Supplementary Table 2. Percent of TAR hits that score better when carrying out EBVS against $E_{0,4rdc}$ compared to the tRNA structure (PDB 1EHZ).

	% TAR hits selective for TAR	% Cell-active hits selective for TAR
Full Library	99% (76/78)	95% (18/19)
Filtered Library	85% (22/26)	75% (6/8)
Optimized Library	93% (13/14)	100% (5/5)

Supplementary Note 1. Generation of small molecule libraries for EBVS evaluation.

A virtual library of the same 103,498 molecules used in HTS was downloaded from the CCG and saved in sdf file format. The library was enriched with 170 molecules drawn in ChemDraw (CambridgeSoft) and saved in sdf file format that were previously reported to bind TAR *in vitro* (Supplementary Table 1). These molecules were identified through a literature search of TAR binders and limited to non-peptide, non-metal binding molecules with reported binding affinity (K_D, K_i, or CD_{50}) less than 100 μM (Supplementary Table 1). Several molecules with affinities above and below this cutoff were purchased and tested in the Tat displacement assay and, as expected, all nine molecules with affinities within the cutoff were active in our assay while all six molecules with weaker affinities were inactive (Supplementary Table 1). These results suggest that our affinity cutoff is correctly selecting TAR binders that bind tightly enough to have been considered hits in HTS. However, we acknowledge that we were not able to validate all additional hits in our assay. We chose to keep molecules with reported high affinity despite technical issues such as solubility or fluorescence interference in our assay conditions. Furthermore, we tested molecules previously shown to bind TAR but that did not have reported affinities. Two molecules previously shown to bind TAR via electric linear dichroism were found to be active in our assay at low micromolar affinities and all molecules from this study were included in our library (Supplementary Table 1). An arginine mimic that was previously shown to bind TAR via NMR was found to weakly (CD_{50} > 100 μM) displace Tat in our assay and thus it and all other molecules from this study were excluded from our library (Supplementary Table 1). For papers reporting many chemically similar molecules, only the tightest

binding derivatives were included to maintain chemical diversity in our library. We further increased chemical diversity by clustering the hits using the Bemis-Murcko atomic framework using the JKlustor package (ChemAxon) and only keeping the most active hit from each scaffold cluster.

From this Full library, we generated the “Filtered” sub-library by removing all molecules with outlier properties defined as any chemical property (molecular weight, formal charge, LogP, hydrogen bond donor count, hydrogen bond acceptor count, and rotatable bond count) with a value outside three standard deviations of the average value of the non-hit library. Chemical properties were determined using the Calculator plugins (ChemAxon) for the predicted protonation state at pH 7. We also generated an “Optimized” sub-library using the general protocol for decoy generation used in the DUD-E (Mysinger, M.M., Carchia, M., Irwin, J.J. & Shoichet, B.K. *J. Med. Chem.* **55,** 6582–94 (2012)). First, we calculated the Tanimoto similarity metric between all hits and non-hits using the ScreenMD package (ChemAxon) set for chemical fingerprint molecular descriptors and removed the 75% of non-hits that are the most topologically similar to any hit. As in the DUD-E, this step decreases the probability of including non-hits that actually do bind TAR either as weak binders or that were false negatives in our assay. From this reduced pool of non-hits, we selected 50 property-matched molecules per hit within a specified similarity cutoff using a Euclidean distance equation for the scaled chemical properties (molecular weight, formal charge, LogP, hydrogen bond donor count, hydrogen bond acceptor count, and rotatable bond count). When there were not enough unique non-hits within the cutoff for a given hit, the hit was excluded. Varying the similarity cutoff or the number of actives selected per hit has a small effect on the chemical property distributions and enrichment scores (Supplementary Fig. 6a and 6b). We determined that a cutoff of 0.08 gave unbiased distributions of chemical properties for hits and non-hits while maintaining a reasonable number of hits.

Supplementary Note 2. Complicating factors of evaluating EBVS pose predictions.

Several factors complicate comparison of ligand-bound poses predicted using EBVS and the NMR structure for the six TAR-ligand complexes. First, EBVS predicts an ensemble of conformations,

not a single structure. Second, the fact that our docked structure is different from the NMR structure complicates alignment and interpretation of ligand bound poses using the conventional RMSD method. In Figure 5, we have chosen to align the RNA binding pockets and ligands prior to measuring the heavy-atom RMSD between ligands. Third, prior studies have shown that TAR retains considerable flexibility when bound to ligands and this flexibility may not be fully captured in the NOE-based NMR structures (Bardaro, M.F. Jr, Shajani, Z., Patora-Komisarska, K., Robinson, J.A. & Varani, G. *Nucleic Acids Res.* **37,** 1529–40 (2009); Zhang, Q., Stelzer, A.C., Fisher, C.K. & Al-Hashimi, H.M. *Nature* **450,** 1263–7 (2007); Pitt, S.W., Majumdar, A., Serganov, A., Patel, D.J. & Al-Hashimi, H.M. *J. Mol. Biol.* **338,** 7–16 (2004)). For example, RDC studies have shown that TAR retains a considerably high degree of inter-helical flexibility when bound to acetylpromazine (Pitt, S.W., Zhang, Q., Patel, D.J. & Al-Hashimi, H.M. *Angew. Chem. Int. Ed. Engl.* **44,** 3412–5 (2005)) while NMR spin relaxation studies show increased levels of motions at residues U23 and C24 near the ligand-binding site in the TAR-arginine complex (Hansen, A.L. & Al-Hashimi, H.M. *J. Am. Chem. Soc.* **129**, 16072–82 (2007)). Finally, we cannot rule out significant uncertainty in the atomic details of the NOE-based NMR structures, determined without RDCs, which are relevant for assessing ligand pose predictions. There are now several studies highlighting the uncertainty in NOE-based NMR structures of RNA determined without RDC restraints and these problems will only be exacerbated in dynamic RNA-ligand complexes (Bermejo, G.A., Clore, G.M. & Schwieters, C.D. *Structure* **24,** 806–815 (2016); Tolbert, B.S. *et al. J. Biomol. NMR* **47,** 205–19 (2010)). Indeed, prior RDC studies on three of these TAR-ligand complexes (arginine, acetylpromazine and neomycin B) show that even for the well-formed A-form helices, the local geometry in the NOE-based structure poorly fits the RDCs whereas much better fits are obtained with an idealized A-form helix (Pitt, S.W., Majumdar, A., Serganov, A., Patel, D.J. & Al-Hashimi, H.M. *J. Mol. Biol.* **338,** 7–16 (2004); Pitt, S.W., Zhang, Q., Patel, D.J. & Al-Hashimi, H.M. *Angew. Chem. Int. Ed. Engl.* **44,** 3412–5 (2005); Hansen, A.L. & Al-Hashimi, H.M. *J. Am. Chem. Soc.* **129**, 16072–82 (2007)). The RDC data measured for these three complexes including the bulge also exhibit a poor fit to these NOE-based NMR structures

(Supplementary Fig. 10a) again reflecting uncertainty or unaccounted dynamics/flexibility. The NOE-based NMR structure of TAR-arginine does not feature a base-triple, which has been reported in several independent studies, and there is also evidence that arginine binds to more than one site on HIV-1 TAR (Davis, B. et al. J. Mol. Biol. 336, 343–356 (2004)). In addition, ICM much more poorly predicts the ligand poses (which, excluding neomycin B, have N_{flex} <11) for these TAR complexes when re-docking the ligands against their NMR structure (average RMSD = 8.0 ± 2.4 Å) relative to benchmarks employing NMR (average RMSD = 6.1 ± 3.5 Å) and X-ray structures (average RMSD = 3.2 ± 3.5 Å) of other RNA-ligand complexes (Fig. 5).