

A high-throughput screening strategy identifies cardiotoxic steroids as alternative splicing modulators

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Alternative splicing has emerged as a promising therapeutic target in a number of human disorders. However, the discovery of compounds that target the splicing reaction has been hindered by the lack of suitable high-throughput screening assays. Conversely, the effects of known drugs on the splicing reaction are mostly unclear and not routinely assessed. We have developed a two-color fluorescent reporter for cellular assays of exon inclusion that can accommodate nearly any cassette exon and minimizes interfering effects from changes in transcription and translation. We used microtubule-associated protein tau (MAPT) exon 10, whose missplicing causes frontotemporal dementia, to test the reporter in screening libraries of known bioactive compounds. These screens yielded several compounds that alter the splicing of the exon, both in the reporter and in the endogenous MAPT mRNA. One compound, digoxin, has long been used in the treatment of heart failure, but was not known to modulate splicing. The positive compounds target different signal transduction pathways, and microarray analysis shows that each compound affects the splicing of a different set of exons in addition to MAPT exon 10. Our results identify currently prescribed cardiotoxic steroids as modulators of alternative splicing and demonstrate the feasibility of screening for drugs that alter exon inclusion.

digoxin | fluorescent reporter | plant steroids | MAPT | FTDP-17

Disruption of exon recognition and misregulation of alternative splicing are a common cause of human disease. Conditions linked to errors in pre-mRNA splicing include autoimmune disorders, neurodegenerative diseases, cystic fibrosis, growth hormone deficiency, muscular dystrophy, and cancer progression (1–3). Thus, the RNA splicing machinery is an important potential target for drug development. However, few drugs have been identified that specifically target the splicing reaction, and the impact of existing drugs on splicing regulation is not routinely examined.

One disease stemming from misregulation of alternative splicing is frontotemporal dementia with parkinsonism on chromosome 17 (FTDP-17) (4). FTDP-17 is caused by mutations affecting exon 10 of the microtubule-associated protein tau (MAPT) gene that increase inclusion of exon 10 in the mRNA. Exon 10 encodes the fourth microtubule-binding domain of the protein, and elevated levels of the four-domain form of tau evidently lead to neurofibrillary tangles and subsequent neurodegeneration. Thus, there is significant therapeutic interest in modulating the splicing of this exon. Multiple factors are known to affect MAPT exon 10 splicing (4–9). This combinatorial control is typical of alternative splicing patterns and points to the difficulties in developing splicing-targeted therapeutics. Interfering with individual factors often only partially affects a given splicing pattern, and each factor can affect many unrelated exons in addition to the therapeutic target.

Finding compounds that modulate alternative splicing requires effective high-throughput screening assays. Small molecules that alter the splicing of particular exons have been identified by using several strategies, including RT-PCR, reporters producing luciferase or GFP, and a topoisomerase I phosphorylation assay (10–14). Each of these assays has limitations in the high-throughput

screening of large chemical libraries. RT-PCR is costly and scales up poorly. Most *in vivo* splicing reporters have poor dynamic range or do not distinguish compounds affecting splicing from those altering transcription or translation. Two recent studies demonstrate the utility of dual color reporter systems in improving the dynamic range and discriminating changes in alternative splicing from changes in transcription or translation (15, 16). These systems may require modification of a test exon to adapt it to the reporter, which may change its regulatory properties.

We developed a sensitive dual-reporter system for alternative splicing that can accommodate most cassette exons and many alternative 5' and 3' splice sites. We report the implementation of this design, which uses red and green fluorescent proteins (RFP and GFP) as reporters to screen chemical libraries for molecules that modulate the splicing of MAPT exon 10. We identified structurally diverse compounds that modulate MAPT exon 10 splicing through different apparent molecular targets. Most notably, we find that cardiac glycosides, widely used in the treatment of heart failure and atrial fibrillation, are potent modulators of alternative splicing.

Results

Development of a Versatile Two-Color Splicing Reporter. A reporter system suitable for high-throughput screening of substances that modulate alternative splicing must meet several requirements. First, it should have a broad dynamic range, allowing measurement of small changes in exon inclusion even at the ends of the range when an exon is mostly included or mostly skipped in the mature transcripts. Second, it should distinguish changes in alternative splicing patterns from changes in transcription and translation and from general inhibition of splicing. Finally, the system should accommodate a variety of test exons from different genes. With these goals, we constructed a minigene that consists of ORFs for destabilized GFP and RFP expressed from a single promoter as a bicistronic transcript (Fig. 1A). The start codon of the GFP ORF is split between two constitutive exons by an alternative exon cassette with its flanking introns. If the alternative exon is skipped, the start codon of the GFP ORF is formed and GFP is expressed from the mature transcript. In this case, the translation of RFP is repressed because eukaryotic ribosomes initiate poorly at downstream ORFs. However, when the alternative exon is included in the mRNA, the GFP ORF loses its start codon and is not translated. The initiating ribosomes will scan for the first available AUG codon that resides in a Kozak consensus sequence, which is the AUG codon of the downstream RFP ORF. To achieve this, we mutated 10 AUG codons upstream of the RFP reporter [supporting information (SI) Table S1]. For two AUGs encoding methionines that

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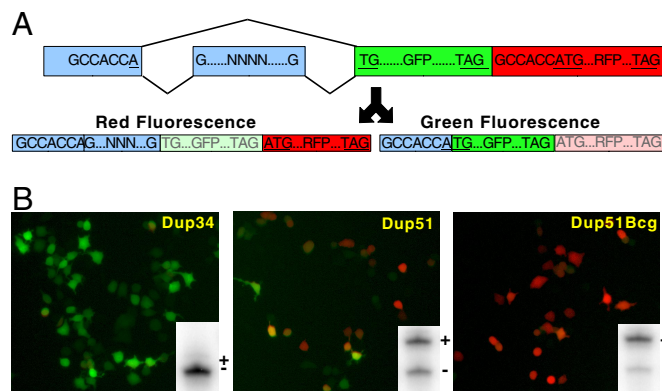


Fig. 1. Reporter design and testing. (A) Schematic of the reporter design used here, which accommodates exons without translation initiation codons and without a 3'-terminal adenosine. The initiation codon for GFP is split between the first and third exons of the reporter. This split results in GFP synthesis when the alternative exon is skipped and RFP synthesis when it is included. (B) Fluorescence microscopy of cells expressing three constructs, each with a different exon inclusion level. The exposures for the GFP and RFP fluorescence were constant across the three images. (Insets) The exon inclusion levels as detected by RT-PCR with the exon-included (+) and the exon-skipped (–) isoforms labeled.

were shown by our mutagenesis to be required for GFP fluorescence, we altered the surrounding nucleotides to remove any match to the Kozak consensus. It is possible that cytoplasmic RNA would be produced that retained one or both of the introns. To prevent RFP expression from these RNAs, we inserted translational start codons into the introns that produce short peptides unrelated to GFP or RFP and thus prevent translation of the two reporter proteins.

As a result of these modifications, two mRNAs are produced from the minigene, depending on the splicing of the alternative cassette. These mRNAs express GFP when the exon is skipped and RFP when the exon is included. Transcripts that retain intronic sequence should not express the fluorescent proteins, reducing the overall reporter expression, but the RFP/GFP ratio will still reflect the level of exon inclusion. Changes in the rate of transcription and translation will also affect the overall expression of GFP and RFP but should not alter the ratio of the mRNAs coding for the two proteins.

The design shown in Fig. 1A requires that the last nucleotide of the test exon is not adenosine and that the test exon does not contain AUG codons within a Kozak consensus sequence. The reporter can be modified to accommodate many other cassette exons, as well as certain mutually exclusive exons and 5' or 3' alternative splice sites. For example, changing the last nucleotide of the first exon to guanosine will allow the assay of exons with a 3' terminal adenosine (Fig. S1). This modification can also accommodate exons with a strong translation initiation codon after adjusting the GFP reading frame to allow translation from this AUG codon when the alternative exon is included in the mRNA.

To test the design, we created three related exons (Fig. S2) by fusing portions of exons 1 and 2 of the β -globin gene and cloned them into the reporter construct. Differences in size and mutations that alter their regulatory elements cause each of these exons to exhibit a different level of splicing in the mature transcript (Fig. 1B Insets). Cells transfected with these reporter constructs showed differences in fluorescent-protein expression corresponding to the inclusion level of the alternative exon. Cells expressing the Dup34 construct, in which the alternative exon is almost completely excluded from the mature transcript, make almost exclusively GFP. In cells transfected with Dup51, which shows intermediate levels of exon inclusion, GFP expression was lower and moderate amounts of RFP were observed. Note that transient expression of the

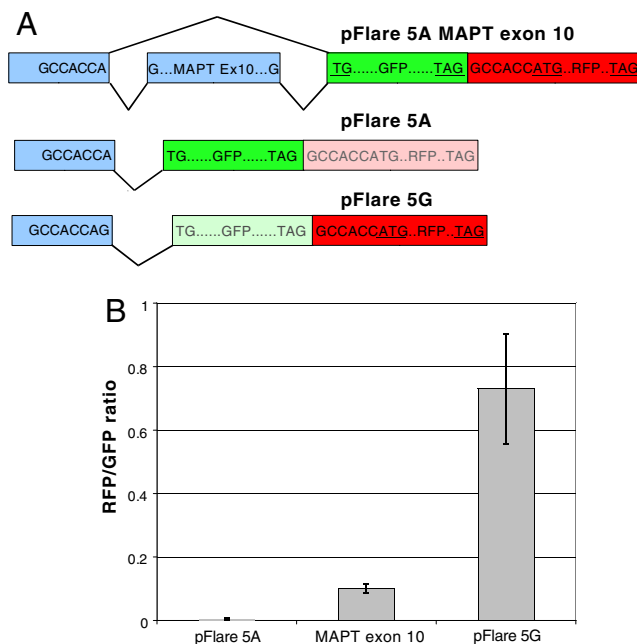


Fig. 2. Dynamic range of the assay. (A) The MAPT exon 10 reporter was compared to two single-intron reporters designed to produce only GFP (pFlare 5A) or RFP (pFlare 5G). (B) Bar plot showing the RFP/GFP ratio for the three reporters. The fluorescence intensity for the two proteins was measured on cell lysates from transient transfections and corrected for background fluorescence as described in *SI Materials and Methods*.

reporters can lead to significant cell-to-cell variation in the protein signals, which we attribute to differences in the stability of the two proteins and in the amount of DNA taken up by each cell. This variability is reduced in stable cell lines expressing the reporter and with reporters where the stability of the two proteins is equalized (see *Discussion* and Fig. S3). Finally, Dup51Bcg-transfected cells, where the alternative exon is mostly included in the transcript, primarily express RFP with only low levels of GFP (Fig. 1B).

Using the Reporter for Screening Chemical Libraries. We chose exon 10 of the human MAPT gene as a test exon for screening. We created three reporter clones (Fig. 2A). pFlare5A MAPT exon 10 contains exon 10 of the MAPT gene as the alternative exon cassette. Two other clones, pFlare5A and pFlare5G, contain a single intron with no alternative exon and are used to define the dynamic range of the assay. Splicing of the pFlare5A transcript creates the GFP start codon, allowing GFP expression. pFlare5G carries an additional G nucleotide at the end of the first exon to eliminate the AUG for GFP and produce only RFP. These clones were used to develop a fluorimetric assay that can be performed on 384-well cell-culture plates (see *Materials and Methods* and *SI Materials and Methods*).

The reporter minigene expresses GFP and RFP in a mutually exclusive manner, depending on the splicing of the alternative exon cassette, allowing the splicing to be monitored as the ratio of the RFP to GFP fluorescence intensities. When the fluorescence-intensity ratio is used to measure the splicing of the reporter, the dynamic range of the assay is the product of the dynamic ranges of the individual reporter proteins. The difference between the two end points of the assay produced by pFlare5A and pFlare5G is ≈ 140 -fold, compared with a difference of 8- to 15-fold when a single reporter protein is monitored (Fig. 2B). The MAPT exon 10 construct showed partial exon inclusion and an intermediate fluorescence ratio. To confirm that changes in exon 10 splicing could be assayed by the fluorescence ratio, we cotransfected the reporter

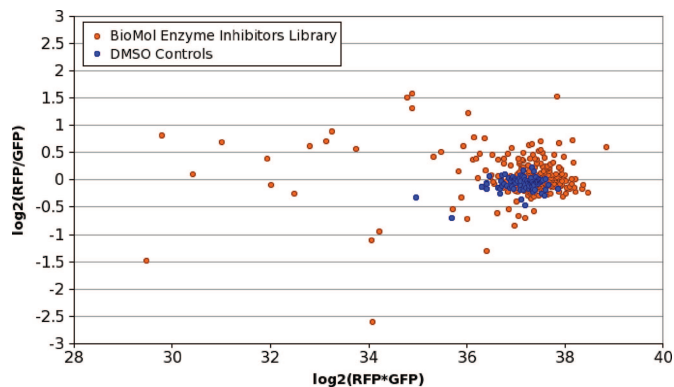


Fig. 3. Biomol library screen. LOWESS-smoothed scatter plots of the log-ratio vs. the log-expression for each compound (orange dots) and the DMSO controls (blue dots). Compounds that change splicing change the log-ratio of the RFP/GFP fluorescence intensities. Compounds that affect overall gene expression change the log-expression but not the log-ratio.

with an expression vector for the protein Tra2-beta1, which is known to positively regulate MAPT exon 10. We found that the RFP/GFP ratio and the splicing of the reporter responded as predicted (data not shown).

To screen for compounds that alter MAPT exon 10 splicing, we created a stable HEK293 cell line expressing the MAPT exon 10 reporter. This cell line showed partial inclusion of exon 10 and the expected fluorescent signals (Fig. S3). We used this line to screen two libraries: a 1,100-compound library (Prestwick) of Food and Drug Administration (FDA)-approved and other drugs and a smaller 340-compound library (BioMol) consisting of enzyme inhibitors and ion-channel antagonists. After plating in 384-well plates, the cells were incubated with the BioMol library for 20 h or with the Prestwick library for 48 h and the fluorescence intensities were determined. The Prestwick library is generally less toxic than the Biomol library, allowing for a longer incubation with the cells. The background intensities were determined for each compound by applying the two libraries to plates containing the parental HEK293 cell line.

Fig. 3 and Fig. S4 show locally weighted least squares (LOWESS)-smoothed scatterplots of the logarithm (base 2) of the RFP/GFP fluorescence intensity ratio (log-ratio) for each compound plotted against the product of the GFP and RFP intensities (log-expression). The DMSO controls cluster within a narrow expression range with a log-ratio close to zero. Most of the compounds in the libraries are grouped close to the DMSO controls. Compounds that change the ratio of RFP/GFP fluorescence are identified by their divergence along the y axis on these plots. Importantly, these compounds are distinguished from compounds that change the overall expression of the reporter and thus diverge along the x axis.

The compounds identified as inducing a significant change in the RFP/GFP ratio are listed in Table S2. These compounds include a significant number of molecules that inhibit transcription (etoposide, doxorubicin) and translation (camptothecin, cephaline, puromycin), as well as compounds that are toxic at the concentrations used in the screen. Thus, some compounds that generally inhibit gene expression can alter the RFP/GFP ratio. We found that this ratio change is due, at least in part, to the different half lives of the destabilized GFP and RFP proteins (Fig. S5A). The more rapid depletion of GFP after the inhibition of transcription or translation results in an increased RFP/GFP ratio. This problem can be corrected either by removing the PEST sequences used to destabilize the reporter proteins (Fig. S5A) or by using destabilized yellow and cyan fluorescent proteins, which exhibit similar degradation rates, instead of GFP and RFP (data not shown).

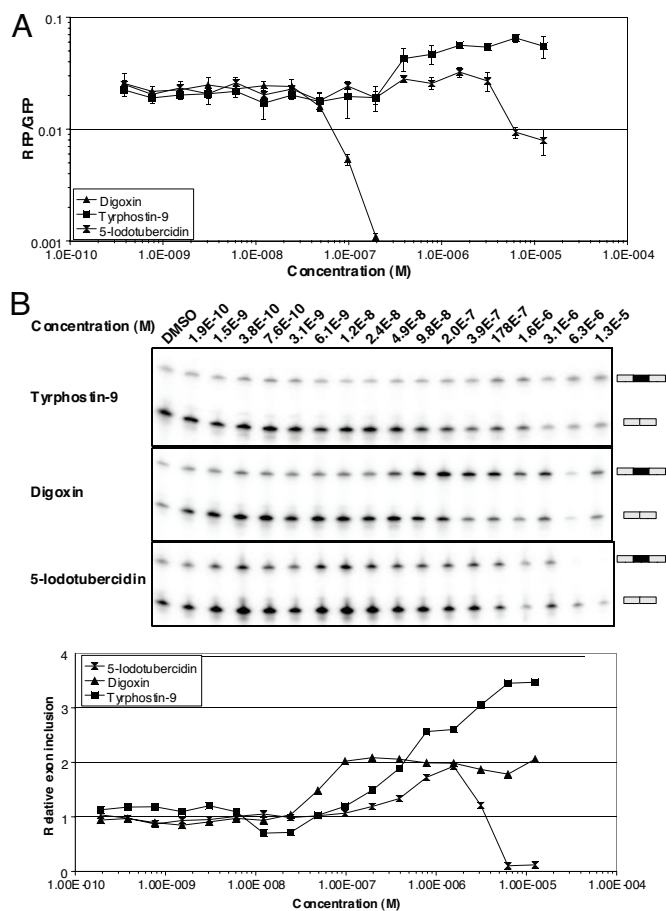


Fig. 4. Dose-response curves. Cells carrying the MAPT exon 10 reporter were treated with increasing amounts of digoxin, tyrphostin-9, and 5-iodotubercidin. (A) Effect of the drugs on the RFP/GFP ratio. (B) Effect of the drugs on the splicing of the MAPT exon 10 reporter relative to the DMSO control as determined by RT-PCR. Tyrphostin-9 caused increased exon inclusion at concentrations >400 nM, 5-iodotubercidin caused increased exon inclusion at concentrations between 800 nM and 1.6 μ M and exon skipping at concentrations >3 μ M, and digoxin caused increased exon inclusion at concentrations >50 nM.

Transcription inhibitors will also lead to false positives if the stabilities of the two mRNA isoforms differ. To test this possibility, we blocked transcription with α -amanitin and assayed the reporter mRNAs by low cycle RT-PCR (Fig. S5B). We found that the exon-10-containing isoform does indeed decay faster than the skipped isoform. As a result, the ratio of the two isoforms shifts after inhibition of transcription, mimicking a change in splicing. This shift can be controlled for in secondary assays, as described in the Discussion. The transcript stability will likely vary between test exons and will need examination in each new application. However, even with these potential sources of false positives, the screen identified a series of potential splicing regulators.

We selected 40 compounds for further evaluation (see Table S2). We performed dose-response experiments with these compounds, monitoring the RFP/GFP ratio by fluorimetry (Fig. 4A) and the splicing of the reporter by RT-PCR (Fig. 4B). Parallel samples were assayed for cell viability by luminometry by using ATP-Glo (Biotium). We found 10 compounds that changed the RFP/GFP ratio in the nanomolar or low micromolar range without having a significant impact on the cell viability (Table S2). A group of four compounds, all of them cardiotonic steroids (Table 1 and Table S2), decreased the RFP/GFP ratio, almost completely eliminating the RFP expression at concentrations of 10–700 nM, depending on the compound (Fig. 4A and data not shown). Another five compounds,

Table 1. Compounds that modulate the splicing of MAPT exon 10

Compound	Effect on the reporter	EC50, μM	Class
Digoxin*	Increased exon inclusion	$\approx 50 \times 10^{-3}$	Cardiotonic steroids
Lanatoside C*	Increased exon inclusion	$\approx 100 \times 10^{-3}$	Cardiotonic steroids
Digitoxigenin*	Increased exon inclusion	$\approx 200 \times 10^{-3}$	Cardiotonic steroids
Ouabain*	Increased exon inclusion	$\approx 20 \times 10^{-3}$	Cardiotonic steroids
Tyrphostin 9	Increased exon inclusion	≈ 1	Tyrphostines
Tyrphostin AG879	Increased exon inclusion	≈ 1	Tyrphostines
5-Iodotubercidin	Inhibits splicing <i>in vivo</i>	>50	Purine ribonucleoside, nucleotide analogue
	Exon skipping	≈ 3	
Rottlerin	Increased exon inclusion	ND	Benzopyranes
Gossypol	Inhibits splicing <i>in vivo</i> and <i>in vitro</i>	≈ 20	Sesquiterpenes
2',4'-Dichlorobenzamil	Inhibits splicing <i>in vivo</i> and <i>in vitro</i>	≈ 40	Pyrazines (amilorides)

*These compounds cause decrease of the RFP/GFP ratio in contrast to their effect on the splicing of MAPT exon 10.

including several kinase inhibitors, increased the RFP/GFP ratio (Fig. 4A and data not shown). Finally, the kinase inhibitor 5-iodotubercidin caused a weak increase in the RFP/GFP ratio at concentrations between 0.7 μM and 1.2 μM and decreased it significantly at higher concentrations (Fig. 4A).

To confirm that the changes in RFP/GFP ratio were because of changes in exon inclusion, we performed RT-PCR experiments. We found that four compounds, including tyrphostin-9 and 5-iodotubercidin, changed the splicing as predicted by the change in the ratio of the two fluorescent proteins (Fig. 4B, Table 1, and data not shown). Three compounds, gossypol, dichlorobenzamil, and AG-879, caused accumulation of unspliced or partially spliced mRNA (Table 1 and data not shown). These compounds are apparently general inhibitors of splicing. To investigate this inhibition, we performed *in vitro* splicing assays by using HeLa nuclear extracts treated with the drugs (data not shown). Both gossypol and dichlorobenzamil inhibited splicing *in vitro* at concentrations similar to those that lead to intron accumulation *in vivo*. AG-879 did not have any discernable effect on splicing *in vitro*.

The performance of high-throughput screening assays is often measured by the Z-factor (17). Unlike most assays, the fluorescence ratio from our reporter can shift in either direction depending on whether exon inclusion increases or decreases. We determined two Z-factors by using 1 μM tyrphostin-9 and 6 μM 5-iodotubercidin as positive controls for increased and reduced exon inclusion, respectively. Because of the relatively low basal level of inclusion of MAPT exon 10 in our reporter, the assay performs better in detecting compounds that cause increased exon inclusion (Z factor = 0.57 for tyrphostin-9), although compounds that decrease exon inclusion can also be assessed (Z factor = 0.18 for 5-iodotubercidin). For other test exons that show primarily inclusion, the assay will perform better for exon skipping.

Cardiotonic Steroids Affect both Splicing and Translation. Four cardiotonic steroids (digoxin, lanatoside C, digitoxigenin, and ouabain) all caused a decrease in the RFP/GFP ratio and were predicted to induce exon skipping (Fig. 4A and data not shown). However, the RT-PCR analysis showed that these compounds increased exon inclusion at similar concentrations to those that decrease the RFP/GFP ratio (Fig. 4B and data not shown). The sharp drop in RFP expression induced by the cardiotonic steroids could result from the inhibition of translation initiation from the far downstream start codon for RFP. To test the expression from this long 5' UTR we examined single intron reporters (Fig. 5A). The construct pFlare5A MAPT Δ I2 generates an mRNA identical to the mRNA that includes exon 10 from the alternative splicing reporter. This transcript will still require translation initiation from the downstream AUG codon (Fig. 5A). The second reporter also lacks alternative splicing and expresses GFP from the upstream

AUG codon, similar to the exon-skipped mRNA generated by pFlare5A MAPT exon 10. We cotransfected these two reporter constructs into HEK293 cells and repeated the dose-response experiments measuring the RFP/GFP ratio. Tyrphostin-9 and 5-iodotubercidin had no effect on the ratio of the two fluorescent proteins, indicating that they do not affect the translation or stability of the two mRNAs (Fig. 5B). In contrast, digoxin caused a sharp drop in the RFP/GFP ratio (Fig. 5B). Similar effects were observed with the other cardiotonic steroids (data not shown). Thus, these compounds may inhibit ribosome initiation at distal AUG codons and thus reduce translation of the downstream RFP reading frame.

Compounds affecting ribosome scanning represent a potential source of false positives in the screen for splicing modulators; however, the cardiotonic steroids also clearly affect the splicing of the reporter in the RT-PCR assays (Fig. 4B) and cause up to a 3-fold increase in exon inclusion when applied in concentrations of 100 nM to 1 μM . Digitoxin and digoxigenin, two compounds that belong to the same structural class but were not present in the compound libraries, were also tested for their effect on splicing.

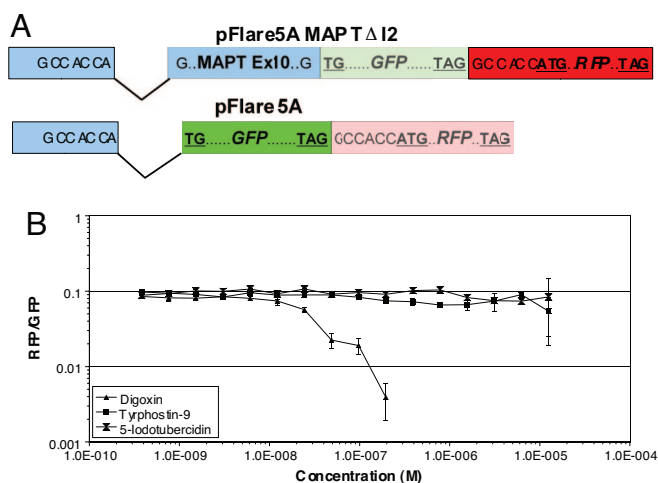


Fig. 5. Digoxin affects the use of downstream translation initiation codons. (A) Schematic diagrams of the two single-intron reporters used to test the effect of the drugs on the translation efficiency. MAPT Δ I2 was derived from the MAPT exon 10 reporter by deleting the second intron. It produces exon 10 containing mRNA equivalent to that generated by the MAPT exon 10 reporter and will result exclusively in RFP expression. The pFlare5A reporter produces exclusively mRNA equivalent to the exon-skipped isoform from the MAPT exon 10 reporter. (B) Dose-response curves of the RFP/GFP fluorescence in cells transiently cotransfected with the two reporters and treated with increasing amounts of digoxin, tyrphostin-9, and 5-iodotubercidin for 16–20 h.

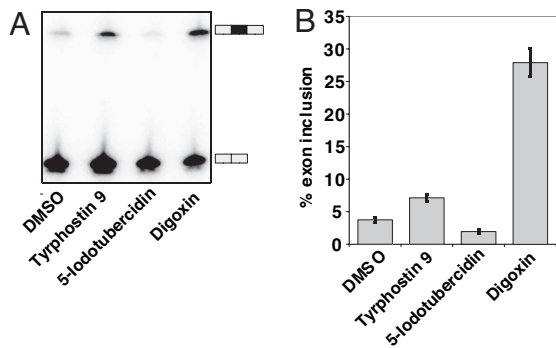


Fig. 6. Effect of digoxin, tyrphostin-9, and 5-iodotubercidin on the splicing of the endogenous MAPT exon 10. Illustrated is RT-PCR showing the splicing of the endogenous MAPT exon 10 in SHSY-5Y cells. (A) Tyrphostin-9 (1.6 μ M) and digoxin (100 nM) cause increased exon 10 inclusion in the mature transcript, whereas 5-iodotubercidin (1.6 μ M) results in increased exon skipping compared with the DMSO controls. (B) Quantification of the splice variants shown in A.

These compounds also lead to increased exon inclusion combined with a decrease in the RFP/GFP ratio (data not shown).

Splicing of the Endogenous MAPT Exon 10 Is Modulated by the Discovered Compounds. We next needed to confirm that the discovered compounds alter the splicing of the endogenous MAPT transcript as they do the minigene transcript. To examine the endogenous MAPT, we treated SHSY-5Y neuroblastoma cells with 1.6 μ M tyrphostin-9, 1.6 μ M 5-iodotubercidin, and 100 nM digoxin for 20 h. RT-PCR analysis showed that tyrphostin-9 and digoxin increased the inclusion of MAPT exon 10 by 2- and 7-fold respectively (Fig. 6), similar to what was seen with the minigene. Treatment with 1.6 μ M 5-iodotubercidine resulted in a 2-fold decrease in exon inclusion (Fig. 6 A and B). This result was in contrast to the increased exon inclusion observed with the minigene in HEK293 cells at this drug concentration but similar to the effect on the minigene at higher concentrations. This altered effect could be because of differences between the endogenous gene and the minigene affecting expression at either the DNA or RNA level or could result from differences in the signaling pathways active in the HEK293 and SHSY-5Y cell lines. It is clear that all three drugs can alter exon inclusion *in vivo*.

Digoxin, Tyrphostin, and 5-Iodotubercidin each Alter Different Subsets of Exons. Substances modulating the splicing of MAPT exon 10 are likely to affect the splicing of other alternative exons. We used splicing sensitive microarrays to identify larger sets of alternative exons in SHSY-5Y cells, whose inclusion is modulated by digoxin, tyrphostin-9, or 5-iodotubercidin. The results indicate that each drug alters the splicing of multiple exons (Table S3). To validate some of these exon targets and determine the false-positive rate of the microarray experiments, we performed RT-PCR analysis on 27 exons showing digoxin-dependent splicing changes on the microarray (Table S4). The splicing of 24 of these exons changed as predicted from the microarray experiments, yielding a validation rate of 89%.

The target exon sets for each compound were distinct but overlapping (see Fig. S6 for examples). Some exons common between the two target sets showed increased inclusion with one drug but increased skipping with another (Table S5). The diversity of exon targets and responses indicate that each drug is acting through a different molecular mechanism.

Discussion

In the course of developing a screen for compounds that alter splicing, we found that digoxin, a drug used in medicine for >200

years, is modulating processes of gene expression that were previously unrecognized. These effects on gene expression will now need to be considered when assessing the physiological effects of this compound. Cardiotonic steroids are high-affinity ligands and inhibitors of the Na^+/K^+ ATPase (18). These drugs lead to increased intracellular calcium concentrations and have long been used in the treatment of heart failure and atrial fibrillation. Recently, endogenous cardiotonic steroids have been shown to regulate blood pressure, gene expression, cell proliferation, and apoptosis. In agreement with our results, a recent report describes a dual effect of cardiotonic steroids on the translation and alternative splicing of the endothelial tissue factor mRNA (19). Interestingly, at the concentrations that alter splicing, these drugs activate several downstream signaling pathways within the cell (20). One pathway results in a slow oscillation in the level of intracellular Ca^{2+} (21). A second begins with the activation of a Src-kinase pool associated with the Na^+/K^+ ATPase (22). Src in turn phosphorylates the EGF receptor to activate the downstream Ras/Raf/MEK/ERK pathway. Both elevated intracellular Ca^{2+} levels and the ERK pathway have previously been shown to regulate the splicing of particular exons (23, 24). A large number of exons are affected by digoxin, including exons in RNA binding proteins (Tables S3 and S4). Modulation of the regulatory loops for splicing factor arginine/serine rich 3, splicing factor arginine/serine rich 5, and hnRNP D by signal transduction pathways is one way by which digoxin and other drugs could affect large sets of exons (25–27).

The EC_{50} for digoxin in our tissue culture assay (50 nM) is comparable with that seen in a patient's heart (108–142 nmol/kg) and skeletal muscle (28–34 nmol/kg) tissue but is higher than the plasma concentration (1–2 nM). However, these two experimental settings are not directly comparable for multiple reasons, and the effect of these drugs on splicing in patients and experimental animals should be examined directly. It is interesting that the relative affinity of the different drugs in this class for the Na^+/K^+ ATPase parallels their effective concentrations for altering splicing.

We describe a versatile system for screening large numbers of compounds for their effect on the alternative splicing of an exon of interest. This system can also be applied to other genetic studies of splicing, such as RNAi library screening. We used this system to identify several compounds that alter the use of MAPT exon 10, whose increased splicing causes frontotemporal dementia with parkinsonism. None of these compounds are likely therapeutics for this disease, as they are either toxic or shift the splicing of exon 10 in the wrong direction. However, these compounds were identified from the screen of a very small library of molecules. Larger scale screens will likely identify many additional candidate drugs. Moreover, the reporter system we describe is applicable to nearly any other disease exon. Diseases that involve the missplicing of an exon to which this system might be applied include spinal muscular atrophy, muscular dystrophy, familial dysautonomia, and many other inherited disorders. There are also exons whose alteration has been implicated in tumor progression that could be subjected to small molecule screens. Splicing microarray analysis provides a means for assessing the specificity of each drug for an exon target. The combination of these two methods comprises a strategy for identifying drugs that modulate specific splicing events.

In performing the screen, we identified several sources of false-positive compounds. In particular, compounds that alter initiation by the ribosome at distal AUG codons can alter the RFP/GFP ratio. The different distances of the RFP and GFP start codons from the 5' end of the mRNA will make RFP more sensitive to the disruption of ribosome scanning. The single intron reporters we describe can be used in a secondary screen to identify and eliminate these compounds. We also found that the differential stability of the GFP and RFP proteins carrying the PEST destabilization sequences led to changes in RFP/GFP values from compounds that simply shutdown gene expression at either the RNA or protein level. This problem can be greatly reduced by equalizing the stabilities of the

two reporter proteins. Another possible source of false positives is the differing stabilities of the two mRNA isoforms generated by the reporter. This effect is a general problem for studies of alternative splicing and in our system is most easily addressed once positive compounds are found. We found that none of the three compounds significantly change the levels of the endogenous MAPT transcripts and that digoxin does not alter the overall levels of the reporter mRNAs. Moreover, tyrphostin-9 and 5-iodotubercidin do not change the RFP/GFP ratio in the single intron reporters, which would be expected if they altered the stability of one mRNA but not the other. Thus, the drugs are likely affecting MAPT splicing rather than mRNA stability.

One question for further study is what kinds of proteins affecting splicing can be targeted with small molecule drugs. Alternative exons are directly regulated by combinations of RNA binding proteins whose interactions or conformation could be altered by bound small molecules. Modulation of transcription elongation rates is also likely to alter the inclusion of some exons (28). The RNA binding proteins involved in pre-mRNA splicing are, in turn, regulated by signal transduction pathways that are already well known drug targets. Indeed, all of the compounds identified here interfere with signal transduction systems. Two compounds, tyrphostin-9 and 5-iodotubercidin, are both protein kinase inhibitors. Tyrphostin-9 inhibits certain tyrosine kinases (29), and 5-iodotubercidin targets several serine/threonine kinases (30). These compounds are relatively nonspecific, and identifying the precise pathways that impact pre-mRNA splicing will require further investigation with drugs with more specific effects.

Whether targeting RNA binding proteins or the signaling pathways that modulate them, most drugs are likely to alter the splicing of multiple exons, as seen here. Splicing-sensitive microarrays allow identification of specific sets of exons altered by each drug. The differences in their target exon sets indicate that the drugs are modulating splicing through different pathways. It will be interesting to expand these exon sets and characterize them by using bioinformatic approaches to assess whether specific sequence features allow targeting by specific drugs.

Finally, it is worth noting that this small-scale screen for effectors of one exon identified multiple compounds that alter its splicing. It will be interesting to determine how many other commonly prescribed drugs also have uncharacterized effects on mRNA maturation and use.

Materials and Methods

The experimental procedures used in this work, including microarray analysis results (Table S6, Table S7, and Table S8) and the plate layouts used for the Biomol and Prestwick libraries (Table S9, Table S10, and Table S11), are described in *SI Materials and Methods*.

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