ACES Asian Chemical Editorial Society

DOI: 10.1002/asia.201901182

CHEMISTRY AN ASIAN JOURNAL Full Paper

Coordination-Accelerated Radical Formation from Acyclic Enediynes for Tumor Cell Suppression

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Abstract: A maleimide-based acyclic enediyne with salicylaldiminato substituents at the alkyne termini was synthesized, which was further chelated with three kinds of metal-ions, Cu^{II}, Zn^{II}, and Mg^{II}, and form metalloenediynes. The cycloaromatization of this thermally inactive enediyne ligand was greatly accelerated through the coordination with metal ions. Specifically, the Cu^{II}-metalloenediyne showed an extremely low onset temperature of 55 °C and underwent spontaneous cycloaromatization at ambient temperature to produce free radicals, followed by generation of reactive oxygen species in the physiological environment. The metalloenediyne exhibited excellent DNA cleavage ability and high cytotoxicity towards HeLa cells, with half-maximal inhibitory concentration values comparable to many commercial antitumor agents. The combination of the electron-withdrawing effect of the maleimide moiety at the ene position and metal coordination at the yne termini provides a new inspiration for designing and synthesizing highly efficient enediyne antitumor agents.

Introduction

Natural enediyne antibiotics with potent antitumor activities and inhibition effects on various tumor cells are acknowledged as the strongest antitumor agents discovered up to date.[1] The cyclic enediyne chromophores in these enediyne antibiotics undergo Bergman-type[2] or Myers-type[3] cycloaromatization in physiological environment to generate highly reactive free radicals and cause DNA damage, providing unique radiomimetic cytotoxic mechanisms. The extremely strong cytotoxicity endows natural enediynes the promising payloads for antibody-drug conjugates (ADC).[4] Indeed, two ADCs with Calicheamicin as the "warhead", Mylotarg® and Besponsa®, have been approved for clinical treatment of leukemia in recent years. Unfortunately, owing to the complicated structures of Calicheamicin and other natural enediynes, the total synthesis and structural modification of them are severely hindered for large-scale preparation.[5]

To this end, the development of non-natural enediyne compounds with potent biological activities has attracted tremendous attentions over the last two decades. The reactivity of an enediyne compound towards cycloaromatization is dictated by many factors. Among them, the separation between two terminal alkyne groups, also known as the critical distance (CD), has been considered as the most important one.[6] Density functional theory studies suggested a CD distance around 3.4 Å is needed for a spontaneous cycloaromatization to take place at ambient temperatures. Several strategies have been proposed to shorten the CD distance of enediynes, including converting acyclic enediynes to highly strained cyclic enediynes[7] and coordinating enediyne ligand with metal-ions.[1d,8] Metal chelation is a simple and straightforward way to bring the two alkyne termini closer, in consequence lowering the cyclization barrier and the onset temperature of acyclic enediynes.[9] Moreover, the valence and crystal field of central metal-ion significantly affect the activation barrier of the cycloaromatization of an enediyne ligand.[10] For example, the thermal cyclization of a Cu^{II}-metalloenediyne is more active than its Cu^{I}-metalloenediyne analogues.[9b,11] In the molecular-level design of highly reactive metalloenediynes, on the other hand, the structure of the enediyne ligand should also be sophisticatedly modulated to enable the cycloaromatization occurring at low temperature.[9b,c,12] Recently, we have demonstrated that the onset temperature of an acyclic enediyne was significantly lowered with the installation of an electron-withdrawing maleimide moiety at the ene position.[13] We also found that the cycloaromatization activities of the enediyne compounds could be regulated by simply adjusting the functional groups at the alkyne termini in order to realize the generation of free diradicals at low temperature.[14] These maleimide-based enediynes showed strong DNA-cleavage and tumor cell suppression activities.[14b,c,15] Furthermore, substituent modifications at the maleimide moiety were applied in tailored synthesis of enediyne compounds employed in drug conjugates[16] and drug delivery.[15,17] By combining the electron-withdrawing effect of maleimide moiety at the ene position and the metal coordination at alkyne termini, herein, we propose a new strategy for the synthesis of highly reactive metalloenediyne. The enediyne

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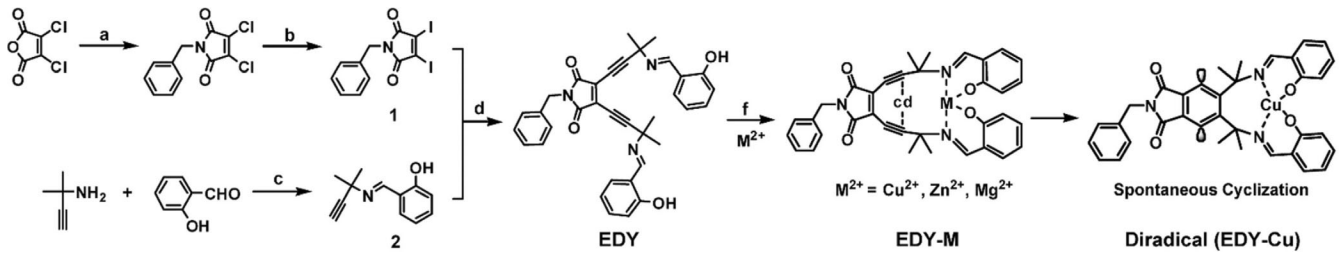
Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under: https://doi.org/10.1002/asia.201901182.



Scheme 1. Synthesis of ligand EDY and metalloenediynes EDY-M. (a) $PhCH_2NH_2$, acetic acid, 40 °C, 40 h; (b) NaI, acetonitrile, 100 °C, 6 h; (c) $MgSO_4$, dichloromethane, 20 °C, 24 h; (d) $Pd(PPh_3)_2Cl_2$, CuI, *N*,*N*-diisopropylethylamine (DIPEA), tetrahydrofuran/toluene, 20 °C, 18 h; (f) dichloromethane/methanol, 1 equiv of $Cu(NO_3)_2$, $Zn(NO_3)_2$ and $Mg(NO_3)_2$, respectively.

compound with salicylaldiminato groups showed low cycloaromatization activity and no DNA-cleavage ability. On the contrary, once chelated with metal ions at the alkyne termini, the thermal reaction was accelerated as demonstrated by differential scanning calorimetry (DSC) experiment. In particular, the cycloaromatization was greatly enhanced to produce radicals when enediyne was chelated with Cu^{II} (Scheme 1), leading to the generation of reactive oxygen species at ambient temperature and showing high DNA-cleavage ability and cytotoxicity.

Results and Discussion

Synthesis and Coordination of Enediyne

3,4-Diiodo-*N*-benzylmaleimide (**1**) was synthesized by a two-step reaction according to previous reports with minor modification (ESI).[18] The terminal alkyne (**2**) with a chelating salicylaldiminato moiety was formed from salicylaldehyde and 1,1-dimethylpropargylamine (ESI). The Sonogashira reaction between **1** and **2** was catalyzed by palladium(II)bis(triphenylphosphine) dichloride and cuprous iodide to produce the enediyne ligand (**EDY**) at ambient temperature (Scheme 1). The obtained **EDY** was characterized by NMR spectroscopy (Figure S2) and high-resolution mass spectroscopy (Figure S3). It is a yellow powder and can be stored stably at room temperature.

The presence of the salicylaldiminato groups at the alkyne termini enables the ligand **EDY** to coordinate with metal-ions to form metalloenediyne complexes (**EDY-M**).[19] Three metalloenediynes were synthesized by mixing **EDY** with $Cu(NO_3)_2$, $Zn(NO_3)_2$ and $Mg(NO_3)_2$ in a solvent mixture of methanol and dichloromethane, respectively, followed by removal of the solvent (Scheme 1). Zaleski et al. reported that the chelation of Mg^{II},[12c,20] Cu^{II},[21] and Zn^{II}[21] with an enediyne ligand, (*Z*)-N,N¢-bis[1-pyridin-2-yl-meth-(*E*)-ylidene]oct-4-ene-2,6-diyne-1,8-diamine, took place at the four N atoms in imines and pyridines. Basak et al proved that[12a] the two N atoms in imines and the two *ortho*-phenolic hydroxyls in salicylaldiminato-enediyne were the coordination sites to form a tetra-coordination complex with a metal-ion. The coordination processes of **EDY** with metal ions were investigated by NMR spectroscopy (Figure S8), electron paramagnetic resonance (EPR) spectroscopy (Figure S9), and infrared (IR) spectroscopy (Figure 1). As shown in Figure 1, the hydroxyl peak (3450 cm^{-1}) in the IR spectrum of ligand **EDY** is absent in that of Zn^{II} complex (**EDY-Zn**), indicating that the hydroxyl groups were involved in the chelation with Zn^{II}. The peaks corresponding to the imine groups are split, implying the coordination of the imine groups with metal ions. Interestingly, while **EDY** and **EDY-Zn** are stable at ambient temperature, the **EDY-Cu** is highly reactive. After 24 h storage at room temperature, the acetylene peak (2216 cm^{-1}) disappeared completely, corresponding to the spontaneous cycloaromatization of the metalloenediyne **EDY-Cu**. Further monitoring the spontaneous cyclization of **EDY-Cu** with FT-IR spectroscopy (Figure S7) showed that the half-life was 27 min. In other words, the thermal cycloaromatization of the salicylaldiminato-enediyne (**EDY**) is significantly accelerated by the chelation with Cu^{II}.

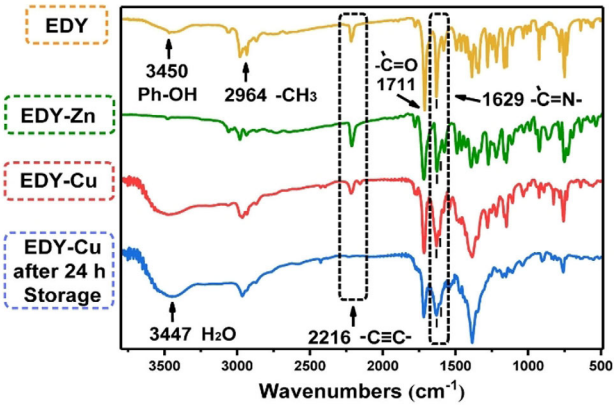


Figure 1. Infrared spectra of ligand **EDY** (yellow line); **EDY-Zn** (green line); **EDY-Cu** (red line); and **EDY-Cu** after 24 hours storage (blue line).

Thermal Reactivity of Enediyne Ligand and Metalloenediynes

The thermal reactivities of the ligand **EDY** and metalloenediyne complexes (**EDY-M**) were evaluated by differential scanning calorimetry (DSC) analysis. As shown in Figure 2, the **EDY** exhibits a sharp endothermal melting peak at 159 °C, followed by an exothermal peak corresponding to the cycloaromatization and the polymerization of free radical intermediates.[22] The onset temperature of ligand **EDY** is 163 °C, suggesting that the ligand **EDY** is thermally inert at room temperature. After the chelation with Cu^{II}, Zn^{II} or Mg^{II}, the onset temperatures significantly decreased to 55 °C (Figure 2B), 125 °C (Figure S4A), and

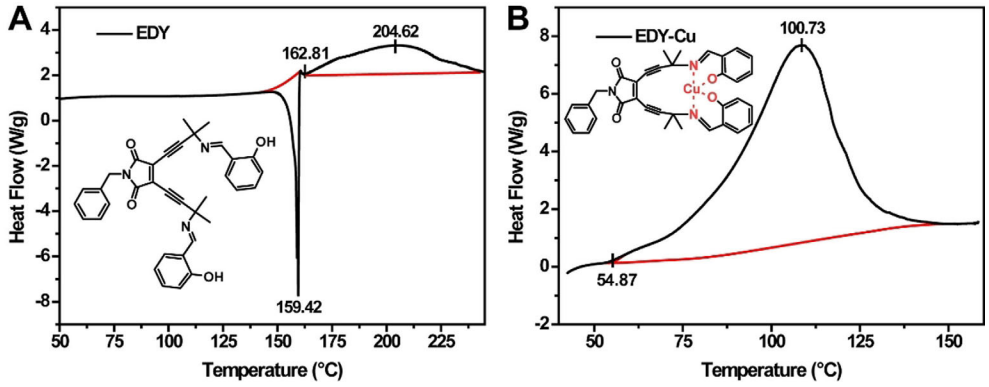


Figure 2. DSC curves of ligand **EDY** (A) and **EDY-Cu** (B). The baselines are marked in red.

100 °C (Figure S4 B), respectively. The greatly accelerated cycloaromatization and the reduction of onset temperature are consistent with the reduction of CD distance after metal coordination with enediyne compounds.[21b] Meanwhile, the charge separation assistance in the metal-ion-catalyzed Bergman cyclization should not be excluded as well.[23] Nevertheless, this study confirmed that the metalloenediyne **EDY-Cu** is highly reactive towards spontaneous cycloaromatization at physiological temperature to produce free diradicals,[14b,15] endowing it with the ability of DNA cleavage and tumor cell suppression as discussed below.

Generation of free diradicals

The radical nature of cycloaromatization is typically verified by electron paramagnetic resonance (EPR) spectroscopy. It is difficult to monitor the free diradicals produced by cycloaromatization in situ because of their high reactivity and short lifetime. However, they can be converted into stable free radicals in the presence of a spin trap like *N*-*tert*-butyl-alpha-phenylnitrone (PBN), 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), or 2-methyl-2-nitrosopropane (MNP).[24] In order to verify the generation of free radical intermediates, the ligand **EDY** and PBN were dissolved in DCM, followed by the addition of a methanol solution of cupric nitrate and stirred at 0 °C or 37 °C for 24 h. As shown in Figure 3, both of the samples exhibit strong radical signals at 3520 G. In particular, for the reaction system at 0 °C, there are two sets of triplet peaks with an intensity ratio of 1:1:1 and a hyperfine splitting constant (A_N) of 14.78 G, similar to that of the PBN adduct of calicheamicin-derived phenyl radical (A_N = 14.6 G),[25] suggesting the formation of two kinds of radical species either from asymmetric cycloaromatization or partial reaction of symmetric diradical intermediates with some donor groups (like solvent molecules). The EPR spectrum of the sample obtained at 37 °C is even more complicated as at least one more set of triplet peaks with similar hyperfine splitting constant shows up. The formation of a complex mixture of PBN-adducts is another evidence of the high reactivity of the diradical intermediates, which might abstract hydrogen or chlorine atoms from the solvent molecules and turn to different kinds of radical species before meeting with PBN. For comparison, no EPR signal was found in **EDY** (Figure S5 A) or **EDY-Zn** (Figure S5 B) under the same conditions. Accordingly, the ligand **EDY** is thermally inactive at ambient temperature, while its chelate with Cu^{II} is capable of undergoing spontaneous thermal cycloaromatization to generate diradicals at 37 °C (physiological temperature) and even at 0 °C, shedding some light on the fabrication of chelation-triggered enediyne antitumor antibiotics system.

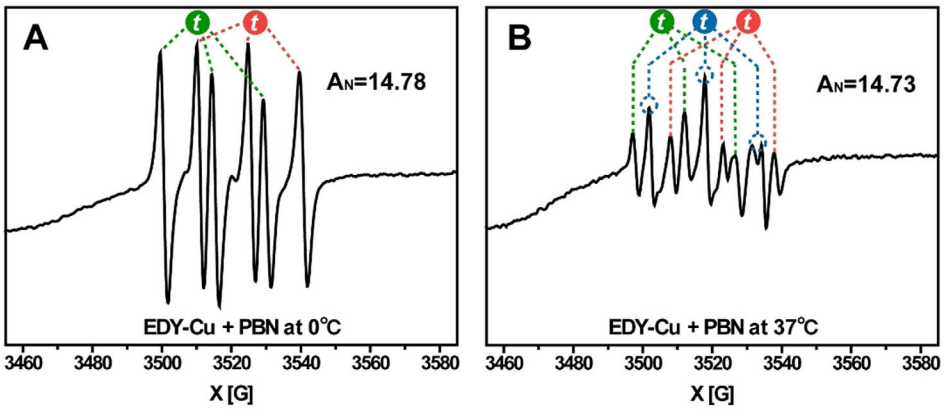


Figure 3. (A) EPR spectrum of **EDY-Cu** (14 mM) in the presence of PBN (140 mM) in DCM:MeOH = 1:1 at 0 °C. (B) EPR spectrum of **EDY-Cu** (14 mM) in the presence of PBN (140 mM) in DCM:MeOH = 1:1 at 37 °C.

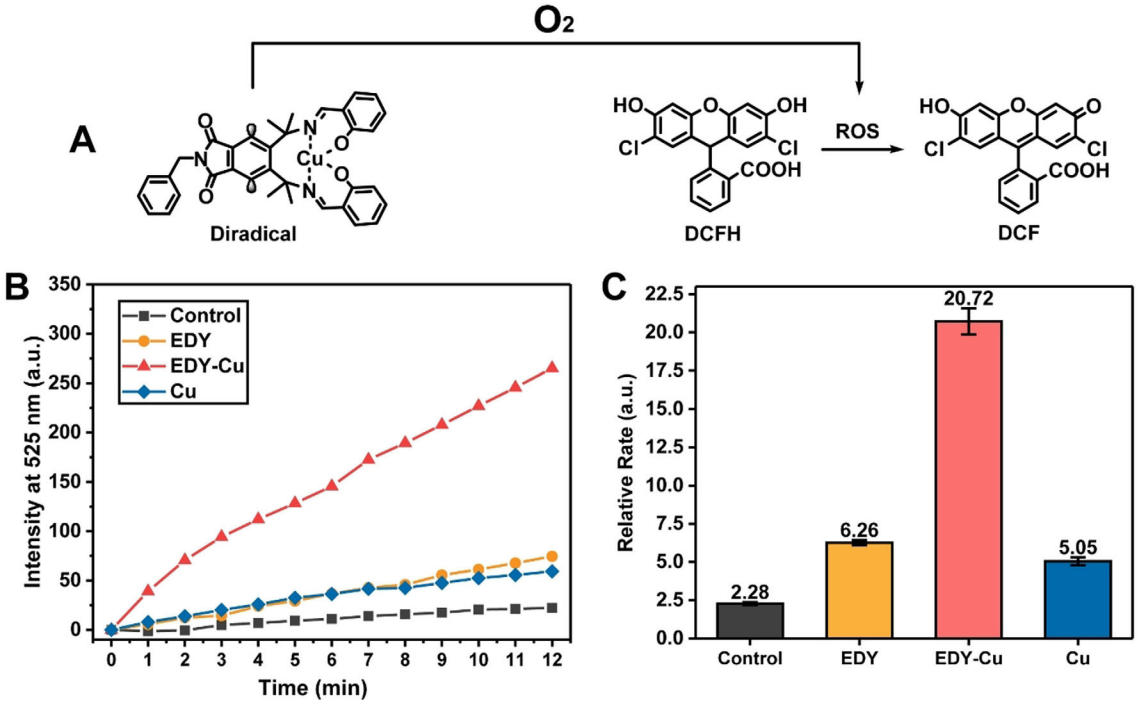


Figure 4. (A) Schematic illustration of the ROS generated by diradicals of **EDY-Cu**. (B) Fluorescence intensities of DCF in PBS (pH 7.4) with control (gray), ligand **EDY** (75 μM, yellow line), **EDY-Cu** (75 μM, red line), and cupric nitrate (75 μM, blue line). [DCFH] = 50 μM. (C) The rates of fluorescence intensity calculated by linear fitting.

Assessment of ROS

Reactive oxygen species (ROS), including singlet oxygen, superoxide, hydrogen peroxide, and hydroxyl radical,[26] are generated from ground state oxygen either by energy or electron transfer reactions. ROS causes oxidative damages to DNA, lipids, and proteins. They are a class of important mediators inducing cytotoxicity of some antitumor agents.[27] To evaluate the level of ROS induced by enediyne, dichlorodihydrofluorescein (DCFH) was used as an indicator in PBS buffer solutions (Figure 4 A). The non-fluorescent DCFH is converted to fluorescent dichlorofluorescein (DCF) in the presence of ROS,[28] and the increase of fluorescence intensity of DCF at 525 nm indicates the production of ROS in the system. As shown in Figure 4, after the addition of **EDY** or **Cu** (cupric nitrate) to DCFH, the fluorescence intensity of DCF slowly increases in 12 min. The calculated increasing rates are 6.26 min^{-1} (**EDY**) and 5.05 min^{-1} (**Cu**), respectively, slightly higher than that of control (2.28 min^{-1}). Interestingly, the fluorescence intensity of DCF in the presence of **EDY-Cu** increases significantly with a rate of 20.72 min^{-1}, suggesting that the radicals formed from the coordination-accelerated cycloaromatization transform O_2 into ROS, which further turns DCFH into fluorescent DCF. These results indicate that the **EDY-Cu** is able to produce ROS in physiological environment, which is essential for DNA-cleavage and tumor cell suppression.

Cleavage of DNA

Supercoiled plasmid DNA was used to evaluate the DNA cleavage activities of **EDY**, metalloenediynes, and metal-ions.[29] The pristine DNA molecule exhibits a double-stranded closed helical structure and is further twisted to form a super-tertiary structure. Once the scissions of one or two strands appear, the supercoiled plasmid DNA becomes open circular forms. Three different DNA forms: native supercoiled form (Form I, intact DNA), circular relaxed form (Form II, single-strand scissions), and linear form (Form III, double-strand scissions) which can be distinguished by gel electrophoresis. The DNA samples (ΦX174 supercoiled plasmid RF1 DNA, 16.7 μg mL^{-1}) in PBS buffer (pH 7.4) were, respectively incubated with **EDY** (1.25 mM), metalloenediynes (1.25 mM), and nitrates (1.25 mM) for 24 hours at 37 °C, and then subjected to agarose gel electrophoresis for photographed scanning analysis. A control sample was prepared with the addition of the same amount of DMSO to the DNA solution. As shown in Figure 5, most of the samples have no obvious DNA cleavage compared with control except **EDY-Cu** and **Cu**. While the native DNA was partially converted to Form II and Form III in the presence of copper nitrate (**Cu**) for an unknown reason, **EDY-Cu** leads to nearly complete scission of DNA into Form II (79%) and Form III (11%) owing to the spontaneous cycloaromatization and the generation of radical species and ROS.

Cytotoxicity Test

Cytotoxicity of ligand **EDY** and metalloenediyne complexes was evaluated by measuring the IC_{50} on HeLa cells via the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) method.[30] The DMSO solution of **EDY**, three **EDY-M**, and three nitrates solutions were diluted to the test concentrations with culture medium and cultured with HeLa cells for 24

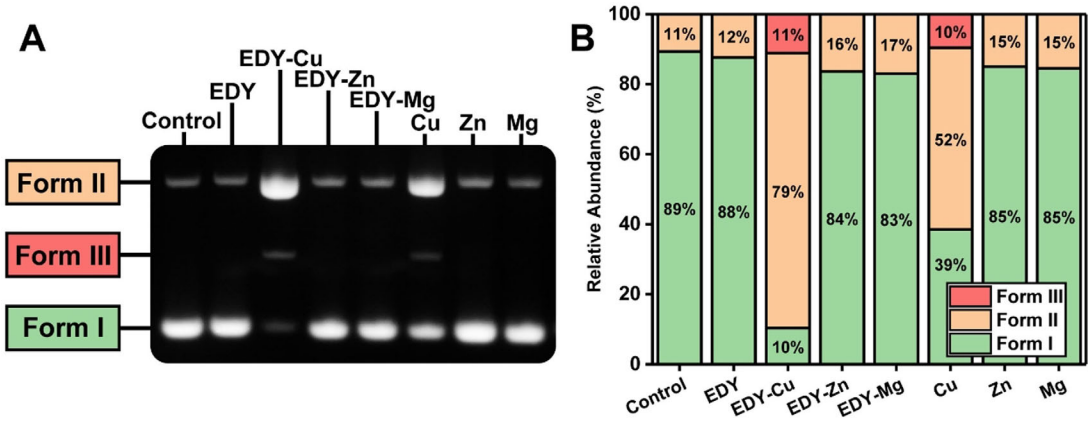


Figure 5. (A) Agarose gel electrophoresis of ΦX174 supercoiled plasmid RF1 DNA. The DNA samples (16.7 μg mL^{-1}) in PBS buffer (pH 7.4) were, respectively incubated with **EDY** (1.25 mM), nitrates (1.25 mM), and metalloenediynes (1.25 mM) at 37 °C for 24 h. (B) Percentage of (i) uncleaved DNA (Form I, supercoiled circular, green block), (ii) single-strand scission (Form II, circular, yellow block), and (iii) double-strand scission (Form III, linear, red block).

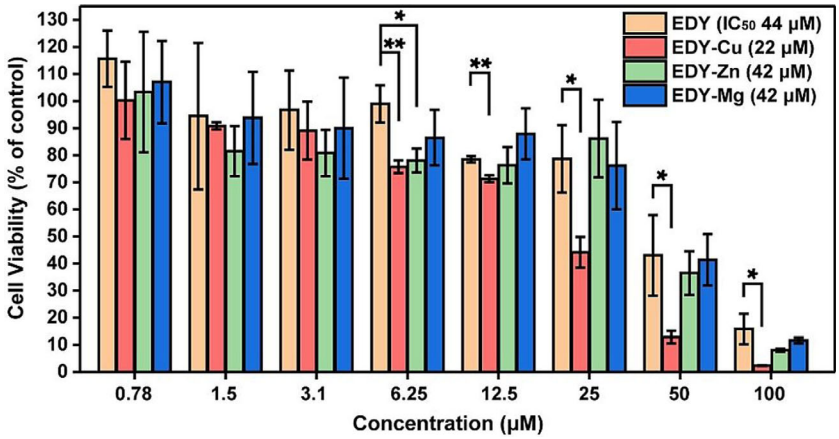


Figure 6. Cell viability of HeLa cells in the presence of **EDY**, **EDY-Cu**, **EDY-Zn**, and **EDY-Mg** after cultured for 24 h at 37 °C with a series of concentrations. HeLa cells incubated without any materials were used as the control. The IC_{50} values are expressed in parentheses. A significant difference in the cell viability compared with the ligand **EDY** is denoted as "*" ($P < 0.05$), "**" ($P < 0.01$).

hours, and then subjected to MTT analysis. The cell viabilities in the presence of **EDY** or **EDY-M** show clear concentration dependence (Figure 6), while the nitrates exhibit negligible cytotoxicity even at the concentration up to 100 μM (Figure S6). The Schiff base structure of the **EDY** ligand might be hydrolyzed in the intracellular environment to give a diamine compound (**EDY 2**) which might endow the ligand with cytotoxicity (Scheme S7). Indeed, the hydrolysate **EDY 2** is a more active enediyne in thermal aromatization (onset temperature = 124 °C, Figure S11 A) and shows a comparable cytotoxicity to the free ligand **EDY** (IC_{50} = 47 μM, Figure S11 B). The IC_{50} levels of **EDY**, **EDY-Zn**, and **EDY-Mg** are all around 40 μM, implying that the coordination of Zn or Mg barely affects the cycloaromatization of the enediyne ligand, which is consistent to the results from EPR and DNA cleavage experiments. It is noteworthy that the cytotoxicity of **EDY-Cu** is much greater than those of the other groups, and the differences between **EDY-Cu** and the ligand **EDY** are statistically significant when the concentration is above 6.25 μM. Almost all Hela cells are destroyed when the concentration of **EDY-Cu** is 100 μM. The **EDY-Cu** shows excellent cytotoxicity with an IC_{50} value of 22 μM, As a control, the cytotoxicity of the cyclized products was much lower (Figure S12), suggesting that the cytotoxicity originates from the spontaneous generation of free radicals and reactive oxygen species at physiological temperature thanks to the coordination effect of Cu^{II} to enediyne ligand.

Conclusions

A maleimide-based acyclic enediyne, which is capable of coordinating with metal ions, has been designed and synthesized with salicylaldiminato substituent groups at the alkyne termini. The ligand **EDY** is inert towards thermal cycloaromatization, while the metalloenediyne **EDY-Cu** spontaneously undergoes cycloaromatization at ambient temperature to produce highly reactive free radicals. The active free radicals induce the generation of reactive oxygen species, causing DNA cleavage by the scissions of single and double strands, resulting in cytotoxicity against HeLa cells. The combination of the electron-withdrawing effect at the ene position and metal coordination effect at the yne termini greatly facilitates the cycloaromatization of an enediyne compound, which would be potentially applied in

the molecular design and synthesis of powerful enediyne compounds for DNA cleavage reagents towards tumor cell suppression.

Experimental Section

Detailed methods are described in the Supporting Information.

Acknowledgements

The authors gratefully acknowledge the financial support from National Natural Science Foundation of China (21871080, 21503078, 21474027), the Fundamental Research Funds for the Central Universities (22221818014), and Shanghai Leading Academic Discipline Project (B502). AH thanks the "Eastern Scholar Professorship" support from Shanghai local government. We thank Prof. Runhui Liu for his kind advice in cellular experiments.

Conflict of interest

The authors declare no conflict of interest.

Keywords: Anticancer agents · Cytotoxicity · DNA cleavage · Enediynes · Metalloenediynes

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Manuscript received: August 22, 2019
Revised manuscript received: October 21, 2019
Version of record online: ■■ ■■, 0000

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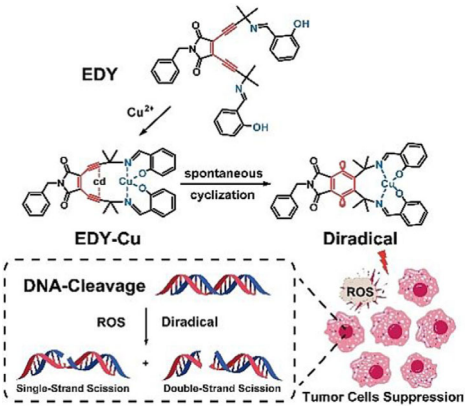

An acyclic enediyne functions via coordination-accelerated radicals to induce ROS and DNA cleavage, thereby inhibiting cancer cell growth.

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Coordination-Accelerated Radical Formation from Acyclic Enediynes for Tumor Cell Suppression