

Oxidative DNA Cleavage with Clip-Phenanthroline Triplex Forming Oligonucleotide Hybrids

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Abstract: We report a systematic study of several new types of hybrids of Cu-chelated Clip-phenanthroline artificial metallonuclease (AMN) with triplex forming oligonucleotides (TFO) for sequence-specific cleavage of double stranded DNA. The synthesis of these AMN-TFO hybrids is based on application of the alkyne-azide cycloaddition click reaction (CuAAC) in the key step. The AMN was attached through different linkers at either the 5'- or 3'-end or in the middle of the TFO stretch. The diverse hybrids efficiently formed triplexes with the target purine-rich sequence and their Cu-complexes were studied for their ability to cleave dsDNA in the presence of ascorbate as reductant. In all cases, we studied the influence of the nature and length of the AMN-TFO, time, conditions and amounts of ascorbate and found optimum conjugates and procedure which gave reasonably efficient (up to 34%) cleavage of the target sequence while rendering an off-target dsDNA intact. The footprint of the cleavage on PAGE was identified only in one case with low conversion meaning that the cleavage does not proceed with single nucleotide precision. On the other hand, these AMN-TFO hybrids are useful for selective degradation of target dsDNA sequences. Future improvements to this design may provide higher resolution and selectivity.

Introduction

Sequence-specific double stranded DNA (dsDNA) cleavage followed by non-homologous end joining or homologous recombination repair can be used for gene editing. Several types of nucleases, e.g. zinc finger nucleases (ZFNs)^[1,2] or transcription activator-like effector nucleases (TALENs)^[3-5] have been studied and applied for DNA cleavage, but in the last decade, the much more specific and reliable CRISPR-Cas9 technologies have dominated the field and are now the gold standard for gene editing. The system relies on a single-guide-RNA (sgRNA) bound to a Cas protein, containing two active nuclease domains, each cutting a strand of the target DNA.^[6-10] Although the CRISPR-Cas9 technologies are very powerful and have been successfully

applied in vivo, non-enzymatic chemical approaches to sequence-specific DNA cleavage are an attractive alternative with potential in therapy.

Artificial metallonucleases (AMNs) have also attracted great attention as tools to introduce dsDNA breaks but sequence specificity remains a major challenge. Diverse metal complexes including iron(II),^[11-16] cerium(IV),^[17] zirconium (IV),^[18] rhenium (I),^[19] rhodium (II),^[20] manganese^[21] and others were linked to modified oligonucleotides (ONs) or to minor-groove binders and were shown to cleave DNA with certain selectivity, which was mostly far below any useful level. Copper(I) complexes with diverse ligands are known to be excellent DNA cleaving agents. Back in 1979, Sigman et al. reported that a bis 1,10-phenanthroline (Phen) cupric complex $[\text{Cu}(\text{Phen})_2]^{2+}$ was able to induce dsDNA cleavage in the presence of molecular oxygen and an external reductant, such as a thiol or ascorbate.^[22,23] $[\text{Cu}(\text{Phen})_2]^+$ reversibly binds to DNA in the minor groove by intercalation and directs the oxidative damage to the sugar backbone of DNA, mainly to the C1' position^[24,25] and, in lower amounts, to the C4' and C5' positions.^[26-28] Cu^{II} -Phen^[29-32] have been attached to short oligonucleotides, to selectively target longer ssDNA containing the complementary sequences, achieving DNA oxidative damage in close proximity to the cutting agent.

To keep the favoured stoichiometry of two Phen ligands bound per Cu ion, which delivers high efficacy DNA cleavage compared to the mono Phen complex, some clamped phenanthroline ligands (Clip-Phen) have been designed and studied, including 2-Clip-Phen (to which we will refer to as Clip-Phen)^[33] and 3-Clip-Phen.^[34] As a consequence of enhanced stability, the oxidative nuclease activity of their Cu^{I} complexes is higher than that of $[\text{Cu}(\text{Phen})_2]^+$; two times higher for Clip-Phen, and higher by a factor of 60 for 3-Clip-Phen.^[35] More recently, the Cu^{II} -Clip-Phen complexes were coupled to minor groove binders or intercalators in order to direct the cleavage to specific regions of dsDNA, but the selectivity was only moderate.^[30,32,36-39]

Here we report a new design of specific DNA cutters based on triplex-forming oligonucleotides (TFOs) coupled to the Clip-Phen ligand at different positions, using click chemistry. TFOs are short oligonucleotides able to bind a polypurine stretches of DNA duplex in its major groove through Hoogsteen base pairs.^[39] The only known examples of TFO-AMN conjugates were based on FeII-EDTA^[11] or Cu(I)-Phen^[29,40,41] linked to the 5' end of a homopyrimidine probe able to form a triplex with the target dsDNA and produce sequence-specific cleavage patterns, with efficiency depending on probe concentration, pH, cation concentration and number of mismatches in the TFO for triplex formation. The higher stability, but also cleaving activity, of Cu-Clip-Phen with respect to Cu-Phen drove our interest towards the possibility to tether this complex to a TFO, for the synthesis of a new DNA-targeting molecular scissors. We envisaged attaching this complex to the TFO probe either at the 5'- or 3'-end or in the middle of the strand (linked to a nucleobase) and designed several linkers of differing

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length, flexibility and polarity. The synthetic strategy relied on the copper catalyzed azide-alkyne cycloaddition, such that different alkyne-modified ONs could be efficiently coupled to an azido-linked Clip-Phen chemical nuclease core.

TFO containing two mismatches, were designed to target this sequence through triple helix formation (for sequences see Figure 1D and Table S1, Supporting Information). We displaced dG nucleotides in the TFOs against the thymidine inversion sites, in

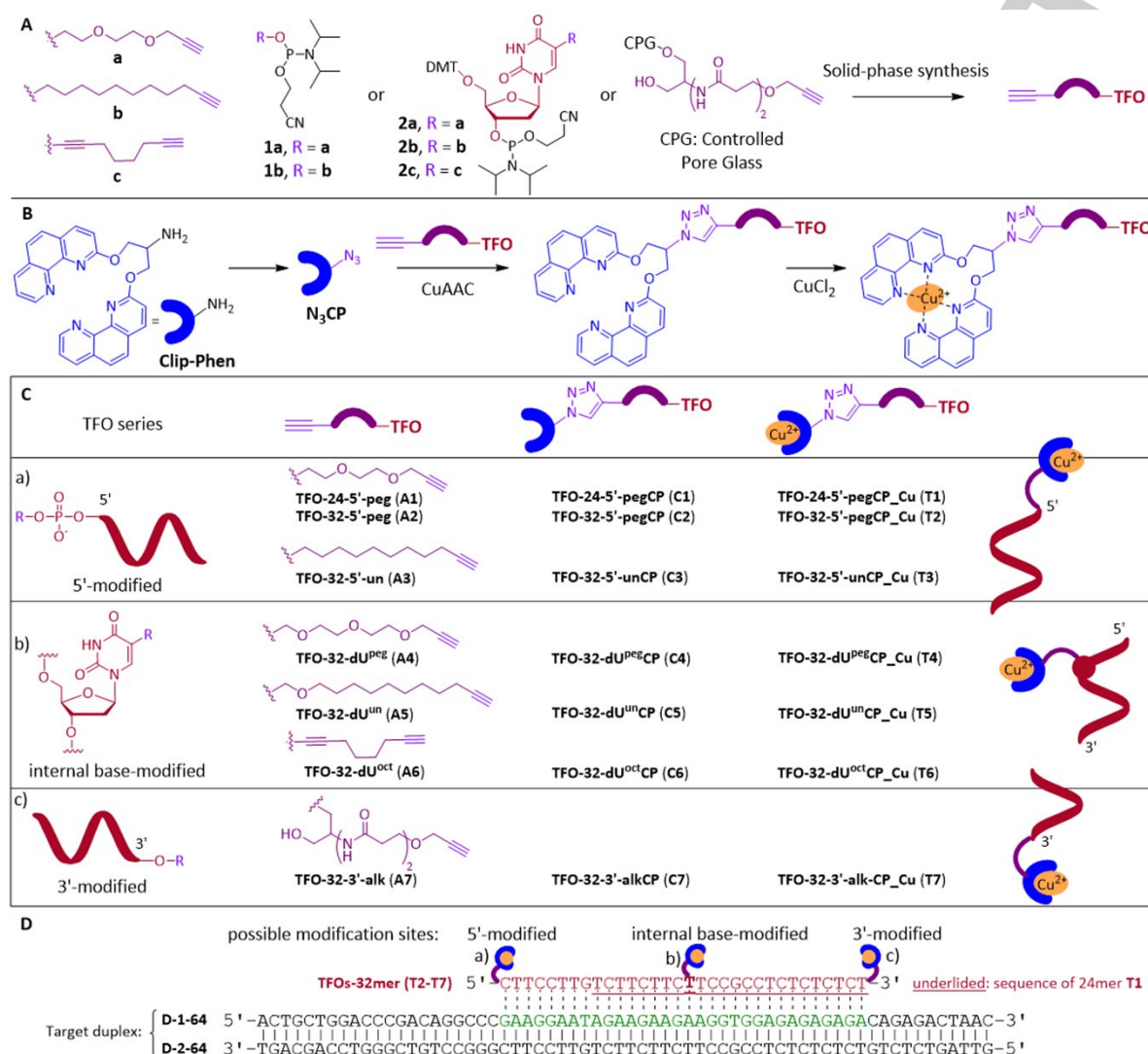


Figure 1. A: Reagents employed in the solid-phase synthesis of alkynyl-modified TFOs A1-A7. B: general scheme of synthesis of Cu-Clip-Phen-modified TFOs T1-T7. C: series of synthesized TFOs carrying the modification at a) the 5' terminal position, b) an internal uridine nucleotide, and c) the 3' terminal. D: Sequences of the employed oligonucleotides; red: TFOs T2-T7, underlined: sequence of the shorter 24mer T1; green: TFO recognition sequence on the polypurine strand of the target duplex.

Results

Design and synthesis of the TFO-AMN conjugates.

As a target DNA duplex for the TFO-AMN molecular scissors, we selected a purine rich segment of the Env-gene, in the HIV-1 genome (sequence in green in Figure 1D), suitable for parallel triplex formation with a third pyrimidine-rich strand. Specifically this was a 32-mer sequence containing only two thymidine nucleotides, which generate mismatches upon triplex formation. A 24-mer TFO containing a single mismatch, as well as a 32-mer

order to minimize the binding destabilization of the third strand, resulting in the highest triplex melting temperatures.^[42,43]

To attach the Clip-Phen moiety to the TFOs, we designed a convenient approach in which the key step consists of a CuAAC^[44-46] between an alkynyl-modified TFO and an azido derivative of Clip-Phen (N₃CP, Figure 1A-B). Alkynyl-modified TFOs were prepared by standard solid-phase synthesis.^[47] To study the influence of the length and polarity of the linker, as well as the position of the modification within the TFO sequences, we designed and synthesized a range of different alkyne-linked ONs. The modifications at the 5'-terminus were attached through a phosphodiester linkage using phosphoramidites **1a-b** in solid-phase ON synthesis to give the desired ONs **A1-A3**. Phosphoramidites **1a** and **1b** were easily prepared in good yields by reacting the relative alcohol carrying the acetylene moiety (2-[2-(prop-2-yn-1-yloxy)ethoxy]ethan-1-ol or undecyn-1-ol) with 2-cyanoethyl-*N,N*-diisopropylchloro-phosphoramidite in presence

of diisopropylethylamine in dichloromethane. To insert internal base-modifications into TFOs **A4** and **A5**, we employed alkynyl-linked thymidine phosphoramites **2a-b**, previously prepared in our lab.^[48] Similarly, TFO **A6** bearing a shorter octadiynyl group was prepared using commercially available octadiynyl-dU phosphoramidite **2c**. For attachment of modifications at the 3' position (through an ether bond) a commercially available alkynyl-CPG was employed in the solid-phase synthesis (Figure 1).

Clip-phen ligand was prepared as previously reported by Pitie et al.^[49] The reaction between Clip-Phen and 1-*H*-imidazole-1-sulfonyl azide, used as a diazo transfer reagent,^[50] provided the azido-modified artificial nuclease **N₃CP** in good yield. CuAAC reactions of **N₃CP** with ONs **A1-A7** provided Clip-Phen-modified TFOs (**C1-C7**). Reactions were performed in a water, DMSO and tert-butanol mixture under a strictly anaerobic atmosphere, and in the absence of sodium ascorbate, to avoid oxidative damage of the ONs in the presence of the AMN **N₃CP**. We employed CuOAc (due to its higher solubility) and TBTA as a ligand to stabilize Cu(I) in solution. The addition of an excess of EDTA served to quench reactions and to displace Cu from the Clip-Phen moiety. Using membrane-based spin columns for the purification of the resulting ONs **C1-C7**, it was possible to wash the EDTA-Cu complexes away, and elute products, without need of any further purification. Resulting Clip-Phen-TFOs were characterized by MALDI-TOF mass spectrometry and no traces of the starting ONs **A1-A7** were found in the spectra, suggesting a quantitative conversion of CuAAC on all the employed substrates. Final yields were in the range 30-45% in all cases and were calculated based on the concentration of the clicked oligonucleotides solutions, measured through absorbance at 260 nm. We attributed the significant loss of yield to the inefficiency of the spin-column purification step. The final step to reach the desired DNA-targeting TFOs (**T1-T7**) involved a Cu²⁺ cation complexation, accomplished by adding one equiv. of CuCl₂ to Clip-Phen-TFOs (**C1-C7**) (Figure 1B). In order to avoid long exposure of the synthesized TFOs to potential damage caused by Cu ions, the addition of CuCl₂ always preceded triplex formation with the target duplex by only short periods of time, typically 1 hour. The Cu-chelated ONs **T1-T7** were used for DNA cleavage experiments without further purification or characterization.

Triplex formation and UV melting temperatures.

Thermal melting experiments were conducted to prove the synthesized TFOs to efficiently target the target duplex. Since parallel DNA triplexes are formed through Hoogsteen base pairing, an acidic pH is required for cytidine N-3 protonation. Cations, and especially divalent cations such as Mg²⁺, are known to contribute to DNA triplex stabilization.^[51] Therefore, we obtained triplexes by annealing synthesized TFOs with the target duplex containing the recognition sequence for the TFOs (Figure 2A), in 10 mM phosphate buffer (pH 6.2) containing 250 mM NaCl and 1 mM MgCl₂. To prove that the annealing between all TFOs and the target duplex successfully occurred, we characterized the resulting triplexes by determining UV melting temperatures (*T_M*). In all cases, the synthesized TFOs formed stable triplexes with *T_M* values between 24.1 and 50.2 °C (Figure 2B). As expected, short 24-mer TFOs **A1**, **C1** and **T1** showed the lowest denaturing

temperatures, although they contain a single mismatch within their sequence. Longer 32-mer TFOs possess a second mismatch, but higher stabilization is gained due to the presence of 7 additional Hoogsteen base pairs. All the 32-mer TFOs, when annealed to the target duplex produced triplexes with melting temperatures higher than 37 °C, potentially allowing in-vivo applications. In general, the Clip-Phen modified TFOs **C1-C7** were found to form more stable triplexes than their corresponding alkynyl precursors **A1-A7**, probably due to some intercalation of the 1,10-phenanthroline moiety into the target duplex. The addition of Cu²⁺ ions further helped, even though not as strongly as expected, with a small gain of 0.2 to 5.8 °C (Figure 2C). TFOs with internal modifications formed the most stable triplexes, and in particular **T6**, bearing the octadiynyl-dU modifications, gave the highest *T_M* (50.2 °C).

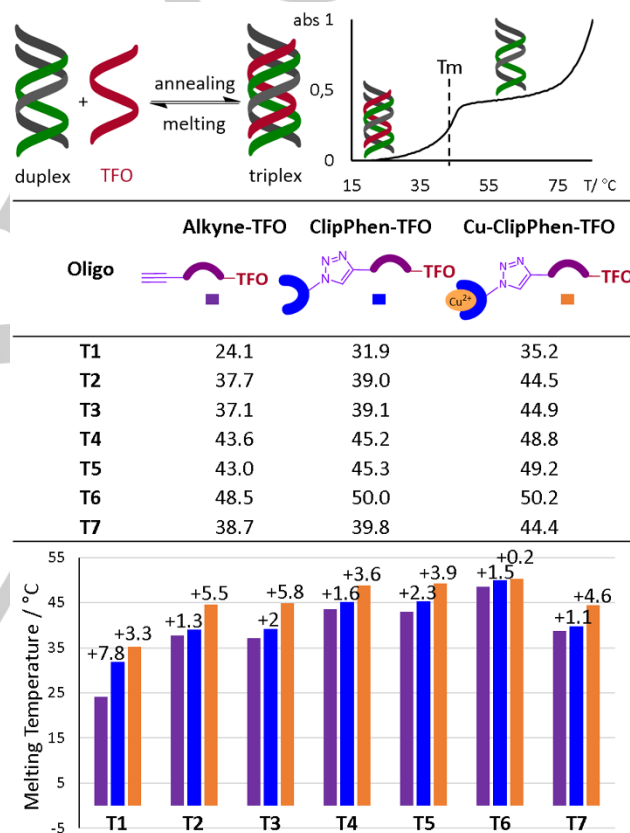


Figure 2. A: General scheme of triplex formation. B: table of triplexes UV melting temperatures. C: Chart showing how *T_M* increases when Clip-Phen, and further Cu-Clip-Phen modifications are present on TFOs.

DNA cleavage assays.

DNA cleavage experiments were performed with the AMN-modified TFOs **T1-T7** complexed to Cu²⁺. They were annealed to the target duplexes to form triplexes and then addition of Na-*L*-ascorbate activated the nuclease through reduction of Cu²⁺ to Cu⁺ (Figure 3A). Although it has been reported with a similar system that cleavage occurs only when the nuclease comes into contact with the target DNA already in presence of the reducing

environment,^[40] we found our hybrids to cleave more efficiently when sodium ascorbate was added as the last reagent, after the triplex was annealed. Therefore, we first incubated the duplex and one of the Cu-Clip-Phen-TFOs **T1-T7** at 10 °C for 1 h, then added ascorbate in a second step. All cleavage experiments were performed in phosphate buffer at pH 6.2, containing 250 mM NaCl and 1 mM MgCl₂, to ensure triplex formation, as proved through triplex melting curves. Reactions were quenched by addition of a stop solution containing EDTA, to complex Copper out of the nuclease.

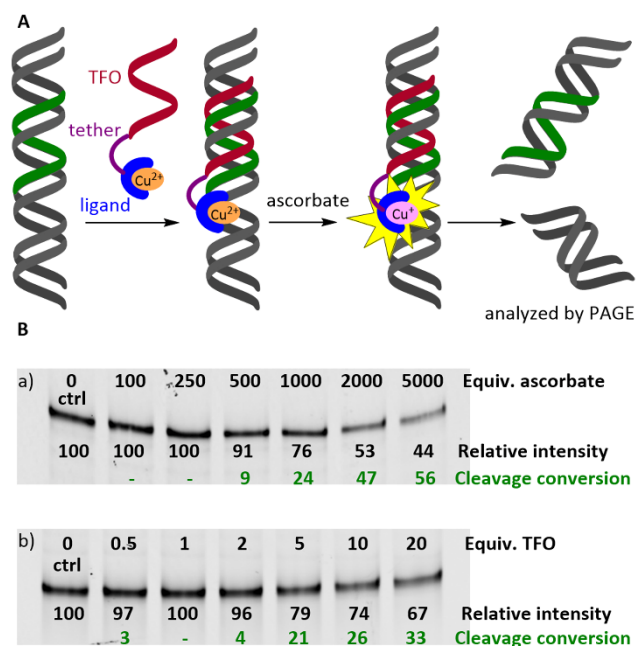


Figure 3. A: General strategy designed for triplex-directed dsDNA cleavage by TFOs **T1-T7**. **B:** Examples of denaturing PAGE analysis of preliminary screening experiments performed by incubating the triplex formed between target duplex and TFO **T4** with a) increasing equiv. of Na-L-ascorbate, or b) increasing equiv. of TFO. In both cases reaction were carried out at 25 °C for 12 h. The relative intensities of the spot are measured by densitometric analysis on gels and referenced to the control spot (triplex incubated in the absence of ascorbate (a) and TFO (b)). They constitute the percentage of un-cleaved DNA left after experiments. Conversions are calculate subtracting the relative intensity from 100.

At first, we performed a short optimization to select suitable conditions to use in the study, using **T4** as the model substrate. As the target duplex, the 64 bp 6-FAM labelled duplex obtained by annealing oligonucleotides **D-1-64-FAM** and **D-2-64** (for sequences see Table S1, Supporting Information) was employed. The duplex was labelled with fluorescent 6-FAM at the 5'-position, and in this way it was possible to follow the cleavage by denaturing PAGE. From preliminary experiments, we noticed that oxidative cleavage was slowly occurring when we incubated the triplex in the presence of ascorbate at temperatures below 25 °C, or for a short period of time (below 3 h) [data not shown]. In order to minimize possible off-target oxidative DNA damage, we investigated the possibility of using lower amounts of ascorbate and catalytic or sub-stoichiometric amounts of TFO. Unfortunately, no substantial cleavage occurred when less than 5 equiv. of **T4**

were used, but when using 5 equiv. of TFO and 1000 equiv. of ascorbate, we achieved significant cleavage (21-26%). When the amount of ascorbate was increased, cleavage was further enhanced (e.g. 56% with 5000 equiv. of ascorbate) but, at the same time, unwanted off-target damage also increased (following paragraph, Figures S11-12, Supporting Information). In a similar way, we observed rather enhanced cleavage when the target duplex was incubated with more than 5 equiv. of **T4**, although still enhancing the risk of non-specific cleavage due to high concentration of free nuclease-TFOs in solution (Figure 3B).

Cleavage selectivity towards dsDNA containing the target sequence.

To test the selectivity of our molecular scissors towards the cleavage of a selected DNA duplex containing the TFO target sequences, we designed an experiment using an equimolar mixture of the target duplex (**D**) and an off-target duplex (**off**, which does not contain the TFO recognition sequence), (Figure 4). In both **D** and **off**, one strand was labelled with fluorescent 6-FAM at the 5'-position, in order to be able to easily follow and measure the cleavage by denaturing PAGE. The off-target dsDNA **off** was 12 nucleosides shorter than **D**, leading to a good separation on gels (Table S1, Supporting Information).

Densitometric analysis of PAGE gels allowed us to calculate the percentage of cleavage (disappearing or weakening of the corresponding spot on PAGE) of each **D** and **off** independently, in comparison to a reference experiment in which neither TFO, nor ascorbate, were employed. We calculated the selectivity of cleavage as the cleavage percentage of **D**, divided by the total cleavage (cleavage of **D** + cleavage of **off**).

Starting from conditions selected during the pilot study, we screened the cleavage of all synthesized TFOs (**T1-T7**) towards **D**, aiming for the highest selectivity, ideally leaving off fully un-cleaved. The influence of several parameters, such as TFO and ascorbate concentrations, or incubation temperature and duration, was evaluated. All results are shown in Table S3 (Supporting Information) and all PAGE gels are shown in Figure S3-13, Supporting Information. In general we observed very low cleavage, of both **D** and **off**, when less than 5 equiv. of TFO, or less than 1000 equiv. of ascorbate, were employed (entries 1,2 and 10-13, Table S3, Supporting Information). On the contrary, employing 2000 or 5000 equiv. of ascorbate (entries 3 and 14-17 respectively in Table S3, Supporting Information), the systems were too active and a high percentage of off-target cleavage was recorded. We observed very similar situations when the mixture of duplexes was exposed to the TFOs for long periods of time, such as 24 or 48 h (entries 10-13 and 17,18, Table S3, Supporting Information). The best results were obtained when an equimolar mixture of **D** and **off** was incubated for 12 h at 25 °C, with 5 equiv. of TFOs **T1-T7**, and 1000 equiv. of ascorbate. Under these conditions, no detectable cleavage of the off-target duplex was observed, whereas all TFOs **T1-T7** efficiently cleaved the target duplex **D** (gel in Figure 4). The shorter 24-mer TFO **T1** was the least selective, probably due to the lower stability of the formed triplex. We did not find significant difference between the cleaving ability and selectivity of TFOs **T2-T5**. All of them were able to considerably cleave **D** to an extent of 25-30 % (with respect to the

reference control). TFO **T6** was only slightly less effective, and gave 21 % cleavage, while, surprisingly, the 3'-modified **T7** was less efficient and gave only 16% cleavage (Table in Figure 4, column: 12h, 25 °C). Raising the temperature to 37 °C only slightly influenced the results: short **T1** and octadiynyl-linked **T6** increased their cleaving ability to 20 and 35 % respectively, although at the expense of selectivity, with some off-target damage occurring. All other TFOs left **off** almost un-cleaved (Table in Figure 4, column: 12h, 37 °C). Reducing incubation time to 3 h, unfortunately did not influence the selectivity (Table in Figure 4, column: 3h, 37 °C) and, as expected, lower selectivity was observed when we incubated annealed triplexes for longer times, such as 24 h (Table in Figure 4, last column). In control experiments, the cleavage of the target sequence was performed in presence of an excess of the off-target sequence (5 or 10 equiv. of **off** with respect to **D**). The selectivity slightly decreased but still the preference in cleavage of the target duplex was maintained (Figure S16, Supporting Information).

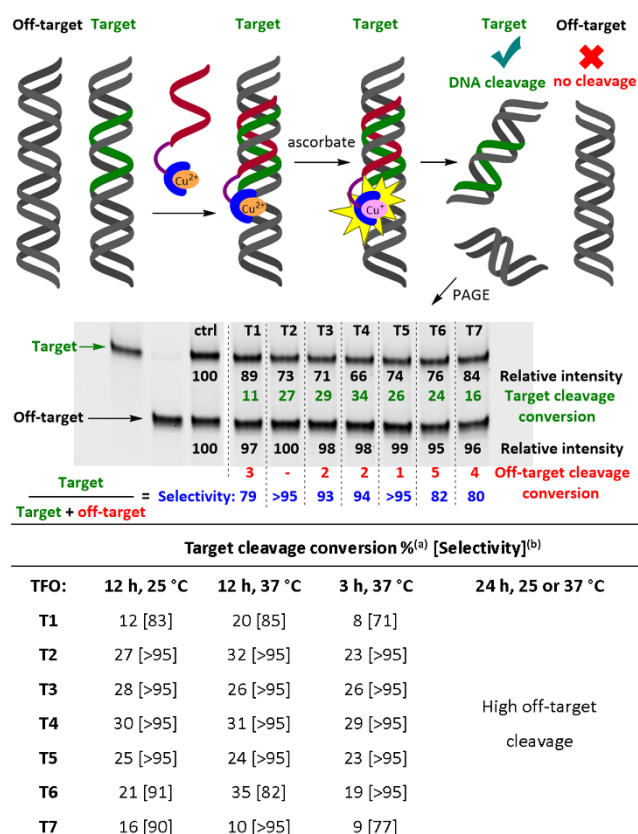


Figure 4. General scheme and example of denaturing PAGE analysis for the measurement of cleavage selectivity towards DNA containing the TFO recognition sequence, in the presence of an off-target duplex. The relative intensities of the spots on gel are measured by densitometric analysis and referenced to the control spot. They constitute the percentage of uncleaved DNA left after experiments. Conversions are calculated subtracting the relative intensity from 100. Reactions are performed employing 2 pmol of both duplexes, 5 equivalents of TFO and 1000 equivalents of Na-L-ascorbate. (a) percentage of dsDNA cleavage occurred at the target duplex with respect to the intensity of the reference spot. (b) the selectivity is calculated dividing the cleavage at the target duplex by the total cleavage (target + off-target duplexes): $\text{Selectivity} = 100 \times \frac{\text{Cleavage}(\text{target})}{\text{Cleavage}(\text{target}) + \text{Cleavage}(\text{off-target})}$

Control experiments

To prove that the presence of the nuclease is essential for dsDNA cleavage, we treated alkynyl-modified TFOs **A1-A7** with CuCl_2 , and employed them in cleavage assays, in conditions in which **T1-T7** gave high cleavage. No cleavage was detected, neither at **D**, nor at **off**, when the mixture of duplexes was treated with 5 equiv. of **A1-A7** and subsequently incubated with 1000 equiv. of ascorbate at 37 °C for 24 h (gel in Figure S14, Supporting Information). In a similar way, we have shown Clip-Phen-modified TFOs **C1-C7** to be unable to cleave dsDNA in the absence of copper. After annealing the ligand-modified TFOs with the duplex, and further treatment with ascorbate, we incubated the mixture at 37 °C for 24 h, without observing any cleavage, proving copper to be essential for nuclease activity. With these control experiments, we proved that all the reagents, i.e. the Clip-Phen-modified TFO complexed to copper, and ascorbate, are crucial to achieve dsDNA cleavage (gel in Figure S14, Supporting Information). Finally, the equimolar mixture of **D** and **off** was treated with different amounts of free Cu complex of **N₃CP** in the presence of Na-L-ascorbate, at 37 °C for 12 hours. An identical extent of oxidative cleavage occurred at both **D** and **off** in all cases, proving that the selectivity observed in previous experiments was induced by triplex formation (see Figure S17, Supporting Information).

Footprinting of cleavage products.

In order to footprint the positions of dsDNA cleavage, we needed to visualize all potential DNA fragments on PAGE.

To achieve that, we made radioactively labelled 98 bp dsDNA containing the TFO target sequence by PCR employing 1 % radiolabelled $\alpha\text{-}^{32}\text{P}$ -dATP (Figure 5A). The polymerase incorporates radioactive $\alpha\text{-}^{32}\text{P}$ -dA statistically at every position against thymine bases and, therefore, all the cleavage products are detectable on denaturing PAGE gel.

A radioactively labelled DNA ladder was also produced through PCR, using the same template but with primers designed to obtain products of 90, 85, 80, 75 and 70 bp lengths. TFO **T2**, presenting the nuclease attached to the 5' position, was found to cleave the target duplex 2 to 5 bp from the TFO recognition sequence, giving a clearly visible cleavage product of approx. 76-79 bp (Figure 5B). The full-length DNA band broadening in cleavage conditions is most probably due to oxidative ssDNA damage and alkali-labile sites, induced by exposure to the AMN.

The first control consisted of the target duplex in reaction buffer (**ctrl1** on gel), the second consisted of the target duplex treated with ascorbate and CuCl_2 (**ctrl2**), while the third control (**ctrl3**) contained target duplex treated with **T2** in the absence of Na-L-ascorbate. All controls together proved that the shorter 76-79 bp product was generated by cleavage by **T2** in the presence of ascorbate, with all the cleaving partners necessary for formation of this product.

In the tested experimental setup, as well as in those employed previously to analyze the cleavage of FAM-labelled duplexes, it was not possible to establish whether a double-strand cleavage or single-strand nicking has occurred (when we labelled each of the strands with different fluorophore, we observed only disappearance of both spots but no fragments: see Figure S18, Supporting Information). Nevertheless, in analogy to previous

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literature,^[17,25,29] we assume that the nature of the Cu-Phen AMN employed suggests formation of both ss- and dsDNA breaks (formed through two successive ssDNA breaks, should be the major cleavage pathway).

The best results were obtained when only 1 equiv. of **T2** was annealed to the target duplex, and the system treated with 1000 equiv. of ascorbate. When the cleavage was pushed to higher extent, either increasing the equivalents of TFO (gels in Figure S19, Supporting Information) or with higher amounts of ascorbate (gel in Figure S21, Supporting Information), only indiscriminate cleavage was observed, and we were not able to detect any distinct fragments. Experiments with **T4** and **T7**, carrying the nuclease at an internal position or at the 3' position respectively, did not provide distinct cleavage products (gels in Figure S20, Supporting Information).

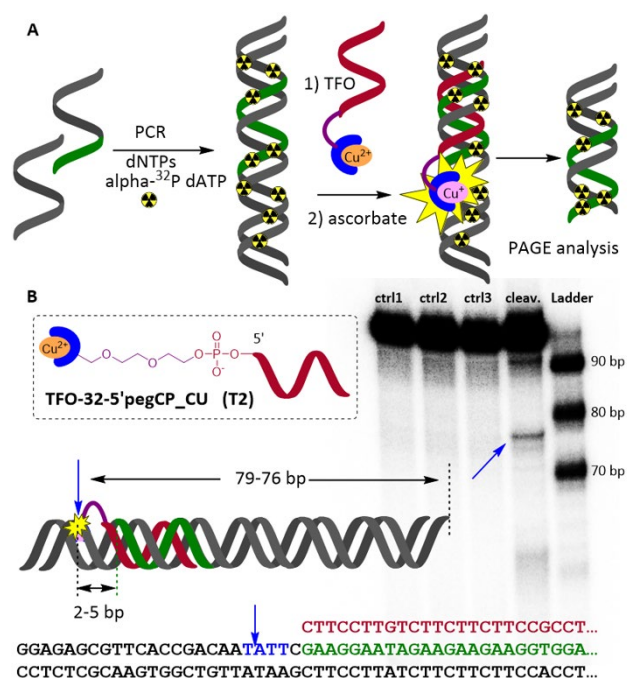


Figure 5. A: The system employed for the detection of cleavage products. B: Cleavage pattern produced by **T2** is found by denaturing PAGE analysis.

Discussion

Influence of the TFO structure on selectivity

The investigation of the influence of the length of the TFO sequence revealed that triplex stability is of crucial importance for the specificity of the cleavage. **T1** and **T2** differ only by their length and in all trials the shorter 24-mer TFO **T1** always cuts the target DNA with lower selectivity. In the reaction buffer, the triplex obtained by annealing **D** and **T1** denatures at 35.2 °C, suggesting that only slightly more than 50% of the duplex is annealed to the TFO at 37 °C (Figure S26, Supporting Information). Accordingly, cleavage experiments performed at 25 °C with **T1** showed higher selectivity, although remaining inferior to other TFOs. Interestingly, not much difference in cleavage efficiency and selectivity was

observed when comparing TFOs containing 5'-terminal or internal modifications. Indeed, 5'-modified TFOs **T2** and **T3** were found to be only slightly more efficient than base-modified **T4-T6** and this is probably due to the higher degree of freedom of the nuclease such that it can more easily reach the sugar backbone. The 3'-modified TFO **T7** displayed poor cleavage ability and low selectivity compared to the other TFOs. However, in this case the linker was different, so a direct comparison is impossible.

Influence of the nature of the linker on cleavage selectivity

The nature of the linker may lead to a modulation of the cleavage selectivity by affecting triplex stability,^[52] or providing optimal length and/or flexibility such that suitable positioning of the Clip-Phen nuclease is obtained. Internal modifications gave higher triplex melting temperatures and, among them, the octadiynyl-linked **T6** provided the highest stability. However, the lower flexibility of this shorter linker compared to the longer and fully saturated peg- and undecynyl- tethers of **T4** and **T5**, resulted in lower selectivity of **T6**. In most cases, TFOs **T2** and **T4**, bearing the AMN attached through a more polar peg linker were the most active and selective, although the differences were small.

Influence of the reaction parameters on the cleavage activity

The amount of ascorbate employed in cleavage reactions played a crucial role in the activity of our DNA-targeting nucleases and its fine-tuning was a key step in the study. When triplexes were incubated with low concentrations of ascorbate, almost no cleavage was detected, as Cu(I) would quickly re-oxidise to Cu(II) in aerobic conditions. In contrast, if high concentrations of ascorbate were used, a significant level of off-target oxidative damage was observed due to high concentration of diffusible free radicals generated (See Table S3 and Figure S15, Supporting Information).^[53,54] Similarly, sub-stoichiometric or equimolar AMN-TFO with respect to the duplex led to very little oxidative cleavage, which increased proportionally with the amount of AMN-TFO employed. In all cases the best compromise between activity and selectivity was found when 5 equiv. of AMN-TFO were used.

Temperature and time of reactions were also important parameters. Preliminary experiments showed that very low cleavage occurred at 10 °C, however, not much difference in terms of activity and selectivity was found between experiments conducted at 25 or 37 °C. Optimized incubation times were also crucial as a significant drop in selectivity was always observed when triplexes were incubated with ascorbate for more than 12 h. Footprinting experiments were performed in mild conditions (e.g. 1 equiv. of TFO and 1000 equiv. of ascorbate) and clearly showed that local site-selective cleavage is achieved only at the very beginning of the reaction, although the flexibility of the peg linker prevented specificity at the single nucleotide level. We believe a similar situation occurs when an equimolar mixture of target and off-target duplexes is subjected to the action of the nuclease-modified TFOs. Cleavage initially occurs only at the target duplex, however, after a certain extent of cleavage is achieved, discrimination between target and off-target duplex drops quickly, most probably due to the combination of higher concentration of

free AMN-TFO in solution and increased relative concentration of off with respect to D.

Conclusions

We have synthesized a range of diverse Cu-chelated Clip-phenanthroline-linked AMN-TFO hybrids and systematically studied their hybridization with target dsDNA and the efficiency and selectivity of cleavage of target versus off-target DNA. Under optimized conditions, the AMN-linked through a flexible linker to 5'-end or to an internal thymine base showed significant cleavage of the target duplex (up to 34%) and no off-target cleavage. Higher cleavage was also possible by optimizing reactions, however off-target DNA cleavage was also enhanced. In most cases, we have not observed clear footprinting cleavage bands showing that the flexible linker apparently allows cleavage at several adjacent positions. Hence, this type of AMN-TFO hybrid can be used for selective degradation of complementary target dsDNA sequence. However, cleavage does not proceed with single nucleotide precision due to diffusible free radical generation by copper. This work nonetheless shows how optimal placement of a chemical nuclease under the guidance of a TFO can induce targeted damage. Indeed, this type of targeted approach has been identified by Glazer *et al.* whereby a photoactivated crosslinking agent (psoralen) coupled to a TFO site-specifically induces mutagenesis in plasmid targets *in vitro* and in mammalian cell culture lines.^[55] At this stage, the AMN-TFO nucleases presented here do not have the specificity of enzymatic methods (i.e. CRISPR-Cas), which would be needed for their use in gene editing. On the other hand, for the damage of genomic DNA at specific loci such that it cannot be repaired, the nature of the damage caused by Cu(I) and the extent of damage could make them superior to CRISPR-Cas which is relatively easy to repair. Further optimization of the structures of AMN-TFO hybrids and investigation of other metals is needed in order to further improve the specificity and efficiency of this approach.

Experimental Section

Materials and methods

Reagents and solvents were purchased from Fluorochem, Sigma-Aldrich and AlfaAesar. CH_2Cl_2 was dried by distillation over CaH_2 . *N,N*-diisopropylethylamine (DIPEA), *N,N*-dimethylformamide (DMF) and pyridine (Py) were dried by distillation at reduced pressure over CaH_2 . Unless otherwise stated, all the reactions were performed under a positive atmosphere of argon by standard syringe, cannula and septa techniques. 2-Clip-Phenanthroline (**Clip-Phen**),^[33] 1-*H*-imidazole-1-sulfonyl azide,^[50] phosphoramidites **2a** and **2b**,^[48] and 2-[2-(prop-2-yn-1-yloxy)ethoxy]ethan-1-ol^[56] were prepared as described previously. The reactions were monitored by thin-layer chromatography (TLC) using Merck silica gel 60 F₂₅₄ plates and visualized by UV (254 nm). Column chromatography was performed using silica gel (40–63 μm). Reverse-phase high-performance flash chromatography (HPFC) purifications were

carried out on a Biotage SP1 apparatus with C-18 columns. TFO **A6** and all natural and FAM-labelled DNA duplexes, primers and templates were purchased from Generi biotech, Czech Republic. NMR spectra were measured on a Bruker AVANCE 600 III HD (^1H at 600.1 MHz, ^{13}C at 150.9 MHz), Bruker 500 III HD (^1H at 500.0 MHz, ^{13}C at 125.7 MHz) and Bruker Avance 400 III HD (^{31}P at 162.3 MHz) in $\text{DMSO}-d_6$ or CD_3CN solutions at 25 °C. Chemical shifts (in ppm, δ scale) were referenced to the residual solvent signal in ^1H spectra or to the solvent signal in ^{13}C spectra. Coupling constants (*J*) are given in Hz. The complete assignment of ^1H and ^{13}C signals (for numbering see Supporting Information) was performed by an analysis of the correlated homonuclear H,H-COSY, and heteronuclear H,C-HSQC and H,C-HMBC spectra. High resolution mass spectra were measured on a LTQ Orbitrap XL spectrometer (Thermo Fisher Scientific). IR spectra were measured on a Bruker Alpha FT-IR spectrometer using attenuated total reflection (ATR).

Synthesis of **N₃CP**.

2-Clip-phenanthroline (**Clip-Phen**, 200mg, 0.447 mmol), 1-*H*-imidazole-1-sulfonyl azide (141 mg, 0.670 mmol), K_2CO_3 (46.6 mg, 0.335 mmol) and CuSO_4 pentahydrate (1.3 mg, 0.005 mmol) were stirred in MeOH (5 mL) at room temperature for 12 hours under aerobic atmosphere. After evaporation of the solvent under reduced pressure, the crude product was purified by flash chromatography (Eluent EtOAc : MeOH 9:1) to afford a yellowish solid. The obtained solid was suspended in hot MeOH and stirred for 20 min, after filtration **N₃CP** was recovered as a pale yellow solid (118 mg, 56%). m.p.: 96.1 – 98.9 °C with decomposition. ^1H NMR (500.0 MHz, $\text{DMSO}-d_6$): 4.68 (tt, 1H, $^3J = 6.7, 4.2$, CHN); 4.93 (dd, 1H, $^2J = 11.4, ^3J = 6.7$, $\text{CH}_a\text{H}_b\text{O}$); 5.01 (dd, 1H, $^2J = 11.4, ^3J = 4.2$, $\text{CH}_a\text{H}_b\text{O}$); 7.33 (d, 2H, $J_{3,4} = 8.7$, H-3); 7.73 (dd, 2H, $J_{8,7} = 8.1, J_{8,9} = 4.3$, H-8); 7.87 (d, 2H, $J_{6,5} = 8.7$, H-6); 7.96 (d, 2H, $J_{5,6} = 8.7$, H-5); 8.44 (d, 2H, $J_{4,3} = 8.7$, H-4); 8.46 (dd, 2H, $J_{7,8} = 8.1, J_{7,9} = 1.8$, H-7); 9.10 (dd, 2H, $J_{9,8} = 4.3, J_{9,7} = 1.8$, H-9). ^{13}C NMR (125.7 MHz, $\text{DMSO}-d_6$): 59.32 (CHN); 65.59 (CH_2O); 113.44 (CH-3); 123.21 (CH-8); 124.40 (CH-6); 125.07 (C-4a); 126.42 (CH-5); 129.02 (C-6a); 136.32 (CH-7); 140.22 (CH-4); 143.75 (C-10b); 144.62 (C-10a); 149.90 (CH-9); 161.60 (C-2). HRMS calc. for $\text{C}_{27}\text{H}_{20}\text{O}_2\text{N}_7$: 474.16730, found: 474.16762. The IR spectrum is shown at page S48, Supporting Information.

Synthesis of phosphoramidite **1a**.

2-[2-(Prop-2-yn-1-yloxy)ethoxy]ethan-1-ol (238 mg, 1.651 mmol) was co-evaporated three times with anhydrous pyridine and dissolved in 5 mL of anhydrous CH_2Cl_2 . Freshly distilled DIPEA (2.5 equiv., 4.127 mmol, 719 μL) was added, followed by 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (1.2 equiv, 1.981 mmol, 442 μL), and the reaction mixture was stirred at room temperature for 1.5 hours. The crude product was diluted with anhydrous CH_2Cl_2 (50 mL), quickly washed with saturated aqueous KCl (20 mL) and dried over Na_2SO_4 . Flash chromatography (cyclohexane : EtOAc 1:3) provided the desired compound as a clear oil (332 mg, 59%). ^1H NMR (600.1 MHz, CD_3CN): 1.14 – 1.19 (m, 12H, $(\text{CH}_3)_2\text{CH}$); 2.60 – 2.68 (m, 2H, H-14); 2.69 (t, 1H, $J_{1,3} = 2.5$, H-1); 3.56 – 3.64 (m, 8H, H-5,6,8,

(CH₃)₂CH); 3.65 – 3.71 (m, 1H, H-9b); 3.72 – 3.84 (m, 3H, H-9a,13); 4.15 (d, 2H, $J_{3,1}$ = 2.5, H-3). ¹³C NMR (150.9 MHz, CD₃CN): 20.90 (d, $J_{C,P}$ = 6.8, CH₂-14); 24.86, 24.88 (d, $J_{C,P}$ = 7.3, (CH₃)₂CH); 43.68 (d, $J_{C,P}$ = 12.4, (CH₃)₂CH); 58.61 (CH₂-3); 59.31 (d, $J_{C,P}$ = 18.9, CH₂-13); 63.51 (d, $J_{C,P}$ = 17.3, CH₂-9); 69.80 (CH₂-5); 70.86 (CH₂-6); 71.77 (d, $J_{C,P}$ = 7.5, CH₂-8); 75.58 (CH-1); 80.80 (C-2); 119.39 (C-15). ³¹P{¹H} NMR (162.3 MHz, CD₃CN): 150.70. HRMS (ESI⁺): calculated for C₁₆H₃₀O₄N₂P = 345.19377; found: 345.19384. Calculated for C₁₆H₂₉O₄N₂NaP = 367.17572; found: 367.17572.

Synthesis of phosphoramidite 1b.

10-Undecyn-1-ol (320 mg, 1,902 mmol) was co-evaporated three times with anhydrous pyridine and dissolved in 5 mL of anhydrous CH₂Cl₂. Freshly distilled DIPEA (2.5 equiv., 4.754 mmol, 828 μ L) was added, followed by 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (1.2 equiv, 2.282 mmol, 510 μ L) and the reaction mixture was stirred at room temperature for 1.5 hours. The crude product was diluted with anhydrous CH₂Cl₂ (50 mL), quickly washed with saturated aqueous KCl (20 mL) and dried over Na₂SO₄. Flash chromatography (cyclohexane : EtOAc 85:15) provided the desired compound as a clear oil (526 mg, 75%). ¹H NMR (600.1 MHz, CD₃CN): 1.16, 1.17 (2 \times d, 2 \times 6H, (CH₃)₂CH); 1.27 – 1.41 (m, 10H, H-5,6,7,8,9); 1.44 – 1.53 (m, 2H, H-4); 1.54 – 1.61 (m, 2H, H-10); 2.11 (t, 1H, $J_{1,3}$ = 2.7, H-1); 2.16 (td, 2H, $J_{3,4}$ = 7.1, $J_{3,1}$ = 2.7, H-3); 2.57 – 2.68 (m, 2H, H-16); 3.54 – 3.68 (m, 4H, H-11, (CH₃)₂CH); 3.69 – 3.83 (m, 2H, H-15). ¹³C NMR (150.9 MHz, CD₃CN): 18.73 (CH₂-3); 20.98 (d, $J_{C,P}$ = 6.9, CH₂-16); 24.90 (d, $J_{C,P}$ = 7.3, (CH₃)₂CH); 26.59, 29.23, 29.37, 29.68, 29.89, 30.12 (CH₂-4,5,6,7,8,9); 31.89 (d, $J_{C,P}$ = 7.2, CH₂-10); 43.65 (d, $J_{C,P}$ = 12.3, (CH₃)₂CH); 59.13 (d, $J_{C,P}$ = 19.0, CH₂-15); 64.26 (d, $J_{C,P}$ = 17.2, CH₂-11); 69.48 (CH-1); 85.33 (C-2); 119.31 (C-17). ³¹P{¹H} NMR (162.3 MHz, CD₃CN): 149.60. HRMS (ESI⁺): calculated for C₂₀H₃₇O₂N₂P = 369.26654; found: 369.26666. Calculated for C₂₀H₃₈O₂N₂NaP = 391.24849; found: 391.24862.

Solid-phase synthesis and purification of alkynyl-TFOs (A1-A7).

Solvents and reagents for the solid-phase synthesis of oligonucleotides were purchased from Link Technologies, Sigma-Aldrich and Thermo Fisher-Scientifics. Natural phosphoramidites were purchased from Sigma-Aldrich. Modified alkyne-CPG for the synthesis of 3'-alkynyl TFOs was purchased from Baseclick GmbH. Modified TFOs **A1-A5** and **A7** were synthesized through standard phosphoramidite chemistry with an automated DNA synthesizer (ABI 3400, Applied Biosystems) employing the modified amidites **1a-b** and **2a-b**, or alkyne-CPG on the solid support. Deprotections were carried out by incubating the reaction products in 30% aqueous NH₃ at 55 °C for 6 hours. Purification of prepared oligonucleotides was performed using semi-preparative HPLC (Waters modular HPLC system) on a column packed with 10 μ m C18 reversed phase (Phenomenex, Luna C18 (2) 100 Å), using a linear gradient of ACN (3.5→40%) in 0.1 M TEAA buffer. The products were analyzed by MALDI-TOF MS; spectra are reported in Figures S45-50, Supporting Information. Concentration of DNA solutions was calculated using A260

values measured on a Cary100 Bio UV-Vis spectrophotometer (Varian). Sequences are shown in Table S1, Supporting Information.

CuAAC reaction of alkynyl-TFOs and N₃CP.

To 50 μ L of a water solution of the starting oligonucleotide (100 μ M, 5 nmol) were added 25 μ L of a DMSO/ ^tBuOH 3:1 mixture, 5 μ L of a DMSO solution of N₃CP (5 mM, 5 equiv.) and 25 μ L of a freshly prepared DMSO solution of CuOAc (500 μ M) and TBTA (2.5 mM). The reaction mixture was stirred overnight at room temperature, and an aqueous solution of EDTA (100 μ L, 100 mM) was added to chelate the copper. The mixtures was freeze-dried, and the crude solid was re-suspended in 100 μ L of milli-Q water to be purified on QIAQUICK Nucleotide Removal kit. The products (ONs **C1-C7**) were analyzed by MALDI-TOF MS (spectra in Figures S51-57, Supporting Information).

UV triplex melting curves.

Melting curves of DNA triplexes were recorded on a Cary 100 Bio UV-Vis spectrophotometer (Varian), in 1 cm path-length quartz cells, monitoring absorbance at 260 nm while slowly increasing temperature.^[57] All the solutions of duplexes or triplexes were prepared in 10 mM phosphate buffer, containing 250 mM NaCl and 1 mM MgCl₂ (pH 6.2). In a typical experiment, an equimolar solution of target duplex and TFO (1 μ M each) was pre-annealed by incubation at 10 °C for 1 hour. For measurements of Cu-Clip-Phen modified TFOs **T1-T7**, solutions contained also 1 μ M CuCl₂. The melting run was performed with a temperature increase of 1 °C/min, in the range of 15 °C - 85 °C. After each run, samples were cooled back to 15 °C with a rate of 0.2 °C/min. The denaturation-annealing process was repeated 3 times. UV melting curves are shown in Figures S23-44, Supporting Information.

DNA cleavage assays.

All stock solutions of duplexes, TFOs or triplexes were prepared in 10 mM phosphate buffer, containing 250 mM NaCl and 1 mM MgCl₂ (pH 6.2). In a typical experiment, 2 μ L of a 1:1 mixture of FAM-labelled target duplex (**D**, 1 μ M, 2 pmol) and FAM-labelled off-target duplex (**off**, 1 μ M, 2 pmol) were incubated for 1 h at 10 °C, in the presence of a premixed solution of Clip-Phen-modified TFO (**C1-C7**, 1 μ L, concentration in dependence of the equiv. needed, see Table S3, Supporting Information) and CuCl₂ (always 1 equiv. with respect to the TFO). A solution of Na-L-ascorbate (1 μ L, concentration in dependence of the equiv. needed, see Table S3) in buffer, was added and the mixture was incubated at the desired temperature for the desired time (see Table S3). Control experiments were performed testing: A) a lack of copper (control **Cu**) in the presence of Na-L-ascorbate and Clip-Phen-modified TFO (**T2** was chosen for the control, as being one of the most active); B) a lack of Na-L-ascorbate (control **Asc**) in the presence of Clip-Phen-modified TFO and CuCl₂; C) a lack of TFO (control **TFO** or **ctrl**) in the presence of Na-L-ascorbate and CuCl₂. Reactions were quenched with 10 μ L of a stop solution containing 80% (v/v) formamide, 100 mM EDTA, 0.025% (w/v) bromophenol blue, 0.025% (w/v) xylene cyanol in water and analyzed by denaturing polyacrylamide gel electrophoresis

(PAGE). Cleavage selectivity was calculated based on the target duplex spot intensity, with respect to the off-target duplex one. PAGE gels are shown in Figures S1-13, Supporting Information. Some experiments were repeated using the same procedure on a 1:5 **D:off** mixture (Figure S16 A and B, Supporting Information) or a 1:10 **D:off** mixture, to test the selectivity in the presence of excess of off-target DNA. To avoid a large error in densitometric analysis, due to too low intensity of the **D** spot with respect to the **off** spot, only 10% of **off** was FAM-labelled (Figure S16 C and D, Supporting Information).

Cleavage activity of alkynyl-TFOs (A1-A7) and Clip-Phen-TFOs (C1-C7).

All stock solutions of duplexes, TFOs or triplexes were prepared in 10 mM phosphate buffer, containing 250 mM NaCl and 1 mM MgCl₂ (pH 6.2). 2 µL of a 1:1 mixture of FAM-labelled target duplex (**D**, 1 µM) and FAM-labelled off-target duplex (**off**, 1 µM) were incubated for 1 h at 10 °C in the presence of A) a premixed solution of alkynyl-modified TFOs (**A1-A7**, 1 µL, 10 µM, 5 equiv.) and CuCl₂ (1 equiv. with respect to the TFO); B) in the presence of Clip-Phen-modified TFOs (**C1-C7**, 1 µL, 10 µM, 5 equiv.) in the absence of CuCl₂. Na-L-ascorbate (1 µL, 2 mM, 1000 equiv.) was added and the mixture was incubated at 37 °C for 12 h. Denaturing PAGE gels are shown in Figure S14, Supporting Information.

PCR synthesis of radioactively labelled 98-bp target duplex.

The PCR mixture (50 µL) contained KOD XL DNA polymerase (1 U) natural dNTPs (32 µM), α-³²P dATP (0.25 mCi, 0.33 µM), primers **PrimFOR** and **PrimREV** (2 µM), template **TempPCR** (20 nM, for sequences of primers and template see Table S1, Supporting Information) in KOD XL polymerase reaction buffer supplied by the manufacturer). After preheating at 94 °C for 2 min, 24 PCR cycles were run under the following conditions: denaturation at 94 °C for 30 sec, annealing at 49 °C for 30 sec, extension at 72 °C for 30 sec, followed by final extension step at 72 °C 30 sec. PCR products were purified by QIAQUICK PCR purification kits. PCR optimization was performed in the absence of α-³²P dATP. DNA concentrations were measured on non-radioactively labelled PCR products, assuming them to be identical to the radioactively labelled PCR products obtained with the same protocol. For the synthesis of the radioactively labelled DNA ladder, primers Prim90, Prim85, Prim80, Prim75 and **Prim 70** were employed instead of PrimFOR, using the same template and conditions.

DNA cleavage assays employing the radioactively labelled target duplex.

All stock solutions of duplex and TFOs were prepared in 10 mM phosphate buffer, containing 250 mM NaCl and 1 mM MgCl₂ (pH 6.2). In a typical experiment 2.5 µL of target duplex (120 ng/µL, 5 pmol) were incubated for 1 h at 10 °C, in the presence of a premixed solution of Clip-Phen-modified TFO (**C1-C7**, 1 µL, concentration dependent on the equiv. needed, see gels in Figures S19-22), and CuCl₂ (always 1 equiv. with respect to the TFO). A solution of Na-L-ascorbate (1 µL, concentration dependent on the equiv. needed) in buffer, was added and the

mixture was incubated at 25 °C for 12 h. The reactions were quenched by adding 10 µL of a stop solution containing 80% (v/v) formamide, 100 mM EDTA, 0.025% (w/v) bromophenol blue, 0.025% (w/v) xylene cyanol in water. The reactions were analyzed by polyacrylamide electrophoresis; Denaturing PAGE gels are shown in Figures S19-22, Supporting Information.

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Conflicts of Interest

The authors declare no conflict of interest.

Keywords: DNA cleavage • nucleases • oligonucleotides • CuAAC click reactions • Cu complexes

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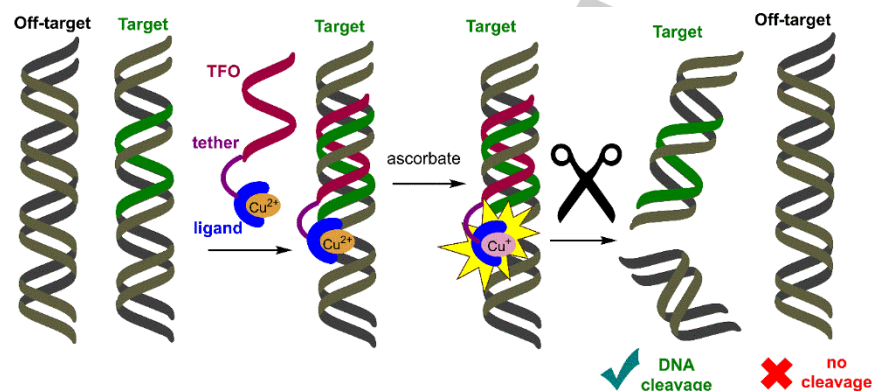
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