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FULL PAPER

A Click Chemistry Approach to Developing Molecularly Targeted DNA Scissors

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Abstract: Nucleic acid click chemistry was used to prepare a family of chemically modified triplex forming oligonucleotides (TFOs) for application as a new gene-targeted technology. Azide-bearing phenanthrene ligands—designed to promote triplex stability and copper binding—were 'clicked' to alkyne-modified parallel TFOs. Using this approach, a library of TFO hybrids was prepared and shown to effectively target purine-rich genetic elements *in vitro*. Several of the hybrids provide significant stabilisation toward melting in parallel triplexes (>20 °C) and DNA damage can be triggered upon copper binding in the presence of added reductant. Therefore, the TFO and 'clicked' ligands work synergistically to provide sequence-selectivity to the copper cutting unit which, in turn, confers high stabilisation to the DNA triplex. To extend the boundaries of this hybrid system further, a click chemistry-based *di*-copper binding ligand was developed to accommodate designer ancillary ligands such as DPQ and DPPZ. When this ligand was inserted into a TFO, a dramatic improvement in targeted oxidative cleavage is afforded.

Introduction

DNA is predominantly found as a right-handed B-form duplex but a variety of tertiary structures including A- and Z-DNA along with more complex structures like G-quadruplexes,[1] i-motifs[2] and Holliday junctions[3] are known. Another tertiary structure is triplex DNA which is formed by the sequence-specific binding of a triplex forming oligonucleotide (TFO) in the major groove of duplex DNA.[4] The base-base recognition properties of TFOs rely on binding to oligopurine-oligopyrimidine target sequences. Two TFO motifs are possible: (a) parallel triplexes which form in acidic conditions when a pyrimidine-rich TFO binds (in parallel orientation) to form T-AT and C^+-GC Hoogsteen bonds with AT and GC base pairs; and (b) antiparallel triplexes arise when a purine-rich TFO binds in reverse-Hoogsteen orientation to form A-AT and G-GC triplets with AT and GC base pairs.[5] Studies indicate over 97% of human genes contain at least one triplex forming sequence with ~86% having a unique configuration.[6] For this reason—combined with their programmability and selectivity—the design of effective TFOs is an important challenge as it provides a method to directly modify gene expression[7] with further applications in bionanotechnology and synthetic biology.[8]

Despite their potential, native TFOs are restricted by several factors including low binding affinity, pH-dependence (for parallel triplexes) and an oligopurine-oligopyrimidine target. To overcome these limitations, chemical modifications to the base,[9] sugar[10] and phosphate[11] groups of TFOs have enabled improvements. One successful strategy relies on inserting intercalating agents in the backbone or nucleobase of TFOs (Figure 1a).[12] Early studies showed phosphate-modified pyrene-based twisted intercalating nucleic acids (TINAs)[13] and aryl-phenanthroimidazole (ImPhen)[14] ligands could stabilise parallel triplexes (Figure 1b). However, recent strategies are focused on base-modified thiazole orange (TO) intercalators (Figure 1c) which stabilise parallel TFOs at pH 7.0 and exert an additive effect where multiple TO units can enhance binding to the point where triplex stability surpasses the underlying duplex.[15] The shape and size of the conjugated intercalator is therefore important since it must not interrupt triplex formation and, conversely, triplex recognition should not preclude intercalative base stacking interactions.

A variety of metal binding ligands have been conjugated to TFOs with the aim of generating metal complex-oligonucleotide hybrids. Several hybrids containing 1,10-phenanthroline (Phen) ligands can, in the presence of coordinated copper ions, direct strand cutting to specific sequences.[16] The cutting method relies

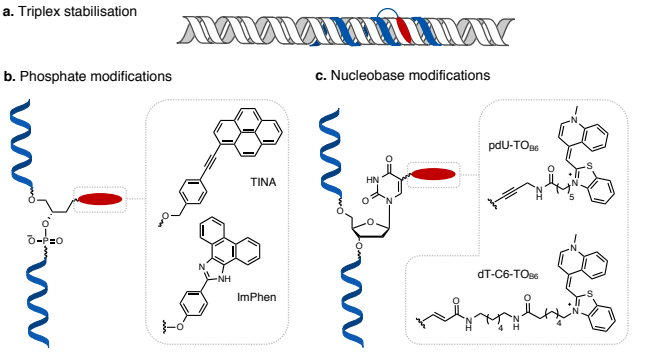

Figure 1. **a**) DNA triplexes can be stabilised by intercalating modifications (TFO in blue, intercalating group in red). **b**) Phosphate modified intercalators such as TINA and ImPhen ligands afford enhanced triplex stability. **c**) Thiazole orange (TO) functionalised bases have recently been employed to enhance parallel DNA triplex stability.

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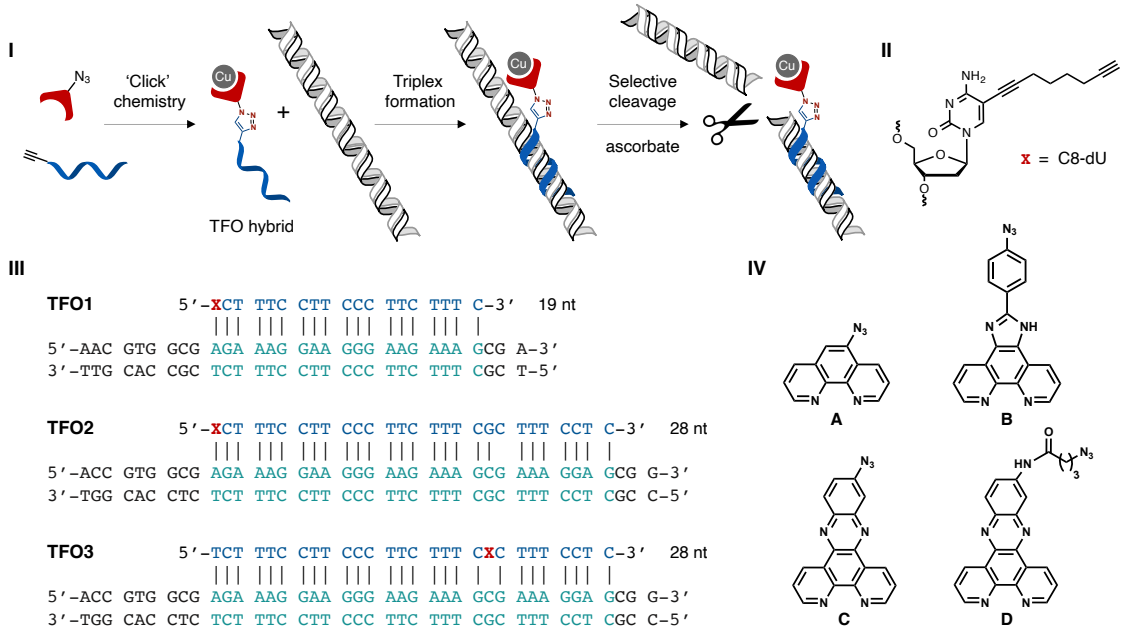

Figure 2. I) Generation of phenanthrene TFOs via nucleic acid 'click' chemistry. Coordination of Cu(II) to TFO hybrids enables targeted DNA cleavage in the presence of ascorbate. **II)** Molecular structure of the alkyne-modified C8-dU inserted at 5'-terminal or internal position (**X**) of TFO probes. **III)** Parallel TFO sequences 1-3 and their binding to target duplexes. **IV)** Library of azide-phenanthrene ligands **A-D**.

on generating a Cu(II)-Phen-TFO complex *in situ* prior to the addition of a reductant to generate the active Cu(I)-Phen agent, which acts as a targeted chemical nuclease. A higher order ligand called clip-Phen—containing two serinol-linked Phen molecules—was subsequently developed[17] and attempts to successfully guide this cutter focused on conjugation to acridine intercalators along with groove binding distamycin or spermine analogs.[18] More recently, a clip-Phen-TFO was developed and targeted to the Env gene in the HIV-1 genome (Supplementary Figure S1).[19] Although this hybrid cleaves the target gene by up to 34%, moderate stability effects indicate difficulty for clip-Phen to bind appropriately in the major groove. Therefore, despite recent progress in the preparation of targeted Phen conjugates (including clip-Phen), TFO hybrids continue to suffer from drawbacks including inefficient target binding, off-target (non-specific) DNA damage, complex preparation methods, and a reliance on a cationic adjuvant (e.g. spermine) to stabilise the negatively charged triple-helical structure. In an effort to overcome some of these limitations and to provide new molecularly targeted chemical nucleases where the guiding oligonucleotide is directly coordinated to the cutting unit, we report a new class of phenanthrene-modified TFO that stabilises parallel DNA triplexes and promote targeted oxidative cutting. Their design relies on nucleic acid click chemistry[20] whereby copper catalysed azide-alkyne cycloaddition (CuAAC) and strain promoted (SPAAC) reactions are used to couple azide-modified phenanthrene ligands to alkyne base-modified parallel TFOs. Using this approach, the rapid generation of phenanthrene-TFO libraries targeted to a portion of the green fluorescent protein (GFP) cloning vector were tested in a first attempt to generate synthetic metal-based gene cleavage systems.

Results and Discussion

Design of hybrid AMN-TFO systems

TFO hybrids were constructed using oligonucleotides (ONs) containing a single alkyne-base modification and an intercalating phenanthrene group bearing an azide modification (Figure 2-I). The C8-alkyne-dU base (Figure 2-II) was inserted either at the 5' terminus or internally within the TFO strand (Figure 2-III). TFOs were designed to bind with a specific portion of the green fluorescent protein (GFP) cloning vector and three types were developed (Figure 2-III): a 5'-terminal base-modified 19 nt ON with no mismatch or inversion sites (TFO1); a 5'-terminal base-modified 28 nt ON with a single inversion site (TFO2); and an internally base modified 28 nt ON with a single inversion site (TFO3).

Four copper-binding phenanthrene ligands containing either aromatic or aliphatic azide linkers (**A-D**) were prepared (Figure 3 and Supplementary S1, Figure S2-S15). **A** and **C** are simple azide-modified Phen and DPPZ ligands, respectively, while **B** is an azide-aryl-phenanthroimidazole (N_3-ImPhen) that provides rotational freedom between the copper binding N,N-unit and azide-aryl group. The final ligand **D** is a DPPZ molecule with a flexible azide-butanamide linker. Single crystal structure analyses were carried out for ligands **B**, **C** and Cu(**A**)$(NO_3)_2$, a Cu(II) complex of ligand **A** (Figure 4). In each case, the ligand has the expected, essentially planar structure and the azide group is unambiguously located. In the complex Cu(**A**)$(NO_3)_2$ the nitrate anions also coordinate to the axial sites of neighbouring copper ions, so that the structure comprises a 1-D polymer of Cu(**A**)$(NO_3)_2$ units linked by Cu–O–Cu and Cu–O-N-O–Cu bridges (for extensive crystal structure analysis, see Supplementary S2, Figure S16-S29). Although, no crystals were obtained for azide-butanamide ligand **D**, a reference compound containing a pentanamide linker was prepared with X-ray structural analysis indicating the relative orientation of the azide

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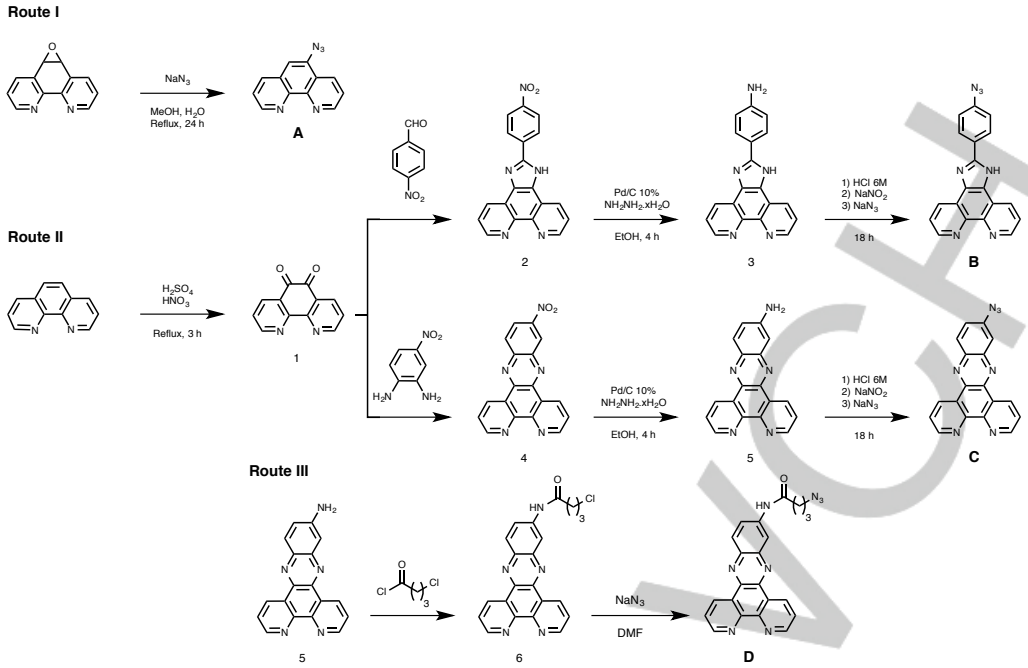


Figure 3. Synthetic routes to phenanthrene derivatives bearing azide handles (ligands **A**-**D**). **Route I**: Azidation of epoxy-1,10-phenanthroline to generate ligand **A**. **Route II**: Diazotization for the generation of ligands **B** and **C**. **Route III**: Treatment of amino-DPPZ (**5**) with an acyl chloride followed by azidation provided ligand **D**

moiety and flexibility of the aliphatic linker (see Supplementary S2, Figure S27-S29). Alkyne-TFOs were synthesised using a solid-phase oligo synthesiser, purified by HPLC, and clicked to the organic azides **A-D** to yield a library of hybrids via copper-catalysed click chemistry (Supplementary S3).

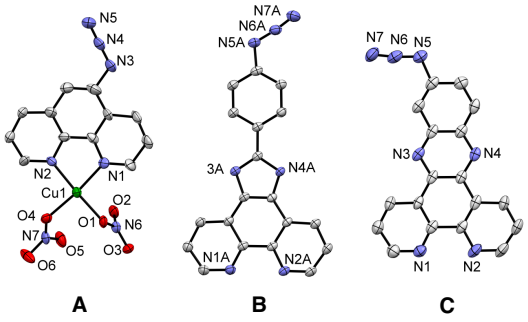


Figure 4. Single crystal X-ray structures of ligands **B**, **C** and the complex Cu(**A**)$(NO_3)_2$ showing 50% probability ellipsoids. Hydrogen atoms omitted for clarity.

Triplex melting and targeted DNA cleavage analysis

Triplex formation was monitored by UV melting where absorbance at 260 nm was recorded at pH 6.0 to ensure stable C^+-GC triplet formation. Clicked phenanthrene ligands conferred, in most cases, significant stabilisation when compared to control alkyne-TFOs (Figure 5-I). The Phen ligand (**A**) provided high stability to the fully complementary TFO (TFO1A) but was not particularly effective in TFO2 and 3. ImPhen modification **B**, on the other hand, provided stabilisation to all triplexes the greatest being >20 °C for TFO2. The DPPZ ligand (**C**) followed a broadly similar trend to Phen modification (**A**) but was better at stabilising the 5'-modified TFO2. Significantly, when the azide modification is positioned on an aliphatic linker connected to DPPZ (**D**), a notable increase in the melting temperatures of both TFO1 (>20 °C) and 3 was identified. Overall, **B** and **D** are the most stabilising ligands and are effective within internally modified TFO3; this is likely due to their enhanced flexibility minimising the strain in central regions of the duplex GFP target.

Targeted nuclease activity was studied with hybrids of TFO2 in the presence of copper(II) nitrate and ascorbate—a reductant natively found in biological systems. A ratio of 1:10:10 was selected for the duplex:TFO:Cu^{2+} mixture and cleavage reactions were initiated upon the addition of ascorbate (*cf*. Figure 5-II). Results show controlled depletion of the triplex with higher ascorbate concentrations almost completely ablating the target. To compare the cleavage activity of hybrids **A-D**, triplicate experiments with 100 equivalents of ascorbate to TFO were examined (Supplementary S5, Figure S30). Band densitometry

I Thermal melting (T_M) values of TFO-hybrids

		5'-mod.		Internal mod.
	Ligand	**TFO1**	**TFO2**	**TFO3**
T_M (°C)	TFO–≡	43.3 ± 0.3	46.1 ± 0.0	44.2 ± 0.2
ΔT_M (°C)	**A**	13.9 ± 0.6	4.3 ± 0.4	< 5.0
	B	8.4 ± 0.1	20.7 ± 0.5	18.8 ± 0.4
	C	14.7 ± 0.2	10.0 ± 0.7	5.2 ± 0.2
	D	22.0 ± 0.5	9.8 ± 0.6	18.9 ± 0.4

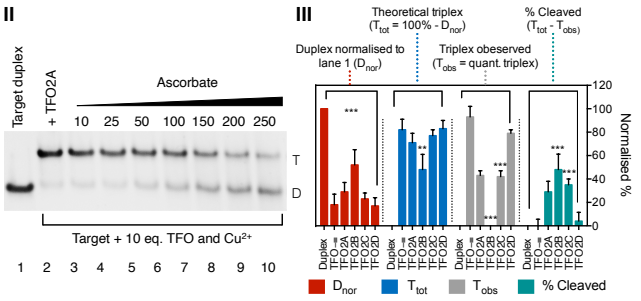


Figure 5. I) T_M values of TFO-hybrids containing ligands **A-D** in TFO1-3 along with control alkyne-modified TFOs (TFO−≡). **II)** Cleavage of duplex target (1.25 pmol, lane 1) was examined with TFO2A (12.5 pmol), Cu(II) nitrate (12.5 pmol) and increasing equivalents of ascorbate. T= triplex, D= duplex. **III)** Band

densitometry analysis of cleavage reactions with TFO2 hybrids (12.5 pmol) in the presence of Cu(II) nitrate (12.5 pmol) and 100 eq. of ascorbate (1.25 nmol).

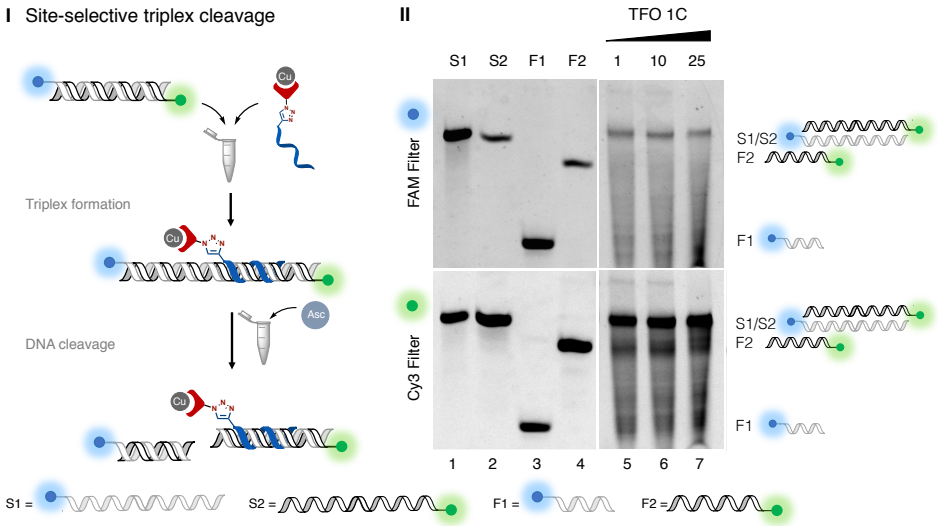


Figure 6. I) Cleavage experiments were designed using fluorescently tagged complementary single strands of the target duplex (S1 labelled with FAM and S2 labelled with Cy3, lanes 1 and 2) and proposed cleavage-site probes (F1 and F2, lanes 3 and 4). **II)** Denaturing PAGE experiments with the S1:S2 target treated with varying amounts of TFO (1-25 equivalents) in lanes 5-7.

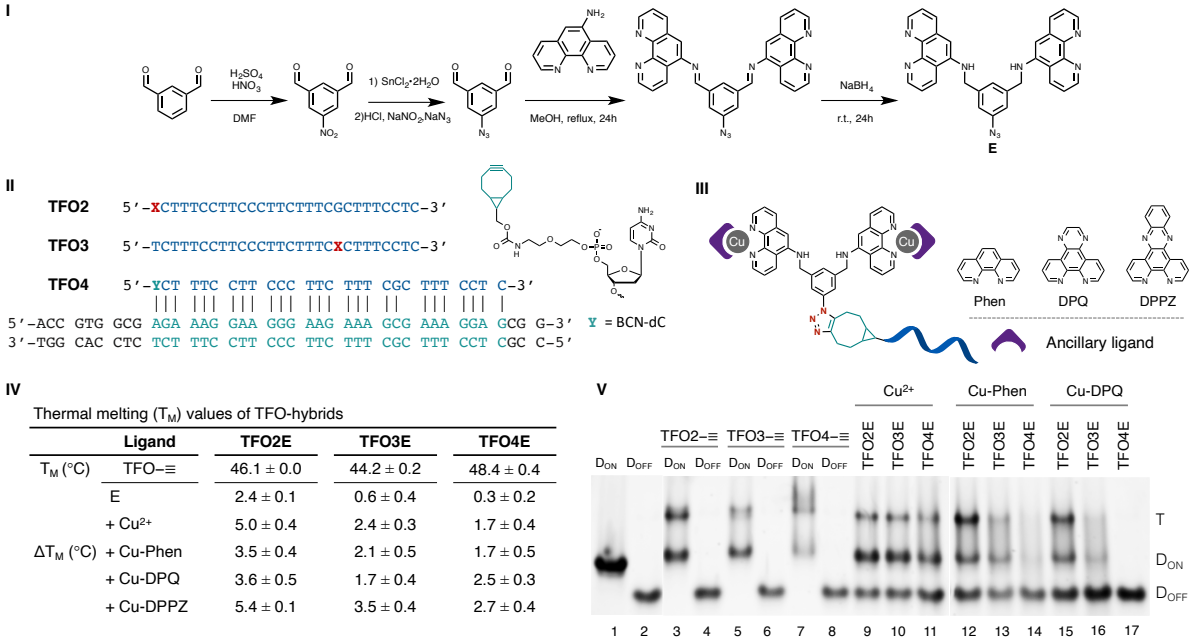
analysis was applied to provide information on triplex depletion (Figure 5-III) with modification **B** displaying the highest target depletion under these conditions.

Mapping oxidative cleavage with fluorophore-labelled sequences

To help identify evidence of site selective scission, denaturing experiments with a fluorophore-labelled target were attempted. The experimental design is outlined in Figure 6-I where complementary FAM (6-carboxyfluorescein) and Cy3 (cyanine 3) labelled strands containing the recognition sequence for TFO1C were used (Figure 6-II, S1 and S2, lanes 1-2). Single stranded ONs—matching the potential fragmentation pattern of the S1:S2 duplex—were also designed and labelled with FAM and Cy3 fluorophores (F1 and F2, lanes 3-4). Cleavage experiments were then performed with 1-25 equivalents of TFO1C in the presence of Cu(II) and ascorbate (Figure 6-II, lanes 5-7). At low reductant loading (lanes 5-7), the S1:S2 duplex was extensively sheared and upon imaging with the FAM filter, it was possible to visualise degradation products aligning with fluorophore fragment F1 (lane 3). The intensity of this band increased with the Cy3 filter and a second fragmentation band close to the expected F2 standard (lane 4) was identified. Overall, it appears that specific strand breaks of the fluorophore-tagged target along with widespread shearing occur with hybrid TFO1C.

Development of a novel *di*-copper click ligand and its application within targeted AMN-TFO hybrids

Although encouraging results with TFO hybrids have been identified to this point, some non-specific cleavage activity towards off-target duplexes was detected (*cf.* Supplementary S5, Figure S31). This is likely due to the high oxidizing capacity associated with labile sites on the metal centre since copper is strongly bound only to a single phenanthrene ligand. To develop more efficient copper chemical nucleases, our recent efforts have focused on (a) *di*-nuclear systems that afford higher nuclease efficiency and enhanced sequence discrimination,[21] and (b) the introduction of designer intercalating phenazine ligands for superior DNA binding affinity.[22] With this in mind, a novel chemical nuclease comprising a *bis*-phenanthroline *di*-copper binding ligand was specifically developed (**E**; Figure 7-I) since it retains capacity to bind two metals that can be functionalised at both coordination sites with designer intercalators. This ligand was clicked to TFO2 and TFO3 by CuAAC and by copper-free strain-promoted click chemistry (SPAAC) to TFO4—a probe containing a 5'-BCN (bicyclo[6.1.0]non-4-yne) modification (Figure 7-II). The three new hybrids TFO2E, 3E and 4E were then functionalised with either simple copper(II) nitrate or copper(II) complexes of Phen, DPQ and DPPZ (Figure 7-III). In contrast to ligands **A-D**, negligible triplex stabilisation was observed for TFOs modified with **E** (Figure 7-IV) and although some stabilisation was observed upon the introduction of copper(II) ions or the Cu-phenanthrenes, the overall effect remains low when compared with to the earlier mono-nuclear hybrids prepared. Nuclease experiments with ascorbate in the presence of a target duplex (D_{ON}) and an off-target duplex (D_{OFF}) were then investigated (Figure 7-V). Although modest target cleavage was identified in the presence of Cu(II) nitrate, activity was dramatically improved by the complexation of either Cu-Phen or Cu-DPQ. Here, TFO3E and in particular TFO4E ablated D_{ON} with no apparent D_{OFF} cleavage (lanes 14 & 17). In contrast, TFO-**E** with coordinated Cu-DPPZ was found to have significantly attenuated cleavage activity (Supplementary S5, Figure S32). Overall, these results indicate that although there is a trade-off in triplex stability by using the *di*-nuclear ligand **E**, highly effective knockout with limited off-target effects are accessible when ancillary Cu-Phen or Cu-DPQ complexes are coordinated. Future avenues of study may now involve broadening the type of ancillary groups coordinated to ligand **E** in order to further extend the boundaries of this hybrid TFO system.



	Ligand	TFO2E	TFO3E	TFO4E
T_M (°C)	TFO–≡	46.1 ± 0.0	44.2 ± 0.2	48.4 ± 0.4
	E	2.4 ± 0.1	0.6 ± 0.4	0.3 ± 0.2
	+ Cu^{2+}	5.0 ± 0.4	2.4 ± 0.3	1.7 ± 0.4
ΔT_M (°C)	+ Cu-Phen	3.5 ± 0.4	2.1 ± 0.5	1.7 ± 0.5
	+ Cu-DPQ	3.6 ± 0.5	1.7 ± 0.4	2.5 ± 0.3
	+ Cu-DPPZ	5.4 ± 0.1	3.5 ± 0.4	2.7 ± 0.4

Figure 7. **I**) Synthetic route to ligand **E**. **II**) Alkyne-modified TFO2, 3 and 4—a 5′-modified BCN-dC, **Y**. **III**) TFO hybrids with coordinated Cu^{2+} and ancillary phenanthrene ligands. **IV**) Thermal melting values of TFO-hybrids of ligand **E** in the presence of Cu^{2+} and Cu-phenanthrenes. **IV**) PAGE chemical nuclease analysis: TFO hybrids were co-treated with a target duplex (on-target, D_{ON}) and a non-target duplex (off-target, D_{OFF}). Cleavage experiments were conducted at target:TFO:Cu^{2+}:ascorbate ratios of 1:10:20:1000. T = triplex.

Conclusion

Nucleic acid click chemistry was applied to develop new molecularly targeted chemical nucleases where the guiding oligonucleotide (TFO) was directly coordinated to the cutting unit (copper phenanthrene). To help overcome stability limitations inherent with DNA triplexes, a library of intercalating ligands was developed with extended aromatic structures or flexible aliphatic spacers. Several hybrids provided significant stabilisation toward melting in parallel triplexes with melting temperatures increasing by greater than +20 °C. The ligands which provide this level of stabilisation include an azide-aryl-phenanthroimidazole (N_3-ImPhen), which enables rotational freedom between the copper binding N,N-unit and azide-aryl group, and a DPPZ molecule containing a flexible azide-butanamide linker. In the presence of copper(II) and ascorbate, TFO hybrids display effective chemical nuclease activity. Significantly, there was no requirement for complex preparation methods, stabilisers (*e.g.* spermine) or excessively high TFO:copper(II) loading in the development and application of the hybrids. DNA cutting is instead controlled by a predictable ratio of Cu(II)-TFO:target in the presence of ascorbate. Triplex formation is based on equilibrium binding and is dependent on reductant (ascorbate) concentration where excessive amounts, far exceeding blood concentrations of 50-100 μM,[23] appear to induce extensive damage whereby the TFO can no longer recognise the target duplex (Figure 8-I).

To the best of our knowledge, this is the first example of a combined triplex stabilising and cutting system based on the copper phenanthrene chemotype. The use of fluorescently tagged oligomers provided support for some sequence-specific cleavage by mononuclear TFOs but high-resolution cleavage at the single nucleotide level was not, at this point, achieved. Site-selective cleavage is possible in the bulge region of RNA substrates by copper(II)-neocuproine conjugated to a peptide nucleic acid recognition moiety in antisense RNAases mimics, most likely through hydrolytic cleavage, with catalytic turnovers displaying enzymatic behaviour.[24] Pin-point activation and single nucleotide scission is currently limited in stand-alone copper-oxidative systems, however cleavage specificity and sequence recognition can be tailored by the coordination environment.[21, 25] To help address this limitation, we recently developed discrete and targeted mononuclear copper(II) complexes using caged *tris*-(2-pyridyl-methyl)amine (TMPA) or *di*-(2-pycolylamine) (DPA) ligands and explored their cutting mechanisms using radical trapping experiments.[26] Oxidative cutting by the Cu(II)-phenanthrene TFO hybrids reported here are, most likely, mediated through a similar superoxide ($O_2^{\bullet-}$) radical mechanism that is outlined in Figure 8-II.

To enhance cleavage selectivity, a novel *bis*-Phen *di*-copper binding ligand was prepared and conjugated to TFOs. Significantly, this ligand can accommodate designer intercalating ancillary ligands and can be coupled to specific nucleic acid vectors via click chemistry. A library of TFO hybrids was developed with this ligand and when this probe was ligated with specific copper-bound ancillary ligands (Phen or DPQ), excellent targeting effects were achieved. This ligand clearly differs from the well-studied clip-Phen molecule[17b, 18a]—which contains two independent Phen molecules linked by a serinol bridge—particularly since the orientation of both Phen moieties allows for: (a) increasing the number of metal ion coordination sites, and (b) the attachment of ancillary ligands that can tailor the chemical nuclease properties. A future avenue for this technology may now lie in the preparation of therapeutically relevant copper phenanthrene-TFO combinations for *in cellulo* validation. These efforts will, undoubtedly, require backbone or ribose modifications (*cf.* Figure 8-III) that protect against cellular deactivation barriers and intrinsic oxidation of the copper bound TFO hybrids. One approach could involve locked nucleic acids (LNA) [10b] to enhance TFO recognition and binding affinity while circumventing self-oxidation through H-abstraction. A further option may involve

using unlocked nucleic acid (UNA) analogs to enhance target stability.[27] Finally, a biocompatible triazole backbone modification (clicked-NA), which is resistant to enzymatic nuclease activity and assists cellular delivery and nuclear uptake, may also be considered.[28] Recent advancements in antisense technologies have combined several of these modifications to combat enzymatic degradation and address stability limitations.[29] Adopting a similar approach here may accelerate copper-phenanthrene TFO hybrid development towards higher precision artificial gene editing applications.

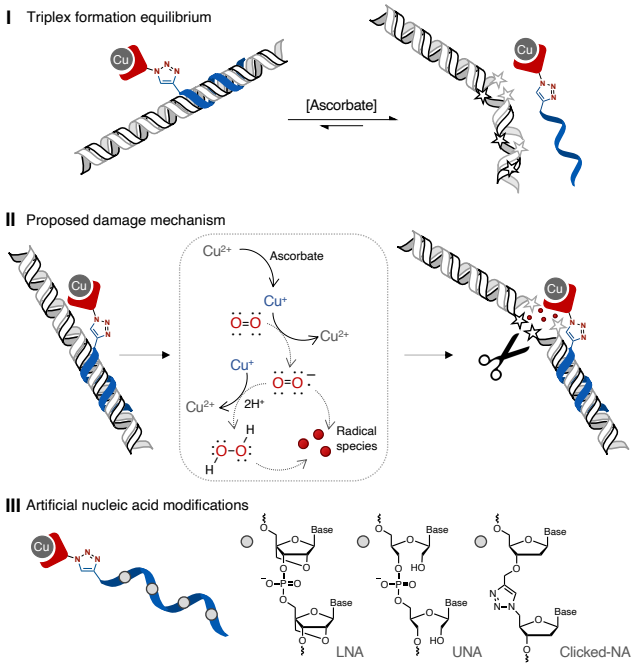


Figure 8. I) Triplex equilibrium binding is influenced by ascorbate concentration. **II**) Simplified DNA cleavage mechanism via the generation of superoxide radical anion intermediates. **III**) TFO constructs containing backbone and ribose modifications: locked nucleic acids (LNA); unlocked nucleic acids (UNA); clicked nucleic acids (clicked-NA).

Experimental Section

Materials and methods

All chemical reagents and solvents were obtained from Sigma-Aldrich (Ireland) Ltd. and unless otherwise stated were used without further purification. C8-Alkyne-dU-CEP was purchased from BaseClick GmbH. NMR spectra were recorded on Bruker AC 400 MHz or 600 MHz spectrometers (Supplementary S1). FT-IR spectra were collected on Perkin Elmer Spectrum Two spectrometer. ESI-MS analysis was performed on a Bruker HCT Mass Spectrometer. pH was monitored using a Mettler Toledo InLab Expert Pro-ISM pH probe. Crystallographic data was collected at 100(1)K on a Synergy Dualflex, AtlasS2 diffractometer (Supplementary S2). The structure was solved by dual space methods and refined on F2 using all the reflections (SHELXL-2018). Mass analysis of oligonucleotides was characterised at Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) on a Bruker Daltonics Autoflex II instrument (Supplementary S3). Thermal melting experiments were conducted on Agilent Cary 100 UV-Vis dual beam spectrophotometer equipped with a 6 × 6 Peltier multicell system with temperature controller. DNA was quantified on a Jasco UV–Vis spectrophotometer. Polyacrylamide gels were imaged on Syngene G:Box mini 9 gel documentation system.

Route I: Synthesis of azide-functionalised ligand A

5-azido-1,10-phenanthroline (**A**). A three-step method for the synthesis and isolation of **A** has been reported,[30] however, a more efficient route was developed as follows. To a solution of 5,6-epoxy-5,6-dihydro-1,10-phenanthroline (100 mg, 0.51 mmol) in a 4:1 MeOH/H_2O mixture (8 mL of MeOH and 2 mL of H_2O), NaN_3 (99 mg, 1.53 mmol) was added and refluxed at 80 °C for 24 h. The resulting precipitate was removed by vacuum filtration and the filtrate was reduced to precipitate the product. Yield = 113 mg, quantitative yield. ^{1}H NMR and ^{13}C NMR data are consistent with those reported in literature.[30]

Route II: Synthesis of azide-functionalised ligand B and C

1,10-phenanthroline-5,6-dione (**1**)

Compound **1** was prepared according to methods reported in literature.[22a] 1,10-Phenanthroline (4.00 g, 22.19 mmol) and KBr (4.00 g, 33.6 mmol) were mixed and slowly added to an ice-cold mixture of H_2SO_4 and HNO_3 (2:1; 40 mL of H_2SO_4 and 20 mL of HNO_3). The solution was refluxed for 3 h at 160 °C and, after cooling to room temperature, it was poured over crushed ice and adjusted with an aqueous NaOH solution to pH 4.0, yielding a yellow solution. The solution was extracted with $CHCl_3$ (in 8 × 100 mL portions) and the organic layers combined, dried over anhydrous magnesium sulfate and filtered before reducing to dryness to isolate a yellow powder. Yield: 4.20 g, 90%. ^{1}H NMR and ^{13}C NMR data are consistent with those reported in literature.[22a]

2-(4-nitrophenyl)-1H-imidazo-[4,5-f]-1,10-phenanthroline (**2**)

Compound **2** was prepared according to methods reported in literature.[31] Compound **1** (500 mg, 2.38 mmol), 4-nitrobenzaldehyde (540 mg, 3.57 mmol) and ammonium acetate were stirred in acetic acid (16 mL) under reflux for 2 h. The resulting solution was neutralised with ammonia and the product was recrystallised in the same mixture. Yield: 570 mg, 70%. ^{1}H NMR and ^{13}C NMR data are consistent with those reported in literature.[31]

4-(1H-imidazo-[4,5-f]-1,10-phenanthrolin-2-yl)aniline (**3**)

Compound **3** was prepared according to methods reported in literature with slight modifications.[32] Compound **2** (500 mg, 1.47 mmol) was treated with Pd/C 10% (0.1% m/m) in 110 mL of MeOH. An excess of hydrazine hydrate (5 mL) was added dropwise and refluxed for 2 h. The mixture was cooled to room temperature and stirred for 2 h. The solution was filtered on celite® and reduced until a yellow precipitate formed. ^{1}H NMR and ^{13}C NMR data are consistent with those reported in literature.[32]

2-(4-azidophenyl)-1H-imidazo[4,5-f]-1,10-phenanthroline (**B**)

Ligand **B** was prepared according to methods reported in literature with slight modifications.[32] Compound **3** (200 mg, 0.64 mmol) was stirred in a 6 M HCl aqueous solution (10 mL) for 20 min at 0°C. $NaNO_2$ (66 mg, 0.96 mmol) in H_2O (1 mL) was added dropwise and stirred for 30 min, after which an aqueous solution of NaN_3 (125 mg, 1.92 mmol, 1 mL of H_2O) was added dropwise and stirred for a further 2 h. The mixture was neutralised with ammonia and the yellow product was isolated by vacuum filtration. ^{1}H NMR and ^{13}C NMR data are consistent with those reported in literature.[32]

11-nitrodipyrido[3,2-a:2',3'-c]phenazine (**4**)

Compound **1** (500 mg, 2.38 mmol) and 4-nitrobenzene-1,2-diamine (547 mg, 3.57 mmol) were stirred in a mixture ethyl acetate/MeOH (7:1, 35 mL of ethyl acetate and 5 mL of MeOH) under reflux for 4 h. The resulting solid was recrystallised in the same mixture. Yield: 662 mg, 85%. ^{1}H NMR (600 MHz, $CDCl_3$) δ [ppm]: 9.63 (ddd, *J* = 8.0, 7.3, 1.8 Hz, 2H), 9.32 (td, *J* = 4.4, 1.8 Hz, 2H), 9.28 (d, *J* = 2.4 Hz, 1H), 8.67 (dd, *J* = 9.2, 2.5 Hz, 1H), 8.50 (d, *J* = 9.2 Hz, 1H), 7.84 (dt, *J* = 8.2, 4.2 Hz, 2H).^{13}C NMR (151 MHz, $CDCl_3$) δ [ppm]: 153.72, 153.54, 149.16, 148.81, 148.22, 144.29, 143.64, 143.22, 140.95, 134.40, 134.15, 131.28, 126.92, 126.87, 126.08, 124.60, 124.51, 123.74.

dipyrido[3,2-a:2',3'-c]phenazin-11-amine (**5**)

Compound **5** was prepared according to previously reported methods[33] with some modifications as follows: compound **4** (500 mg, 1.53 mmol) was treated with Pd/C 10% (0.1% m/m) in EtOH (60 mL). An excess of

hydrazine hydrate (10 mL) was added dropwise and refluxed for 1 h. The hot solution was filtered on celite® and filtrate was reduced to isolate a red precipitate. Yield: 409 mg, 90%. ^{1}H NMR and ^{13}C NMR data are consistent with those reported in literature.[33]

11-azidodipyrido[3,2-a:2',3'-c]phenazine (**C**)
Compound **5 (**250 mg, 0.84 mmol) was treated with 10 mL of 6 M HCl solution for 20 min at 70 °C. The solution was cooled to 0 °C and aqueous $NaNO_2$ (174 mg, 2.52 mmol, 1 mL of H_2O) was added dropwise and stirred for 30 min. The resulting solution was added dropwise to NaN_3 dissolved in H_2O (164 mg, 2.52 mmol, 1 mL of H_2O) and stirred for a further 2 h. The mixture was neutralised with ammonia and the red-brownish precipitate was isolated by filtration. Yield: 250 mg, 92%. ^{1}H-NMR (600 MHz, $CDCl_3$) δ [ppm]: 9.57 (td, *J* = 8.2, 1.8 Hz, 2H), 9.27 (ddd, *J* = 8.7, 4.4, 1.8 Hz, 2H), 8.29 (d, *J* = 9.0 Hz, 1H), 7.93 (d, *J* = 2.5 Hz, 1H), 7.79 (ddd, *J* = 7.8, 4.4, 3.3 Hz, 2H), 7.54 (dd, *J* = 9.0, 2.5 Hz, 1H). ^{13}C NMR (151 MHz, $CDCl_3$) δ [ppm]: 152.94, 152.67, 148.70, 148.35, 143.12, 142.98, 141.90, 140.60, 140.58, 133.96, 133.69, 131.46, 127.62, 127.45, 124.56, 124.34, 124.32, 116.44. FT-IR (ATR, cm^{-1}): 2111, 1613, 1578, 1490, 1408, 1358, 1265, 1215, 1110, 1071, 809, 739. ESI-MS: *m*/*z* calcd., 324.1 $[M+H]^+$; found, 324.0.

Route III: Synthesis of azide-functionalised ligand D
4-chloro-N-(dipyrido[3,2-a:2',3'-c]phenazin-11-yl)butanamide (**6**)
Compound **6** was synthesised using a method previously reported in literature with slight modification.[33a] Compound **5** (100 mg, 0.34 mmol) was treated with TEA (0.68 mmol) in dry $CHCl_3$ (10 mL) and stirred for 10 min at room temperature. The mixture was cooled and 4-chlorobuteryl acid chloride (0.34 mmol) was added dropwise at 0 °C and stirred at room temperature overnight. The precipitate was collected by vacuum filtration, washed with chloroform and used immediately to prepare ligand **D**. Yield: 131 mg, 97%. ^{1}H NMR (600 MHz, DMSO-d_6) δ [ppm]: 10.81 (s, 1H), 9.79-9.59 (dd, J = 20.7, 7.0 Hz, 1H), 9.28 (ddd, J = 7.6, 2H), 8.91 (d, J = 2.2, 1H), 8.37 (d, J = 9.2 Hz, 1H), 8.12 (ddd, J = 11.6, 8.6, 3.4 Hz, 3H), 3.79 (t, J = 6.5 Hz, 2H), 2.66 (t, J=2.68, 2H), 2.14 (quint, J = 13.0, 6.7 Hz, 2H).

4-azido-N-(dipyrido[3,2-a:2',3'-c]phenazin-11-yl)butanamide (**D**)
Compound **6** (100 mg, 0.249 mmol) was treated NaN_3 (16 mg, 0.249 mmol) in dry DMF (10 mL) and refluxed at 85 °C overnight. The mixture was poured over ice and vacuum filtered. Yield: 101 mg, quantitative yield. ^{1}H NMR (600 MHz, DMSO-d_6) δ [ppm]: 10.64 (s, 1H), 9.49 (dd, J = 8.1, 1.7 Hz, 1H), 9.45 (dd, J = 8.1, 1.7 Hz, 1H), 9.18 (ddd, J = 11.0, 4.3, 1.7 Hz, 2H), 8.81 (d, J = 2.2 Hz, 1H), 8.26 (d, J = 9.1 Hz, 1H), 8.00 (d, J = 2.3 Hz, 1H), 7.91 (ddd, J = 8.0, 4.3, 1.9 Hz, 2H), 3.48 (t, J = 6.8 Hz, 2H), 2.58 (t, J = 7.3 Hz, 2H), 1.94 (p, J = 7.0 Hz, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ [ppm]: 171.97, 152.69, 152.35, 148.26, 147.87, 143.26, 141.63, 141.35, 139.34, 133.57, 133.11, 130.14, 127.54, 127.36, 125.95, 124.90, 114.84, 50.78, 34.02, 24.61. FT-IR (ATR, cm^{-1}): 2100, 1674, 1624, 1558, 1495, 1446, 1360, 1215, 1072, 1031. ESI-MS: m/z calcd., 407.1 $[M-H]^-$; found, 407.0.

Route IV: Synthesis of azide-functionalised ligand E
5-nitro-1,10-phenanthroline (**7**)
Compound **7** was synthesised using a method previously reported in literature.[34] 1,10-Phenanthroline (4.00 g, 22.19 mmol) was dissolved in ice-cold mixture of 2:1 H_2SO_4:HNO_3 (24 mL of H_2SO_4 and 12 mL of HNO_3) was slowly added. The solution was refluxed for 3 h at 160 °C, cooled down to room temperature and poured over crushed ice. The pH was adjusted (pH = 3) with aqueous NaOH to obtain a pale-yellow precipitate, which was then vacuum filtered and washed with H_2O. Yield: 5.00 g, quantitative yield. ^{1}H NMR and ^{13}C NMR data are consistent with those reported in literature.[34]

5-amino-1,10-phenanthroline (**8**)
Compound **8** was synthesised using a method previously reported in literature with slight modifications.[34] Compound **7** (2.00 g, 8.88 mmol) was treated with Pd/C 10% (0.1% m/m) in the minimum amount of EtOH (40 mL). An excess of hydrazine hydrate (10 mL) was added dropwise and refluxed for 4 h. The hot solution was filtered on celite® and the filtrate was reduced yielding a yellow precipitate. Yield: 1.70 g, 98%. ^{1}H NMR and ^{13}C NMR data are consistent with those reported in literature.[35]

5-nitroisophthalaldehyde (**9**)
5-nitroisophthalaldehyde was prepared according to a method reported in literature with some modifications.[36] To a mixture of 2.00 g (15.13 mmol) of ammonium sulfate in 3.30 mL of sulfuric acid and 0.70 mL of nitric acid, 500 mg (3.73 mmol) of isophthalaldehyde solubilised in 3.30 mL of concentrated sulphuric acid were added. After 2 days the mixture was poured into ice to afford precipitation of the white product which was filtered and washed with copious H_2O. Yield: 334 mg, 50%. ^{1}H NMR and ^{13}C NMR data are consistent with those reported in literature.[36]

5-azidoisophthalaldehyde (**10**)
Compound **9** (200 mg, 1.12 mmol), $SnCl_2.2H_2O$ (1.26 g, 5.6 mmol) and EtOH (10 mL) were refluxed at 70 °C for 3 h. The reaction mixture was then poured over ice and quenched with a saturated solution of sodium bicarbonate. The product was then extracted several times with ethyl acetate, before adjusting to pH 5 with acetic acid. The product was then extracted again with ethyl acetate. The combined organic layers were concentrated using the rotary evaporator and dried under vacuum overnight yielding a dark yellow powder. 100 mg (0.67 mmol) of the product was treated with 6 M HCl (5 mL) solution for 1 h on an ice bath. Afterwards, 3 equivalents of sodium nitrite (140 mg, 2.011 mmol) in a minimum amount of cold H_2O (1 mL) was added dropwise with stirring, giving a brownish solution. After 1 h, a solution of NaN_3 (130 mg, 2.011 mmol, 1 mL of H_2O) was added dropwise and stirred overnight on ice. The mixture of reaction was allowed to equilibrate to room temperature before adjustment to pH 7.0 with ammonia. The solution was extracted 10 times with ethyl acetate. It was then concentrated (in darkness) to yield a dark orange-yellowish oil. Yield: 170 mg, 87%. ^{1}H NMR (600 MHz, DMSO-d_6) δ [ppm]: 10.08 (s, 2H), 8.14 (t, 1H), 7.78 (d, 2H). FTIR-ATR (neat, cm^{-1}): 3066, 2924, 2853, 2131, 1689, 1595, 1463, 1392, 1379, 1316.

(1*E*,1'*E*)-1,1'-(5-azido-1,3-phenylene)bis(*N*-(1,10-phenanthrolin-5-yl)methanimine*)* (**11**)
The compound **11** was synthesised using a similar procedure reported in literature with some modifications.[37] Compound **10** (100 mg, 0.57 mmol) and **7** (330 mg, 1.71 mmol) were stirred in 10 mL dry MeOH under reflux and overnight with catalytic amount of glacial acetic acid. Afterwards, a hot filtration was performed. The solid was collected, dried under vacuum and recrystallised with MeOH. Yield: 302 mg, quantitative yield. ^{1}H NMR (600 MHz, $CDCl_3$): δ 9.26 (dd, J = 4.3, 1.8 Hz, 2H), 9.16 (dd, J = 4.3, 1.7 Hz, 2H), 8.81 – 8.78 (m, 4H), 8.41 (t, J = 1.4 Hz, 1H), 8.27 – 8.25 (m, 2H), 7.94 (d, J = 1.4 Hz, 2H), 7.70 (dd, J = 8.2, 4.3 Hz, 2H), 7.64 (dd, J = 8.0, 4.3 Hz, 2H), 7.38 (s, 2H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 159.67, 151.07, 149.89, 149.84, 147.23, 146.53, 145.65, 142.38, 138.40, 135.98, 132.77, 128.88, 127.12, 126.83, 126.04, 123.56, 123.15, 122.05, 111.04. FT-IR (ATR, cm^{-1}): 3332, 3030, 2109, 1631, 1581, 1420, 1312, 1136, 1060, 890.

N,*N*'-((5-azido-1,3-phenylene)bis(methylene))bis(1,10-phenanthrolin-5-amine) (**E**)
The ligand **E** was synthesised using a similar procedure reported in literature with some modifications.[37] Compound **11** (100 mg, 0.19 mmol) was treated with $NaBH_4$ (21 mg, 0.56 mmol) in 10 mL of dry MeOH at room temperature overnight. The mixture was dried under vacuum and purified with silica gel column chromatography in DCM:MeOH with gradient 9:1 – 8:2 and 1% ammonia. Yield: 60 mg, 60%. ^{1}H NMR (600 MHz, MeOD-d4 + $CDCl_3$): δ 8.95 (d, J = 4.3 Hz, 2H), 8.58 (d, J = 4.4 Hz, 2H), 8.47 (d, J = 8.4 Hz, 2H), 7.61 – 7.52 (m, 4H), 7.40 – 7.26 (m, 3H), 7.05 (s, 2H), 6.34 (s, 2H), 4.61 (s, 4H). FT-IR (ATR, cm^{-1}): 3280, 2104, 1611, 1593, 1536, 1410, 1299, 1219, 1165, 1106. ESI-MS: *m*/*z* calcd. 534.22 $[M+H]^+$; found 534.21.

Preparation of [Cu(**A**)$(NO_3)_2$]
A solution of **A** (80 mg, 0.36 mmol) in 5 mL of MeOH was added dropwise to a solution of copper(II) nitrate trihydrate (100 mg, 0.41 mmol,) in 10 mL MeOH. The solution was left stirring overnight where the colour changed

from light blue to green. The product was filtered, washed with diethyl ether and dried by desiccation. Yield: 100 mg, 93%. FT-IR [neat, cm^{-1}]: 2131, 2062, 1431, 1471, 1396, 1277, 1015, 879, 807, 728. ESI-MS: *m*/*z* calcd. 345.99 $[M-(NO_3)]^+$; found, 345.99. Dark green crystals formed in EtOH/H_2O (1:1) solution when left to stand with slow evaporation at room temperature for 15 days.

Hybrid generation and molecular biology procedures

Crystallography

Data for all the structures were collected at 100(1)K on a Synergy Dualflex, AtlasS2 diffractometer using Cu*K*α radiation (λ = 1.54184 Å) and the *CrysAlis PRO* suite. The structures were solved by dual space methods (SHELXT)[38] and refined on F^2 using all the reflections (SHELXL-2018/3).[39] Full occupancy non-hydrogen atoms were refined using anisotropic atomic displacement parameters and hydrogen atoms were inserted at calculated positions using a riding model. Crystal data, data collection and structure refinement details are in Supplementary S2. CCDC 1952707-1952710 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Oligonucleotide synthesis

TFOs were synthesised on an Applied Biosystems Incorporated 394 automated synthesizer.[40] Phosphoramidites and solid supports columns were purchased from Glen Research, Link Technology, and ChemGene Corporation. The synthesis of alkyne-bearing oligonucleotides was performed in a 1 µmol scale with the standard DNA synthesis cycles DMT-off (trityl off mode). Post synthesis, sequences were deprotected using 1 mL of AMA (ammonium hydroxide:methylamine, 50:50), vortexed for 5 min, heated for 15 min at 65 °C, cooled on ice and centrifuged for 3 min, removed the supernatant and decanted with 0.5 mL of Milli-Q® H_2O, vortexed for 3 min and collected the supernatant. The addition of Milli-Q® H_2O and the collection of the supernatant were performed three times and the supernatant was evaporated using a SpeedVac concentrator. Sequences bearing the benzoyl group were treated with the protocol above however utilising ammonium hydroxide in place of AMA for two days. For desalting, the sequences were solubilised with 100 µL of sodium acetate, precipitated with 1 mL of cold EtOH (with 5% of diethyl ether) and centrifuged for 1 h and 30 min at 0 °C. Afterwards, the supernatant was removed and the oligos were then freeze-dried. Preliminary analysis was conducted on analytical RP-HPLC using a *Macherey-Nagel* Nucleodur, 100-3 C18ec column, on 2695 Separation Module, equipped with a Waters Alliance 2996 Photodiode Array Detector (flow 0.5 mL/min). Afterwards, the TFOs were purified with a semi-preparative RP-HPLC, performed using a *Macherey-Nagel* C18 column (5 mm, 9.4 × 250 mm) using a Waters Breeze 2487 Dual λ Array Detector, 1525 Binary HPLC Pump. Alkyne-modified TFOs and hybrids were isolated using a mobile phase gradient buffer A to buffer B (0-40%) where buffer A is 0.1 M TEAA (triethylammonium acetate) in H_2O and buffer B is 0.1 M TEAA in 80% ACN. The collected fractions were characterised by MALDI-ToF. The concentration of the oligonucleotide solutions was determined by UV absorbance at 260 nm using a Nanodrop ND-1000 spectrophotometer.

Generation of TFO-hybrid materials through click chemistry

CuAAC-Small scale: A 5 µL solution of alkyne-modified TFO (5 nmol, 1 eq) and azide ligand (50 nmol, 10 eq) were prepared in a 1:1 DMSO/H_2O mixture (85.25 µL) and stirred. A freshly prepared solution of tris(3-hydroxypropyltriazolylmethyl)amine (THPTA, 50 nmol, 50 eq) and $CuSO_4$ (75 nmol, 15 eq) was then added, followed by the addition of 2.5 µL of a Na-*L*-ascorbate solution (250 nmol, 50 eq). The solution was mixed for 2 h at room temperature after which time 9 µL of a 50 mM solution of EDTA was added. After diluting the reaction mixture with H_2O to reduce the amount of DMSO to 5% the product was freeze-dried and purified by RP-HPLC (TEAA buffer, pH = 8.0) or directly using Amicon Ultra 0.5 mL centrifugal filters (MWCO = 3000) according to operating instructions. Briefly, the diluted fractions were spun down using the mini-columns to remove excess organic azide, ascorbate, THPTA, copper and EDTA. The pure product was then recovered in a collection tube by centrifuging the inverted column.

CuAAC-Large scale: Alkyne-modified TFOs (25 nmol) and 25 µL of 10 mM DMSO solutions of azides **A**-**D** (1 eq) were prepared in 475.25 µL of a 1:1 DMSO/H_2O mixture. The reactions were catalysed with the addition of $CuSO_4$ (625 nmol, 25 eq) THPTA (1.25 µmol, 50 eq) and ascorbate (1.25 µmol). For ligand **E**, TFO (25 nmol) and 59.5 µL of a 4.2 mM DMSO solution of **E** was prepared in 440.7 µL of 1:1 DMSO/H_2O mixture. The reaction was catalysed with the addition of $CuSO_4$ (1.25 µmol, 50 eq) THPTA (2.5 µmol, 100 eq) and ascorbate (1.25 µmol). After 5 hours the mixture was quenched with 45 µL of excess EDTA disodium salt aqueous solution (50 mM) and freeze-dried overnight. The solid was re-dissolved in H_2O and purified using Amicon Ultra 0.5 mL centrifugal filters (MWCO = 3000). All hybrid materials were quantified *via* the Nanodrop spectrometer, characterised by MALDI-ToF, where the TFO showed no signs of self-degradation (see Supplementary S3).

Thermal melting experiments

Custom duplex targets (D) where purchased from Integrated DNA Technologies (D1: 31-mer, TFO1; D2: 40-mer, TFO2, TFO3 and TFO4). Oligonucleotides were quantified (1 µM) and samples were prepared of D:TFO 1:2.5 in 10 mM phosphate buffer with 150 mM NaCl and 2 mM $MgCl_2$ (pH = 6.0). For experiments investigating the stabilisation effects of TFOs modified with ligand **E** and copper-modified ancillary ligands, D:TFO:Cu^{2+} ratios were reduced to 1:1.25:2.5. Oligonucleotides were denatured using a heating ramp from 20−90 °C at 10 °C/min (held at 90 °C for 10 min). Sequences were annealed by cooling from 90−12 °C at 0.5 °C/min (held for 2 min at every 0.5 °C). UV melting curves were then recorded at 260 nm upon heating from 12–90 °C at 0.5 °C/min (held for 10 min at 90 °C). Melting curves were recorded for three consecutive heating cycles. Non-clicked controls are shown in Supplementary S4, Table S6.

Triplex Formation and Nuclease Activity

PAGE gels were prepared using an Invitrogen SureCast™ system and reagents. All PAGE reactions followed the general procedure unless otherwise stated. Cleavage reactions were performed with Cu^{2+} and ascorbate present, while triplex formation experiments were conducted in the absence of both. TFOs (12.5 pmol) were incubated with Cu^{2+} ($CuSO_4$) for 30 min at 37 °C and then added to a solution containing the target duplex (1.25 pmol) in the presence or absence of ascorbate (12.5 nmol). All samples were prepared in a final volume of 5.5 µL using 10 mM phosphate buffer with 150 mM NaCl and 2 mM $MgCl_2$ (pH 6.0). Unless otherwise stated, experiments with varying ratios of target duplex (D) and TFO, the relative amount of Cu^{2+} and ascorbate were kept to 1 and 100 eq of TFO, respectively (i.e. D:TFO:Cu^{2+}:ascorbate = 1:X:X:100X). Cleavage experiments involving on- (D_{ON}) and off-targets (D_{OFF}) were conducted in a similar manner to the cleavage reactions mentioned above. For TFO hybrids containing ligand **E**, samples were prepared at a ratio of 1:10:20:1000 D:TFO:Cu^{2+}:ascorbate. Reactions were incubated at 37 °C for either 6, 12 or 24 h and then loaded onto a 22% PAGE gel (50 mM Tris acetate, 150 mM NaCl, 5 mM $MgCl_2$) with 6× loading dye and subjected to electrophoresis for 14 h at 50 V in 50 mM tris-acetate running buffer (pH 6.0). Gels were soaked in a SybrGold solution for 30 min and visualised using a Syngene G:Box Mini 9 system.

Site-selective cleavage with fluorescently labelled DNA

The target duplex D2 was appropriately fluorescently-functionalised to probe site-selective DNA damage. The template strands S1 and S2 of the fluorescently-tagged strands of target duplex were labelled at the 5'-position with FAM and with Cy3 respectively. Corresponding fragment probes of DNA damage were also rationally designed (F1 and F2) based on the proposed cleavage site giving FAM- and Cy3-labelled sequences as fragmentary products of S1 and S2, all of which were purchased from Sigma Aldrich Ltd (S1: 5'-[6FAM]AACGTGGCGGAAAGGAAGGGAAGAAAGCGA-3'; S2: 5'-[Cy3]TCGCTTTCTTCCCTTCCTTTCTCGCCACGTT-3'; F1: 5'-[6FAM] AACGTGGCG-3'; F2: 5'-[Cy3]TCGCTTTCTTCCCTTCCTTTCT-3'). Target strands (S1 and S2, 10 pmol) were annealed and treated with varying amounts TFO (D:TFO:Cu^{2+}:ascorbate = 1:X:2X:1500X, where X =

1-25 eq). All reactions were carried out as stated above with 40 h incubation. The samples were loaded onto 22.5% denaturing PAGE gel (1× TBE with 7.4 M urea, pH 8.3) and subjected to electrophoresis for 1.5 h at 15 mA in 1× TBE buffer.

Data analysis

Melting curves were plotted in GraphPad Prism V6.0 and interpolated to sigmoidal curve models, with thermal melting values obtained from first derivatives (where n = 3 ± S.D.). The extent of triplex formation was quantified with cleaved DNA calculated by band densitometry using Syngene GeneTools analysis software and mean ± S.D. (n = 3) were plotted in GraphPad Prism V6.0. For statistical analysis, comparisons between datasets were analysed with two-way ANOVAs in GraphPad Prism. Differences between means were analysed post-hoc with Tukey's test at 95% confidence level. Differences between groups were considered to be statistically significant if $P \leq 0.05$ ($^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$).

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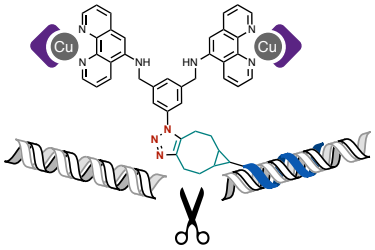

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