Supporting Information

Multifaceted Nucleic Acid Probing with A Rationally Upgraded Molecular Rotor

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1. General Method:

Instrumentation: NMR spectra were acquired on an AVIII 400 (400 MHz) (Bruker) spectrometer and chemical shift reported in parts per million (δ) relative to internal standard TMS (0 ppm). Probe mass spectra was measured on a LCQ Fleet LCMS (Thermo Scientific). High-resolution mass spectrometric data was obtained using 6546 LC-QTOF (Agilent). Oligonucleotide mass spectra were acquired by MALDI-TOF using a JEOL SpiralTOF JMS-S3000 in negative ion mode. Absorbance spectrum were recorded on Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific) and Cary 3500 Compact UV-Vis spectrometer (Agilent). Measurement of DNA melting point was conducted with Cary 3500 Compact UV-Vis spectrometer (Agilent). Fluorescence spectrum, fluorescence time courses were recorded on Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific) with black, non-binding 384-well plate. Oligonucleotide concentrations were determined by UV-absorption on a NanoDrop One Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific). Agilent 1260 Infinity II Prime LC connected to Agilent Poroshell 120 EC-C18 column was used to analyse the reaction conversion between DNA and probe. Isothermal Titration Calorimetry (ITC) experiments were performed on MicroCal iTC200 Isothermal titration calorimetry (GE Healthcare, Biosciences ltd., Sweden).

Synthesis and Chemicals: All chemicals were purchased from Sigma-Aldrich, TCI chemicals and BLDPharm and used without further purification. Analytical TLC was performed on ready-to-use plates with silica gel 60 (Merck, F254), Flash column chromatography was performed over Fisher Scientific silica gel (grade 60, 230-400 mesh). All reagents were weighed and handled in air, while anhydrous solvents were handled under N_2 atmosphere. Approved Oncology Drugs Set X library, plate 4893/63 (80 compounds) was retrieved from National Cancer Institute (NIH). dUTP and dNTP set were purchased from New England Biolabs and Thermo Fisher, respectively.

Enzymes: *E. coli* Uracil DNA Glycosylase (UDG), Methyl Purine Glycosylase (MPG), Human single-stranded selective monofunctional uracil DNA glycosylase (hSMUG1) and Klenow fragment (exo-) were purchased from New England Biolabs.

General procedure for preparation of AP site in DNA:[1] A dU-containing oligonucleotide (50 μM) was dissolved in 50 mM Tris pH 7.0 and 100 mM NaCl. The solution was treated with *E. Coli* UDG (50 U/mL) for 0.5 h at 37 °C. The generated AP site was directly proceeded to next step without purification.

General procedure for preparation of DNA-probe conjugates:[1] To a solution of AP site-containing oligonucleotide (25 μM) was added probes (CCVJ-1 or CCVJ-H) (500 μM) and incubated at room temperature overnight. The conjugate was then purified by ethanol precipitation by adding 0.1x (v:v) of 3 M AcONa pH 5.2, 3.75x (v:v) of absolute EtOH. The mixture was incubated at -80 °C for 3 h, and the conjugate pellet was obtained by centrifuging (21,000 xg) for 1 h at 4 °C. The supernatant was removed, and the pellet was washed 3 times with 75% EtOH and centrifuged (21,000 xg) for 10 min at 4 °C. The supernatant was removed, and the pellet was dried in air for 0.5 h. The conjugate was dissolved in nuclease-free water and stored at -20 °C for further analysis.

General method for fluorescence real-time response experiment: The 384-well plate was firstly added oligonucleotides in buffer solutions with probes (CCVJ-1 or CCVJ-H) and placed on ice for 10 min. Note that DMSO percentage should not exceed 4% and the reaction volume is 50 μL. Corresponding DNA glycosylase (UDG, MPG or SMUG1) was then added, and the plate was inserted to microplate reader immediately to record fluorescence in 2-hour time course at 37 °C.

General method for profiling interactions between small molecules and nucleic acids: The 384-well plate was firstly added oligonucleotides in buffer solutions with small molecules and incubated at room temperature for 15 min. The plate was then added CCVJ-H and placed on ice for 10 min. Note that DMSO percentage should not exceed 4% and the reaction volume is 50 μL. UDG was then added, and the plate was inserted to microplate reader immediately to record fluorescence in 2-hour time course at 37 °C.

General procedure for library screening for small-molecule binder identification of $(CTG)_7$/$(CAG)_7$ and 23-mer DNA/RNA duplex: A 80-member Approved Oncology Drugs Set X library, plate 4893/63 was chosen for this proof-of-concept study. Each compound labelled as well number (10 mM) was diluted with DMSO to a final concentration of 1.0 mM. The screenings were conducted in 20 mM Tris pH 8.0, 1.0 mM DTT, 1.0 mM EDTA with a total volume of 50 μL in a 384-well plate. Oligonucleotide duplex (2.0 μM) in buffer solution was incubated with compound (20 μM) at room temperature for 15 min. CCVJ-H (20 μM) was then added to the solution and incubated at room temperature for additionally 10 min. UDG (5.0 U/mL) was added to the mixture and the fluorescent response was recorded over the 2-hour time course. For $(CTG)_7$/$(CAG)_7$, six compounds were chosen for the second-round screening with the concentration of 2.0 μM with the same condition of first-round screening. The relative fluorescent intensity or the relative initial rate constant was used to evaluate the binding affinity of small molecules with DNA, and calculated by the ratio of fluorescence intensity or initial rate constant, between the reaction with the presence of the small molecules to the absence (DMSO only) of the small molecules.

Procedure for BETr labelling:[2] To a template oligonucleotide (1.0 μM) dissolved in 20 mM Tris pH 7.0, 10 mM $MgCl_2$, 1 mM DTT, 50 μg/mL BSA, 5.0 μM primer, 50 μM dNTPs (dA, dU, dG, dC), 10 μM CCVJ-H, and 20 U/mL UDG (New England Biolabs) were added. Upon the addition of 2.0 U/mL polymerase (Klenow), the fluorescent real-time response was recorded at 37 °C.

General procedure for isothermal titration calorimetry (ITC): The DNA (8.0 μM–12 uM) samples were taken in the cell, which was prepared in 10 mM potassium phosphate buffer containing 50 mM Tris pH 7.0, 100 mM NaCl and the DNA-binder solution prepared in the same buffer (500 μM-1 mM) kept in a syringe. The 1.78 μL DNA-binder solutions were injected into DNA inside the cell in each titration by using an automated syringe. Prior to curve fitting, the heat of dilution was removed from the corresponding binding assays. In Origin 7.0, the thermograms (integrated heat/injection data) acquired in the ITC trials were fitted using a suitable model. The collected data was analyzed using MicroCal Origin software and fitted using a two-mode fitting model for the study of the association constant (Ka) and various thermodynamic parameters change in enthalpy (ΔH) and change in entropy (ΔS).

2. List of Oligonucleotides

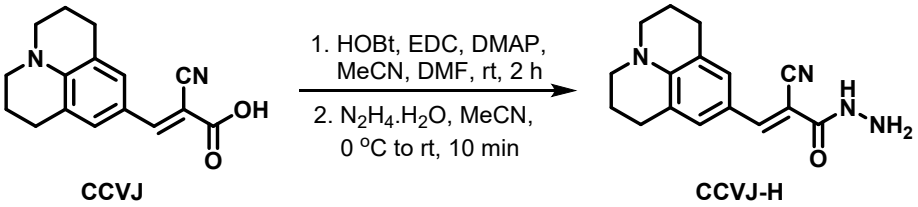
All oligonucleotides were purchased from Integrated DNA Technologies (IDT) with standard desalting purification and used without further purification.

Oligo #	Name	Sequence (5' → 3')
1	1dU 23-mer DNA	GTG TCA TCA CUC TGA ATA CCA AT
2	23-mer DNA	ATT GGT ATT CAG TGT GAT GAC AC
3	23-mer RNA	auu ggu auu cag ugu gau gac ac
4	dU 17-mer hpDNA (oppo C)	CGC UGA GGA ACT CCG CG
5	dU 17-mer hpDNA (oppo A)	CGC UGA GGA ACT CAG CG
6	dU 17-mer hpDNA (oppo T)	CGC UGA GGA ACT CTG CG
7	dU 17-mer hpDNA (oppo G)	CGC UGA GGA ACT CGG CG
8	dI 23-mer DNA	GTG TCA TCA CIC TGA ATA CCA AT
9	dI 25-mer DNA	CGA TAG CAT CCT ICC TTC TCT CCA T
10	25-mer cDNA (oppo A)	ATG GAG AGA AGG AAG GAT GCT ATC G
11	25-mer cDNA (oppo T)	ATG GAG AGA AGG TAG GAT GCT ATC G
12	25-mer cDNA (oppo G)	ATG GAG AGA AGG GAG GAT GCT ATC G
13	25-mer cDNA (oppo C)	ATG GAG AGA AGG CAG GAT GCT ATC G
14	2 dA	TCG CCT GGT ACT GCC GCT CGC GTC CGT CAT CGG CGT CTG TGG CCG CCA TGC CGT AGC CAG
15	5 dA	TCG CAT GGT TCT ACC GCT CGA GTC CGT CAT CGG CGT CAG TGG CCG CCA TGC CGT AGC CAG
16	9 dA	TCG CAT GGT ACT TAC GCT AGG ATC CAT CGT AGG CGA CCG AGG CCG CCA TGC CGT AGC CAG
17	11 dA	TCG CAT GAT TAC TAC CGA TCG AGT CAG TCA TCG ACG ACC GAG GCC GCC ATG CCG TAG CCA G
18	Primer	CTG GCT ACG GCA TGG CGG
19	cDNA-oppo A, A	GTG TCA UCA CAC TGA AUA CCA AT
20	cDNA-oppo U, U	GTG TCU TCA CAC TGA ATU CCA AT
21	cDNA-oppo A, U	GTG TCA UCA CUC TGA ATA CCA AT
22	Spectator RNA 1	agg aau uaa gag aag caa cau
23	Spectator RNA 2	caa gcu gac ccu gaa guu c
24	dU-KRAS-cDNA	TGG AUC TGT TG
25	WT-KRAS DNA	TTG CCT ACG CCA CCA GCT CCA AC
26	G12V-KRAS DNA	TTG CCT ACG CCA ACA GCT CCA AC
27	dU-near 33-mer DNA	CGC TGC AAA UTT GCG GAA CGC AAA TTT GCA GCG
28	dU-far 33-mer DNA	CGC UGC AAA TTT GCG GAA CGC AAA

		TTT GCA GCG
29	DA-5bp-1	CGA CGC CAG UTT GAA GGT TCG TTC GCA GGT GTG GAG TGA CGT CG
30	DA-5bp-2	CGA CGC CAG TTT GAA GGT TCG TUC GCA GGT GTG GAG TGA CGT CG
31	DA-5bp-3	CGA CGC CAG TTT GAA GGT TCG TTC GCA GGT GTG GAG UGA CGT CG
32	DA-5bp-4	CGA CGC CAG TTT GAA GGT TCG TTC GCA GGT GTG GAG TGA CGU CG
33	dU-$(CTG)_7$	CTG CTG CTG CUG CTG CTG CTG
34	$(CAG)_7$	CAG CAG CAG CAG CAG CAG CAG

3. Synthesis of CCVJ-H

CCVJ was prepared as described by Rumble and co-workers.[3] The synthesis of CCVJ-H was based on method reported by Zhang and co-workers.[4]



CCVJ **CCVJ-H**

To a solution of CCVJ (66.7 mg, 0.250 mmol) in anhydrous MeCN (4 mL) and anhydrous DMF (2 mL) at room temperature under N_2 was added HOBt.H_2O (47.7 mg, 0.310 mmol), EDC.HCl (59.3 mg, 0.310 mmol) and DMAP (4.3 mg, 0.036 mmol) orderly. The mixture was stirred at room temperature. After 15 min, the red precipitate appeared, and the solution was allowed to stir at room temperature for 2 h until the acid activation completed. The solution of $N_2H_4.H_2O$ (18.2 μL, 0.375 mmol) in anhydrous MeCN (2 mL) was added to the mixture at 0 °C. The solution became cleared, and was stirred at room temperature for additional 10 min. Water (10 mL) was added, and the mixture was extracted with ethyl acetate (20 mL x 2). The organic layer was then separated, washed with saturated $NaHCO_3$ solution (20 mL x 2), and water (20 mL). The organic layer was dried with Na_2SO_4 and concentrated *in vacuo*. The resulting solid was triturated with minimal amount of cold diethyl ether to afford CCVJ-H as a reddish orange solid (43.0 mg, 61%). The compound was stored at -20 °C for further usage.

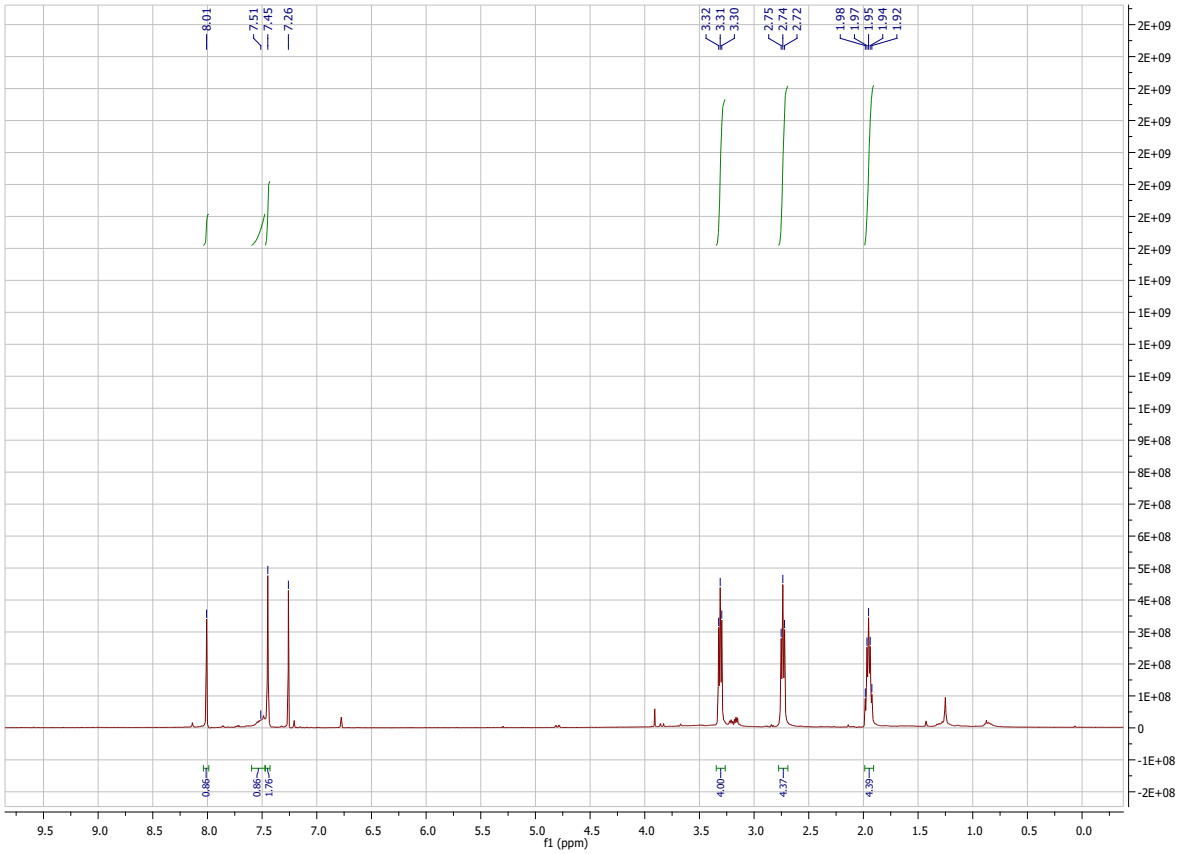
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.01 (s, 1H), 7.53 (brs, 1H), 7.45 (s, 2H), 3.32 – 3.30 (m, 4H), 2.74 (t, J = 6.2 Hz, 4H), 1.98 – 1.92 (m, 4H).

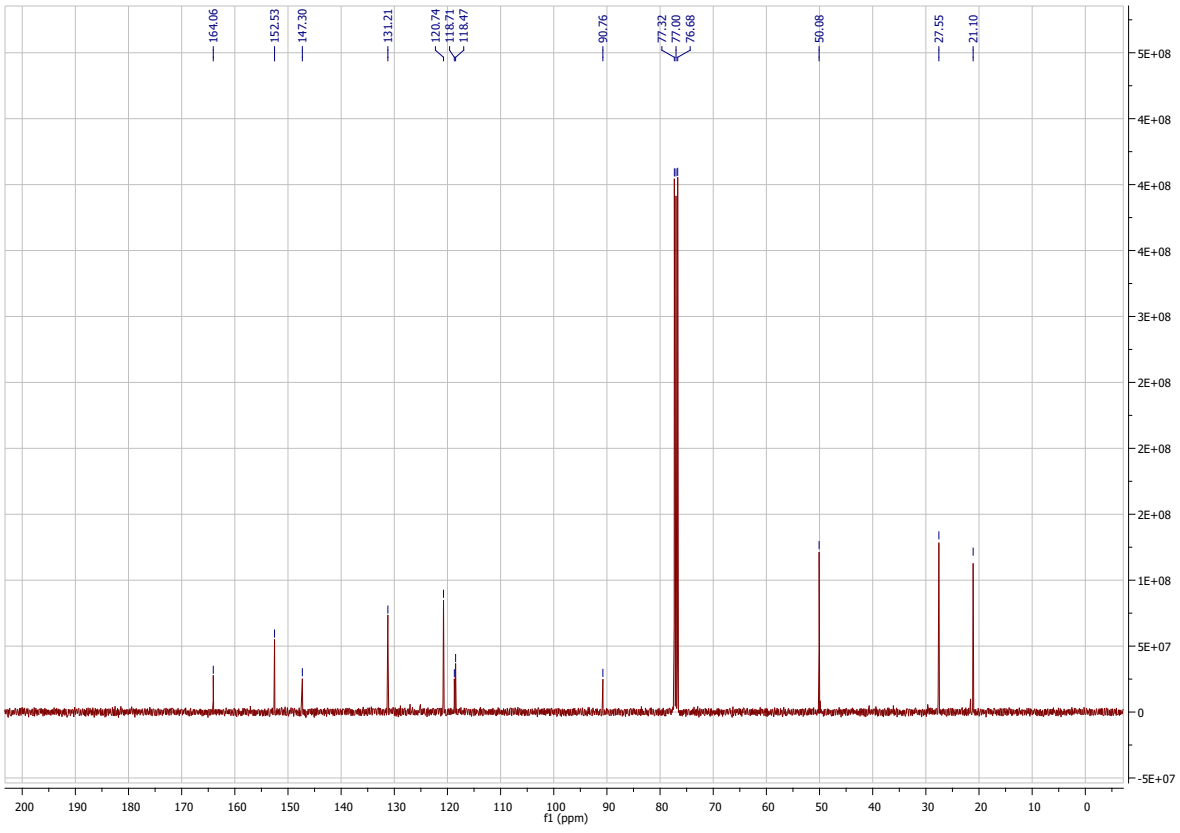
^{13}C NMR (101 MHz, $CDCl_3$) δ 164.06, 152.53, 147.30, 131.21, 120.74, 118.71, 118.47, 90.76, 77.3, 77.00, 76.68, 50.08, 27.55, 21.10.

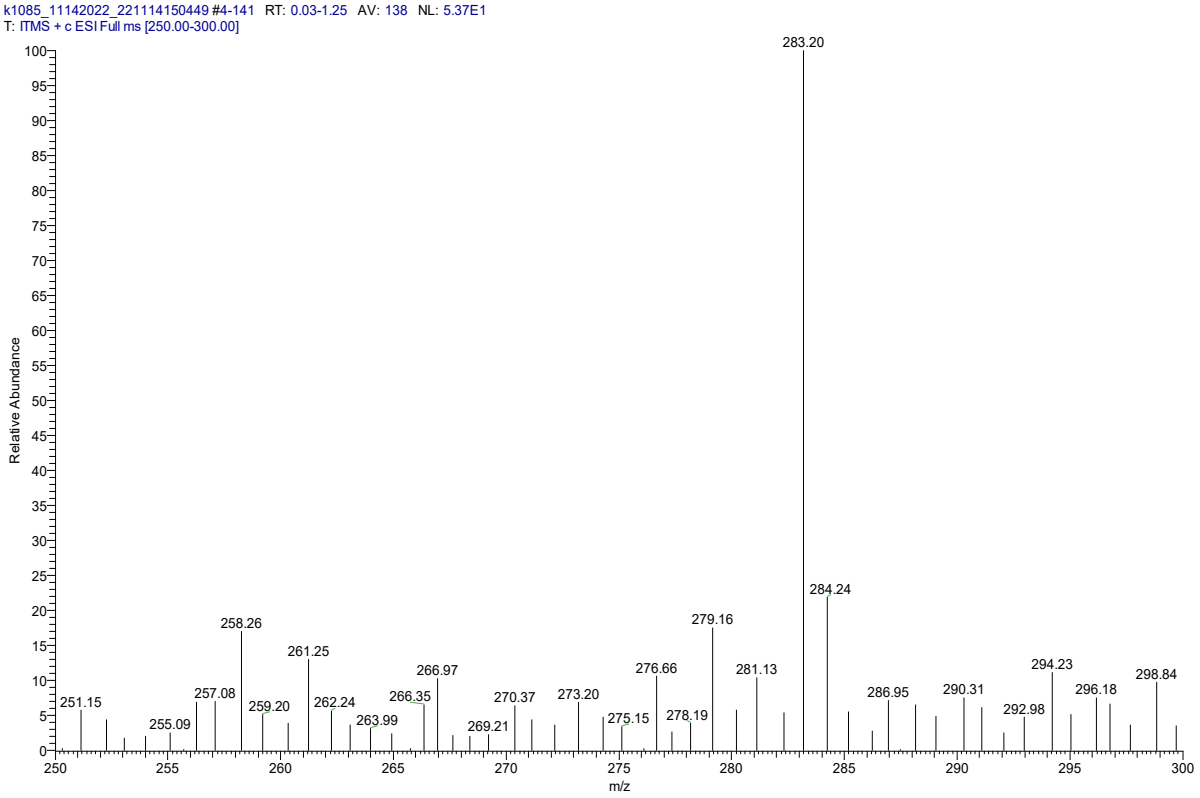
ESI-MS $[M+H]^+$ calcd for $C_{16}H_{19}N_4O$: 283.16, found: 283.20.

HRMS $[M+H]^+$ calcd for $C_{16}H_{19}N_4O$: 283.1553; found: 283.1556.

4. NMR spectrum and MS of CCVJ-H



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Relative Abundance
m/z
283.20
284.24
279.16
258.26
261.25
266.97
281.13
276.66
294.23
298.84
251.15
257.08
259.20
262.24
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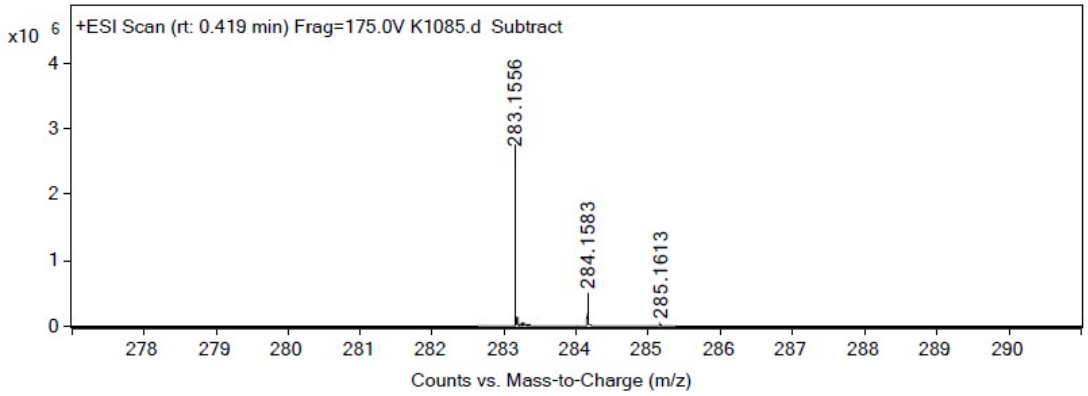
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+ESI Scan (rt: 0.419 min) Frag=175.0V K1085.d Subtract
283.1556
284.1583
285.1613
Counts vs. Mass-to-Charge (m/z)

5. Supplementary Figures:

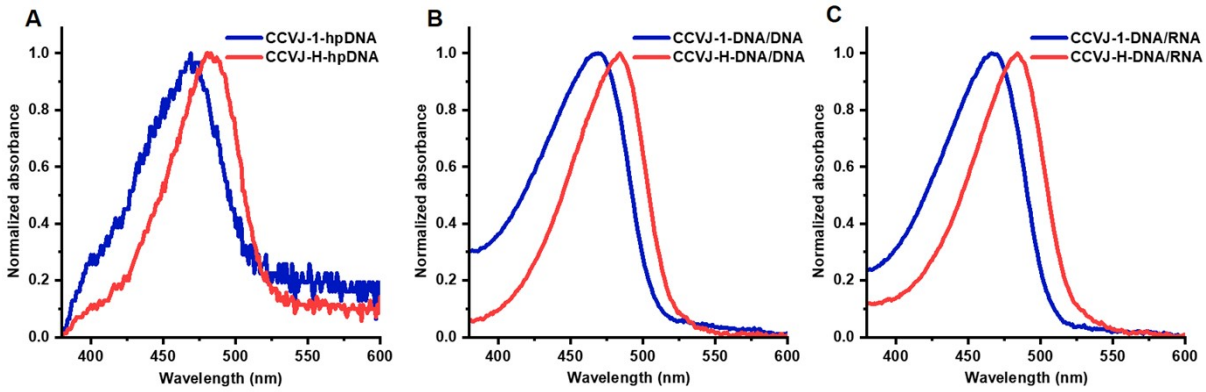

Figure S1. Normalized absorbance spectra of conjugates between CCVJ-1 or CCVJ-H and (A) 17-mer hpDNA, (B) 23-mer DNA/DNA, and (C) 23-mer DNA/RNA. Concentration of all conjugates are 5.0 μM.

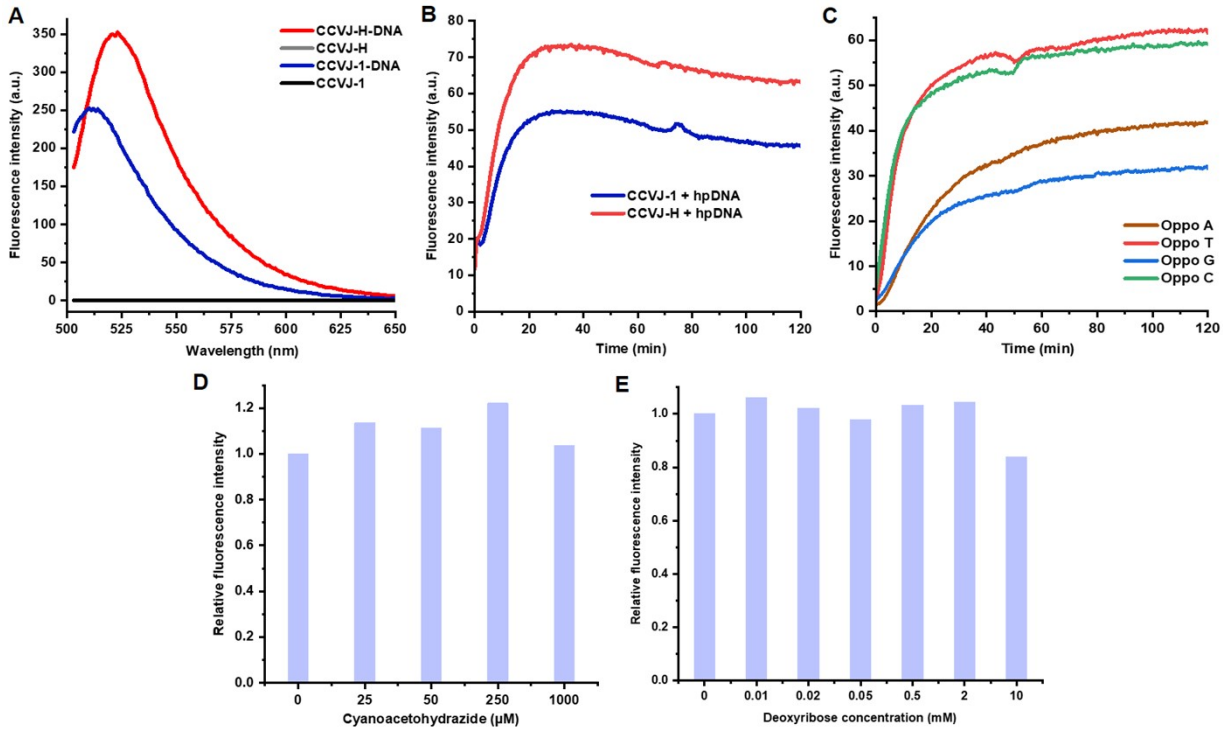

Figure S2. Fluorescence response of CCVJ-H in conjugation with hpDNA. (A) Emission spectra of CCVJ-1 and CCVJ-H-conjugated hpDNA (2.0 μM) at 50 mM Tris pH 7.0. (B) Fluorescence real-time response of 1dU hpDNA with CCVJ-1 or CCVJ-H and UDG in 2 hours. (C) The steric effect of opposite base of dU can affect the fluorescence intensity, as smaller opposite pyrimidine bases (C, T) yielded higher fluorescence intensity than purine bases (A, G). (D-E) Effect of competing cyanoacetohydrazide (D) and deoxyribose (E) to the maximum fluorescence signal of reaction between CCVJ-H and hpDNA. External hydrazide or carbohydrate did not significantly affect the fluorescence enhancement which indicates high selectivity of CCVJ-H towards DNA abasic site. It can be explained by the pre-association of molecular rotor CCVJ with the AP site that lead to the ultrafast hydrazone formation (B-E) The excitation wavelength and emission wavelength were 485 nm and 530 nm, respectively. The reactions were conducted with 50 U/mL UDG in 50 mM Tris pH 7.0, 100 mM NaCl at 37 ^{o}C in 2 hours. 1dU 17-mer hpDNA (oppo C) was used in B, D, E, and 1dU 17-mer hpDNA (oppo A, T, G, C) were used in C. (B, C) [DNA] = 2.0 μM, [CCVJ-H] = 20 μM (D, E) [DNA] = 10 μM, [CCVJ-H] = 2.5 μM.

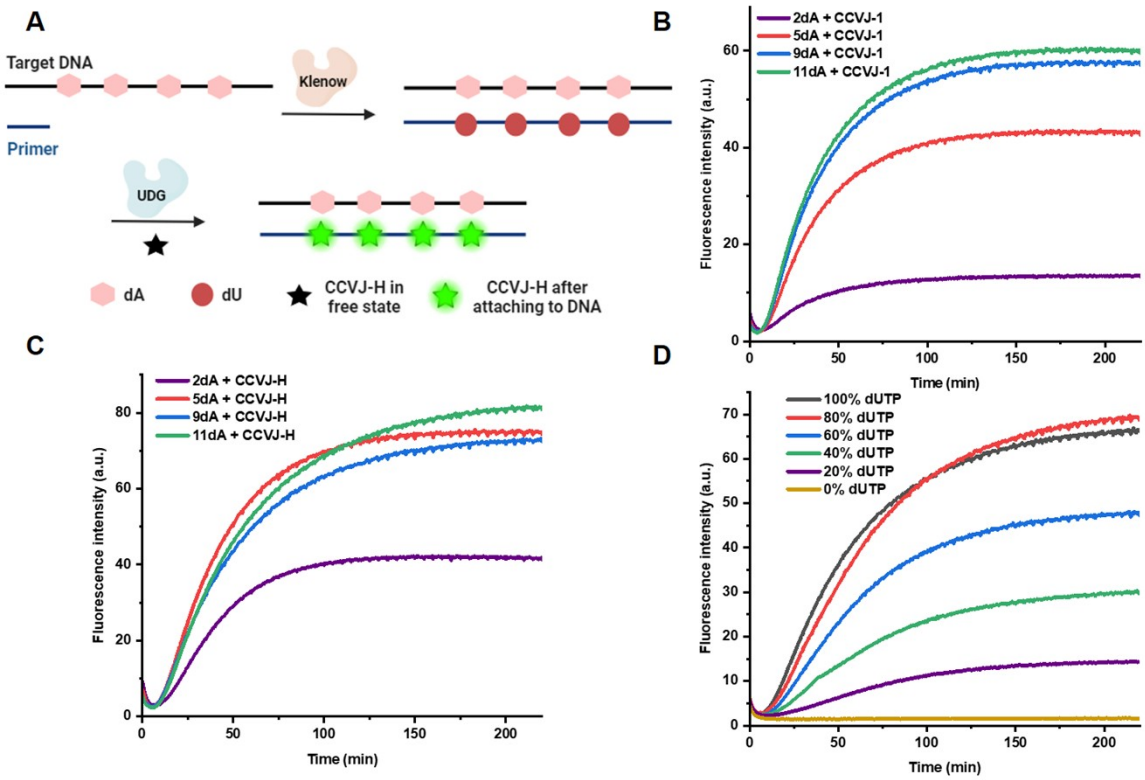

Figure S3. Application of CCVJ-H in DNA fluorescent labeling through base excision trapping (BETr).[2] (A) Illustration scheme of DNA fluorescent labeling by BETr. Deoxyuracil is firstly incorporated to the DNA strand by the activity of DNA polymerase (Klenow). dU then can be recognized and cleaved by UDG to form AP site, which is subsequently labeled by CCVJ-H. (B) Fluorescent real-time response of CCVJ-1 when conjugated with DNA bearing different dA number. (C) Fluorescent response of CCVJ-H when conjugated with DNA with different dA number. Fluorescence intensity from CCVJ-H was higher than CCVJ-1, which indicates the possibility of CCVJ-H in nucleic acid labeling. (D) Fluorescent response of CCVJ-H in the labeling of 9dA-DNA with different dUTP:dTTP ratio. (B-D) Experiment condition: [DNA] = 1.0 μM, [dNTP] = 50 μM (for each A, U, G, C), [primer] = 5 μM, [probe] = 20 μM, [UDG] = 20 U/mL, [Klenow] = 2 U/mL in 20 mM Tris pH 7.0, 10 mM $MgCl_2$, 1 mM DTT, 50 μg/mL BSA. Excitation wavelength and emission wavelength was 485 and 530 nm, respectively.

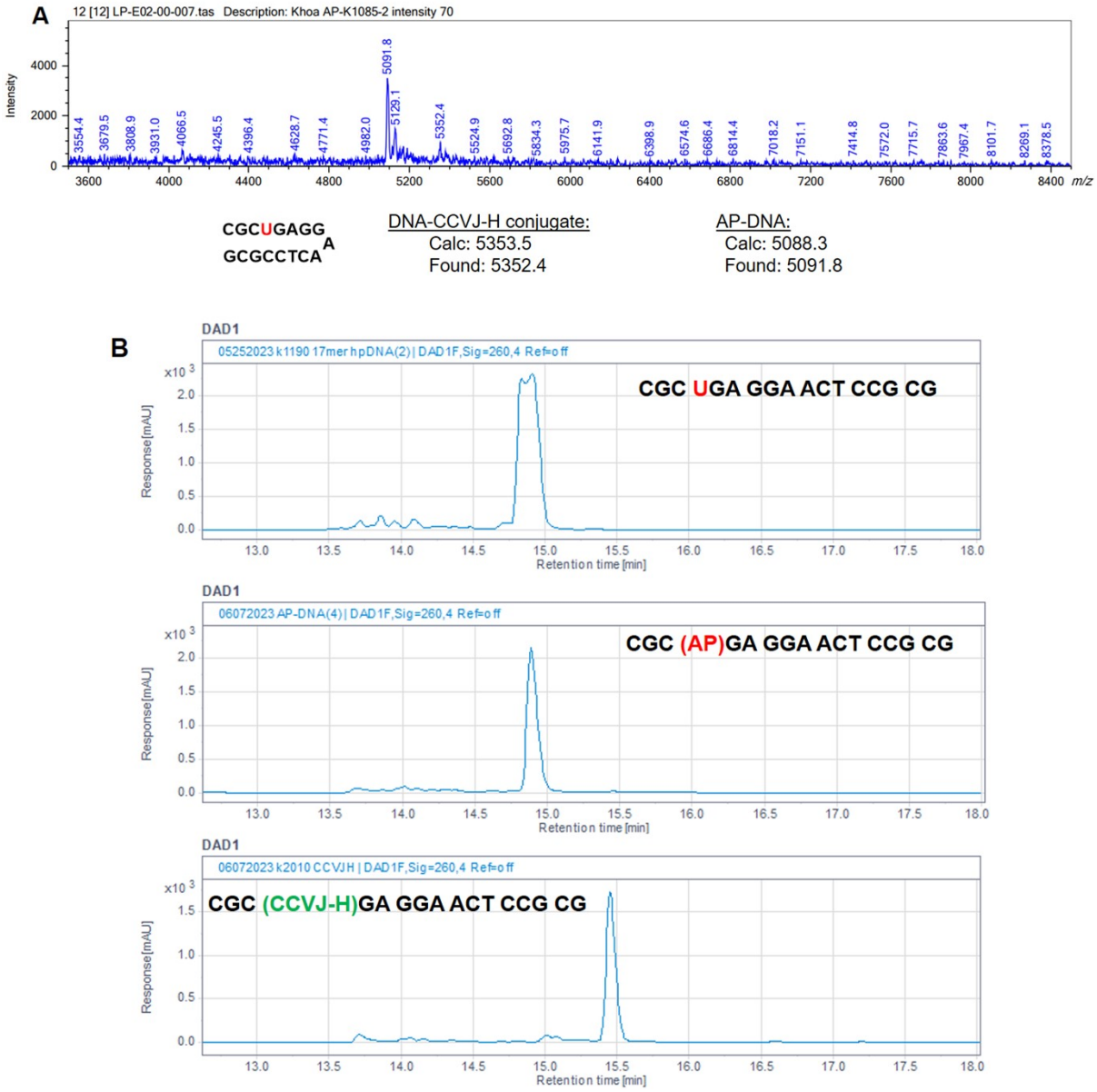

Figure S4. Characterization of 17-mer hpDNA-CCVJ-H conjugate. (A) MALDI-TOF analysis of hydrazone conjugate shows significant amount of AP site while the signal corresponding to conjugate is low. It is probably due to the fragmentation of hydrazone under the strong power of MALDI laser in acidic matrix. (B) HPLC analysis of the dU hpDNA, AP site and conjugate from reaction between DNA (25 μM) with CCVJ-H (500 μM) confirms the full conversion of hydrazone bioconjugation between CCVJ-H and AP-DNA.

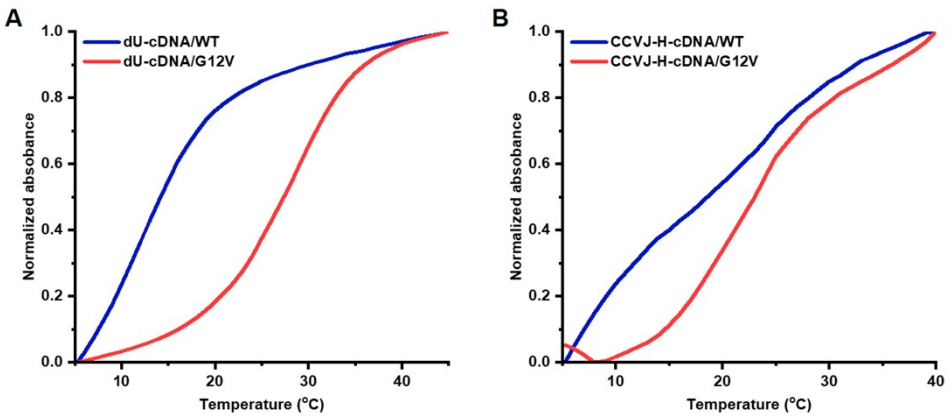

Figure S5. Melting point measurement of dU-cDNA/KRAS (WT and G12V) and CCVJ-H-cDNA/KRAS (WT and G12V) at 5.0 μM in PBS buffer.

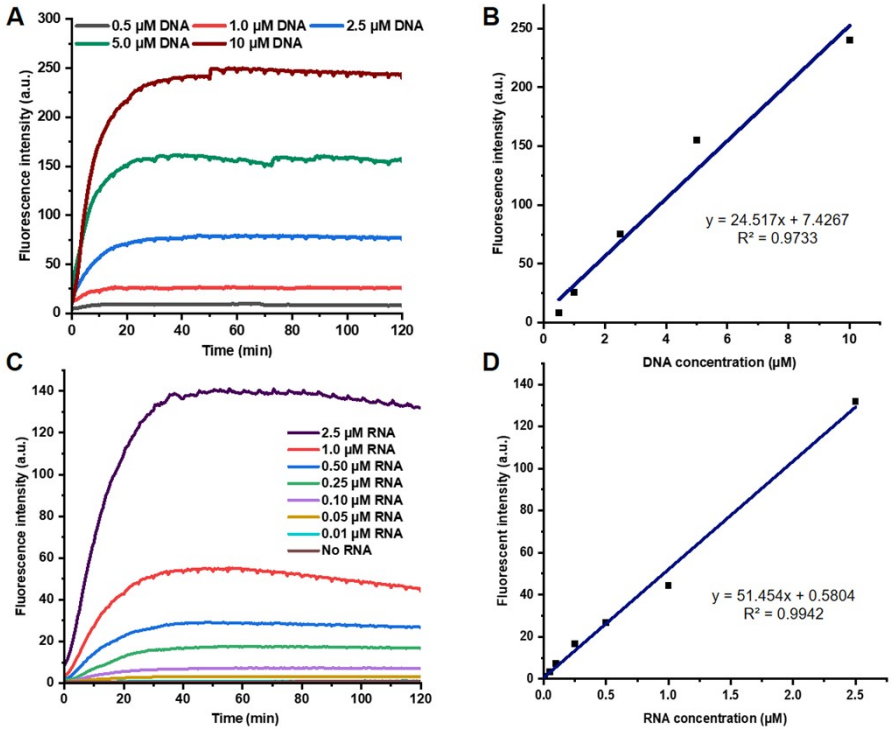

Figure S6. (A) Fluorescent real-time response of different 1dU 17-mer hpDNA (oppo C) concentration with CCVJ-H (20 μM). (B) Linear relationship between the fluorescent intensity with different DNA concentration. (C) Fluorescent real-time response of 1dU 23-mer RNA (5 μM) with CCVJ-H (20 μM) in the presence of UDG and different RNA concentration. (D) Linear relationship between the fluorescent intensity with different RNA concentration. (A-D) The fluorescence intensities were recorded at emission wavelength 530 nm. The reactions were conducted in the presence of 50 U/mL UDG in 50 mM Tris pH 7.0, 100 mM NaCl at 37 °C.

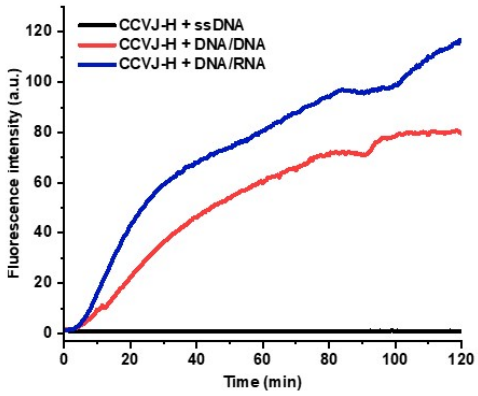

Figure S7. Evaluation of SMUG1 activity by CCVJ-H. Reaction conditions were [DNA/DNA] or [DNA/RNA] = 2 μM, [probe] = 20 μM, [SMUG1] = 50 U/mL in 50 mM Tris pH 7.0, 100 mM NaCl. Excitation wavelength and emission wavelength was 485 and 530 nm, respectively. The reactions were conducted at 37 °C. dU 23-mer DNA and its complementary DNA or RNA (23-mer DNA and 23-mer RNA) were used.

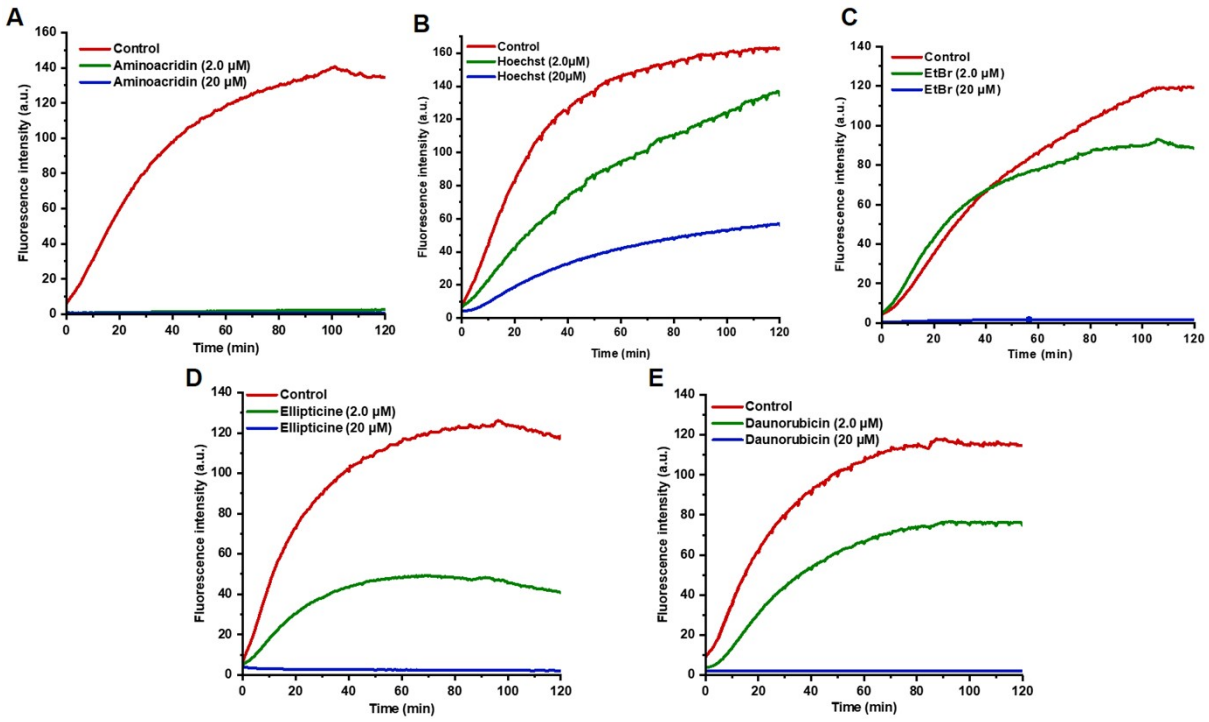

Figure S8. Fluorescent response of CCVJ-H (20 μM) with dU-containing 17-mer hpDNA (2.0 μM) in the presence of UDG (50 U/mL) and known DNA-binding small molecules (2.0 μM or 20 μM). The reactions were conducted in 20 mM Tris pH 8.0, 1.0 mM DTT, 1.0 mM EDTA at 37 °C. Excitation wavelength and emission wavelength was 485 and 530 nm, respectively.

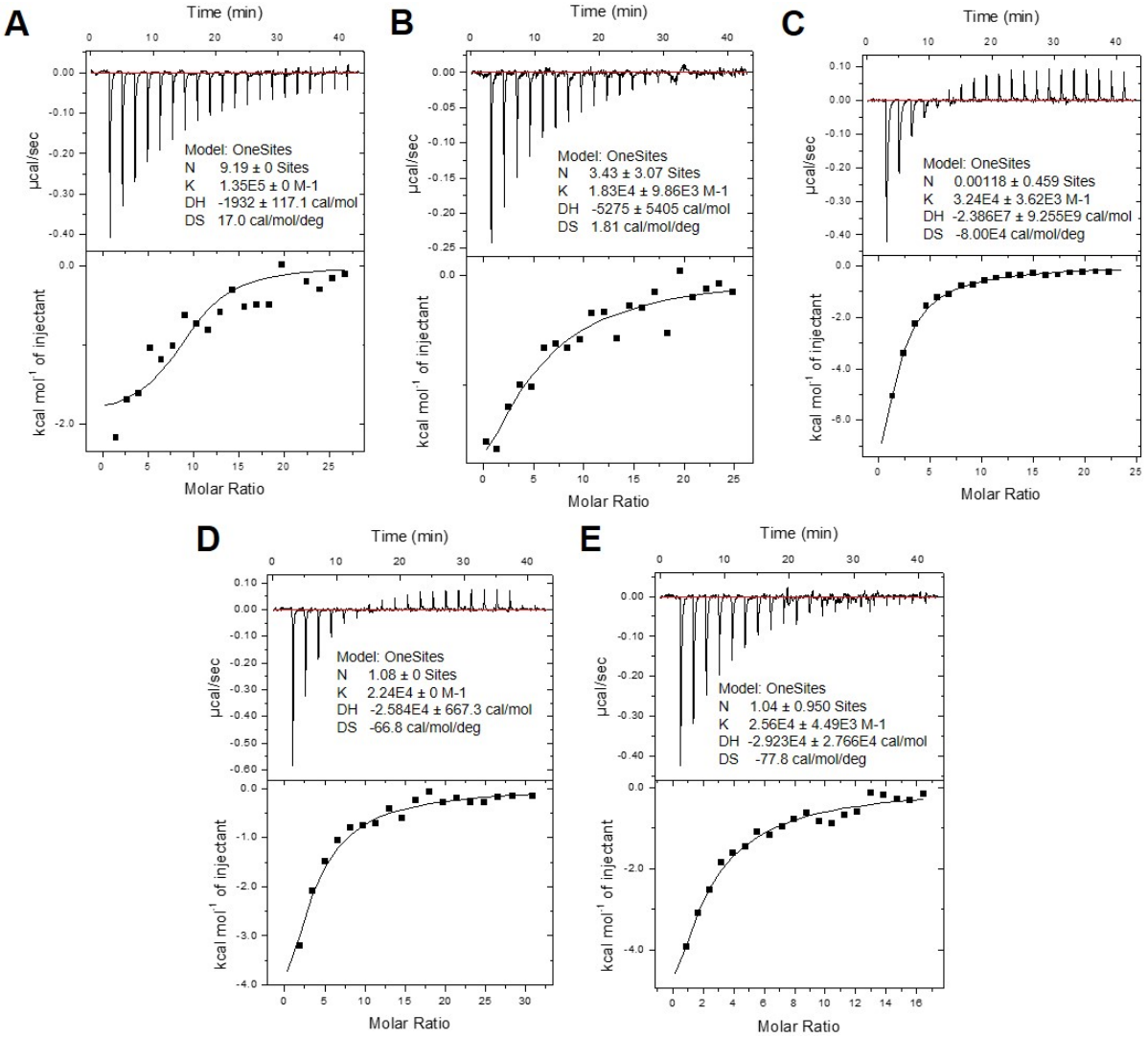

Figure S9. ITC binding curves of 17-mer dU hpDNA with small molecules. (A) 9-Aminoacridine. (B) Hoechst. (C) Ethidium bromide. (D) Ellipticine. (E) Daunorubicin.

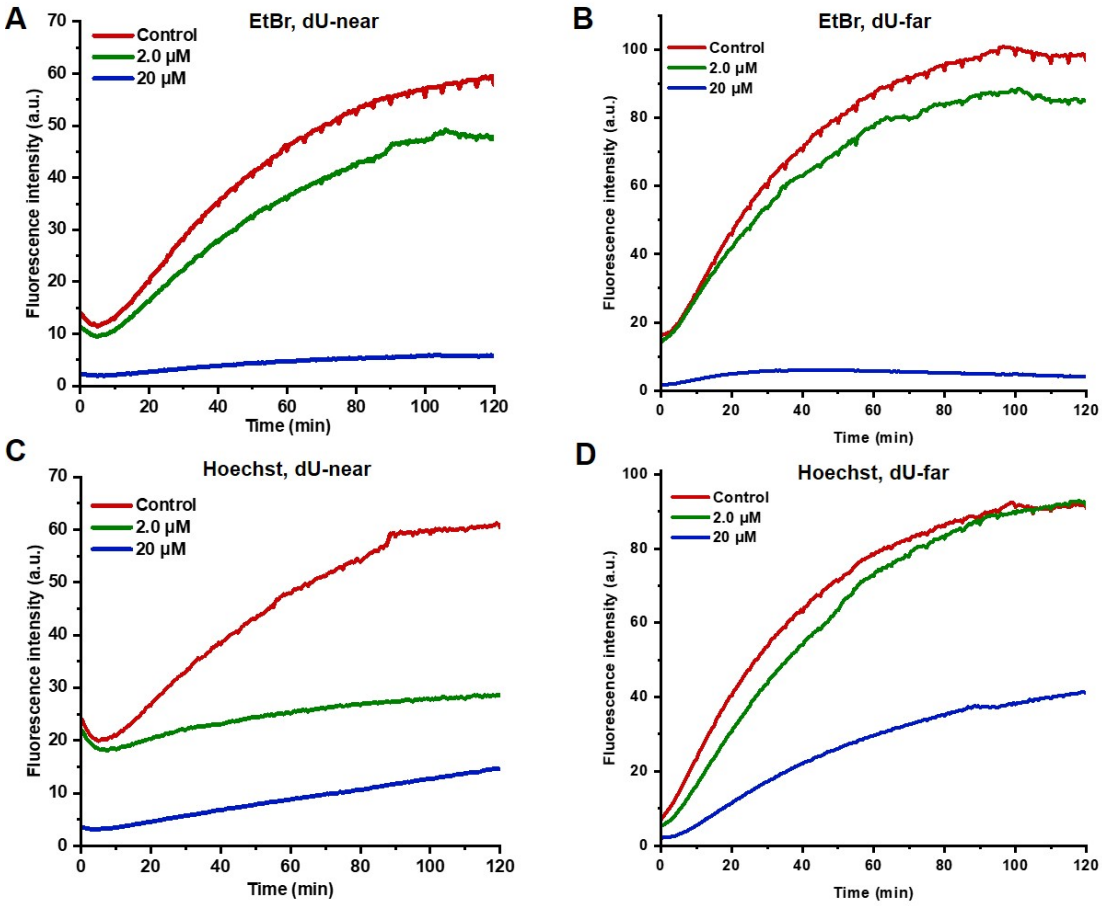

Figure 10. The effect of Hoechst (binding preference to AT-rich region) and EtBr on fluorescence response when dU is placed inside (dU-near) or outside (dU-far) AT-rich region. Experiment condition: [DNA] = 2.0 μM, [CCVJ-H] = 20 μM, [UDG] = 50 U/mL, [small molecule] = 2.0 or 20 μM in 20 mM Tris pH 8.0, 1.0 mM DTT, 1.0 mM EDTA at 37 °C. Excitation wavelength and emission wavelength was 485 and 530 nm, respectively.

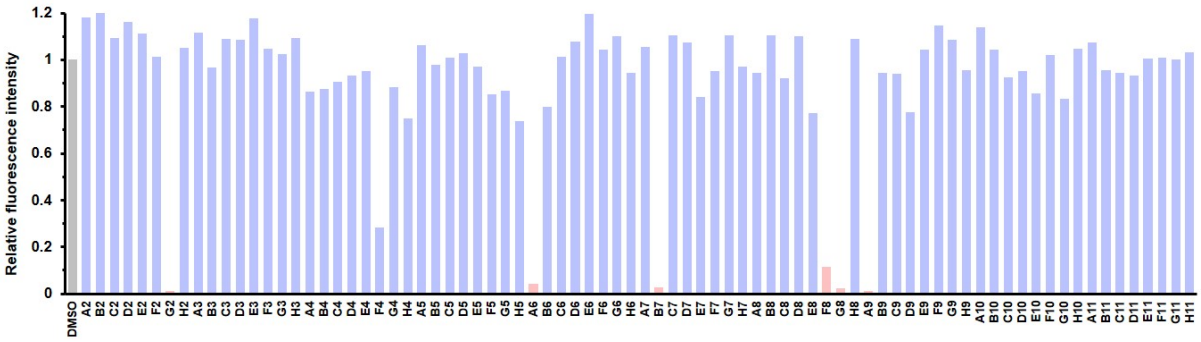

Figure S11. First round screening data of small-molecule binders to the DNA repeat expansion $(CTG)_7/(CAG)_7$ sequence. The first-round screening condition was: [small molecule] = 20 μM, [DNA duplex] = 2.0 μM, [CCVJ-H] = 20 μM, [UDG] = 5.0 U/mL in 20 mM Tris pH 8.0, 1.0 mM DTT, 1.0 mM EDTA at 37 °C. Excitation wavelength and emission wavelength was 485 and 530 nm, respectively. Six molecules highlighted in red color were chosen for the second round validation.

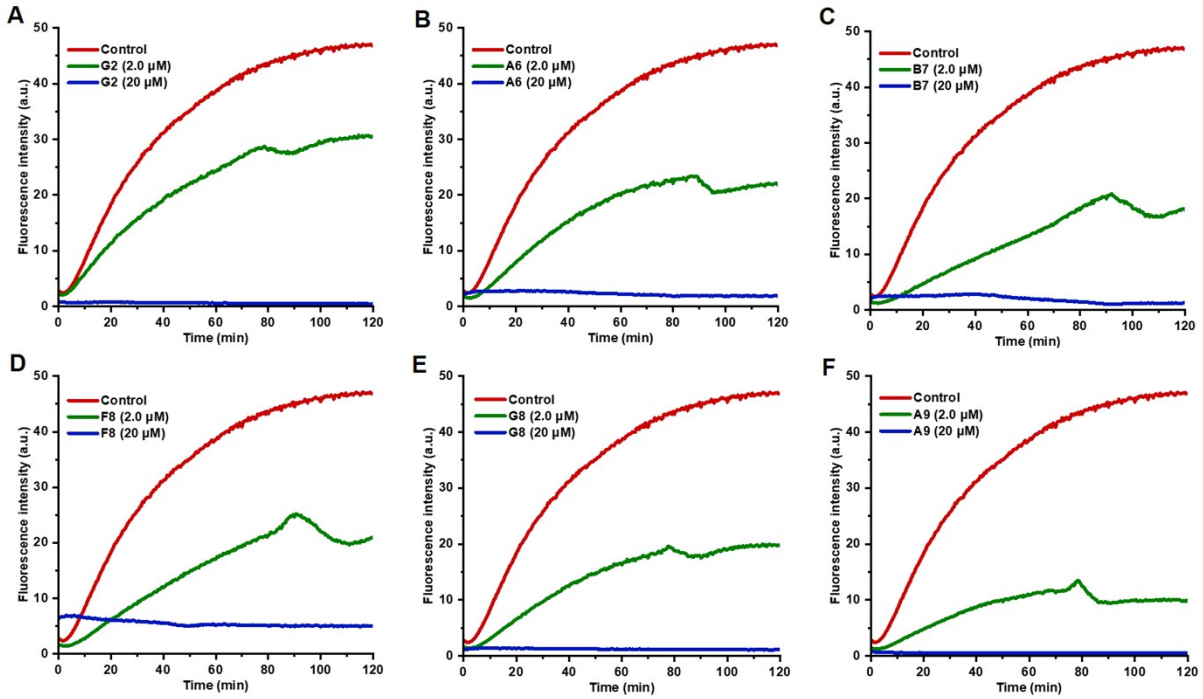

Figure S12. Fluorescent response of CCVJ-H (20 μM) with dU-containing $(CTG)_7$/$(CAG)_7$ (2.0 μM) in the presence of UDG (5.0 U/mL) and selected small molecules (2.0 μM or 20 μM). The reaction occurred in 20 mM Tris pH 8.0, 1.0 mM DTT, 1.0 mM EDTA at 37 °C. Excitation wavelength and emission wavelength was 485 and 530 nm, respectively.

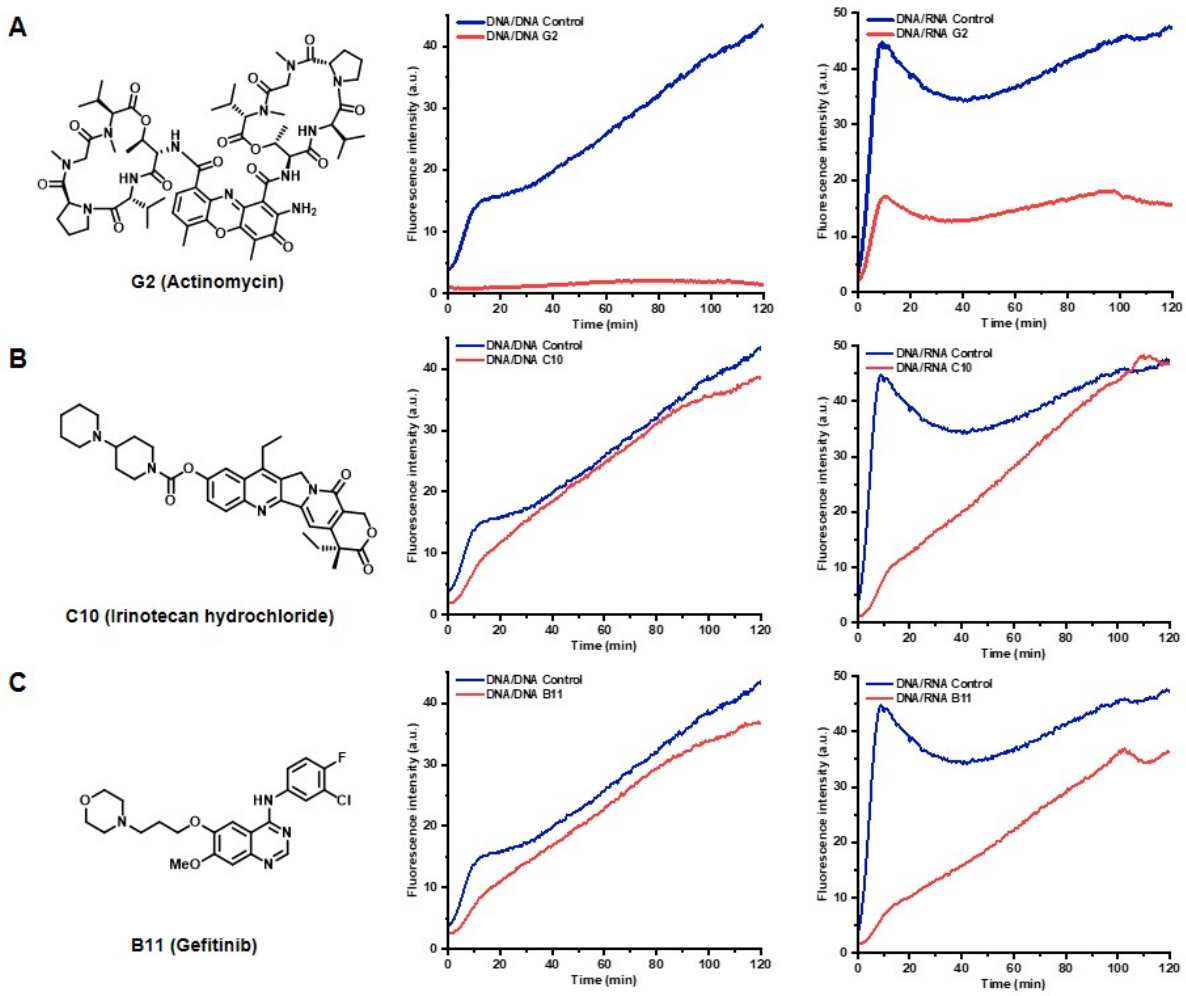

Figure S13. Fluorescent response of CCVJ-H (20 μM) with DNA/DNA or DNA/RNA (2.0 μM) in the presence of UDG (5.0 U/mL) and selected small molecules (20 μM). The reaction occurred in 20 mM Tris pH 8.0, 1.0 mM DTT, 1.0 mM EDTA at 37 °C. Excitation wavelength and emission wavelength was 485 and 530 nm, respectively. 23-mer 1 dU-DNA and its complementary strand, 23-mer DNA or 23-mer RNA were used.

6. References:

(1) Wilson, D. L.; Kool, E. T. Ultrafast Oxime Formation Enables Efficient Fluorescence Light-up Measurement of DNA Base Excision. *J. Am. Chem. Soc.* **2019**, *141* (49), 19379–19388.

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