SAR by MS: A Ligand Based Technique for Drug Lead Discovery Against Structured RNA Targets

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Abstract: A technique for lead discovery vs RNA targets utilizing mass spectrometry (MS) screening methods is described. The structure−activity relationships (SAR) derived from assaying weak binding motifs allows the pharmacophores discovered to be elaborated via "SAR by MS" to higher affinity ligands. Application of this strategy to a subdomain of the 23S rRNA afforded a new class of compounds with functional activity.

Ribonucleic acids (RNAs) are attractive targets for drug discovery,[1] as their complex 3-dimensional structures play critical roles in bacterial, viral, and human disease processes. Directed screening and analogue synthesis efforts against RNA targets have improved affinities of known classes of ligands such as peptides,[2] aromatic cations,[3] and aminoglycosides.[4] Attempts to discover new compound classes via high-throughput screening (HTS) against RNA targets have yielded active compounds at much lower rates than those generally observed for protein targets.[5] The identification of new RNA ligands remains difficult, and low micromolar RNA binding compounds that fall outside of the aforementioned classes are extremely rare.

The low hit rates in traditional HTS assays can be traced to difficulties in detecting and accurately measuring low affinity interactions between small molecules and RNA, and we have developed a high-throughput mass spectrometry (MS)-based assay to address these issues.[6] In contrast to traditional HTS assays, the MS assay accurately quantifies binding affinity, stoichiometry, and specificity over a wide range of ligand K_D values and can be used to provide unique information on the simultaneous interaction of multiple ligands with an RNA target.[7]

We now describe how drug design approaches that exploit structure−activity relationships (SAR) derived from studying weak ligand−target interactions[8] such as "SAR by NMR"[9] can be extended utilizing these advances in MS screening methods. This "SAR by MS" process (Figure 1) begins by screening a set of compounds to identify hits against an RNA target of interest. The SAR and specific nature of ligand binding are probed through chemical elaboration and/or additional MS experiments. The accumulated SAR suggests a pharmacophore hypothesis incorporating key structural features of two or more classes of ligands. Appropriate linking of two or more low affinity motifs has been demonstrated to provide a large gain in binding energy[10] in several applications of the SAR by NMR strategy,[11] as well as in structure-[12] and combinatorial-based[13] drug design approaches.

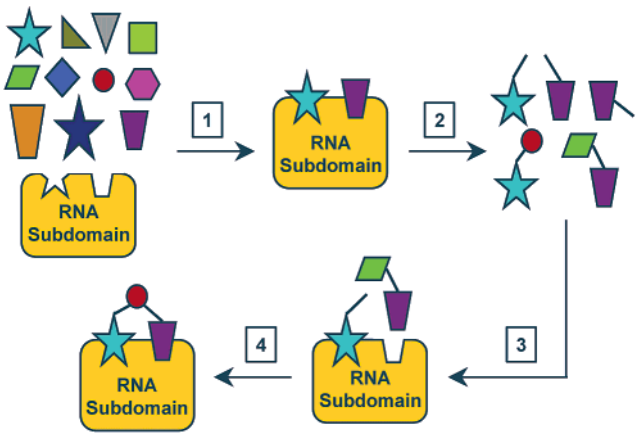

Figure 1. SAR by MS ligand-based lead discovery strategy. (i) A panel of motifs is screened for binding to an RNA subdomain, and ligands for the target are identified. Motifs that bind the target at different locations (purple trapezoid, blue star) are evident as a ternary complex in the MS assay. (ii) Simple derivatives of the most interesting ligands are then prepared. (iii) The compounds are then rescreened using the MS assay. From the changes observed in binding, extensive knowledge of the RNA target structure is obtained. For example, if a simple chemical change converts a pair of ligands from being concurrent binders to being competitive with each other, it implies that the two ligands now occupy overlapping spaces on the binding site. (iv) The aggregate information is used to guide the linking of motifs into a single structure with higher affinity for the target.

The SAR by MS method has been used to identify a new class of ligands for the 1061 region of bacterial 23S rRNA. This subdomain of the rRNA interacts with the protein L11 and is the site of binding for the antibiotic thiostrepton.[14] Our initial attempts at lead discovery for this antibacterial target via traditional HTS assays[15] afforded extremely low hit rates. Although a crystal structure of the RNA−protein interaction is available,[16] it is not amenable to traditional structure-based rational drug design approaches due to the large and complex nature of the interaction, making it an ideal target for the ligand-based approach offered by the SAR by MS strategy.

A screen of compound libraries[17] revealed two classes of motifs (Figure 2) that displayed an interesting SAR toward the U1061A RNA target[18] (Figure 3). For the D-amino acid class (series A), a positively charged side chain is clearly beneficial for binding, as unsubstituted and uncharged derivatives bound poorly. The N-substitution on the amino acid was less important, as A1, A5, and A6 all showed similar affinity. In the second class (series B), the quinoxalin-2,3-dione of B5 emerged as an important pharmacophore, as other aryl piperazines such as B6 showed no affinity. Furthermore, various substitutions off the quinoxalin-2,3-dione carboxyl group are tolerated, with larger substituents preferred, indicating that pendant groups can be attached at the carboxylic acid position.

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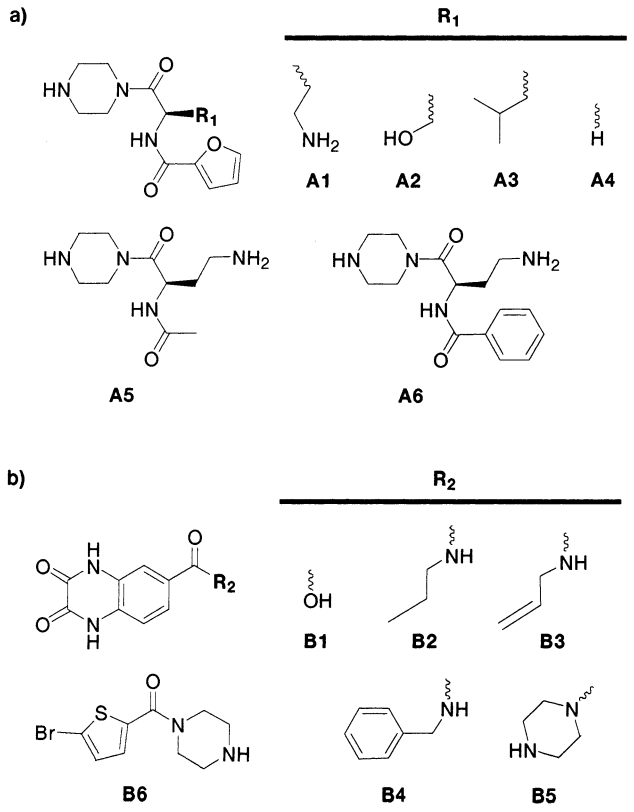


Figure 2. Structures of selected motif classes screened against the U1061A RNA. (a) D-Amino acid motifs (series A) and (b) quinoxalin-2,3-diones (series B).

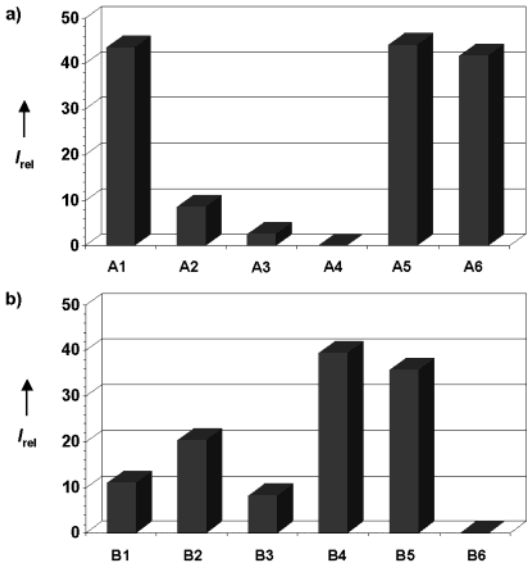


Figure 3. Binding affinity for a subset of motifs screened against the U1061A RNA chosen to highlight the observed SAR trends. Binding is expressed as normalized percent MS ion intensity of the RNA:ligand complex relative to the parent U1061A RNA such that a value of 100 indicates 50% of the target is bound by ligand. (a) 50 μM series A ligands + 2 μM RNA. (b) 150 μM series B ligands + 2 μM RNA.

To further study the spatial relationships of motif binding to the U1061A construct, MS competition experiments were performed between ligand classes that bind to the target when examined singly. Because A and B are structurally different, it was postulated that they bind at distinct sites on the target RNA. This was supported by a competition experiment between ligands

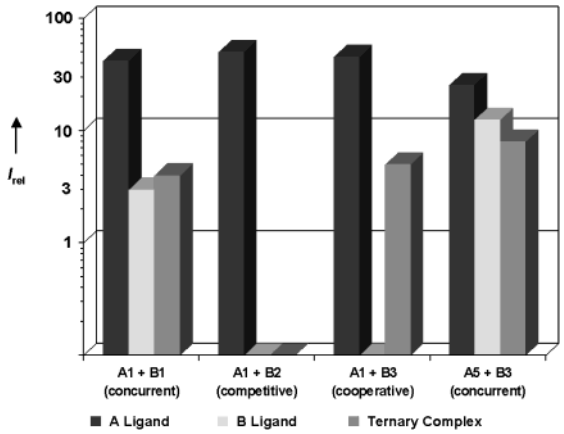


Figure 4. Competition experiments with key motifs. A1 and B1 bind concurrently to the target as the presence of the U1061A:A1:B1 ternary complex is evident. In contrast, A1 and B2 are competitive, with only the U1061A:A1 complex observed. A1 and B3 exhibit cooperative binding, as the U1061A:B3 complex is not observed in the presence of A1 while the U1061A:A1:B3 is clearly evident. A5 and B3 are concurrent but not cooperative. The propyl and allyl groups of B2 and B3 thus serve as molecular rulers, placing the optimal separation of ligands A1 and B1 at approximately 3−4 atoms.

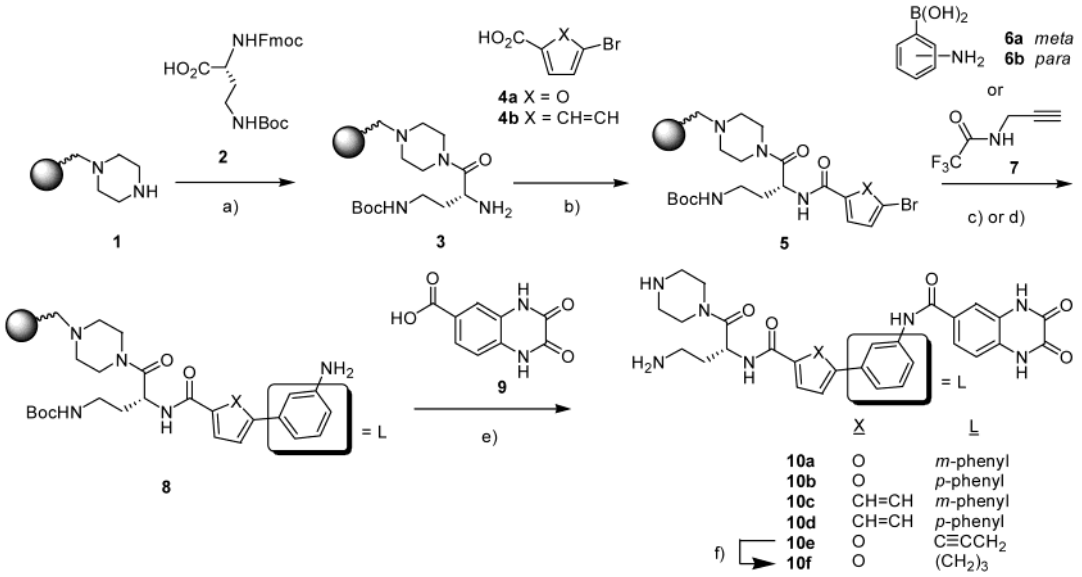
A1 and B1, in which a ternary complex consisting of the U1061A RNA, A1, and B1 was observed (concurrent binding, Figure 4). In contrast, the propyl-substituted ligand (B2) was completely displaced from the target RNA by the higher affinity A1, and no ternary complex was observed (competitive binding).

Interestingly, the allyl-substituted ligand (B3) forms the ternary complex U1061A:A1:B3 in the presence of A1, but no U1061A:B3 complex is observed. This is suggestive of cooperative binding, as B3 does not bind the target in a competitive setting unless A1 is also bound. A possible explanation for the differences found between the allyl- and the propyl-substituted ligands is that the alkene of the allyl group interacts favorably with the aromatic furan of A1, driving the formation of a ternary complex despite the close proximity of binding sites indicated by the competition of A1 and B2. This hypothesis is supported by the observation of concurrent, but not cooperative binding, between motifs A5 and B3, which indicates that the binding sites do not overlap with an acetyl substituent on the series A ligand.

The distance constraints derived from these simple competition experiments provides a “molecular ruler”, which suggests that the furan portion of A1 is separated by roughly three atoms from the carboxyl function of B1. The observed SAR for each ligand series, combined with results of the competition experiments, indicates that the aryl group of A1 and the amide moiety of B are available as potential linking positions. To test this hypothesis, several fused compounds were prepared via a solid phase synthesis employing palladium-catalyzed cross-coupling reactions as the key linker assembly step (Scheme 1). They were evaluated for both binding affinity to the U1061A target, as well as for their ability to inhibit bacterial transcription/translation in a cell-free functional assay (Figure 5).

The linked compounds **10** all bound markedly tighter to the target RNA than the parent motifs (K_D of 6−50 μM vs >100 μM for the motifs, as determined by MS[19]).

Scheme 1[a]



[a] Reagents and conditions: (a) Compound **2**, HATU/collidine/DMF, and then piperidine/DMF. (b) Compound **4**, HATU/collidine/DMF. (c) Compound **6**, $Pd(Ph_3P)_4/Na_2CO_3$/EtOH/DME, 70 °C. (d) Compound **7**, $Pd(PPh_3)_4$/CuI/THF/Et_3N, and then 10% K_2CO_3/H_2O. (e) Compound **9**, HATU/collidine/DMF, and then 5% triisopropylsilane/TFA. (f) H_2/Pd/C/MeOH (**10e** not isolated).

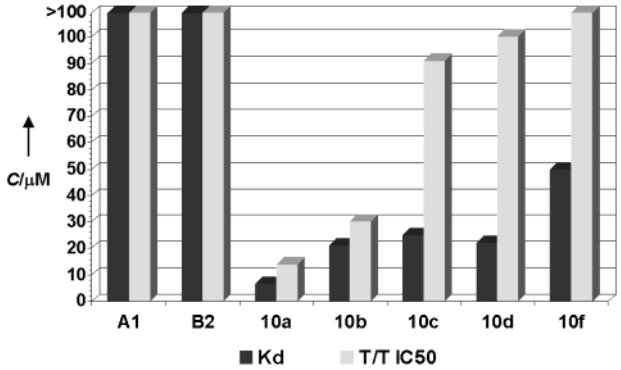


Figure 5. MS determined K_D values for U1061A RNA subdomain (determined as described previously[19]) and bacterial transcription/translation IC_{50} values (determined as described previously[20]) for selected structures.

The rigid biaryl-linked **10a** displays a 20-fold enhancement in affinity for the RNA target relative to the motif ligands, with a K_D of 6.5 μM. Furthermore, this compound has similar activity ($IC_{50} = 14\ \mu$M) in the related functional assay,[20] indicating that **10a** may bind to the target RNA in a manner that interferes with ribosomal function. Altering the orientation and/or structure of the linker in compounds **10b**−**d** resulted in a modest 4-fold reduction in binding. The increased flexibility of the RNA target relative to most proteins may allow it to accommodate these moderate changes in conformation. In contrast, binding of the more flexible **10f** is reduced almost 10-fold relative to **10a** and shows no functional activity up to 100 μM. This highlights the importance of providing a rigid framework for binding elements. For **10a**,**b**, the affinity and functional activity are well-correlated; however, this association does not hold for **10c**,**d**,**f**. The observed 3−4-fold reduction in functional activity relative to affinity may reflect differences in target structure and rigidity between the U1061A subdomain and the wild-type 23S rRNA target.

In conclusion, a ligand-based lead discovery strategy utilizing MS screening methods has been developed and demonstrated. The high-throughput and low cost of the MS assay, coupled with the ability to perform quantitative competition experiments between ligands of different chemical classes, make the technique extraordinarily useful. Application of the method has afforded a novel compound class with good affinity and functional activity for an RNA target, which failed to provide leads using conventional HTS approaches. These compounds can then serve as a starting point for more traditional lead optimization approaches. MS-based assay methods are suited to both nucleic acids and proteins,[21] and the SAR by MS method can be applied to targets that have proven intractable using more traditional lead discovery approaches.

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Supporting Information Available: Genreral procedures and synthetic details for the preparation of **A1**−**6**, **B2**−**5**, and **10a**−**d**,**f**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(18) The RNA used for screening was the U1061A mutant 58mer utilized for crystallization studies by Prof. Draper's lab and was kindly supplied by Prof. Draper. This U1061A substitution stabilizes the proper fold of the 58mer subdomain (ref 16a).

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