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Supporting Information

Deciphering the Mechanism of action of 5FDQD and design of new neutral analogues for FMN riboswitch: A Well-tempered metadynamics simulation study

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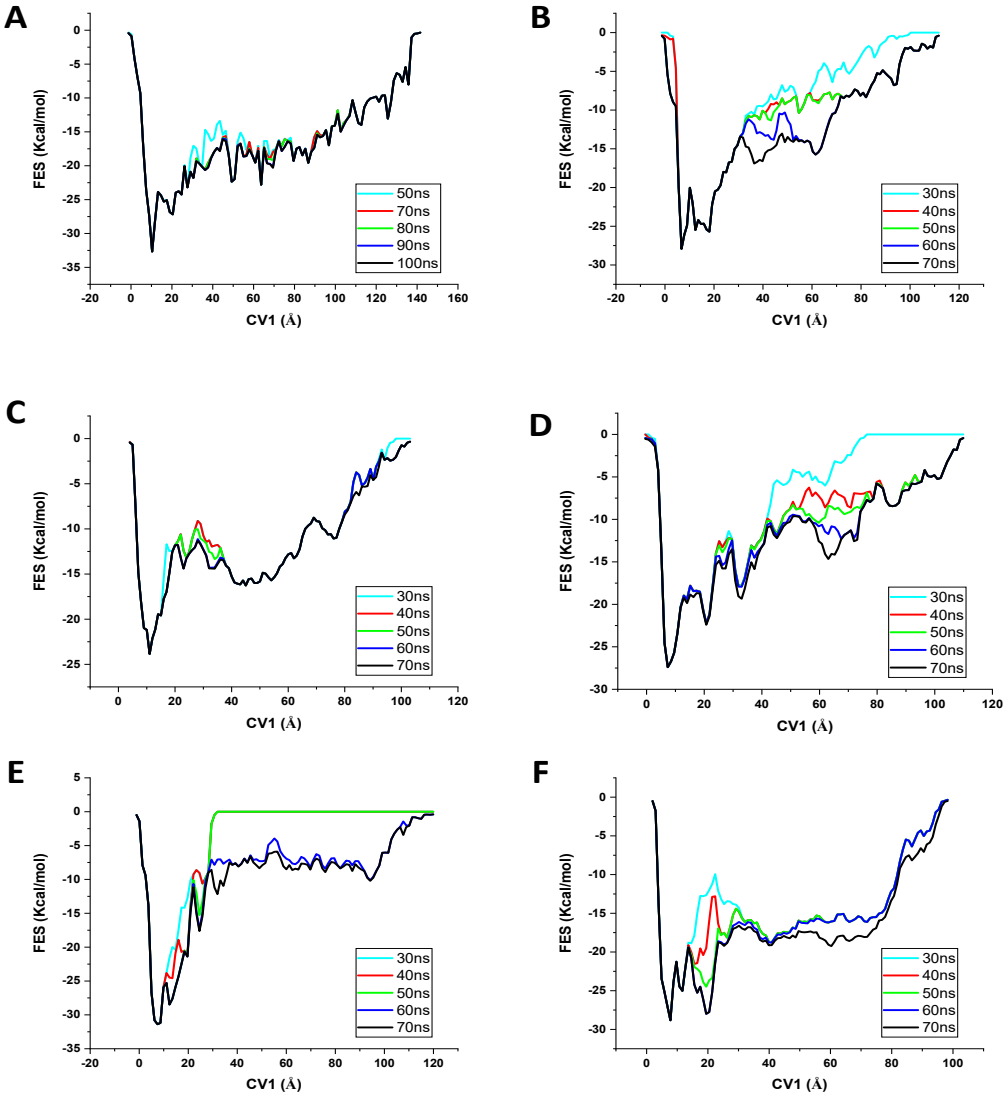

Figure S1: Convergence plot of the Free Energy landscape at different time interval for **A**) FMN-fluoro (5FDQD) B) FMN-benzene **C**) FMN-difluoro1 **D**) FMN-difluoro2 **E**) FMN-trifluoro **F**) FMN-amino.

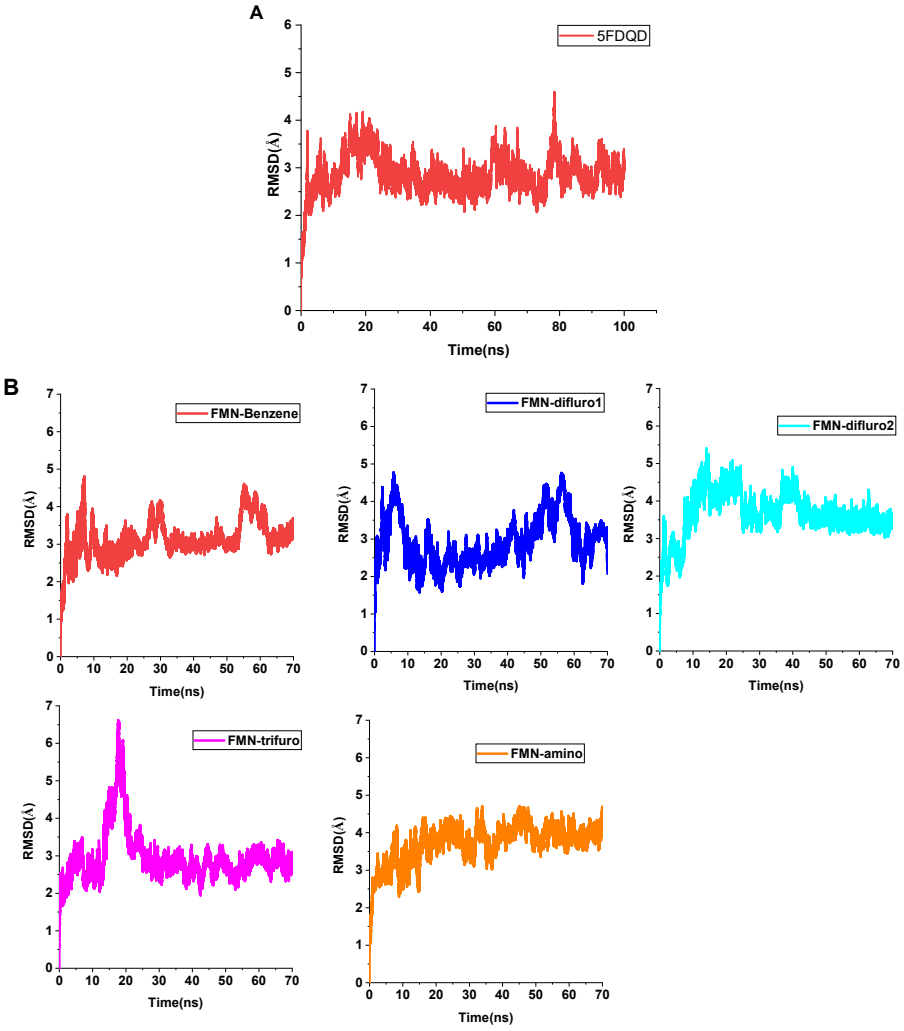

Figure S2: Root means square deviation for all ligand-bound FMN riboswitch is plotted as a function of time.

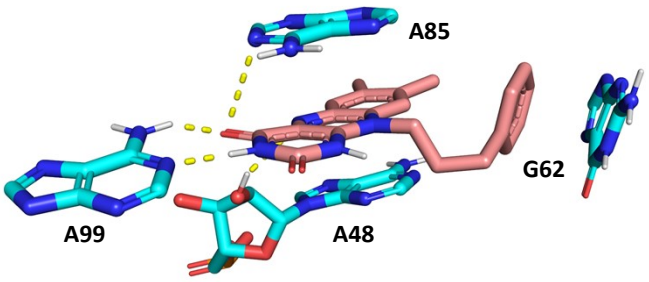

Figure S3 Binding site interaction of BRX1555 with riboswitch in crystal structure.

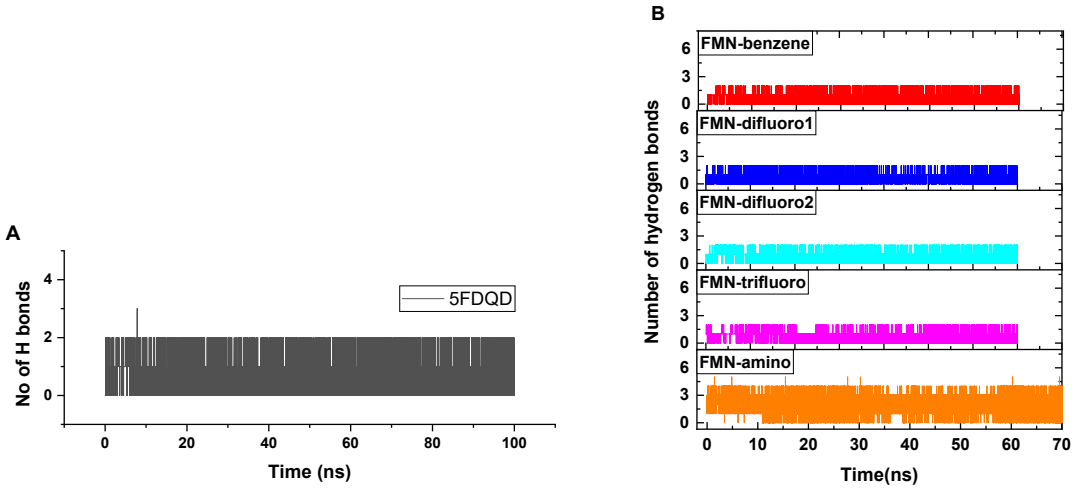

Figure S4: Number of hydrogen bonds form by ligands with the riboswitch during simulation are plotted against the time.

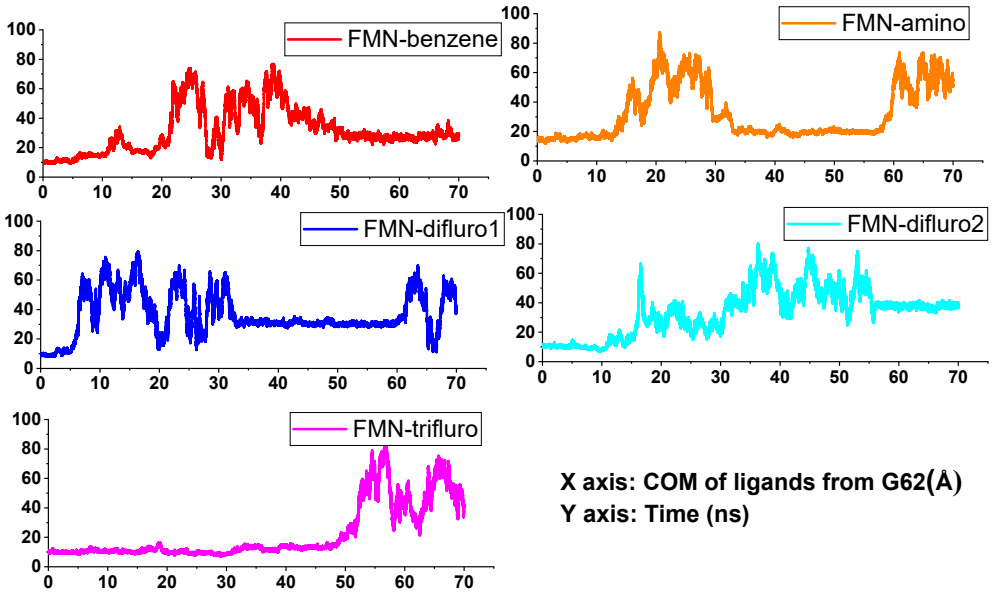

Figure S5: COM distance between the base G62 and ligand.

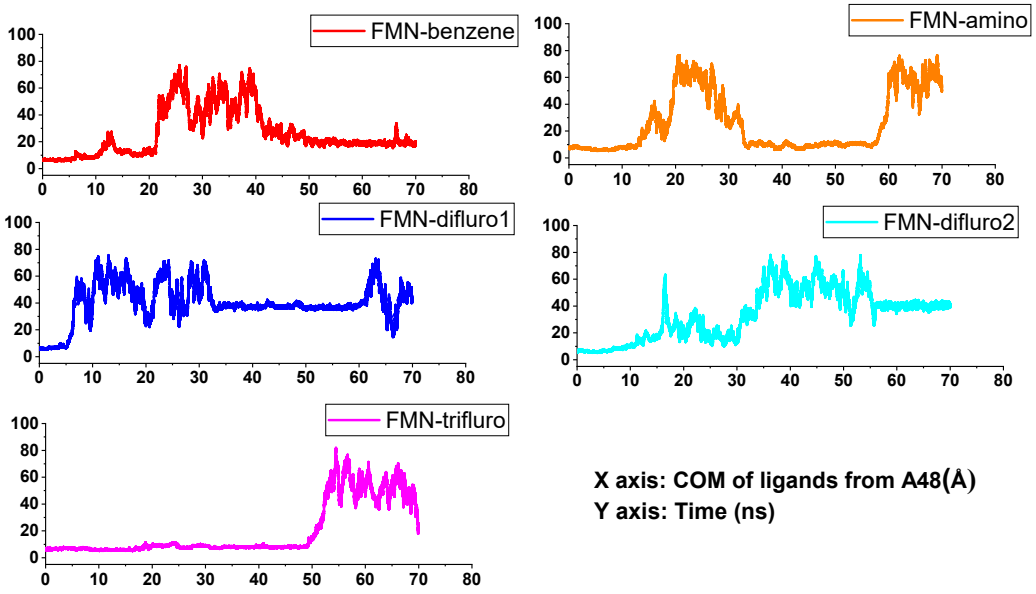

Figure S6: COM distance between the base A48 and ligand.

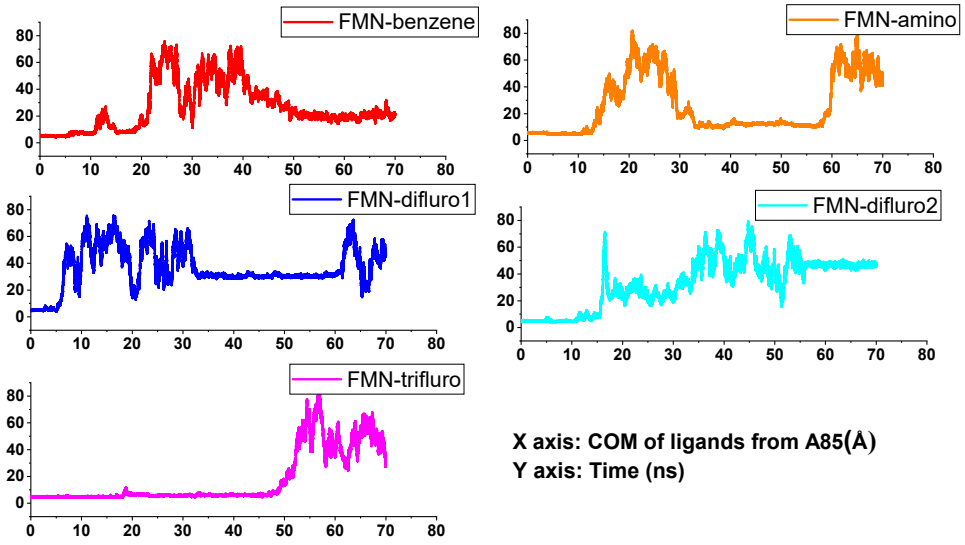

Figure S7: COM distance between the base A85 and ligand.

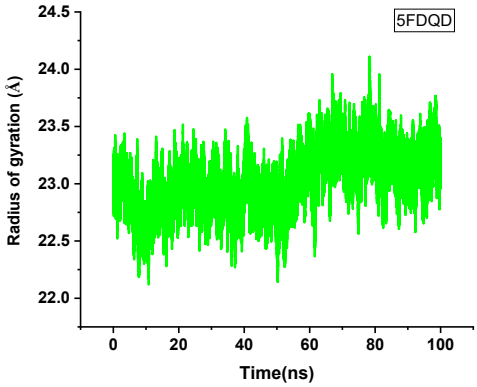

Figure S8: Radius of gyration values for riboswith bound to 5FDQD is plotted against the simulation time.

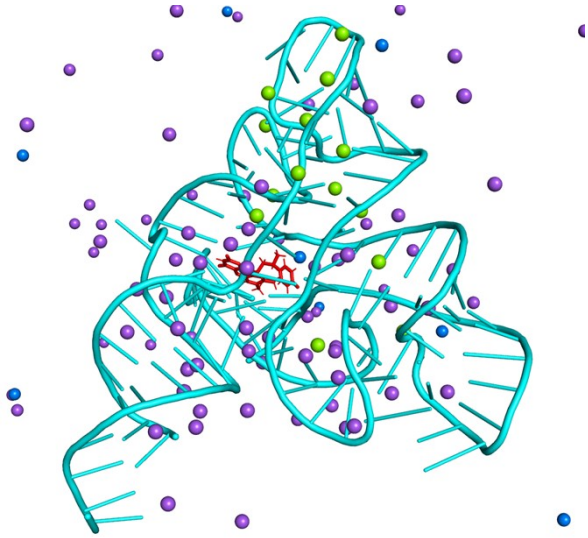

Figure S9: Interaction of metal ions with the riboswitch backbone 5FDQD.

Table S1: Ligand Charges with AM1-BCC and DFT calculations at B3LYP/6-31g(d) method.

Ligands	Ligand Charges with AM1-BCC	Ligand Charges with DFT calculations at B3LYP/6-31g(d)
FMN-benzene	C 1 -0.130000 C1 2 -0.127000 C2 3 -0.127000 C3 4 -0.124500 C4 5 -0.124500 C5 6 -0.089100 C6 7 -0.015400 C7 8 -0.097400 C8 9 -0.181000 C9 10 -0.100300 C10 11 -0.051800 C11 12 -0.064800 C12 13 -0.007300 C13 14 -0.110300 C14 15 -0.093300 C15 16 -0.003600 C16 17 0.286600 C17 18 0.025300 C18 19 0.083400 C19 20 0.301800 N 21 -0.415400 H 22 0.348500 N1 23 -0.516001 O 24 -0.586501 O1 25 -0.571501	1 C -0.127225 2 C -0.131362 3 C -0.134158 4 C -0.182777 5 C -0.186724 6 C -0.465034 7 C -0.205777 8 C -0.269219 9 C -0.204230 10 C -0.219883 11 C -0.532858 12 C -0.539690 13 C 0.113244 14 C 0.125639 15 C 0.161850 16 C 0.367573 17 C 0.254158 18 C -0.188671 19 C 0.564633 20 C 0.183749 21 N -0.599050 22 H 0.350389 23 N -0.514419 24 O -0.495472 25 O -0.481587

	N2 26 -0.615501 C20 27 0.610001 N3 28 -0.105100 C21 29 0.850901 H2 30 0.132000 H3 31 0.135000 H4 32 0.135000 H5 33 0.136500 H6 34 0.136500 H7 35 0.040367 H8 36 0.040367 H9 37 0.040367 H10 38 0.044700 H11 39 0.059700 H12 40 0.059700 H13 41 0.150000 H14 42 0.163000 H15 43 0.050033 H16 44 0.050033 H17 45 0.050033 H18 46 0.054700 H19 47 0.054700 H20 48 0.054700 H21 49 0.081700 H22 50 0.081700	26 N -0.677934 27 C 0.617036 28 N -0.614511 29 C 0.696288 30 H 0.132209 31 H 0.135031 32 H 0.131868 33 H 0.130660 34 H 0.129305 35 H 0.155242 36 H 0.144845 37 H 0.161619 38 H 0.167024 39 H 0.158913 40 H 0.176610 41 H 0.115950 42 H 0.159604 43 H 0.158531 44 H 0.176926 45 H 0.176586 46 H 0.164579 47 H 0.176064 48 H 0.177301 49 H 0.234359 50 H 0.172800
5FDQD (FMN-fluro)	C 1 -0.107000 C1 2 -0.107000 C2 3 -0.089100 C3 4 -0.019400 C4 5 -0.098400 C5 6 -0.183000 C6 7 -0.101300 C7 8 -0.165500 C8 9 -0.165500 C9 10 -0.052800 C10 11 -0.067800 C11 12 -0.006300 C12 13 -0.109300 C13 14 -0.097300 C14 15 -0.004600 C15 16 0.286600 C16 17 0.123900 C17 18 0.026300 C18 19 0.083400 C19 20 0.301800 N 21 -0.417400 H 22 0.348500 F 23 -0.138900 N1 24 -0.515001 O 25 -0.584501 O1 26 -0.571501 N2 27 -0.615501	1 C -0.179257 2 C -0.179430 3 C -0.455636 4 C -0.172005 5 C -0.298983 6 C -0.235313 7 C -0.214595 8 C -0.190891 9 C -0.206004 10 C -0.527342 11 C -0.538242 12 C 0.110628 13 C 0.115584 14 C 0.159761 15 C 0.378994 16 C 0.253155 17 C 0.376593 18 C -0.178473 19 C 0.566697 20 C 0.182311 21 N -0.600894 22 H 0.350437 23 F -0.300195 24 N -0.514326 25 O -0.496227 26 O -0.481372 27 N -0.678234

	C20 28 0.610001 N3 29 -0.106100 C21 30 0.848901 H1 31 0.055700 H2 32 0.055700 H3 33 0.055700 H4 34 0.050033 H5 35 0.050033 H6 36 0.050033 H7 37 0.157000 H8 38 0.163000 H9 39 0.080200 H10 40 0.080200 H11 41 0.062200 H12 42 0.062200 H13 43 0.061700 H14 44 0.042367 H15 45 0.042367 H16 46 0.042367 H17 47 0.141000 H18 48 0.150500 H19 49 0.150500 H20 50 0.141000	28 C 0.617350 29 N -0.612306 30 C 0.696910 31 H 0.166563 32 H 0.183781 33 H 0.177305 34 H 0.160237 35 H 0.179215 36 H 0.179088 37 H 0.144853 38 H 0.157358 39 H 0.171138 40 H 0.235231 41 H 0.150192 42 H 0.159761 43 H 0.164624 44 H 0.160594 45 H 0.151328 46 H 0.157308 47 H 0.137528 48 H 0.150666 49 H 0.144306 50 H 0.120228
FMN- difluro1	N 1 -0.419400 C 2 -0.094400 O 3 -0.583501 C1 4 -0.191000 N1 5 -0.516001 O1 6 -0.570501 C2 7 -0.051100 N2 8 -0.614501 C3 9 -0.100300 N3 10 -0.108100 C4 11 -0.052800 C5 12 -0.069800 C6 13 -0.191000 C7 14 -0.191000 C8 15 -0.028300 C9 16 -0.005300 C10 17 -0.110300 C11 18 -0.000600 C12 19 -0.208000 C13 20 0.285600 C14 21 0.157400 C15 22 0.157400 C16 23 0.032300 C17 24 0.081400 C18 25 0.300800 C19 26 0.610001 C20 27 0.849900 F 28 -0.133400 F1 29 -0.133400	1 C 0.138549 2 C -0.263723 3 C 0.402122 4 C 0.270258 5 C -0.237462 6 C 0.140138 7 C 0.203104 8 C 0.590697 9 C 0.696610 10 C 0.627036 11 H 0.177407 12 H 0.164263 13 C -0.542432 14 H 0.177757 15 H 0.177869 16 H 0.174471 17 C -0.544115 18 H 0.184169 19 H 0.184235 20 H 0.177065 21 C -0.168563 22 H 0.189401 23 H 0.206202 24 C -0.272968 25 H 0.164623 26 H 0.171174 27 C -0.358811 28 H 0.167154 29 C 0.157505

	H 30 0.349500 H2 31 0.072200 H3 32 0.072200 H4 33 0.154000 H5 34 0.063200 H6 35 0.063200 H7 36 0.164000 H8 37 0.050700 H9 38 0.050700 H10 39 0.050700 H11 40 0.057367 H12 41 0.057367 H13 42 0.057367 H14 43 0.156000 H15 44 0.156000 H16 45 0.168000 H17 46 0.077700 H18 47 0.077700	30 C -0.265264 31 C -0.254512 32 C 0.387852 33 H 0.162849 34 C 0.384723 35 H 0.163275 36 C -0.282079 37 N -0.557474 38 N -0.600415 39 N -0.630997 40 N -0.677509 41 H 0.362102 42 O -0.533269 43 O -0.553612 44 H 0.173552 45 H 0.176817 46 F -0.304794 47 F -0.304982
FMN- difluro2	N 1 -0.417400 C 2 -0.133000 O 3 -0.581501 C1 4 -0.094400 N1 5 -0.516001 O1 6 -0.569501 C2 7 -0.189000 N2 8 -0.614501 C3 9 -0.048100 N3 10 -0.108100 C4 11 -0.099300 C5 12 -0.141000 C6 13 -0.052800 C7 14 -0.066800 C8 15 -0.145000 C9 16 -0.008300 C10 17 -0.110300 C11 18 -0.068300 C12 19 0.001400 C13 20 0.285600 C14 21 0.032300 C15 22 0.091900 C16 23 0.101900 C17 24 0.081400 C18 25 0.302800 C19 26 0.610001 C20 27 0.846901 F 28 -0.122900 F1 29 -0.122900 H 30 0.349500 H2 31 0.141000 H3 32 0.071700 H4 33 0.071700 H5 34 0.152000	1 C -0.193995 2 C -0.247219 3 C -0.251055 4 C -0.383063 5 C -0.290046 6 C -0.206211 7 C -0.533521 8 C -0.533149 9 C -0.265412 10 C 0.093053 11 C 0.138087 12 C 0.172414 13 C 0.347017 14 C 0.381802 15 C -0.197808 16 C 0.318464 17 C 0.330305 18 C 0.618454 19 C 0.242751 20 N -0.760589 21 H 0.357623 22 F -0.283355 23 F -0.283368 24 N -0.841282 25 O -0.523108 26 O -0.542420 27 N -0.689435 28 C 0.582172 29 N -0.649668 30 C 0.757767 31 H 0.154890 32 H 0.174655 33 H 0.144256 34 H 0.116080

	H6 35 0.060200 H7 36 0.060200 H8 37 0.165000 H9 38 0.154000 H10 39 0.050367 H11 40 0.050367 H12 41 0.050367 H13 42 0.055033 H14 43 0.055033 H15 44 0.055033 H16 45 0.158000 H17 46 0.077700 H18 47 0.077700	35 H 0.171825 36 H 0.156741 37 H 0.118299 38 H 0.161726 39 H 0.154458 40 H 0.162968 41 H 0.163652 42 H 0.154420 43 H 0.155993 44 H 0.156019 45 H 0.154032 46 H 0.161515 47 H 0.150052
FMN-trifluro	C 1 -0.098100 C1 2 -0.012400 C2 3 -0.160300 C3 4 -0.093400 C4 5 -0.181000 C5 6 -0.100300 C6 7 -0.052800 C7 8 -0.065800 C8 9 -0.007300 C9 10 -0.110300 C10 11 -0.002600 C11 12 0.287600 C12 13 -0.222500 C13 14 -0.222500 C14 15 0.177900 C15 16 0.174400 C16 17 0.174400 C17 18 0.027300 C18 19 0.083400 C19 20 0.301800 N 21 -0.415400 H 22 0.348500 F 23 -0.126900 F1 24 -0.126900 F2 25 -0.124900 N1 26 -0.516001 O 27 -0.583501 O1 28 -0.570501 N2 29 -0.615501 C20 30 0.610001 N3 31 -0.106100 C21 32 0.848901 H2 33 0.049700 H3 34 0.049700 H4 35 0.049700 H5 36 0.071700 H6 37 0.062700 H7 38 0.062700	1 C -0.462322 2 C -0.180273 3 C 0.023395 4 C -0.267797 5 C -0.201317 6 C -0.220404 7 C -0.532606 8 C -0.537792 9 C 0.101339 10 C 0.125029 11 C 0.367846 12 C 0.255925 13 C -0.274652 14 C -0.266032 15 C 0.406001 16 C 0.350181 17 C 0.313375 18 C -0.210433 19 C 0.557969 20 C 0.185068 21 N -0.599930 22 H 0.349701 23 F -0.286371 24 F -0.289478 25 F -0.291422 26 N -0.515291 27 O -0.496835 28 O -0.482423 29 N -0.677014 30 C 0.616248 31 N -0.609097 32 C 0.694113 33 H 0.153218 34 H 0.161469 35 H 0.170375 36 H 0.161701 37 H 0.153199 38 H 0.180676

	H8 39 0.149000 H9 40 0.163000 H10 41 0.050033 H11 42 0.050033 H12 43 0.050033 H13 44 0.055367 H14 45 0.055367 H15 46 0.055367 H16 47 0.172500 H17 48 0.172500 H18 49 0.080700 H19 50 0.080700	39 H 0.132973 40 H 0.158232 41 H 0.158518 42 H 0.175745 43 H 0.175797 44 H 0.162086 45 H 0.177552 46 H 0.167935 47 H 0.176185 48 H 0.174666 49 H 0.218766 50 H 0.196208
FMN-amino	C 1 0.145600 C1 2 -0.091500 C2 3 -0.091500 C3 4 -0.088100 C4 5 -0.013400 C5 6 -0.194500 C6 7 -0.194500 C7 8 -0.097400 C8 9 -0.182000 C9 10 -0.102300 C10 11 -0.051800 C11 12 -0.066800 C12 13 -0.009300 C13 14 -0.111300 C14 15 -0.131300 C15 16 -0.004600 C16 17 0.286600 H 18 0.393300 H3 19 0.393300 C17 20 0.024300 C18 21 0.083400 C19 22 0.301800 N 23 -0.416400 H24 24 0.348500 N1 25 -0.517001 O 26 -0.584501 O1 27 -0.572501 N2 28 -0.615501 C20 29 0.610001 N3 30 -0.823201 N4 31 -0.104100 C21 32 0.852899 H4 33 0.133000 H5 34 0.133000 H6 35 0.040367 H7 36 0.040367 H8 37 0.040367 H9 38 0.057700 H10 39 0.133000 H11 40 0.133000	1 C 0.301211 2 C -0.195125 3 C -0.191849 4 C -0.464261 5 C -0.178405 6 C -0.169173 7 C -0.180998 8 C -0.251989 9 C -0.251327 10 C -0.220518 11 C -0.533275 12 C -0.534185 13 C 0.120501 14 C 0.127456 15 C 0.150459 16 C 0.384872 17 C 0.256378 18 H 0.316639 19 H 0.319519 20 C -0.183418 21 C 0.557898 22 C 0.184087 23 N -0.597563 24 H 0.350263 25 N -0.515260 26 O -0.496671 27 O -0.482364 28 N -0.676821 29 C 0.616223 30 N -0.788078 31 N -0.608562 32 C 0.693401 33 H 0.128509 34 H 0.124424 35 H 0.152288 36 H 0.146169 37 H 0.158603 38 H 0.169605 39 H 0.121487 40 H 0.113871

	H12 41 0.059200 H13 42 0.059200 H14 43 0.161000 H15 44 0.162000 H16 45 0.048700 H17 46 0.048700 H18 47 0.048700 H19 48 0.054367 H20 49 0.054367 H21 50 0.054367 H22 51 0.081200 H23 52 0.081200	41 H 0.158573 42 H 0.141446 43 H 0.150744 44 H 0.159568 45 H 0.158220 46 H 0.176431 47 H 0.176559 48 H 0.163356 49 H 0.175468 50 H 0.176706 51 H 0.170493 52 H 0.218416

Table S2**:** Hydrogen bond energy (ΔE**)** calculated at the DFT level of theory B3LYP/6-31g(d) in water solvent.

Ligand	ΔE(kcal/mol)
FMN-benzene	-11.5
FMN-fluoro	-8.5
FMN-difluoro1	-3.1
FMN-difluoro2	-8.4
FMN-trifluoro	-12.0
FMN-amino	-10.1

Table S3. Dihedral angle C1, C2, C3, C4 at Basin I in all neutral the ligands

Ligands	C1,C2,C3,C4 dihedral angle(degrees)
FMN-benzene	50.0°
FMN-fluoro	73.6°
FMN-difluoro1	175.2°
FMN-difluoro2	60.3°
FMN-trifluoro	76.3°
FMN-amino	85.5°

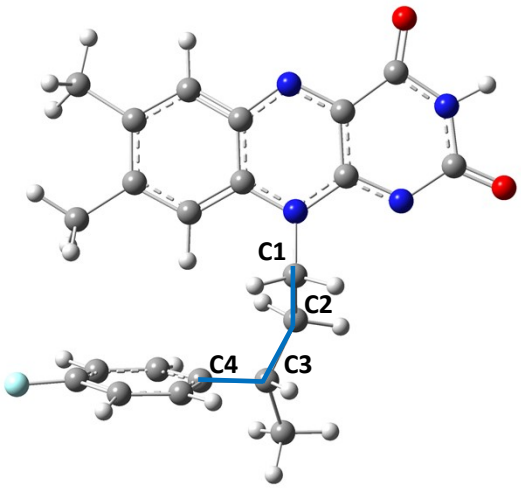

Table S4: Electrostatic energy between ligands and the metal ions in the system, calculated with MMPBSA approach.

Ligands	Electrostatic energy (kcal/mol)
FMN-benzene	-2.94± 8.15
FMN-fluoro	0.69±6.2
FMN-difluoro1	-1.51±7.4
FMN-difluoro2	-1.98±10.5
FMN-trifluoro	-0.21±10.1
FMN-amino	-1.02±6.7

Table S5: Van der Waals and Electrostatic energy contributions in the ligand binding free energy with riboswitch calculated with the MMPBSA approach.

Ligands	Van der Waals energy (kcal/mol)	Electrostatic energy (kcal/mol)
FMN-benzene	-13.84±22.3	-0.53±8.9
FMN-fluoro	-3.09±11.8	-1.23±6.4
FMN-difluoro1	-4.57±15.0	-0.72±7.7
FMN-difluoro2	-16.12±20.5	-3.97±17.5
FMN-trifluoro	-40.13±24.5	-8.40±13.6
FMN-amino	-11.80±21.2	-4.36±11.0

Computational Methodology:

Alchemical free energy (AFE) calculations with free energy perturbation (FEP):

The alchemical free energy simulation was performed in Gromacs 4.6 package with Amber99sb_ParmBSC0 force field. The ligand-riboswitch complex along with the water box

were used same as the Wt-MTD details given in main text. A time step of 2 fs was used in all MD runs. To make the configuration suitable for simulation, we have first minimized its energy with steepest descent minimizer. The electrostatic and van der Waals interactions were calculated using the particle mesh Ewald (PME) method. Minimized system then used for the global equilibration with turning on pressure and temperature coupling. Temperature coupling using velocity rescaling with a stochastic term with time constant tau-t set to 0.2. Pressure coupling with berendsen method with the compressibility set to 4.5 x 10-5 bar-1 and time constant tau-p 5. The rlist, rvdw and rcoulomb were set to 1. In free energy perturbation methods, we force the system by coupling the interaction strength between a molecule of interest and the rest of the system to a variable λ. The final simulation couples the ligand from λ=0 where it doesn't interact with the system, to the situation at λ=1, where the riboswitch is bound to the ligand. After the equilibration we split the system into different fep-lambda points 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 0.99, 0.999, 0.9999, 1. Gromacs uses 'soft-core' interactions to make sure that while the normal (Lennard-Jones and Coulomb) interactions are being turned off. The sc-power, sc-sigma and sc-alpha were set to 1, 0.3, 1 respectively. For each window 500000 production steps after 100000 equilibration steps was performed. The simulation was carried out for both ligands 5FDQD and FMN-Difluro2 in both aqueous solution and the binding site. The free energy change was calculated by using of the Bennett acceptance ratio (BAR)[1] method.

Table S6. Free energy values obtained with the alchemical free energy calculations.

States	5FDQD (ΔG kJ/mol)	FMN-Difluro2 (ΔG kJ/mol)
0-1	-51.86±0.71	-42.49±1.21
1-2	-48.54±0.32	-44.87±0.74
2-3	-37.19±0.37	-32.17±0.69
3-4	-18.37±0.62	-14.29±0.86
4-5	6.71±0.73	6.32±1.10
5-6	35.28±2.57	30.70±2.40
6-7	7.01±5.20	5.82±1.50
7-8	-6.47±1.83	-10.74±0.44
8-9	-13.36±1.40	-17.49±0.73
9-10	-34.40±0.85	-37.99±0.73
10-11	-10.65±0.18	-9.32±0.15
11-12	-1.26±0.02	-1.14±0.02
12-13	-0.12±0.00	-0.13±0.00
Final ΔG kJ/mol	-173.21±11.54	-167.80±2.24

Final ΔG kcal/mol	-41.40	-40.10

1 C. H. Bennett, Efficient estimation of free energy differences from Monte Carlo data, *J. Comput. Phys.*, 1976, **22**, 245–268.