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A cross-linking approach to map small molecule-RNA binding sites in cells

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Abstract

Methods to identify RNAs bound by small molecules in cells are sparse. Herein, an advance to identify the direct RNA targets of small molecules in cells is described. The approach, dubbed Chemical Cross-Linking and Isolation by Pull-down to Map Small Molecule-RNA Binding Sites (Chem-CLIP-Map-Seq), appends a cross-linker and a purification tag onto a small molecule. In cells, the compound binds to RNA and undergoes a proximity-based reaction. The cross-linked RNA is purified and then amplified using a universal reverse transcription (RT) primer and gene-specific PCR primers. At nucleotides proximal to the binding site, RT “stops” are observed. This approach has broad utility in identifying and validating the RNA targets and binding sites of small molecules in the context of a complex cellular system.

Keywords

RNA; Small molecules; Target validation; Target profiling; Oncogenic microRNA

As aberrant expression and mutation of human RNAs are linked to diseases, RNA is a desirable target for small molecule therapeutics.^{1,2} Although it is difficult to drug RNA, salient examples of success include compounds that target bacterial riboswitches³ and human pre-mRNAs,^{4,5} identified from phenotypic screens. There are even fewer examples of the purposeful design of small molecules that directly engage RNA targets and modulate biological activity in cells, largely for RNA repeat expansions and microRNA (miRNA) precursors.⁶ As the RNA targeting area continues to evolve and develop, there is a need for approaches to define the *bona fide* targets of RNA-directed small molecules in intact living systems, to establish modes of action, and to facilitate development of improved chemical probes that could emerge into medicines.

Indeed, these types of methods have been well established for protein drug targets. For example, covalent approaches, such as Cross-Linking and ImmunoPrecipitation (CLIP), have been used to characterize RNA-protein complexes.⁷ In CLIP, proteins are cross-linked to their bound RNAs by irradiation with light. These studies have transformed the understanding of how proteins regulate RNA including their binding site localization and the

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Appendix A. Supplementary data

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exclusion and inclusion of exons. Activity-based protein profiling (ABPP) and related approaches can assign enzymatic activity across the proteome and elucidate “ligand-ability” on a proteome-wide scale.^{8,9}

Recently, covalent approaches for small molecule recognition of nucleic acids have been described. For example, by using derivatives of *cis*-platin, it was found that this important anti-cancer compound recognizes the ribosome and affects its activity.¹⁰ Modular approaches have also been developed to probe the RNA targets of designer small molecules, dubbed Chemical Cross-Linking and Isolation by Pull-down (Chem-CLIP).^{11,12} In Chem-CLIP, a chemical cross linker and biotin purification tag are appended to an RNA-binding small molecule (Fig. 1). When the small molecule binds an RNA, the cross linker is brought into high effective concentration and undergoes a proximity-based reaction.^{11,12} The covalent adducts can be purified from cells via biotin to identify the small molecule’s cellular targets. In previous studies, this approach has been used, for example, to map ligand binding sites onto repeating transcripts.¹³ In that application, total RNA was incubated with an antisense oligonucleotide complementary to the target of interest and RNase H, digesting the RNA target into fragments. Fragments that reacted with the Chem-CLIP probe were isolated with streptavidin beads and analyzed by RT-qPCR with a set of gene-specific primers.¹³

RNA repeat expansions, however, are atypical targets of small molecules. We therefore sought to develop a Chem-CLIP-based mapping approach for RNAs with more diverse composites of structures such as microRNA (miRNA) precursors, named Chem-CLIP-Map-Seq. To do so, we used the primary transcript of oncogenic microRNA-96 (pri-miR-96) as a test case (Fig. 1). The small molecule Targaprimir-96 (1) binds selectively to the Drosha processing site of pri-miR-96 and selectively inhibits its biogenesis in triple negative breast cancer (TNBC) cells and an *in vivo* xenograft model.¹⁴ The Chem-CLIP probe for Targaprimir-96 (2) and a derivative of 2 lacking the RNA-binding modules (control; 3) (Fig. 2) were synthesized and studied in MDA-MB-231 TNBC cells.

When MDA-MB-231 cells were treated with the Chem-CLIP probe 2 (50 nM), which is capable of cross-linking to pri-miR-96, mature miR-96 levels were reduced to a similar extent as the parent compound 1 (Fig. 3A). No effect was observed with control compound 3 (Fig. 3A). To confirm that both 1 and 2 reduced mature miR-96 levels by inhibiting Drosha processing, we measured pri-miR-96 levels by RT-qPCR, the results of which showed that both compounds increased the RNA’s abundance by ca. 1.5-fold at 50 nM (Fig. 3A).

Since miR-96 suppresses apoptosis in cancer cells by repressing the translation of Forkhead box O1 (FOXO1) transcription factor,¹⁵ the effects of 1, 2, and 3 on FOXO1 protein levels were measured in MDA-MB-231 cells. As expected, based on RT-qPCR analysis, only 1 and 2, but not 3, enhanced the expression of FOXO1 protein (Fig. 3B). To validate that the increase in FOXO1 levels was mediated by inhibition of miR-96, we studied the effects of 1 and 2 on all 15 miRNAs predicted to target the FOXO1 3’ untranslated region (UTR) by TargetScan (Fig. 3C). Only miR-96 was inhibited significantly, supporting that the increase in FOXO1 protein expression is due to 1’s and 2’s inhibitory effect on miR-96 biogenesis (Fig. 3C). To further probe 2’s selectivity for pri-miR-96, we performed an unbiased

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profiling experiment on 373 miRNAs expressed in MDA-MB-231 TNBC cells (based on Qiagen miRNA Human miRbase profiler HC). As shown in the volcano plot in Fig. 3D, miR-96 was the most affected, indicating the selectivity of covalent compound 2. Further, the levels of only ten other miRNAs were affected by > 25% and were statistically significant ($p < 0.05$). We have previously shown that binding small molecules and small molecules that cleave pri-miR-96 are selective.^{14,16} The modest selectivity with the covalent compound could be due to a variety of factors, most likely the non-specific reactivity of the chlorambucil module, as it is highly reactive. However, as is demonstrated in this study, cross-linking compounds are quite useful to define targets and target sites that the molecules directly engage.

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We next studied the ability of 1 and 2 to trigger apoptosis, i.e., reverse phenotype, in MDA-MB-231 by measuring Caspase activity. Analogous experiments were completed in healthy breast epithelial MCF-10A cells that do not aberrantly express miR-96. That is, miR-96 is expressed at such low levels in MCF-10A cells that it is not engaged by the target and therefore does not induce apoptosis.¹⁷ Both 1- and 2-treated MDA-MB-231 cells had increased Caspase activity indicative of apoptosis (Fig. 3E). Compound 3 had no effect on Caspase activity, as expected. Importantly, neither 1 nor 2 had any effect on MCF-10A cells (Fig. 3F). Thus, the covalent compounds show relative selectivity for cancerous over healthy breast cells.

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We previously studied the engagement of pri-miR-96 *in vitro* by incubating radioactively labeled pri-miR-96 RNA with 2 and then pulling down 2-pri-miR-96 adducts with streptavidin beads.¹⁴ Engagement of pri-miR-96 by 2 was observed, and no pull-down was observed with 3 which lacks RNA-binding modules.¹⁴ To further show that the RNA-binding modules drive recognition of the pri-miR-96 target, we previously completed an *in vitro* competitive (C-)Chem CLIP experiment in which pri-miR-96 was incubated with a constant concentration of 2 (1000 nM) and increasing amounts of 1. Indeed, a 1-dependent decrease in the amount of RNA pulled down by 2 was observed (Fig. S2).

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To the study the engagement of pri-miR-96 by 2 in cells, we previously pulled down the RNAs with which 2 reacted with streptavidin beads and quantified pri-miR-96's enrichment via RT-qPCR.¹⁴ A 5-fold enrichment of pri-miRNA-96 was observed in the pulled down fraction (as compared to the amount present in the sample prior to incubation with streptavidin beads) (Fig. S3). Akin to *in vitro* studies, we completed a cellular C-Chem-CLIP study to show that the RNA-binding modules are required for reaction. As expected for a selective interaction, the amount of pri-miR-96 pulled down decreased as a function of the concentration of 1.¹⁴

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Given the enrichment of pri-miR-96 in the pulled down fraction and 2's pro-apoptotic properties, we mapped 2's binding site within pri-miR-96 in cells. [Please note that for these experiments, a significantly higher concentration of 2 was required to afford sufficient material for analysis, 1000 nM or 20 times higher than that required to inhibit miR-96 biogenesis and trigger apoptosis.] After reverse transcription, ssDNA adaptors were ligated to the cDNA, which was then cloned into NEB's pminiT 2.0 vector and sequenced (Fig. 4A). Mirroring the predicted and validated (via nuclease mapping¹⁴) binding site, 2's

binding site was localized to nucleotides that are proximal to pri-miR-96's Drosha site (Fig. 4B). In particular, the most occupied binding site observed is a C residue four nucleotides upstream of the UU internal loop bound by 1 (54%; highlighted in red in Fig. 4). Two other reactions sites were observed: (i) a G residue in the GG internal loop that binds 1 (14%); and (ii) a U residue 13 nucleotides upstream of the UU internal loop that is part of the Drosha processing site (24%), in agreement with primer extension studies completed *in vitro* (Fig. S2). We note that differences between *in vitro* and cellular mapping experiments can be observed due to the presence of proteins in the latter. To support our cellular mapping experiments, we modeled 2 bound to pri-miR-96 (Fig. 4B). Indeed, the distance between 2's cross-linking module and the most reacted nucleotides match precisely. In Fig. 4C, we further analyzed the distances between chlorambucil's reactive Cl and N7 of guanine (3.3 Å and 2.8 Å), suggesting the alkylation of guanine's N7 by the cross-linking module.

We have developed a cleavage-based approach named small molecule nucleic acid profiling by cleavage applied to RNA, or RiboSNAP, that is complementary to Chem-CLIP-Map-Seq.¹³ In RiboSNAP, an RNA-binding small molecule is conjugated to bleomycin A5. Conjugation of the RNA binder to bleomycin A5 attenuates bleomycin's ability to bind and cleave DNA, directing it towards the desired RNA target. RiboSNAP was used to identify the binding site of 1 within pri-miR-96, or RiboSNAP-Map.¹⁸ As expected, both Chem-CLIP-Map-Seq and RiboSNAP-Map localized the binding site of 1 to pri-miR-96's Drosha site.¹⁸ Collectively, these studies demonstrate that Chem-CLIP-Map-Seq is a powerful method to investigate target engagement in cells as well as define small molecule binding sites within a cellular RNA target and can be used synergistically. Although herein the target was known from previous studies, Chem-CLIP-Map-Seq could be used in a target agnostic fashion by using universal primers and RNA-seq analysis, rather than gene-specific primers. Such an approach, however, would require an additional data set to identify specific target engagement (i.e., identify targets that non-selectively react with the cross-linking module): a competitive Chem-CLIP study in which cells are co-treated with the parent compound and the Chem-CLIP probe. *Bona fide* targets are depleted in the pulled down fraction as compared to treatment with the Chem-CLIP probe alone.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.
Secondary structure of pri-miR-96 with small molecule binding sites indicated (top) and the miR-96-FOXO1 pathway (bottom).

Fig. 2. Chemical structures of the compounds used in this study. Compound 1 is parent compound "Targaprimir-96"; 2 is the Targaprimir-96 Chem-CUP probe; and 3 is a control compound containing reactive and purification modules but lacking RNA-binding modules.

Fig. 3.

Effects of 1, 2, and 3 in MDA-MB-231 TNBC and MCF-10A (non[^]malignant mammary epithelial breast) cells. A, Effects of 1 and 2 (50 nM) on mature miR-96 and pri-miR-96 levels. B, Effects of 1 and 2 (50 nM) on FOXO1 protein expression. C, Profiling of miRNAs predicted to target *FOXO1* mRNA by TargetScan in 2-treated MDA-MB-231 cells. D, Volcano plot profiling the effect of 2 on all miRNAs that are expressed in MDA-MB-231 cells [blue circles are miRNAs predicted to regulate *FOXO1* mRNA by TargetScan, purple squares indicate the ten miRNAs that were affected by > 25% and were statistically significant ($p < 0.05$)]. E, Effects of 1, 2, and 3 on Caspase 3/7 activity, indicative of apoptosis, in MDA-MB-231 TNBC cells. F, Effects of 1, 2, and 3 on Caspase 3/7 activity, indicative of apoptosis, in MCF-10A normal breast epithelial cells.

Fig. 4. Mapping the binding sites of **2** within pri-miR-96 in MDA-MB-231 TNBC cells A, Scheme of amplification approach to identify the small molecule binding site in pri-miR-96. LM-PCR is ligation-mediated-polymerase chain reaction. B, *In cellulo* mapping of the binding site for **2** within pri-miR-96 in MDA-MB-231 TNBC cells. Percentages indicate the number of sequences observed terminating at the respective nucleotide shown in red. C, A close-up of the interaction of the cross-linking module with pri-miR-96 (the CG and GC base pairs). The distances between chlorambucil's reactive Cl and N7 of guanine are indicated as dotted lines.