



Understanding the Exon Target Space of Splicing Modifying Compounds

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Background: Modulation of pre-mRNA splicing has emerged as an established therapeutic strategy, enabling selective regulation of gene expression through targeted exon inclusion. Small-molecule splicing modifiers such as Risdiplam and Branaplam promote inclusion of exons that are typically skipped due to weak 5' splice sites. Although these compounds show motif preferences (e.g., ANGA for Risdiplam and AGA for Branaplam), the determinants underlying their specificity remain incompletely understood, as many similar exons are not responsive. **Method:** We investigated the molecular basis of splicing-modulator specificity using biochemical assays, whole-transcriptome analyses, and genetic perturbation approaches. A cellular genetic perturbation system was developed to systematically reprogram U1 snRNA pairing across all possible exon endings and assess transcriptome-wide exon inclusion. Integrative analyses of sequence features, RNA structure, and regulatory elements were performed, alongside machine-learning modeling using an XGBoost classifier. **Results:** We identified primary sequence co-dependencies that shape RNA secondary structure and govern splice-site responsiveness to small-molecule induction. Reprogramming U1 snRNA demonstrated that splicing-modulator specificity can be altered. Transcriptome-wide profiling revealed thousands of inducible exons across multiple sequence classes, substantially expanding known targets. Additional determinants of inducibility included enhancer/silencer enrichment and splice-site accessibility. A predictive classifier accurately identified compound-responsive exons across different cell lines. **Conclusion:** This study provides a comprehensive mechanistic and predictive framework for understanding small-molecule splicing modulation. The findings enable improved target identification, assessment of on-mechanism effects, and rational design of next-generation splicing therapeutics.