Selective inhibition of ADAR2-catalyzed editing of the serotonin 2c receptor pre-mRNA by a helix-threading peptide

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RNA editing by adenosine deamination is a form of epigenetic control of gene expression wherein the ADAR enzymes convert adenosine to inosine in RNA often changing the meaning of codons. The pre-mRNA for the 2c subtype of serotonin receptor (5-HT2cR) is shown here to support small molecule binding near known editing sites. Furthermore, a helix-threading peptide binds this site and inhibits the *in vitro* reaction of ADAR2 in an RNA-substrate selective manner. This is the first example of substrate-selective inhibition of editing by an RNA-binding small molecule and sets the stage for the development of new reagents capable of controlling gene function through manipulation of mRNA editing.

Introduction

The prominent roles different RNAs play in the cell makes targeting RNA processing an attractive approach for controlling cellular events with small molecules. Indeed, recent reports describe the discovery of small molecule inhibitors of pre-mRNA splicing, mRNA capping, miRNA maturation, and a DEAD box RNA helicase involved in translation.[1–14] Furthermore, molecules capable of perturbing RNA processing pathways have therapeutic potential. For instance, antisense oligonucleotides and small molecules able to induce exon skipping in the splicing of the dystrophin pre-mRNA are being pursued as therapeutics for Duchenne Muscular Dystrophy, a disease caused by frame shift mutations in the dystrophin gene leading to nonfunctional proteins.[15,16] Induced skipping of the mutated exon leads to a shorter, yet functional, dystrophin spice variant.[17,18]

RNA editing is a type of processing wherein protein enzymes change the sequence of transcripts.[19] Adenosine deamination catalyzed by the ADAR enzymes generates inosine at the corresponding nucleotide position in the strand. Since inosine is decoded as guanosine during translation, this modification can lead to codon changes (recoding) and the introduction of amino acids into a gene product not encoded in the gene.[20,21] Several recoding sites are found in mRNAs for proteins important in the central nervous system (CNS) such as glutamate receptors[21] and serotonin receptors.[20] Recoding within these mRNAs contributes to the protein structural diversity required for proper CNS function. Furthermore, altered editing of these RNAs has been linked to CNS disorders.[22–27] Despite this, efforts to develop editing inhibitors as a means of controlling protein function are just beginning.[22] In addition, given the number of different editing substrates and their diverse functions, substrate-selective inhibitors may ultimately be the most useful as probe molecules or therapeutics.[23] Therefore, we endeavor to develop such inhibitors and have focused our initial efforts on editing sites found in the pre-mRNA for the 2c subtype of serotonin receptor (5-HT2cR) (Fig. 1). Deamination of five adenosines (sites A–E) within exon five of the 5-HT2cR pre-mRNA leads to changes at three codon positions. These three amino acids are located in an intracellular loop that binds G proteins and complete editing changes the genomically encoded IRNPI to the sequence VRGPV, modulating serotonin receptor function (Fig. 1).[20] 5-HT2cR has been linked to numerous neurological disorders, including those correlated with hyper editing (*e.g.* severe depression and Prader Willi syndrome).[24–26] In addition, one of the editing site adenosines (the D site) is present in a 5′-UpA-3′ base paired step adjacent to a five nucleotide internal loop (Fig. 1). This structure is similar to known RNA binding sites for helix-threading peptides (HTPs) developed previously in our laboratory.[27–32] These compounds have an aromatic heterocycle modified at opposite edges such that binding by intercalation places substituents in both the minor and major grooves at duplex binding sites (*i.e.* threading intercalation).[27–29] Our previous studies demonstrated that compounds of this type bind preferentially to sites in duplex RNA bearing a 5′-PyPu-3′ sequence flanked on each 3′ side by bulges or loops.[28] Selective binding could also be

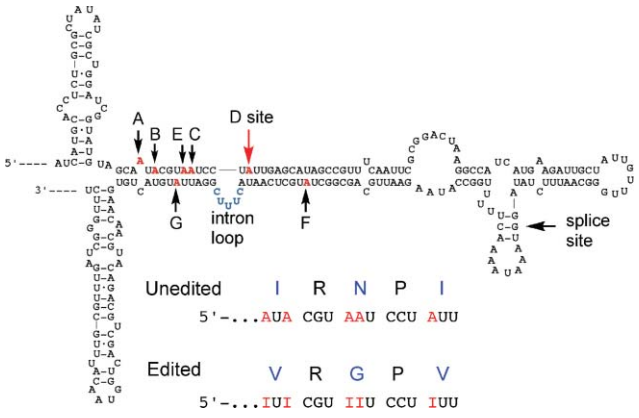

Fig. 1 The 5-HT2cR pre-mRNA editing sites (red) shown on the predicted secondary structure surrounding these sites including the location of a five nucleotide loop in the intron sequence (blue). Also shown is the change in protein sequence resulting from complete editing on this substrate.

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observed if a single large loop was present 3′ to a base paired 5′-PyPu-3′ sequence.[29] The presence of such a secondary structural element near the D editing site suggested that the 5-HT2cR pre-mRNA might be predisposed to bind HTPs. Here we describe the interaction of a macrocyclic HTP with this RNA editing substrate and the effect this molecule has on the ADAR2-catalyzed editing reaction.

Results

To evaluate small molecule binding, we used a ribonuclease footprinting assay with an RNA duplex including the five nucleotide internal loop adjacent to the D site (Fig. 2). As a control, we generated an otherwise identical structure lacking the five-nucleotide loop. Our previous studies have shown that helix defects 3′ to the 5′-PyPu-3′ step are necessary for selective HTP binding.[28–32] We evaluated the binding of cyclic HTP **1** shown previously to occupy high affinity sites present on an *in vitro* selected RNA and a pre-miRNA (Fig. 2).[32] We found that HTP **1** protects specific nucleotides on the 5′ side of the five-nucleotide loop near the D site including the uridine paired with the D site adenosine. Protection is also observed at a nucleotide approximately one helical turn from the loop. Furthermore, binding of HTP **1** to this RNA causes an increase in reactivity for S1 nuclease at the central uridine of the loop (Fig. 2). A quantitative nuclease footprint titration was used to measure a $K_D = 4.4 \pm 0.2$ μM for HTP **1** on this RNA under these conditions (Fig. 2). Importantly, we did not observe protection of the control RNA from nuclease cleavage at these concentrations of HTP **1**, illustrating its binding selectivity (Fig. 2).

To further characterize HTP binding to the 5-HT2cR RNA, we synthesized a derivative of HTP **1** bearing an EDTA-Fe modification on the macrocycle (HTP **2**, Fig. 3). The inclusion of EDTA-Fe in the structure allows one to reveal selective binding sites by identifying nucleotides that correspond to sites of EDTA-Fe-promoted hydroxyl radical cleavage in the complex.[33,34] HTP **2** was synthesized using a solid phase strategy with our previously reported 2-(*p*-allylphenyl)quinoline amino acid (Scheme 1).[32] A

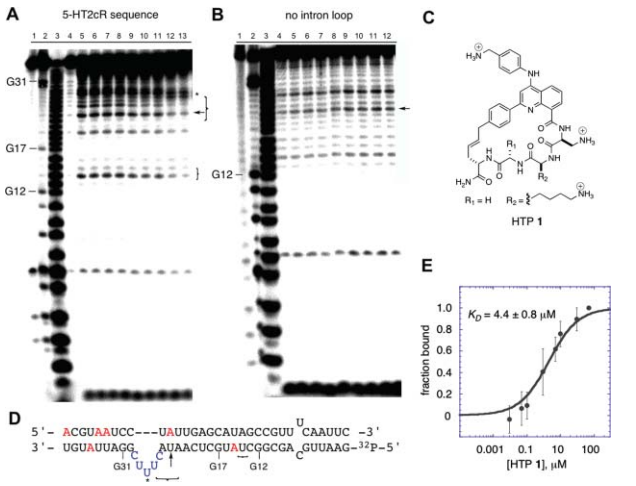


Fig. 2 Ribonuclease footprinting analysis of HTP **1** binding. **A**. Autoradiogram of gel used to resolve nuclease cleavage products for loop substrate. Lane 1: 5′-end labeled RNA strand with no treatment, with lane 2: denaturing T1 digestion and lane 3: alkaline hydrolysis. Lane 4: RNA duplex in the absence of S1 and HTP **1**. Lane 5: nuclease S1 digestion in the absence of HTP **1**, while lanes 6 through 13 contain increasing concentrations of HTP **1** (300 nM, 700 nM, 1 μM, 3 μM, 7 μM, 10 μM, 30 μM, and 70 μM respectively). Brackets indicate nucleotides protected from nuclease S1 digestion. Star indicates a nucleotide rendered hyper-reactive to S1 digestion with increasing concentrations of HTP **1**. Arrow indicates quantified nucleotide protected from S1 digestion with increasing concentrations of HTP **1**. **B**. Autoradiogram of gel used to resolve nuclease cleavage products for no loop substrate. Lane 1: 5′-end labeled RNA strand with no treatment, with lane 2: denaturing T1 digestion and lane 3: alkaline hydrolysis. Lane 4 is S1 nuclease digestion in the absence of HTP **1**, while lanes 5 through 13 contain increasing concentrations of HTP **1** (10 nM, 30 nM, 100 nM, 300 nM, 1 μM, 3 μM, 10 μM, 30 μM, and 100 μM respectively). Arrow indicates nucleotide corresponding to that quantified in **A**. **C**. Structure of HTP **1**. **D**. Sequence of oligonucleotides used for these studies with editing sites shown in red. Nucleotides removed from the loop substrate to generate the control RNA are shown in blue. Arrow indicates quantified nucleotide protected from S1 nuclease cleavage by HTP **1** for the loop substrate and bracket indicates all nucleotides protected from S1 nuclease digestion. No protection is apparent for the uridine opposite the D editing site (arrow) in the no loop control RNA. Star indicates a nucleotide rendered hyper-reactive in the presence of HTP **1**. Location of ^{32}P indicates labeled end for the gels depicted in **A** and **B**. **E**. Plot of fraction of RNA bound as a function of [HTP **1**].

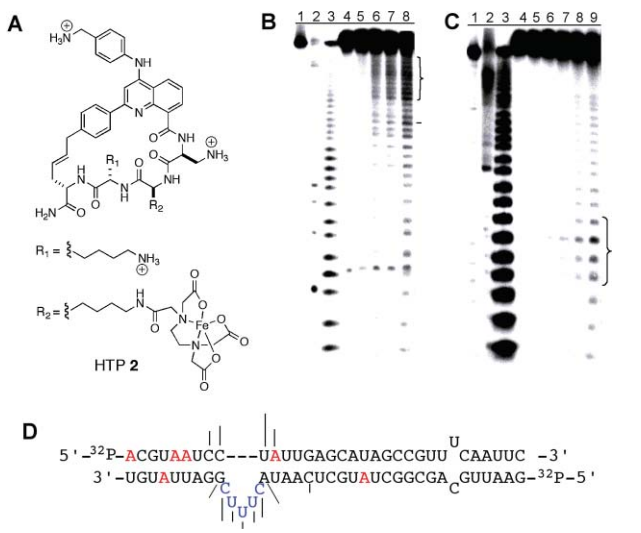
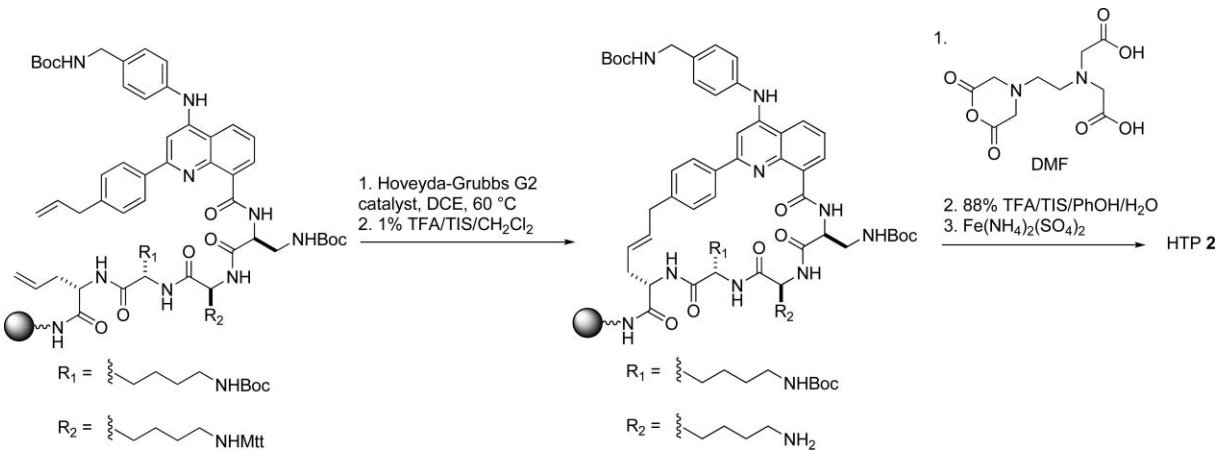


Fig. 3 Directed hydroxyl radical cleavage with HTP **2**. **A**. HTP **2** structure. **B**. Autoradiogram of gel used to resolve directed hydroxyl radical cleavage products, intron strand 5′ end labeled. Lane 1: RNA with no treatment, with lane 2: denaturing T1 digestion and lane 3: alkaline hydrolysis. Control lane 4 : 20 mM sodium ascorbate and control lane 5 : 100 μM EDTA-Fe. Experimental lanes 6, 7, and 8: increasing concentrations of HTP **2** (1 μM, 3 μM, and 30 μM respectively). Bracket indicates nucleotides cleaved by HTP **2** directed hydroxyl radical cleavage. **C**. Autoradiogram of gel used to resolve directed hydroxyl radical cleavage products, exon strand 5′ end labeled. Lane 1: RNA with no treatment, with lane 2: denaturing T1 digestion and lane 3: alkaline hydrolysis. Control lane 4 : 20 mM sodium ascorbate and control lane 5 : 100 μM EDTA-Fe. Experimental lanes 6 through 9: increasing concentrations of HTP **2** (1 μM, 3 μM, 10 μM, and 30 μM respectively). Bracket indicates nucleotides cleaved by HTP **2** directed hydroxyl radical cleavage. **D**. Summary of directed hydroxyl radical cleavage results. Line indicates site of cleavage by HTP **2** and edited nucleotides are shown in red. Location of ^{32}P indicates labeled end for the gels depicted in **B** and **C**.



Scheme 1

resin-bound bis-olefin linear peptide was generated with an internal Mtt-protected lysine residue. Ring closing metathesis on the solid phase with Hoveyda-Grubbs second-generation ruthenium catalyst was followed by removal of the Mtt group with 1% TFA. EDTA monoanhydride was coupled to the resulting free amine and the product was cleaved from the resin and purified by HPLC. Loading the tethered EDTA by treatment with $Fe(NH_4)_2(SO_4)_2$ generated HTP **2** for directed hydroxyl radical cleavage experiments. Consistent with the footprinting results with HTP **1**, HTP **2** cleaved the 5-HT2cR duplex between the C and D editing sites on one strand and in the five nucleotide loop and adjacent nucleotides of the other (Fig. 3). These observations further establish the location of a selective HTP binding site on this RNA near the intron loop between the D and C editing sites.

Although the studies described above identified an HTP binding site on the 5-HT2cR RNA, it was not clear how ligand binding at this site would affect the editing reaction. To evaluate the effect of HTP binding on 5-HT2cR editing, we developed a cell free assay that uses a longer RNA substrate (~ 350 nt) encompassing all the 5-HT2cR editing sites (see Experimental Section, Fig. 1). We assessed the editing efficiency by sequencing RT-PCR products using the editing reaction product RNA as the template. This method of evaluating editing has been shown to give quantitative results.[35] As a control, we generated a substrate lacking the intron loop nucleotides near the editing sites. We initially characterized ADAR2 editing on these two substrate RNAs by quantifying the % editing at each of the known sites (Fig. 4). Under the conditions of our assay, we observed efficient ADAR2 editing at the D site for the native 5-HT2cR sequence (89% inosine) and low levels of editing at the F (12%) and G (7%) sites located in the intron sequence opposite sites A–E in the secondary structure (Fig. 1 and 4).[20,36–38] All other sites were edited by ADAR2 to less than 5% on this RNA (Fig. 4). Interestingly, the editing efficiency of ADAR2 was greater at all sites except the D site in the control RNA lacking the intron loop. ADAR2 deaminates the D site in this RNA to a level of 72% under these conditions (Fig. 4). Thus, while the intron loop clearly influences the ADAR2 reaction on this substrate, RNA lacking this loop supports efficient editing at the D site. This provided us with a functional control RNA for inhibitor studies since selective HTP binding requires the intron loop (Fig. 2).

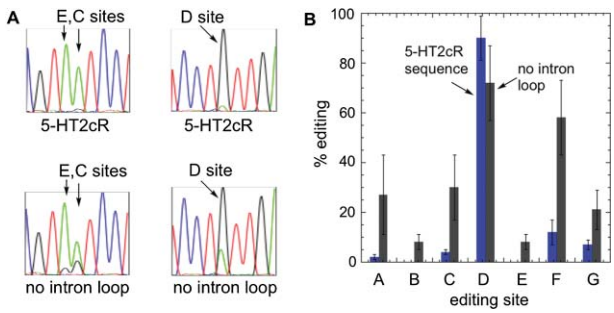


Fig. 4 Editing efficiency at the different 5-HT2cR editing sites on two substrate RNAs used for this study. **A**. Sequencing traces show editing at the E, C and D sites on the RNA substrates with and without the intron loop. **B**. Percent editing of sites A–G on the RNA substrates with and without the intron loop.

The binding of HTP **1** to the 5-HT2cR RNA inhibits editing at the D site. ADAR2 deamination of the D site adenosine was reduced from 89% inosine in the absence of compound to 75% at 10 μM HTP **1** and 6% at 30 μM HTP **1** (Fig. 5). Importantly, editing on the control RNA lacking the intron loop is less sensitive to HTP **1** at these concentrations. For this RNA, no change is seen up to 10 μM HTP **1** and a less than a two-fold reduction to 42% editing is observed at 30 μM HTP **1** (Fig. 5). In stark contrast to these results, neomycin, a promiscuous RNA-binding aminoglycoside, inhibits editing on both RNAs showing equal potency for these two substrates (Fig. 5).[39–42]

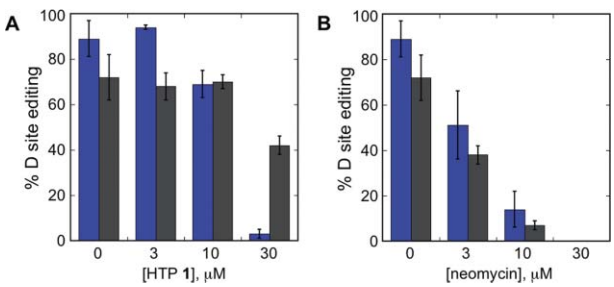


Fig. 5 D site editing on 5-HT2cR substrate RNAs in presence of varying concentrations of HTP **1** (**A**) or neomycin (**B**). Blue: wild type sequence, Gray: sequence lacking intron loop.

Discussion

In this study, we have shown that the pre-mRNA for the 2c subtype of serotonin receptor (5-HT2cR) harbors a selective binding site for a small molecule near the edited adenosines and that this compound inhibits the *in vitro* reaction of the RNA editing adenosine deaminase ADAR2. Furthermore, the inhibition was shown to be substrate selective since editing on a control RNA with diminished binding to the small molecule was inhibited less effectively. This is the first demonstration of substrate-selective inhibition of an editing reaction by an RNA-binding small molecule. The editing inhibitor (HTP **1**) is a macrocyclic helix-threading peptide synthesized in our laboratory and shown previously to bind certain duplex RNA binding sites selectively. We tested this compound for binding to 5-HT2cR pre-mRNA due to secondary structure elements shared by this RNA and other RNAs with known high affinity sites for HTPs.[28–30,32] Our results have implications for the development of molecular probes for RNA editing biology and possible therapeutic leads. For instance, compounds targeting this site capable of modulating editing levels on the 5-HT2cR message will be useful in the study and possible control of conditions associated with editing perturbations on this RNA, such as severe depression, Prader-Willi syndrome and interferon therapy.[24–26,43–45]

The affinity and substrate selectivity observed for HTP **1** are modest (Fig. 2 and 5). However, no optimization for binding to 5-HT2cR pre-mRNA has yet been carried out. Indeed, this same compound was shown previously to bind a human pre-miRNA with similar affinity to that measured here for the 5-HT2cR site.[32] Nevertheless, the HTP scaffold appears to be superior to that of the aminoglycosides as a starting point for the development of potent and selective inhibitors of 5-HT2cR editing given the complete lack of substrate selectivity observed for inhibition by neomycin (Fig. 5). The modular synthesis of HTP **1** will facilitate structural changes designed to enhance affinity for the 5-HT2cR RNA and decrease nonselective binding. Indeed, the route described here to HTP **2** involving elaboration of a lysine side chain while the molecule remained solid phase immobilized will be advantageous for generating additional analogs. Efforts are currently underway to optimize HTP binding to 5-HT2cR RNA and control editing on this substrate in live cells.

We generated an RNA editing substrate lacking the five-nucleotide loop in the intron as a control for inhibitor studies since this change reduces HTP affinity (Fig. 2 and 4). While this change also reduces editing efficiency slightly at the D site (91% to 75% under these conditions), increases in editing efficiency were observed at all other sites and particularly the A, C, F and G sites (Fig. 4). It is apparent that this loop enhances ADAR2 selectivity for the D site, likely by reducing the number of binding sites available for interaction with ADAR2′s two double stranded RNA binding motifs (dsRBMs).[46] This may be important for regulation of editing at these sites since ADAR1 is known to edit the A, B and C sites *in vivo*.[45] Without the intron loop, the A and C sites would likely also be efficiently edited by ADAR2.

Conclusions

In conclusion, we have shown for the first time that one can inhibit an RNA editing reaction in a substrate-selective manner with an RNA-binding small molecule. Since RNA editing manipulates protein structure through changes in codon meaning, editing inhibitors provide a novel route to the control of gene function. An HTP can be used to selectively inhibit editing on the 5-HT2cR pre-mRNA whereas the aminogylcoside neomycin lacks selectivity for this substrate. Therefore, the HTP scaffold appears to be a useful starting point for the development of potent and selective inhibitors of 5-HT2cR editing.

Experimental Section

General

All commercial reagents were used as purchased with no further purification unless otherwise noted. Amino acids and reagents used in solid phase synthesis were obtained from NovaBiochem, with the exception of Fmoc protected (*S*)-allyl glycine that was purchased from Acros Organics. Polypropylene columns for solid phase synthesis were purchased from BioRad. Hoyveda Grubb's second-generation catalyst for macrocyclic HTP ring-closing metathesis was purchased from Sigma-Aldrich. Chemical reactions were performed under argon gas and liquids were added *via* oven-dried syringes. The synthesis of HTP **1** was reported previously.[32] EDTA monanhydride for the modification of HTP **2** was prepared as described by Ebright *et al.*[34] Nuclease-free water was purchased from Ambion and was used for all RNA purification, quantitative S1 footprinting, and affinity cleavage experiments. For *in vitro* transcription, *in vitro* editing and PCR, nuclease-free water from Molecular Biologicals, Inc was used. For protein overexpression and purification, Milli-Q water was used. A 10X ADAR reaction buffer (150 mM Tris, pH 7.4, 15 mM EDTA, 1 M KCl, 30 mM $MgCl_2$, 30% glycerol, 0.03% Nonidet P-40) was used for footprinting, affinity cleavage, and *in vitro* editing experiments. Intron loop and no loop oligonucleotides for footprinting and affinity cleavage were purchased from the DNA/Peptide Core Facility at University of Utah and the exon strand was purchased from Dharmacon, Inc. γ-[^{32}P]ATP (6000 Ci/mmol) was purchased from Perkin-Elmer Life Sciences. Primers were purchased from Bioneer. Reagents for RNA footprinting, affinity cleavage, *in vitro* transcription, *in vitro* editing, and PCR amplification were purchased from USB Corporation: nuclease S1, Sigma Aldrich: $Fe(NH_4)_2(SO_4)_2{\cdot}(H_20)_6$, sodium ascorbate, neomycin sulfate, glycerol and phenol:chloroform, GE Healthcare: MicroSpin G-25 columns, Promega: yeast $tRNA^{Phe}$, RNasin, Access RT-PCR kit, New England Biolabs: T4 polynucleotide kinase, BamHI, DpnI. Gels were imaged using storage phosphor imaging plates from Molecular Dynamics on a Molecular Dynamics 9400 Typhoon phosphorimager. Data was analyzed using Molecular Dynamics ImageQuant 5.2 software. RNA editing was quantified using 4Peaks and ImageJ software (Abramoff, *et al.* 2004).

RNA end labeling and duplex formation

All RNA was obtained as a solid pellet. RNA from Dharmacon, Inc arrived PAGE purified and was re-suspended in water. RNA from the University of Utah DNA/Core Peptide Facility was re-suspended in water and loaded onto a 19% denaturing polyacrylamide gel. The RNA was visualized with UV shadowing, cut from the gel, and isolated using the crush and soak method.[47,48] The

isolated oligonucleotides were PhOH:$CHCl_3$ extracted, ethanol precipitated, and pelleted *via* centrifugation. The pelleted RNA was dried by lyophilization and re-suspended in water. RNA concentration was determined by measuring absorbance at 260 nm. For the preparation of 5′-[^{32}P] end labeled RNA, purified RNA (60 pmol) was treated with γ-[^{32}P]ATP (3 mCi/mL) and T4 polynucleotide kinase (2 U) and incubated at 37 °C for 1 h. Unreacted γ-[^{32}P]ATP was removed with a size exclusion G-25 column and 5′-end labeled RNA was purified on a 19% denaturing polyacrylamide gel. Radioactive bands were imaged and cut from the gel. RNA was isolated using the crush and soak method. The RNA duplex was formed by hybridization with the addition of 0.5 equivalents cold RNA and 1.5 equivalents of cold opposite strand to the 5′-end labeled RNA. The RNA mixture was heat denatured at 95 °C for 5 min and slow cooled to room temperature.

Quantitative ribonuclease footprinting

To determine binding affinity of the HTPs, 5′-end labeled RNA duplexes were digested with nuclease S1 (3 U for quantification experiments and 30 U for gels shown in Fig. 2). The experiments were carried out in 1X ADAR reaction buffer with 10 μg mL^{-1} yeast $tRNA^{Phe}$, and 100 μM $ZnCl_2$ to a final reaction volume of 20 μL for 45 min at room temperature. The reaction was quenched with 10 μL of loading buffer, heat denatured at 95 °C for 5 min, and loaded onto a 19% denaturing polyacrylamide gel. To quantify binding of HTP **1** to the RNA, the footprint at the U base paired with the D-site A was monitored with respect to a constant S1-dependent band at a C located 3 nucleotides to the 5′ end of the footprinted U. The RNA cleavage data was converted to binding data such that maximum cleavage was assumed to be 0% HTP **1** occupancy and minimum cleavage was assumed to be 100% HTP occupancy at the binding site. The fraction of RNA bound was plotted as a function of HTP **1** concentration and fitted with the equation: fraction RNA bound = [HTP]/([HTP]+K_d). The binding affinity of HTP **1** was reported as an average and standard deviation of three different experiments.

Directed hydroxyl radical cleavage

Hydroxyl radical affinity cleavage was carried out using a modified approach from a procedure described previously by our lab.[49] RNA duplexes were 5′-end labeled as described above. Varying concentrations of HTP **2** or EDTA (100 μM) control were added to 5′-end labeled RNA in 1X ADAR reaction buffer to a final reaction volume of 20 μL. To form the EDTA·Fe complex, $Fe(NH_4)_2(SO_4)_2{\cdot}(H_20)_6$ was added such that the final concentration was twice that of HTP **2** concentration. The mixtures were allowed to incubate for 20 min at room temperature to allow the HTP/RNA complexes to reach equilibrium. Affinity cleavage was initiated by the addition of sodium ascorbate to a final concentration of 20 mM. To quench the reaction, 80 μL of hot water was added and the mixture was extracted with PhOH:$CHCl_3$. The organic layer was collected and extracted again with ethyl ether. The aqueous layer was lyophilized to dryness, re-suspended, and loaded onto a 19% denaturing polyacrylamide gel.

Synthesis of HTP 2

Methyl-2-(4′-allylphenyl)-4-(4′-methylamino *t*-butyloxycarbamate) anilino-quinoline-8-carboxylate was synthesized as described previously.[32,50] The macrocyclic HTP was synthesized using standard 9-fluoronylmethoxycarbonyl (Fmoc)-protected amino acid solid phase peptide synthesis (SPPS) protocols. Amino acids were immobilized using Novagel Rink Amide resin (0.63 mmol g^{-1} loading, 35 mg, 0.022 mmol) and a polypropylene column. Allylglycine (37 mg, 5 equivalents, 0.110 mmol), lysine(Boc) (52 mg, 5 equivalents, 0.110 mmol), lysine(Mtt) (69 mg, 5 equivalents, 0.110 mmol), Dpr(Boc) (47 mg, 5 equivalents, 0.110 mmol), and the quinoline acid (42 mg, 3.7 equivalents, 0.080 mmol) were coupled to the solid support respectively as described previously.[32] The resin was washed with CH_2Cl_2 (3 × 5 mL), DMF (3 × 5 mL), MeOH (3 × 5 mL) and diethyl ether (3 × 5 mL) and lyophilized for 12 h. The resin was transferred to a flame-dried round bottom flask and the macrocycle was formed while on solid phase using ring-closing metathesis as described previously.[32,50] The peptides were transferred back to the column, washed with CH_2Cl_2 (3 × 5 mL), 10% 1,3-bis(diphenylphosphino)propane in CH_2Cl_2 (1 × 5 mL) and MeOH (3 × 5 mL), and lyophilized for 12 h. The resin was then swollen in DMF for 30 min, drained, suspended in a 1.1% TFA/5% TIS in CH_2Cl_2 solution (5 mL), and shaken for 5 min (5 × 5 mL) to effect cleavage of the Mtt protecting group. The resin was then washed with CH_2Cl_2 (3 × 5 mL), DMF (3 × 5 mL), and MeOH (3 × 5 mL). A solution of EDTA-monoanhydride (60 mg, 10 equivalents, 0.220 mmol) in DMF (5 mL) was added to the column and coupling to the deprotected lysine was carried out by shaking the suspension for 15 h. The resin was then washed with H_2O (3 × 5 mL), DMF (3 × 5 mL), and MeOH (3 × 5 mL) and lyophilized for 12 h. The peptide was deprotected and cleaved from the solid support by suspension in TFA/TIS/PhOH/H_2O (88 : 5 : 5 : 2, 3 mL) and shaking for 3.5 h. The solution was collected and concentrated under reduced pressure. Ether precipitation and extraction with water yielded the crude peptide. The lyophilized crude product was HPLC-purified on a reverse phase C-18 column (4.6 × 250 mm, Vydac) eluting with 0–60% CH_3CN in H_2O over 20 min with a flow rate of 1.5 mL min^{-1}. ESI-MS calculated mass for $C_{54}H_{72}N_{13}O_{12}$: 1093.5, found $[M+2H]^{2+}$ 548.5 m/z.

ADAR2 overexpression and purification

Plasmid hADAR2a-LV$(H)_6$ was used for overexpression of human ADAR2 in *Saccharomyces cereviseae.*[51,52] ADAR2 was purified from yeast lysates as previously described.[52]

Preparation of RNAs for deamination assay

A plasmid (plasmid SerLIVT) was ordered from Biomatik containing the following sequence within the SmaI site of a pGE plasmid:

CG TAC AAGCTT ACCTAGATAT TTGTGCCCCG TCT-GGATTTC TTTAGATGTT TTATTTTCAA CAGCGTCCAT CATGCACCTC TGCGCTATAT CGCTGGATCG GTAT-GTAGCA ATACGTAATC CTATTGAGCA TAGCCGTTTC AATTCGCGGA CTAAGGCCAT CATGAAGATT GCTATT-GTTT GGGCAATTTC TATAGGTAAA TAAAACTTTT

TGGCCATAAG AATTGCAGCG GCTATGCTCAA TACTTTCGGA TTATGTACTG TGAACAACGT ACAGACGTCG ACTGGTAACA TTTGCGTTTG ATCGGGTTCT GGATC CGTGTC. The plasmid was linearized with BamHI. T7 RNA polymerase was prepared by overexpression in *E. coli.* *In vitro* transcription was carried out by incubating the linearized plasmid with T7 RNA polymerase with transcription buffer (80 mM HEPES, 25 mM $MgCl_2$, 2 mM spermidine, 30 mM DTT, pH 7.5), NTPs (8 mM), and RNasin (1 U/μL) for 2 h at 42 °C. The product was purified by urea-polyacrylamide gel electrophoresis (PAGE) (5%). After electrophoresis, the RNA bands were visualized by UV shadowing and extracted from the gel *via* the crush and soak method. Polyacrylamide particles were removed using a Centrex filter (0.2 μm) followed by a PhOH:$CHCl_3$ extraction and ethanol precipitation. The RNA solution was lyophilized to dryness, resuspended in a solution of 1 X TE buffer and 100 mM NaCl and quantified by absorbance measurements at 260 nm. The solution was diluted in the same buffer to 180 nM. The RNA was refolded by heating to 95 °C for 5 min and then allowing to slowly cool to room temperature. To prepare a control RNA without the internal loop, a mutagenic PCR was carried out on the plasmid SerLIVT using the following primers: NLF (AATTGCAGCGGCTATGCTCAATAGGATTATGTACTGTGAACAACGT) and NLR (ACGTTGTTCACAGTACATAATCCTATTGAGCATAGCCGCTGCAATT)

The PCR was done according to the following program: 95 °C for 30 s; 18 cycles of 95 °C for 30 s, 55 °C for 1 min, 68 °C for 4 min; 68 °C for 7 min; 4 °C hold. After the PCR, the product was digested with DpnI to remove the parent plasmid. The plasmid was transformed into XL10 Gold cells (Stratagene) and sequenced to confirm that the loop sequence was removed. RNA was prepared from this plasmid as for the sequence containing the loop.

Deamination assay

Editing of the substrate RNA either with or without the intron loop was evaluated by the following reaction. ADAR2 (18 nM final concentration) was mixed with 10 nM RNA in assay buffer containing 1X ADAR buffer, 0.5 mM DTT, 160U/mL RNasin. and 1.0 ug/mL yeast $tRNA^{Phe}$. The reaction was carried out at 30 °C for 15 min. The RNA was purified by PhOH:$CHCl_3$ extraction and ethanol precipitation and resuspended in 400 μL nuclease free water. RT-PCR was done using 2 μL of template, primers SQF (TGGGTACGAATTCCCCGTACAAGCTT) and SQR (AGAACCCGATCAAACGCAAATGTTAC) and the Access RT-PCR System according to the manufacturer's instructions. The PCR was carried out using the following program: 45 °C for 45 min; 94 °C for 2 min; 25 cycles of 94 °C for 30 s, 60 °C for 1 min and 68 °C for 2 min; 68 °C for 7 min; 4 °C hold. PCR products were gel purified using the Qiaquick Gel Extraction Kit from Qiagen. Extent of editing was determined by DNA sequencing using primer SQF (above).

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References

1 B. J. Albert, P. A. McPherson, K. O'Brien, N. L. Czaicki, V. Destefino, S. Osman, M. Li, B. W. Day, P. J. Grabowski, M. J. Moore, A. Vogt and K. Koide, *Mol. Cancer Ther.*, 2009, **8**, 2308–2318.
2 K. G. Aukema, K. K. Chohan, G. L. Plourde, K. B. Reimer and S. D. Rader, *ACS Chem. Biol.*, 2009, **4**, 759–768.
3 M. E. Bordeleau, J. Matthews, J. M. Wojnar, L. Lindqvist, O. Novac, E. Jankowsky, N. Sonenberg, P. Northcote, P. Teesdale-Spittle and J. Pelletier, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 10460–10465.
4 M. E. Bordeleau, A. Mori, M. Oberer, L. Lindqvist, L. S. Chard, T. Higa, G. J. Belsham, G. Wagner, J. Tanaka and J. Pelletier, *Nat. Chem. Biol.*, 2006, **2**, 213–220.
5 S. Chirayil, R. Chirayil and K. J. Luebke, *Nucleic Acids Res.*, 2009, **37**, 5486–5497.
6 K. Gumireddy, D. D. Young, X. Xiong, J. B. Hogenesch, Q. Huang and A. Deiters, *Angew. Chem., Int. Ed.*, 2008, **47**, 7482–7484.
7 D. Kaida, H. Motoyoshi, E. Tashiro, T. Nojima, M. Hagiwara, K. Ishigami, H. Watanabe, T. Kitahara, T. Yoshida, H. Nakajima, T. Tani, S. Horinouchi and M. Yoshida, *Nat. Chem. Biol.*, 2007, **3**, 576–583.
8 Y. Kotake, K. Sagane, T. Owa, Y. Mimori-Kiyosue, H. Shimizu, M. Uesugi, Y. Ishihama, M. Iwata and Y. Mizui, *Nat. Chem. Biol.*, 2007, **3**, 570–575.
9 A. N. Kuhn, M. A. van Santen, A. Schwienhorst, H. Urlaub and R. Luhrmann, *RNA*, 2008, **15**, 153–175.
10 C. Lagisetti, A. Pourpak, T. Goronga, Q. Jiang, X. Cui, J. Hyle, J. M. Lahti, S. W. Morris and T. R. Webb, *J. Med. Chem.*, 2009, **52**, 6979–6990.
11 W. K. Low, Y. Dang, T. Schneider-Poetsch, Z. Shi, N. S. Choi, W. C. Merrick, D. Romo and J. O. Liu, *Mol. Cell*, 2005, **20**, 709–722.
12 K. O'Brien, A. J. Matlin, A. M. Lowell and M. J. Moore, *J. Biol. Chem.*, 2008, **283**, 33147–33154.
13 G. A. Roybal and M. S. Jurica, *Nucleic Acids Res*, 2010, DOI: 10.1093/nar/gkq1494.
14 N. S. Wickramasinghe, T. T. Manavalan, S. M. Dougherty, K. A. Riggs, Y. Li and C. M. Klinge, *Nucleic Acids Res.*, 2009, **37**, 2584–2595.
15 A. Aartsma-Rus and G. J. van Ommen, *RNA*, 2007, **13**, 1609–1624.
16 J. R. Chamberlain and J. S. Chamberlain, *Nat. Med.*, 2010, **16**, 170–171.
17 M. G. Dunckley, M. Manoharan, P. Villiet, I. C. Eperon and G. Dickson, *Hum. Mol. Genet.*, 1998, **7**, 1083–1090.
18 Z. Dominski and R. Kole, *Proc. Natl. Acad. Sci. U. S. A.*, 1993, **90**, 8673–8677.
19 H. Grosjean and R. Benne, *Modification and Editing of RNA*, ASM Press, Washington, D.C., 1998.
20 C. M. Burns, H. Chu, S. M. Rueter, L. K. Hutchinson, H. Canton, E. Sanders-Bush and R. B. Emeson, *Nature*, 1997, **387**, 303–308.
21 M. Higuchi, F. N. Single, M. Kohler, B. Sommer, R. Sprengel and P. H. Seeburg, *Cell*, 1993, **75**, 1361–1370.
22 N. T. Pham, M. Tohda, Y. Tezuka and K. Matsumoto, *Biol. Pharm. Bull.*, 2010, **33**, 527–529.
23 M. V. Morabito and R. B. Emeson, *Neuropsychopharmacology*, 2009, **34**, 246.
24 C. M. Doe, D. Relkovic, A. S. Garfield, J. W. Dalley, D. E. Theobald, T. Humby, L. S. Wilkinson and A. R. Isles, *Hum. Mol. Genet.*, 2009, **18**, 2140–2148.
25 K. Iwamoto and T. Kato, *Neurosci. Lett.*, 2003, **346**, 169–172.
26 M. V. Morabito, A. I. Abbas, J. L. Hood, R. A. Kesterson, M. M. Jacobs, D. S. Kump, D. L. Hachey, B. L. Roth and R. B. Emeson, *Neurobiol. Dis.*, 2010, **39**, 169–180.
27 C. B. Carlson and P. A. Beal, *Bioorg. Med. Chem. Lett.*, 2000, **10**, 1979–1982.
28 C. B. Carlson, M. Vuyisich, B. D. Gooch and P. A. Beal, *Chem. Biol.*, 2003, **10**, 663–672.
29 B. D. Gooch and P. A. Beal, *J. Am. Chem. Soc.*, 2004, **126**, 10603–10610.
30 B. D. Gooch, M. Krishnamurthy, M. Shadid and P. A. Beal, *ChemBioChem*, 2005, **6**, 2247–2254.
31 M. Krishnamurthy, B. D. Gooch and P. A. Beal, *Org. Biomol. Chem.*, 2006, **4**, 639–645.
32 M. Krishnamurthy, K. Simon, A. M. Orendt and P. A. Beal, *Angew. Chem., Int. Ed.*, 2007, **46**, 7044–7047.

33 R. P. Hertzberg and P. B. Dervan, *J. Am. Chem. Soc.*, 1982, **104**, 313–315.
34 Y. W. Ebright, Y. Chen, P. S. Pendergrast and R. H. Ebright, *Biochemistry*, 1992, **31**, 10664–10670.
35 A. Nakae, T. Tanaka, K. Miyake, M. Hase and T. Mashimo, *Int. J. Biol. Sci.*, 2008, **4**, 397–405.
36 R. Flomen, J. Knight, P. Sham, R. Kerwin and A. Makoff, *Nucleic Acids Res.*, 2004, **32**, 2113–2122.
37 C. M. Niswender, S. C. Copeland, K. Herrick-Davis, R. B. Emeson and E. Sanders-Bush, *J. Biol. Chem.*, 1999, **274**, 9472–9478.
38 P. Vitali, E. Basyuk, E. Le Meur, E. Bertrand, F. Muscatelli, J. Cavaille and A. Huttenhofer, *J. Cell Biol.*, 2005, **169**, 745–753.
39 J. R. Thomas and P. J. Hergenrother, *Chem. Rev.*, 2008, **108**, 1171–1224.
40 T. Hermann, *Cell. Mol. Life Sci.*, 2007, **64**, 1841–1852.
41 Y. Tor, *Angew. Chem., Int. Ed.*, 1999, **38**, 1579–1582.
42 Y. Tor, *ChemBioChem*, 2003, **4**, 998–1007.
43 I. Gurevich, H. Tamir, V. Arango, A. J. Dwork, J. J. Mann and C. Schmauss, *Neuron*, 2002, **34**, 349–356.
44 C. M. Niswender, K. Herrick-Davis, G. E. Dilley, H. Y. Meltzer, J. C. Overholser, C. A. Stockmeier, R. B. Emeson and E. Sanders-Bush, *Neuropsychopharmacology*, 2001, **24**, 478–491.
45 W. Yang, Q. Wang, S. J. Kanes, J. M. Murray and K. Nishikura, *Mol. Brain Res.*, 2004, **124**, 70–78.
46 K. A. Lehmann and B. L. Bass, *J. Mol. Biol.*, 1999, **291**, 1–13.
47 O. Maydanovych, L. M. Easterwood, T. Cui, E. A. Veliz, S. Pokharel and P. A. Beal, *Methods Enzymol.*, 2007, **424**, 369–386.
48 J. Sambrook, E. F. Fritsch and T. Maniantis, *Molecular Cloning: A Laboratory Manual*, 2nd edn, Cold Spring Harbor Laboratory Press, New York, 1989.
49 N. V. Jammi and P. A. Beal, *Nucleic Acids Res.*, 2001, **29**, 3020–3029.
50 M. Krishnamurthy, N. T. Schirle and P. A. Beal, *Bioorg. Med. Chem.*, 2008, **16**, 8914–8921.
51 B. L. Haudenschild, O. Maydanovych, E. A. Veliz, M. R. Macbeth, B. L. Bass and P. A. Beal, *J. Am. Chem. Soc.*, 2004, **126**, 11213–11219.
52 M. R. Macbeth, A. T. Lingam and B. L. Bass, *RNA*, 2004, **10**, 1563–1571.