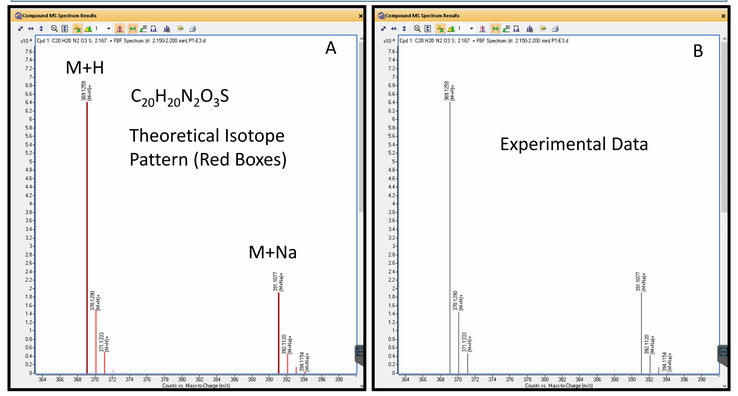


Figure 3: Hit identification is performed by 2 sets of filters. The first filter is an isotope pattern match of the theoretical isotope distribution for the protonated and/or sodiated adducts for the molecular formula of a given test compound (red lines in panel A) with the experimentally determined data (shown in panel B). The match is scored on a scale from 1 (no match) to 100 (perfect match) and any cutoff can be user selected in SW (default is 70). The second filter is a selectivity filter that ensures the hits are in the correct wells only.

Experimental Details

Structurally similar test compounds from a hit expansion study were prepared as 10 mM stock solutions in DMSO. Equal volumes of 8 test compounds and a single tool compound were mixed together and diluted 27.8x with DMSO to yield a 9-plex pool where each test compound is at a final concentration of 40 uM. A total of nine 250 nL aliquots of each pool were dispensed into a "U" bottom polypropylene plate using a Tecan D300e liquid handler. The target RNA was melted and annealed to ensure proper folding as described previously[2]. An 8 point serial dilution of the RNA target plus a 9th buffer-only sample was prepared at a starting concentration of 10 uM. A 10 uL aliquot of each target concentration was added to each of the nine 250 nL pools of test compounds in DMSO. Upon addition of the target solution the test compounds are diluted 40x to a final concentration of 1 uM each. The test compound/target mixture was incubated for 30 minutes at room temperature and each well was interrogated by ASMS. All data analysis was performed by Agilent Mass Hunter and proprietary software. At the highest target concentration of 10 uM there is enough capacity for all 9 test compounds at 1 uM each to bind the target without competition. However, as the target concentration is titrated lower the total amount of test compound becomes greater than the binding capacity of the target. This results in competition between the test compounds such that tighter binders displace weaker ones.

References

1) Annis DA et al, (2004) International Journal of Mass Spectrometry **128**(2), 77-83
2) Rizvi, NF *et al*, (2018) ACS Chem Biol **13**(3), 820-831
3) Annis, DA et al, JACS 126, 15495-15503

Tool Compound Characterization

The binding affinity of a known ligand of the target was determined by running a ½ log dilution series starting at 50 uM against 1 uM target RNA. The K_d was determined to be 1.1 uM

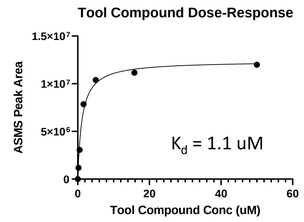
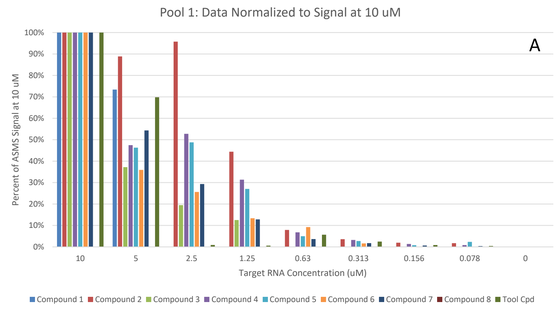


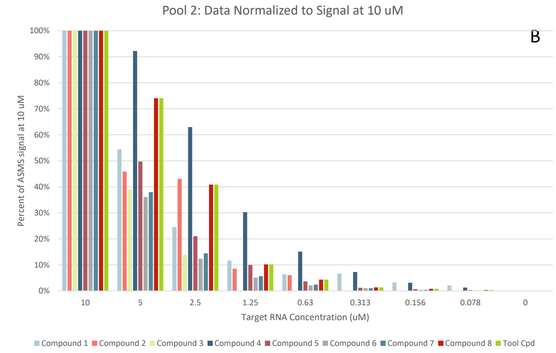
Figure 4: Saturating dose-response experiment of a ligand against the target RNA indicates selective and stoichiometric binding. The Kd was calculated to be 1.1 uM using non-linear curve fitting (GraphPad Prism).

ACE50 Experiments

Pools of 8 test compounds plus the tool compound at 1 uM each were interrogated at 9 different target RNA concentrations. At 10 uM target there is enough binding capacity for all 9 compounds to bind without competition. As the RNA concentration is lowered tighter binders begin to compete away weaker ones. The data below shows the ASMS peak area of each compound normalized to the maximum signal at 10 uM RNA. The signals can be rank ordered from tightest to weakest binder as shown in the inset tables. Compounds highlighted in green are tighter binders than the tool compound while those in red are weaker binders.



Cpd ID	Rank
Cpd 1	8
Cpd 2	1
Cpd 3	7
Cpd 4	3
Cpd 5	2
Cpd 6	6
Cpd 7	5
Cpd 8	9
Tool Cpd	4



Cpd ID	Rank
Cpd 1	1
Cpd 2	7
Cpd 3	8
Cpd 4	2
Cpd 5	6
Cpd 6	5
Cpd 7	4
Cpd 8	9
Tool Cpd	3

Figure 6: ASMS peak areas for each test compound are shown for two example pools in panels A and B. The peak areas have been normalized to the signal at 10 uM which is the concentration at which there is enough target capacity for all 9 test compounds to bind without competition. The table inset shows the rank ordering of the test compounds in each pool, compounds highlighted in green are tighter binders than the tool compounds while those in red are weaker binders. No binding at all was observed from one compound in each of the 2 example pools

Conclusions

Through the use of a pooling strategy the binding affinity of 8 test compounds were determined and benchmarked to a well characterized tool compound with just 9 ASMS injections. This enables the rapid assessment of a hit expansion study in which test compounds with structural similarity to hits identified in a high-throughput screen. The approach can also be used to streamline structure-activity relationship studies such that only the tightest binders need to be followed up with full dose-response experiments.

Summary of ASMS

- Label-free, solution-based binding with native target: target or test compound does not need a tag, modification or immobilization on a surface (particularly important for RNA targets)
- Ability to interrogate very large targets and target complexes
- Straightforward assay development: generally limited to buffer optimization
- High throughput: 100K test compound in pools of 250 can be screened in 36 hours
- Low target usage: Typical screens requires 50 pmoles of target per injection (2.5 µg of a 50 KDa target protein/RNA or ~1 mg of target for a 100K test compound screen)
- Single method for HTS and hit characterization minimizes assay development
- Compound quantification is dependent on molecular formula eliminating false results due to impurities or test compound breakdown/oxidation