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Bottom-up design of small molecules that stimulate exon 10 skipping in mutant *MAPT* pre-mRNA

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One challenge in chemical biology is to develop small molecules that control cellular protein content. The amount and identity of proteins are influenced by the RNAs that encode them; thus, protein content in a cell could be affected by targeting mRNA. However, RNA has been traditionally difficult to target with small molecules.^[1] In this report, we describe controlling the protein products of the mutated microtubule-associate protein tau (*MAPT*) mature mRNA (Figure 1) with a small molecule. *MAPT* mutations in exon 10 are associated with inherited frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17), an incurable disease that is directly caused by increased inclusion of exon 10 in *MAPT* mRNA.^[2] Recent studies have shown that mutations within a hairpin at the *MAPT* exon 10-intron junction destabilize the RNA's thermodynamic stability, increasing binding to U1 snRNP and thus exon 10 inclusion.^[2-3] Therefore, we designed small molecules that bind and stabilize a mutant *MAPT* using information about RNA-small molecule interactions.^[4] The optimal compound selectively binds the mutant *MAPT* hairpin and thermodynamically stabilizes its folding, facilitating exon 10 exclusion.

RNA is an important target of chemical probes due to its diverse cellular functions.^[1] For example, microRNAs target mRNAs for degradation or translational silencing to decrease the amount of a protein in a cell. Exon inclusion/exclusion affects which protein variants are produced and their relative concentrations. Deregulation of microRNA expression levels^[2] and single nucleotide polymorphisms that affect splice site selection^[3] are associated with disease. In particular, mutations in the *MAPT* pre-mRNA at the exon 10–intron junction deregulate pre-mRNA splicing and cause FTDP-17. These mutations thermodynamically destabilize an RNA hairpin structure that controls splice site selection (Figure 1).^[4,5] That is, the less thermodynamically stable RNA hairpins bind U1 snRNP to a greater extent, abnormally increasing exon 10 inclusion in the mature mRNA.^[4]

Antisense oligonucleotides have been successfully employed to affect pre-mRNA splicing in cells.^[6] However, alternative strategies are desirable. In our case, we chose to explore the

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therapeutic potential of small molecules that bind mutant *MAPT* mRNA as small molecules can have advantages over oligonucleotides.^[7] The development of small molecules targeting RNA is challenging, however, given that it is difficult to identify selective lead small molecules and that many small molecule collections (chemical screening libraries) are generally not enriched in RNA binders. In an effort to design small molecules that bind RNA and modulate function, our research group developed a bottom-up strategy in which we use a database of RNA motif (hairpins, internal loops, bulges, etc.)-small molecule interactions to inform lead compound design.^[4, 8] The interactions in the database are derived from two-dimensional combinatorial screening (2DCS) experiments,^[9] in which a small molecule library is probed for binding to a library of RNA motifs such as the bulge library^[10] shown in Figure 2. 2DCS selects the highest affinity and most selective RNA motifs that bind a small molecule. Rational design is enabled when a motif present in the target RNA's structure is also found in our database of RNA motif-small molecule interactions; the corresponding lead small molecules serve as lead compounds (Figure 2).

We queried the secondary structure of the exon 10-intron junction in a mutant *MAPT* pre-mRNA against our database of RNA-small molecule interactions. The mutant, known as DDPAC, has a C to U mutation at position +14 relative to the splice site donor (Figure 1). This single nucleotide change alters a GC base pair to a GU base pair, destabilizing the hairpin stem and leading to FTDP-17 via increasing exon 10 inclusion. We identified four lead compounds (**1** – **4**) that bind to an A bulge motif that is similar to the one in the *MAPT* hairpin (Figure 2). Ideally, these small molecules would bind the mutant RNA and thermodynamically stabilize it, thereby excluding exon 10 in the mature *MAPT* mRNA.^[2-3]

The binding affinities of **1** – **4** were measured for the A bulge present in DDPAC *MAPT* as its closing base pairs are different than the RNA motifs in the database (Table 1 and Figure 2). Compounds **1**, **2**, and **4** bind the A bulge with micromolar affinities while compound **3** does not bind with measureable affinity (Table 1 and Supplementary Figure S1).

The activities of compounds **1** – **4** were evaluated in a cell-based assay to assess if they affect protein production coupled to exon 10 inclusion. The DDPAC mini-gene was inserted upstream of firefly luciferase, which is translated in-frame with exon 10 (Figure 3A).^[3] That is, when exon 10 is included, luciferase is produced and mimics production of a tau protein that causes FTDP-17. Optimal compounds stabilize the DDPAC RNA, leading to exon 10 exclusion and a decrease in luciferase activity relative to an untreated control (Figure 3A). Interestingly, the compounds that bind the DDPAC RNA *in vitro* (**1**, **2**, and **4**) decrease luciferase production in the cell-based assay while compound **3**, which does not bind *in vitro*, has no effect on luciferase expression (Figure 3B). Moreover, the highest affinity compound, **2**, is also the most potent *in vivo*. Statistically significant decreases in luciferase expression were observed when cells were treated with 80 μ M of **1** (~40% reduction; $p < 0.05$) and with 30 μ M (~45% reduction; $p < 0.05$) or 60 μ M of **2** (~60% reduction; $p < 0.01$). A slight decrease in luciferase production (~20%) was observed when cells were treated with 100 μ M of **4**. The effect of **2** on protein production was further confirmed using a dual-luciferase assay in which cells were cotransfected with the DDPAC-luciferase construct and a plasmid encoding Renilla luciferase, which was used for normalization of firefly luciferase activity (Supplementary Figure S2).

Next, we confirmed that the decrease in luciferase activity caused by compound **2** is due to alteration of pre-mRNA splicing outcomes. Indeed, RT-PCR analysis shows that **2** alters the ratio of 4R (exon 10 inclusion; disease): 3R (exon 10 exclusion; healthy) (Figures 3A & 4). A dose response was observed; that is, decreasing exon 10 inclusion was observed as the concentration of compound increased. Statistically significant changes in the splicing ratio were observed when cells were treated with 60 μ M of **2**. We also confirmed the effect of **2** on alternative splicing by quantitative real-time PCR (qPCR), which mirrored the results of our dose response from end point RT-PCR (Supplementary Figure S3A and B). It should be noted that the efficacy/potency of **2** to improve the splicing of endogenous *MAPT* mutants could be different than the effect observed on the splicing of *MAPT* mini-genes. Indeed, we are currently further characterizing the effect of compound **2** and its derivatives on inhibition of exon 10 splicing derived from endogenous tau pre-mRNA.

One extremely important property of any small molecule that serves as a chemical probe of function or therapeutic is selectivity. We studied the selectivity of **2** both *in vitro* and *in vivo*. First, the affinity of **2** for two related *MAPT* hairpins was measured, the wild type (WT) hairpin and a DDPAC + I17T mutant (Figure 1). The latter mutant has an additional mutation in which the A bulge is converted to an AU pair. Compound **2** binds to the WT RNA and the DDPAC mutant with similar affinities (K_{ds} = 14 μ M and 10 μ M, respectively; Table 1) while binding to the DDPAC + I17T mutant was not detectable (K_d > 100 μ M). These results support that **2** binds the A bulge, as designed.

The *in vivo* selectivity of **2** was then studied in HeLa cells using mini-genes that express WT or DDPAC + I17T *MAPT* RNA. No statistically significant effect on exon 10 inclusion was observed when cells that express DDPAC + I17T were treated with **2** (Supplementary Figure S3 and 4), in agreement with binding studies. Interestingly, there is also no statistically significant effect on the alternative splicing of WT *MAPT* when cells were treated with 60 μ M of **2** (Supplementary Figure S3 and 4). This suggests that further stabilization of the WT RNA by **2** does not affect U1 snRNP binding. We also tested the effect of **2** on the alternative splicing of an endogenously expressed mRNA, *CAMKK2* (calcium/calmodulin-dependent protein kinase kinase 2).^[11] No effect was observed (Supplementary Figure S5). Taken together, these results indicate that **2** exhibits at least modest selectivity *in vivo*.

To investigate if **2** stabilizes the DDPAC *MAPT* mutant to a greater extent than WT *MAPT*, we completed optical melting experiments (Table 2). These studies show that the **2**-DDPAC RNA complex is 1.4 kcal/mol more stable than the RNA alone, corresponding to an increase in K_{eq} (or the folding of the hairpin) by ~10-fold at 37 °C. The enhancement in stability is traced to increased enthalpic contributions as the H° decreases by 26 kcal/mol whereas the entropic contribution is more unfavorable by 80 eu (Table 2). Only a small difference is observed in the T_m in the presence and absence of **2**, suggesting that full melting analyses should be completed to study the impact of ligand binding. The T_m 's may not reflect the effect of heat capacity on the system, which could afford a significant change in G°_{37} . Taken together with the measured binding affinity (Table 1) and cell-based assays (Figures 3 & 4), these data suggest that the compound's mode of action is binding and stabilization of the DDPAC *MAPT* mutant.

Likewise, we measured if **2** stabilizes the WT *MAPT* RNA. Even though **2** binds with similar affinity to the DDPAC and WT *MAPT* RNAs (Table 1), it has little effect on the stability of WT *MAPT*, only a ΔG_{37}° of -0.2 kcal/mol. (Table 2). These results suggest that no statistically significant change in alternative splicing of the WT *MAPT* is observed when cells are treated with **2** because small molecule binding does not significantly alter the RNAs' thermodynamic stability.

Small molecules have been identified that bind *MAPT* exon 10 hairpins by screening chemical libraries including aminoglycosides^[12] and mitoxantrone, which is used clinically to treat cancer.^[13] Mitoxantrone was further optimized for binding the *MAPT* RNA by using medicinal chemistry, which identified features that are important for binding.^[14] Dynamic combinatorial chemistry was also employed in which aminoglycoside and heterocyclic RNA binding modules were used.^[15] In each of these extremely important, pioneering studies, the activity of small molecules in cellular models of disease were not reported. A cell-based screen in which exon 10 inclusion/exclusion affected the production of reporter identified several cardiotonic steroids that affect pre-mRNA splicing. However, the steroids also differentially affected splicing of other transcripts, suggesting that activity may be due to multiple mechanisms that include binding to proteins involved in pre-mRNA splicing.^[16] Herein, we used a rational, bottom-up strategy to identify small molecules that target a pre-mRNA and induce exon skipping. These studies may validate our approach as a strategy to identify lead small molecules that control splice site selection for other pre-mRNAs. This rational approach may prove more expedient than traditional high-throughput screening.^[17]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. a Thomas JR, Hergenrother PJ. *Chem. Rev.* 2008; 108:1171–1224. [PubMed: 18361529] b Guan L, Disney MD. *ACS Chem. Biol.* 2012; 7:73–86. [PubMed: 22185671]
2. Calin GA, Croce CM. *Oncogene.* 2006; 25:6202–6210. [PubMed: 17028600]
3. Kole R, Krainer AR, Altman S. *Nat. Rev. Drug Discov.* 2012; 11:125–140. [PubMed: 22262036]
4. Jiang Z, Cote J, Kwon JM, Goate AM, Wu JY. *Mol. Cell. Biol.* 2000; 20:4036–4048. [PubMed: 10805746]
5. Donahue CP, Muratore C, Wu JY, Kosik KS, Wolfe MS. *J. Biol. Chem.* 2006; 281:23302–23306. [PubMed: 16782711]
6. Rayburn ER, Zhang R. *Drug Discov. Today.* 2008; 13:513–521. [PubMed: 18549978]
7. Velagapudi SP, Gallo SM, Disney MD. *Nat. Chem. Biol.* 2014; 10:291–297. [PubMed: 24509821]
8. Kumar A, Parkesh R, Sznajder LJ, Childs-Disney JL, Sobczak K, Disney MD. *ACS Chem. Biol.* 2012; 7:496–505. [PubMed: 22252896]
9. Childs-Disney JL, Wu M, Pushechnikov A, Aminova O, Disney MD. *ACS Chem. Biol.* 2007; 2:745–754. [PubMed: 17975888]

10. Tran T, Disney MD. Nat. Commun. 2012; 3:1125. [PubMed: 23047683]
11. Warf MB, Nakamori M, Matthys CM, Thornton CA, Berglund JA. Proc. Natl. Acad. Sci. U. S. A. 2009; 106:18551–18556. [PubMed: 19822739]
12. a Lopez-Senin P, Artigas G, Marchan V. Org. Biomol. Chem. 2012; 10:9243–9254. [PubMed: 23108673] b Varani L, Spillantini MG, Goedert M, Varani G. Nucleic Acids Res. 2000; 28:710–719. [PubMed: 10637322]
13. Donahue CP, Ni J, Rozners E, Glicksman MA, Wolfe MS. J. Biomol. Screen. 2007; 12:789–799. [PubMed: 17525136]
14. a Zheng S, Chen Y, Donahue CP, Wolfe MS, Varani G. Chem. Biol. 2009; 16:557–566. [PubMed: 19477420] b Liu Y, Peacey E, Dickson J, Donahue CP, Zheng S, Varani G, Wolfe MS. J. Med. Chem. 2009; 52:6523–6526. [PubMed: 19839622]
15. Lopez-Senin P, Gomez-Pinto I, Grandas A, Marchan V. Chemistry. 2011; 17:1946–1953. [PubMed: 21274946]
16. Stoilov P, Lin CH, Damoiseaux R, Nikolic J, Black DL. Proc. Natl. Acad. Sci. U. S. A. 2008; 105:11218–11223. [PubMed: 18678901]
17. Younis I, Berg M, Kaida D, Dittmar K, Wang C, Dreyfuss G. Mol. Cell. Biol. 2010; 30:1718–1728. [PubMed: 20123975]

Figure 1.

Deregulation of the alternative pre-mRNA splicing of *MAPT* causes FTDP-17. Single nucleotide changes at the exon 10-intron junction destabilize its hairpin structure, thereby increasing U1 snRNP binding and hence exon 10 inclusion.^[2-3] Small molecules that bind mutant exon 10-intron hairpins could increase their stabilities and effect exon 10 exclusion.

Figure 2.

Lead compounds for the DDPAC *MAPT* mutant were identified from a database of RNA motif-small molecule interactions. The interactions in the database were identified by Two-Dimensional Combinatorial Screening (2DCS), a small molecule library-vs.-RNA library selection approach.^[9] Compounds **1** – **4** were selected to bind an A bulge similar to that in the DDPAC *MAPT* mutant.^[10]

Figure 3.

Studies on the effect of compounds on exon 10 inclusion as determined using by a firefly luciferase reporter.^[3] (A) Schematic of the reporter. Luciferase is in-frame with exon 10. Therefore, luciferase activity is a measure of exon 10 inclusion (disease). Compounds that stabilize the DDPAC *MAPT* mutant should inhibit binding to U1 snRNP and result in decreased luciferase activity. (B) Quantification of luciferase activity from cells that express the DDPAC *MAPT* mutant in the presence or absence of compound. Data are reported as the average $\pm$ standard deviation (n=4). *, $p < 0.05$; **, $p < 0.01$

Figure 4. Splicing analysis of DDPAC *MAPT* mutant (C+14U) when cells are treated with different concentrations of **2**.^[3] **(A)** Representative gel image of exon 10 alternative splicing in the presence of compound **2**. “4R” indicates exon 10 inclusion (disease) while “3R” indicates exon 10 exclusion (healthy). **(B)** Quantification of RT-PCR analysis of exon 10 alternative splicing, reported as the ratio of 4R/3R. As expected, **2** significantly affects exon 10 inclusion. Data are reported as the average $\pm$ standard deviation (n=4). *, $p < 0.05$

Table 1

Binding constants of small molecules to various RNAs.

Binding of 1 - 4 to DDPAC (C+14U)	
Small Molecule	K _d (μM)
1	14 ± 2
2	10 ± 1
3	> 100
4	53 ± 8
Binding of 2 to DDPAC + I17T	
2	>100
Binding of 2 to Wild Type	
2	14 ± 2

Data reported are the averages and errors are standard deviations (n=3).

Table 2

The binding of compound 2 increases the thermodynamic stability of the DDPAC *MAPT* RNA to a much greater extent than WT *MAPT* RNA.

Cmpd.	H° (kcal/mol)	S° (eu)	G°37 (kcal/mol)	T _m (°C)
DDPAC MAPT Mutant (C+14U)				
None	-52±4	-160±11	-2.5±0.1	52
2	-78±3	-240±11	-3.9±0.1	53
Wild Type <i>MAPT</i>				
None	-64±3	-188±8	-6.1±0.4	69
2	-66±4	-193±13	-6.3±0.4	70

Data reported are the averages and errors are standard deviations (n=3).