Electronic Supplementary Information

Two rhenium compounds with benzimidazole ligands: synthesis, properties and G4 DNA-interactions

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Experimental section

Reagents and instrumentation

All reagents were obtained from commercial sources. ^{1}H NMR spectra were recorded on Bruker Avance DPX 300 MHz spectrometer operating at 299.95 MHz. Mass spectra were recorded on TripleTOF™ 5600 LC/MS/MS System, (AB SCIEX) mass spectrometer under temperature: 400 ºC and Ion Spray Voltage (ISVF): 5500, electronic absorption spectra (EAS) were recorded on a Cary 100 UV-Vis spectrophotometer. IR-spectra were recorded on ALPHA II FTIR spectrometer (Bruker). X-ray diffractometer was Bruker D8 Venture X-ray diffractometer using MoKα radiation (λ=0.71073Å) equipped with an Oxford low temperature unit operating at 120K. The emission spectra were recorded on Varian Cary Eclipse spectrometer. Fluorescence resonance energy transfer (FRET) analysis was performed on a PCR Stratagene Mx3005P instrument (Agilent Technologies).

All the oligonucleotides were purchased from IDT DNA. The sequences for the unlabeled oligonucleotides were: cMyc (5'-TGAGGGTGGGTAGGGTGGGTAA-3'), ckit1 (5'-AGGGAGGGCGCTGGGAGGAGGG3'), ckit2 (5'-CGGGCGGGCGCGAGGGAGGGG-3'), Bcl-2 (5'-GGGCGCGGGAGGAATTGGGCGGG-3'), and ds26 (5'-CAATCGGATCGAATTCGATCCGATTG- 3'). All the oligonucleotides are single stranded DNA sequences which are well-known to fold into a G-quadruplex or duplex structures.
The sequences for the labeled oligonucleotides were the same, including 5'-FAM (carboxyfluorescein) and 3'-TAMRA (carboxytetramethylrhodamine). Ligands were dissolved in miliQ water to give 2 mM stock solutions. All solutions were stored at -20 ºC. Before use, they were defrosted and diluted to yield the appropriate concentrations.

Cell Culture

Murine macrophage Raw 264.7 cell line was obtained from the Central Support Service for Experimental Research (SCSIE) of the University of Valencia (ATCC, Raw 264.7 - TIB-71). Raw cells were maintained in DMEM High Glucose (4.5 g/l) with L-glutamine (GIBCO) supplemented with 10% heat-inactivated foetal bovine serum, 1% of penicillin/streptomycin (100 units/ml) and 0.1% of Fungizone (GIBCO). Cells were grown subconfluently in a humidified incubator at 37º C with 5% CO_2 and passaged, routinely tested for mycoplasma contamination and subjected to frequent morphological examination and growth curve analysis as quality-control assessments. In our experimental protocol to conduct cell viability assays, we routinely perform the control experiments using the same percentage of solvents (i.e. < 0.1% DMSO) for all the solutions of incubation with the rhenium-benzimidazole compounds concentration. We therefore incubate cells (by triplicate) with the solvent used for dissolving the rhenium compounds which is assigned as the negative control.

Synthesis of the complexes

Synthesis of cis-$[Re_2(Benzim)_4Cl_4(CH_3CN)_2]Cl_2$ (I). (Benzim = benzimidazole). **I** was obtained according to our previous published procedure under inert atmosphere.[1] The yield of **I** was 75%.

Synthesis of $[ReO_2(Benzim)_4]$ (II). The synthesis of **II** was accomplished by a similar method as **I** but without inert atmosphere and using as a reagent Benzimidazole base: a mixture of 0,05g (0,044 mmol) of $(Bu_4N)_2Re_2Cl_8$ and 0,07g (0,59 mmol) Benzim was stirred for 25 h in chloroform. Red crystalline product, which come out after evaporation of chloroform was recrystallized from dichloromethane. Yield: 78 % (0,032 mg, 0,034 mmol). EAS (CH_3CN) (ν, cm^{-1}): 20504; ε= ($l/M^{-1}\cdot cm^{-1}$), 28249; ε= ($l/M^{-1}\cdot cm^{-1}$); IR (ν_{max}/cm^{-1}): 3446 (ν (N(3)-H), 1586 (νN1-C5),1474 (ν N3-C4), also 1428, 1182, 1056, 888, other peaks are referred to deformation vibrations of Benzim core, The strongest peaks at 765cm^{-1} and average one at 920 cm^{-1} indicates on the vibrations O=Re=O and Re=O accordingly. ^{1}H NMR (300 MHz, DMSO-d_6, ppm): 7.25 - 7.57 (m), 9.02 (s). Mass spectra of **II** (DMSO)(m/z): 686.55, 572.52, 456.39, 338.34, 242.28.

Crystallographic structure determination

Single-crystals of **II** suitable for diffraction were measured in a Bruker D8 Venture X-ray diffractometer using MoKα radiation (λ=0.71073Å) equipped with an Oxford low temperature unit operating at 120K. Indexing, strategy and data collection were performed with APEX3 software suite. OLEX2[2] was used as frontend for solving and refining. Initial structure was solved with direct methods using SHELXS and then refined with SHELXL2018[3]. Initially, an isotropic refinement was performed on the non-hydrogen atoms. Then, anisotropic refinement was done for non-hydrogen atoms.

Fluorescence titration and FRET assays

Fluorescence titrations: unlabeled DNA was dissolved in potassium cacodylate buffer (100 mm KCl, 10 mm LiCac, pH 7.3) and annealed at 95 °C for 5 min, before cooling to room temperature overnight. The concentration of DNA was checked by using the molar extinction coefficients. Annealing concentrations were approximately 250 μm. For fluorescence emission titrations, complexes (1 μm) in the same buffer were titrated with the corresponding DNA until saturation of fluorescence. The emission spectra were recorded in 1 cm path-length quartz cuvettes. Spectra were smoothed by using the Savitzky-Golay algorithm and emission maxima were fitted to a 1:1 binding model with the Levenberg–Marquardt algorithm and equations.[4]

FRET melting assays were performed according to procedure described in.[5] Analysis was performed on a PCR analyzer with excitation at 450–495 nm and detection at 515–545 nm. Readings were taken in the temperature range 25–95 °C (intervals of 0.5 °C). Each measurement was performed in triplicate. Labelled DNA was dissolved in milliQ water to give 20 μM stock solutions. The solutions were then further diluted to 0.4 μM by using the appropriate buffer and annealed at 95 °C for 5 min. Finally, they were allowed to slowly cool to room temperature overnight. The buffer used for the DNA was 10 mM KCl/90 mM LiCl/10 mM LiCac. Ligands were diluted from stock solutions (see above) in the same buffer as the tested sequence to yield specific final concentrations. Each well of a 96-well plate (Applied Biosystem) was filled with a total volume of 60 μL with a final DNA concentration of 200 nM and increasing concentrations

of ligands (0-4 μM). The normalized fluorescence signal was plotted against the compound concentration and the ΔT_m values were determined.

Molecular Dynamics Simulation

Molecular Dynamics for complex **II** were carried with the GROMACS 2023[6] software using the natively supported AMBER 03 forcefield.[7] The complex **II** was parametrised for this forcefield using AmberTools22[8] tool MCPB.py.[9] The Seminario method[10] was chosen for the parameter refinement and GAMESS[11] as auxiliary tool. The tool amb2gmx was used to convert AMBER parmeters to GROMACS format. Initial structures for G4 DNA tetraplex c-kit promoters were retrieved from the Nucleic Acids Knowledge Bank with ids 2O3M[12] and 2KYP.[13] Initial files were prepared with the nucleic acid in the center of the simulation box and the complex **II** located on one side of the box. Water, TIP3P model was used as solvent. Water bonds and angles were restrained with SETTLE.[14] KCl 0.15 M was choosen to balance the charge and to fill the box as electrolite. The initial structure was relaxed with steep descent energy minimisation followed by an 200 ps NVT run and a 400 ps NPT run at 300 K and 1 atm. In these runs the complex position was restrained and a 2 ft step was used. When the system was properly equilibrated the restraints were removed and the production MD was run for 10 ns with a 2 fs step. The obtained trajectories was analysed with MDAnalysis.[15] Visual output was produced with VMD.[16]

Cell viability assay

Raw 264.7 cells were plated in 96-well plates at a density of 10.000 cells/well. After 24 hours of adherence, the cells were treated with **I** and **II** at 10, 50 and 100 μM for 48 hours.[17] The number of viable cells in culture was determined by quantification of ATP, which signals the presence of metabolically active cells, using the Cell Titer-Glo® luminescence assay kit (Promega, Madison, WI). According to the manufacturer's instructions, the Cell Titer-Glo reagent was added to cells cultured with treatment medium in the 96-well plate, and luminescence was read using the BioTek Synergy H1 Multimode Reader. Samples were normalized to untreated controls to obtain percentages of cell viability and graphs were generated using GraphPad Prism 6 software.

Quantification of rhenium cellular uptake

Raw cells were seeded in a 6-well plate (200.000 cells/well) in DMEM medium and incubated for 24 h. After the adherence, a fresh medium containing 20 μM **I** and **II** was added and incubated for a further 24 hours. After washing with 1× PBS (Phospho-Buffered Saline, GIBCO), Raw cells were harvested by trypsinization, and 1×10^6 cells were collected by centrifugation and washed twice with PBS. The cell pellet was digested under acidic conditions and the total rhenium uptake was measured by Inductively Coupled Plasma Mass Spectrometry (ICP-MS7900, Agilent Technologies).

Table S1.- EAS characteristics of **I** and **II**

Substance	Optical characteristics	
	λ_{max} (nm)	ε ($l \cdot mol^{-1} \cdot cm^{-1}$)
I	601	78,8
	360 (inflection point)	130,6
	273	4635,3
	266	4982,9
	260	4309,5
	233	5112,9
II	476	213,3
	308 (inflection point)	1452,8
	277	12757,3
	270	13398,2
	264	12008,3
	241	14052,1
	214	13126,1

Table S2.- Cell viability (%) of the murine macrophage Raw 264.7 cell line upon addition of **I** and **II** at final concentrations of 5, 10, 100 µM for 48 h; amount (ng) of rhenium in Raw 264.7 cells treated for 24 h with 20 µM **I** and **II**. The amount of Re was quantified by ICP-MS.

Substance	**Concentration (µM)**	**Cell viability (%)**	**Amount of rhenium (ng/10^6 cells)**
I	10	106,80 ± 5,62	2,04 ± 0.03
	50	63,04 ± 2,88	
	100	38,93 ± 1,50	
II	10	111,94 ± 2,78	0.29 ± 0.001
	50	113,29 ± 0,50	
	100	109,20 ± 2,34	

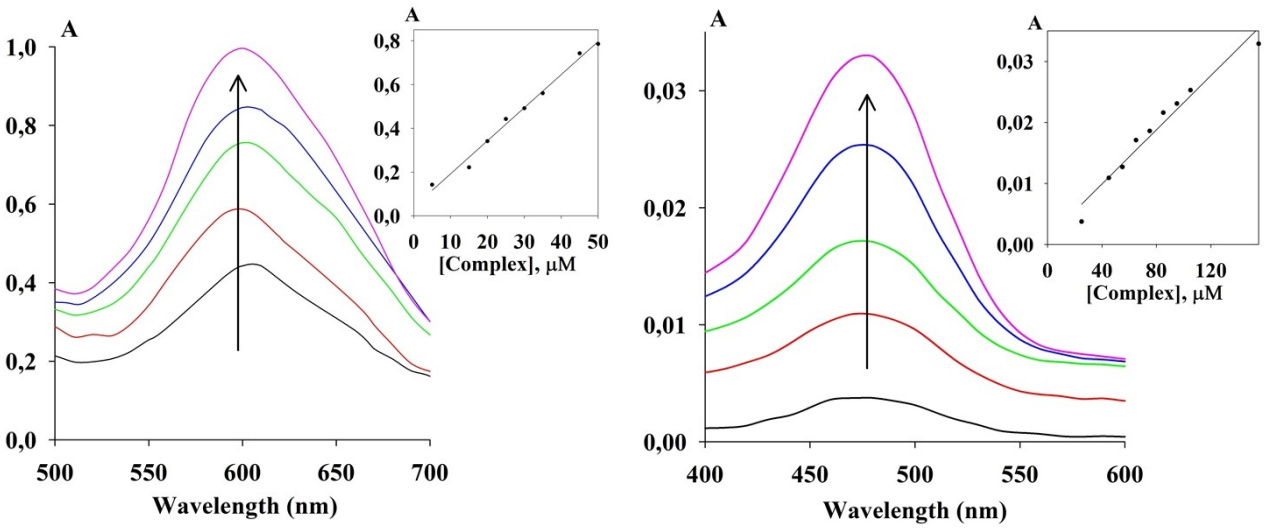


Fig. S1 UV-Vis spectra of **I** (left panel) and **II** (right pane) at increasing concentrations in LiCac buffer. Insets: Plot of the absorbance at the maxima *vs.* [complex].

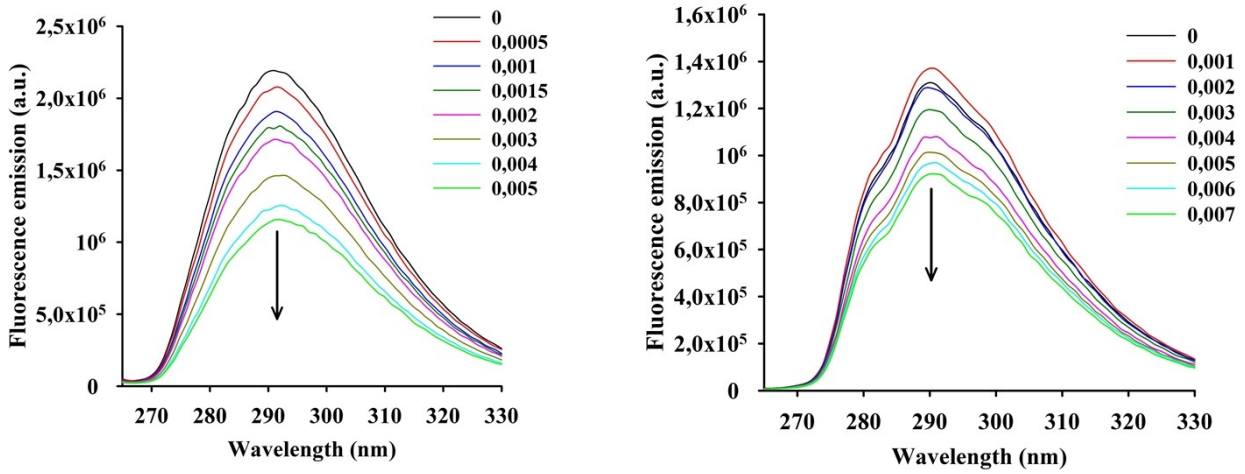


Fig. S2 Fluorescent titration spectra of **I** (left panel) and **II** (right pane) with Bcl-2 G4 DNA at increasing concentrations in LiCac 10 mM, KCl 100 mm KCl, pH 7.3).

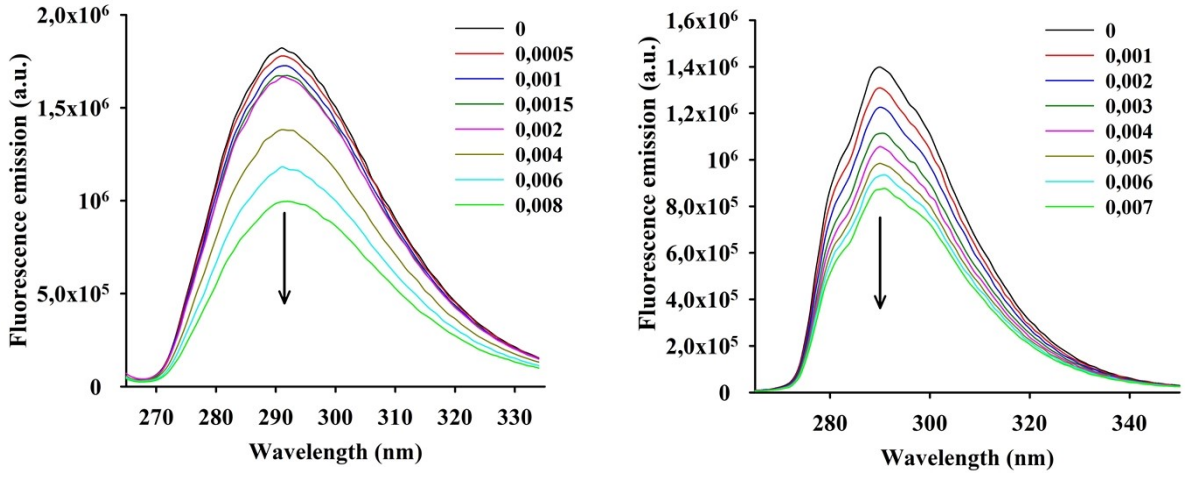


Fig. S3 Fluorimetric titration of **I** (left panel) and **II** (right pane) with c-kit2 G4 DNA at increasing concentrations in LiCac 10 mM, KCl 100 mm KCl, pH 7.3).

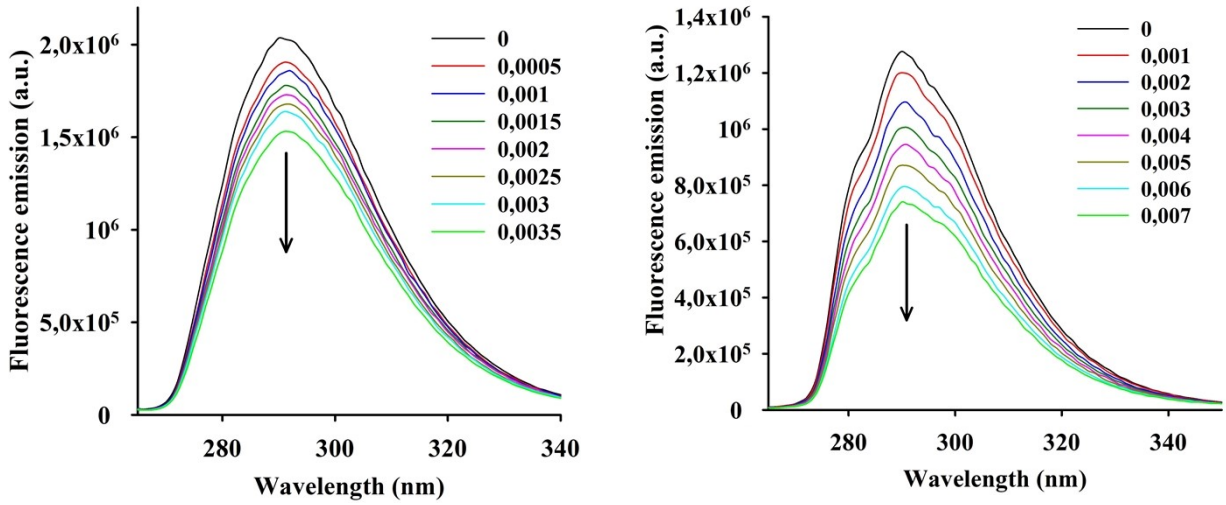


Fig. S4 Fluorimetric titration of **I** (left panel) and **II** (right pane) with cMyc G4 DNA at increasing concentrations in LiCac 10 mM, KCl 100 mm KCl, pH 7.3).

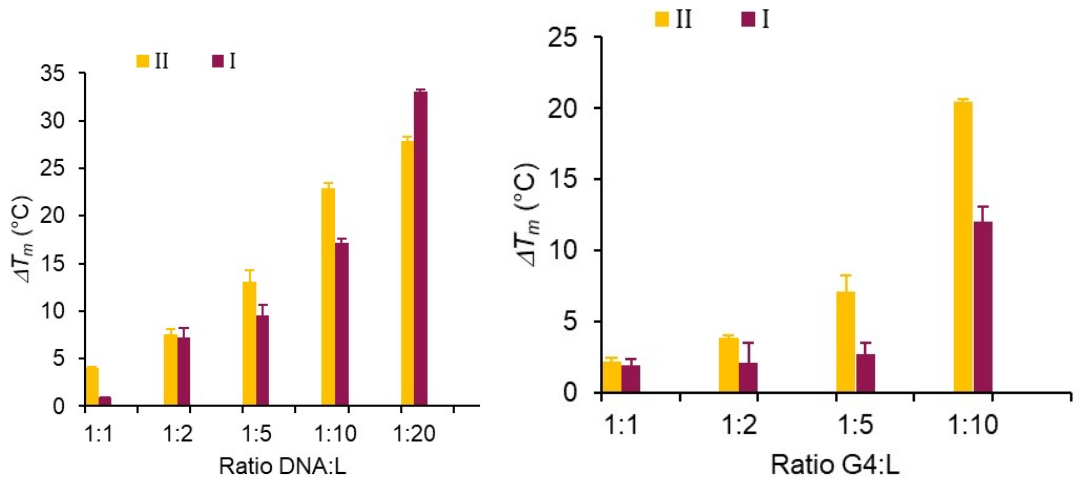


Fig. S5 Values of ΔT_m (°C) of G4 DNAs *ckit1* (left panel) and *ckit2* (right panel) under different concentrations of **I** and **II**.

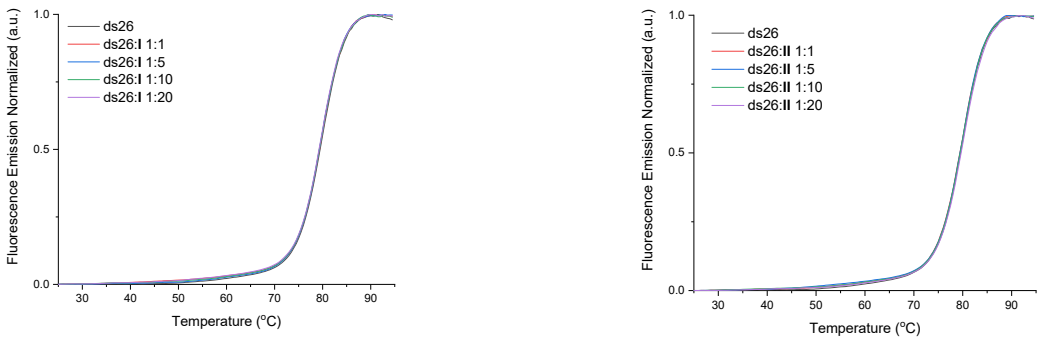


Fig. S6 Stabilization curves of ds26 in the presence of **I** (left panel) and **II** (right panel) at different molar ratio DNA:complex.

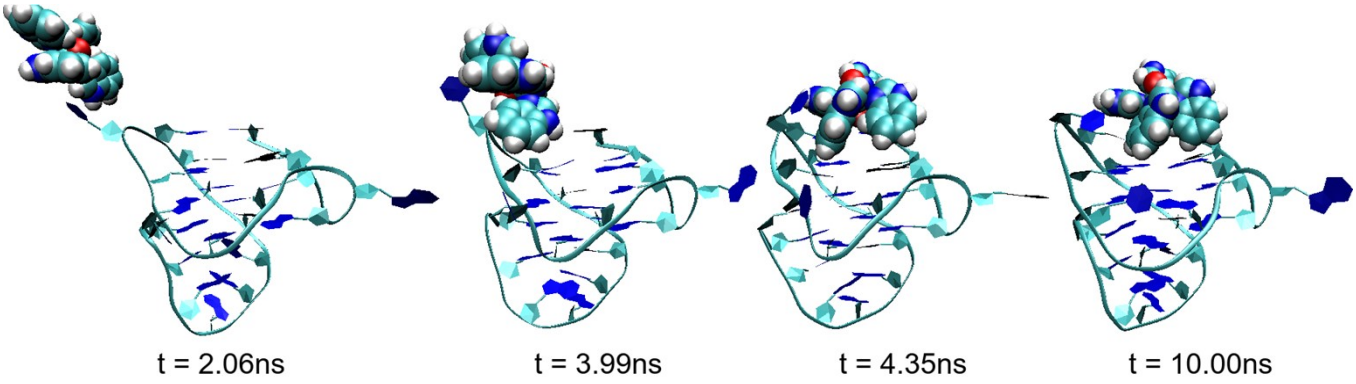


Fig. S7 Conformations of *c-kit1* and **II**

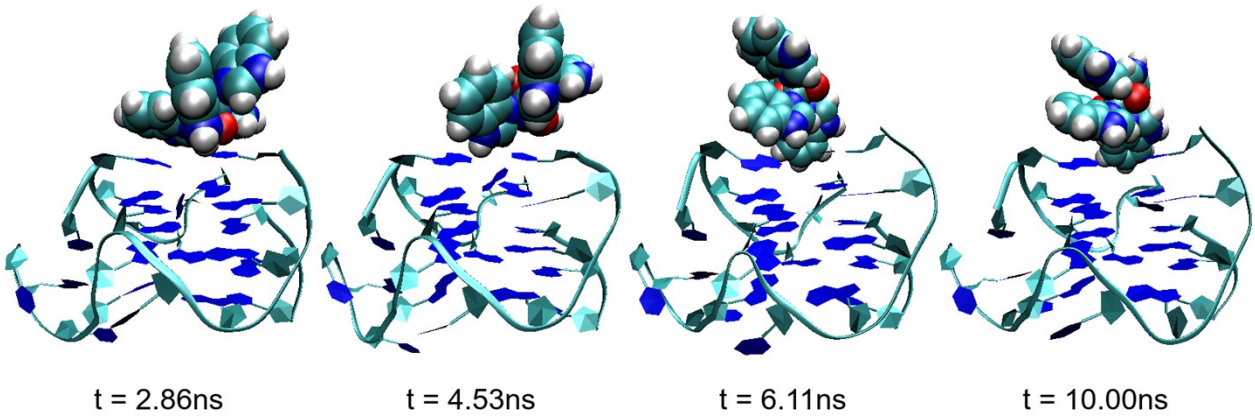
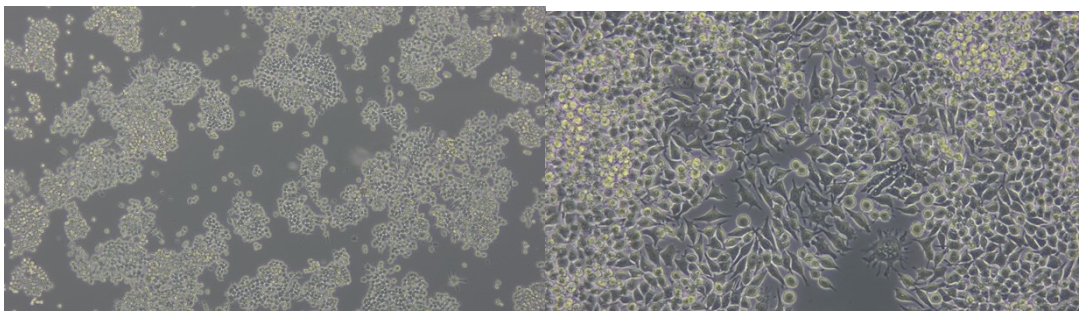


Fig. S8 Conformations of *c-kit2* and **II**



A B

Fig. S9 Representative phase-contrast photomicrographs of Raw 264.7 cells untreated (A) or treated with 50 μM **I** (B) after 48 h exposure; original

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