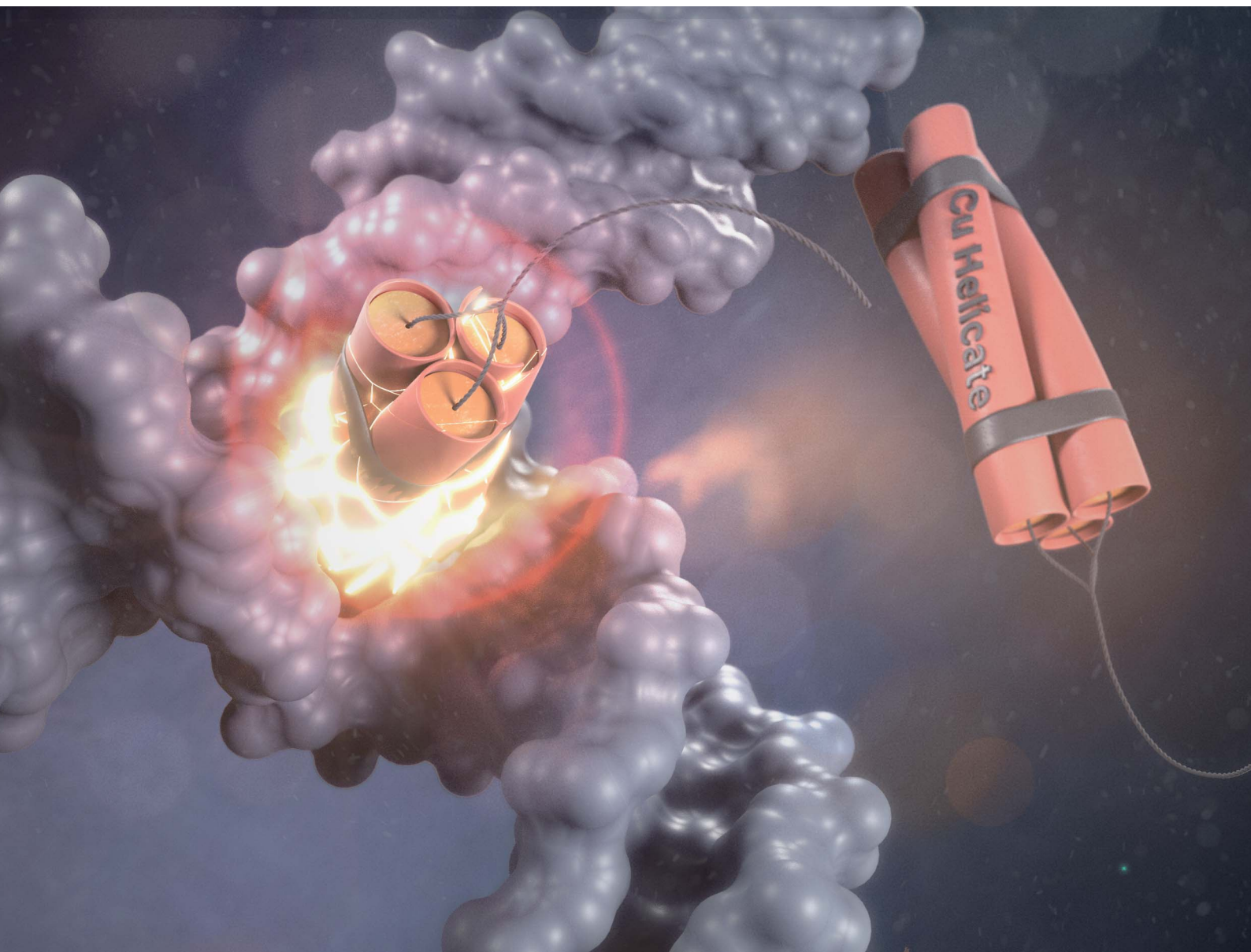


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A copper(II) peptide helicate selectively cleaves DNA replication foci in mammalian cells†

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The use of copper-based artificial nucleases as potential anticancer agents has been hampered by their poor selectivity in the oxidative DNA cleavage process. An alternative strategy to solve this problem is to design systems capable of selectively damaging noncanonical DNA structures that play crucial roles in the cell cycle. We designed an oligocationic Cu^{II} peptide helicate that selectively binds and cleaves DNA three-way junctions (3WJs) and induces oxidative DNA damage via a ROS-mediated pathway both *in vitro* and *in cellulo*, specifically at DNA replication foci of the cell nucleus, where this DNA structure is transiently generated. To our knowledge, this is the first example of a targeted chemical nuclease that can discriminate with high selectivity 3WJs from other forms of DNA both *in vitro* and in mammalian cells. Since the DNA replication process is deregulated in cancer cells, this approach may pave the way for the development of a new class of anticancer agents based on copper-based artificial nucleases.

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Introduction

The discovery of the cytotoxic properties of cisplatin by Barnett Rosenberg in 1965¹ gave rise to the new field of canonical dsDNA-targeted metallodrugs for cancer therapy.² More recently, as our knowledge of the structural polymorphism and biology of DNA expanded, the bioinorganic field has shifted towards targeting noncanonical DNA structures, including single-stranded DNA, i-motifs, G-quadruplexes (G4s), DNA triplexes and DNA junctions, such as three-way (3WJs) and four-way (Holliday) junctions, among others.^{3–13} Here, we present a Cu^{II} peptide helicate that selective recognises 3WJs both *in*

vitro and at DNA replication foci in mammalian cells. This is the first time such specificity has been achieved.

3WJs are the simplest branched DNA structures,¹⁴ consisting of symmetrical assemblies formed by three convergent dsDNA units that meet at a central point called the branch point, which forms a hydrophobic cavity approximately 12 Å in diameter that is defined by the terminal base pairs of each of the three B-DNA arms.¹⁵ Metallosupramolecular helicates are the most promising agents for the selective recognition of these noncanonical DNA structures.^{6–12} One of the key factors for their selectivity is the high complementarity between their shape and the trigonal symmetry of the branch point of the 3WJs. Indeed, this is a key structural feature of other 3WJ binders, such as triptycene derivatives,¹⁶ C₃-symmetric cationic azacryptands,¹⁷ self-assembled supramolecular Fe^{II} tetrahedral metallocages¹⁸ and three-fold symmetric tripeptides.¹⁹

3WJs participate in a number of key biological processes,^{20–22} and notably they are transiently formed in the replication fork during DNA replication.²³ Therefore, 3WJ-binding ligands are expected to stall the replication fork and elicit genotoxic replication stress in rapidly dividing cells, such as tumoral ones.^{24,25} For these reasons, 3WJs are currently considered a promising target for the development of new anticancer drugs with unique therapeutic properties.^{26–31}

On the other hand, artificial Cu^{II} metallonucleases have been investigated as nucleic acid targeting drug molecules,^{32,33} and there are several examples in the literature of Cu^{II} complexes^{34–36} with remarkable anticancer activity combined with lower off-target toxicity than classical platinum drugs.³⁷ Copper

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nucleases produce reactive oxygen species (ROS) that can cleave dsDNA through two alternative mechanisms: hydrolysis of the phosphodiester backbone, or oxidative H-atom abstraction from the deoxyribose ring.³⁸ Copper-based nucleases themselves have little or no sequence selectivity,³⁹ which limits their therapeutic potential. Consequently extensive research has been performed to generate hybrid chemical nucleases that can target specific nucleic acid structures and sequences. This has previously involved the attachment of well-studied chemical nucleases to nucleic acid recognition moieties such as dsDNA groove binders,^{40–43} non canonical DNA binders (specifically triplex DNA⁴⁴ and G-quadruplexes⁴⁵) or sequence specific dsDNA targeting agents.^{46–49} However, to date, no chemical nuclease, copper-derived or not, specifically directed against 3WJs has been described.

In 2021, we reported the first example of a chemical compound that selectively recognizes 3WJs in cells.⁵⁰ The oligocationic ligand precursor of the metal complex that exhibited such activity was equipped with six artificial 2,2'-bipyridine-derived metal-binding residues that can fold predictably in the presence of Fe^{II} ions giving rise to a stable, water soluble, chiral dinuclear peptide helicate. That Fe^{II} peptide helicate, $\Delta\Delta\text{-Fe}^{\text{II}}_2\text{LLD}$ (Scheme 1), showed high affinity and selectivity towards 3WJs compared with canonical DNA *in vitro* and, importantly, selectively labelled DNA replication foci in the cell nuclei.⁵⁰ Inspired by this precedent, herein we describe a redox active Cu^{II} peptide helicate that exhibit highly precise nuclease activity towards 3WJs *in vitro*, and can selectively cleaves DNA replication foci in mammalian cells. To our knowledge, the system reported herein is the first example of a chemical nuclease that exhibits high cleavage selectivity for 3WJs from other forms of DNA both *in vitro* and in mammalian cells. Since

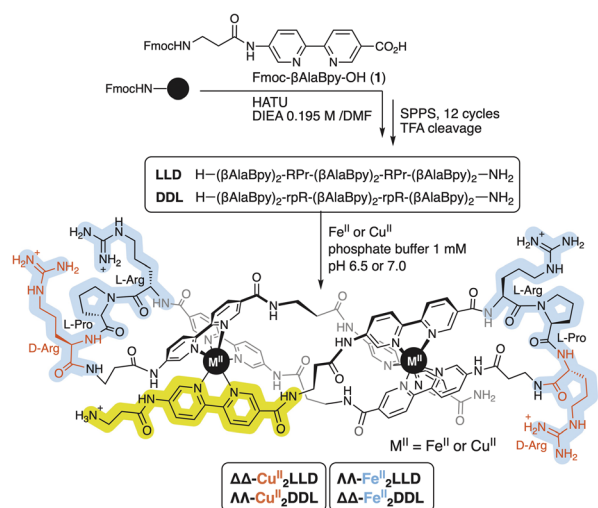
cancer cells have a deregulated DNA replication process, this approach may lead to the development of a new class of anti-cancer agents based on copper-based artificial nucleases.

Results and discussion

Synthesis and characterization of the Cu^{II} peptide helicate

The precursor peptide ligand **LLD** was assembled using standard Fmoc solid-phase peptide synthesis procedures.⁵⁰ The Bpy ligand was introduced as an Fmoc-protected building block, Fmoc- β AlaBpy-OH (**1**; Scheme 1). The final peptide ligand was purified by HPLC, and its identity was confirmed by mass spectrometry (Fig. S1 and S5†).

The formation in solution of the Cu^{II}₂LLD peptide helicate (Scheme 1) was characterized by IR and UV spectroscopies (Fig. S3†) and by monitoring the quenching in the fluorescence emission band of the Bpy fluorophores present in **LLD** upon metal coordination (Fig. 1a and S4†). The resulting titration profile was fitted to a 1 : 2 (**LLD** : Cu^{II}) model with a global dissociation constant of approximately 34.3 (9.4) nM. MALDI spectra of the solution at saturating concentrations of Cu^{II} ions showed a peak at $m/z = 2555.97$, consistent with the formation of the Cu^{II}₂LLD peptide helicate (Fig. S4†).



Scheme 1 Sequences of the peptide ligands **LLD** and **DDL** equipped with six units of the Fmoc- β AlaBpy-OH (**1**) coordinating residue. After their synthesis by SPPS methods, cleavage, and purification, the Cu^{II} helicates are stereoselectively folded under thermodynamic control in the presence of Cu^{II} ions. L-amino acids are indicated in upper-case, and D-amino acids in lower case (*i.e.*, R, P for L-Arg and L-Pro, and r for D-Arg). Heterochiral β -turn loops are highlighted in blue and one of the six coordinating residues (**1**) in yellow.

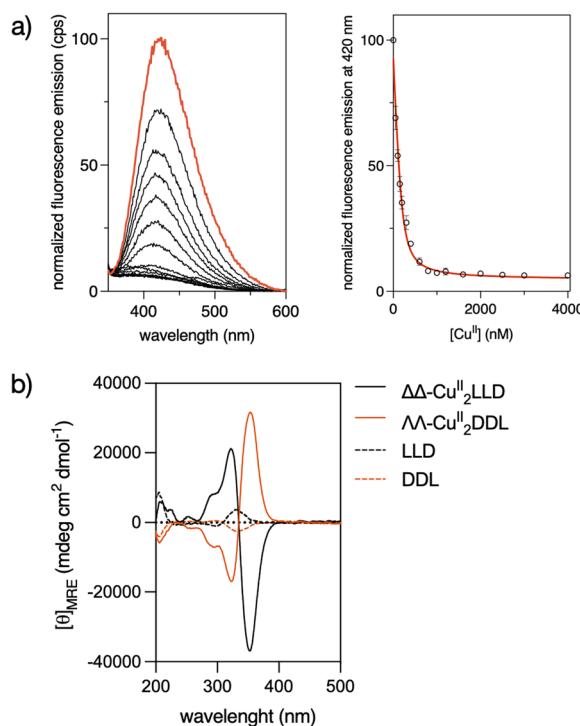


Fig. 1 (a) (Left) Normalized emission spectrum of a 200 nM solution of **LLD** in phosphate buffer (1 mM, NaCl 10 mM, pH 7.0, orange line), and spectra of the same solution in the presence of increasing concentrations of Cu^{II} ions (black lines). (Right) Titration profile of three independent fluorometric titration experiments at 420 nm and best fit according to the 1 : 2 model in DynaFit (same range as main plot). $\lambda_{\text{exc}} = 308$ nm. (b) CD spectra of 5 μ M solutions of **LLD** (black dashed line) and **DDL** (orange dashed line) ligands in phosphate buffer (1 mM, 10 mM NaCl, pH 7.0), and in the presence of 25 μ M Cu^{II} ions (same colours, continuous lines).

Circular Dichroism studies revealed a spectrum for the **L****L****D** peptide ligand that was dominated by a positive Cotton effect band at *ca.* 320 nm. This indicated an important preorganization of the peptide chain and the effective chiral induction of the heterochiral β -turn loops in combination with π -stacking interactions between the bipyridine units. Interestingly, the addition of Cu^{II} ions to a solution of **L****L****D** resulted in an inverted Cotton effect at this wavelength, accompanied with a large increase in its intensity. This is consistent with the formation of a $\Delta\Delta$ - (or P) helicate (Fig. 1b). Surprisingly, this is the opposite chirality to that induced by Fe^{II} ions with the same **L****L****D** peptide ligand, which induce the formation of the diastomeric (M) helicate.⁵⁰ We also synthesised the isomeric **Cu**^{II}**_2DDL** peptide helicate (Scheme 1), which, as expected, showed a mirror image CD spectrum to that of **Cu**^{II}**_2LLD**, consistent with a $\Lambda\Lambda$ configuration of the Cu^{II} metallopeptide (*i.e.*, with the formation of a M helicate) (Fig. 1b).

It is known that the d^9 configuration of Cu^{II} complexes results in pronounced Jahn–Teller distortion for six-coordinate systems and subsequent kinetic lability of axial ligands, even in chelated systems.^{51,52} It appears that the differences in the

coordination behavior of Fe^{II} and Cu^{II} lead to different arrangements of the peptide ligand around the metal centres, leading to opposite helical chirality in the corresponding peptide helicates. To better define the coordination of Cu^{II} ions in the helicates, we performed EPR spectra at low (120 K) and room temperature (300 K) using a 200 μM solution of $\Delta\Delta$ -**Cu**^{II}**_2LLD** in phosphate buffer (Fig. S6†). At low temperature (*i.e.*, frozen solution), the metallo-peptide spectra displayed a relatively symmetric signal, with an isotropic $g = 2.197$ that suggests regular coordination, indicative of octahedral or trigonal prismatic environments. In contrast, at room temperature, the narrowing of the lines suggests a possible rhombic distortion (Fig. S6†), as observed in other Cu^{II} trisbidentate octahedral complexes due to Jahn–Teller distortion.⁵³

Cu^{II} peptide helicates recognize three-way DNA junctions *in vitro*

To study the DNA binding properties of the new Cu^{II} peptide helicates, we used a fluorescein-labelled 3WJ (Flu-3WJ; see ESI† Section 2.1 for DNA sequences) and measured its emission in the presence of increasing concentrations of the preformed $\Delta\Delta$ -**Cu**^{II}**_2LLD** or $\Lambda\Lambda$ -**Cu**^{II}**_2DDL** helicates. The progressive quenching of the fluorescein emission at 515 nm could be fitted to a 1 : 1 binding mode (Flu-3WJ/helicate) plus nonspecific interactions, with an apparent dissociation constant $K_D = 260$ nM for $\Delta\Delta$ -**Cu**^{II}**_2LLD** and $K_D = 654$ nM for $\Delta\Delta$ -**Cu**^{II}**_2DDL** (Fig. 2). This indicates that the P helicate, $\Delta\Delta$ -**Cu**^{II}**_2LLD**, has higher affinity for the 3WJ. We also attempted to investigate the K_D of the interaction of $\Delta\Delta$ -**Cu**^{II}**_2LLD** with fluorescein-labelled dsDNA (Flu-dsDNA), but the mixture precipitated during the titration, which suggests a nonspecific interaction between the peptide helicate and dsDNA.

To ensure that the $\Delta\Delta$ -**Cu**^{II}**_2LLD** could bind with 3WJs of different sequence compositions (*i.e.* different GC content) the fluorescein emission assay was repeated with a GC-rich 3WJ sequence (Flu-GC-3WJ, see ESI† Section 2.1† for DNA sequences). A K_D of 300 nM was obtained for the interaction between $\Delta\Delta$ -**Cu**^{II}**_2LLD** and Flu-GC-3WJ (Fig. S7†), which indicates that the affinity of this Cu^{II} peptide helicate for 3WJs is not limited to a single DNA sequence. The key role played by metal ions in the recognition of 3WJ by these family of peptide helicates was demonstrated in our previous work. There, we demonstrated by means of fluorescence anisotropy experiments the absence of interaction between the free peptide ligand **L****L****D** and 3WJ.⁵⁰

Kinetic inertness of the $\Delta\Delta$ -**Cu**^{II} peptide helicate

Next, we investigated the stability of Cu^{II} peptide helicate in solution, through a competitive binding assay with EDTA—an organic ligand that readily forms complexes with copper ions with extraordinary thermodynamic stability.⁵⁴ Specifically, five equivalents of Cu^{II} ions were added to a 2 μM solution of **L****L****D** ligand, and fluorescence spectra were recorded before and after the addition of the metal ions, that is, before and after the assembly of the peptide helicate $\Delta\Delta$ -**Cu**^{II}**_2LLD**. Then, two equivalents of EDTA (with respect to the **L****L****D** peptide ligand) were added and the fluorescence spectra were recorded for

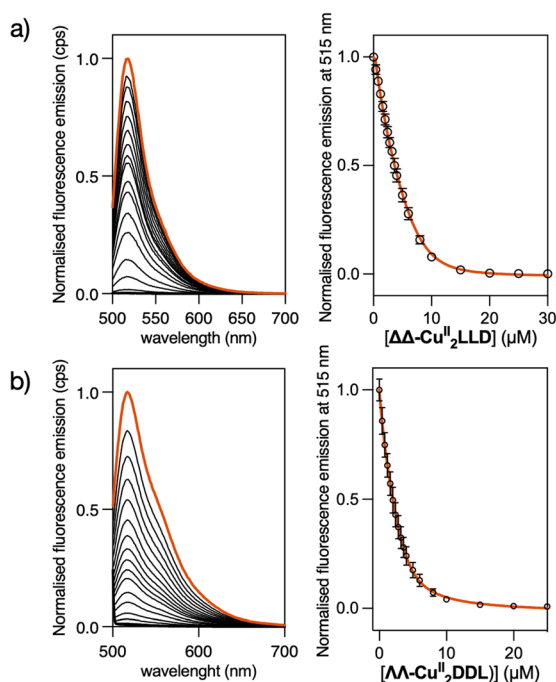


Fig. 2 (a) (Left) Emission spectra of a 2 μM solution of the fluorescein-labelled 3WJ (Flu-3WJ) in absence (orange line) and in the presence of increasing concentrations of $\Delta\Delta$ -**Cu**^{II}**_2LLD** (black lines of decreasing intensity). (Right) Profile of the titration with the best fit (orange line) according to a 1 : 1 model plus unspecific interaction in DynaFit, with an apparent $K_D = 260$ nM. (b) (Left) Emission spectra of a 2 μM solution of Flu-3WJ in absence and in the presence of increasing concentrations of $\Lambda\Lambda$ -**Cu**^{II}**_2DDL** (decreasing intensity). (Right) Profile of the titration with the best fit (orange line) according to a 1 : 1 model plus unspecific interaction in DynaFit, with an apparent $K_D = 654$ nM. Conditions: 1 mM phosphate buffer, 10 mM NaCl, pH 7.0, $\lambda_{\text{exc}} = 490$ nm.

60 min. The experiment was repeated in the presence of 3WJ. It is necessary to clarify that the peptide ligand **L**L Δ is able to coordinate two Cu^{II} ions while the EDTA molecule is only able to coordinate a single metal ion, so to perform this classical competition study the stoichiometric ratio between the two ligands must be 1 : 2 (**L**L Δ : EDTA) against a fixed amount of Cu^{II} ions. The data obtained showed that the ligand substitution reaction takes ≥ 5 minutes to occur in the absence of 3WJ, but it slows to ≥ 20 minutes in the presence of 3WJ, which indicates that $\Delta\Delta$ -Cu^{II}₂**L**L Δ possesses, in addition to its high thermodynamic stability, a remarkable kinetic inertness in water media and probably also *in cellulo* (Fig. S8†).

Analysis of three-way DNA junction recognition of the $\Delta\Delta$ -Cu^{II} peptide helicate by PAGE

To further analyse the interaction of $\Delta\Delta$ -Cu^{II}₂**L**L Δ with 3WJ and dsDNA, we performed polyacrylamide gel electrophoresis (PAGE) experiments (Fig. 3a).^{55–57} In agreement with the fluorescence studies, we observed that incubation of 3WJ with increasing concentrations of preformed $\Delta\Delta$ -Cu^{II}₂**L**L Δ resulted in the appearance of a slower-migrating band consistent with the formation of the 3WJ/ $\Delta\Delta$ -Cu^{II}₂**L**L Δ adduct (Fig. 3b). Here, almost all the 3WJ has been complexed with $\Delta\Delta$ -Cu^{II}₂**L**L Δ , even at the lowest concentration of helicate tested, which is expected for a high-affinity interaction. The intensity of this 3WJ band increases with the concentration of the Cu^{II} helicate (Fig. S9†), while the intensity of the single-stranded DNA band decreases. In contrast, no changes in the position or intensity of the dsDNA were observed upon incubation of the preformed $\Delta\Delta$ -Cu^{II}₂**L**L Δ helicate with dsDNA in the presence of 3WJ, confirming the low affinity this metalloprotein has for canonical dsDNA (Fig. 3b).

The $\Delta\Delta$ -Cu^{II} peptide helicate selectively cleaves three-way DNA junctions *in vitro*

To provide further clarity on 3WJ formation and enable ease of visualisation of target 3WJ and off-target dsDNA, PAGE experiments were also performed with fluorophore labelled sequences

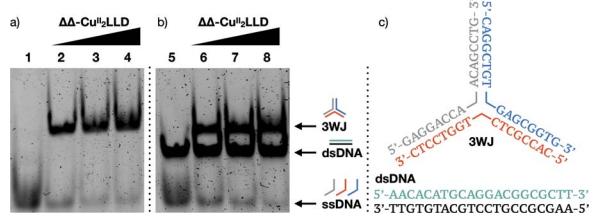


Fig. 3 (a) 10% native PAGE analysis of 3WJ strands in the absence (lane 1) and presence 1, 2 and 3 equivalents of $\Delta\Delta$ -Cu^{II}₂**L**L Δ (lanes 2–4); (b) 3WJ strands and dsDNA in the absence (lane 5) and presence of $\Delta\Delta$ -Cu^{II}₂**L**L Δ (1–3 eq. in lanes 6–8, respectively). 3WJ remains as single-stranded DNA in the absence of $\Delta\Delta$ -Cu^{II}₂**L**L Δ and forms the 3WJ when $\Delta\Delta$ -Cu^{II}₂**L**L Δ is present. In contrast, dsDNA can be visualised in lanes 5–8 and remains unchanged in the presence of increasing concentrations of the Cu^{II} peptide helicate, demonstrating the low affinity of the metalloprotein for canonical DNA; (c) 3WJ and dsDNA sequences used for PAGE analysis.

(Fig. 4a).⁵⁸ 3WJ strands were labelled at their 5' end with FAM, ROX or Cy5, while the dsDNA strands were labelled with FAM and Cy5. Fluorophore 3WJ and dsDNA strands were then incubated with $\Delta\Delta$ -Cu^{II}₂**L**L Δ and visualised using a multiplex fluorescent assay (Fig. 4a). The blue (FAM), green (ROX) and red (Cy5) signal of the 3WJ sequences were combined to produce a yellow 3WJ band, while the blue (FAM) and red (Cy5) dsDNA strands appear pink. This multiplex binding assay was next applied for the analysis of the cleavage profile of $\Delta\Delta$ -Cu^{II}₂**L**L Δ .

The ability of $\Delta\Delta$ -Cu^{II}₂**L**L Δ to selectively recognise and cleave 3WJs *in vitro* was analysed by PAGE (Fig. 4b). The fluorescently labelled target 3WJ and off-target dsDNA oligos (see

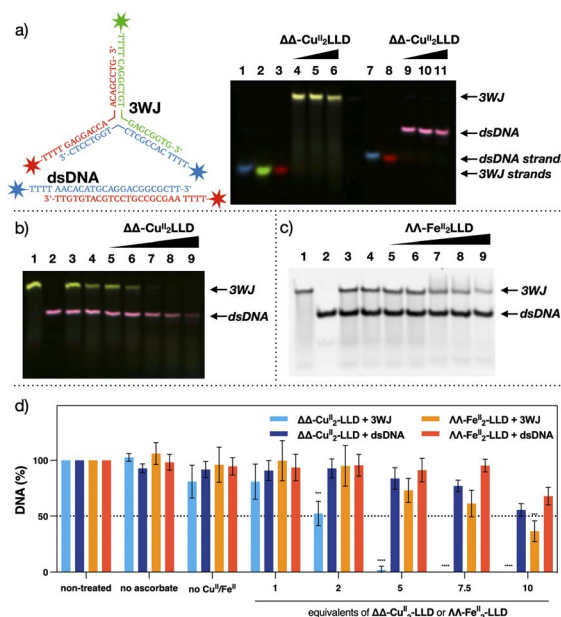


Fig. 4 (a) Fluorescently labelled target 3WJ and off-target dsDNA. Blue = FAM, Green = ROX and Red = Cy5 (see ESI Section 2.3† for DNA sequences and experimental conditions). Multiplex image from 20% native analysis of the formation of 3WJ in the presence of increasing concentrations of $\Delta\Delta$ -Cu^{II}₂**L**L Δ . Lane 1: FAM-labelled single strand (ss) of 3WJ. Lane 2: ROX-labelled ss. Lane 3: Cy5-labelled ss of 3WJ. Lane 4–6: 3WJ strands incubated with 1, 2 or 3 eq. of $\Delta\Delta$ -Cu^{II}₂**L**L Δ , respectively. Lane 7: FAM-labelled ss of dsDNA. Lane 8: Cy5-labelled ss of dsDNA. Lanes 9–11: dsDNA incubated with 1, 2 or 3 eq. of $\Delta\Delta$ -Cu^{II}₂**L**L Δ , respectively. See Fig. S10† for individual images from multiplex assay. (b) PAGE analysis of the DNA damage inflicted by $\Delta\Delta$ -Cu^{II}₂**L**L Δ . Fluorophore labelled DNA was utilised to ensure clarity of target 3WJ DNA (yellow) and off-target dsDNA (pink). Lane 1: 3WJ. Lane 2: dsDNA. Lane 3: 3WJ and dsDNA incubated with $\Delta\Delta$ -Cu^{II}₂**L**L Δ . Lane 4: Target and off-target with LLD peptide ligand (no Cu^{II} present). Lanes 5–9: 3WJ and dsDNA incubated with 1, 2, 5, 7.5 or 10 eq. of $\Delta\Delta$ -Cu^{II}₂**L**L Δ and Na-L-ascorbate (10 eq. to $\Delta\Delta$ -Cu^{II}₂**L**L Δ). Individual images available in Fig. S11†. (c) Control cleavage experiment performed with $\Delta\Delta$ -Fe^{II}₂**L**L Δ . Lane 1: 3WJ DNA. Lane 2: dsDNA off-target. Lane 3: control of 3WJ and dsDNA incubated with $\Delta\Delta$ -Fe^{II}₂**L**L Δ . Lane 4: 3WJ and dsDNA with non-metalated LLD peptide ligand. Lanes 5–9: 3WJ and dsDNA incubated with 1, 2, 5, 7.5 or 10 eq. of $\Delta\Delta$ -Fe^{II}₂**L**L Δ and Na-L-ascorbate (10 eq. to $\Delta\Delta$ -Fe^{II}₂**L**L Δ). (d) Band densitometry analysis of cleavage induced upon 3WJ and dsDNA by $\Delta\Delta$ -Cu^{II}₂**L**L Δ (Fig. S12†) and $\Delta\Delta$ -Fe^{II}₂**L**L Δ (Fig. 4c and S13†). * = $P \leq 0.05$ ** = $P \leq 0.01$ *** = $P \leq 0.001$ and **** = $P \leq 0.0001$. Na-L-ascorbate concentration is 10 eq. to $\Delta\Delta$ -Cu^{II}₂**L**L Δ or $\Delta\Delta$ -Fe^{II}₂**L**L Δ .

ESI 2.3† for DNA sequences) were incubated with increasing equivalents $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$ and Na-L-ascorbate at 37 °C for 24 hours. PAGE analysis revealed that the Cu^{II} peptide helicate selectively cleaves 3WJ, which fully disappears when exposed to 5 eq. of $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$, while the dsDNA present in the sample remains intact when exposed up to 7.5 equivalents of the Cu^{II} peptide helicate. A control cleavage experiment was also performed with $\Delta\Delta\text{-Fe}^{\text{II}}_2\text{LLD}$. Here, 3WJ and dsDNA were incubated with the Fe^{II} helicate and Na-L-ascorbate under identical conditions to that of the $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$ assay, and the cleavage profile was visualised by PAGE (Fig. 4c). Band densitometry revealed a significant difference between the cleavage $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$ induced towards 3WJ compared to the off-target dsDNA; for example, just 2% of the 3WJ remained *versus* 80% of off-target dsDNA following exposure to 5 eq. of $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$ (Fig. 4d and S14†). By contrast, $\Delta\Delta\text{-Fe}^{\text{II}}_2\text{LLD}$ did not show selectivity in the cleavage of 3WJ *versus* dsDNA across all tested concentrations (Fig. 4d and S14†). Moreover, the Fe^{II} peptide helicate is only able to cleave 70% of 3WJ at the highest concentration of metalloprotein tested (10 eq.), which highlights its lack of efficiency.

A time point study was performed to determine the incubation period required to achieve 3WJ selective damage (Fig. S14†). This revealed that cleavage initiates within 1 hour and increases up to 12 hours; from 12 to 24 hours no significant increase in cleavage was observed. This indicates that $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$ can rapidly and selectively damage DNA 3WJs, and that off-target dsDNA damage would not be expected upon longer incubation. Combining the results of Fig. 3, 4 and S10–S15,† it can be concluded that $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$ can discriminate with great efficiency between 3WJ and dsDNA, and selectively cleave 3WJ in the presence of canonical DNA. To our knowledge, this is the first example of such activity for a chemical nuclease *in vitro*.

Next, the cleavage mechanism for the $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$ helicate was investigated using reactive oxygen species (ROS) scavengers.^{59,60} Here, 3WJ was incubated with $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$, Na-L-ascorbate, and a series of selective ROS scavengers (L-histidine, D-mannitol, L-methionine, and tiron (sodium catechol sulfate)),

and the resulting cleavage profiles were visualized by PAGE (Fig. 5). The presence of tiron significantly impeded the activity of the peptide helicate, indicating that superoxide radicals ($\text{O}_2^{\cdot-}$)⁶¹ play a dominant role in the 3WJ cleavage inflicted by $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$. To probe this finding further, we determined if diffusible superoxide radicals were directly contributing to DNA cleavage by examining the activity in the presence of 4-hydroxy-TEMPO, a known scavenger of diffusible $\text{O}_2^{\cdot-}$ (Fig. S16†).⁶² TEMPO resulted in negligible cleavage inhibition indicating that diffusible $\text{O}_2^{\cdot-}$ plays a minimal role in $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$ cleavage and that, instead, oxidative damage is likely mediated by a copper bound superoxo radical species ($\text{Cu-O}_2^{\cdot-}$) proximate to the 3WJ. The scavenger L-histidine also impeded 3WJ damage, which suggested that singlet oxygen ($^1\text{O}_2$) is contributing to the cleavage mechanism. However, since $^1\text{O}_2$ is usually generated under photoactivated conditions we further investigated the role of this ROS in $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$ activity. The scavenger assay was therefore expanded to include deuterium oxide (D_2O)—a known $^1\text{O}_2$ stabiliser—and sodium azide (NaN_3)—a known scavenger of $^1\text{O}_2$ (Fig. 5a). The presence of D_2O and NaN_3 did not perturb 3WJ cleavage and consequently it is not clear at this point how $^1\text{O}_2$ mediates $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$ activity. It should be noted that several recent studies reporting the dsDNA damaging effects of polynuclear Cu^{II} metallonucleases have predicted $^1\text{O}_2$ -mediated DNA damage and identified L-histidine as viable scavenger *in vitro* and *in cellulo*.^{63,64}

A rhodamine-labelled $\Delta\Delta\text{-Cu}^{\text{II}}_2$ peptide helicate selective labels DNA-replication sites in mammalian cells

To this point we have demonstrated that the $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$ readily forms in solution, its stability in aqueous environments, and that $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$ can selectively bind and damage 3WJ *in vitro*. Next, *in cellulo* studies were performed to investigate the ability of the Cu^{II} helicate to selectively target DNA replication centres (*i.e.*, 3WJs in the cell nuclei). Preliminary experiments indicated, as was the case with Fe^{II} analogues,⁵⁰ that a TAMRA-labelled Cu^{II} peptide helicate, $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{TAMRA-LLD}$ (Fig. S2†), did not internalise in Vero cells. However, it readily translocated in digitonin-treated cells where it showed a diffused

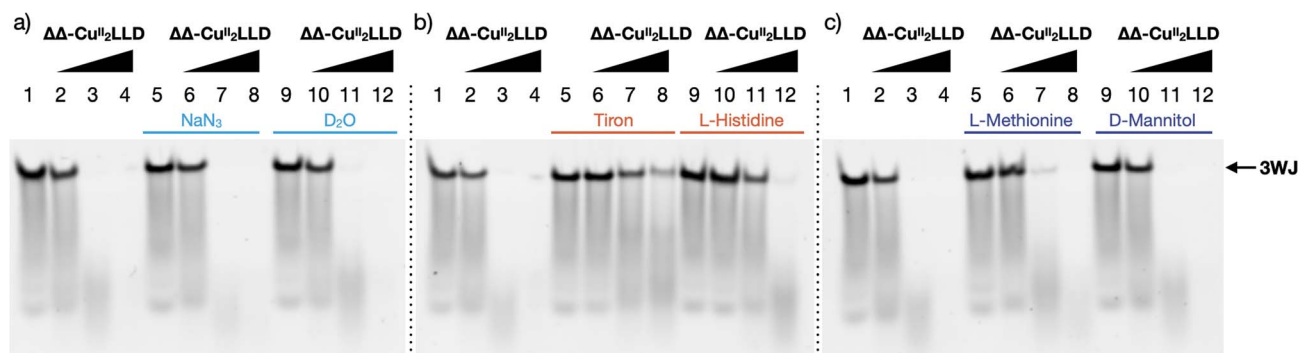


Fig. 5 20% native PAGE showing the cleavage profile of the $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$ in the presence of ROS scavengers: (a) NaN_3 and D_2O ; (b) tiron and L-histidine; (c) L-methionine and D-mannitol. Lane 1–4: control (no scavenger). Lanes 5–8: (a) NaN_3 ; (b) tiron; (c) L-methionine. Lanes 9–12: (a) D_2O ; (b) L-histidine; (c) D-mannitol. Lanes 1, 5, 9: 3WJ control. Lanes 2, 6, 10: 1 eq. $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$. Lanes 3, 7, 11: 5 eq. $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$. Lanes 4, 8, 12: 10 eq. $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$. All lanes (excluding controls) contain Na-L-ascorbate (10 eq. to $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$).

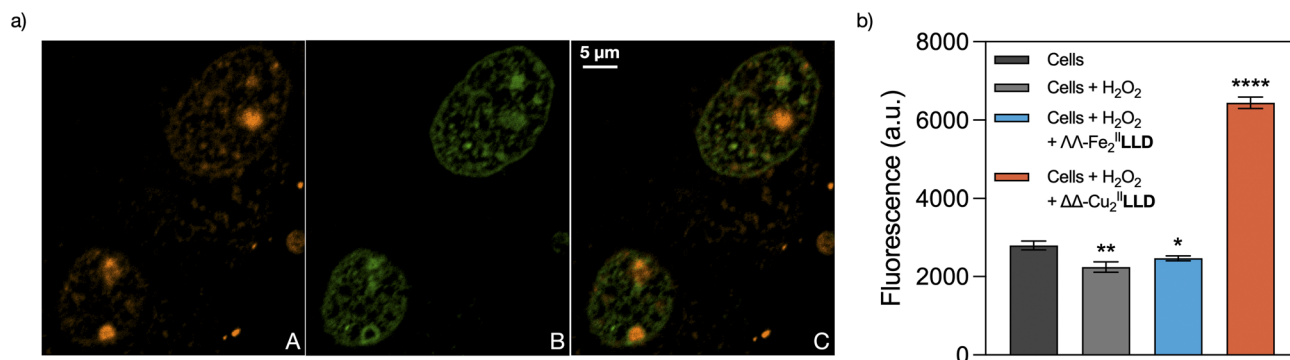


Fig. 6 (a) $\Delta\Delta$ -Cu^{II}₂TAMRA-LLD selectively stains DNA replication sites at the cell nuclei. Vero cells expressing protein GFP-PCNAL2 were incubated in the presence of 25 $\mu\text{g mL}^{-1}$ of digitonin for 3 min and then in the presence of 5 μM of $\Delta\Delta$ -Cu^{II}₂TAMRA-LLD for 15 min. The Cu^{II} peptide helicate used in these studies was previously assembled before the internalization experiment by adding 5 eq. of CuCl₂·2H₂O to an aqueous solution of TAMRA-LLD peptide ligand. (A) Red channel emission showing the distribution of the TAMRA-labelled helicate; (B) green channel, corresponding to the emission of the GFP-PCNAL2 probe that labels DNA replication foci; (C) overlay of the green and red channels, which shows that $\Delta\Delta$ -Cu^{II}₂TAMRA-LLD has localised at the replication foci. Nucleoli are clearly labelled as red spots inside the cell nuclei. The images show a representative confocal section of the Vero cells. (b) Detection of free DNA ends by TUNEL assay. S-phase synchronized Vero cells were incubated with H₂O₂ alone and with $\Lambda\Lambda$ -Fe^{II}₂LLD or $\Delta\Delta$ -Cu^{II}₂LLD plus H₂O₂ at 37 °C for 2 hours and then subjected to TUNEL analysis for the detection of free DNA ends. Results are represented as the mean with standard deviation of a triplicate experiment * = $P \leq 0.05$ ** = $P \leq 0.01$ *** = $P \leq 0.001$ and **** = $P \leq 0.0001$.

distribution in the cytosol, and a strong punctuated distribution in the nucleus, as well as an evident concentration in the nucleolus (Fig. 6a(A)—red and Fig. S18†). Most of the nuclear spots co-localized with GFP-tagged Proliferating Nuclear Antigen (PCNA), which specifically labels DNA replication foci in actively dividing cells (Fig. 6a(B)—green).⁶⁵ While its cytosolic distribution and association with the nucleoli might reflect some off-target RNA-binding of the helicate, replisomes labelled by PCNA are enriched in 3WJs due to the presence of replication forks. Thus, our results strongly suggest the direct association of the Cu^{II} peptide helicate with 3WJs structures in functional cell nuclei. Moreover, control experiments show that the free LLD peptide ligand does not localise in the DNA replication centres (Fig. S17†). As already suggested by the results obtained in the competition studies with EDTA described above, this control experiment further demonstrates that the $\Delta\Delta$ -Cu^{II}₂LLD helicate is stable in cell medium and that it only binds DNA replication centres in the nucleus as a discrete metalloprotein.

The $\Delta\Delta$ -Cu^{II} peptide helicate selective cleaves DNA-replication foci in mammalian cells

Having demonstrated that $\Delta\Delta$ -Cu^{II}₂LLD is stable *in cellulo* and associates with 3WJ-enriched foci in cellular DNA, we next investigated if it could exert its nuclease activity upon chromatin-associated 3WJs in cells. To maximize the number of cells undergoing DNA replication, and thus increase the chances of detecting free DNA ends obtained by nuclease cleavage of 3WJs, we used Vero cells that were previously synchronised by 42 h of serum deprivation.⁶⁶ 16 h after re-supplementation of the cultures with 10% serum—when most of the cells were in S phase—the cells were pre-treated with digitonin and then incubated with H₂O₂ in the presence or absence of $\Delta\Delta$ -Cu^{II}₂-LLD. Cells were then incubated for 2 h at 37 °C and subjected to

a Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labelling (TUNEL) assay. The TUNEL assay uses TdT polymerase to label the free ends of DNA with a fluorescent dUTP, and thereby enables detection of cleavage sites. Here, the free DNA ends in the cell nuclei DNA (*i.e.*, DNA sites cleaved by $\Delta\Delta$ -Cu^{II}₂LLD) were detected through Alexa Fluor 488™ fluorescence.⁶⁷ Very importantly, the results show that incubation of $\Delta\Delta$ -Cu^{II}₂LLD in the presence of H₂O₂ produced a significant increase for the TUNEL fluorescent signal (Fig. 6b), which indicates the presence of free DNA ends and thus, nuclease activity *in cellulo*. A control experiment was also performed with $\Lambda\Lambda$ -Fe^{II}₂LLD in the presence of H₂O₂. Significantly, the increase in fluorescent signal—indicative of DNA cleavage—was not observed for the Fe^{II} helicate, which agrees with the above-described *in vitro* studies, which suggested a lack of nuclease activity. To our knowledge, this is the first example of such activity for a chemical nuclease *in cellulo*.

Conclusion

We reported herein the synthesis of two new chiral dinuclear oligocationic Cu^{II} peptide helicates derived from two enantiomeric peptide ligands equipped with six chelating 2,2'-bipyridine residues, named LLD and DDL, following a SPPS methodology.³⁰ Spectrofluorimetric and PAGE DNA binding studies indicate that both Cu^{II} peptide helicates can recognize 3WJs *in vitro* with high affinity and selectivity, even in the presence of canonical DNA, showing chiral discrimination between the two diastereoisomers, being $\Delta\Delta$ -Cu^{II}₂LLD the metalloprotein with higher 3WJ affinity of the two ($K_D = 260$ nm vs. 654 nm). Moreover, the kinetic inertness of $\Delta\Delta$ -Cu^{II}₂LLD in water was investigated through a competition study with EDTA in presence and absence of 3WJ, suggesting that $\Delta\Delta$ -Cu^{II}₂LLD is very stable *in vitro* and *in cellulo*.

The studies carried out clearly show that $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$ is an efficient and selective *in vitro* agent for the cleavage of 3WJs, even in the presence of canonical DNA. The mechanism through which this 3WJ cleavage by $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$ occurred was also investigated. Studies using ROS scavengers showed that metal-bound superoxide radicals ($\text{Cu-O}_2^{\cdot-}$) play the key role in the cleavage process and suggest that singlet oxygen ($^1\text{O}_2$) is also involved in some way.

The ability $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$ to internalise in cells was studied using of Cu^{II} helicate labelled with TAMRA ($\Delta\Delta\text{-Cu}^{\text{II}}_2\text{TAMRA-LLD}$). This helicate was effectively internalised by digitonin-treated mammalian cells and was localised into the nucleus. Its nuclear distribution matched the localization of DNA replication sites, demonstrating that $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$ binds with high affinity and selectivity to 3WJ *in cellulo*. Finally, the selective cleavage of DNA replication foci in cells was evaluated by TUNEL assay. The studies performed indicate that $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$ produced a significant increase of the TUNEL fluorescent signal in the presence of H_2O_2 , which indicates the presence of free DNA ends and thus, nuclease activity *in cellulo*.

To our knowledge, $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$ is the first example of a chemical nuclease that selectively cleaves 3WJ both *in vitro* and *in cellulo*. As DNA replication is deregulated in cancer cells, we strongly believe that this work will form the basis of a new class of anticancer metallodrug that target and damage with high efficiency and selectivity this noncanonical DNA structure that is transiently generated in the DNA replication foci of cell nuclei.

Data availability

Raw data for Fig. 1–6 are published in the figshare repository <https://doi.org/10.6084/m9.figshare.22754600>.

Author contributions

M. V. L., J. M.-C. and A. K. conceived and designed the investigations. M. E. V. deeply assisted in the design of the investigations, interpretation of experimental data and proof-reading of the manuscript. A. A.-O., N. B.-P., B. McG. and J. G.-G. performed the experiments. D. B. assisted in conducting DNA-binding studies. F. R. conducted the EPR studies. M. V. L., J. M.-C. and A. K. wrote the manuscript.

Conflicts of interest

There are no conflicts to declare.

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- 67 TUNEL Assay (commercial) Kit detects the DNA fragmentation of apoptotic cells by exploiting the fact that the DNA breaks expose many 3'-hydroxyl ends. These hydroxyl groups can then serve as starting points for terminal deoxynucleotidyl transferase (TdT), which adds deoxyribonucleotides in a template-independent fashion. Addition of the deoxythymidine analog 5-bromo-2'-deoxyuridine 5'-triphosphate (BrdUTP) to the TdT reaction serves to label the break sites. Once incorporated into the DNA, BrdU can be detected by an anti-BrdU antibody using standard immunohistochemical technique.

Electronic Supplementary Information

A Copper(II) Peptide Helicate Selectively Cleaves DNA Replication Foci in Mammalian Cells

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Author attributions

The following table describes the contribution of each author according to the CRediT taxonomy as described in A. Brand, L. Allen, M. Altman, M. Hlava, J. Scott, *Learn. Publ.* **2015**, *28*, 151–155. This taxonomy provides a detailed classification of the various roles performed by each author. The degree of contribution is coded as *lead* (black), *equal* (dark grey), or *supporting* (light grey).

	AAO	NBP	BM	JGG	DB	FR	MEV	AK	JMC	MVL
Conceptualization. Ideas, formulation of overarching research goals and aims.										
Methodology. Development or design of methodology; creation of models.										
Validation. Verification of the overall reproducibility of results and research outputs.										
Formal Analysis. Application of formal techniques to analyze the data.										
Investigation: helicate synthesis, metal binding, in vitro DNA binding studies.										
Investigation: EPR studies.										
Investigation: In vitro nuclease activity studies.										
Investigation: In vivo fluorescence microscopy.										
Resources. Provision of reagents, materials, instrumentation, and analysis tools.										
Data Curation. Management to annotate, scrub and maintain research data.										
Writing: Original Draft. Preparation of the paper, specifically writing the initial draft.										
Writing: Review & Editing. Critical review, commentary, or revision.										
Visualization. Preparation, of the paper, specifically visualization &/or presentation.										
Supervision. Oversight responsibility for the research planning & execution.										
Project Administration. Management and coordination of the research.										
Funding Acquisition. Acquisition of the financial support leading to this publication.										

1. Reagents

The solvents and reagents used in the synthesis of the Fmoc- β Ala5Bpy-OH were obtained from *Fisher Chemical* (acetonitrile for HPLC, chloroform, toluene, DMF, methanol, NaOH), *Scharlau* (absolute ethanol), *Sigma Aldrich* (5,5'-dimethyl-2,2'-bipyridine, KMnO_4 , SOCl_2 , hydrazine monohydrate, xylene, DIPEA), *Panreac* (celite and NaNO_2). For the synthesis of the peptide ligands, all the amino acids were provided by *Sigma Aldrich* and *IRIS Biotech GmbH*. All the amino acids were used as their Fmoc protected derivatives. The resin employed for the SPPS was H-Rink-Amide *ChemMatrix* 35-100 mesh particle size from *Sigma Aldrich*. For the Alloc deprotection, $\text{Pd(PPh}_3)_4$, PhSiH_3 were obtained from *Sigma Aldrich*. $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ employed in the obtention of the Cu^{II} metallopeptides was acquired to *Acros Organics*. Mohr's salt, $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, employed in the obtention of the Fe^{II} metallopeptides as a source of Fe^{II} ions, was provided by *Sigma Aldrich*. 6-Carboxytetramethylrhodamine succinimidyl ester (TAMRA) was provided by *Carbosynth*.

2. Experimental Methods

2.1. DNA sequences used in the fluorescent DNA binding studies and (fluorophore) non-labelled PAGE binding studies.

Fluorescein-labelled 3WJ (Flu-3WJ):

Y1: 5'-FAM-TTT TCA CCG CTC TGG TCC TC-3'

Y2: 5'-CAG GCT GTG AGC GGT G-3'

Y3: 5'-GAG GAC CAA CAG CCT G-3'

3WJ:

Y1: 5'-CAC CGC TCT GGT CCT C-3'

Y2: 5'-CAG GCT GTG AGC GGT G-3'

Y3: 5'-GAG GAC CAA CAG CCT G-3'

Fluorescein-labelled GC-3WJ (Flu-GC-3WJ):

Y1: 5'-FAM-TTTT CAC CGG GCC CCG GGG CCC CCT C-3'

Y2: 5'-CAG GGG GCC CCG GGG CCC GGT G-3'

Y3: 5'-GAG GGG GCC CCG GGG CCC CCT G-3'

Fluorescein-labelled dsDNA (Flu-dsDNA):

Z1: 5'-FAM-TTT TAA CAC ATG CAG GAC GGC GCT T-3'

Z2: 5'-AAG CGC CGT CCT GCA TGT GTT-3'

dsDNA:

Z1: 5'-AAC ACA TGC AGG ACG GCG CTT-3'

Z2: 5'-AAG CGC CGT CCT GCA TGT GTT-3'

2.2. Example of Dynafit script

```
[task]                                ;semicolons indicate comments
task = fit                            ;nature of the calculation to be performed
data = equilibria

[mechanism]                           ;Free-form 1:1 binding model with Kd
R + L <=> RL : Kd dissoc              ;to be calculated as dissociation constant

[constants]                           ;Initial Kd value for iteration
Kd = 1.0 ?                            ;the "?" indicates that this will be optimized

[concentrations]                      ;Fixed conc. of the DNA during the peptide titration
R = 2.0

[responses]                           ;contribution to the spectroscopic signal of each
R = 0.1 ?                             ;of the different components of the equilibrium
RL = 1.5 ?                            ;these will be optimized ("?" after the values).

[data]                                ;location of files and information about the data
variable L                            ;the species that changes conc. during the titration
offset auto ?
directory ./exp/brHis                 ;file path (relative to DynaFit program location)
extension txt                          ;name of the experimental data file
file f1

[output]                              ;path indicating location of DynaFit output files
directory ./exp/brHis/out

[settings]                            ;cosmetic settings that control DynaFit graphics
{Output}                              ;fits were exported & finally plotted with
XAxisUnit = uM                        ;GraphPad Prism 7.0c. GraphPad Software,
BlackBackground = n                   ;La Jolla California, www.graphpad.com
XAxisLabel = [peptide]
YAxisLabel = anisotropy
WriteTXT = y
```

2.3. DNA sequences used in fluorophore labelled 3WJ and dsDNA PAGE binding studies.

3WJ:

Y1: 5'-FAM-TTT TCA CCG CTC TGG TCC TC-3'

Y2: 5'-ROX-TTT TCA GGC TGT GAG CGG TG-3'

Y3: 5'-Cy5-TTT TGA GGA CCA ACA GCC TG-3'

dsDNA:

Z1: 5'-FAM-TTT TAA CAC ATG CAG GAC GGC GCT T-3'

Z2: 5'-Cy5-TTT TAA GCG CCG TCC TGC ATG TGT T-3'

2.4 Mass spectrometry

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) was performed with a Bruker Autoflex MALDI-TOF model in positive scan mode by direct irradiation of the matrix-adsorbed peptide. 4-HCCA (α -cyano-4-hydroxycinnamic acid) was the selected matrix for all these experiments.

2.5 UV-Vis spectroscopy

UV measurements were made in a Jasco V-630 spectrophotometer coupled to a Jasco ETC-717 temperature controller, using a standard Hellma semi-micro cuvette (108.002-QS) with a light path of 10 mm. Measurements were made at 20 °C. Acquisition parameters were: 220-700 nm range, scan speed of 200 nm/min, resolution of 0.2 nm.

2.6. IR spectroscopy

FT-IR spectra were recorded on a Perkin-Elmer Spectrum Two equipped with a UATR accessory. Measurements were made at 20 °C. The acquisition parameters were in the 4000-400 cm^{-1} range.

2.7. Circular Dichroism

Circular dichroism measurements were made with a Jasco J-715 coupled to a Neslab RTE-111 thermostated water bath, using a Hellma 100-QS cuvette (2 mm light pass). Scan speed was 200 nm/min and the obtained spectra are the mean of three accumulations.

2.8. EPR studies

EPR measurements were made in a Bruker EMX spectrometer.

2.9. HPLC and UHPLC

a) UHPLC-MS

Peptide analysis was performed by analytical UHPLC-MS with an Agilent 1200 series LC/MS using a SB C18 (1.8 μm , 2.1 $\times$ 50mm) analytical column from Phenomenex. Standard conditions for analytical UHPLC consisted of a linear gradient from 5% to 95% of solvent B in 12 min at a flow rate of 0.35 mL/min (A: H_2O 0.1% TFA, B: CH_3CN 0.1% TFA). Compounds were detected by UV absorption at 222, 270 and 330 nm. Electrospray Ionization Mass Spectrometry (ESI/MS) was performed with an Agilent 6120 Quadrupole LC/MS model in positive scan mode using direct injection of the purified peptide solution into the MS detector.

b) Preparative HPLC

Peptide purification was performed by preparative RP- HPLC with a Waters 1500 series Liquid Chromatograph using a Sunfire Prep C18 OBD (5 μm , 19 $\times$ 150 mm) reverse-phase column from Waters. Standard conditions for preparative RP- HPLC consisted of an isocratic regime during the first 5 min, followed by different linear gradients of solvent B during 30 min (A: H_2O 0.1% TFA, B: CH_3CN 0.1% TFA). The gradients were adjusted for each peptide.

2.10. DNA hybridization

Oligonucleotides were obtained from Biomers. Concentration of the oligonucleotides was measured by UV-VIS by using the extinction coefficient given by the Commercial House Biomers.

For the hybridization process, a stoichiometric mixture of the DNA strands was prepared in H_2O MQ and heated at 90°C for 10 minutes. After this time the mixture was slowly cooled down until room temperature, obtaining the resulting 3WJ/ds hybridized DNA.

The DNA sequences used can be found in the Supporting Information (Section 2.1).

2.11. Solid phase peptide synthesis

a) *LLD and DDL peptide ligand synthesis*

C-terminal amide **LLD** and **DDL** ligands were synthesized following standard Fmoc-peptide synthesis protocols on a 0.1 mmol scale using a 0.5 mmol/g loading H-Rink amide ChemMatrix resin (35–100 mesh size particle). The peptides were synthesized by hand, using HBTU as activating agent for the natural amino acids and HATU for the Fmoc- β Ala5Bpy-OH (**1**) couplings. Each amino acid was activated for 1 min in DIEA/DMF 0.2 M (4 mL) before being added onto the resin. These manual couplings were conducted for 60 min. Deprotection of the temporal Fmoc protecting group was performed by treating the resin with 20% piperidine in DMF for 20 min. All the couplings were carried out in a 5-fold excess of the corresponding amino acid for the natural ones and in a 3.5-fold excess for the Fmoc- β Ala5Bpy-OH (**1**).

N-capping with TAMRA was performed by reacting the N-terminal deprotected amine with the activated fluorophore —6-Carboxytetramethylrhodamine succinimidyl ester (TAMRA-OSu) in 1 mL DIEA/DMF 0.2 M during 2 h (approximately 7.5 mg of the reactive fluorophore/40 mg of resin).

b) *Cleavage from the resin and final deprotection*

Cleavage and deprotection of the peptides were simultaneously performed using standard conditions by incubating the resin for 2.5 h with an acidic mixture containing 50 μ L CH_2Cl_2 , 25 μ L of H_2O , 25 μ L of TIS (triisopropylsilane), and 900 μ L of TFA. The resin was filtered, and the TFA filtrate was concentrated under a nitrogen stream to an approximate volume of 1 mL, and then added onto ice-cold diethyl ether (20 mL). After 10–30 min, the precipitate was centrifuged and washed again with 5 mL of ice-cold ether. The obtained residue was then dissolved in a mixture of H_2O and CH_3CN and purified by preparative HPLC for the obtention of the pure compounds.

2.12. Fluorescence spectroscopy

Luminescence experiments were made with a Varian Cary Eclipse Fluorescence Spectrophotometer coupled to a Cary Single Cell Peltier accessory (Agilent Technologies) temperature controller. All measurements were made with a Hellma semi-micro cuvette (108F-QS) at 20 °C. The settings for these measurements were adapted depending on the studied system.

a) *Metal binding studies*

A fluorescence titration of a 2 μ M solution of **LLD** or **DDL** peptide in phosphate buffer (1 mM, 10 mM NaCl, pH 7.0) with increasing concentrations of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ resulted in a series of emission spectra with decreasing emission intensity due to the quenching of the bipyridine residues upon coordination of the Cu^{II} ions. The resulting titration profiles at 420 nm could be fitted to a 1:2 binding mode using Dynafit 4.0 software.

b) *DNA binding studies*

The fluorescence emission of a 2 μ M solution of the fluorescein-labelled 3WJ/dsDNA in phosphate buffer (1 mM, 10 mM NaCl, pH 7.0) was monitored after the addition of increasing amounts of the preformed $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$ peptide helicate. The quenching effect in the fluorescein emission band at 515 nm could be fitted to a 1:1 binding mode (Flu-DNA/helicate) plus nonspecific interactions using Dynafit 4.0 software.

c) *Studies on the kinetic inertness of the Cu^{II} peptide helicate*

The fluorescence emission at 420 nm of a 2 μ M solution of $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$ and 4 μ M of EDTA in phosphate buffer (1 mM, 10 mM NaCl, pH 7.0) was monitored during 60 min. The resulting complex half time was $t_{1/2} = 5.815$ minutes and was calculated using Prism 9 software (by Dotmatics). The same experiment was repeated in the presence of 0.267 μ M of 3WJ. The resulting complex half time was $t_{1/2} = 23.12$ minutes and was also calculated using Prism 9 software (by Dotmatics).

d) *Data analysis in fluorescence titrations*

Fluorescence titration data were analyzed using the DynaFit 4.0 software, which characterizes the reacting system in terms of stoichiometric equations instead of mathematical notation; the chemical equations are then translated into the underlying mathematical equations using matrix theory.

DynaFit is available at <http://www.biokin.com/dynafit/>. Dynafit requires plain text files (scripts) that contain the chemical model underlying the experimental data, the values of model parameters, such as starting concentrations of reactants, as well as information about location of the files. A typical script used in the analysis titrations is included in Supporting Information (Section 2.2.)

2.13. Polyacrylamide gel electrophoretic analysis (PAGE)

PAGE experiments with fluorophore-labelled DNA were performed using an Invitrogen mini gel tank. Gels were cast using a SureCastTM gel casting system (Invitrogen) and powered by a BioRad basic power

pacTM. PAGE gels were visualised using a G:Box 9 mini gel documentation system and analysed with GeneSys software (Syngene). The multiplex assay was performed on GeneSys software (Syngene); an image with the excitation and emission for each fluorophore was captured, the images were overlaid, and a false colour applied for each fluorescent signal. Band densitometry was performed in triplicate on gels post-stained with SYBR gold (1X, Invitrogen) using GeneTools software (Syngene). Statistical analysis was performed using GraphPad Prism.

PAGE experiments with non-labelled DNA were performed with a BioRad Mini Protean gel system, powered by an electrophoresis power supplies PowerPac Basic model, maximum power 150 V, frequency 50–60 Hz at 130 V (constant V).

a) Non-labelled 3WJ and dsDNA binding studies

Non-labelled DNA sequences used in these studies can be found in ESI (Section 2.1). Binding reactions were performed over 30 min in 18 mM Tris-HCl (pH 7.5), 90 mM KCl, 1.8 mM MgCl₂, 9% glycerol, 0.11 mg/mL BSA, and 2.2% NP-40. For the experiments we used 200 nM of the DNAs (3WJ and dsDNA), different quantities of $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$ (200, 400 or 600 mM) and a total incubation volume of 20 μL . After incubation for 30 min at room temperature, products were resolved by PAGE using a 10% non-denaturing polyacrylamide gel and 0.5 $\times$ TBE buffer (0.445 M Tris, 0.445 M Boric acid) for 35 min at 25 $^{\circ}\text{C}$ and analyzed by staining with SyBrGold (Molecular Probes: 5 μL in 50 mL of 1 $\times$ TBE) for 10 min and visualized by fluorescence (BioRad GelDoc XR+ molecular imager). The area of each band in the gel was measured using the Fiji ImageJ implementation using the Gel Analysis method outlined in the ImageJ documentation (<https://imagej.nih.gov/ij/docs/menus/analyze.html#gels>).

b) Fluorophore labelled 3WJ and dsDNA binding studies

Fluorescently labelled 3WJ (5 pmol) and dsDNA (5 pmol) sequences (ESI, Section 2.3) were incubated with 1, 2 or 3 eq. of $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$ (5, 10 or 30 pmol) in HEPES buffer (10 mM HEPES, 750 mM NaCl, 50 mM MgCl₂, pH 7) at 37 $^{\circ}\text{C}$ for 2 hours prior to 20% native PAGE analysis. The gel was run in 50 mM Tris-acetate buffer at 70 V for 240 min and visualised with a multiplex assay. FAM fluorescence was observed with blue light and 525 nm filter, ROX was excited by UV fluorescence and Cy5 was visualised with red light and a 705 nm filter.

c) In vitro nuclease activity studies

Nuclease activity was analysed by PAGE. Equal concentrations of each strand of the 3WJ were mixed in HEPES buffer (10 mM HEPES, 750 mM NaCl, 50 mM MgCl₂, pH 7) and heated to 95 $^{\circ}\text{C}$ before being cooled in 5 $^{\circ}\text{C}$ increments to 4 $^{\circ}\text{C}$. The annealed 3WJ (5 pmol), and an off-target DNA duplex (5 pmol), were incubated at 37 $^{\circ}\text{C}$ for 24 hours with $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$ (1, 2, 5, 7.5 or 10 equivalents, that is, 5, 10, 25, 37.5 or 50 pmol) and Na-L-ascorbate (10 eq. to $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$, that is, 50, 125, 250, 375 or 500 pmol). The reaction was quenched with loading dye (6X, 10 mM Tris-HCl, pH 7.6, 0.03% bromophenol blue, 0.03% xylene cyanol, 60 mM EDTA) and loaded onto a 20% native PAGE gel (50 mM Tris-acetate, 75 mM NaCl, 5 mM MgCl₂, pH 6.1). The gel was run in 50 mM Tris-acetate buffer at 70 V for 240 min, and post-stained with SYBR gold (1X, Invitrogen). Gel was visualized under UV fluorescence. Band densitometry was performed in triplicate, and the significance of the data was analysed by Two-way ANOVA with Tukey's multiple comparisons.

Cleavage activity on fluorescently labelled DNA. Samples were prepared as described above, but fluorophore labelled 3WJ and dsDNA strands were utilised. Cleavage assay was quenched and loaded onto a 20% native PAGE gel (50 mM Tris-acetate, 75 mM NaCl, 5 mM MgCl₂, pH 6.1). The gel was run in 50 mM Tris-acetate buffer at 70 V for 240 min and visualised using the multiplex assay described above.

$\Delta\Delta\text{-Fe}^{\text{II}}_2\text{LLD}$ cleavage (negative control). Samples containing 3WJ and dsDNA were prepared as previously described but $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$ was replaced with $\Delta\Delta\text{-Fe}^{\text{II}}_2\text{LLD}$. All other reaction components were kept consistent with the Cu^{II} helicate cleavage assay.

Time-point study. Samples containing 3WJ, dsDNA, $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$ (1, 5 and 10 eq. to DNA) and Na-L-ascorbate were prepared as described above, and incubated at various time points (1 hour, 3 hours and 12 hours) at 37 $^{\circ}\text{C}$ before being quenched with loading dye (6X, 10 mM Tris-HCl, pH 7.6, 0.03% bromophenol blue, 0.03% xylene cyanol, 60 mM EDTA) and loaded onto a 20% native PAGE gel (50 mM Tris-acetate, 75 mM NaCl, 5 mM MgCl₂, pH 6.1). Gel was run at 70 V for 240 min, post-stained with SYBR gold (1X, Invitrogen) and visualised by UV fluorescence.

Reactive oxygen species (ROS) analysis. The 3WJ and $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$ samples were prepared as previously described, but the dsDNA off-target was replaced with a reactive oxygen species scavenger (50 nmol); L-histidine, D-mannitol, L-methionine, tiron (4,5-dihydroxy-1,3-benzendisulfonic acid), sodium azide (NaN₃), deuterium oxide (D₂O) or 4-hydroxy-TEMPO and incubated at 37 $^{\circ}\text{C}$ for 24 hours. Products were analysed by 20% Tris-acetate PAGE (50 mM Tris-acetate, 75 mM NaCl, 5 mM MgCl₂, pH 6.1).

2.14. Cell internalization studies

Vero or HeLa cells were grown in DMEM (Dulbecco modified Eagle Medium) with 10% of fetal bovine serum in glass-bottom 35 mm tissue culture plates (IBIDI). One day before the internalization experiment, the cells were transfected with plasmid pc653-pENeGFPCNAL2mut (H. Leonhardt, 2000) using PEI Polyethylenimine HCl MAX (PEI_{max}®, obtained from Polysciences) following the instructions from the manufacturer. That plasmid drives the expression of GFP-tagged Proliferating Cellular Nuclear Antigen (PCNA) to label DNA replication factories. The next day the cells were washed twice with transfer buffer (25 mM HEPES, 125 mM KOAc, 2 mM Mg(AcO)₂, 1 mM EDTA). Then, cells were treated with a digitonin solution (25 µg/mL) in the same previous buffer at 0 °C for 3 minutes. After this time cells were washed again twice with transfer buffer containing 10 mg/mL of BSA. Cells were then incubated in transfer buffer with 5 µM of **ΔΔ-Cu^{II}₂TAMRA-LLD** for 15 minutes. After this time cells were washed three times with transfer buffer containing 10 mg/mL of BSA and finally covered with DMEM supplemented with 10% of fetal bovine serum.

Images were obtained with an Andor Dragonfly spinning disk confocal system mounted on a Nikon TiE microscope equipped with a Zyla 4.2 PLUS camera (Andor).

2.15. Nuclease activity assays in cells

a) Cell synchronization

4x10⁶ Vero cells were grown in T175 flasks. After 24 hours of incubation at 37 °C with 5% CO₂, the medium was removed, and cells were washed three times with PBS 1X and incubated in DMEM-HEPES with 1% antibiotics and without Glutamine and FBS. After 42 hours, the medium was replaced by DMEM + 10% FBS and 1% glutamine + antibiotics. After 16 hours the cells were trypsinized and counted.

b) In cellulo nuclease assay

Vero cells were pelleted (500 x g, 5 min 4 °C) and washed three times with Transfer Buffer (25 mM HEPES pH 7.9, 125 mM KOAc, 2 mM Mg(AcO)₂, 1 mM EDTA). Subsequently, cells were treated with 25 µg/ml digitonin in transfer buffer for 3 min on ice. After this time cells were centrifuged, and the supernatant were removed. Cells were resuspended according to the treatment in: transfer buffer, transfer buffer + 2.5 mM H₂O₂, transfer buffer + 2.5 mM H₂O₂ + 5 µM **ΔΔ-Fe^{II}₂LLD** and transfer buffer + 2.5 mM H₂O₂ + 5 µM **ΔΔ-Cu^{II}₂LLD** and incubated 2 hours at 37 °C shaking every 15 minutes. After this time, cells were centrifuged and washed twice with Transfer buffer + 10 mg/ml BSA and twice with PBS 1X. Finally, cells were fixed for the TUNEL Assay.

c) TUNEL Assay

Vero cells were fixed with 5 mL of 1% PFA (paraformaldehyde) in PBS for 15 min at 4 °C. Subsequently, cells were centrifuged (500 x g, 5 min, 4 °C) and washed twice with PBS 1X. Then, the cells were resuspended in 5 mL of ice-cold 70% ethanol and incubate 5 days at -20 °C. The cells were then collected by centrifugation and subjected to TUNEL assay following the manufacturer's instructions (APO-BrdUTM TUNEL Assay Kit, Thermo Fisher Scientific). Briefly, they were washed with 1 mL of Wash buffer and then incubated with DNA-labelling solution overnight at RT on a nutator mixer. Next, the cells were washed twice with Rinse Buffer and incubated with Alexa Fluor 488TM dye-labelled anti-BrdU antibody for 30 min at RT on a nutator and analysed by flow cytometry using a Guava easyCyte HT Millipore flow cytometer equipped with a 488 nm blue laser coupled with a 525/30 nm filter. One-way ANOVA with Dunnett's multiple comparisons was performed to determine significance of data collected.

2.16. Cytotoxicity studies

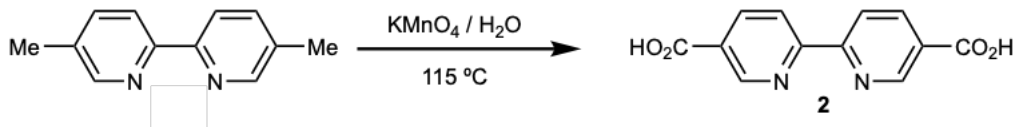
The effect of **ΔΔ-Cu^{II}₂LLD** on Vero cell viability was determined with the Cell Counting Kit-8 (CCK-8, TargetMol) (E. Oner, 2023).

3. Experimental data

3.1. Synthesis of Fmoc- β Ala5Bpy-OH (8)

The synthesis of this chelating residue was carried out following the methodology recently published by our group.[1] All the obtained products were analyzed by HPLC-MS to test their purity.

3.1.1. Synthesis of 2,2'-bipyridine-5,5'-dicarboxylic acid (2)



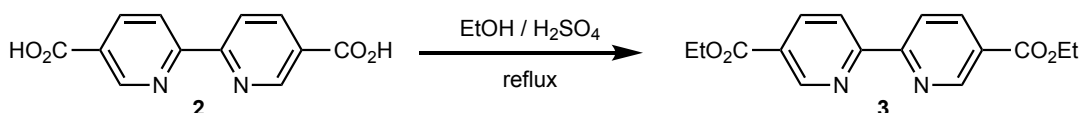
A mixture of 39 g of potassium permanganate and 7 g of 5,5'-dimethyl-2,2'-bipyridine in 250 mL of H_2O was heated for 2 h (115°C), cooled at room temperature and filtrated trough celite. The filtrate was cooled to 4°C and acidified with HCl until precipitation of a white solid, which was filtrated, washed with water and lyophilized to afford the desired product in a 93% yield (8.7 g)

$^1\text{H-NMR}$ (500 MHz, $\text{DMSO-}d_6$, δ): 13.50 (br); 9.18 (dd, $^4J = 2.15$, $^5J = 0.8$ Hz 2H); 8.55 (dd, $^3J = 8.3$; $^5J = 0.8$ Hz, 2H); 8.44 (dd, $^3J = 8.3$; $^4J = 2.15$ Hz, 2H).

$^{13}\text{C-NMR}$ (125 MHz, $\text{DMSO-}d_6$, δ): 165.49, 156.84, 149.82, 137.97, 126.65, 120.62.

MALDI-TOF (m/z) $[\text{M}+\text{H}]^+$ calculated for $[\text{C}_{12}\text{H}_8\text{N}_2\text{O}_4]$ 245.0; found 245.0.

3.1.2. Synthesis of diethyl [2,2'-bipyridine]-5,5'-dicarboxylate (3)



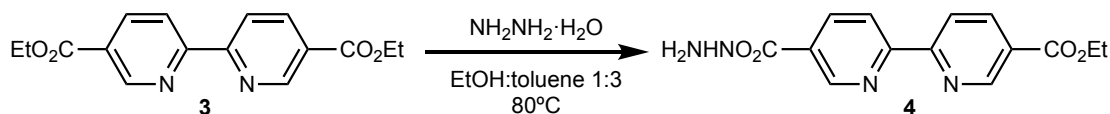
2,2'-bipyridine-5,5'-dicarboxylic acid (10.0 g, 41 mmol) was suspended in 150 mL of absolute ethanol. Concentrated sulfuric acid (20.0 mL) was slowly added, and the resulting mixture was refluxed for 18 h. The solution was cooled at room temperature and added over 400 mL of water at 4°C causing the precipitation of a white solid, which was filtered, washed with water and lyophilized. 11.5 g (93.0 %)

$^1\text{H-NMR}$ (500 MHz, $\text{DMSO-}d_6$, δ): 9.20 (dd, $^4J = 2.15$, $^5J = 0.8$ Hz 2H); 8.57 (dd, $^3J = 8.3$; $^5J = 0.8$ Hz, 2H); 8.46 (dd, $^3J = 8.3$; $^4J = 2.15$ Hz, 2H); 4.0 (q, $^3J = 7.1$ Hz, 4H); 1.37 (t, $^3J = 7.1$ Hz, 6H).

$^{13}\text{C-NMR}$ (125 MHz, $\text{DMSO-}d_6$, δ): 164.2, 157.2, 149.8, 138.0, 126.2, 121.0, 61.1, 13.9.

MALDI-TOF (m/z) $[\text{M}+\text{H}]^+$ calculated for $[\text{C}_{16}\text{H}_{16}\text{N}_2\text{O}_4]$ 301.1; found 301.1.

3.1.3. Synthesis of Ethyl 5'-Carbohydrazido-2,2'-bipyridine-5-carboxylate (4)



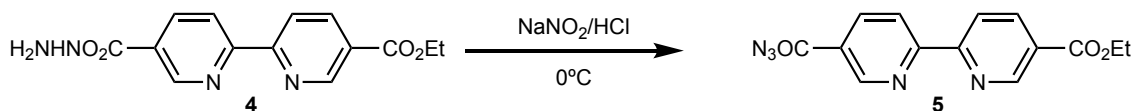
A mixture of diethyl 2,2'-bipyridine-5,5'-dicarboxylate (15.00 g, 50 mmol) and hydrazine hydrate (3.75 mL, 55 mmol) in a solution of EtOH (42 mL) and toluene (128 mL) was heated at 80°C for 48 h. The precipitate was filtered, washed with CHCl_3 and dried under vacuum 11.4 g (80%). The unreacted diethyl ester was concentrated and mixed again to obtain a global yield of (90%).

$^1\text{H-NMR}$ (500 MHz, $\text{DMSO-}d_6$, δ): 1.35 (t, 3H), 4.37 (q, 2H), 8.35 (d, $^3J = 8.4$ Hz, 1H), 8.45 (dd, $^3J = 8.4$ Hz, $^4J = 2$ Hz, 1H), 8.52 (d, $^3J = 8.4$ Hz, 1H), 8.57 (d, $^3J = 8.4$ Hz, 1H), 9.1 (d, $^4J = 2$ Hz, 1H), 9.2 (d, $^4J = 2$ Hz, 1H), 10.1 (br, 1H).

$^{13}\text{C-NMR}$ (125 MHz, $\text{DMSO-}d_6$, δ): 14.1, 61.3, 120.9, 120.9, 126.1, 129.6, 136.2, 138.2, 148.1, 150.0, 155.8, 157.8, 163.8, 164.5.

MALDI-TOF (m/z) $[\text{M}+\text{H}]^+$ calculated for $[\text{C}_{14}\text{H}_{14}\text{N}_4\text{O}_3]$ 287.1; found 287.1.

3.1.4. Synthesis of Ethyl 5'-Carbohydrazido-2,2'-bipyridine-5-carboxylate (5)



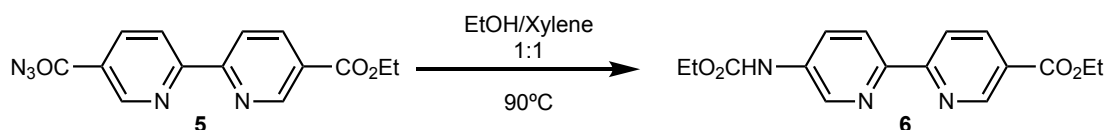
A stirred solution of 5-(ethoxycarbonyl)-5'-carbohydrazido-2,2'-bipyridine (5.7 g, 20 mmol) in concentrated HCl (100 mL) was cooled to 0°C , and then an aqueous solution of NaNO_2 (1.73 g, 25 mmol; 15 mL) was added dropwise, maintaining the temperature at 0°C . After 60 min, the yellow solution was diluted with water (300 mL) to precipitate the monoester 5 as a white powder, which was filtered, washed with water, and lyophilized 5.5 g (92 %).

$^1\text{H-NMR}$ (500 MHz, Acetone- d_6 , δ): 9.13 (d, $^4J = 1.91$ Hz, 1H), 9.10 (d, $^4J = 1.91$ Hz, 1H), 8.71 (d, $^3J = 12.5$ Hz, 1H), 8.69 (d, $^3J = 12.5$ Hz, 1H), 8.517 (m, 2H; 4.31 (q, $^3J = 7.15$ Hz, 2H); 1.29 (t, $^3J = 7.15$, 3H).

$^{13}\text{C-NMR}$ (125 MHz, Acetone- d_6 , δ): 170.8, 164.5, 159.2, 157.7, 150.3, 150.0, 138.0, 137.9, 127.0, 121.3, 61.2, 13.6.

MALDI-TOF (m/z) $[\text{M}+\text{H}]^+$ calculated for $[\text{C}_{14}\text{H}_{11}\text{N}_5\text{O}_3]$ 298.1; found 298.1

3.1.5. Synthesis of Ethyl 5'-(ethoxycarbonyl)amino-2,2'-bipyridine-5-carboxylate (6)



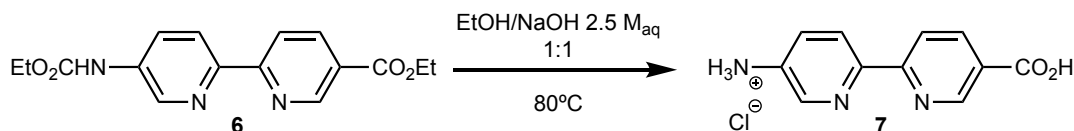
A solution of ethyl 5'-carbazido-2,2'-bipyridine-5-carboxylate (8.6 g, 29 mmol) in a mixture of EtOH (100 mL) and xylene (100 mL) was heated at 90°C for 4 h. The solvent was evaporated under reduced pressure, and the yellow residue was washed with EtOH and dried in vacuo. 8.21 g (90%).

$^1\text{H-NMR}$ (500 MHz, DMSO- d_6 , δ): 1.27 (t, $J = 7.2$ Hz, 3H), 1.35 (t, $J = 7.2$ Hz, 3H), 4.17 (q, $J = 7.2$ Hz, 2H), 4.36 (q, $J = 7.2$ Hz, 2H), 8.08 (dd, $^3J = 8$ Hz, $^4J = 2$ Hz, 1H), 8.35 (dd, $^3J = 8.3$ Hz, $^4J = 2$ Hz, 1H), 8.40 (dd, $^3J = 8.3$ Hz, $^5J = 1$ Hz, 1H), 8.45 (d, $^3J = 8$ Hz, 1H), 8.70 (d, $^4J = 2$ Hz, 1H), 9.12 (d, $^4J = 2$ Hz, $^5J = 1$ Hz, 1H), 10.13 (s, 1H).

$^{13}\text{C-NMR}$ (125 MHz, DMSO- d_6 , δ): 13.6, 13.9, 60.3, 60.6, 119.1, 121.2, 124.46, 124.9, 136.7, 137.4, 139.0, 147.4, 149.4, 153.1, 158.1, 164.2.

MALDI-TOF (m/z) $[\text{M}+\text{H}]^+$ calculated for $[\text{C}_{16}\text{H}_{17}\text{N}_3\text{O}_4]$ 316.1; found 316.1.

3.1.6. Synthesis of 5'-Amino-2,2'-bipyridine-5-carboxylic acid hydrochloride (7)



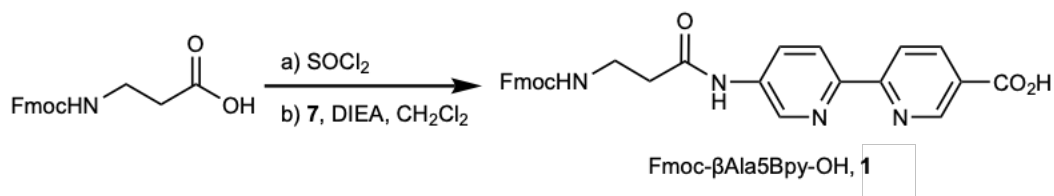
A stirred solution of ethyl 5'-[(ethoxycarbonyl)amino]-2,2'-bipyridine-5-carboxylate (14.55 g, 45.9 mmol) in a mixture of EtOH (50 mL) and 2.5 M aqueous NaOH (50 mL) was heated at 75°C for 14 h. The EtOH was concentrated in vacuum, and the aqueous solution was acidified with HCl to afford a bright yellow precipitate, which was filtered, washed with cold water, and lyophilized 9.80 g (85%).

$^1\text{H-NMR}$ (500 MHz, D_2O , δ): 7.00 (dd, $^3J = 8.5$ Hz, $^4J = 2.8$ Hz, 1H), 7.55, 7.61 (d, $^3J = 8.5$ Hz, 2H), 7.81 (d, $^4J = 2.9$ Hz, 1H), 7.98 (dd, $^3J = 8.5$ Hz, $^4J = 2.9$ Hz, 1H), 8.65 (d, $^4J = 2.8$ Hz, 1H)

$^{13}\text{C-NMR}$ (125 MHz, D_2O , δ): 123.3d, 126.2, 126.7, 133.8, 139.8, 141.34, 147.5, 147.4, 152.4, 159.6, 176.04

MALDI-TOF (m/z) $[\text{M}+\text{H}]^+$ calculated for $[\text{C}_{11}\text{H}_9\text{N}_3\text{O}_2]$ 216.1; found 216.1

3.1.7. Synthesis of Fmoc- β Ala5Bpy-OH (1)



Over 4.58 g (14.72 mmol) of Fmoc- β Ala 10 mL of SOCl_2 was added carefully at room temperature. The solution was stirred magnetically for 30 min, the thionyl chloride was evaporated in vacuum and the yellow solid was washed with CH_2Cl_2 (3x10 mL), and dried under reduced pressure. The compound (7) 3.7 g (14.8

mmol), 20 mL of CH₂Cl₂ and 5 mL of DIEA were added. The suspension was stirred at room temperature overnight. The solvent was evaporated, the solid was suspended in acetonitrile 0.1 %TFA/H₂O (2:1), centrifuged and washed with acetonitrile and H₂O and lyophilized 6.70 g (89 %).

Regarding the nomenclature of the coordinating residue **1**, Fmoc is the amine protecting group necessary to introduce the 2,2'-bipyridine residue into a peptide sequence by SPPS methodology. βAla is the amino acid attached to the amino group of the 2,2'-bipyridine amino acid. This extra amino acid is key to being able to integrate the 2,2'-bipyridine amino acid into the peptide sequence, as its amino group is not basic enough to form peptide bonds by a SPPS methodology. The number 5 indicates that the substitutions of the 2,2'-bipyridine molecule to transform it into an artificial amino acid have been made at their 5 and 5' positions.

¹H-NMR (300 MHz, DMSO-*d*₆, δ): 10.44 (s, 1H); 9.13 (d, ⁴*J* = 1.36 Hz, 1H); 8.89 (d, ⁴*J* = 2.12 Hz, 1H); 8.42 (m, 3H); 8.25(dd, ³*J* = 8.48 Hz, ⁴*J* = 2.12 Hz, 1H); 7.88 (d, ³*J* = 7.46 Hz, 2H); 7.67 (d, ³*J* = 7.38 Hz, 2H); 7.49 (t, ³*J* = 5.34 Hz, NH); 7.40 (t, ³*J* = 7.21 Hz, 2H); 7.30 (t, ³*J* = 7.38 Hz, 2H); 4.30 (d, ³*J* = 6.95 Hz, 2H); 4.21 (t, ³*J* = 6.95 Hz, 1H) 3.32 (t, ³*J* = 6.70 Hz, 2H); 2.60 (t, ³*J* = 6.70 Hz, 2H).

¹³C-NMR (75 MHz, DMSO-*d*₆, δ): 169.7 (C), 168.6 (C), 157.2 (C), 150.9 (C), 149.2 (C), 144.7 (C), 143.4 (C), 142.2 (C), 141.6 (C), 140.3 (CH), 138.3 (CH), 138.1 (CH), 129.7 (CH), 128.1 (CH), 125.9 (CH), 122.1 (CH), 120.9 (CH), 120.8 (CH), 118.7 (CH), 110.3 (CH), 67.4 (CH₂), 63.8 (CH₂), 47.6 (CH), 41.7 (CH₂).

MALDI-TOF (m/z) [M+H]⁺ calculated for [C₂₉H₂₅N₄O₅] 509.1; found 509.1.

3.2. UHPLC-MS of the purified peptide ligands

3.2.1. LLD: H-(βAla5Bpy)₂RPr(βAla5Bpy)₂RPr(βAla5Bpy)₂-NH₂

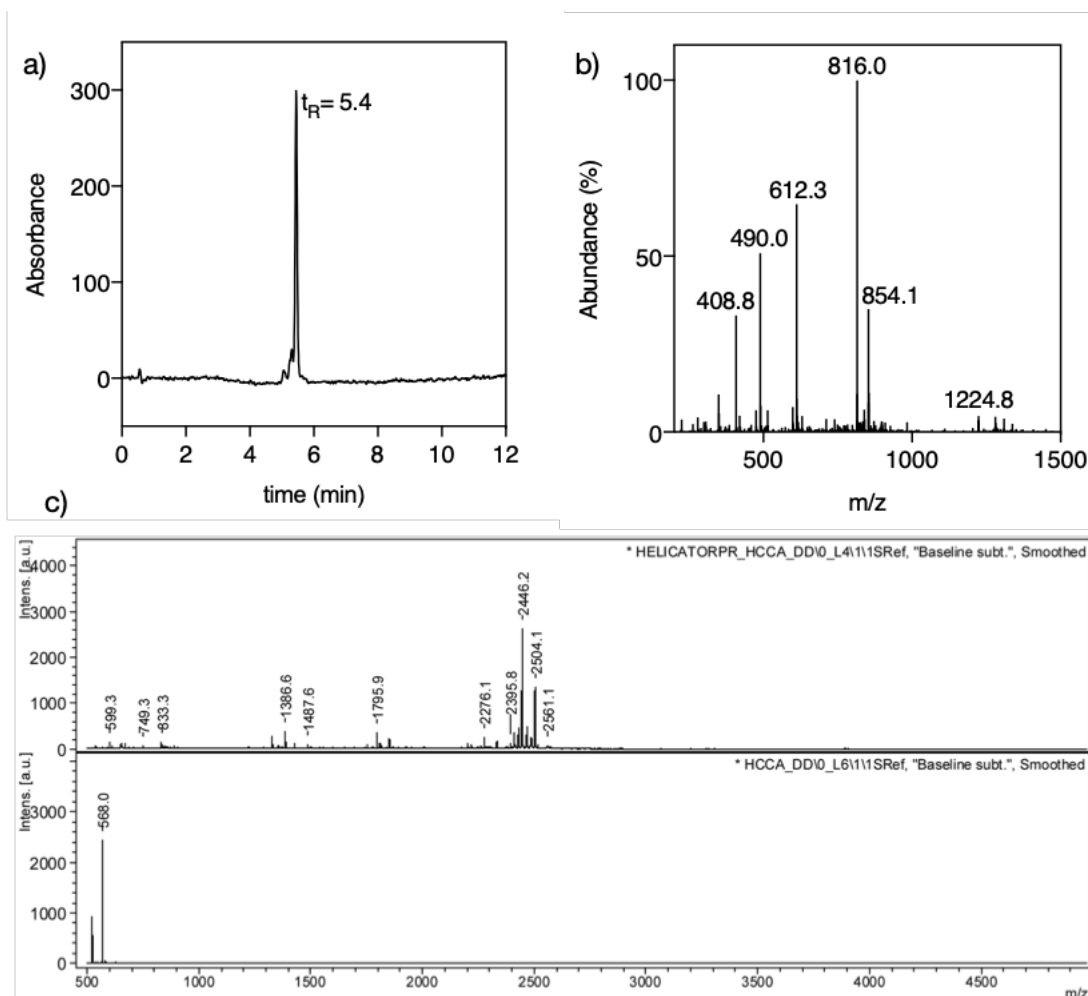


Figure S1. a) Chromatogram of the purified **LLD** peptide ligand. b) ESI mass spectra corresponding to the peak with *t_R* of 5.4 min. c) MALDI mass spectra of **LLD**.

HPLC-MS (ESI): (5-95% B, *t_R* = 5.4 min) Calculated for C₁₁₈H₁₃₇N₄₃O₁₈ = 2444.11; found [M+2H+TFAH]²⁺ = 1289.7; [M+3H]³⁺ = 816.0; [M+3H+TFAH]³⁺ = 854.1; [M+4H]⁴⁺ = 612.3; [M+5H]⁵⁺ = 490.0; [M+6H]⁶⁺ = 408.8.

MS (MALDI-TOF): Calculated for $C_{118}H_{137}N_{43}O_{18} = 2444.11$; found: $[M+H]^+ = 2446.21$.

3.2.2. DDL: $H-(\beta Ala5Bpy)_2rpR(\beta Ala5Bpy)_2rpR(\beta Ala5Bpy)_2-NH_2$

HPLC-MS (ESI): (5-95% B, $t_R = 5.4$ min) Calculated for $C_{118}H_{137}N_{43}O_{18} = 2444.11$; found $[M+2H+TFAH]^{2+} = 1289.7$; $[M+3H]^{3+} = 816.0$; $[M+3H+TFAH]^{3+} = 854.1$; $[M+4H]^{4+} = 612.3$; $[M+5H]^{5+} = 490.0$; $[M+6H]^{6+} = 408.8$.

MS (MALDI-TOF): Calculated for $C_{118}H_{137}N_{43}O_{18} = 2444.11$; found: $[M+H]^+ = 2446.21$.

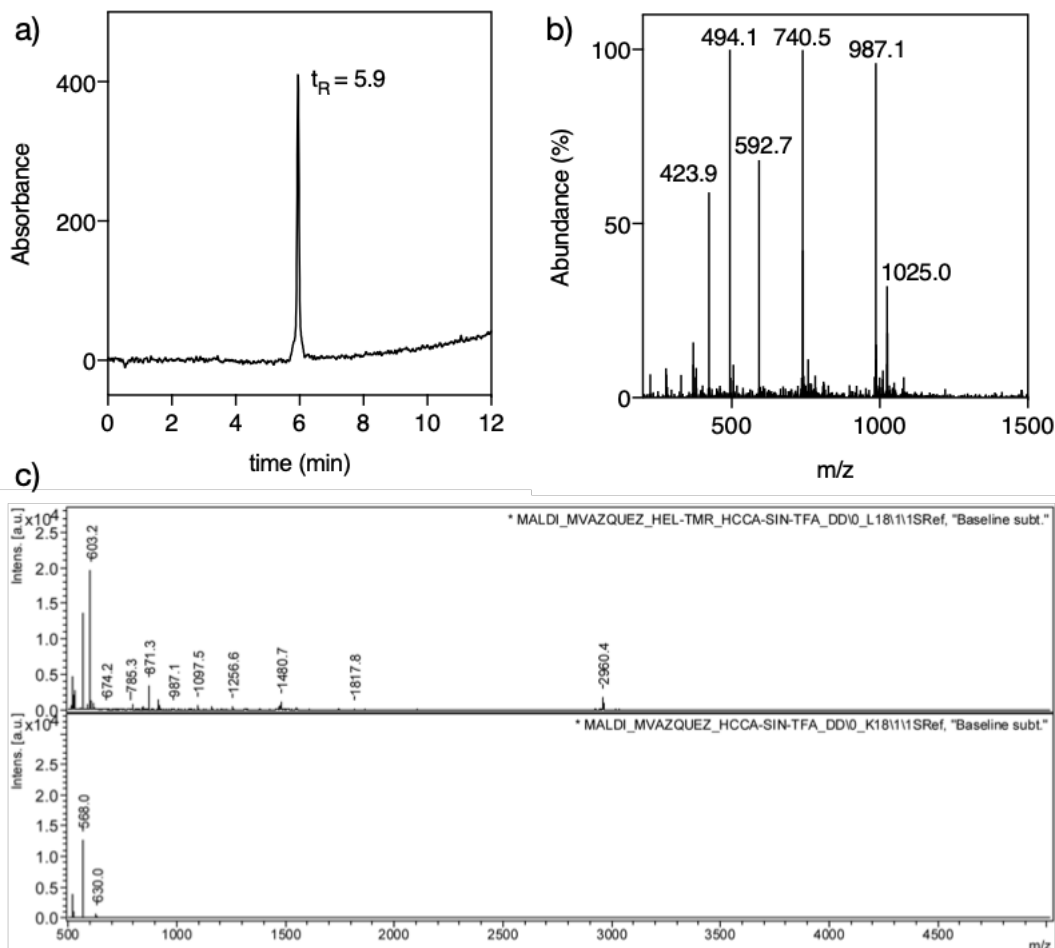


Figure S2. a) Chromatogram of the purified **TAMRA-LLD** peptide ligand. b) ESI mass spectra corresponding to the peak with t_R of 5.9 min. c) MALDI mass spectra of **TAMRA-LLD**.

HPLC-MS (ESI): (5-95% B, $t_R = 5.9$ min) Calculated for $C_{147}H_{166}N_{46}O_{24} = 2959.31$; found $[M+3H+TFAH]^{3+} = 1025.0$; $[M+3H]^{3+} = 987.1$; $[M+4H]^{4+} = 740.5$; $[M+5H]^{5+} = 592.7$; $[M+6H]^{6+} = 494.1$; $[M+7H]^{7+} = 423.9$.

MS (MALDI-TOF): Calculated for $C_{147}H_{166}N_{46}O_{24} = 2959.31$; found: $[M+H]^+ = 2960.43$.

3.3. Synthesis of the peptide helicates

3.3.1. Synthesis of the Fe^{II} peptide helicates

The synthesis of $\Lambda\Lambda$ - Fe^{II}_2 LLD and $\Lambda\Lambda$ - Fe^{II}_2 TAMRA-LLD metallopeptides were made following the procedure reported in our previous paper (J. Gómez-González, 2021).

3.3.2. Synthesis of the Cu^{II} peptide helicates

Onto a solution of LLD, DDL or TAMRA-LLD in phosphate buffer (1 mM, 10 mM NaCl, pH 7.0), $CuCl_2 \cdot 2H_2O$ was added as source of Cu^{II} ions until saturating conditions (5 eq).

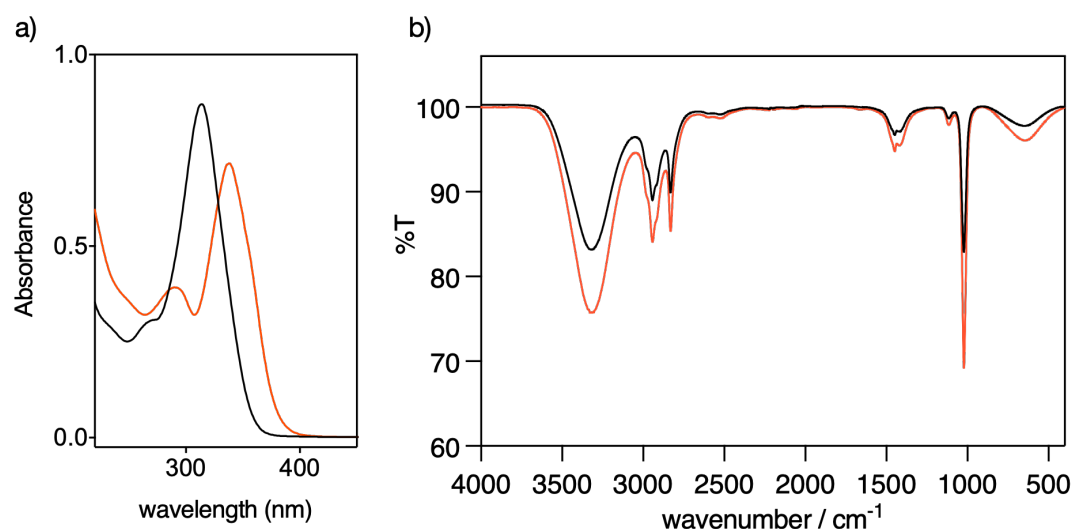


Figure S3. a) UV-Vis spectrum of a 5 μM solution of **LLD** peptide ligand (1 mM phosphate buffer, 10 mM NaCl, pH 7.0) before (black solid line) and after the addition of 5 equivalents of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (orange line); b) IR spectrum of a 5 μM solution of **LLD** peptide ligand in MeOH before (black line) and after the addition of 5 equivalents of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (orange line).

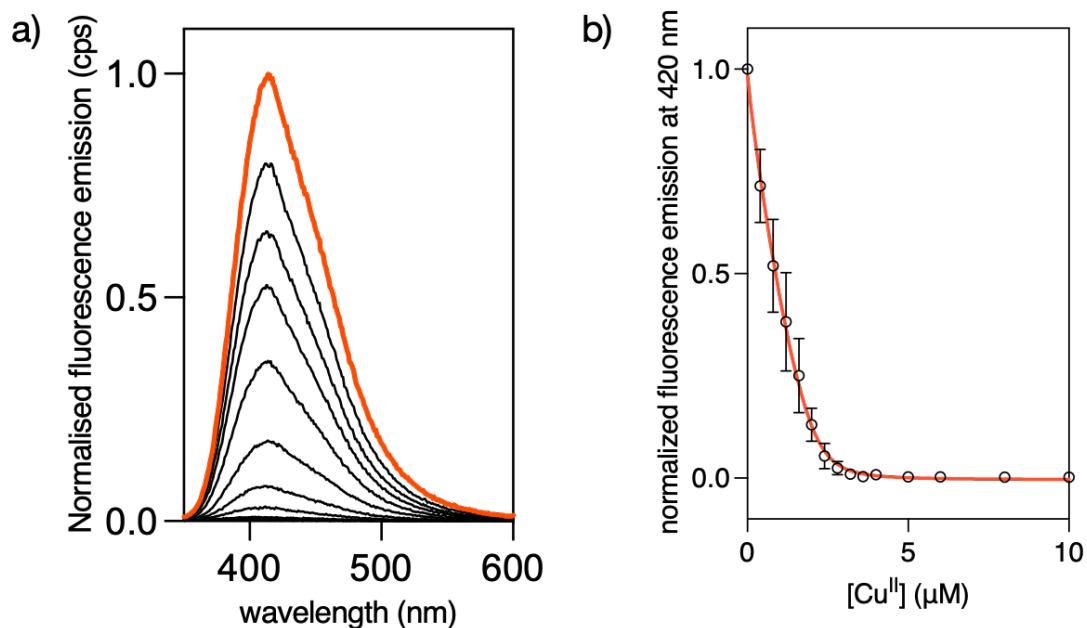


Figure S4. a) Normalized emission spectrum of a 2 μM solution of **DDL** in phosphate buffer (1 mM, NaCl 10 mM, pH 7.0, orange line), and spectra of the same solution in the presence of increasing concentrations of Cu^{II} ions (black lines). b) Titration profile of three independent fluorometric titration experiments at 420 nm and best fit according to a 1:2 model in DynaFit (same range as main plot) with stepwise dissociation constants of approximately 0.3 and 0.15 μM for the first and the second Cu^{II} ions. $\lambda_{\text{exc}} = 308 \text{ nm}$.

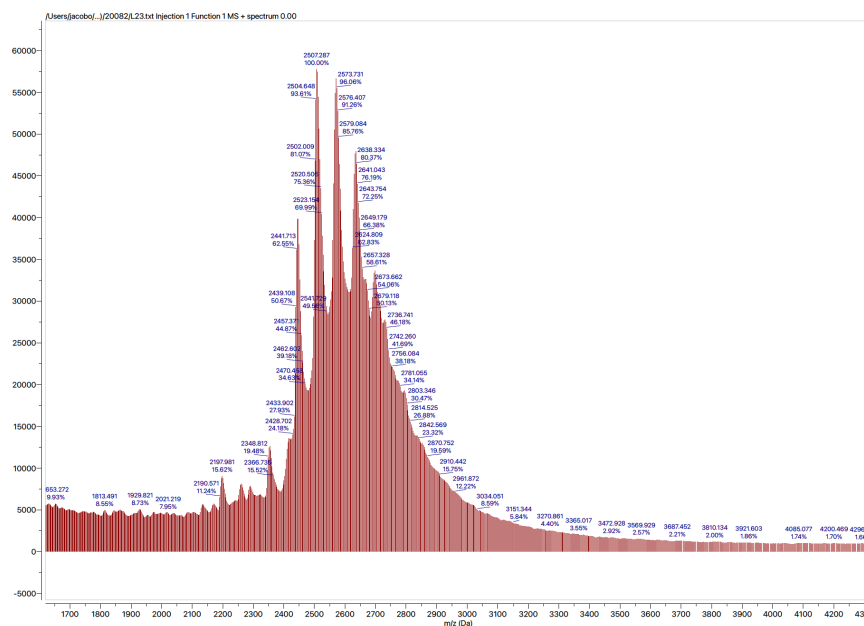


Figure S5. MALDI spectra of $\Delta\Delta\text{-Cu}_2\text{LLD}$

MS (MALDI-TOF): Calculated for $\Delta\Delta\text{-Cu}_2\text{LLD}$ $\text{C}_{118}\text{Cu}_2\text{H}_{137}\text{N}_{43}\text{O}_{18} = 2572.77$; found: $[\text{M}+2\text{Cu}+\text{H}]^+ = 2573.73$.

MS (MALDI-TOF): Calculated for $\Delta\Delta\text{-Cu}_2\text{DDL}$ $\text{C}_{118}\text{Cu}_2\text{H}_{137}\text{N}_{43}\text{O}_{18} = 2572.77$; found: $[\text{M}+2\text{Cu}+\text{H}]^+ = 2573.73$.

MS (MALDI-TOF): Calculated for $\Delta\Delta\text{-Cu}_2\text{TAMRA-LLD}$ $\text{C}_{147}\text{Cu}_2\text{H}_{166}\text{N}_{46}\text{O}_{24} = 3086.32$; found: $[\text{M}+2\text{Cu}] = 3086.75$.

3.4. EPR studies

X-band EPR spectra of the $\Delta\Delta\text{-Cu}_2\text{LLD}$ metalloprotein were obtained in solution [200 μM of metalloprotein in phosphate buffer (1 mM, 10 mM NaCl, pH 7.0)] at 300 K and 120 K.

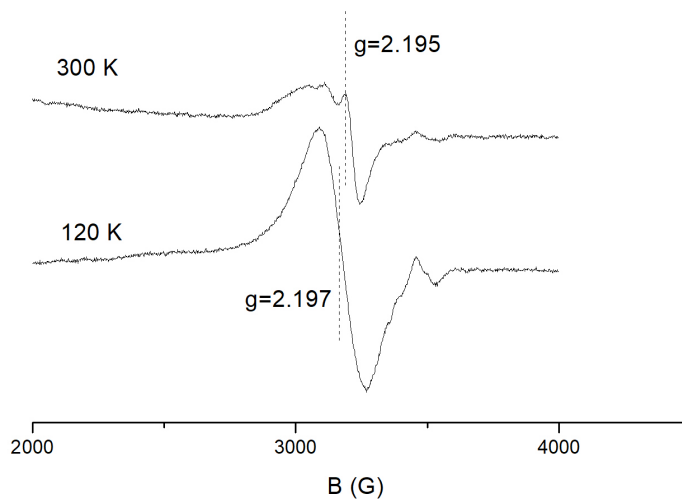


Figure S6. X-band Cu^{II} EPR spectra of the $\Delta\Delta\text{-Cu}_2\text{LLD}$ peptide helicate measured in solution (200 μM of metalloprotein in phosphate buffer 1 mM, 10 mM NaCl, pH 7.0) at 120 K (bottom) and 300 K (top).

3.5. Fluorescence titrations experiments with GC-3WJ DNA

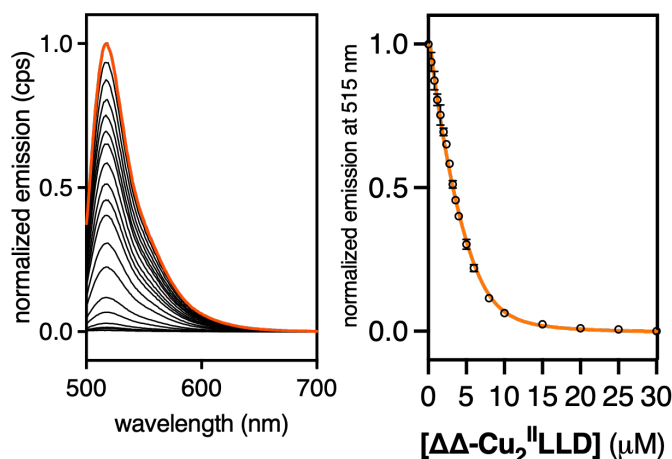


Figure S7. Left: full emission spectra of a 2 μM solution of fluorescein-labelled GC-3WJ (Flu-GC-3WJ) in absence and in presence of increasing concentrations of $\Delta\Delta\text{-Cu}^{\text{II}}\text{LLD}$ (decreasing intensity). Right: profile of the full titration with the best fit (orange line) according to the 1:1 model plus unspecific interactions in DynaFit, with an apparent dissociation constant $K_D = 300$ nM. Conditions: phosphate buffer 1 mM, NaCl 10 mM, pH 7.0.

3.6. Studies on the kinetic inertness of $\Delta\Delta\text{-Cu}^{\text{II}}\text{LLD}$ in physiological media

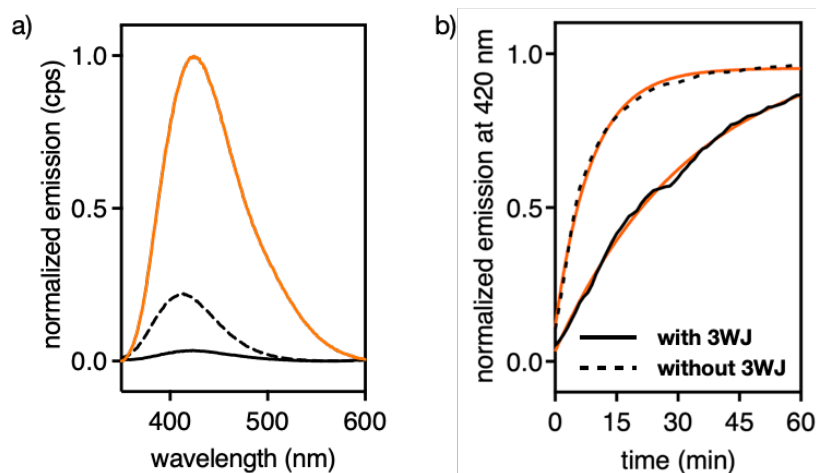


Figure S8. a) Full emission spectra of a 2 μM solution of **LLD** (orange line), and spectra of the same solution with 10 μM of Cu^{II} ions (black line), and with 4 μM of EDTA (S. Chaberek Jr, 1955) after 60 min of stabilization (black dashed line); b) fluorescence emission at 420 nm of the 2 μM solution of $\Delta\Delta\text{-Cu}^{\text{II}}\text{LLD}$ and 4 μM of EDTA without (black continuous line) and with (black dashed line) the presence of 0.267 μM of 3WJ during 60 minutes, together with their respective best fits (orange lines) resulting in a complex half time $t_{1/2} = 5.815$ and 23.12 minutes, respectively. Conditions: phosphate buffer 1 mM, NaCl 10 mM, pH 7.0.

3.7. 3WJ-binding properties of the free peptide ligand LLD

The key role played by metal ions in the recognition of 3WJ by the helicates derived from the peptide ligand **LLD** was demonstrated in our previous work in ACIE (J. Gómez-González, 2021) through a fluorescence anisotropy titration of a 2 μM solution of the fluorescein-labelled **LLD** peptide ligand with increasing concentrations of 3WJ. The descending and oscillating pattern of the spots obtained in the titration demonstrated the absence of interaction between the free peptide ligand **LLD** and 3WJ.

3.8. Gel electrophoresis: DNA binding studies

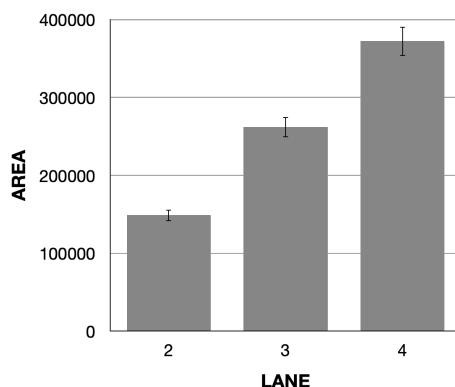


Figure S9. The area of each band in the gel of Figure 3b of the manuscript was measured using the Fiji ImageJ implementation (C. A. Schneider, 2012; J. Schindelin, 2012) using the Gel Analysis method outlined in the ImageJ documentation (<https://imagej.nih.gov/ij/docs/menus/analyze.html#gels>). The area of each band clearly shows a saturation profile and concentration dependent signal, consistent with the progressive saturation of 3WJ binding with the Cu^{II} helicate.

3.9. Gel electrophoresis: *In vitro* nuclease activity studies

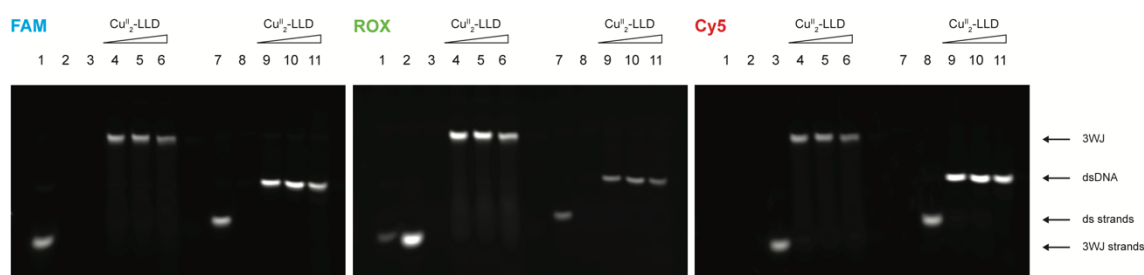


Figure S10. Images obtained during multiplex assay with FAM (left), ROX (middle) and Cy5 (right) excitation and emission filters.

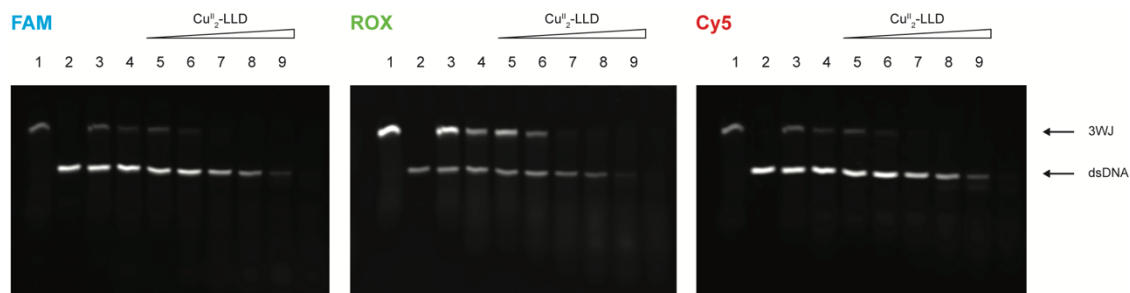


Figure S11. FAM (left), ROX (middle) and Cy5 (right) images for fluorophore cleavage assay.

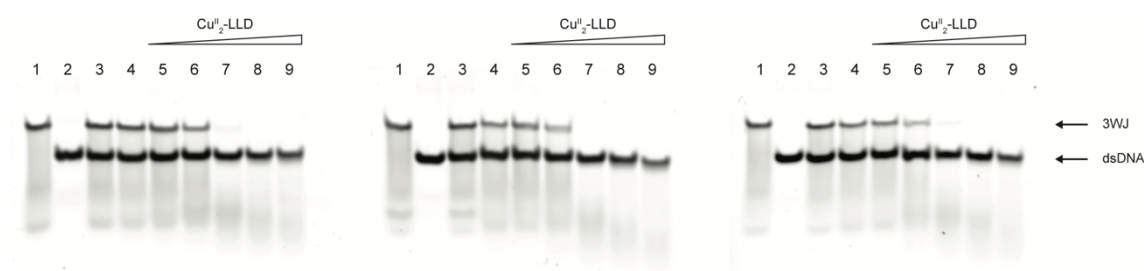


Figure S12. 20% native page of cleavage inflicted by $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$ in triplicate for densitometry. Lane 1; 3WJ. Lane 2; dsDNA. Lane 3; 3WJ and dsDNA incubated with $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$. Lane 4: Target and off-target with LLD (no Cu^{II} present). Lanes 5-9; 3WJ and dsDNA incubated with 1, 2, 5, 7.5 or 10 eq. of $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$ and Na-L-ascorbate (10 eq. to $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$).

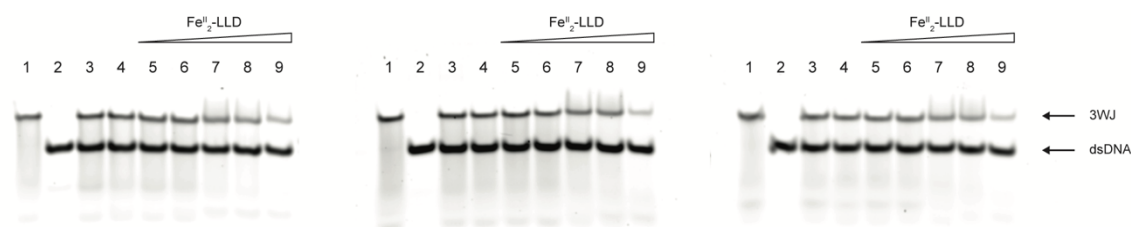


Figure S13. $\Lambda\Lambda\text{-Fe}^{\text{II}}_2\text{LLD}$ cleavage for densitometry. $\Lambda\Lambda\text{-Fe}^{\text{II}}_2\text{LLD}$ cleavage assay was performed in triplicate for band densitometry analysis. Lane 1; 3WJ. Lane 2; dsDNA. Lane 3; Target 3WJ and off-target dsDNA incubated with $\Lambda\Lambda\text{-Fe}^{\text{II}}_2\text{LLD}$. Lane 4; 3WJ and dsDNA with LLD (no Fe^{II} present). Lanes 5-9; Target 3WJ and off-target dsDNA with 1, 2, 5, 7.5 or 10 eq. of $\Lambda\Lambda\text{-Fe}^{\text{II}}_2\text{LLD}$ and Na-L-ascorbate (10 eq. to $\Lambda\Lambda\text{-Fe}^{\text{II}}_2\text{LLD}$). Gel on the left is also shown in Figure 4c.

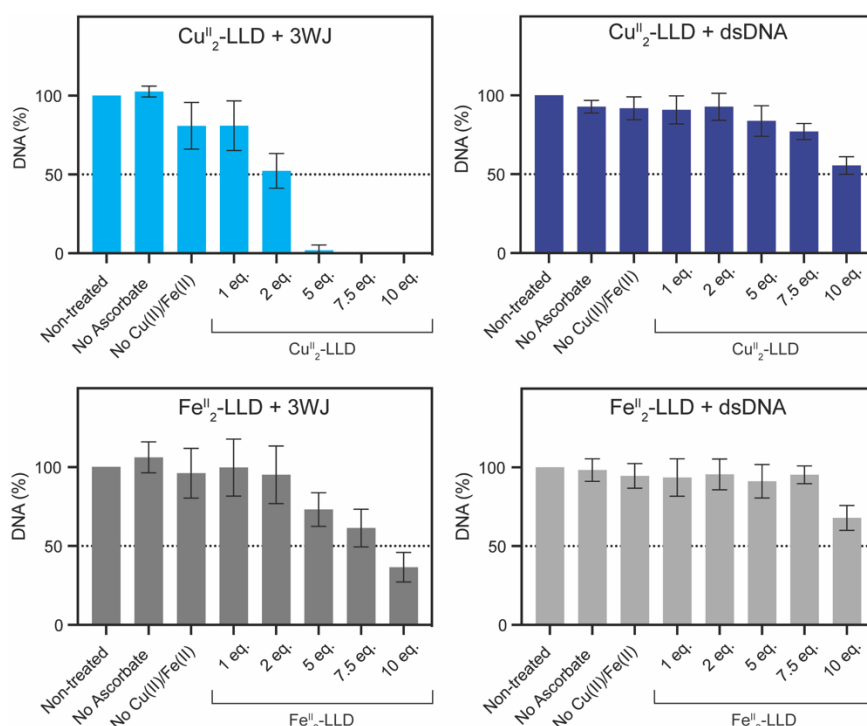


Figure S14. Band densitometry analysis of cleavage inflicted by $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$ and $\Lambda\Lambda\text{-Fe}^{\text{II}}_2\text{LLD}$ on target 3WJ and off-target dsDNA. Densitometry was performed in triplicate, average DNA remaining, and standard deviation were calculated and plotted. This data is also shown in Figure 4d of the manuscript.

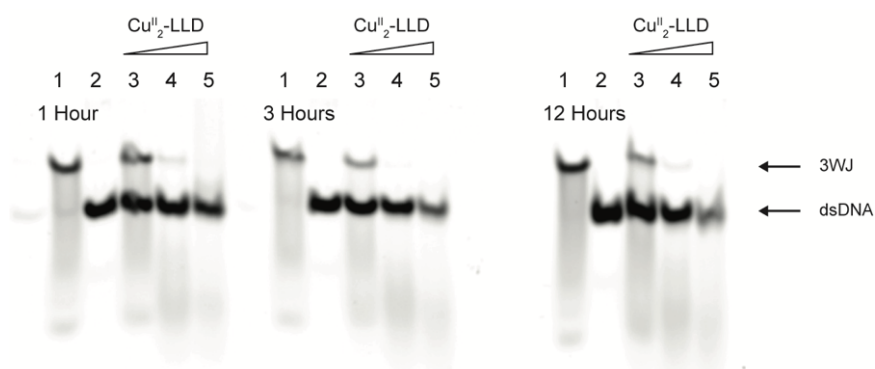


Figure S15. PAGE analysis of cleavage profile at 1, 3 and 12 hours. Lane 1; Target 3WJ. Lane 2; off-target dsDNA. Lane 3-5; 3WJ and dsDNA with 1, 5 or 10 eq. $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$ and Na-L-ascorbate (10 eq. to $\Delta\Delta\text{-Cu}^{\text{II}}_2\text{LLD}$).

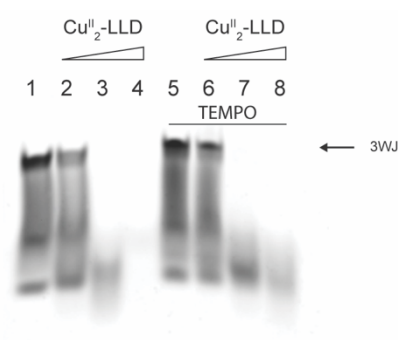


Figure S16. PAGE analysis of cleavage profile of $\Delta\Delta$ - $\text{Cu}^{\text{II}}_2\text{LLD}$ in the presence of 4-hydroxy-TEMPO. Lane 1-4 3WJ incubated with 0, 1, 5 and 10 eq. $\Delta\Delta$ - $\text{Cu}^{\text{II}}_2\text{LLD}$ and Na-L-ascorbate. Lane 5-8 3WJ incubated with 0, 1, 5 and 10 eq. $\Delta\Delta$ - $\text{Cu}^{\text{II}}_2\text{LLD}$, Na-L-ascorbate and 4-hydroxy-TEMPO, a diffusible superoxide scavenger.

3.10. Cell-internalization studies

3.10.1. Cell-internalization of the peptide ligand TAMRA-LLD in Vero cells

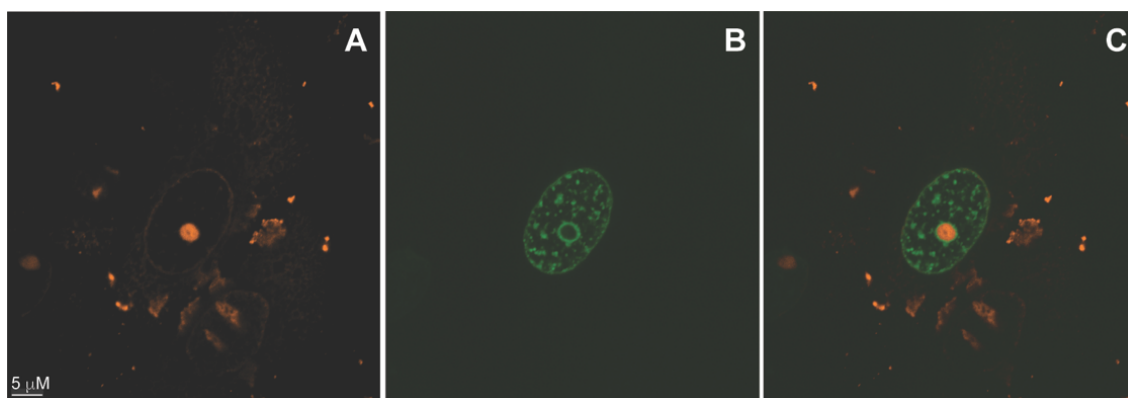


Figure S17. Free peptide ligand **TAMRA-LLD** alone enters the cell nucleus but cannot recognize DNA replication sites. Vero cells expressing protein GFP-PCNAL2 were incubated in the presence of $25 \mu\text{g mL}^{-1}$ of Digitonin for 3 min and then in the presence of $5 \mu\text{M}$ of **TAMRA-LLD** for 15 min (these reported concentrations of digitonin, and peptide ligand refer to the final amount of these compounds in the cell culture transfer buffer after their addition from more concentrated stock solutions). A. Red channel emission showing the distribution of the rhodamine-labelled peptide ligand; B. green channel, corresponding to the emission of the GFP-PCNAL2 probe labelling the DNA replication foci; C. overlay of the green and red channels. Nucleoli are clearly labelled as big red spots inside the cell nuclei. The images show a representative confocal section of the Vero cells.

3.10.2. Cell-internalization of the Cu^{II} peptide helicate in HeLa cells

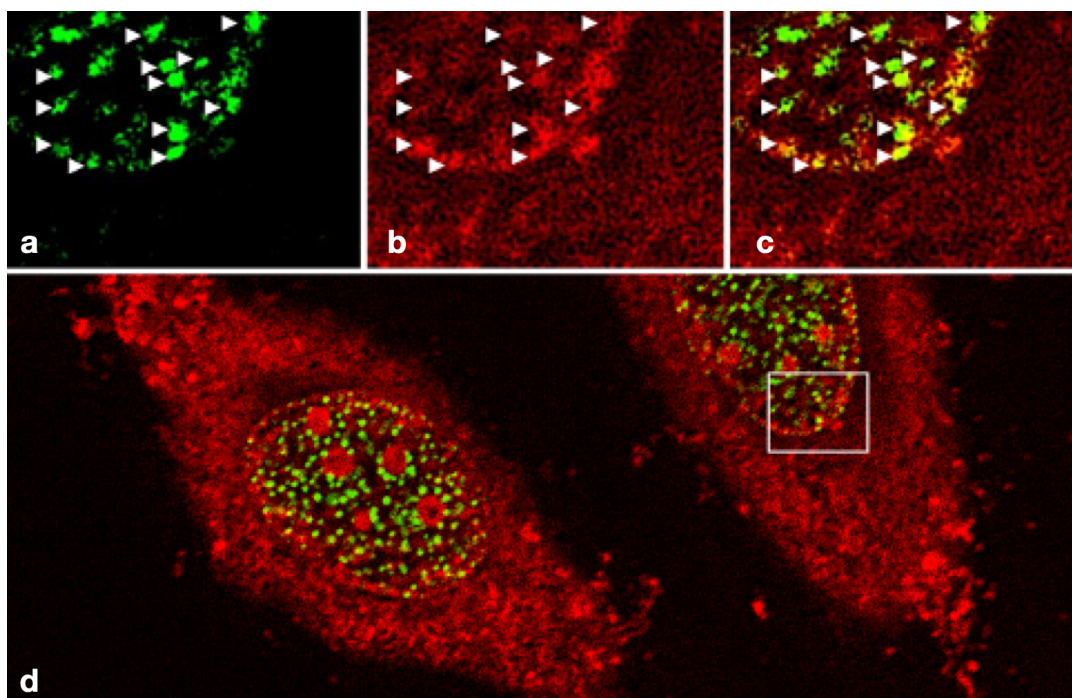


Figure S18. $\Delta\Delta$ -Cu^{II}-TAMRA-LLD selectively stains DNA replication sites. HeLa cells expressing protein GFP-PCNAL2 were incubated in the presence of 25 $\mu\text{g mL}^{-1}$ of digitonin for 3 min and then in the presence of 5 μM of TAMRA-LLD for 15 min (these reported concentrations of digitonin and helicate refer to the final amount of these compounds in the cell culture transfer buffer after their addition from more concentrated stock solutions). The Cu^{II} peptide helicate used in these studies was pre-formed in an independent Eppendorf vial just before the internalization experiment by adding 5 equivalents of CuCl₂·2H₂O to an aqueous solution of TAMRA-LLD peptide ligand. a) Red channel emission showing the distribution of the rhodamine-labelled helicate; b) green channel, corresponding to the emission of the GFP-PCNAL2 probe labelling the DNA replication foci; c) overlay of the green and red channels of the square region shown in d). Arrows highlight some foci where the staining of GFP-PCNAL2 and $\Delta\Delta$ -Cu^{II}-TAMRA-LLD overlap. Nucleoli are clearly labelled as big red spots inside the cell nuclei. The images show a representative confocal section of the HeLa cells.

3.11. Cytotoxicity studies

As can be seen from the data shown in Figure S19, at the concentrations and incubation time used in the cell viability assay (1.25, 2.5, 5.0 and 10.0 μM of helicate, and 20 hours of incubation), $\Delta\Delta$ -Cu^{II}-LLD presents considerable cytotoxicity. On the other hand, we have observed that at the incubation time used in the *in cellulo* nuclease studies reported in this work (2 hours), we were not able to detect the compound inside cells, which is the reason for using digitonin permeabilization. Although it might be interesting to determine the cause for the detected cytotoxicity, it is far beyond the scope of this study, as it might be related to the DNA damage caused by the Cu^{II} peptide helicate activity, or to any other still unknown effect on the integrity of the membrane, cell homeostasis, etc.

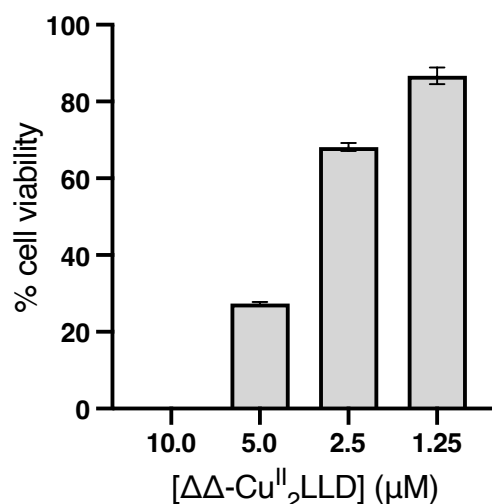


Figure S19. Vero cells were plated at 4000 cells/well in a 96-well plate and incubated 24 hours in a humidified incubator at 37°C, 5% CO₂. Then, different concentrations (10.0 μM, 5.0 μM, 2.5 μM and 1.25 μM) of $\Delta\Delta$ -Cu^{II}₂LLD were added to the cells, that were incubated for additional 20 hours at 37°C. 1% Triton X-100 was used as positive (killing) and medium as negative (100% viability) controls. After incubation, 10 μl/well of CCK-8 solution (Target Mol) was added and incubated for 2 hours at 37°C, 5% CO₂. Absorbance at 450 nm was then measured in a microplate reader Tecan Infiniti 200 PRO and the percentage of cell viability was calculated by comparison with controls.

4. References

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