Supplementary Information for:

Expanding the DNA Damaging Potential of Artificial Metallo-Nucleases through Click Chemistry

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General Remarks

Chemicals and reagents were sourced from Sigma-Aldrich and Tokyo Chemical Industry (TCI) and were used without any further purification. HPLC grade chloroform and methanol were used without further purification. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker AC 600 MHz NMR spectrometer and processed in MNova (MastreLab). Thermal melting analysis was performed on a Roche LightCycler 480 II. Fluorescence quenching assays were performed using commercial EtBr, Hoechst 34580 (sigma) and Methyl green (TCI) and plates were read on a TECAN Spark® microplate reader. Circular dichroism data was collected on an Applied Photophysics Chirascan Plus. Microscale thermophoresis experiments were conducted on a Nanotemper Monolith® instrument using standard capillaries. Human topoisomerase I was acquired from Sigma.

Caution! Sodium azide is acutely toxic and is an explosion hazard. Refer to organic azide stability prior to the preparation of any azide compounds. The total number of nitrogen atoms in a final organic azide should not exceed that of carbon. Organic azides with a C/N ratio of <1 should never be isolated. It may be synthesised if the azide is a transient intermediate species and the limiting reagent in the reaction mixture has a maximum quantity of 1 g. Each azide compound should be individually evaluated.

Synthesis

TC Ligands were prepared using the copper catalysed alkyne-azide cycloaddition (CuAAC) of 2,4,6-Tris-(azidomethyl)-mesitylene (Triazide) with a variety of commercially available alkynes in the presence of the co-catalyst tris-hydroxypropyltriazolylmethylamine (THPTA). In all cases the aqueous solutions of active catalyst was prepared in situ by dissolving appropriate quantities of Cu(II) sulphate (1 mol %) and THPTA (1 mol %) in 0.5 mL of water followed by addition of 0.5 mL aqueous Na-L-ascorbate (5 mol %).

2,4,6-Tris-(azidomethyl)-mesitylene (triazide):

Synthesis was modified from previously reported procedure To a solution of 2,4,6-tris-(bromomethyl)-mesitylene (1.025 g, 2.56 mmol) in acetone (50 mL) sodium azide (1.00 g, 15.38 mmol) was added in portions over ice over a period of 20 min. (Caution! Sodium azide is acutely toxic and is an explosion hazard. Refer to organic azide stability prior to the preparation of any azido compounds). The reaction was refluxed overnight and allowed to cool to room temperature. The suspension was gravity filtered and the filtrate was dried under reduced pressure to yield the title product as a crystalline white solid. NMR data was in line with previously reported results.

(((2,4,6-trimethylbenzene-1,3,5-triyl)tris(methylene))tris(1H-1,2,3-triazole-1,4-diyl))trimethanol (TC-OH):

The active catalyst solution was added to a solution of triazide (100mg, 0.35mmol) in DMF (5 mL) and allowed to stir for 10 minutes. The resulting solution was then added dropwise to a stirring solution of propargyl alcohol (196mg, 3.5mmol) in DMF (5mL). The flask was flushed with nitrogen and allowed to stir overnight at room temperature. The bright yellow solution was dried under a stream of nitrogen. The yellow solid was suspended in acetone (5mL) with sonication. The title product (125mg, 81%) was isolated by vacuum filtration and washing with ice cold acetone (5 x 5 mL). 1H NMR (DMSO, 600 MHz): δ 2.42 (s, 9H) 4.48 (d, 6H, J = 5.5 Hz) 5.13 (t, 3H, J = 5.5 Hz) 5.66 (s, 6H) 7.76 (s, 3H). 13C NMR (DMSO, 151 MHz) δ: 16.78, 48.73, 55.45, 122.80, 131.42, 139.64, 148.36. ESI-MS positive ionization mode:

[M+H]+ Calculated m/z = 454.23 found m/z = 454.23, [M+Na] calculated m/z = 476.21 found m/z = 476.21.

1,1',1''-((2,4,6-trimethylbenzene-1,3,5-triyl)tris(methylene))tris(1H-1,2,3-triazole-4-carboxylic acid) (TC-Acid):

The active catalyst solution was added a solution of triazide (100mg, 0.35mmol) in methanol (10 mL) and stirred at room temperature for 10 minutes. The resulting solution was added dropwise to a solution of propiolic acid (98 mg, 1.4mmol) in methanol (5 mL). The reaction flask was flushed with nitrogen, sealed and the reaction was stirred vigorously overnight. A white precipitate was removed by vacuum filtration. The filtrate was dried under reduced pressure to yield a white solid that was then suspended in acetone (5mL) with sonitcation and isolated by decanting. The remaining solid was dried over desicant to yield the title product as a white solid. Yield = 145mg (83 %). ^{1}H NMR (DMSO 600 MHz): δ 2.38 (s, 9H), 7.74 (s, 6H) 8.55 (s, 3H) 13.14 (s, 3H) ESI-MS (negative ionization mode): ESI-MS negative ionization mode: $[M + H]^{+}$ calcualted m/z = 496.17 found m/z = 496.17. $[M+Na]^{+}$ calculated m/z = 518.15 found m/z = 518.15.

1,1',1''-((2,4,6-trimethylbenzene-1,3,5-triyl)tris(methylene))tris(1H-1,2,3-triazole-4-carboxamide) (TC-Amide):

The active catalyst solution was added a solution of triazide (159.2mg, 0.56mmol) in degassed ACN (8mL). The mixture was allowed to stir for 15 minutes before the dropwise addition of propiolamide (115mg, 1.67 mmol). The reaction mixture was allowed to stir at room temperature for 3 hours. The title product was collected by vacuum filtration washed with cold acetonitrile (3 x 5 mL) and dried over desiccant. yield = 270mg (98 %). 1H NMR (DMSO, 600 MHz): δ 2.38 (s, 9H), 5.74 (s, 6H), 8.34 (s, 3H). ESI-MS: [M+Na]+ m/z calculated = 515.20 found m/z = 515.20.

2,2',2''-(((2,4,6-trimethylbenzene-1,3,5-triyl)tris(methylene))tris(1H-1,2,3-triazole-1,4-diyl))tripyrimidine (TC-Pyrm):

The active catalyst solution was added to a stirring solution of triazide (91mg, 0.32mmol) in methanol (8mL). The mixture was allowed to stir at room temperature for 15 minutes before dropwise addition of 2-ethynyl pyrimidine (100mg, 0.96mmol) in methanol (1mL). The title product was isolated as a off-white solid by vacuum filtration, washing with 5mL 0.1M aqueous EDTA solution and 5mL acetone. Yield = 170mg, 89%.1H NMR (DMSO, 600 MHz): δ 2.48 (s, 9H), 5.76 (s, 6H), 7.42 (t, 3H J = 4.96Hz) 8.57 (s, 3H), 8.83 (d, 6H, J = 4.88Hz). ESI-MS: [M+H]+ calculated m/z = 598.327 found m/z = 598.26 [M+Na]+ calculated mz/ = 620.25 found m/z = 620.25

2,2',2''-(((2,4,6-trimethylbenzene-1,3,5-triyl)tris(methylene))tris(1H-1,2,3-triazole-1,4-diyl))tripyridine (TC-Py): An activated catalyst solution consisting of 1mol% CuSO4 and 5 mol% Na-L-Ascorbate was added to a stirring solution of triazide (120mg, 0.42mmol) in methanol (8mL). The mixture was allowed to stir at room temperature for 15 minutes before dropwise addition of 2-ethynyl pyridine (132mg, 1.28mmol) in methanol (2mL). The reaction mixture was allowed to stir for 16 hours at room temperature. The title product was isolated as a white solid by vacuum filtration and washing with 5mL 0.1M aqueous EDTA solution, 5mL acetone and 20 mL Et_2O. Yield = 225mg, 90%. 1H NMR (DMSO, 600 MHz): δ 2.48 (s, 9H), 2.53 (s, 6H), 7.32 (m, 3H) 7.87 (td, 3H, J1 = 7.4Hz, J2 = 1.4Hz) 7.99 (dt, 3H, J1 = 7.94 Hz, J2 = 0.84 Hz) 8.38 (s, 3H) 8.54 (m, 3H). ESI-MS: Found [M+H]+ m/z = 595.28 calculated [M+H]+ = 595.28.

2,2',2''-(((2,4,6-trimethylbenzene-1,3,5-triyl)tris(methylene))tris(1H-1,2,3-triazole-1,4-diyl))tris(benzo[d]thiazole) (TC-Benzo):

To a stirring solution of triazide (119mg, 0.4mmol) in methanol (20 mL) , were added solutions of CuSO4 (5 mol%) and (+)-Sodium L-ascorbate (20 mol %) in water (0.5 ml each). The mixture was allowed to stir

for 15 minutes before being added dropwise to a solution of 2-ethynyl benzothiazole (200 mg, 1.2 mmol) in methanol (5 mL). The flask was flushed with nitrogen, sealed, and allowed to stir overnight at room temperature. A yellow precipitate formed. The reaction volume was reduced to 3 mL and the title product collected by vacuum filtration (310 mg, 99%). 1H NMR (DMSO, 600 MHz): δ 5.85 (s, 6H), 7.45 (t, 3H, J = 7.41 Hz), 7,52 (t, 3H, J = 8.19 Hz), 7.97 (d, 3H, J = 7.73 Hz), 8.14 (d, J = 7.41 Hz), 8.79 (s, 3H) ESI-MS: [M+H]+ calculated = 763.20 found m/z = 763.19 [M+Na]+ calculated m/z = 785.18 found m/z = 785.18.

Crystallography

The data were collected at 100(1)K on a Synergy, Dualflex, AtlasS2 diffractometer using CuKα radiation (λ = 1.54184 Å) and the CrysAlis PRO suite.[1] Using shelXle[2] and Olex2[3] the structure was solved by dual space methods (SHELXT[4]) and refined on F^2 using all the reflections (SHELXL-2019/2[5]). All the non-hydrogen atoms were refined using anisotropic atomic displacement parameters and hydrogen atoms were inserted at calculated positions using a riding model. Crystal parameters, data collection and structure refinement details are summarised in **Table S1**.

Verification of In-Situ Complexation by ESI-MS

Formation of TC-Cu_3 complexes *in-situ* was investigated using ESI-MS. Solutions of TC ligands were prepared in DMF prior to addition of three equivalents of aqueous Copper(II) nitrate trihydrate. Samples were vortexed and incubated at 37 °C for 30 minutes before further dilution (as required) and analysis on a MaXis HD quadrupole electrospray time-of-flight (ESIQTOF) mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany), using a glass syringe (Hamilton) and syringe pump (KD Scientific, Model 781100) for infusions at a flow rate of 3 mL/min.

Fluorescence Quenching

Competitive ethidium bromide displacement was conducted as previously reported with slight modification.[6] Briefly, a triplicate serial dilution of test compound was prepared on a 96 well plate to a volume of 50 µL. 50 µL of a working solution of EtBr (25.2 µM) and ctDNA (25 µM) was then added to give a final volume of 100 µL, 12.5 µM EtBr, 12.5µM ctDNA. All solutions were prepared in 80mM HEPES, 25 mM NaCl, 5% DMSO. Control wells contained EtBr and ctDNA at equivalent concentration to the test. Blank wells contained EtBr only in the same buffer. Fluorescence intensity due to DNA / EtBr binding was calculated using equation 1.

1) $$F = (F_s - F_b)/(F_c - F_b)$$

Where F is the (fractional) normalised intensity of fluorescence due DNA-bound EtBr. F_s is the observed fluorescence of the sample well, F_b is the fluorescence of the blank well and F_c is the fluorescence observed in the control well. Experiments conducted with Hoechst 34580 and Methyl green were conducted under equivalent conditions but with 5 µM fluorophore. Data was collected on a TECAN® Spark microplate reader. Fluorescence quenching of methyl Green and Hoechst 34580 were conducted in an equivalent manner but with final fluorophore concentrations of 5 µM. Excitation emission wavelengths used were 530/590 nm for EtBr, 350/450 nm for Hoescht 34580 and 630/670 nm for MG.

Microscale Thermophoresis

Samples for MST analysis were prepared to contain 500nM F-DDH in 80 mM HEPES, 25 mM NaCl, 5% DMSO with Cu(II)-TC-Py at varying concentrations. MST power was set to medium and excitation power was set to automatic mode in the red channel, typical excitation power was approximately 14%. The laser-on time was set to 20 seconds and with data being recorded for two seconds prior to the laser being turned on and for 2 seconds post being turned off.

Fluorescence Melting

Thermal melting analysis was performed on a Roche LightCycler®480 II using 80mM HEPES 25mM NaCl buffer. Prior to analysis FRET-DDH was denatured by heating to 90°C (10°C/min, 2 min hold) and reannealed at 12°C(0.5°C/min, 20min hold). Sample tubes for analysis were prepared to contain 1 μM FRET-DDH and Cu(II)-TC-Py at various r loadings. (r = Cu(II)-TC-Thio / [FRET-DDH]). Melting was conducted in triplicate at a ramp rate of 0.5 °C min^{-1} up to a maximum of 95°C. Tm values were taken as the midpoint of the melting curves.

Single Molecule Analysis – DNA Binding

Sample Preparation

Samples for analysis were prepared to contain 5 μM (base pairs) of lambda DNA and 5 μM YOYO-1 with varied concentrations of Cu(II)-TC-Py in a total volume of 10 μL and incubated for 30 minutes at room temperature. Before imaging, 0.5 μL of β-mercapto ethanol was added to minimize YOYO-1 photocleavage and the sample was diluted to 50 μL to prevent overloading of nanochannels.

DNA Stretching and Imaging

Samples were added to nanofluidic chips that were fabricated as described elsewhere. Compressed air was used to move DNA molecules from the sample well, into the microchannel and to accumulate at the nanochannel interface. Air pressure was then increased manually to drive DNA molecules into nanochannels where images were taken at the excitation and emission wavelengths of YOYO-1 with 2% excitation laser intensity, collecting 20 frames at 100 millisecond exposure times each to give 2 second videos of each imaging field.

Data Processing

Data analysis was performed with the freeware ImageJ and a custom-written MatLab based software. For DNA confined in nanochannels a kymograph (timetrace) was extracted for each movie using ImageJ and the average distance between either end of the DNA molecule and the average pixel intensity along the DNA molecule was found using the custom MatLab software. For mapping, the initial alignment is done on the center of the molecule to eliminate the effects of drift along the nanochannel. The intensity profile from the molecule is fitted to a linear combination of error functions. A finer alignment procedure is subsequently performed on distinct features (peaks or dips in intensity) along the DNA.

Docking Studies

Docking studies of Cu(II)-TC-Py with a B-DNA dodecamer (PDB: 1BNA) was performed using AutoDock Vina.[7] The Cu(II)-TC-Py was prepared by first removing nitrates from the DFT structure. Atomic charges and rotatable bonds were then defined in Autodock tools (version 1.5.7) and the structure output file saved in .pdbqt format. The DNA receptor was prepared by removing water molecules, building in missing hydrogen atoms, and assigning atomic charges. Grid boxes were sized to incorporate the entire DNA fragment and 10 docking poses were tested and ranked in terms of stability using the scoring function in AutoDock Vina

Molecular Dynamics

MD simulations were performed using the GROMACS-2018.4 code.[8,9] The topology and parameters for the DNA model were defined by the CHARMM36m classical mechanics force field.[10] The topology and parameters for Cu(II)-TC-Py were obtained from density functional theory (DFT) calculations using the

Gaussian 16 package followed by CHARMM-compatible charge fitting with Antechamber7 using Restrained Electrostatic Potential (RESP) calculations.[11]

The Cu(II)-TC-Py–DNA complex was solvated in a large cuboid of TIP3P water molecules with counterions added to balance any formal charge. 0.15 M NaCl was added to model physiological salt concentration. Energy minimisation was followed by thermalisation and equilibration under constant volume (NVT) and then constant pressure (NPT) conditions. Production MD simulations were carried out for 4 μs for each complex, starting with PCU in the minor groove or PCU in the major groove, for a total of 8 μs of free dynamics.

Characterisation of interactions

We performed the characterisation of interactions in Cu_3-TC-Py -DNA complexes by using the protein–ligand interaction profiler (PLIP)[12], a web service for visualization and detection of non-covalent (hydrogen bonds, metal complexes, hydrophobic interactions, stacking and salt bridge) binary contacts in 3D complex structures. The analysis was carried out in two parts: first, for the full 4 μs trajectory of each system accounting for frames every 100 ns, and second, for the last 1 μs of converged trajectory accounting for frames every 1 ns.

National Cancer Institute 60 Cell Line Screening (NCI-60)

TC-1 (NSC: 843989), TC-Thio (NSC: 843990), and TC-Py (NSC: 843991) were submitted to the U.S. National Cancer Institute (NCI) Developmental Therapeutics Program (DTP) for 60 human cancer cell line screening. Growth inhibition (GI_{50}) data were identified using a five-dose exposure level and are shown in **Table S3**. These results are categorised by cancer type across leukaemia, non-small cell lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, renal cancer, prostate cancer, and breast cancer and visualised in a heat map using GraphPad Prism as shown in **Figure S23**.

ICP-MS Studies of Intracellular Cu

ICP-MS experiments were performed on MDA-MB-231 cells. An adequate number of cells were seeded in a 150 mm diameter Petri dish using DMEM medium with 10% Feotal bovine serum and penicillin streptomycin (100 unit/ml) and incubated in at 37 °C, 5% CO^2. When the cell confluency reached around 80–90%, a fresh medium containing 20 μM of Cu(II)-TC-Py was added and incubated for 48 hours at 37 °C, 5% CO_2. Cells were harvested by trypsinization. The medium was removed by centrifugation, and the cell pellet was washed twice with cold DPBS. The nucleus and DNA of treated cells were then isolated using the standard kit protocols mentioned below. The exact number of cells was always counted using the hemocytometer. All experiments were performed in duplicate.

Total cells: To determine the total copper uptake, 2×10^6 cells were collected and digested using 500 μL of 70% nitric acid at 65 °C for 12 h. 100 μl of the acid solution was further diluted in 3000 μL of MilliQ water. Finally, the copper concentration was measured using ICP-MS. Each experiment was performed in triplicate

Nuclei: The nuclei isolation kit, NE-PER nuclear and cytoplasmic extraction reagent (Thermo-scientific) was used to isolate the nuclei from the complex-treated cells, and the standard kit protocol was employed for the isolation process. 2×10^6 of the complex-treated cells were used to isolate the nuclei pellet using the protocol. We followed the first step of the supplier protocol and we stopped at the nuclear pellet step. Then, nuclear pellet is dissolved in 500 μL of 70% nitric acid at 65 °C for 12 h.

Bacterial Assays

Bacterial growth conditions and compounds preparation

Unless stated otherwise, *Bacillus subtilis* strains were aerobically grown in Mueller Hinton Broth (MHB). All tested compounds except for ciprofloxacin were dissolved and diluted in DMSO. Ciprofloxacin was dissolved and diluted in water. Unless stated otherwise, all assays were performed in biological triplicates. Minimal inhibitory concentrations (MICs) were determined in a broth microdilution assay according to Clinical and Laboratory Standards Institute (CLSI) guidelines. The MIC was defined as the lowest concentration inhibiting growth of a 5×10^5 colony forming units (CFU)/mL inoculum after a 16-h incubation period at 37 °C.

Bacterial cytological profiling (BCP)

Bacterial cytological profiling was performed as described by Wenzel *et al.*[13] In short, *B. subtilis* UG10 (*trpC2 amyE::Pxyl-recA-mgfp*) was grown in Muller Hinton Broth (MHB) supplemented with 0.1% (w/v) xylose at 30 °C. After reaching an OD_{600} of 0.3, cells were treated with 0.5× and 1× MIC of the respective compounds for 10 and 30 min. Ciprofloxacin and nitrofurantoin were used as positive controls. Immediately prior to imaging, cells were stained with 1 µg/mL of the DNA dye DAPI for 5 min. Samples (0.5 µL) were spotted on glass slides covered with a thin film of 1.2% agarose, covered with a poly-L-dopamine-coated coverslip,[14] and imaged using a Nikon Eclipse Ti2 inverted fluorescence microscope equipped with a CFI Plan Apochromat objective (DM Lambda 100X Oil N.A. 1.45, W.D. 0.13mm, Ph3), a Lumencor Sola SE II FISH 365 light source, a Photometrics PRIME BSI camera, an Okolab incubator, and Nis ELEMENTS AR 5.21.03 software.

BCP image analysis

Images were processed and analyzed with Fiji and the ImageJ plugin MicrobeJ.[15,16] The MicrobeJ parameters for bacterial detection in the phase contrast were set to smoothed segmentation with an area of 1 μm^2-max. The width and circularity were adjusted accordingly to ensure proper detection while other parameters remained at default settings. All chained cells were assessed and separated using the MicrobeJ manual editing interface.[16] For DNA compaction analysis, the maxima of foci detection were used as described previously.[17] The parameters within the maxima detection remained at default settings. The *Z*-score and tolerance were adjusted manually to ensure fitting DNA detection. The compaction was calculated based on the quotient of the cell area divided by the DNA area. To quantify RecA-GFP foci, the total number of cells showing the foci and the total number of analysed cells were counted manually. All unfocused and visibly lysed cells were excluded from the analysis. P values were calculated using either heteroscedastic or nested t-tests, as specified in the figure legends, using OriginPro (OriginLab Corporation version 2023) or GraphPad Prism.

Bacterial chromosomal DNA preparation

Overnight cultures of *B. subtilis* 168CA[18] were diluted in 20 mL MHB and grown at 30 °C until reaching an OD_{600} of 0.3. Cells were then split and treated with either 64 µg/mL nitrofurantoin, 0.75 mM AG1, or left untreated as control. After 30 min of treatment, chromosomal DNA was isolated from all samples following a standard phenol-chloroform protocol.[19] DNA was quantified using a Qubit 4 fluorometer following the protocol of the dsDNA Broad Range Assay kit (Invitrogen, Thermo Fisher, USA). Wide bore pipette tips were used throughout the experiment to minimize shear-induced fragmentation of the DNA.

Pulse field gel electrophoresis (PFGE)

Isolated DNA was separated in 20-50 kb resolution by PFGE using a 1% agarose gel run in a CHEF III DR System (Bio-Rad Laboratories, Hercules, CA, United States) with 0.5× TBE (45 mM Tris-HCl, pH 8.0; 45 mM boric acid; 1 mM EDTA) as running buffer. Electrophoresis was performed for 24 h at 14 °C. The forward and reverse voltages were 9 and 6 V/cm, respectively, with an initial switch time of 0.86 s

and final switch time of 0.92 s, with a 180° angle. The gel was stained with 1× concentrated SYBR Safe DNA gel stain (Invitrogen) for at least 30 min before imaging in a UV transilluminator with default auto optimal exposure settings for 590/110 SYBR safe gels (BioRad ChemiDoc MP imaging system, ImageLab Touch software v 2.3.0.07).

Bacterial DNA fluorescence microscopy

Silanization of coverslips

Stretching of DNA was performed on silanized glass coverslips. The silanization was done as follows: 18 × 18 mm coverslips were placed in a coverslip rack and carefully submerged in a mixture of 1% (3-aminopropyl)triethoxysilane (Sigma Aldrich) and 1% allyltrimethoxysilane (Sigma Aldrich) in acetone solution, and silanized overnight. The activated coverslips were rinsed with acetone:water solution (2:1 v/v) and dried under a nitrogen gas flow immediately before DNA stretching.

DNA staining, stretching and imaging

100 ng DNA were stained with 320 nM YOYO-1 (Invitrogen) in 0.5× TBE and supplemented with 2% β-mercaptoethanol (BME, Sigma Aldrich) in a final volume of 50 µL. 3.2 µL of stained DNA sample were placed at the interface of the silanized coverslip and a clean microscopy slide (VWR), causing the capillary force between the silanized coverslip and the microscope slide to stretch the DNA. Extended DNA molecules were visualized using a Zeiss Observer.Z1 fluorescence microscope equipped with an Andor iXON Ultra EMCCD camera and a Colibri 7 LED illumination system. For imaging YOYO-1, band-pass excitation filters (475/40 nm) and band-pass emission filters (530/50 nm) were utilized.

Data analysis

A custom-made MATLAB software was used to analyse DNA fragments. Stretched DNA molecules were detected and the length of each molecule was measured in microns. The software was set to exclude overlapping DNA strands from the analysis. One-way ANOVA statistical significance was determined using Tukey's model for multiple comparisons with a confidence level of 95%. P-values are represented using the GraphPad PRISM style; ****P < 0.0001. The distribution graph was created in OriginPro (OriginLab Corporation version 2023).

DNA Cleavage by Agarose Gel Electrophoresis

Cleavage reactions were prepared to a final volume of 20 µL in 100 µL Eppendorf tubes and contained 400ng supercoiled pUC19 DNA, 1 mM Na-L-ascorbate (where indicated) and 25 mM NaCl in 80 mM HEPES buffer (pH = 7.4). Cleavage reactions with ROS scavengers were prepared to contain 10 mM of the ROS scavenger by addition of 1 µL of a 200 mM stock solution prior to DNA addition. Reactions probing the cleavage site were prepared by preparing reaction mixtures to contain 16 µM and 8 µM methyl green and netropsin respectively from stock solutions prepared in 80 mM HEPES buffer (pH = 7.4). Upon addition of pUC19 the samples were incubated for 30 minutes at 37 °C, quenched with 6X loading dye (Thermo Fisher R0611) and loaded on to a 1.3 % agarose gel, prepared using 1 X TAE buffer and run at 70v for 90 minutes.

Repair Assisted Damage Detection

The protocol was adapted from Singh et al.[20,21] The use of blood samples was approved by the Regional Ethical Review Board in Gothenburg (Dnr: 246-07 and Dnr: 308-08).

Blood Sample Collection

Samples were collected from individuals with normal blood count from the Hematology Lab at the Clinical Chemistry Department at Sahlgrenska University Hospital, Gothenburg, Sweden Lymphoprep (Axis-Shield PoC AS, Oslo, Norway) was used to harvest peripheral blood mononuclear cells (PBMCs) via density gradient-based separation. The blood-based study was performed with mixed blood samples collected from different individuals and pooled together.

Treatment of PBMCs with Cu(II)-TC-Py and antioxidants

5×10^5 PBMCs were treated with 200 μM Cu(II)-TC-Py and incubated for 2 h on a thermal block at 37°C. For antioxidant-treated samples, PBMCs were pretreated with 1 mM of tiron, L-histidine, L-Methionine, D-mannitol, and sodium pyruvate for 2 h prior to Cu(II)-TC-Py treatment.

Extraction of DNA

DNA was extracted using GenElute-Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich) following manufacturer's instructions, after drug treatment and free radical scavenger incubation. DNA concentrations were quantified using a NanoDrop 1000 spectrophotometer. Care was taken to avoid excess shearing of DNA by using wide-bore tips.

Fluorescent labeling of Cu_3-TC-Py induced DNA damage

DNA (100 ng) was incubated with Endo III (2.5 U), Endo IV (2.5 U), Endo VIII (2.5 U), APE1(2.5 U), Fpg (2.5 U) and AAG (2.5 U) in 1× CutSmart Buffer for 1 h at 37°C for *in vitro* DNA repair initiation. dNTPs (1 μM of dATP, dGTP, dCTP, 025 μM dTTP and 0.25 μM aminoallyl-dUTP-ATTO-647N) were incorporated at the damage sites in 1× NEBuffer 2 using DNA polymerase I (1.25 U) at 20°C. Subsequently, the reaction was terminated with 2.5 μl of 0.25 M EDTA.

Salinization of coverslips

18 × 18 mm glass coverslips (Thermo Fischer Scientific) were placed in a coverslip rack and carefully put into an acetone solution containing 1% (3-aminopropyl) triethoxysilane (APTES) and 1% allyltrimethoxysilane (ATMS) (v/v) and coated overnight. [22] Coated coverslips were rinsed with acetone:water solution (2:1 v/v) to remove residues for reproducible stretching of DNA. The slides were always used on the day they were produced.

Stretching of DNA and imaging

The Aminoallyl-dUTP-ATTO-647N incorporated fluorescent DNA samples were diluted with 0.5× TBE and stained with 320 nM YOYO-1 (Invitrogen) in a total volume of 50 μl. β-mercaptoethanol (2% v/v, Sigma-Aldrich) was added prior to image acquisition to minimize photodamage. The DNA samples were stretched by placing 3.2 μl at the interface of the silane-activated coverslip and a microscopy slide (VWR Frosted). Zeiss Observer.Z1, equipped with an Andor iXON Ultra EMCCD camera and a Colibri 7 LED illumination system was used to obtain the fluorescence images of the stretched DNA molecules. Each image consisted of two colours, YOYO-1 (green channel), and aminoallyl-dUTP-ATTO-647N (red channel) having appropriate band-pass excitation filters (475/40 and 640/30 nm) and bandpass emission filters (530/50 and 690/50 nm).

Software analysis

A custom-made MATLAB script was used to analyse the data which estimates the length of the stretched DNA molecule in micron (μm) and counts the total number of Aminoallyl-dUTP-ATTO-647N dots along the DNA. The results were expressed as Dots.MBp^{-1} by stretching lambda DNA (48502 bp, New England Biolabs) in a similar buffered conditions to determine 1 μm stretched DNA to be ~3000 bp. DNA molecules that overlapped as wells as dots at the end of the DNA molecule which could have resulted from DNA strand breaks during the DNA extraction process were excluded from the analysis.

Statistics

Experiments were conducted in technical duplicates and analyzed using GraphPad Prism. Statistical significance was evaluated with one-way ANOVA, and p-values are presented in the GraphPad Prism format: ns (not significant) ****P <0.0001

Supplementary Figures and data:

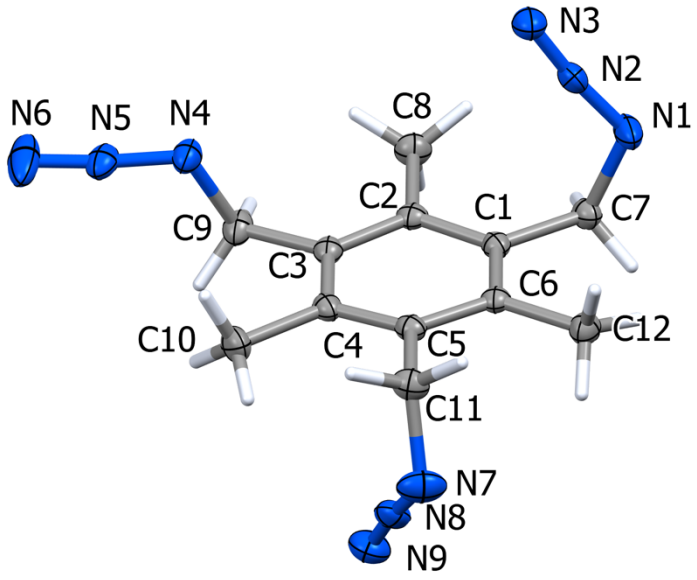

Figure S1: Crystal structure of Triazide showing 50% probability atomic displacement ellipsoids

Table S1: Crystal data and structure refinement Triazide

CCDC code	2409062
Empirical formula	$C_{12}H_{15}N_9$
Formula weight	285.33
Temperature/K	100.00(10)
Crystal system	monoclinic
Space group	$P2_1/c$
a/Å	12.80290(10)
b/Å	10.80650(10)
c/Å	10.06400(10)
α/°	90
β/°	90.7580(10)
γ/°	90
Volume/Å^3	1392.28(2)
Z	4
ρ_{calc} g/cm^3	1.361
μ/mm^{-1}	0.760
F(000)	600.0
Crystal size/mm^3	0.17 × 0.07 × 0.06
Radiation	Cu Kα (λ = 1.54184)

2θ range for data collection/°	6.904 to 148.86
Reflections collected	39527
Independent reflections	2838 [R_{int} = 0.0236, R_{sigma} = 0.0087]
Data/restraints/parameters	2838/0/193
Goodness-of-fit on F^2	1.034
Final R indexes [I>=2σ (I)]	R_1 = 0.0352, wR_2 = 0.0940
Final R indexes [all data]	R_1 = 0.0376, wR_2 = 0.0963
Largest diff. peak/hole / e $Å^{-3}$	0.25/-0.24

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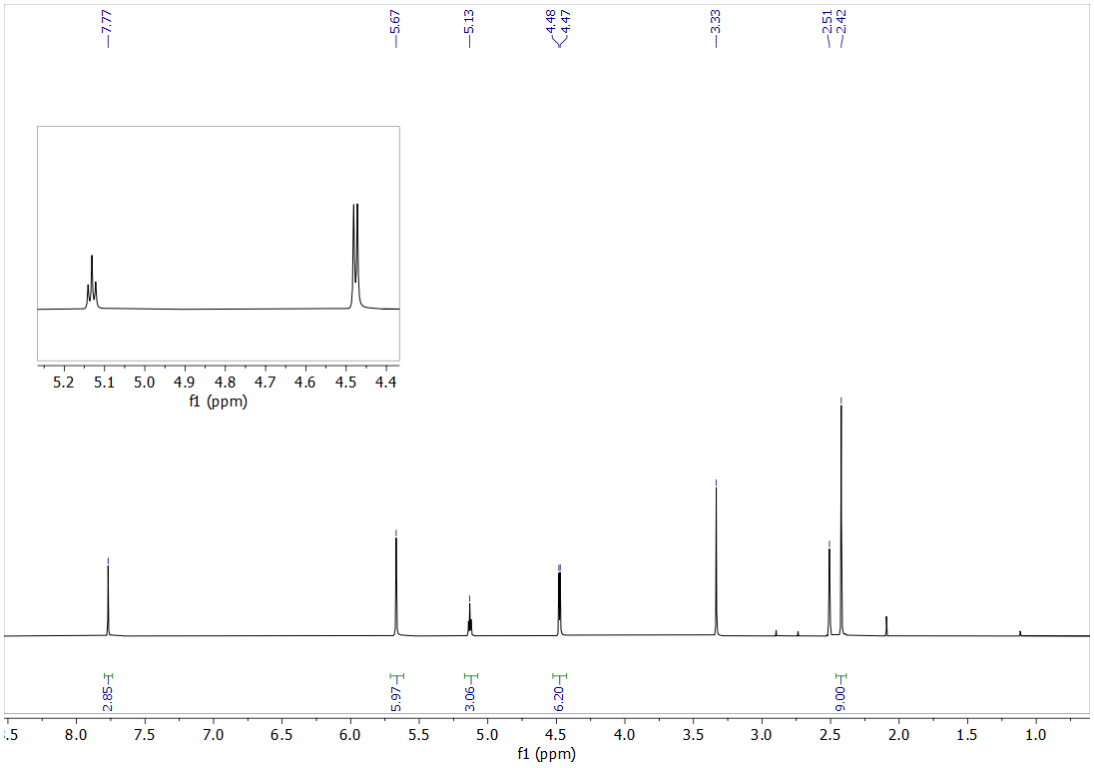

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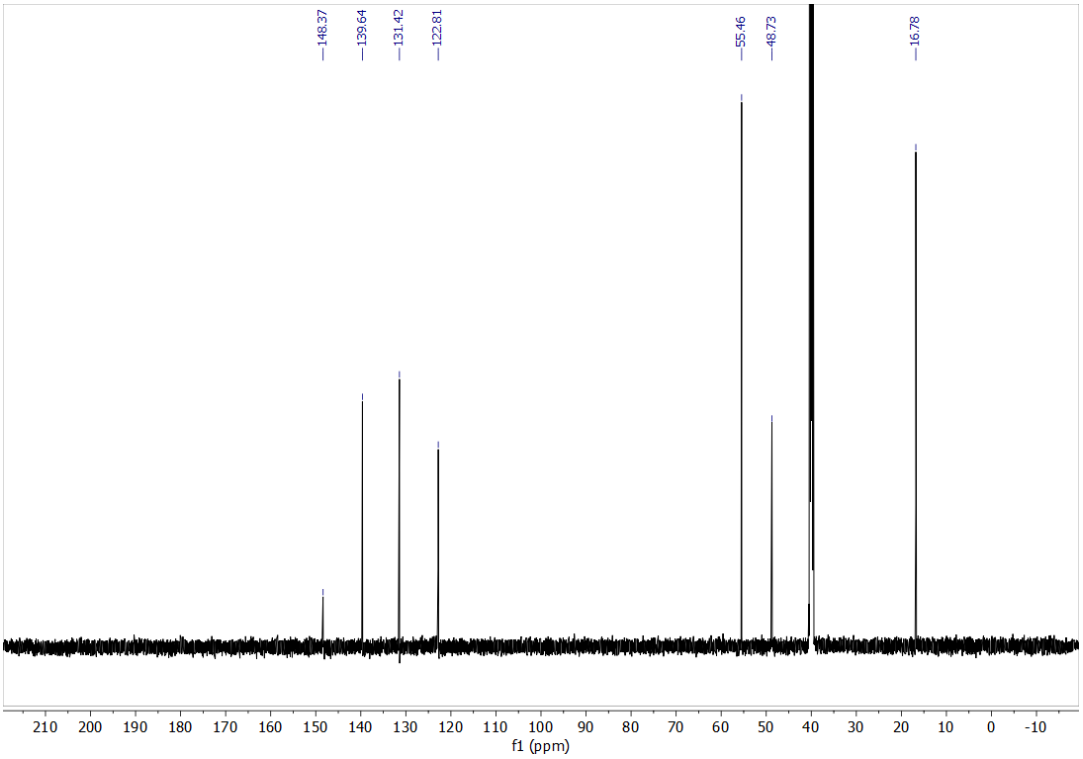
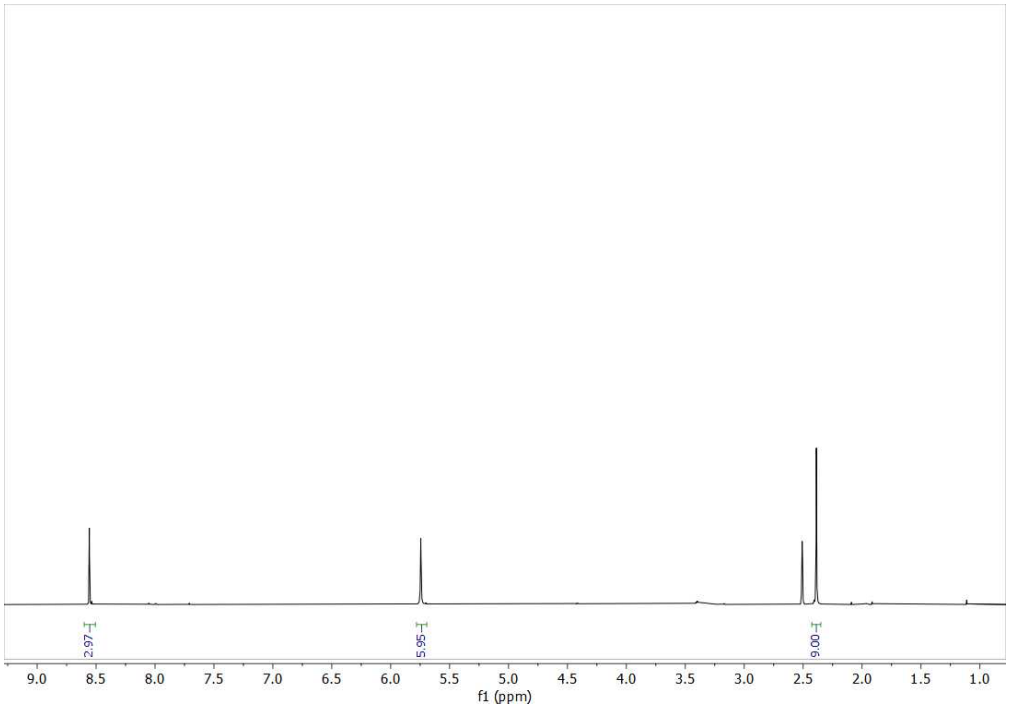
430 **Figure S2:** ^1H NMR Spectra of TC-OH in DMSO-d_6 600 MHz



431

432 **Figure S3:** 13C NMR of TC-OH



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434 **Figure S4:** ^{1}HNMR Spectra of TC-Acid in DMSO-d_6 600MHZ

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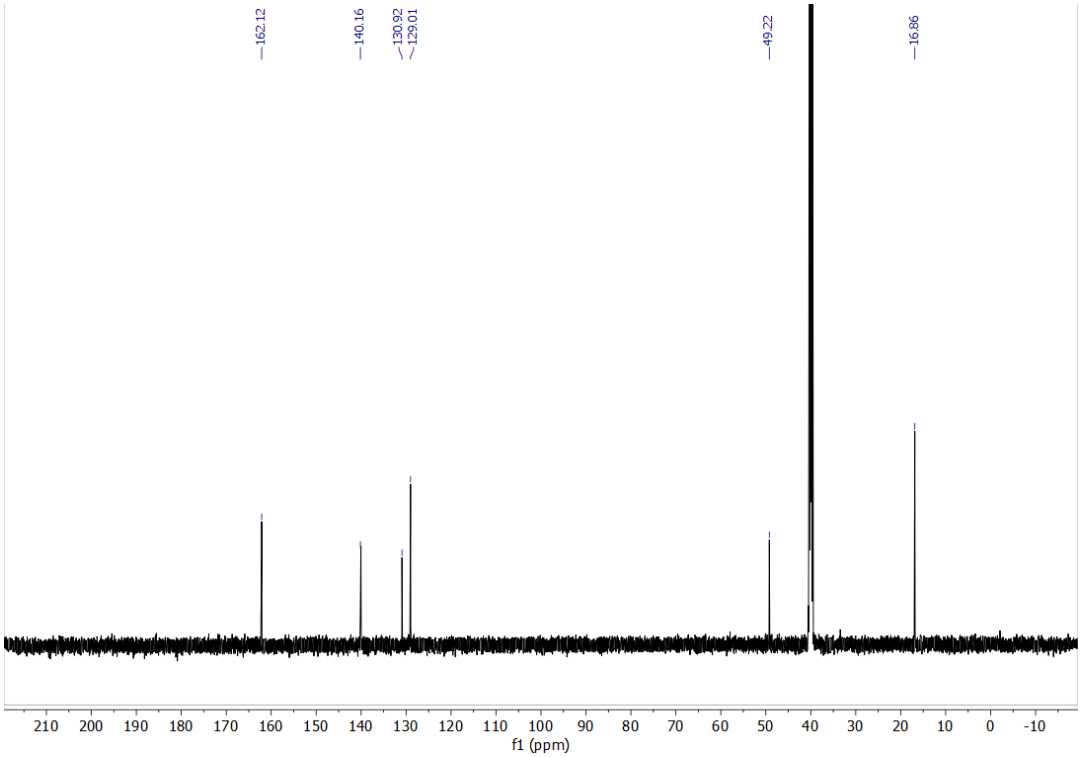

436

437 **Figure S5:** ^{13}C NMR of TC-ACID in DMSO-d6

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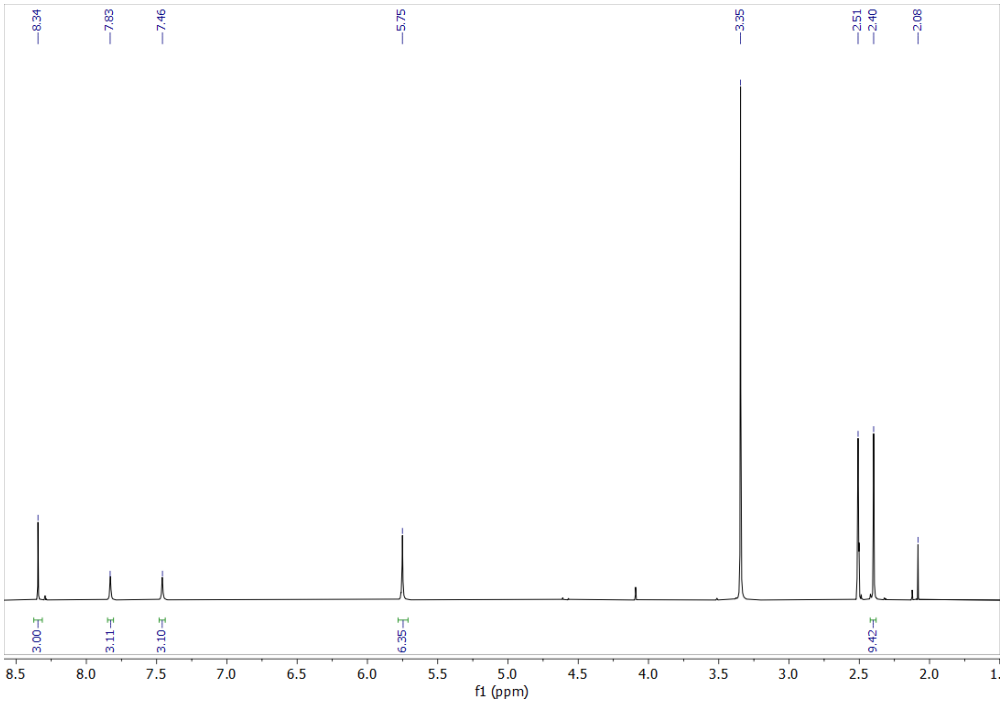

440

441 **Figure S6:** 1H NMR of TC-Amide in DMSO-d6

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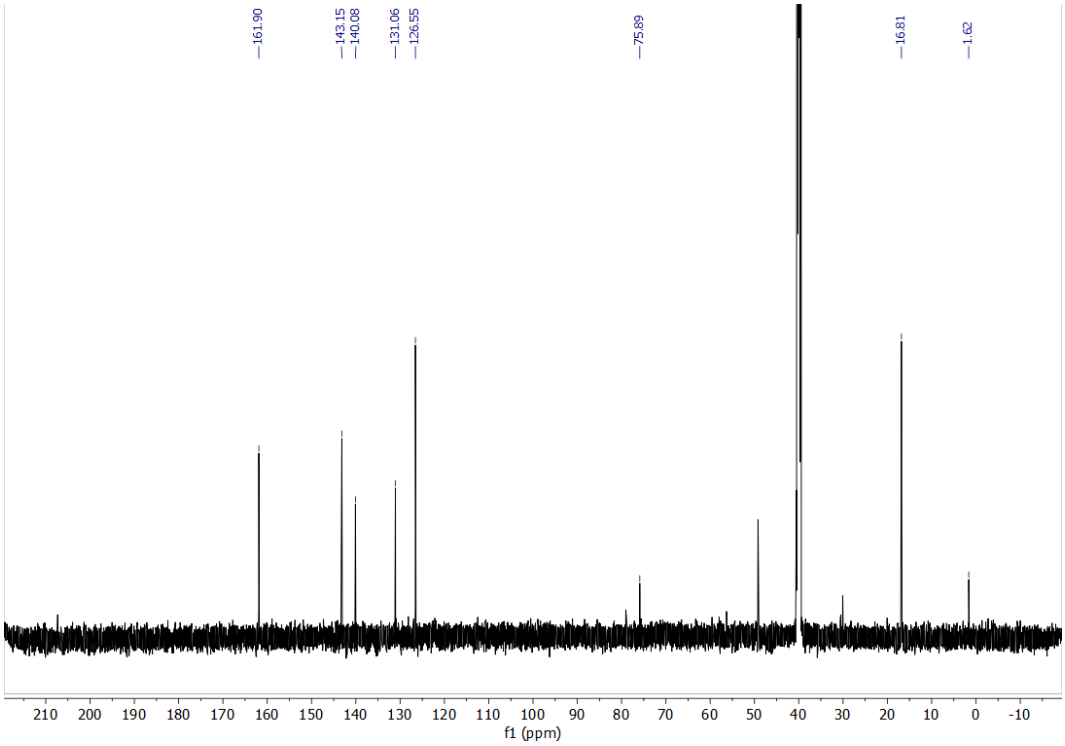

443

444 **Figure S7:** 13C NMR of TC-Amide in DMSO-d6

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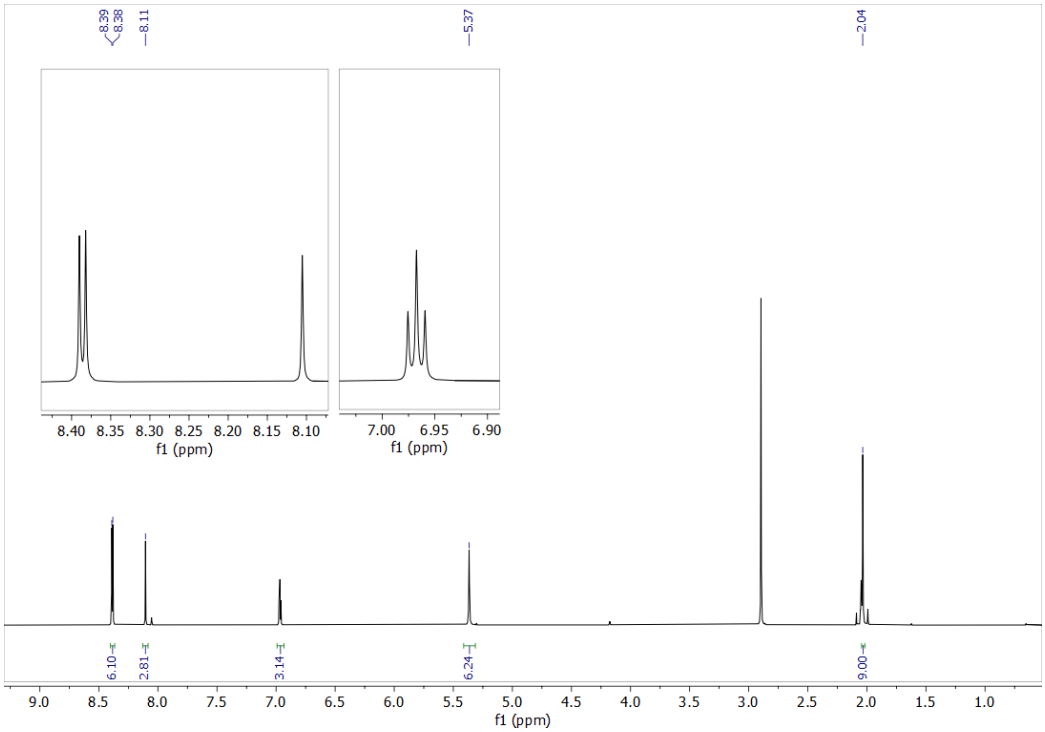

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447 **Figure S8:** 1H NMR Spectra of TC-Pyrm in DMSO-d6 600MHz

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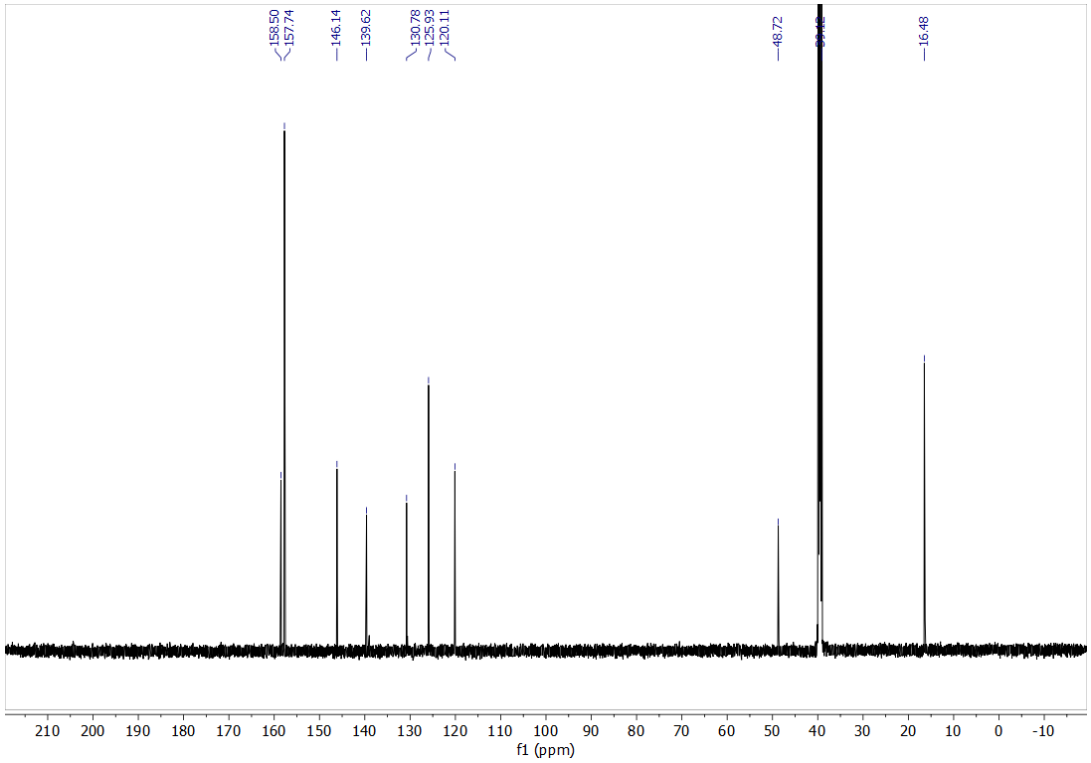

Figure S9: 13C NMR of TC-Amide in DMSO-d6

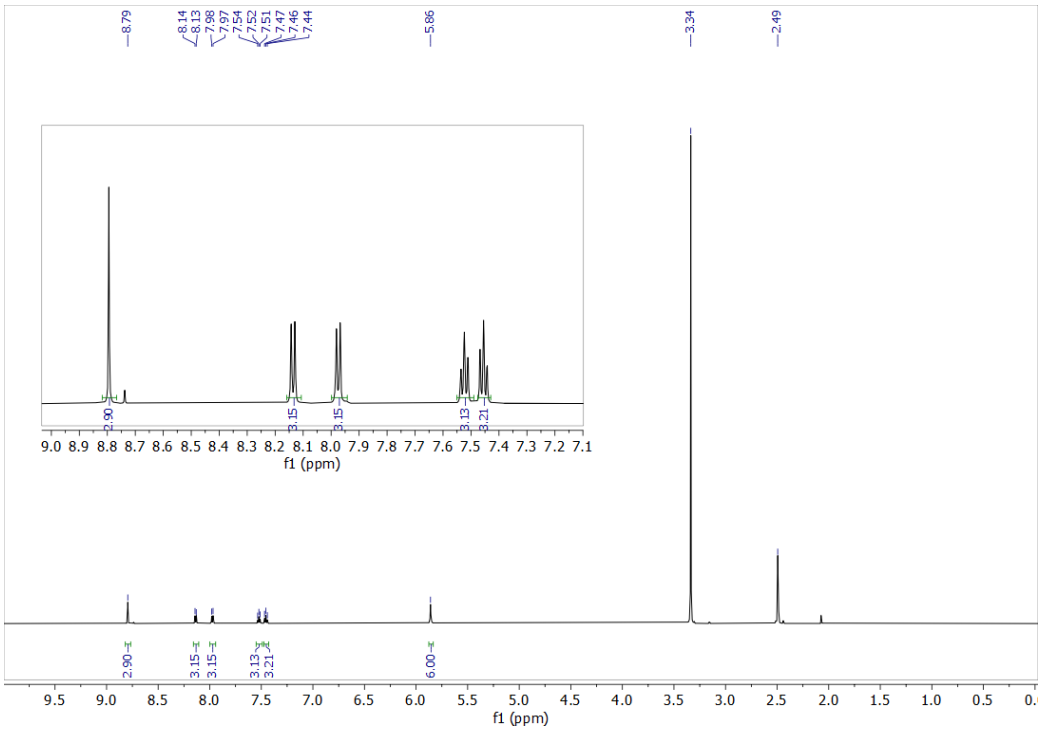

Figure S10: 1H NMR of TC-Benzo in DMSO-d6 600 MHz

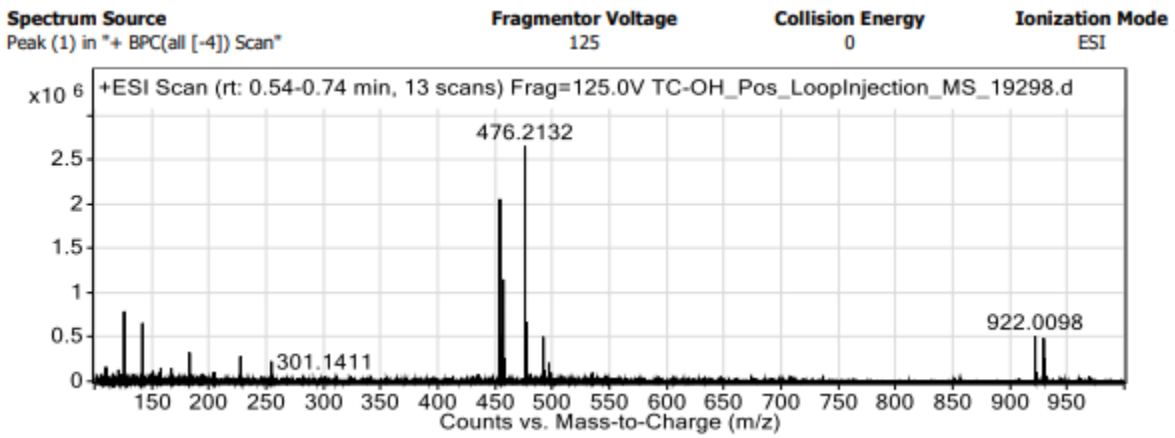

Figure S11: ESI-MS spectrum of TC-OH in positive ionization mode

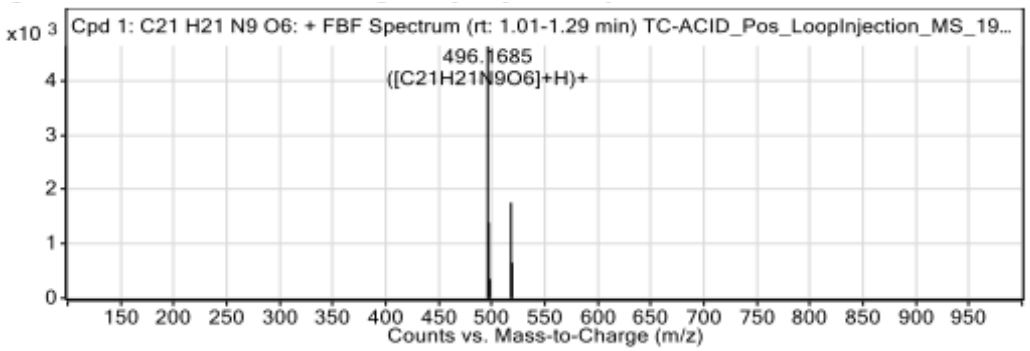

Figure S12: ESI-MS spectrum of TC-Acid in positive ionization mode

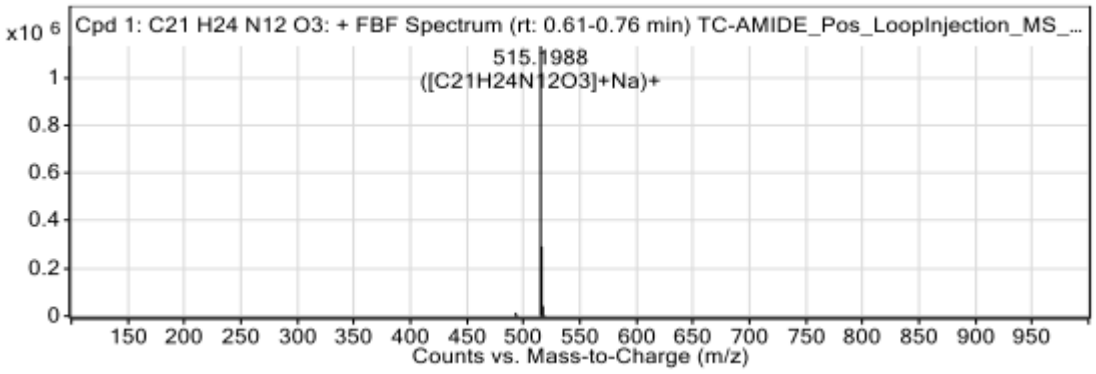

Figure S13: ESI-MS spectrum of TC-Amide in positive ionization mode.

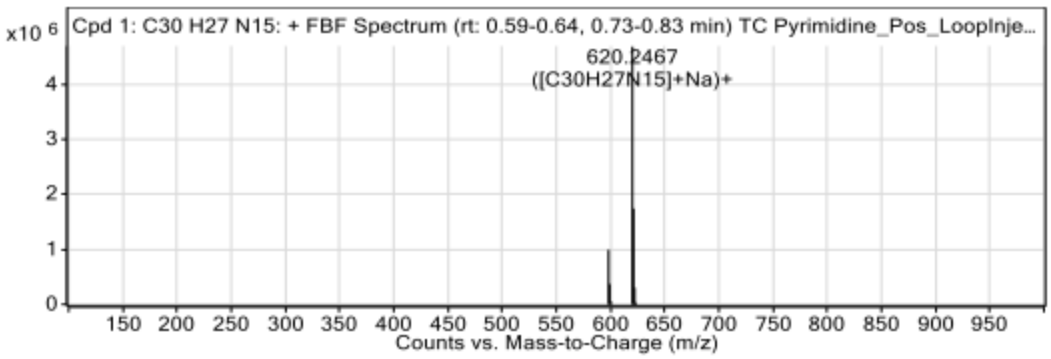

Figure S14: ESI-MS spectrum of TC-Pyrm in positive ionization mode

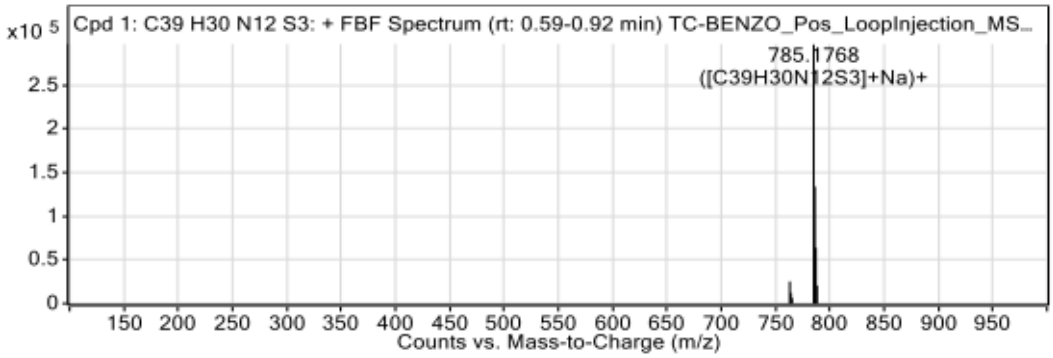

Figure S15: ESI-MS spectrum of TC-Benzo in positive ionization mode

Table S2: TC-Cu species identified by ESI-MS. Listed are molecular and elemental formulae with corresponding expected and found monoisotopic m/z values.

Complex / adduct (Formula)	Expected m/z	Found m/z
TC-Py + 3Cu		
$[Cu_3(TC\text{-}Py)(NO_3)_5]+$ ($C_{33}H_{30}Cu_3N_{17}O_{15}$)	1092.99	1092.98
$[Cu_3(TC\text{-}Py)(NO_3)_4]2+$ ($C_{33}H_{30}Cu_3N_{16}O_{12}$)	515.50	515.50
$[Cu_3(TC\text{-}Py)(NO_3)_6+H]+$ ($C_{33}H_{31}Cu_3N_{19}O_{21}$)	1217.98	1217.91
$[Cu_3(TC\text{-}Py)(NO_3)6+HNO_3]+$ ($C_{33}H_{31}Cu_3N_{20}O_{24}$)	1279.91	1279.88
TC-Pyrm + 3Cu		
$[Cu_3(TC\text{-}Pyrm)(NO_3)_5]+$ ($C_{30}H_{27}Cu_3N_{20}O_{15}$)	1095.98	1095.97
$[Cu_3(TC\text{-}Pyrm)(NO_3)_4]2+$ ($C_{30}H_{27}Cu_3N_{19}O_{12}$)	517.00	516.99
$[Cu_3(TC\text{-}Pyrm)(NO_3)_6Na]+$ ($C_{30}H_{27}Cu_3N_{21}O_{18}Na$)	1180.96	1180.94
$[Cu_2(TC\text{-}Pyrm)(NO_3)_3]+$ ($C_{30}H_{27}Cu_2N_{18}O_9$)	909.08	909.07

TC-Benzo + 3Cu		
$[Cu_3(TC\text{-}Benzo)(NO_3)_5]+$ $(C_{39}H_{30}Cu_3N_{17}O_{15}S_3)$	1260.91	1260.89
TC-OH + 3Cu		
$[Cu_3(TC\text{-}OH)(NO_3)_5\text{-}2H+2DMF]+$ $(C_{24}H_{32}Cu_3N_{15}O_{19})$	1022.98	1022.91
$[Cu_3(TC\text{-}OH)(NO_3)_2\text{-}3H]+$ $(C_{21}H_{24}Cu_3N_{11}O_9)$	762.96	762.96
TC-Acid + 3Cu		
$[Cu_3(TC\text{-}ACID)(NO_3)_5$ - 2H + 2DMF]+ $(C_{27}H_{33}Cu_3N_{16}O_{23})$	1137.97	1137.90
$[Cu_3(TC\text{-}ACID)(NO_3)_5$ -2H + 1DMF]+ $(C_{24}H_{26}Cu_3N_{15}O_{22})$	1064.93	1064.85
$[Cu_3(TC\text{-}ACID)(NO_3)_2$ -3H + 1DMF]+ $(C_{24}H_{25}Cu_3N_{12}O_{13})$	877.95	877.95

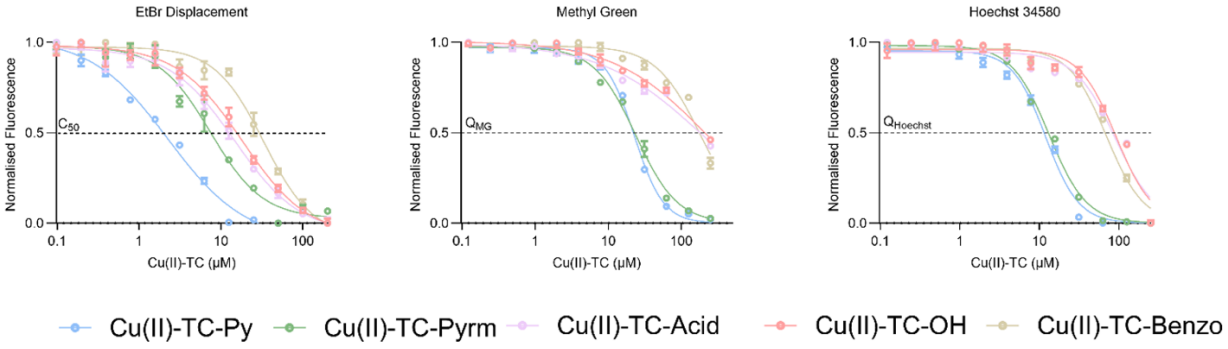

Figure S16: Fluorescence melting curves. Fluorophores indicated above each plot were kept constant while Cu-TC complexes were titrated. Plots were fit with nonlinear regression in Graphpad Prism to find C_{50}.

*FDDH: 5'-**F**-CGCGAATTCGCGAAAAACGCGAATTCGCG-3'*

*FRET-DDH: 5'-**F**-CGCGAATTCGCGAAAAACGCGAATTCGCG-**Q**-3'*

Figure S17: Sequences of hairpins used for MST and fluorescence melting experiments. Loop region is highlighted grey. F = AlexaFluor 647, Q = Iowa Black.

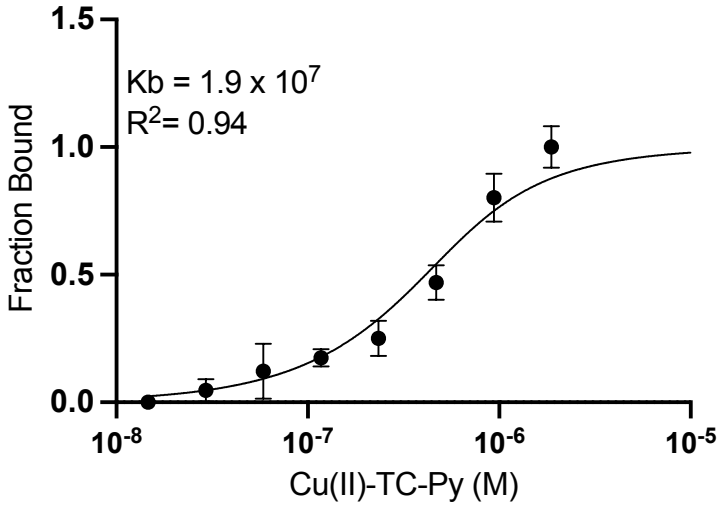

Figure S18: Fitting of the Bard equation t the binding region of MST data with *n* constrained to 2.

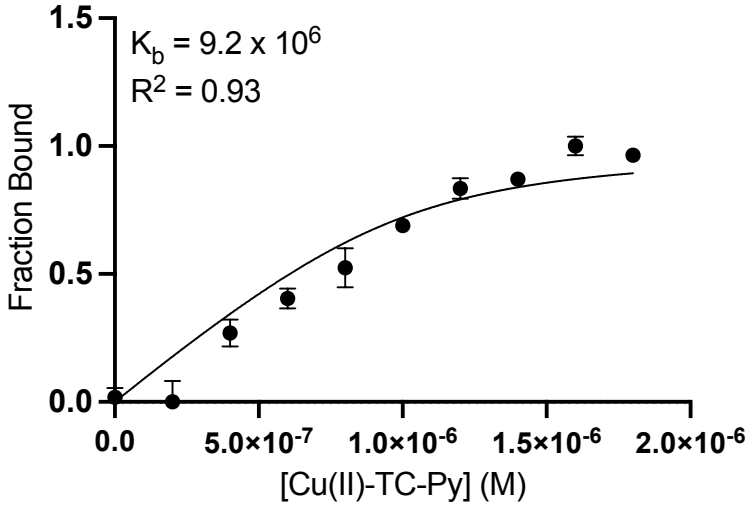

Figure S19: Fitting of the Bard equation to the binding region of thermal melting data with n constrained to 2.

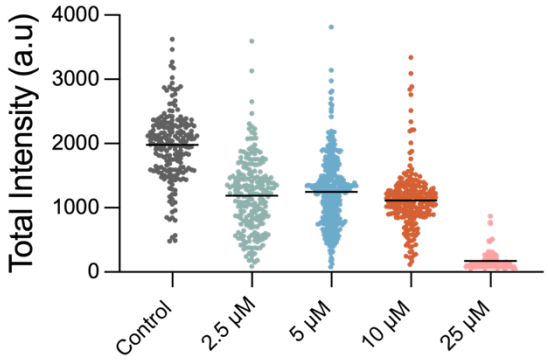

Figure S20: Plot of total fluorescence intensity observed along DNA molecules treated with 5µM YOYO and varying concentration of Cu_3-TC-Py

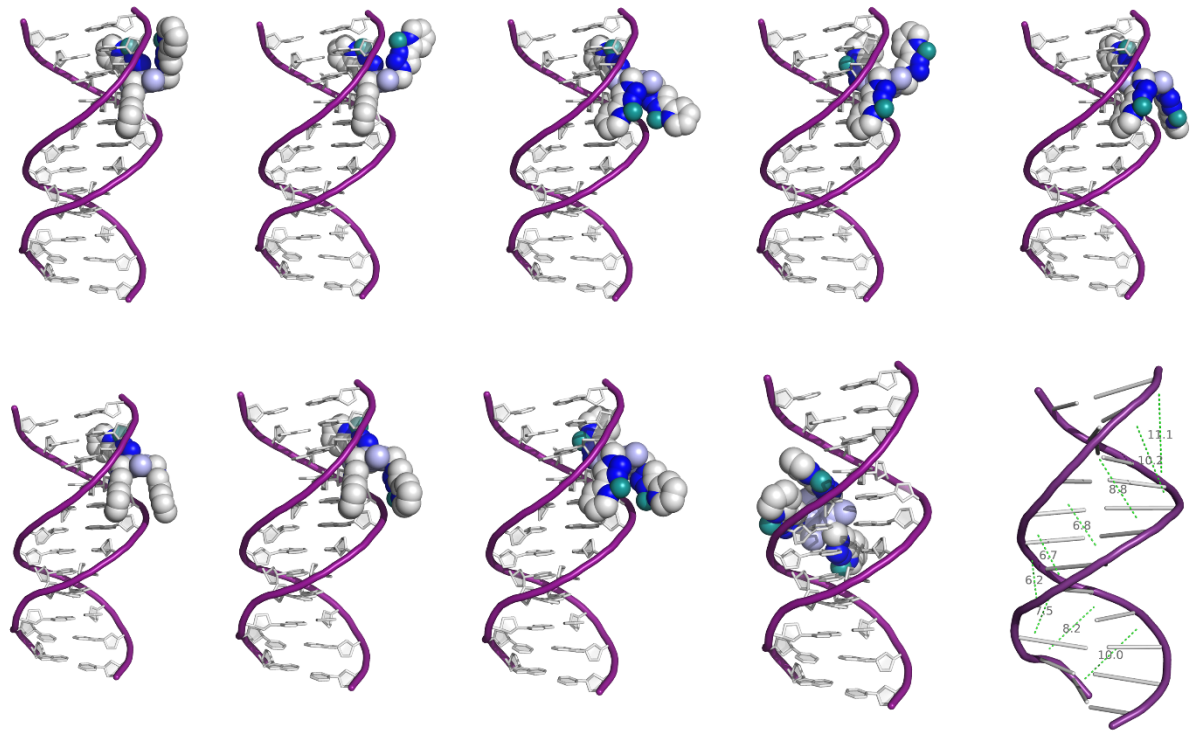

Figure S21: Nine output poses from docking studies of DNA (PDB: 1BNA) with modelled Cu(II)-TC-Pyr. Measurements of minor groove widths along the 1BNA structure, measured paths shown as dashed green lines.

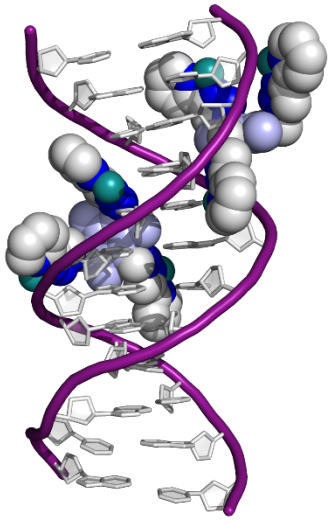

Figure S22: Top ranked output from double docking experiment. Generated by using the top ranked pose from the original docking study, treated as a rigid structure, with an additional Cu(II)-TC-Py molecule.

Table S3. NCI-60 50% growth inhibition (GI_{50}) results of TC-Py and TC-Thio identified using the five-dose response experimental results.

	TC-Py	***TC-Thio***
Cell line	**GI_{50} (μM)**	**GI_{50} (μM)**
CCRF-CEM	> 100	> 100
HL-60(TB)	> 100	> 100
K-562	> 100	> 100
MOLT-4	> 100	> 100
RPMI-8226	50.12	50.12
SR	93.33	93.33
A549/ATCC	30.20	79.43
EKVX	12.88	67.61
HOP-62	2.34	18.20
HOP-92	1.20	11.48
NCI-H226	3.09	22.91
NCI-H23	4.47	16.22
NCI-H322M	42.66	52.48
NCI-H460	24.55	50.12
NCI-H522	10.47	31.62
COLO 205	> 100	> 100
HCC-2998	28.18	> 100
HCT-116	7.41	20.89
HCT-15	77.62	> 100
HT29	> 100	> 100
KM12	> 100	> 100
SW-620	36.31	> 100
SF-268	6.61	22.91
SF-295	4.90	17.78

SF-539	2.95	15.85
SNB-19	3.72	19.05
SNB-75	2.24	11.48
U251	2.24	16.60
LOX IMVI	14.79	70.79
MALME-3M	19.05	10.96
M14	27.54	> 100
MDA-MB-435	58.88	> 100
SK-MEL-2	14.45	14.79
SK-MEL-28	56.23	> 100
SK-MEL-5	18.20	> 100
UACC-257	25.12	53.70
UACC-62	17.38	38.90
IGROV1	13.49	18.62
OVCAR-3	10.72	33.88
OVCAR-4	0.95	60.26
OVCAR-5	69.18	> 100
OVCAR-8	2.69	23.99
NCI/ADR-RES	12.02	36.31
SK-OV-3	3.16	14.79
786-0	2.88	19.05
A498	25.12	21.38
ACHN	21.38	25.70
CAKI-1	3.98	28.18
RXF 393	7.76	17.78
SN12C	12.02	33.11
TK-10	18.62	16.22
UO-31	13.80	> 100

PC-3	14.45	58.88
DU-145	27.54	52.48
MCF7	9.12	72.44
MDA-MB-231/ATCC	7.24	17.78
HS 578T	3.47	25.12
BT-549	12.59	15.49
T-47D	6.03	22.39
MDA-MB-468	10.72	20.42

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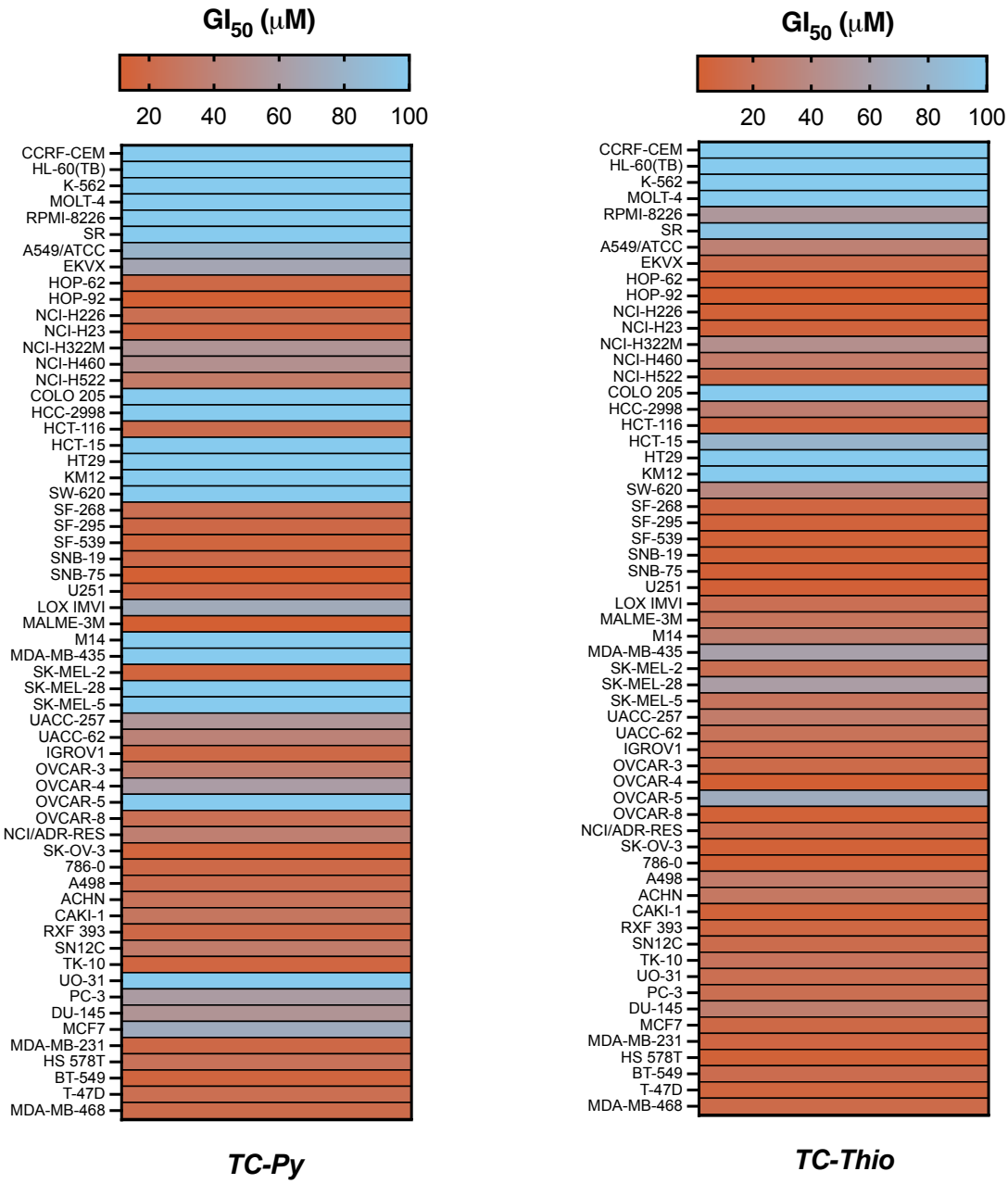

515 **Figure S23**. NCI-60 data for TC-Py and TC-Thio. Data presented as a heatmap showing 50% growth
516 inhibition (GI_{50}) results arising from exposure of selected human cancer cell lines to the complex upon
517 five-dose exposure.

518

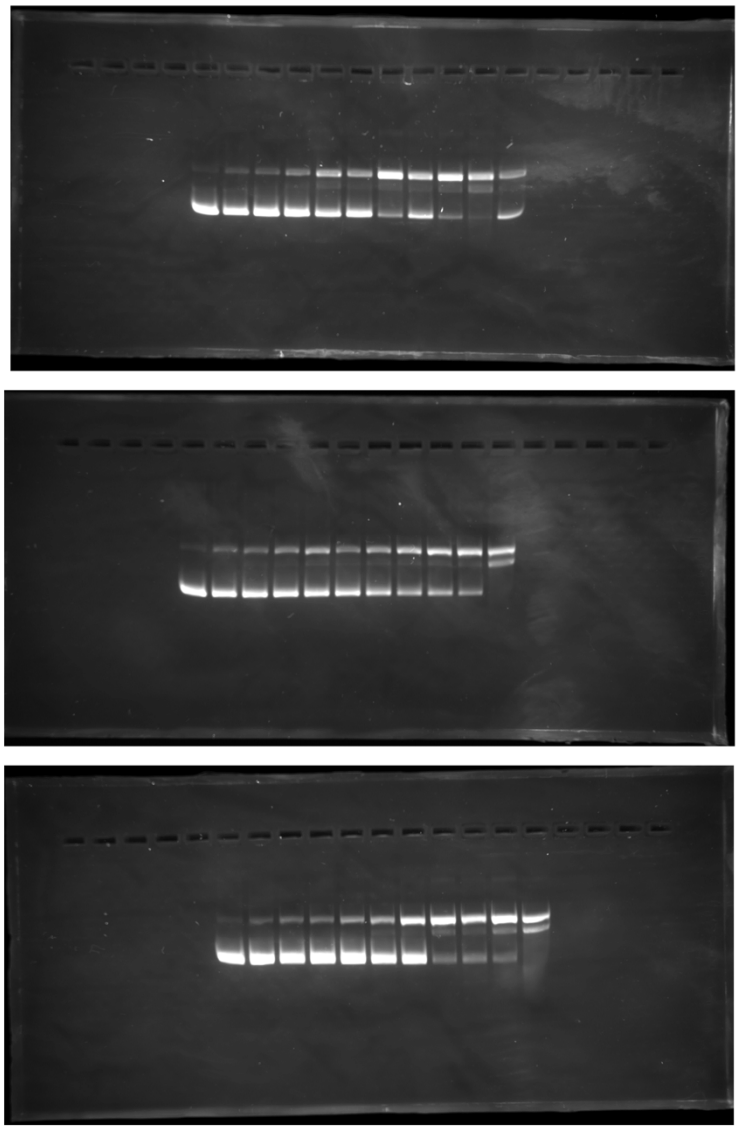

Figure S24: Complete gels used for densitometry. All lanes contained 1mM Na-L-ascorbate, 400ng pUC19 DNA and 25mM NaCl in 80mM HEPES pH 7.2. Lanes 1-11 contained 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 μM Cu(II)-TC-Py respectively.

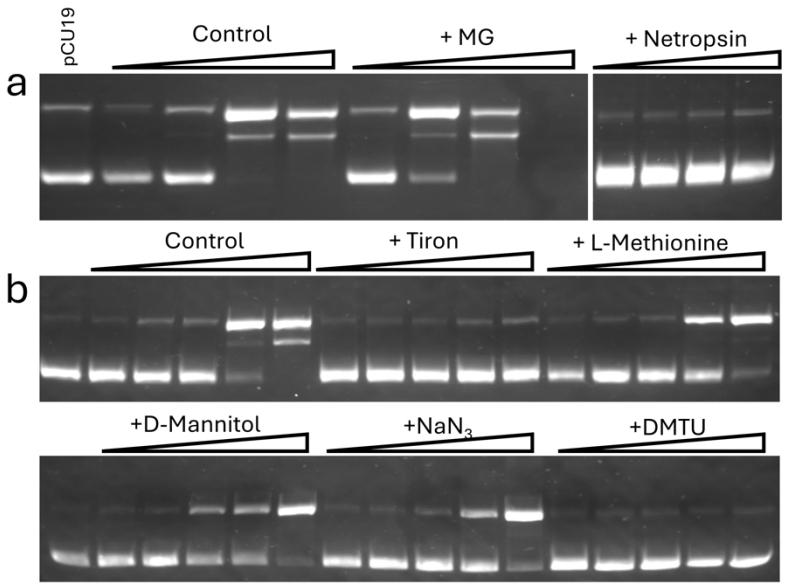

Figure S25: (a) Cleavage experiments in the presence of groove blocking agents, methyl green (16 μM) and netropsin (8 μM). Wedges above gels indicate increasing Cu_3-TC-Py concentrations of 4, 6, 8 and 10 μM. Lane 1 contained pUC19 DNA only. Control ramp contained pUC19 DNA and Cu_3-TC-Py only with no groove blocking agent. (b) DNA cleavage experiment in the presence of antioxidants (10mM). Each wedge indicates increasing Cu(II)-TC-Py concentration at 2, 4, 6, 8, 10 μM. Lane 1 contained pUC19 DNA only. The control gradient contained Cu_3-TC-Py and pUC19 with no antioxidant. All experiments contained 1mM Na-L-ascorbate.

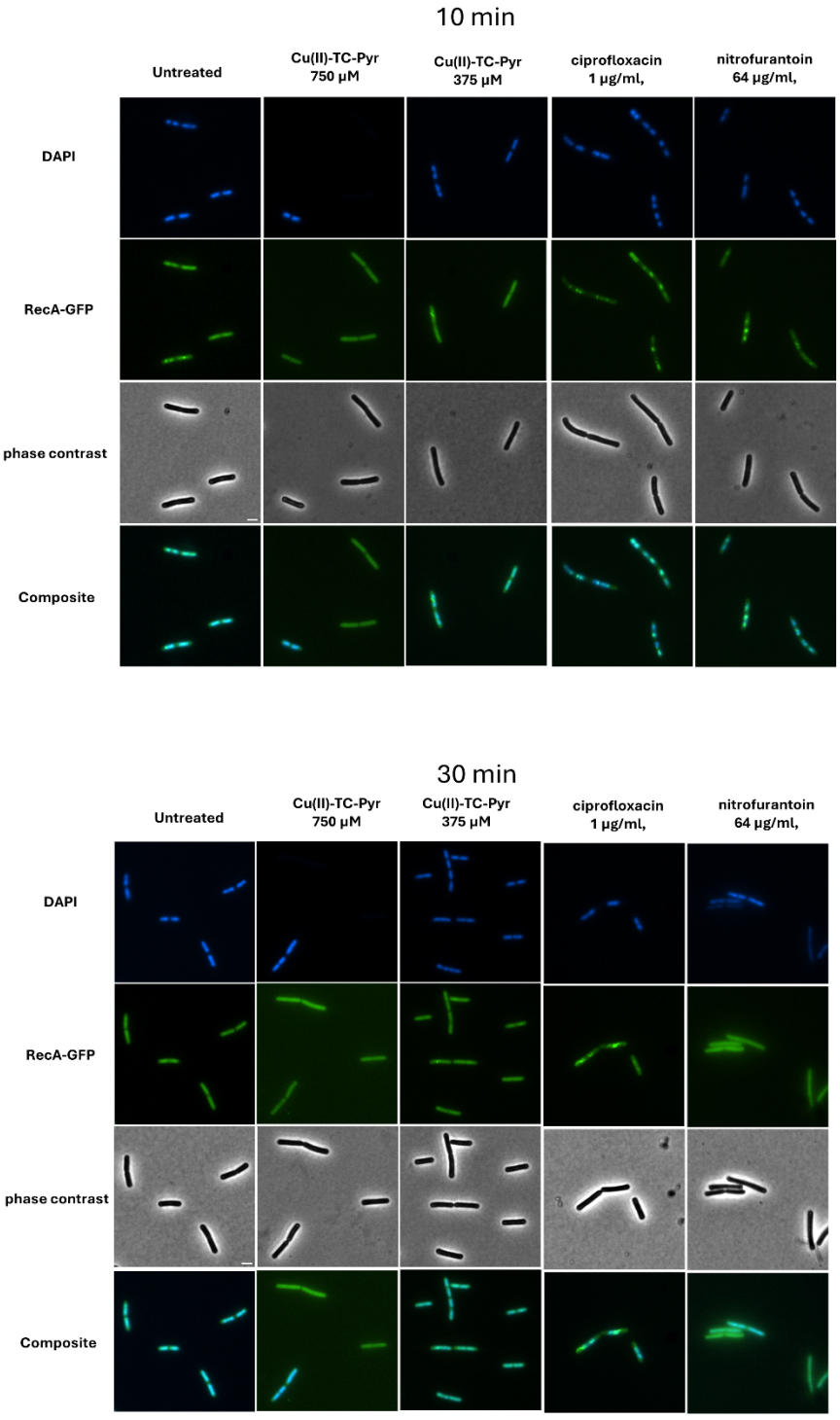

Figure S26: Microscopy images collected form bacterial cytological profiling experiments of cells treated with Cu_3-TC-Py ciprofloxacin or nitrofurantoin for 10 or 30 minutes. Cells contained a GFP-tagged Rec A and were stained with DAPI prior to imaging.

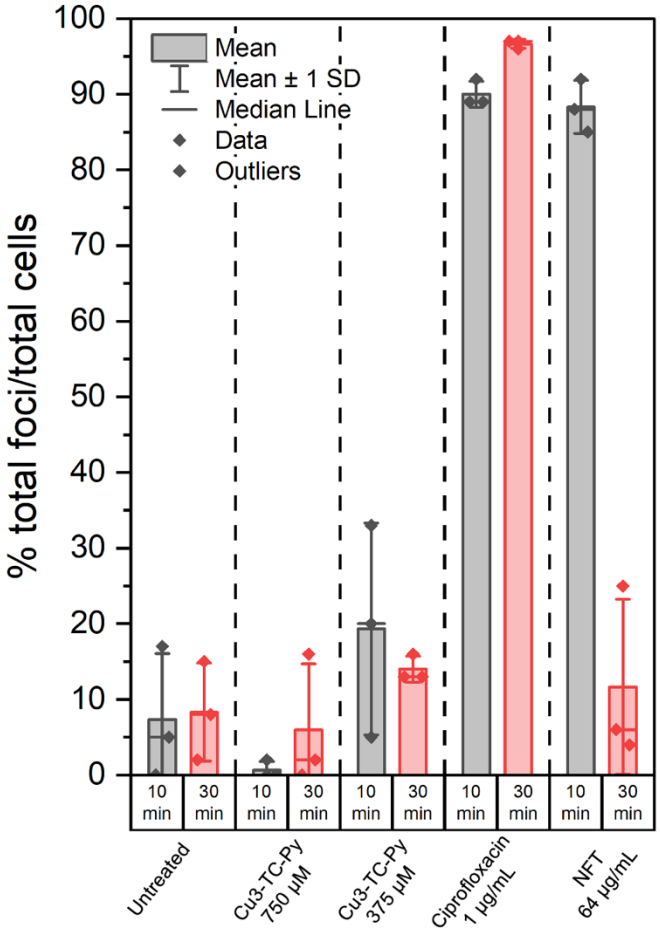

Figure S27: Quantification of RecA Foci in cells treated with varying Cu_3-TC-Py concentrations and fixed concentrations of ciprofloxacin and NFT.

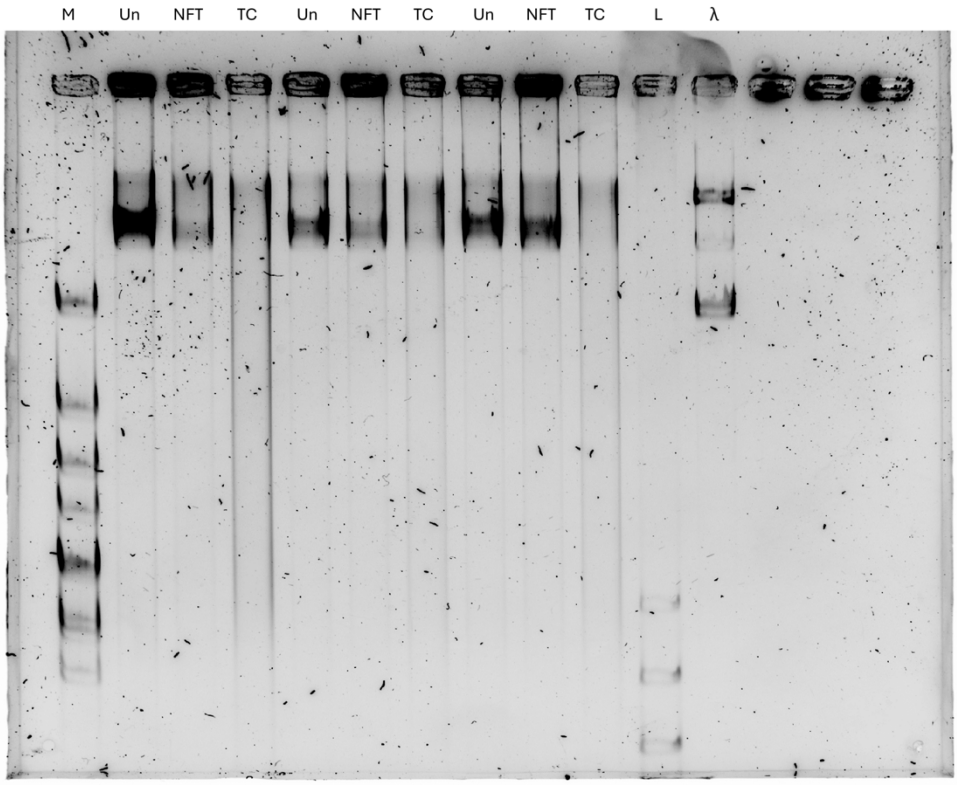

Figure S28: Pusle field electrophoresis. DNA was extracted from treated and untreated cells. Treated cells were incubated with 64 μg/mL (268 μM) nitrofurantoin (labelled NFT) or 750 μM Cu(II)-TC-Py (labelled TC) for 30 mins. Lane labelled M contained an NEB λ DNA monocut mix ladder, lane L contained GeneRuler 1 kb plus DNA ladder and the rightmost lane contained λ DNA as control for verifying accuracy of DNA ladders. Untreated samples are labelled Un.

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