Metal-mediated diradical tuning for DNA replication arrest via template strand scission

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A series of M(PyED)-X (X = 2Cl−, SO42−) pyridine-metalloenediyne complexes [M = Cu(II), Fe(II), or Zn(II)] and their independently synthesized, cyclized analogs have been prepared to investigate their potential as radical-generating DNA-damaging agents. All complexes possess a 1:1 metal-to-ligand stoichiometry as determined by electronic absorption spectroscopy and X-ray diffraction. Solution structural analysis reveals a per Cl → Cu(II) LMCT (22,026 cm−1) for Cu(PyED)-2Cl, indicating three nitrogens and a chloride in the pseudooctahedral plane with the remaining pyridine nitrogen and solvent in axial positions. EPR spectra of the Cu(II) complexes exhibit an axially elongated octahedron. This spectroscopic evidence, together with density functional theory computed geometries, suggest six-coordinate structures for Cu(II) and Fe(II) complexes and a five-coordinate environment for Zn(II) analogs. Bergman cyclization via thermal activation of these constructs yields benzannulated product indicative of diradical generation in all complexes within 3 h at 37°C. A significant metal dependence on the rate of the reaction is observed [Cu(II) > Fe(II) > Zn(II)], which is mirrored in in vitro DNA-damaging outcomes. Whereas in situ chelation of PyED leads to considerable degradation in the presence of all metals within 1 h under hyperthermia conditions, Cu(II) activation produces >50% compromised DNA within 5 min. Additionally, Cu(II) chelated PyED outcompetes DNA polymerase I to successfully inhibit template strand extension. Exposure of HeLa cells to Cu(PyBD)-SO4 (IC50 = 10 μM) results in a G2/M arrest compared with untreated samples, indicating significant DNA damage. These results demonstrate metal-controlled radical generation for degradation of biopolymers under physiologically relevant temperatures on short timescales.

Significance

Pharmaceuticals often act within a lock-and-key model whereby molecules bind their targets nearly irreversibly, either stalling or initiating biological processes. Here, the agent itself performs no chemical transformation on its target but rather triggers an initiating biological event or cascade. However, unwanted side effects become more likely as the reactivity of these molecules increases. In contrast, molecular compounds may irreversibly damage biological targets using metal-mediated radical chemistry, but controlling the onset and extent of reaction is challenging. Even so, multiple examples of metal-containing or metal-radical paradigms have been used clinically for imaging and chemotherapy. Within this framework we report a class of metal-mediated radical generators that attack DNA, outcompete DNA polymerase, and are cytotoxic in short times and modest concentrations.


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subsequently replaced by Fe(II) (20). Fe(II)-BLM abstracts an H-atom from the C4′ deoxyribose sugar to produce a radical, leading to a gapped DNA site in the presence of O2. Subsequent single- and double-strand DNA breaks (21), and cell cycle arrest at the G2/M checkpoint (22, 23). Although the BLM analogs phleomycin and zorbamycin have differing DNA sequence selectivity (24), each acts along a similar metal-chelation, radical-generation pathway, ultimately leading to overreplication following prevention of the G2/M transition by DNA damage and/or incompletely replicated DNA (25). Although many DNA-damaging reagents display a small degree of G2 arrest as the cell provides itself an opportunity to repair the damage (25–27), cellular toxicity may arise from either the induction of apoptosis or permanent cell cycle arrest (26).

In addition to the BLM family of therapeutics, small molecules such as hydralazine, doxorubicin, and ascorbic acid have also been shown to produce radical oxygen species (ROS) through various mechanisms upon interaction with Cu(II) (28, 29). Controlled ROS generation can provide a potent means of damage in tumor cells; however, when unregulated, it can lead to significant problems in healthy cells (30). Therefore, an alternative approach harnessing direct H-atom abstraction without the need for ROS formation would allow for activation of radical generators in hypoxic environments and circumvent the issues associated with uncontrolled ROS production. In the interest of metal chelation leading to radical generation, Cu(II) is an attractive choice due to the increased Cu(II) levels observed in malignancies (31, 32) arising from Cu(II)-mediated processes for tumorigenesis, such as BRAF signaling (33). This suggests that once inside the cell a ligand would have the opportunity to chelate Cu(II) if needed for activation.

Natural products containing a distinctive enediyne framework provide a unique mechanism for damaging DNA. The cycloaromatization of the enediyne unit produces a potent 1,4-diradical species capable of H-atom abstraction from the closely positioned ribose ring of DNA. Unfortunately, the ability to control the onset of the radical formation event has proven challenging. Our current approach toward the development of small molecule, chelation-induced diradical generators capable of DNA damage is influenced by metal-mediated therapeutics such as Fe–BLM and hydroxyl radical footprinting reagents that act via Fenton chemistry or H-atom abstraction, leading to oxidative DNA strand scission (34, 35). From this conceptual platform, we have prepared the versatile tetradeinate ligand \((Z)\)-N,N-bis[1-pyridin-2-yl-meth(E)-ylidene]octa-4-ene-2,6-diyne-1,8-diamine (PyED), which demonstrates facile Bergman cyclization and 1,4-benzenoid diradical formation within 2.5 h at room temperature upon chelation of Mg(II) in methanolic solution (36).

In aqueous media, hyperthermal (42.5 °C) treatment of HeLa cells with PyED (20 μM) in the presence of divalent metal ions for 1 h leads to a surviving cell fraction of only 0.5 (37). Moreover, this diradical-induced degradation approach is also functional against peptidic biopolymer frameworks such as Αβ fibrils, which can be disaggregated upon Cu(II) or Zn(II) chelation within 2 h under physiological conditions (38).

Thematically, structure-dependent diradical formation and its influence on substrate activation in real time represents an alternative therapeutic paradigm and has significant potential for influencing kinetically dominant events during cell proliferation. To this end, we report the preparation of PyED–metaladoenediyne complexes containing Cu(II), Fe(II), and Zn(II) in an effort to evaluate the geometric structure/Bergman cyclization relationship under physiological conditions and probe dynamic arrest of DNA extension processes via diradical degradation.

**Results and Discussion**

**Preparation of Pyridine Metaladoenediynes and Cyclized Analogs.** The tetradeinate enediyne ligand, PyED, and cyclized analog, PyBD, were prepared according to published literature procedures and characterized by their 1H and 13C NMR and mass signatures (36). A series of air-stable metaladoenediyne complexes was synthesized by the addition of PyED to a solution of MCl2·xH2O or MSO4·xH2O [M = Cu(II), Fe(II), or Zn(II)] in MeOH at 0 °C. After stirring for 4 h, the solvent was removed in vacuo, the remaining solid stirred in Et2O at the same temperature, and the final product isolated by filtration (SI-Appendix, Scheme S1). The cyclized PyBD derivatives were generated under identical conditions to assist in the elucidation of solution structures for the reactive metaladoenediynes. The chloride complexes are moderately soluble in water and methanol, whereas the sulfate derivatives demonstrate excellent water solubility but are insoluble in all organic solvents other than DMSO. Magnetic moments of the copper complexes (1.7–1.8 μB) confirm d0 Cu(II) centers, whereas those of the iron analogs (chlorides: 4.2–4.6 μB; sulfates: 2.4–3.2 μB) suggest that the Fe(II) systems are not purely low- or high-spin at room temperature due to a possible temperature-dependent spin cross-over for these complexes.

The IR spectra for the PyED and PyBD complexes are comparable due to the high structural similarity between the ligands; however, the metaladoenediyne spectra all contain a diagnostic alkyne stretch at \(\sim 2,100 \text{ cm}^{-1}\) [chlorides: Cu(II) = 2,171, Fe(II) = 2,179, Zn(II) = 2,181 cm\(^{-1}\)]; sulfates: Cu(II) = 2,174, Fe(II) = 2,173, Zn(II) = 2,180 cm\(^{-1}\)], confirming the integrity of the enediyne functionality in the isolated products. Although
the Raman spectrum of Fe(PyED)-2Cl is not as clearly resolved as that of Fe(PyBD)-2Cl even at reduced temperature, the presence of the intact enediyne is confirmed via a weak alkylene vibration at 2,176 cm\(^{-1}\) (39) (Fig. 1). Additionally, C=N and C=C in-plane vibrations are observed at 1,554, 1,471, and 1,021 cm\(^{-1}\), whereas the imine stretches at 1,613 and 1,591 cm\(^{-1}\) are broadened due to overlap with the C=C stretch of the enediyne functional group (39, 40) (Fig. 1).

Job plots for the stable, cyclized PyBD complexes were generated using the continuous variation method (41) for determination of solution stoichiometry upon binding to Cu(II) and Fe(II) in methanol. A 1:1 metal-to-ligand ratio is observed in solution for both Cu(PyBD)-2Cl and Fe(PyBD)-2Cl (SI Appendix, Fig. S1) and confirmed using high-resolution electrospray mass spectrometry (HRMS-ESI) performed immediately after complex dissolution. Analysis of HRMS-ESI data reveals a 1:1 metal-to-ligand relationship for the Zn(II) species as well. The chloride derivatives for all complexes were generated in high vacuum. The presence of a bound chloride is supported by the Raman spectrum of Fe(PyED)·2Cl and Fe(PyBD)·2Cl even at reduced temperature, the presence of the chloride (42). In contrast, the chloride ligand bound to the metal center even under high vacuum. The presence of a bound chloride is supported by electronic spectroscopy measurements (discussed below) for the Cu(II) and Fe(II) derivatives and is in good agreement with previously proposed structures of analogous quinoline-containing metalloenediyne (36).

Structural Evaluation via Electronic Absorption Spectroscopy. The wealth of information available in the literature regarding the electronic spectra of Cu(II) with N-donor ligands allows for a qualitative analysis of the solution structures via their electronic absorption spectra (43). Ligand field absorption profiles can be used to assess the coordination number/geometry relationship, because square planar complexes display ligand field transitions in the range of 17,000–20,000 cm\(^{-1}\) with decreasing energies as the geometry varies from square planar to octahedral to tetrahedral (44–46). The electronic spectra for Cu(PyED)-2Cl and Cu(PyBD)-2Cl acquired in MeOH at room temperature reveal low-energy \(d-d\) transitions centered at 13,300 cm\(^{-1}\), indicative of a six-coordinate, distorted octahedral structure for both complexes (45, 47, 48) (Fig. 2, Inset).

Although these ligand field transitions provide information regarding the solution coordination number, they do not lend insight into the spatial arrangement of the ligands. However, the interaction of the equatorial ligand field with the \(d_{2z^2-r^2}\) Cu-centered SOMO is accessible by charge transfer electronic spectroscopy. In the electronic spectrum of Cu(PyED)-2Cl, a \(p\tau Cl \rightarrow Cu(II)\) LMCT transition is observed at 22,026 cm\(^{-1}\) (\(\varepsilon = 1,032\) M\(^{-1}\)cm\(^{-1}\)) (Fig. 2, Inset), confirming the presence of chloride in the equatorial plane (45, 49–53) (Fig. 3d). This LMCT transition is not observed in the Cu(PyBD)-2Cl spectrum, suggesting that the equatorial chloride is displaced by MeOH in solution (Fig. 3b). This smaller size and decreased flexibility of the PyBD chelate results in stronger binding to the metal center compared with PyED, leading to more tightly coordinated imine donors in the equatorial plane that weaken the \(trans\)-Cl bond, subsequently increasing substitution by coordinating solvents. A qualitative analysis correlates the higher-energy absorption bands centered at 38,461 cm\(^{-1}\) (\(\varepsilon = 9,280\) M\(^{-1}\)cm\(^{-1}\)), PyED) to the expected \(pn\) Cl \(\rightarrow d_{2z^2-r^2}\) Cu(II) (49–51) transitions that overlap with the lower-energy \(pn\) (imine) \(\rightarrow d_{2z^2-r^2}\) (Cu(II) LMCT (45, 54) features, whereas the band at 34,722 cm\(^{-1}\) (\(\varepsilon = 9,467\) M\(^{-1}\)cm\(^{-1}\)), PyBD) is attributed to \(\pi\) (imine) \(\rightarrow d_{2z^2-r^2}\) Cu(II) LMCT because no Cl \(\rightarrow Cu(II)\) LMCT transitions are observed. These charge transfer transitions are overlaid on the tail end of intense, higher-energy features from the \(pn\) (imine) \(\rightarrow d_{x^2-y^2}\) Cu(II) LMCT (45, 55, 56) and pyridine \(\pi\rightarrow\pi^*\) (45, 57, 58) (Fig. 2). Overall, the proposed Cu(N\(_2\))(N\(_3\))Cl ligand field environment for both constructs is supported by these spectroscopic signatures, as well as the previously reported, energy-minimized structure for the Mg(II)-pyridine metalloenediyne (36).

The electronic absorption spectra of Fe(PyED)-2Cl and Fe(PyBD)-2Cl obtained in MeOH display characteristic bands corresponding to the \(\pi\rightarrow\pi^*\) transitions for dimine ligands coordinated to Fe(II) (59–61) at 36,101 cm\(^{-1}\) (\(\varepsilon = 14,777\) M\(^{-1}\)cm\(^{-1}\)), PyED) and 35,842 cm\(^{-1}\) (\(\varepsilon = 14,083\) M\(^{-1}\)cm\(^{-1}\)), PyBD) (Fig. 4), in agreement with other Fe(II) complexes containing pyridine and/or dimine ligands (62, 63). Multiple MLCT absorption features are observed in the visible region of the spectrum via excitation of an electron from the \(t^3\_g\) orbitals on the Fe(II) center to the empty \(\pi^*\) pyridyl-imine

Fig. 3. Proposed solution structures for Cu(PyED)-2Cl and Cu(PyBD)-2Cl. In the chelated ligand, N represents the imine nitrogens and N’ represents the pyridyl nitrogens. A indicates both pyridyl nitrogens and chloride bound to the metal center, however, in solution either the chloride (A) or the axial pyridyl ligand (C) may be displaced by solvent.

Fig. 4. Electronic spectra of Fe(PyED)-Cl\(_2\) (red) and Fe(PyBD)-Cl\(_2\) (blue) obtained in MeOH with the visible region shown (Inset).

Fig. 5. X-band EPR spectra of Cu(PyBD)-2Cl (600 \(\mu\)M, blue trace) and Cu(PyED)-2Cl (600 \(\mu\)M, red trace) at 77 K in MeOH. Simulated spectra are shown in black.
orbital set on the ligand (45, 61, 64, 65). Overlapping with these intense π-π* ligand transitions are MLCT transitions [PyED: 29,498 cm⁻¹, ε = 5,246 M⁻¹cm⁻¹; PyBD: 28,490 cm⁻¹, ε = 2,740 M⁻¹cm⁻¹] (Fig. 4). A second MLCT band for each complex is observed with a shoulder at higher energy [PyED: 19,880 cm⁻¹, ε = 1,647 M⁻¹cm⁻¹ and 18,115 cm⁻¹, ε = 1,833 M⁻¹cm⁻¹; PyBD: 19,305 cm⁻¹, ε = 2,672 M⁻¹cm⁻¹ and 17,857 cm⁻¹, ε = 3,478 M⁻¹cm⁻¹] (59) (Fig. 4, Inset).

Copper Complex Structure from EPR Spectroscopy. EPR spectra of Cu(PyED)·2Cl and the cyclized control Cu(PyBD)·2Cl in MeOH were obtained at 77 K to elucidate the solution geometry and ligand binding about the Cu(II) centers (Fig. 5). Both complexes exhibit axial symmetry (g∥ > g⊥ > 2.0023) with a Cu(II) d⁷-τ,τ' ground state (66–68). The Cu(PyBD)·2Cl spectrum reveals a major species with g∥ = 2.25 and g⊥ = 2.06, whereas the enediyne analog exhibits a major species with g∥ = 2.25 and g⊥ = 2.07 (Table 1) that is consistent with aza-aromatic nitrogen-donor ligands such as pyridine (69). These larger g∥ values result from increased ligand anisotropy in the xy plane due to the presence of either bound chloride [Cu(PyBD)·2Cl] or an electron-withdrawing oxygen from methanolic solvent [Cu(PyED)·2Cl] in an equatorial position (Fig. 3A and B).

The resulting spin Hamiltonian parameters obtained from spectral simulation for Cu(PyBD)·2Cl show the presence of a minor species (~15%) with a reduced g∥ value (g∥ = 2.22). This likely corresponds to the Cu(PyED)·2Cl structure with the chloride bound in the equatorial position (Fig. 3A), because the timescale for sample preparation (10 min) lies between HRMS analysis (2 min) documenting inner sphere chloride and electronic spectral confirmation of chloride displacement by MeOH within 30 min (Fig. 2, Inset). Although the chloride-bound Cu(PyED)·2Cl complex EPR spectrum also exhibits a minor species (~35%), the larger g∥ value (g∥ = 2.27) indicates a more oxygen-rich coordination environment upon displacement of either chloride (Fig. 3B) or the weakly coordinated axial pyridine by MeOH (Fig. 3C).

From these spin Hamiltonian parameters, G values, G = (g∥ – 2.0023)(g⊥ – 2.0023), can be directly calculated to shed light on the deviation from planarity of the equatorial ligand set. Values greater than 4 signify the equatorial M–L bonds are in the same plane or only slightly misaligned, whereas values less than 4 suggest a significant dihedral distortion (70). The G value for Cu(PyBD)·2Cl (G = 4.17) containing a pyridyl nitrogen in the axial position reveals only slight dihedral distortion from the xy plane. However, once the chloride has been displaced by solvent the distortion increases (G = 3.67). In contrast, Cu(PyED)·2Cl (G = 3.57) exhibits a larger distortion of the equatorial plane that is relaxed upon replacement of the axial pyridine by solvent (G = 3.86) (Table 1). Distortion of the dihedral angle around the metal center is supported by reduced A∥ values, which denote a decrease in direct overlap between the equatorial ligand set and the Cu(II) d⁷-τ,τ' SOMO (69, 71, 72).

Because deviation of g∥ from the free electron value (2.0023) originates from spin-orbit coupling, variations in these values can be used to interpret the ionic vs. covalent character of ligand coordination (70). Because g∥ values >2.3 are symptomatic of more ionic bonding, the g∥ values of both the cyclized and uncyclized analogs are indicative of a more covalent character (70, 73). In this vein, analysis of the chloride-bound forms of both species suggests a tighter and more covalent ligand–Cu(II) interaction for the cyclized complex (g∥ = 2.22) compared with the uncyclized enediyne (g∥ = 2.25) (66, 67). This higher degree of covalency stems from the constricted binding pocket of the cyclized ligand compared with the more flexible chelation of the enediyne analog.

Crystallographic and Computational Structure Determination. Obtaining crystallographic information from stable, cyclized controls is paramount for insight into the conformations of their highly reactive enediyne-containing counterparts. Within this theme, X-ray crystallographic analysis of Cu(PyBD)·2Cl reveals a five-coordinate geometry between trigonal bipyramidal and square pyramidal (τ = 0.549) containing a chloride, both imines, and one pyridine nitrogen bound in a pseudoequatorial plane with the remaining pyridine donor coordinated axially, consistent with other N₄-chelated compounds (74, 75) (Fig. 6). The equatorial Cu–N distances range from 1.99 to 2.01 Å, typical for Cu-imine single bonds (76–78), whereas the axial pyridine shows weaker coordination (2.16 Å) (SI Appendix, Table S1). This solid-state geometry provides an open coordination site trans to the axial pyridine for solvent coordination, forming a tetragonally elongated octahedron that accounts for the low-energy ligand field transitions observed in the electronic absorption spectrum. Additionally, the long Cu–Cl bond (2.29 Å) trans to a short Cu–imine distance (2.01 Å) correlates well with the absence of Cl → Cu(II) LMCT in the electronic absorption spectrum due to chloride displacement by solvent. The Zn(PyBD)·SO₄ crystal structure adopts a weak six-coordinate geometry containing an asymmetric sulfate chelate with one shorter Zn–O1 bond (2.18 Å), which is in agreement with all other six-coordinate chelated sulfate zinc structures (79–81), and a longer Zn–O2 distance at 2.21 Å. Parallel to the Cu(PyBD)·2Cl structure described above, the two imine nitrogens and one pyridine nitrogen coordinate in the equatorial plane, with the remaining pyridine nitrogen in an axial position (Fig. 7).

Due to the absence of crystallographic data on the reactive PyED complexes, density functional theory calculations were used to probe the structures and predict the Bergman cyclization reactivity of these complexes. The computed structures of
were obtained by minimization of the crystallographic coordinates to ensure reliable computational methods for modeling the reactive PyED complexes. Geometry optimizations, vibrational analyses, and solvation calculations (MeOH, dielectric constant ε = 32.613) using the PCM model (SI Appendix, Table S1a and S2) were performed in Gaussian 09 (87) with the (U)BPW91 (88) density functional/basis set combination and an ultrafine grid, performed in Gaussian 09 (87) with the (U)BPW91 (88)
dielectric constant

Fig. 8. Computed structures of ligand–metal bonding interactions for M (PyED) complexes [M = Cu(II), Fe(II), and Zn(II)], where d is the interalkynyl distance. Monodentate ligands are presented as either MeOH or Cl−.

Cu(PyBD)·2Cl and Zn(PyBD)·SO4 were obtained by minimization of the crystallographic coordinates to ensure reliable computational methods for modeling the reactive PyED complexes. Geometry optimizations, vibrational analyses, and solvation calculations (MeOH, dielectric constant ε = 32.613) using the PCM model (82–86) were performed in Gaussian 09 (87) with the (U)BPW91 (88–93)/6-31G** density functional/basis set combination and an ultrafine grid, applying the SDD (94–98) pseudopotential to all metal atoms. Although all calculated bond lengths for both Cu(PyBD)·2Cl and Zn(PyBD)·SO4 are slightly underestimated, these minor deviations are anticipated as the computed structures account for solvation (SI Appendix, Tables S1 and S2).

Although the bond lengths for the computed Cu(PyBD)·2Cl complex are consistent with the X-ray structure, deviations of 11–13° in the N3–Cul–N1 and N3–Cul–ClI bond angles are observed (SI Appendix, Table S1), implying that upon solvation Cu(PyBD)·2Cl adopts a more square pyramidal geometry (τ = 0.338) relative to the solid-state structure (τ = 0.549). This pyramidalization allows for coordination of a sixth ligand from solvent, in line with the electronic absorption spectrum of Cu(PyBD)·2Cl indicating a distorted octahedron in solution. In light of this proposed solution structure, computational analysis of a six-coordinate solvated Cu(PyBD)·2Cl reveals an axially elongated octahedral complex (Fig. 8). The differences in coordination number notwithstanding, an axial pyridine is consistent with the X-ray structure of the five-coordinate cyclized analog. However, the C1–N3 bond is lengthened by 0.11 Å, indicating that PyED acts as a weaker σ donor relative to PyBD. Additionally, the C1–N1 bond is found to lengthen dramatically (~0.16 Å) in the enediyne-containing compound, consistent with the minor product in the Cu(PyED)·2Cl EPR spectrum where the axial pyridine has been displaced by solvent (Fig. 3C).

Unlike Cu(II), d10 Zn(II) centers demonstrate only modest electronic barriers between different coordination numbers and geometries. In this vein, the crystallographic and computed structures for Zn(PyBD)·SO4 show a weakly chelated sulfate (SI Appendix, Table S2), implying that in the presence of coordinating solvent the anion can be displaced to generate a four-coordinate or weak five-coordinate complex. Examination of the 1H NMR spectra for Zn(PyBD)·X (X = 2Cl−; SO42−) reveals a highly symmetric splitting pattern that is identical for both analogs, supporting the proposal that in solution the anions are noncoordinating. Based on complex solubility in polar solvent and the nature of the coordinated anion in the Zn (PyBD)·SO4 crystal (Fig. 8), a weakly solvated five-coordinate structure is purported for Zn(PyED)·2Cl. Consistent with this proposal, the computed enediyne structure shows a pseudosquare pyramidal geometry (τ = 0.014) containing an axial pyridine. The Zn1–N1 (2.14 Å) and Zn1–O1 (2.31 Å) bonds are elongated, generating a weak axis (SI Appendix, Table S3). As such, the bound solvent is likely fluxional in solution, allowing the complex to oscillate between a four- and five-coordinate structure. This fluxionality accounts for the highly symmetric, time-averaged NMR spectra observed for both Zn(PyED)·SO4 and Zn(PyED)·2Cl and is in line with the spectroscopic observables for the cyclized controls.

From spectroscopic analyses of the enediyne-containing constructs, six-coordinate solution geometries are proposed for both the Cu(II) and Fe(II) PyED chloride complexes. Uniting the structural datasets reveals that Cu(PyED)·2Cl exhibits tighter M–L bonding in the equatorial plane and weaker axial coordination relative to Fe(PyED)·2Cl due to the tetragonal elongation experienced by the d6 Cu(II) center. This distortion leads to compression of the imine bonds in the xy plane [Cu(II): 2.06, 2.16 Å; Fe(II): 2.23, 2.29 Å] and a decrease in the interalkynyl distance of Cu(PyED)·2Cl. Comparatively, the interalkynyl distance of the Zn(PyED)·2Cl complex (4.17 Å) is significantly larger than both the Cu(II) (3.68 Å) or Fe(II) (3.82 Å) analogs, resulting not from differences in bond lengths but from changes in bond angles (Fig. 8).

Scheme 1. Thermal activation of PyEd at physiological temperature (37 °C) to yield cyclized PyBDH via Bergman cyclization. M = Cu(II), Fe(II), or Zn(II).
The five-coordinate geometry of the Zn(II) analog permits a wider interalkynyl distance (N2−M−N3 = 146.8°) compared with Cu(II) (101.9° and 103.0°, respectively), resulting in a larger interalkynyl distance (SI Appendix, Table S3). Because Bergman cyclization requires the formation of a C–C bond, shorter interalkynyl distances allow for facile formation of the benzannulated cyclization product. Therefore, comparison of the interalkynyl distances for these reactive PyED complexes isolated at 0 °C suggests that upon metal complexation at elevated temperatures thermal Bergman cyclization rates in solution should be observed in the order of Cu(II) > Fe(II) > Zn(II).

**In Situ Chelation-Induced Diradical Reactivity.** The addition of free PyED to a solution of MCl2 [M = Cu(II), Fe(II), or Zn(II)] in MeOH results in the formation of the Bergman cyclized product detectable by HRMS within 3 h at 37 °C (SI Appendix, Table S4) for all complexes. This timescale correlates well with the previously published Mg(II) analog that displays in situ cyclization within 2.5 h in MeOH at ambient temperature (36). The soluble organic reaction products were isolated via reduction of the imine bonds with NaBH4 and extraction of the metal center using an EDTA solution (Scheme 1). Formation of the benzannulated ligand was confirmed by reaction of the independently cyclized PyBD complexes under identical conditions (SI Appendix, Scheme S2) and the HRMS signatures for the cyclized product were established by comparison with the independently prepared amine PyBDH.

Although all complexes display formation of the cyclized product within 3 h at 37 °C, differences in the extent of cyclization within this time period are observed. Ionizability of the reduced ligands was measured by recording mass spectra from an equimolar solution of PyBDH and PyEDH. Based on these measurements, a simple analysis of the ratio of cyclized product vs. starting material of multiple trials by MS was used to probe these variances. The addition of PyED to a solution of CuCl2 yields no detectable starting material after 3 h at 37 °C. In contrast, reaction of PyED with ZnCl2 leads to ~50% formation of the cyclized product, whereas the FeCl2 reaction lies somewhere between these two limits, with roughly 74% formation of cyclized product compared with unreacted enediyne. The reduced product formation for cyclization in the presence of Zn(II) relative to Cu(II) aligns well with the suite of structural data, as well as the disaggregation of preformed amyloid-β aggregates at 37 °C within 2 h in the presence of Cu(II) vs. 8 h with Zn(II) (38). The extent of product formation for all metals increases at 42 °C, although under these hyperthermal conditions the enhanced kinetics of the radical reaction escalates the degree of polymerization and limits detection of the cyclized product. For the MeOH-insoluble, but highly water-soluble sulfate derivatives, cyclized product formation was also confirmed by HRMS for Cu(II), Fe(II), and Zn(II) (SI Appendix, Table S5) with parallel reactivity trends to the chloride salts, although rapid formation of H2 and degradation of NaBH4 to form sodium metaborate in aqueous solution precluded quantitative product analysis.

**Metal-Mediated PyED Radical Damage of DNA.** Although PyED has been shown to promote HeLa cell death after 1 h at 42.5 °C in the presence of divalent metals (37), the mechanism of toxicity has not been established. To examine the ability of PyED to damage DNA as a potential target of this reaction, supercoiled DNA was treated to various concentrations of ligand in the absence or presence of 20 μM Cu(II), Fe(II), or Zn(II) at 42 °C (Fig. 9A). Efficient nicking...
of the supercoiled DNA occurs in the presence of all three divalent ions; however, the extent of the reaction is strongly metal-dependent. Consistent with the structure–activity relationship above, the reaction with Cu(II) is the most effective, followed by Fe(II) and Zn(II). The observed DNA damage by Cu(II)-activated PyED compares favorably with that of phleomycin, a chelation-dependent, radical-generating control, with an equivalent response occurring upon exposure to lower concentrations of PyED (Fig. 9B). The activity displayed by the Cu(II) reaction at 37 °C is similar to that demonstrated at 42 °C (Fig. 9C), with 80% DNA degradation in the presence of 10 μM PyED and DNA nicking occurring at concentrations as low as 2.5 μM after 1 h. Minimal degradation is detected in the presence of either free PyED (20 μM) or PyBD, indicating that the chelation-induced radical-generation event is responsible for the activity observed.

To determine the rate of this radical-induced DNA damage, time-course analyses were conducted by titration of PyED in the presence of Cu(II) (Fig. 10A). Using approximately a 1:1 ratio of PyED (30 μM) to nucleotides (33 μM), over 50% of the DNA is reacted within 5 min. Notably, when PyED is present in excess (40 μM), slower migrating DNA species are also observed corresponding to higher molecular weights (Fig. 10B), which may stem from PyED-induced DNA cross-linking. This type of DNA lesion is extremely toxic to human cells due to the multiple strand cleavage events that are required for repair. Further investigation of these species with a higher molecular weight could allow for potentiation of PyED as a cross-linking strategy toward cancer therapy.

Many chemotherapeutic agents or antibiotics induce DNA damage by inhibiting cell growth through DNA lesions that stall replication. Therefore, the effect of PyED treatment on DNA replication was examined using an in vitro primer-extension assay to monitor extension of 49 nucleotides by *Escherichia coli* DNA polymerase I large fragment (Klenow). This polymerase I large fragment lacks 5'-exonuclease activity but retains full polymerase and 3'-exonuclease proofreading ability. Simultaneous addition of PyED (40 μM) and DNA polymerase I efficiently inhibits primer extension in a Cu(II)-dependent manner, leading to the complete inhibition of 50% of the polymerase reaction (Fig. 11A). The polymerase assay was repeated allowing for preincubation of PyED and Cu(II) with either DNA polymerase I or substrate DNA. Interestingly, preincubation of substrate DNA with 50 μM PyED and Cu(II) completely stalls primer extension, whereas preincubation with DNA polymerase I has minimal effect on the extension reaction (Fig. 11B). Consequently, the effective prevention of the DNA polymerase reaction by PyED is largely due to its reaction with the DNA substrate itself. Although *E. coli* DNA polymerase I is not the most robust of the polymerase family, once engaged it replicates DNA at a speed of 10–20 nucleotides per s. The efficient inhibition of the DNA polymerase reaction upon addition of PyED concurrently with DNA polymerase I suggests the enediyne radical generation event is fast enough to out-compete DNA replication machinery and potentially other DNA metabolic processes. This speed may be an additional attribute...
of PyED as an effective DNA-damaging agent in vivo, because most of the genomic DNA is protected by the formation of chromatin and is only exposed transiently during DNA replication, transcription, and repair.

**Radical-Induced in Vivo Cellular Effects.** To query whether the difference in DNA-damaging capabilities between PyED complexed with Cu(II), Fe(II), Zn(II) correlates with their cellular toxicity, clonogenic assays were conducted to measure HeLa cell survival upon treatment with M(PyED)·SO₄ [M = Cu(II), Fe(II), and Zn(II)] at 37 °C. Cu(PyED)·SO₄, the most effective DNA-damaging agent in vitro, is also the most toxic to HeLa cells among all three compounds tested with a calculated IC₅₀ of 10.5 μM (Fig. 12). Under the maximum concentration used (100 μM) a survival fraction of <0.5% was observed with Cu(PyED)·SO₄, whereas survival fractions of 23.6 ± 1.5% and 76 ± 3.5% were observed with Fe(PyED)·SO₄ and Zn(PyED)·SO₄, respectively. Importantly, the nonreactive M(PyBD)·SO₄ complexes demonstrate minimal toxicity to HeLa cells, indicating that the radical-generating enediyne moiety is essential to cell death.

Cells that have undergone DNA damage, DNA double-strand breaks in particular, often display G2/M delay upon activation of the DNA damage checkpoint response to aid in repair. Treatment of Cu(PyED)·SO₄ generates mostly single-strand breaks on supercoiled DNA in vitro. In cells, however, these single-strand breaks may be converted into double-strand breaks during DNA replication. Thus, cell cycle assays were conducted to answer whether treatment of HeLa cells with Cu(PyED)·SO₄ alters progression through

![Fig. 13. Perturbation of the cell cycle progression in cells treated with Cu(PyED)·SO₄. (A) Asynchronous HeLa cells were either untreated (i) or treated with Cu(PyED)·SO₄ (100 μM) (ii) and incubated at 37 °C before harvest at indicated time intervals for flow cytometry analysis with PI staining. (B) Synchronized HeLa cells with double thymidine block were either untreated (i) or treated with Cu(PyED)·SO₄ (100 μM) at G1/S boundary for 1 h (ii), then released to the drug-free medium for indicated time before harvest for cell cycle analysis.](www.pnas.org/cgi/doi/10.1073/pnas.1621349114)
G2/M phase. Exposure of asynchronous HeLa cell culture to Cu(PyED)SO₄ at 37 °C causes an increase of the G2/M fraction from ~16-31% at 24 h posttreatment, persisting at 48 h posttreatment (Fig. 13A). To ensure the nearly twofold increase in the G2/M population is due to a G2/M delay and not because only a fraction of the cell culture has responded to Cu(PyED)SO₄, the effect of Cu(PyED)SO₄ treatment on cell cycle progression was explored using HeLa cells synchronized at the G1/S border using double treatment of thymidine, a deoxynucleoside that in excess inhibits DNA synthesis in a reversible manner. The HeLa cells were first arrested at the G1/S border followed by treatment with Cu(PyED)SO₄ for 1 h and then released to cell cycle progression by thymidine removal. At 8 h after the release from G1/S arrest the majority of both untreated (53.8%) and Cu(PyED)SO₄-treated (65.4%) HeLa cells had progressed through G2/M phase (Fig. 13B). However, 10 h after the release from G1/S arrest the majority of untreated HeLa cells had progressed through G2/M phase and reached G1 phase (58.2%) and the fraction of cells at G2/M phase reduced to 27.8% (Fig. 13B). In contrast, the Cu(PyED)SO₄-treated samples demonstrate a majority of cells (63.7%) remaining at the G2/M phase (Fig. 13B), clearly indicating that Cu(PyED)SO₄ causes a delay due to Cu(PyED)SO₄ exposure. The findings that the cytotoxicity of (M)PyEDSO₄ correlates with the DNA-damaging efficiency in vitro and Cu(PyED)SO₄ treatment causes a G2/M delay in HeLa cells suggest that the cytotoxicity of (M)PyEDSO₄ is due in part to their DNA-damaging activities. Because these metalloenynes are highly reactive toward radical generation, the majority of cells receiving treatment in these experiments are at the G1 phase or the G1/S border. Given that Cu(PyED)SO₄ inhibits DNA replication in vitro, it will be interesting to examine whether the S-phase cells are hypersensitive to PyED-strand breaks, M(PyED)-X radical-induced DNA damage may also generate DNA cross-links and adducts, both of which are more toxic DNA lesions. Thus, radical-generating compounds of this type may eventually serve as an alternative strategy to DNA interruption of malignant cell proliferation.

Conclusion

To gain insight into the development of reactive, yet controllable, small molecules for biological activity a series of pyridine-metalloenyne complexes and their cyclized analogs have been synthesized and the differences in their solution Bergman cyclization reactivities examined. Electronic and EPR spectroscopic analysis indicates a six-coordinate structure solution for both Cu(II) and Fe(II) complexes, each containing three nitrogens and a chloride in the equatorial plane with a weakly bound pyridine nitrogen and solvent in the axial positions, and a five-coordinate solution structure is determined for the Zn(II) complex.

PyED demonstrates facile Bergman cyclization within 3 h at 37 °C in the presence of divalent Cu, Fe, or Zn with the extent of product formation following the order Cu(II) > Fe(II) > Zn(II). Computational investigation shows the source of the reactivity profile to be structural differences that lead to lengthening of the alkynyl distance and subsequent retardation of cyclization in solution. This trend is mirrored in the ability of PyED to damage DNA by metal-dependent diradical formation within 1 h at 42 °C. Remarkably, efficient inhibition of the DNA polymerase reaction in the presence of Cu(II) suggests the radical-generation event is fast enough to outcompete the replication process. Treatment of HeLa cells with Cu(PyED)SO₄ leads to arrest of the cell cycle at the G2/M checkpoint, indicating that this DNA damage is effective in vivo, and cellular toxicity is observed with an IC₅₀ of 10.5 μM. Thus, metal-mediated PyED activation offers unique control of the potent diradical generation event and thereby a means for potentiation of critical DNA metabolic processes under physiologically relevant temperatures on short timescales.

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