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Discovery of a self-assembling small molecule that sequesters RNA-binding proteins

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ABSTRACT

Biomolecular condensates, also known as membraneless organelles, play a crucial role in cellular organization by concentrating or sequestering biomolecules. Despite their importance, synthetically mimicking these organelles using non-peptidic small organic molecules has posed a significant challenge. The present study reports the discovery of D008, a self-assembling small molecule that sequesters a unique subset of RNA-binding proteins. Analysis and screening of a comprehensive collection of approximately 1 million compounds in the Chinese National Compound Library (Shanghai) identified 44 self-assembling small molecules in aqueous solutions. Subsequent screening of the focused library, coupled with proteome analysis, led to the discovery of D008 as a small organic molecule with the ability to condensate a specific subset of RNA-binding proteins. *In vitro* experiments demonstrated that the D008-induced sequestration of RNA-binding proteins impeded mRNA translation. D008 may offer a unique opportunity for studying the condensations of RNA-binding proteins and for developing an unprecedented class of small molecules that control gene expression.

Membraneless organelles, also known as biomolecular condensates, are essential to cellular organization, concentrating or sequestering biomolecules to regulate biological reactions [1,2]. The extensive engagement of membraneless organelles in cellular functions has stimulated the creation of synthetic molecules emulating their characteristics [3-10]. These artificial structures, often leveraging self-assembling peptides, have provided new avenues for manipulating biological processes [11-20]. Nevertheless, the achievement of comparable outcomes with small non-peptidic organic molecules remains challenging [21-23]. Creating artificial membraneless organelles using small organic molecules offers simple models for recreating or controlling cellular events and unique opportunities for drug discovery.

A promising source of such small molecules is a collection of organic compounds that self-assemble or aggregate in aqueous solutions. To construct such a focused library, we initially selected 1000 divergent small molecules with molecular weights exceeding 700 Da, enriched in aromatic, hydrophobic, or symmetric structures, from a comprehensive collection of approximately 1 million compounds in the Chinese National Compound Library (Shanghai). Self-assembling properties of these molecules were profiled by a method we previously reported using two fluorescent probes, 8-anilino-1-napthlenesufonic acid (ANS) and Nile Red [24]. These environment-sensitive probes, typically nonfluorescent in aqueous solutions, experience fluorescence augmentation when encapsulated in hydrophobic pockets. Given the propensity of self-assembling or aggregating small molecules to form higher-order structures with multiple hydrophobic pockets, the observed increase in fluorescence intensity serves as an indicator for monitoring the presence of molecular condensates in aqueous solutions. Out of the initial 1000 molecules, 44 demonstrated a significant increase in the fluorescence of both probes in phosphate-buffered saline (PBS), prompting their selection for subsequent investigations.

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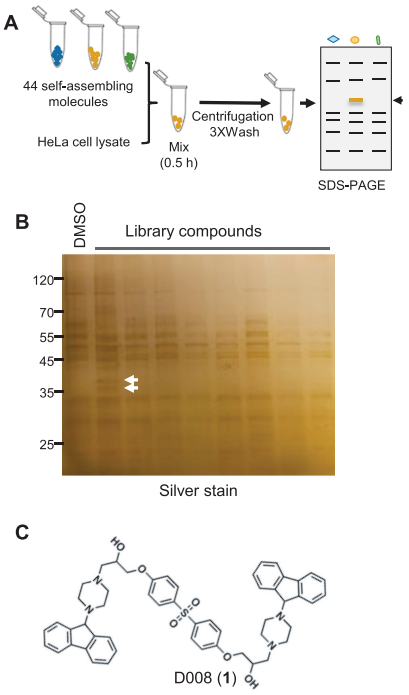

Fig. 1. Discovery of D008 (**1**). (A) Screening of self-assembling molecules. (B) A representing SDS-PAGE gel of the library screening. The arrows indicate bands specific to the D008 sample. (C) Chemical structure of D008 (**1**).

Naturally occurring membraneless organelles typically concentrate or sequester a subset of proteins. We examined the ability of the 44 molecules to interact with selective cellular proteins (Fig. 1A). Each compound (250 μmol/L) was incubated with HeLa cell lysates for 0.5 h. After centrifugation and extensive wash, the proteins co-precipitated with small-molecule assemblies were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Silver staining of the gels showed that the majority of the 44 self-assembling molecules displayed similar patterns of co-precipitated proteins, which either non-specifically interacted with the assembled materials or denatured during the experiments. One compound, D008 (molecule **1**), exhibited selective protein bands within the molecular weight range of 35–40 kDa (Figs. 1B and C). The structures of the compounds used in Fig. 1B are listed in Table S1 (Supporting information). For subsequent in-depth studies, we chemically synthesized D008 (**1**) and confirmed its ability to reproduce the screening result.

Dynamic light scattering (DLS) analysis of D008 confirmed its capability to form assemblies in both PBS and water (Fig. S1 in Supporting information). The diameters of D008 particles in PBS exhibited an increase with concentrations, reaching micrometer sizes (1000–2000 nm). In contrast, the particle diameter of D008 remained nearly constant (~200 nm) with increasing concentration in pure water. These characteristics were further corroborated by confocal microscopy, wherein Nile Red-stained D008 particles were detected in both PBS and water (Fig. S2 in Supporting information). Particle aggregation occurred in PBS with increasing concentrations, whereas dispersed nanoparticles were observed in water. This observation aligns with cryo-transmission electron microscopy (cryo-TEM) results, which depicted spherical particles of D008 in water and their aggregation in PBS (Fig. S3 in Supporting information). Collectively, these results suggest that D008 forms nanoparticles in aqueous solutions, and their higher-order assembly patterns are contingent upon solution conditions.

To identify D008-binding proteins at a proteome level, we performed a comprehensive liquid chromatography with tandem mass

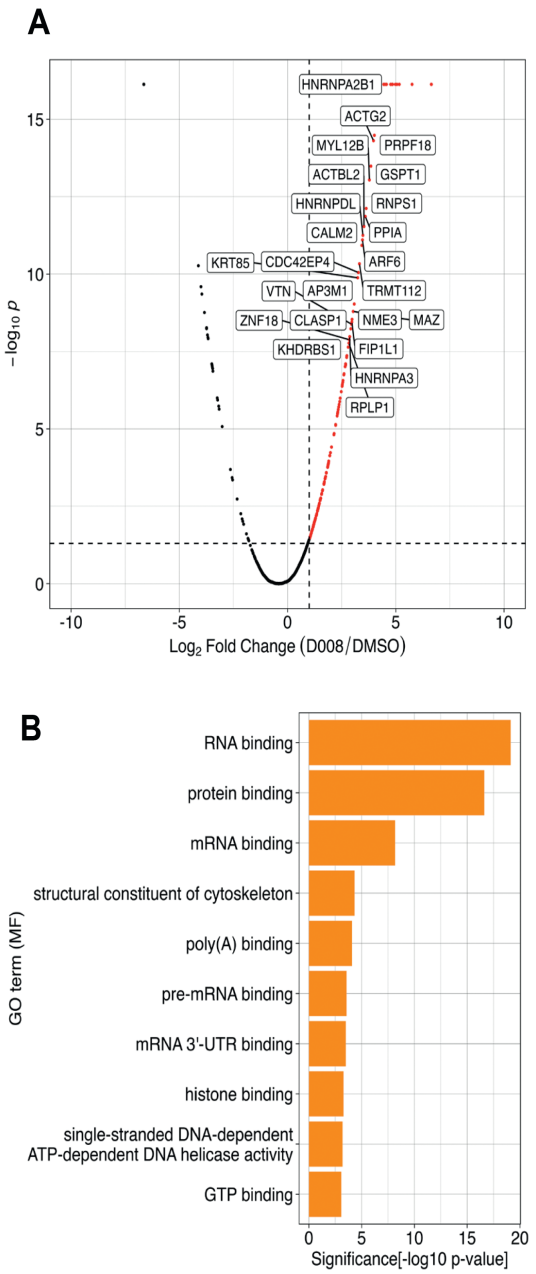

Fig. 2. LC-MS/MS analysis of D008-precipitated proteins. (A) Volcano plots ($\log_2$(D008/DMSO) *vs.* $-\log_{10}$(*P*-value)). Dashed line represents threshold (($\log_2$D008/DMSO) > 1, *P*-value < 0.05). Enriched proteins over the threshold are indicated as red dots. (B) GO enrichment analysis (molecular function) was conducted for the 174 proteins.

spectrometry (LC-MS/MS) analysis as outlined in Fig. S4 (Supporting information). HeLa cell lysates were treated with 50 μmol/L D008 in PBS, followed by centrifugation and washing. The proteins coprecipitated with D008 particles were analyzed by LC-MS/MS for label-free quantification. The criteria for protein enrichment were set as $\log_2$[D008/DMSO] > 1 and $-\log_{10}P$-value > $-\log_{10}0.05$, resulting in the identification of 174 enriched proteins (Fig. 2A, refer also to Supporting information Excel file 1). To gain insights into the functional relevance of the identified D008-binding proteins, a gene ontology (GO) enrichment analysis was performed. The analysis of the 174 D008-binding proteins revealed an enrichment of RNA-binding proteins (Fig. 2B).

Among the highly significant D008-binding proteins, we noticed hnRNPA2B1, an RNA-binding protein known to exhibit a dou-

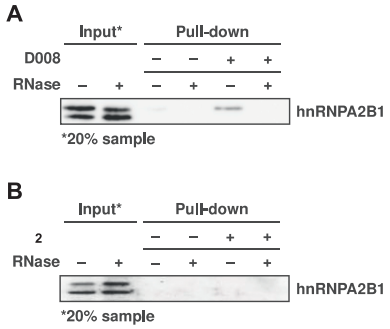

Fig. 3. The interaction of D008 and molecule **2** with hnRNPA2B1. The HeLa cell protein samples co-precipitated with D008 (A) or molecule **2** (B) were subjected to Western blot analysis using an antibody specific to hnRNPA2B1. The impact of RNA removal by RNase was also assessed.

blet band at 36 and 38 kDa on an SDS-PAGE gel, consistent with the bands observed during the library screening. hnRNPA2B1 plays essential roles in mRNA processing, export, translation, and the innate immune response [25-29]. To corroborate the binding of D008 to hnRNPA2B1, the protein samples coprecipitated with D008 were subjected to Western blot analysis using an antibody specific to hnRNPA2B1 (Fig. 3A). The results demonstrated the binding of D008 to hnRNPA2B1. Intriguingly, treatment with RNase abolished this interaction, suggesting the dependence on RNA for the observed binding. These results collectively suggest that D008 interacts selectively with a subset of mRNA-binding proteins, including hnRNPA2B1, and this interaction is mediated through RNA.

The interaction of D008 with RNA-binding proteins prompted us to examine its ability to modulate the translation of mRNA *in vitro*. The impact of D008 on translation efficacy was evaluated through a luciferase translation assay using the Human Cell-Free Protein Expression System (Fig. 4A) [30]. Human cell lysates were subjected to incubation with varying concentrations of D008, spanning from 50 μmol/L to 500 μmol/L, over a duration of 3 h. Subsequently, firefly luciferase mRNA was introduced into the lysate solution. Following an additional 3-h incubation to allow for luciferase translation, the level of luciferase expression within each cell lysate was estimated by measuring the intensity of luciferase luminescence. The results demonstrated a concentration-dependent reduction in luciferase translation by D008, indicating that D008 particles impede mRNA translation, at least within an *in vitro* cell-lysate environment, through the sequestration of RNA-binding proteins.

D008 has two hydroxyl groups attached to the stereocenters. To investigate their effects, we synthesized an achiral analog lacking hydroxyl groups (molecule **2**) (Fig. 4B). Its ability to modulate the translation of mRNA *in vitro* was similarly assessed by the luciferase translation assay (Fig. 4C). Human cell lysate was treated with 200 μmol/L of each compound, and the luminescence intensity was subsequently monitored. The results showed that molecule **2**, lacking hydroxyl groups, exhibited less activity than D008 (Figs. 4C and D), underscoring the importance of hydroxyl groups. DLS analysis of **2** in PBS revealed that it maintains the ability to form particles akin to D008 (Fig. S5 in Supporting information). To evaluate the binding affinity of **2** to hnRNPA2B1, protein samples coprecipitated with **2** were subjected to Western blot analysis. The results revealed no detectable interaction between molecule **2** and hnRNPA2B1 (Fig. 3B). These results suggest that the hydroxyl groups of D008 are dispensable for its self-assembly but play a crucial role in facilitating binding to RNA-binding proteins.

Encouraged by the *in vitro* results, we extended our study to evaluate the impact of D008 in cultured human cells. To monitor

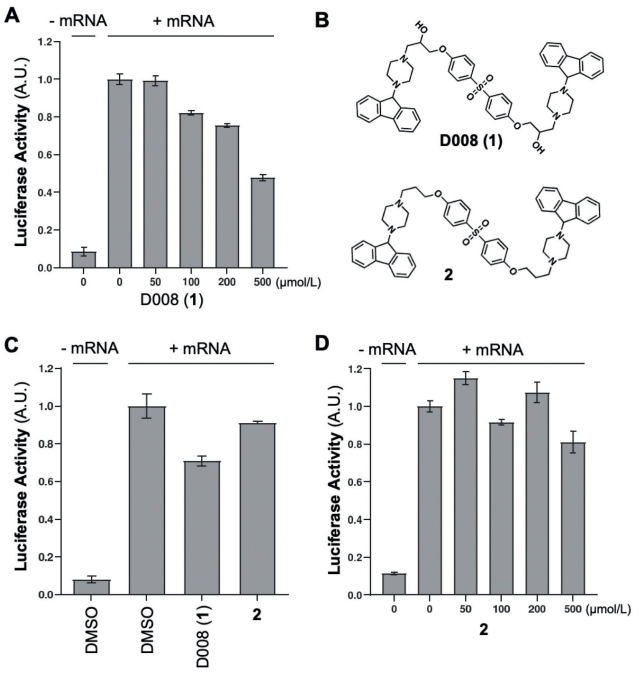

Fig. 4. Effects of D008 and its analogs on *in vitro* translation of mRNA. (A) Human cell lysates were subjected to incubation with varying concentrations of D008 (50 μmol/L to 500 μmol/L) for 3 h. Subsequently, firefly luciferase mRNA was introduced into the lysate solution. Following an additional 3-h incubation to allow for luciferase translation, the level of luciferase expression within each cell lysate was estimated by measuring the intensity of luciferase luminescence. (B) Chemical structures of D008 and **2**. (C) Effects of the hydroxyl group. Human cell lysate was treated with 200 μmol/L of each compound, and the luminescence intensity was subsequently monitored. (D) Effects of molecule **2** on the *in vitro* translation of luciferase mRNA at varying concentrations.

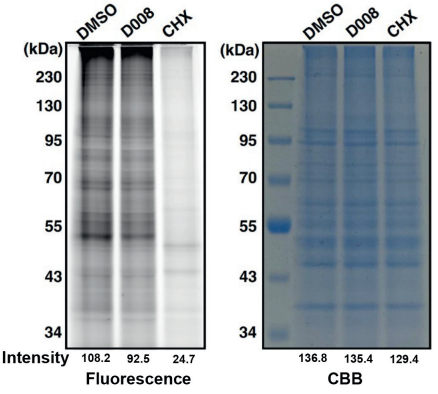

Fig. 5. SDS-PAGE analysis of newly synthesized proteins in the presence of 1% (v/v) DMSO, D008 (50 μmol/L), or cycloheximide (CHX, 50 μmol/L). The figure displays fluorescence scanning images (left) and Coomassie brilliant blue (CBB)-stained images (right). Mean intensities of bands are shown below.

the protein biosynthesis, we employed azidohomoalaine (AHA), an azide-containing methionine analog that is incorporated into newly synthesized proteins. Through a click reaction between the azido group in the incorporated AHA and an alkyne-tagged fluorescent molecule, the newly synthesized proteins can be visualized by fluorescent scanning of an SDS-PAGE gel [31]. While cycloheximide (CHX), a gold-standard translation inhibitor, diminished the fluorescent intensity of protein bands, D008 had no obvious effects (Fig. 5). However, a closer examination of the band intensities suggested that D008 had a marginal effect, if any, on protein translation. The limited cellular activity observed for D008 may be due to the challenges associated with the cell permeability of rapidly

formed particle aggregates of D008. Precise control over D008 self-assembly, including the development of a prodrug, would enhance the cellular activity of D008 in future investigations.

The present study discovered D008 as a self-assembling small molecule that sequesters a subset of RNA-binding proteins to impede mRNA translation. This discovery echoes McKnight and co-workers' serendipitous discovery of b-isox, a self-assembling molecule that forms hydrogel *in vitro* and co-precipitates with RNA-binding proteins containing RNA granule constituents [21]. Although the cellular activity of b-isox remains unexplored, the b-isox-induced RNA granule-like assemblies facilitated *in vitro* analysis of mRNA components within RNA granules [32]. A comparison of b-isox-binding proteins with D008-binding proteins reveals a certain level of overlap, but key RNA granule proteins, including G3GPs, ATXN2, and PABPs, were not found in D008-binding proteins (Fig. S6 in Supporting information). This observation suggests D008's potential utility in isolating a distinct set of RNA-binding proteins. The biological significance of this unique set of RNA-binding proteins remains undetermined. Nevertheless, the present identification of another self-assembling organic molecule that co-precipitates RNA-binding proteins may offer a unique opportunity for analyzing the condensations of RNA-binding proteins and for developing an unprecedented class of small molecules that control gene expression.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Hyoseok Kim: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Changyi Cui:** Methodology, Investigation, Formal analysis, Data curation. **Kohei Toh:** Methodology, Investigation, Formal analysis, Data curation. **Genyir Ado:** Methodology, Investigation, Formal analysis, Data curation. **Tetsuya Ogawa:** Methodology, Investigation, Formal analysis. **Yixin Zhang:** Investigation, Formal analysis. **Shin-ichi Sato:** Supervision. **Yong-Beom Lim:** Supervision, Funding acquisition. **Hiroki Kurata:** Supervision. **Lu Zhou:** Supervision, Resources, Project administration, Funding acquisition, Formal analysis. **Motonari Uesugi:** Writing – review & editing, Writing – original draft, Supervision, Resources, Funding acquisition, Formal analysis, Conceptualization.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2024.110135.

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