Supplementary Information

Development of a New Colorimetric and Red-Emitting Fluorescent Dual Probe for G-Quadruplex Nucleic Acids

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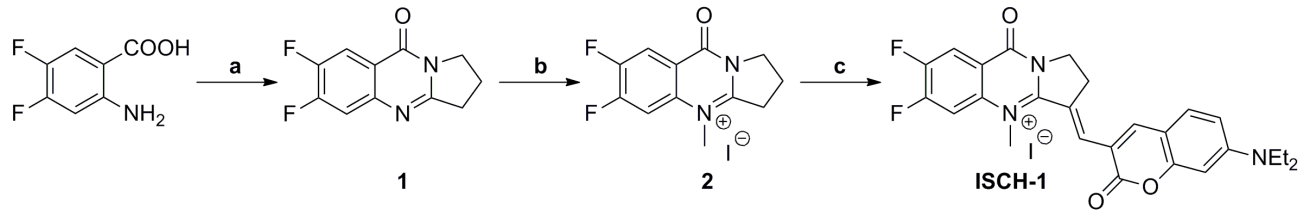
1. Experimental Section

1.1 Synthesis and Characterization

Scheme S1. Synthesis of **ISCH-1**[a]



[a] Reagents and conditions: (a) pyrrolidin-2-one, $POCl_3$, reflux; (b) CH_3I, tetramethylene sulfone, 50 °C; (c) 7-diethylaminocoumarin-3-aldehyde, AcOH, AcONa, reflux.

^1H, ^{13}C and ^{19}F NMR spectra were recorded using TMS or $CFCl_3$ as the internal standard in DMSO-d_6 or $CDCl_3$ with a Bruker BioSpin GmbH spectrometer at 400 MHz, 100 MHz and 376 MHz, respectively. Mass spectra (MS) were recorded on a Shimadzu LCMS-2010A instrument with an ESI or ACPI mass selective detector and high resolution mass spectra (HRMS) on Shimadzu LCMSIT-TOF. Melting points (mp) were determined using a SRS OptiMelt automated melting point instrument without correction. Flash column chromatography was performed with silica gel (200–300 mesh) purchased from Qingdao Haiyang Chemical Co. Ltd. The purity of synthesized compound was confirmed to be higher than 95% by using analytical HPLC performed with a dual pump Shimadzu LC-20 AB system equipped with a Ultimate XB-C18 column (4.6 × 250 mm, 5 μm) and eluted with methanol-water (55 : 45) containing 0.1% TFA at a flow rate of 1.0 mL·min^{-1}. All chemicals were purchased from commercial sources unless otherwise specified. All the solvents were of analytical reagent grade and were used without further purification. Compound 7-*N*,*N*-diethylaminocoumarin-3-aldehyde was synthesized according to previous report.[1]

Synthesis of 6,7-difluoro-2,3-dihydropyrrolo[2,1-b]quinazolin-9(1H)-one (1).[2] To a solution of 2-amino-4,5-difluorobezoic acid (3.34 g, 19.3 mmol) and pyrrolidin-2-one (3.00 mL, 39.5 mmol) was carefully added 45 mL of $POCl_3$ at room temperature. The mixture was then stirred at 110 °C for 7 h. After $POCl_3$ was removed under reduced pressure, the residue was poured into ice water, and then solution of NaOH was added to make the solution basic. The mixture was extracted with 3×50 mL portions of CH_2Cl_2. The combine organic phase was dried over $MgSO_4$ and concentrated under reduced pressure. The crude product was purified by using flash column chromatography with EtOAc/petroleum ether (1 : 4) elution to afford a white solid (**1**, 2.81 g, yield 65%): ^1H NMR (400 MHz, $CDCl_3$) δ 8.02 (t, J = 9.2 Hz, 1H), 7.49-7.34 (m, 1H), 4.20 (t, J = 7.1 Hz, 2H), 3.17 (t, J = 7.8 Hz, 2H), 2.37-2.23 (m, 2H). ESI-MS m/z: 223.1 $[M+H]^{+\bullet}$.

Synthesis of 6,7-difluoro-4-methyl-9-oxo-1,2,3,9-tetrahydropyrrolo[2,1-b]quinazolin-4-ium iodide (2).[3] A solution of **1** (0.71 g, 3.22 mmol) in tetramethylene sulfone (1.0 mL) was treated with CH_3I (2.0 mL, 32 mmol). The mixture was heated at 50 °C for 12 h. After cooling, the mixture was filtered, washed with anhydrous ether and dried under vacuum to afford the product as a white solid (**2**, 0.99 g, yield 85%): ^1H NMR (400 MHz, DMSO-d_6) δ 8.54-8.32 (m, 2H), 4.32 (t, J = 8.0 Hz, 2H), 3.99 (s, 3H), 3.73 (t, J = 7.9 Hz, 2H), 2.42-

2.30 (m, 2H). ESI-MS m/z: 237.1 $[M-I]^{+\bullet}$.

Synthesis of (*E*)-3-((7-(diethylamino)-2-oxo-2H-chromen-3-yl)methylene)-6,7-difluoro-4-methyl-9-oxo-1,2,3,9-tetrahydropyrrolo[2,1-b]quinazolin-4-ium iodide (ISCH-1). A mixture of **2** (0.15 g, 0.43 mmol), 7-*N*,*N*-diethylaminocoumarin-3-aldehyde (0.11 g, 0.45 mmol), AcOH (5 mL), and AcONa (0.02 g) was stirred at reflux temperature for 12 h. After cooling to room temperature, the mixture was poured into water and extracted with CH_2Cl_2. The organic phase was dried over $MgSO_4$ and concentrated under reduced pressure. The crude product was purified by using flash column chromatography with CH_3OH/CH_2Cl_2 (1 : 20) elution to afford brownish black solid **ISCH-1** (0.09 g, yield 36%): mp > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.44-8.29 (m, 3H), 7.88 (s, 1H), 7.70 (d, J = 9.0 Hz, 1H), 6.87 (d, J = 7.8 Hz, 1H), 6.65 (s, 1H), 4.33-4.20 (m, 5H), 3.53 (q, J = 7.1 Hz, 4H), 3.37 (t, J = 6.0 Hz, 2H), 1.17 (t, J = 6.8 Hz, 6H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 160.34, 159.79, 156.96, 156.12, 152.74, 145.41, 139.64, 138.88, 131.83, 126.28, 116.61, 115.60, 115.40, 112.81, 110.41, 109.75, 109.52, 108.48, 96.47, 46.75, 44.49, 41.27, 27.61, 12.38. ^{19}F NMR (376 MHz, DMSO-d_6) δ -122.58, -134.06. ESI-MS m/z: 464.2 $[M-I]^{+\bullet}$. Purity: 97.7% by HPLC. HRMS (ESI): calcd for $[M-I]^{+\bullet}$ ($C_{26}H_{24}F_2N_3O_3^{+\bullet}$) 464.1780, found 464.1786.

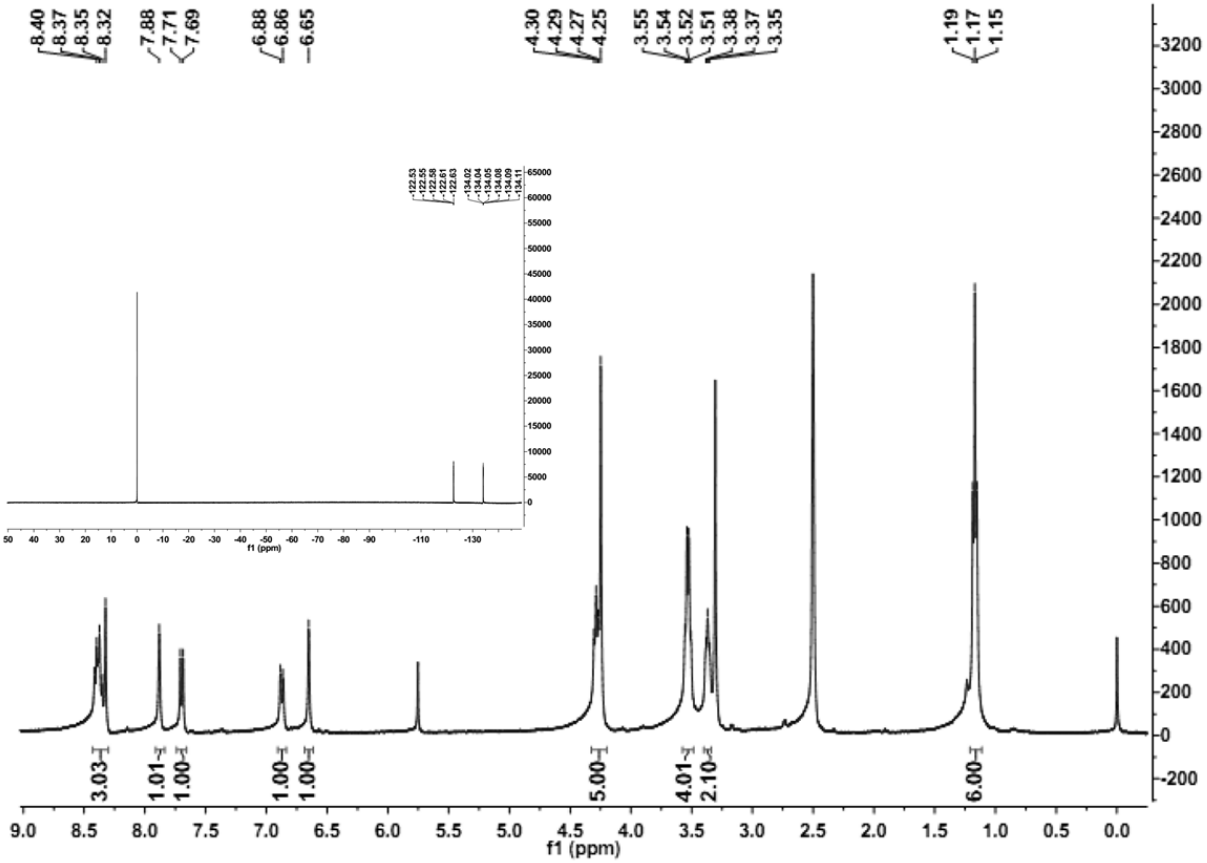


Figure S1. ^{1}H NMR and ^{19}F NMR (inner panel) spectra of **ISCH-1**.

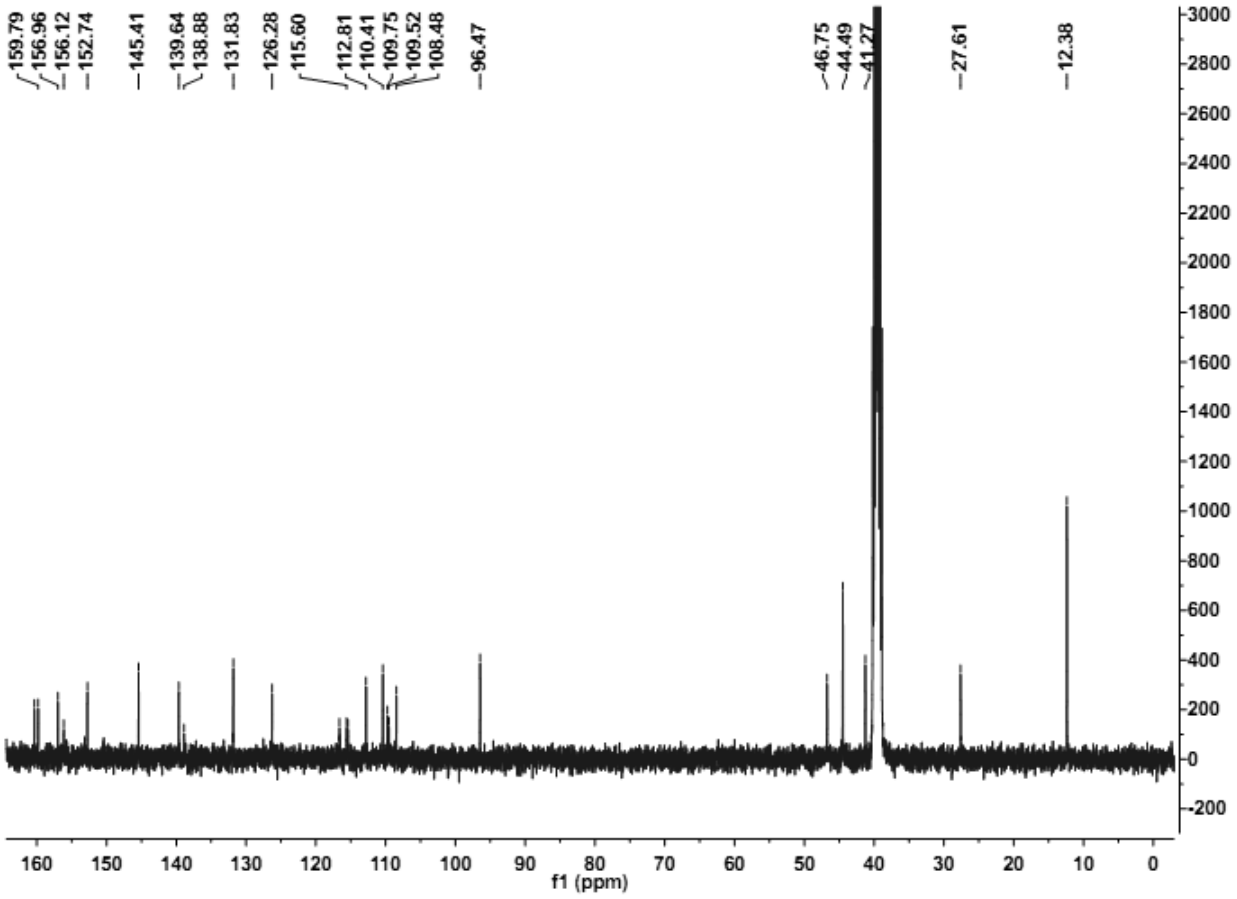


Figure S2. ^{13}C NMR spectrum of **ISCH-1**.

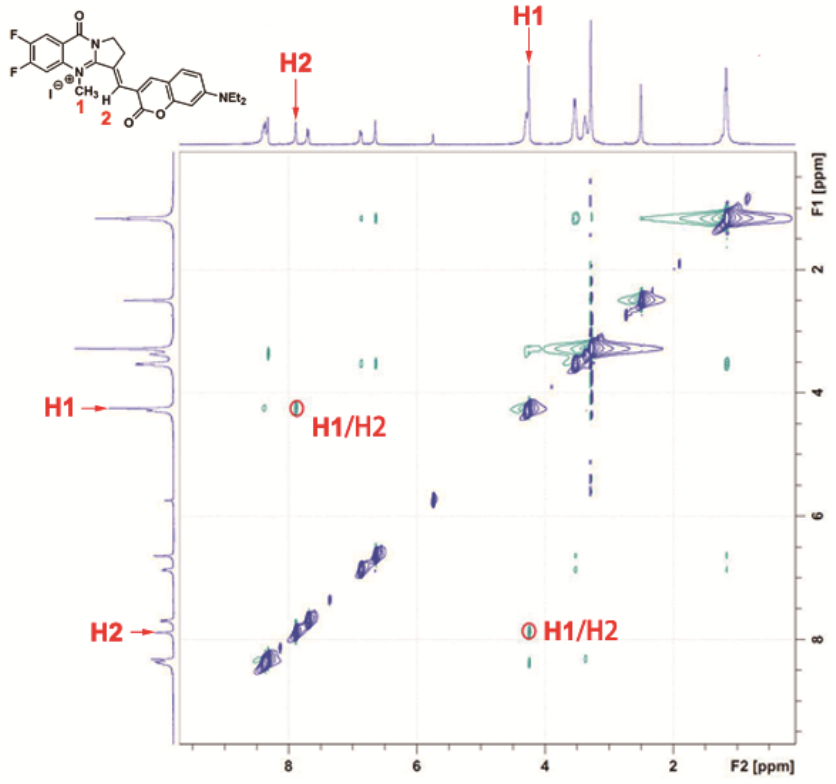


Figure S3. NOESY spectrum of **ISCH-1**.

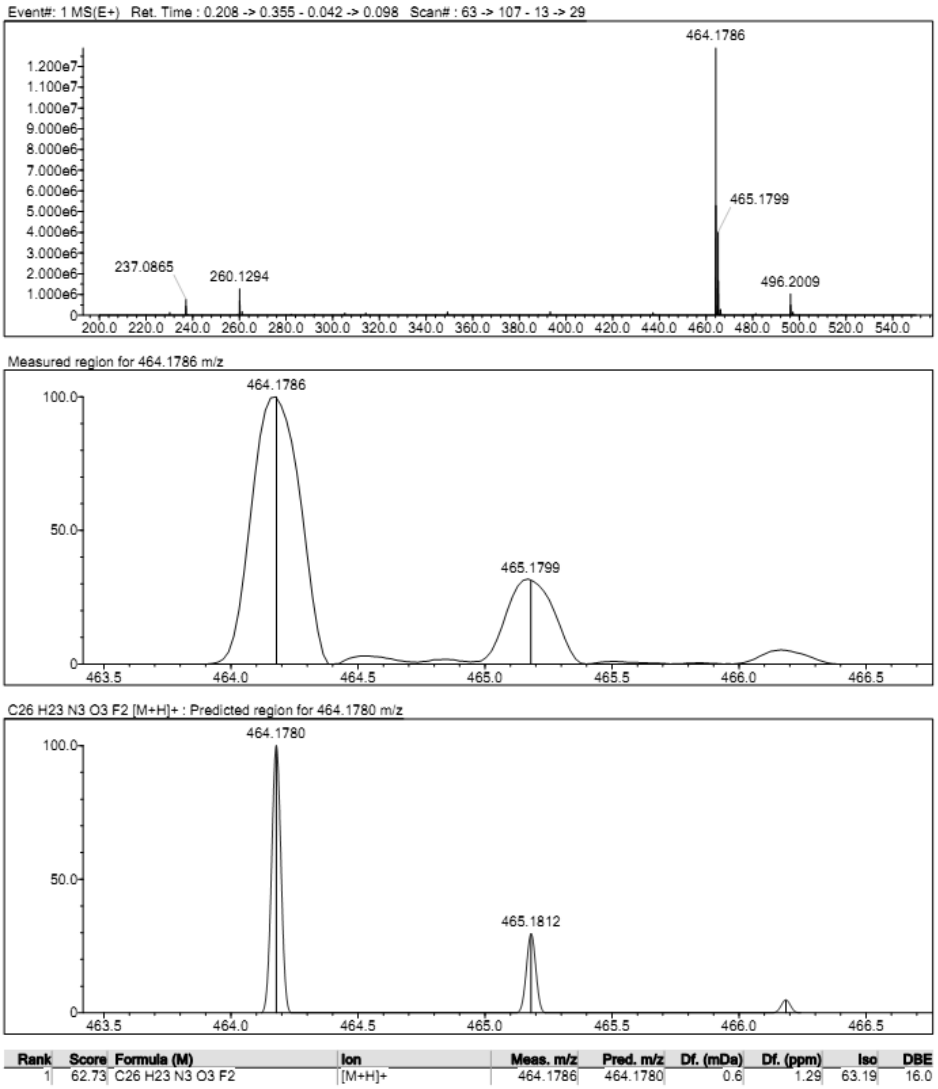
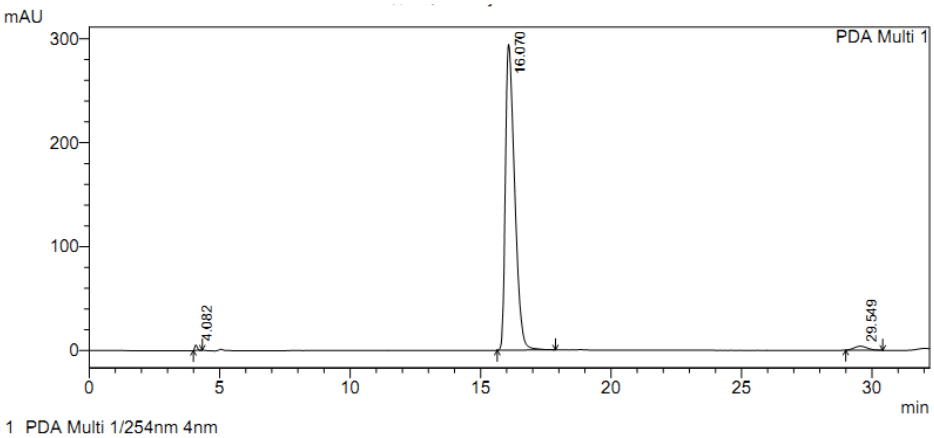


Figure S4. HRMS spectrum of **ISCH-1**.



PeakTable

PDA Ch1 254nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	4.082	32302	5270	0.437	1.737
2	16.070	7232197	294335	97.742	97.008
3	29.549	134740	3809	1.821	1.255
Total		7399239	303414	100.000	100.000

Figure S5. HPLC analysis of **ISCH-1**.

1.2 Materials

All oligonucleotides used in this study were purchased from Invitrogen (Shanghai, China) and Sangon (Shanghai, China). Calf thymus DNA (CT-DNA), bovine serum albumin (BSA) and DAPI were purchased from Sigma-Aldrich (Singapore). RNase-free DNase I, DNase and protease-free RNase A and Pierce Agarose ChIP Kit were purchased from Thermo Scientific (USA). Antibody against nucleolin was purchased from Abcam (USA). Normal rabbit IgG was purchased from Santa Cruz Biotechnology (USA). Tumor cell lines were obtained from the American Type Culture Collection. All the oligonucleotides, CT-DNA and BSA were dissolved in relevant buffer. Their concentrations were determined from the absorbance at 260 nm and 279 nm for DNA and BSA, respectively on the basis of respective molar extinction coefficients using NanoDrop 1000 Spectrophotometer (Thermo Scientific). To obtain G-quadruplex formation, highly concentrated solutions of oligonucleotides were annealed in relevant buffer containing KCl by heating to 90 °C for 5 min, followed by gradual cooling to room temperature and incubation at 4 °C overnight. The oligonucleotides were engaged in G-quadruplex formation, as determined by circular dichroism (CD) measurements (Fig. S6). Stock solutions of **ISCH-1** (10 mM) were dissolved in DMSO and stored at -80 °C. Further dilutions of samples and **ISCH-1** to working concentrations were made with relevant buffer immediately prior to use.

Table S1. Sequences of oligonucleotides used in the present study

Name	Oligonucleotide Sequence
dA21	5′-d[AAAAAAAAAAAAAAAAAAAAA]-3′
dT21	5′-d[TTTTTTTTTTTTTTTTTTTTT]-3′
ds15	5′-d[CGCGCGTTTCGCGCG]-3′
htg21	5′-d[GGGTTAGGGTTAGGGTTAGGG]-3′
htc21	5′-d[CCCTAACCCTAACCCTAACCC]-3′
c-kit1	5′-d[AGGGAGGGCGCTGGGAGGAGGG]-3′
c-kit2	5′-d[GGGCGGGCGCGAGGGAGGGG]-3′
pu18	5′-d[AGGGTGGGGAGGGTGGGG]-3′
pu27	5′-d[TGGGGAGGGTGGGGAGGGTGGGGAAGG]-3′
Rb	5′-d[CGGGGGGTTTTGGGCGGC]-3′
ILPR	5′-d[ACAGGGGTGTGGGGACAGGGGTGTGGGG]-3′
KRAS	5′-d[AGGGCGGTGTGGGAAGAGGGAAGAGGGGGAGG]-3′
VEGF	5′-d[GGGGCGGGCCGGGGGCGGGG]-3′
RET	5′-d[GGGGCGGGGCGGGGCGGGGG]-3′
bcl-2	5′-d[GGGCGCGGGAGGAAGGGGGCGGG]-3′
THTG	5′-d[TTGGGGTTGGGGTTGGGGTTGGGG]-3′
dimer	5′-d[GGGGTTTTGGGG]-3′
tetramer	5′-d[TGGGGT]-3′
HT-6	5′-d[TTAGGG]-3′
hras-1	5′-d[TCGGGTTGCGGGCGCAGGGCACGGGCG]-3′
STAT3	5′-d[GGGCTGGGGATGGGGAGGGGG]-3′
mutated-pu22	5′-d[TGAGCGTGGCGAGCGTGGCGAA]-3′
rDNA-2957	5′-d[GGGTCGGGGGGTGGGGCCCGGGCCGGGG]-3′
rDNA-5701	5′-d[AGGGAGGGAGACGGGGGGG]-3′
rDNA-13079	5′-d[GGGGTGGGGGGGAGGG]-3′
SPR- HTG	5′-biotin-d[GTTAGGGTTAGGGTTAGGGTTAGGGTTAGG]-3′
SPR-c-MYC	5′-biotin-d[ACGTACGTGGGGAGGGTGGGGAGGGTGGGGAAGGTGGGG]-3′
SPR-c-KIT	5′-biotin-d[AGGGAGGGCGCTGGGAGGAGGG]-3′
SPR-Single	5′-biotin-d[AGTTAGAGTTAGAGTTAGAGTTAGAGTTAG]-3′
SPR-Duplex	5′-biotin-d[TTCGCGCGCGTTTTCGCGCGCG]-3′

1.3 UV-Vis Spectroscopic Studies

UV-Vis spectroscopic studies were performed on a UV-2450 spectrophotometer (Shimadzu, Japan) using 1 cm path length quartz cuvette. For titration experiment, all oligonucleotides were firstly prepared through heating at 95 °C for 10 min followed with slow cooling to room temperature. Small aliquots of a stock solution of sample (oligonucleotides, CT-DNA and BSA) were added into the solution containing **ISCH-1** at fixed concentration (5 μM) in Tris-HCl buffer (10 mM, pH 7.4) with 60 mM KCl. The final concentration of sample was varied from 0 to 15 μM. After each addition of sample, the reaction was stirred and allowed to equilibrate for at least 2 min and absorbance measurement was taken.

1.4 Fluorescence Studies[4-5]

Fluorescence studies were performed on a LS-55 luminescence spectrophotometer (Perkin-Elmer, USA). A quartz cuvette with 1 cm × 1 cm path length was used for the spectra recorded at 10 nm excitation and emission slit widths unless otherwise specified. The fluorescence quantum yield (Φ_F) of **ISCH-1** was calculated relative to a standard solution of sulforhodamine 101 in ethanol (Φ_F = 0.95) and was determined using the following formula: $\Phi_u = \Phi_s(I_u A_s / I_s A_u) \times (n_u/n_s)^2$, where Φ is the fluorescence quantum yield, I is the measured integrated emission intensity, n is the refraction index of the solvents, and A is the optical density (absorbance). The u refers to the compound (**ISCH-1**) of unknown quantum yield, and s refers to the reference compound (sulforhodamine 101) of known quantum yield. The fluorescence spectra were recorded at 5 nm excitation and emission slit widths for the determination of Φ.

For titration experiment, all oligonucleotides were firstly prepared through heating at 95 °C for 10 min followed with slow cooling to room temperature. Small aliquots of a stock solution of sample (oligonucleotides, CT-DNA and BSA) were added into the solution containing **ISCH-1** at fixed concentration (5 μM) in Tris-HCl buffer (10 mM, pH 7.4) with 60 mM KCl. The final concentration of sample was varied from 0 to 15 μM. After each addition of sample, the reaction was stirred and allowed to equilibrate for at least 2 min and fluorescence measurement was taken at Ex 570 nm.

1.5 Dual-Channel and Large-Scale Identification of G-Quadruplexes

Probe **ISCH-1** (5 μM) without and with sample (oligonucleotides, CT-DNA and BSA, 5 μM) were dissolved in 150 μL Tris-HCl buffer (10 mM, pH 7.4) containing 60 mM KCl. These mixtures were then added into 96-well plate. After incubation for 4 min at 25 °C, their absorbance at 535 and 600 nm as well as fluorescent intensity at 653 nm (λ_{ex} = 570 nm) were determined using a FlexStation 3 benchtop multi-mode microplate reader (Molecular Devices, USA). Each sample was examined in triplicate.

1.6 SPR Studies

SPR measurements were performed on a ProteOn XPR36 Protein Interaction Array system (Bio-Rad Laboratories, Hercules, CA) using a Streptavidin-coated GLH sensor chip. Biotinylated oligonucleotides (SPR- HTG, SPR-c-MYC, SPR-c-KIT, SPR-Single, SPR-Duplex) were attached to the chip. In a typical experiment, biotinylated DNA was folded in filtered and degassed running buffer (50 mM Tris-HCl, 100 mM

KCl, pH 7.4). The DNA samples were then captured (~1000 RU) in five flow cell, leaving one flow cell as a blank. Ligand solutions were prepared with running buffer through serial dilutions of stock solution. Five concentrations were injected simultaneously at a flow rate of 50 μL·min^{-1} for 400 s of association phase, followed with 400 s of dissociation phase at 25 °C. The GLH sensor chip was regenerated with short injection of 50 mM NaOH between consecutive measurements. The final graphs were obtained by subtracting blank sensorgrams from different DNA sensorgrams. Data were analyzed with ProteOn manager software, using the Langmuir model for fitting kinetic data.

1.7 Live Cell Staining Experiment

The cells were grown in DMEM media containing 10% fetal bovine serum, 100 U·mL^{-1} penicillin with 100 μg·mL^{-1} streptomycin at 37 °C, with 5% CO_2 atmosphere. 2.0 × 10^5 Cells were seeded in 3 cm Petri dishes and grew overnight. Cells were incubated with 8 μM (5 μg·mL^{-1}) of Hoechst 33342 in DMEM media containing 10% fetal bovine serum for 5 min, and subsequently stained with 5 μM **ISCH-1** for 20 min at 37 °C, with 5% CO_2 atmosphere. The cells were imaged using a LSM 710 laser scanning confocal microscope (Carl Zeiss, Germany) with a 60× objective lens at 37 °C, with 5% CO_2 atmosphere. The excitation wavelengths were 405 nm for Hoechst 33342 and 543 nm for **ISCH-1**, respectively. Duplicated experiments were performed.

1.8 DNase and RNase Digest Experiment[6]

The Hela cells were grown in DMEM media containing 10% fetal bovine serum, 100 U·mL^{-1} penicillin with 100 μg·mL^{-1} streptomycin at 37 °C, with 5% CO_2 atmosphere. 2.0 × 10^5 Cells were seeded in 3 cm Petri dishes and grew overnight. Cells were fixed with 4% paraformaldehyde in PBS for 15 min (rinsed with 0.1 M PBS twice). The cell membrane was then permeabilized by immersing the cell in 0.3% Triton X-100 for 30 min (rinsed with 0.1 M PBS twice). Cells were incubated with 100 units·mL^{-1} RNase-free DNase I, or 100 units·mL^{-1} DNase and Protease-free RNase A, or PBS as control, at 37 °C in 5% CO_2 for 2 h. Cells were stained with 5 μM of **ISCH-1** in PBS for 30 min at 37 °C (rinsed with 0.1 M PBS twice), and subsequently stained with 14 μM (5 μg·mL^{-1}) DAPI for 20 min at 37 °C (rinsed with 0.1 M PBS for three times). The cells were imaged using a LSM 710 laser scanning confocal microscope with a 60× objective lens. The excitation wavelengths were 405 nm for DAPI and 543 nm for **ISCH-1**, respectively. Duplicated experiments were performed.

1.9 ChIP Assay[7]

The A549 cells were grown in DMEM media containing 10% fetal bovine serum at 37 °C, with 5% CO_2 atmosphere. ChIP assay was performed using the Pierce Agarose ChIP Kit following the protocol described in the kit. Briefly, A549 cells were treated with 0.1% DMSO or 5 μM **ISCH-1** for 16 h, and then fixed with 1% formaldehyde for 10 min and lysed. Chromatin was sheared to an average size of 200-500 bp using nuclease (provided by the kit). 10% of lysate was used as input control. 3 μg antibody against nucleolin was used to immunoprecipitate chromatin. Normal rabbit IgG was used as negative control. ChIP was performed overnight at 4 °C. Immunoprecipitated DNA samples were amplified by using PCR. The sequences of primers used in the

PCR are as follows: +2121, 5′-d[TGCCTGAGGTTTCTCCCCGA]-3′ and 5′-d[CAACGGACGTGAAGCCGGTG]-3′; +2907, 5′-d[GACGTGTGGCGTGGGTCGAC]-3′ and 5′-d[GACGGGAGGCAGCGACCGG]-3′; +4995, 5′-d[TTCTTAGTTGGTGGAGCGATTTG]-3′ and 5′-d[ATCTAAGGGCATCACAGACCTGT]-3′; +5645, 5′-d[GTTCGCTCGCTCGTTCGTTC]-3′ and 5′-d[CAACGACACGCCCTTCTTTC]-3′; +12323, 5′-d[TTCATAGCGACGTCGCTTTTTG]-3′ and 5′-d[ACCAAATGTCTGAACCTGCGGT]-3′; and +12855, 5′-d[ACCTGGCGCTAAACCATTCGT]-3′ and 5′-d[GGACAAACCCTTGTGTCGAGG]-3′. The primers were used to amplify three regions containing PQSs in the non-template strand of rDNA (rDNA-2957, rDNA-5701, and rDNA-13079) and three corresponding regions that are flanking the three PQSs (rDNA-2121, rDNA-4995, and rDNA-12323) but they are themselves are not PQSs. The amplified products were separated on a 2.5% agarose gel, and photos were taken on a Gel Doc 2000 Imager System (Bio-Rad Laboratories, Hercules, CA). Duplicated experiments were carried out.

Other Supplementary Spectra and Graphs

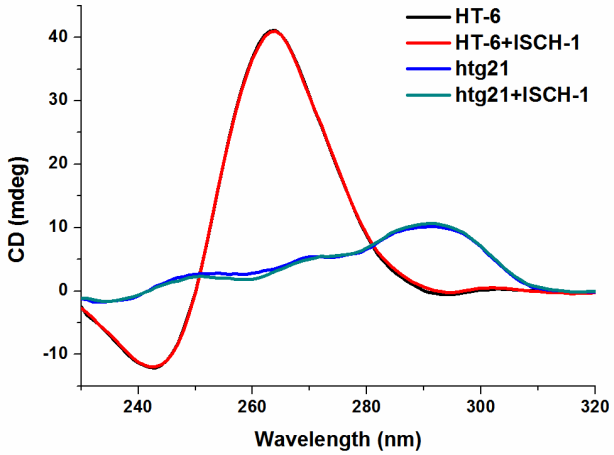


Figure S6. CD spectra of 5 μM G-quadruplex forming oligonucleotides HT-6 and htg21 in 10 mM Tris-HCl buffer, 60 mM KCl, pH 7.4, without and with 5 μM **ISCH-1**. The HT-6 oligonucleotide formed parallel-type G-quadruplex structure and the htg21 oligonucleotide formed hybrid-type G-quadruplex structure. The quadruplex topology of these oligonucleotides was retained with the binding of **ISCH-1**. CD experiments were performed in a Chirascan circular dichroism spectrophotometer (Applied Photophysics, UK). A quartz cuvette with a 4 mm path length was used for the recording of spectra over a wavelength range of 230–320 nm with a 1 nm bandwidth, 1 nm step size and time of 0.5 s per point.

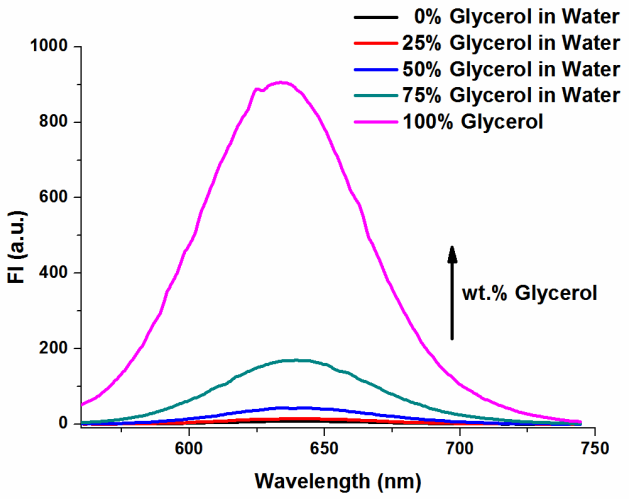
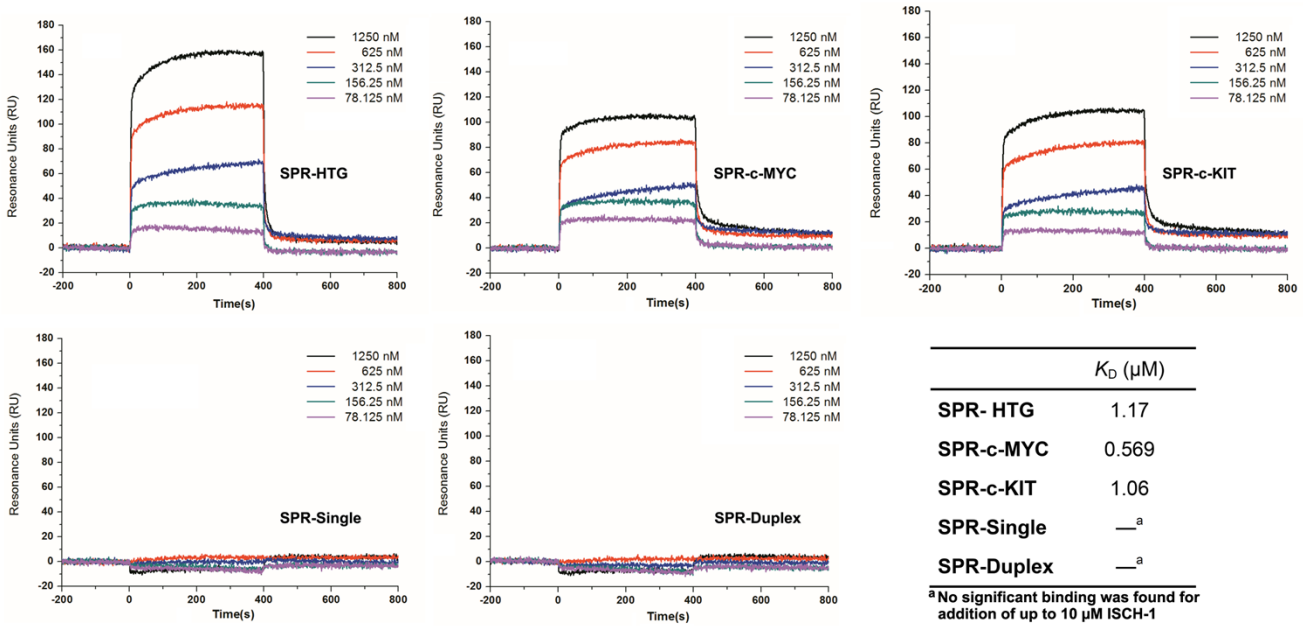


Figure S7. Fluorescence spectra of **ISCH-1** (20 μM) in glycerol–H_2O mixtures (wt.% glycerol: 0, 25, 50, 75, 100%). All samples were excited at 536 nm. The arrow indicated changes in fluorescence intensity upon increasing the glycerol content from 0 to 100%. A quartz cuvette with 0.2 cm × 1 cm path length was used.



	K_D (μM)
SPR- HTG	1.17
SPR-c-MYC	0.569
SPR-c-KIT	1.06
SPR-Single	—[a]
SPR-Duplex	—[a]

[a] No significant binding was found for addition of up to 10 μM ISCH-1

Figure S8. SPR sensorgrams for binding of **ISCH-1** to the immobilized G-quadruplex DNA (SPR-HTG, SPR-c-MYC, SPR-c-KIT), single-strand DNA (SPR-Single), and double-strand DNA (SPR-Duplex). In the plot, the concentrations of **ISCH-1** in the flow solutions were 78.125, 156.25, 312.5, 625, and 1250 nM. Equilibrium binding constants (K_D) for the binding of **ISCH-1** with different DNA were given in the inset table.

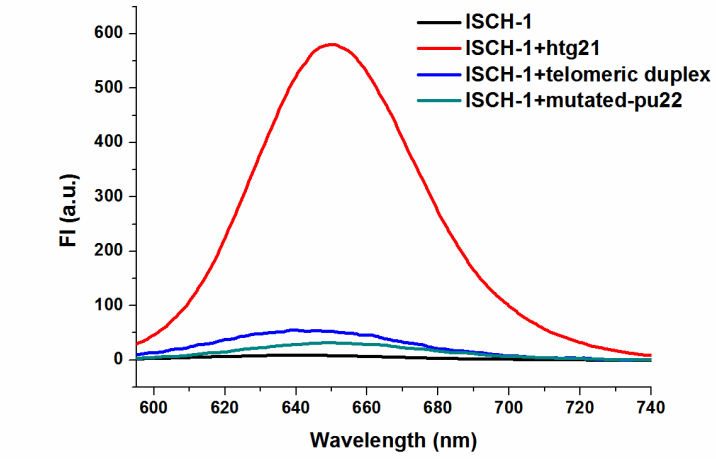


Figure S9. Fluorescence spectra of 5 μM **ISCH-1** with 5 μM different oligonucleotides (htg21, telomeric duplex and mutated-pu22) in 10 mM Tris-HCl buffer, 60 mM KCl, pH 7.4. The htg21 formed hybrid-type G-quadruplex structure. The telomeric duplex was a G-rich double-stranded DNA prepared by 1:1 mix of htg21 and htc21. The mutated-pu22 was a just G-rich single-stranded DNA that could not formed G-quadruplex structure.

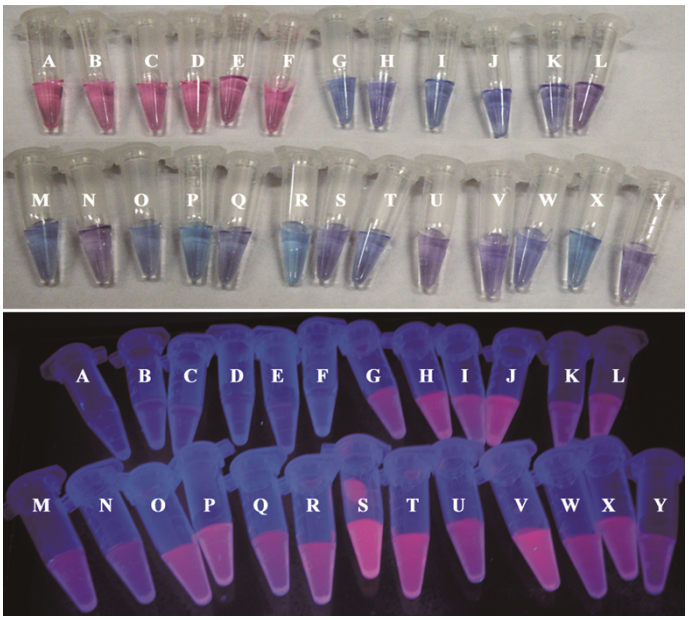


Figure S10. Photograph (taken under ambient light or UV light) of 5 μM **ISCH-1** without (A) and with 5 μM dA21 (B), dT21 (C), CT-DNA (D), ds15 (E), BSA (F), c-kit1 (G), bcl-2 (H), pu27 (I), THTG (J), VEGF (K), Rb (L), c-kit2 (M), RET (N), ILPR (O), KRAS (P), STAT3 (Q), pu18 (R), htg21 (S), dimer (T), tetramer (U), HT-6 (V), rDNA-2957 (W), rDNA-5701 (X), rDNA-13079 (Y).

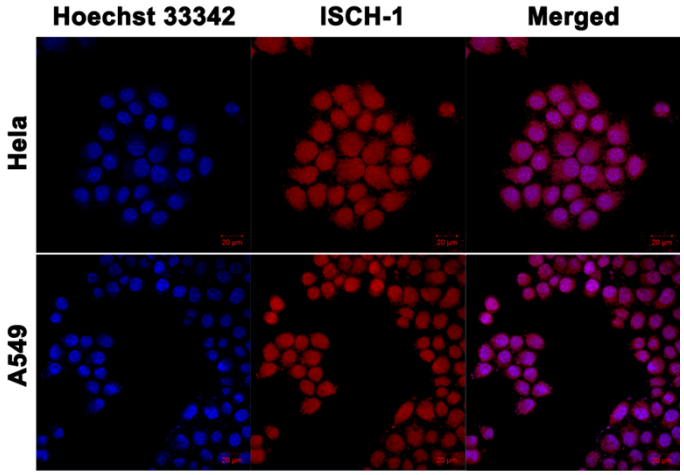


Figure S11. Confocal fluorescence images of live cells using a 543 nm laser. Cells were stained with 5 μM **ISCH-1** and 8 μM (5 μg·mL^{-1}) Hoechst 33342.

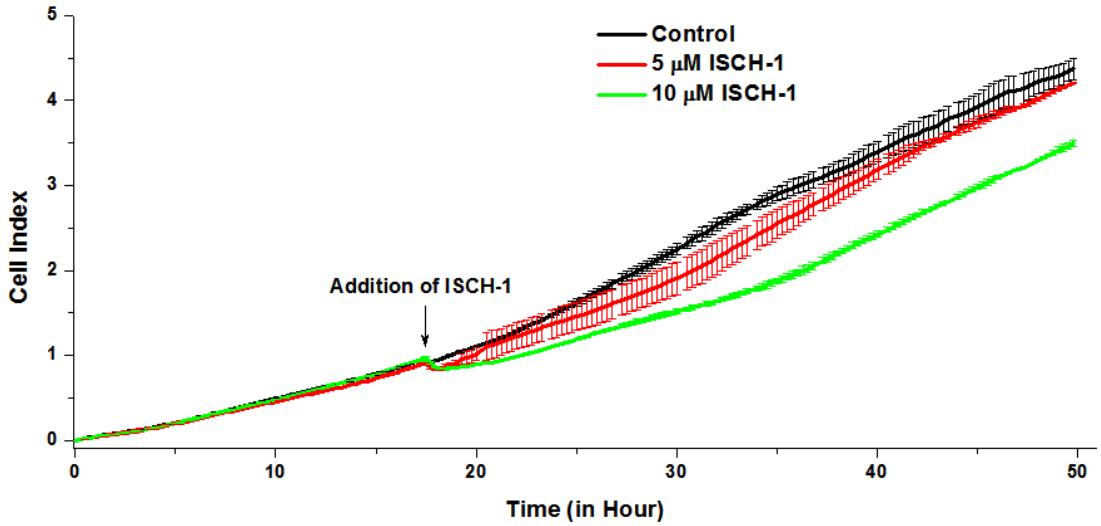


Figure S12. Real-time cell analysis (RTCA) of A549 cells treated with 5 μM and 10 μM **ISCH-1**. RTCA was performed by impedance measuring. The impedance is expressed as the cell index, which is a parameter of cell viability. In this experiment, A549 cells were seeded in 16 well E-plates. The plates were kept in the CO_2 incubator (37 °C in 5 % CO_2) during the experiment. After ~18 h, 0.1% DMSO (control) or **ISCH-1** were added. Cell growth was monitored using the xCELLigence RTCA DP analyzer (Roche, Germany). The 24-hr cytotoxicity (IC_{50}) of **ISCH-1** against A549 cells was 23.5 μM.

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