Hoechst 33258 Selectively Inhibits Group I Intron Self-Splicing by Affecting RNA Folding

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Fungal pathogens are increasing in prevalence due to an increase in resistant strains and the number of immunocompromised humans. Candida albicans *is one of these pathogens, and ~40% of strains contain a group I self-splicing intron, which is a potential RNA drug target, in their large subunit rRNA precursor. Here, we report that Hoechst 33258 and derivatives thereof are selective inhibitors of* C. albicans *group I intron self-splicing with an IC_{50} of 17 μM in 2 mM Mg^{2+}. Chemical probing of the intron in the presence of Hoechst 33258 reveals that the folding of several nucleotides in the P4/P6 region of the intron is affected. A nucleotide near the J4/5 region is protected from chemical modification in the presence of Hoechst 33258 and several nearby are more reactive; this suggests that this region is the molecule's binding site. These results expand the available information on small-molecule targeting of RNA and suggest that the RNA-targeting scaffold provided by Hoechst may prove valuable in designing compounds that inhibit the functions of RNA.*

Introduction

The increasing occurrence of fungal infections and antifungal resistance makes discovering new therapeutics against fungi increasingly important.[1] RNA is one class of potential drug targets in fungi and other pathogenic organisms because many RNAs play critical roles in cellular processes. For example, untranslated regions in mRNAs can regulate translation of the message,[2] tRNAs are primers for HIV reverse transcriptase,[3] and it has been suggested that ribosomal RNA catalyzes peptide-bond formation.[4–7] Several compounds that are clinically used to treat bacterial infections inhibit protein synthesis by binding to rRNA.[8,9] It is likely that other essential RNA activities can be inhibited by intervention with small molecules.

Candida albicans is a significant contributor to mortality in immunocompromised humans, including those infected with HIV.[10] About 40% of *C. albicans* and all *Candida dubliniensis* strains contain a group I intron in their large subunit (LSU) rRNA precursor.[11] This group I intron is an attractive RNA drug target because self-splicing is necessary for maturation of ribosomes.[12] In addition, group I introns have not been found in mammalian genomes, and assays for self-splicing are easily performed.[13,14]

Here, we report that Hoechst 33258 and derivatives thereof are effective inhibitors of group I intron self-splicing; Hoechst 33258 has an IC_{50} of 17 μM in vitro in a buffer that contains 2 mM Mg^{2+}. Inhibition is also selective because addition of 2.9 mM nucleotide solution from Torula Yeast bulk RNA does not affect the IC_{50} of Hoechst 33258. Chemical mapping, in the presence and absence of Hoechst 33258, of a ribozyme derived from the *C. albicans* group I intron shows that the folding of the intron is primarily affected in the P4/P6 domain. In particular, Hoechst 33258 protects a nucleotide adjacent to the J4/5 loop from chemical modification and enhances the reactivity of several nearby nucleotides; this suggests that this region is the molecule's binding site.

Results

Inhibition of self-splicing

Ethidium bromide, DAPI, pentamidine, and various Hoechst derivatives were tested for inhibition of self-splicing in a buffer containing 2 mM Mg^{2+} (Scheme 1, Figure 1, and Table 1). Under these conditions, all tested compounds inhibit self-splicing at concentrations less than 100 μM. The most effective compound is ethidium bromide, $IC_{50} = 3$ μM, and the least effective is pentamidine, $IC_{50} = 98$ μM. Ethidium bromide and methidiumpropyl EDTA have been previously shown to inhibit self-splicing and cyclization of the *Tetrahymena thermophila* ribozyme,[15,16] and pentamidine is known to inhibit self-splicing of the *C. albicans*[17,18] and the *Pneumocystis carinii* group I introns.[19]

The effect of Mg^{2+} concentration on inhibition of self-splicing by pentamidine and Hoechst 33258 was measured (Table 1). Previous results have shown that the amount of spliced precursor depends on Mg^{2+} concentration; the per-

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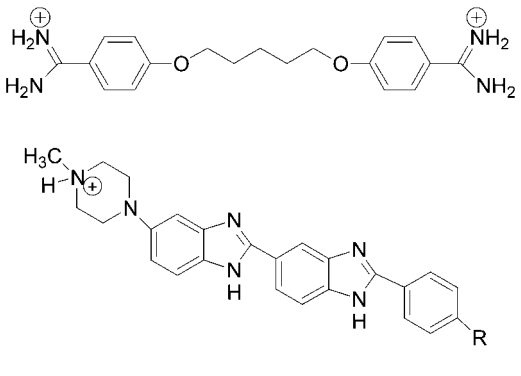

Hoechst	R
33258	OH
S769121	SO_2NH_2
34580	$N(CH_3)_2$

Scheme 1. *Structures of group I intron inhibitors, pentamidine (top) and Hoechst derivatives (bottom). Starting from the methylated nitrogen of the piperazine ring and ending with the terminal hydroxyl group of the phenol ring, the pK_a's of unbound Hoechst 33258 are approximately 9.8, 3.5, 5.5, and 8.5, where 3.5 and 5.5 are for additional protonation of the benzimidazole rings.[66–68] Thus at pH 7.5, Hoechst 33258 has +1 charge, as shown.*

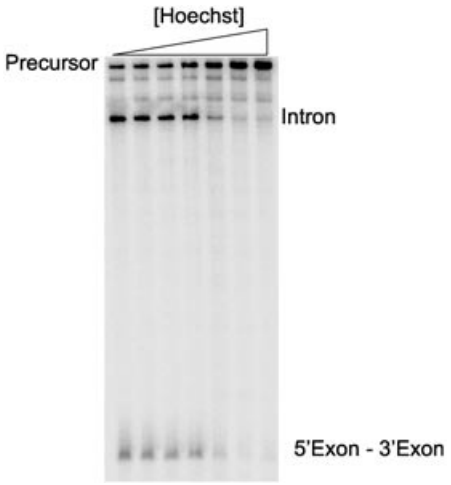

Figure 1. *An autoradiogram of a gel for inhibition of self-splicing by Hoechst 33258 at 2 mM Mg^{2+}. The concentrations from left to right are 0, 0.5, 2, 8, 32, 124, and 248 μM.*

Table 1. *Inhibition of precursor self-splicing by small molecules.*

Compound	[Mg^{2+}] [mM]	[Torula Yeast Bulk RNA] in nucleotides 0 mM	2.9 mM	9.5 mM	29 mM
		IC_{50} [μM] for *C. albicans* precursor			
Ethidium Br	2	3 ± 0.5	8 ± 2	12 ± 3	25 ± 2
DAPI	2	34 ± 10	69 ± 17	156 ± 4	270 ± 19
Pentamidine	2	98 ± 7	255 ± 15	295 ± 70	445 ± 30
	4	213 ± 29	250 ± 50	290 ± 30	440 ± 80
	10	320 ± 50	440 ± 14	532 ± 110	900 ± 100
Hoechst 33258	2	17 ± 3	16 ± 3	27 ± 4	90 ± 20
	4	41 ± 5	39 ± 5	54 ± 4	125 ± 20
	10	80 ± 11	85 ± 20	160 ± 50	200 ± 40
Hoechst S769121	2	6.5 ± 4	20 ± 2	34 ± 5	–
Hoechst 34580	2	6.2 ± 2	17 ± 2	27 ± 3	–

centage of spliced product increases from ~30% at 1 mM Mg^{2+} to ~75% at 2 mM Mg^{2+}.[13] Increasing the Mg^{2+} concentration from 2 to 10 mM increases the IC_{50}'s for pentamidine and Hoechst 33258 by about three- and fivefold, respectively (Table 1). These results mirror those previously reported for pentamidine inhibition of *C. albicans* self-splicing.[18]

To investigate whether inhibition is due to specific or nonspecific binding to the intron, IC_{50}'s were measured in the presence of Torula Yeast bulk RNA (Table 1). At 2 mM Mg^{2+}, IC_{50}'s for ethidium bromide and DAPI increase by about two- and eightfold in the presence of 2.9 or 29 mM nucleotide of bulk RNA, respectively. At 2 mM Mg^{2+}, the IC_{50} for pentamidine increases about three- and fivefold when a 2.9 or 29 mM nucleotide solution of bulk RNA is added, respectively, whereas the IC_{50} for Hoechst 33258 is unchanged at 2.9 mM and increases approximately fivefold at 29 mM bulk RNA. Similar trends are observed for Hoechst 33258 at 4 and 10 mM Mg^{2+}.

Hoechst derivatives S769121 and 34580 were also tested for inhibition of self-splicing (Scheme 1). These compounds are better than Hoechst 33258 for inhibition of self-splicing at 2 mM Mg^{2+} in the absence of competitor RNA. Addition of competitor RNA, however, increases the IC_{50}'s for inhibition of self-splicing more for these derivatives than for Hoechst 33258 (Table 1). Thus, these compounds are less selective for inhibition of self-splicing than Hoechst 33258.

Chemical probing of ribozyme structure in the presence of Hoechst 33258

A ribozyme[13] construct was used to probe the effects of Hoechst 33258 binding to the catalytic fragment of the LSU rRNA precursor. Ribozyme structure in the presence of the 5′ exon mimic, GACUCU, was chemically mapped in the presence and absence of Hoechst 33258. The 5′ exon mimic was added to allow the ribozyme to fold into the conformation required for splice site recognition. Hydroxyl radicals generated from peroxynitrous acid[20] were used to cleave solvent accessible phosphodiester bonds, and diethyl pyrocarbonate (DEPC)[21,22] was used to modify A's, G's, and U's.

Hydroxyl radical mapping of the ribozyme between nucleotides 11 and 362, shows that many nucleotides in the P4, P5, and P5abc regions are more accessible to cleavage by hydroxyl radicals when Hoechst 33258 is present (Figures 2 and 3). The increased solvent accessibility of this region is particularly interesting because experiments with the *T. thermophila* ribozyme have shown that this region folds independently of the rest of the intron[23] and is the first to fold.[24,25]

The entire ribozyme between nucleotides 11 and 362 was also probed by DEPC. Both enhanced and reduced modifications are observed with Hoechst 33258. Nucleotides in which the modification is enhanced at least twofold are situated in J4/5, J5/5a, at the bottom of P6, and in L8 (Figures 3 and 4). Nucleotides in and adjacent to the J4/5 region are both enhanced and protected from chemical modification. In particular, modification of U195, A196, and A197 is enhanced. In contrast, G193 in the P5 helix is protected from modification by DEPC; this suggests that a Hoechst 33258 binding site is near

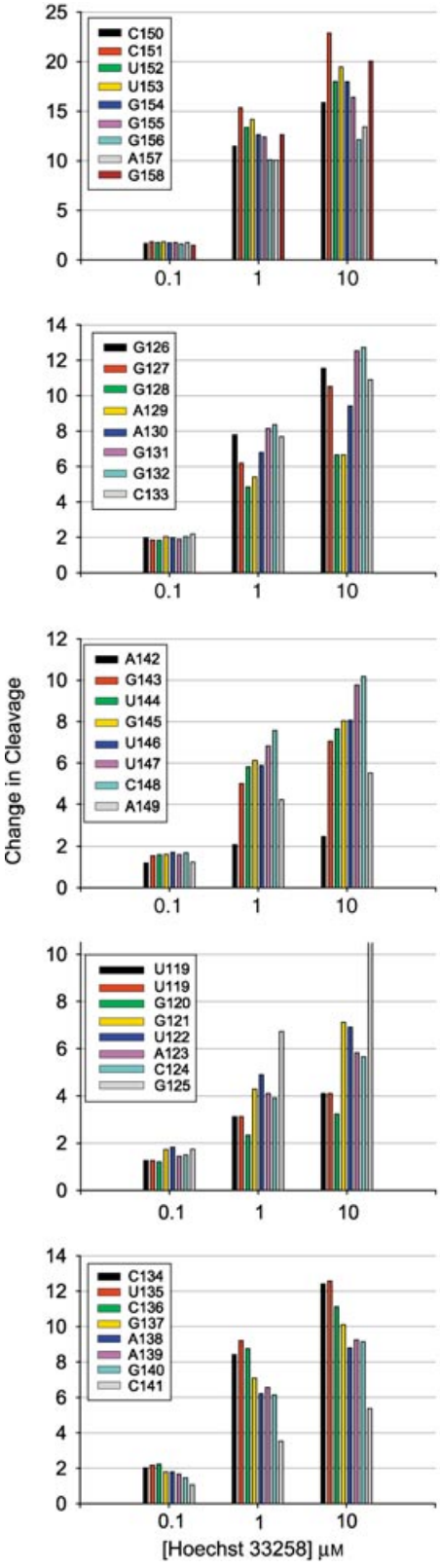

Figure 2. Relative reactivity of phosphodiester bonds for nucleotides in the C. albicans *group I intron that are susceptible to increased hydroxyl radical cleavage at 10 mM Mg^{2+} as a function of Hoechst 33258 concentration. The change in cleavage was determined by dividing the number of radioactive counts for a nucleotide of interest by the number of radioactive counts from a nucleotide in which the cleavage was not affected by addition of peroxynitrous acid.*

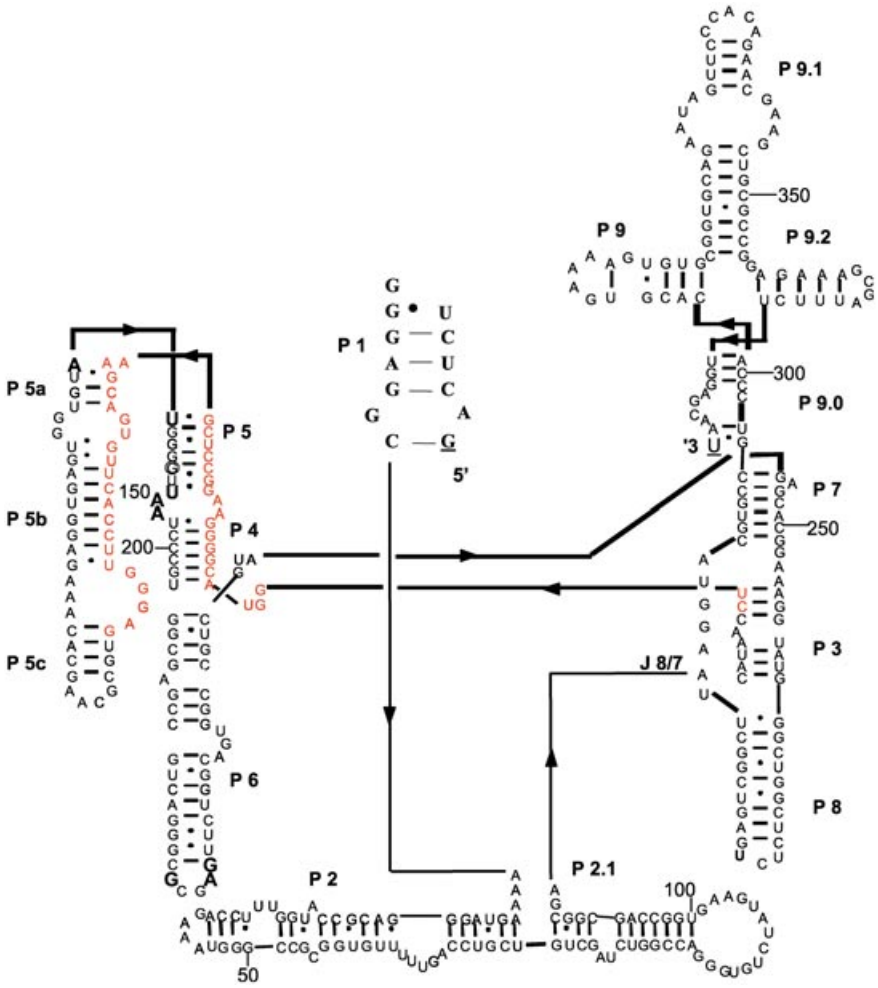

Figure 3. Summary of ribozyme chemical modification in the presence of Hoechst 33258 and 10 mM Mg^{2+}. Positions of enhanced cleavage by hydroxyl radicals upon addition of Hoechst 33258 are in red. Nucleotides with enhanced modification by DEPC upon addition of Hoechst 33258 are shown in bold. The circled nucleotide, G193, is protected from modification by DEPC. The ribozyme was probed between nucleotides 11 and 362 by both hydroxyl radicals and DEPC.

this nucleotide (Figures 3 and 4). Enhanced modification of nucleotides in the J4/5 internal loop of the intron is particularly interesting. By analogy to the *T. thermophila* intron, the J4/5 loop recognizes the exocyclic amine of the G·U pair at the splice site and thus contributes to binding of the 5′ exon substrate, recognition of the splice site, and stabilization of the transition state.[26] Thus, altering the folding of nucleotides in this region can disable the ribozyme. These results show that Hoechst 33258 affects the global folding of the intron and, in particular, nucleotides directly involved in tertiary contacts.

Self-splicing inhibition in the presence of competing J4/5 mimic

To test the hypothesis that the J4/5 region in the intron is a binding site for Hoechst 33258, IC_{50}'s for inhibition of self-splicing by Hoechst 33258 were measured as a function of concentration of a short oligonucleotide mimic of this region (Table 2). Addition of 15 and 30 μM J4/5 mimic oligonucleotide increases the IC_{50} by 13 and 38 μM, respectively. Within experi-

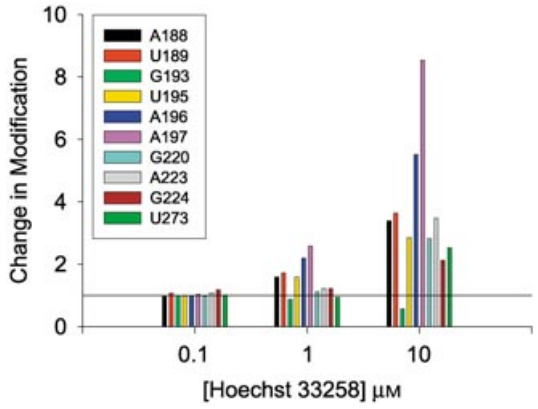

Figure 4. *Relative reactivity of nucleotides in the* C. albicans *group I intron that have increased or decreased modification by DEPC at 10 mM Mg^{2+} as a function of Hoechst 33258 concentration. The change in cleavage was determined by dividing the amount of radioactive counts for a nucleotide of interest by the number of counts for a nucleotide in which the cleavage was not affected by addition of DEPC.*

mental error, this is the increase that is expected if a 1:1 complex forms between Hoechst 33258 and the J4/5 mimic when the binding constant for that complex is much tighter than for the complex between Hoechst 33258 and ribozyme. In contrast, addition of 15 and 30 μM $tRNA^{Phe}$ only increases the IC_{50} by 3 and 14 μM, respectively, which is essentially negligible within experimental error, even though $tRNA^{Phe}$ has ~2.5-fold more nucleotides than the J4/5 mimic (Table 2).

Table 2. *IC_{50}'s [μM] for inhibition of* C. albicans' *group I precursor self-splicing by Hoechst 33258 in the presence of competing RNA in H2Mg buffer.*

Competing RNA	0 μM	15 μM	30 μM
J4/5 Mimic[a]	17 ± 3	30 ± 1	55 ± 10
$tRNA^{Phe}$	17 ± 3	20 ± 6	31 ± 10

[a] The sequence of the J4/5 mimic is: 5′CGGGGAAGGCCUGGAAACGGGUUAAUCCCG; the unpaired nucleotides are underlined.

To determine if the J4/5 mimic or $tRNA^{Phe}$ affects self-splicing, the rate of precursor self-splicing was measured in the presence of either 30 μM J4/5 mimic or $tRNA^{Phe}$. The rate of splicing was similar in the presence and absence of oligonucleotide (data not shown).

Discussion

RNA is used relatively rarely as a therapeutic target, despite the fact that there are many potential RNA drug targets in cells. Group I introns are one class of potential RNA drug targets in fungi because the activity of these introns is essential for the assembly of active ribosomes.[12] Several compounds inhibit group I intron splicing in vitro and in vivo, including 5′ fluorocytosine,[11,27] pentamidine,[17] aminoglycosides,[28–31] and other molecules[13,32–38] Several small-molecules are known to inhibit functions of other RNAs, for example, HIV TAR RNA.[39,40] Understanding the mode of action of small molecules that inhibit group I intron splicing can provide a foundation for designing small molecules to inhibit RNA function and potentially serve as therapeutics.

The Hoechst dyes studied here are based on a relatively simple scaffold that allows for synthesis of derivatives. The pK_a's of the benzimidazole sites in unbound Hoechst 33258 are ~3.5 and ~5.5, which does not add any additional charge at neutral pH, so the overall charge at neutral pH is +1.[41–43] This may minimize nonspecific binding to nucleic acids, which is expected for highly charged cations.[44,45] The IC_{50} for self-splicing changes little upon addition of 9.5 mM nucleotide from Torula yeast bulk RNA (Table 1) or 2.3 mM nucleotide from phenylalanine tRNA (Table 2). Even though the charge is +1, the IC_{50} is 17 μM for inhibition of self-splicing in the presence of 2 mM Mg^{2+}, which is similar to intracellular Mg^{2+} concentrations.[46] These results suggest that Hoechst is a good scaffold that can allow for selective binding of RNA. The results also give insight into future design of RNA binding ligands by using this scaffold. For example, Hoechst S769121 and Hoechst 34580 are both better inhibitors of self-splicing than Hoechst 33258. These compounds, however, are less selective inhibitors of self-splicing (Table 1). This position can also be derivatized with oligonucleotides, however,[47,48] which can potentially increase specificity. Derivatization of the Hoechst scaffold in other positions may allow further enhancement of binding and selectivity.

The type of motif that may be targeted by Hoechst scaffolds is suggested by the chemical modification studies (Figure 3). The only nucleotide in the entire ribozyme that is protected from DEPC modification by Hoechst 33258 is in the P5 helix near the J4/5 internal loop; this suggests that J4/5 and/or the P5 helix is the binding site. Increased modification is observed for nucleotides in this and in distant regions. The effects in the distant regions may be due to additional binding sites but likely reflect perturbations in tertiary structure. The J4/5 region is the docking site for the P1 helix. Presumably, Hoechst 33258 binding inhibits P1 docking, thus altering the tertiary structure of the catalytic core. The observation that competition by a 30 nucleotide mimic of the P4-J4/5-P5 motif increases the IC_{50} for inhibition of self-splicing by Hoechst 33258 is also consistent with this region being a binding site. Interestingly, this binding site differs from those proposed for pentamidine.[18] Aminoglycosides have been shown to bind to and inhibit self-splicing of the td group I intron. Mapping experiments show that the J4/5 internal loop is one of the sites for aminoglycosides binding.[31]

Other studies have shown that Hoechst 33258 binds to internal loops or bulges in RNA. For example, in HIV TAR RNA Hoechst 33258 recognizes a bulged U residue.[49,50] RNA untranslated regions and aptamers have also been shown to bind to Hoechst 33258 and these sequences have conserved pyrimidine rich internal loops.[51,52] These studies and the ones reported here show that Hoechst 33258 binds RNA in or near sites that are not canonically base paired. While further experiments are needed to thoroughly define the selectivity of Hoechst 33258, the results suggest that it provides a useful

scaffold for binding to internal loops in RNA. It is likely that related compounds with tighter binding and higher specificity towards RNA can be made. For example, steric blocks could be introduced that eliminate binding to Watson–Crick paired DNA while retaining binding to RNA internal loops.

This study shows that Hoechst 33258 binds to the *C. albicans* group I intron and inhibits self-splicing. Binding appears to be to a functionally important internal loop. Preliminary experiments indicate that Hoechst 33258 also exhibits activity against *C. albicans* and *C. dubliniensis* strains that harbor a group I intron in their LSU rRNA precursor. Hoechst 33258 and derivatives thereof have potential as broad-spectrum antifungals, since other fungi with group I introns in essential genes include several *Candida* species,[11,13,53] *P. carinii*,[54,55] and *Aspergillus nidulans*.[56] Sequencing of the genomes of many human pathogens is likely to reveal group I introns that are potential therapeutic targets in other organisms.

Studies of molecular recognition of RNA by Hoechst 33258 and derivatives thereof could lead to development of improved therapeutics that target RNA. Several aminoglycosides bind bacterial rRNA and inhibit protein synthesis.[57] Bacteria have gained resistance to these drugs, however, through a variety of mechanisms.[58] For example, bacteria can alter the structure of antibiotics by phosphorylation, thus rendering the compound ineffective at binding its cellular target. Bacteria can also change their rRNA sequence such that the antibiotics no longer bind.[59,60] Insights into new chemical scaffolds that recognize RNA in a sequence-specific manner could lead to the design of new therapeutics that inhibit growth of resistant strains. These new scaffolds could be designed such that they are not substrates for bacterial kinases but are ligands for mutated ribosomes.

Experimental Section

Buffers: HXMg buffer is Hepes (50 mM; 25 mM NaHepes), KCl (135 mM), and $MgCl_2$ ("X" mM) at pH 7.5.[55] TBE buffer is Tris (100 mM), Boric Acid (90 mM), and EDTA (1 mM) at pH 8.4. Stop buffer is urea (12 M), Na_2EDTA (12 mM), and 0.1X TBE buffer.

Instruments and general protocols: All radioactivity was quantified on a Molecular Dynamics phosphorimager with ImageQuaNT version 4.1 software. Oligonucleotides were synthesized on an Applied Biosystems 392 solid-phase synthesizer by using standard phosphoramidite chemistry[61] with monomers purchased from Glen Research (Baltimore, MD). For the oligonucleotide that is a mimic of the J4/5 region in the *C. albicans* ribozyme, the 2′ hydroxyl groups on the phosphoramidite monomers were protected as the triisopropylsilyloxymethyl (TOM) ether;[62] all other monomers were protected as the tertbutyldimethylsilyl (TBDMS) ether.[63] The J4/5 mimic oligonucleotide was purified on a denaturing 20% polyacrylamide gel, and isolated from the gel by electroelution.[64] Samples were desalted with a Sep-Pak column (Waters Corp.) as described.[64] The purity of each oligonucleotide was greater than 95% as determined by analytical kinase[35] or HPLC. The *C. albicans* precursor and ribozyme were as transcribed and purified as described.[13,36]

Chemical mapping experiments: A 50 nM solution of ribozyme was refolded in H10Mg buffer at pH 7.5 and 55 °C as described.[13] After the solution was cooled to 37 °C, a mimic of the ribozyme's 5′ exon substrate, GACUCU, was added to give a final concentration of 5 μM, and the solution was incubated for 30 min at 37 °C to allow equilibration. Various concentrations of Hoechst 33258 were added and the samples incubated at 37 °C for at least 1 h.

For structure probing, ribozyme was chemically modified by addition of 10% (*v*/*v*) DEPC; DEPC modifies A, G, and U nucleotides at pH 7.5.[21,22] Samples were incubated at 37 °C for 20 min and precipitated with 2.5 volumes of ethanol in the presence of carrier tRNA (10 $\mu g\,mL^{-1}$) and resuspended in 200 μL of sterile water. To remove Hoechst 33258, which inhibits reverse transcriptase, the solution containing RNA was extracted at least three times with 100 μL of phenol/chloroform/isoamylalcohol (25:24:1) that was saturated with TE buffer. A final ethanol precipitation of the aqueous layer was used to isolate the ribozyme. After most of the solvent was decanted off, the remaining solvent was evaporated in vacuo.

Ribozyme structure was also probed by cleavage with hydroxyl radicals generated from peroxynitrous acid.[20] Ribozyme was refolded and incubated with Hoechst 33258 as described above, then a 2.5% volume of peroxynitrous acid was added and the samples incubated at 37 °C for 6 min. After the incubation period, samples were immediately placed into a dry ice–ethanol bath. The samples were ethanol precipitated in the presence of carrier tRNA, extracted, and isolated as described above.

Sites of modification were detected by primer extension[21] by using AMV Reverse Transcriptase (Life Sciences) according to manufacturer's protocol except that samples were annealed in $NaOOCCH_3$ (435 mM) instead of water.[33] The ribozyme was sequenced by the Sanger method with reverse transcriptase.[65] The change in modification was determined by normalizing the signals from the position of interest to a nucleotide where the amount of modification is not affected by Hoechst 33258.

Splicing assays: Splicing assays were completed as described.[13,37] In a typical experiment, 2 nM of precursor was refolded in buffer by incubating at 50 °C for 3 min. The sample was placed at 37 °C for at least 2 min to allow the temperature to equilibrate, and then 3 μL of this solution was added to 3 μL of a solution containing 2 mM pG and inhibitor in buffer. Samples were incubated at 37 °C for 1 h and a $^2/_3$ volume of stop buffer was added to quench the reactions. For splicing experiments with competing RNA, precursor was refolded in buffer as described above, and then a solution containing competing RNA in buffer was added. Products were separated on a denaturing 5% polyacrylamide gel and quantified with a phosphorimager.[13] The IC_{50}'s were determined as described.[33]

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