**Materials**

Unless otherwise stated, all chemicals were purchased from Sigma (St. Louis, MO) and were of the highest purity available. Oligonucleotides were purified by polyacrylamide gel electrophoresis (PAGE) and obtained from Midland Certified Reagent Company, Inc (Midland, TX, USA). The following are the sequences of oligonucleotides and their respective molar extinction coefficients (ε, x10^3 M^-1 cm^-1): triplex R2, 5′-CCCCTCCC TTTTT GGGAGGGG CGCTTAT GGGGAGGG-3′ (366.0); triplex GT, 5′-GGGG TTTT GGGG TTTT GGGG AAAA GGGG TTTT CCCC TTTT CCCC-3′ (446.4); hairpin duplex R2D, 5′-CCCCTCCC TTTTT GGGAGGGG-3′ (201.1); linear duplex DS12, 5′-CTT GAG CTC AAG-3′ (129.0); and G4-DNA G4Htel, 5′-TTA GGG TTA GGG TTA GGG TTA-3′ (303.4).

We determined that the full-length oligonucleotides migrated as single bands by using denaturing PAGE [7 M urea, 89 mM Tris-borate, 2 mM EDTA (TBE)] on 20% acrylamide gels stained with SYBR Gold (Thermo Fisher, Waltham, MA). Concentrations of oligonucleotide stock solutions (prepared in nuclease-free water) were calculated using their respective molar extinction coefficients, and their absorbances were measured at 260 nm using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE). Oligonucleotides were kept at -20°C until resuspended. Oligonucleotide solutions were re-suspended in assay buffer and annealed by heating to 95 °C for 5 min, followed by slow cooling to room temperature.

Fluorescence assays were performed in a Corning black 384-well plate obtained from Thermo Scientific (Waltham, MA, catalog #: 3821).

**Procedures**

**Development of the coralyne-based, ‘light-up’ intramolecular triplex assay**

To probe the dose-dependent RFU change of coralyne, we titrated varying concentrations of triplex-forming R2 with 1 µM coralyne in 1x assay buffer composed of 20 mM, Tris, pH 7.4, and 0.01% Tween 20 in a 20 µL assay volume. In detail, 10 µL of various concentrations of R2 (0 –
800 nM) was mixed with 10 µL of a 1 µM coralyne solution and the fluorescence was observed after a 2 h incubation at room temperature. In investigating the binding affinity of coralyne with different structure-forming sequences as substrates, we assayed equal volumes of 100 nM of the DNA substrate with 1 µM coralyne. The assay buffer used depended on the DNA structure: for R2, GT and R2D - 20 mM Tris, pH 7.4, 0.01% Tween 20; for G4-DNA - 20 mM sodium cacodylate pH 7.0, 0.1 mM EDTA, 110 mM KCl, 0.01% Tween 20; for DS12 - 20 mM sodium cacodylate pH 7.0, 100 mM NaCl, 0.1 mM EDTA, 10 mM MgCl₂, 0.01% Tween 20. The fluorescence signal of coralyne was read on a black 384-well plate with excitation and emission at 424/9.0 and 494/9.0 nm, respectively, using a Synergy H4 plate reader (Biotek, Winooski, VT). An unpaired t-test was utilized to compare mean % RFU change between R2 and G4Htel substrates.

The BePI and doxorubicin competition assay was conducted by assaying 10 µL of varying concentrations of BePI (or doxorubicin) dissolved in water, with an equal volume of 2x coralyne: R2 complex solution in 2x buffer (40 mM Tris pH 7.4, 0.02% Tween 20). The final concentrations of coralyne and R2 were 500 and 50 nM, respectively. Solutions were incubated for 2 h at room temperature before reading.

Coralyne affinity and % RFU change experiments in the presence of BePI and Dox using another triplex-forming sequence, the GT triplex, were conducted following the procedures described above.

UV thermal denaturation experiments

To determine the thermal melting temperature of R2 (0.5 µM) in the absence or presence of BePI (0.5 µM) or doxorubicin (0.5 µM), DNA solutions were annealed using Tris buffer (20 mM, Tris, pH 7.4). Thermal denaturation of the DNA solutions in 0.4 cm quartz cuvettes was monitored from 30–100 °C at 260 nm with a ramp rate of 0.4 °C/min using a Cary 4000 UV-Vis equipped with a Peltier temperature controller (Santa Clara, CA). The end point of the melting curve was
normalized to Abs = 1.0, and the thermal melting temperature ($T_m$) calculated as the maximum value of the first derivative of the absorbance versus temperature, using the thermal application of Cary WinUV Ver 6.2 software (Santa Clara, CA).

**FID assay performance validation**

Master mixtures of both positive and negative controls were prepared as follows: the negative control contained 500 nM coralyne and 0.1% DMSO in 1x assay buffer (20 mM, Tris, pH 7.4, 0.01% Tween 20) and the positive control contained 50 nM R2, 500 nM coralyne, and 0.1% DMSO in 1x assay buffer (20 mM, Tris, pH 7.4, 0.01% Tween 20). To a Corning 384-well plate, 5 µL of each control solution was dispensed using an electronic pipettor as illustrated in ESI-Figure 3. Columns 11, 22, 23, and 24 were left empty to monitor instrument stability over the time. Fluorescence signals were monitored at different time points (1, 2, 3, and 24 h) to evaluate room temperature stability.
ESI-Figure 1. Dose-dependent % RFU change of coralyne in the presence of the GT triplex. $[C_{50}] \approx 30 \text{ nM}$.

ESI-Figure 2. Dose-dependent % RFU change of coralyne in the competitive displacement assay using the GT triplex. $[C_{50}]_{\text{BePI}} \approx 0.5 \text{ µM}$, $[C_{50}]_{\text{Dox}} \approx 2.5 \text{ µM}$. 

Supplementary data
**ESI-Figure 3.** Plate layout and typical readouts of the FID assay shown in a 384-well plate format.