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Chemical and photo-induced nuclease activity of a novel minor groove DNA binder Cu(II) complex

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Abstract: A new type of copper(II) metal complex containing 1,10-phenanthroline (phen) and 8-(difluoromethoxy)-3,4-dihydro-2*H*-[1,3]thiazino[3,2-*a*]benzimidazole (dtb) ligands was prepared and characterized. The ds-DNA interaction of the complex was studied by UV–Vis spectrophotometry, competitive fluorometric titration with ethidium bromide (EB) and 4′,6-diamidino-2-phenylindole (DAPI), viscosity measurements and agarose gel electrophoresis. The results show that the complex can bind to ds-DNA in the minor groove by displacing DAPI molecules. DNA cleavage mechanism studies revealed that hydrogen peroxide radicals are responsible for DNA oxidative cleavage.

Keywords: Cu(II)-phenanthroline; chemical nuclease activity; DNA-binding affinity; minor groove binding.

INTRODUCTION

Metal-based chemotherapeutic molecules, such as cisplatin and its derivates, are well defined and currently the most popular anticancer drugs used for clinic applications.[1] However, despite the high therapeutic efficiencies of these metal-based chemotherapeutics, negative side effects limit their anti-cancer profiles. Hence, the development of a new generation of transition metal complexes with reduced negative side effects are an important topic.[2–4] Copper is an essential trace element that plays an important role in many crucial biological mechanisms and is less toxic for the human body than exogenous metals.[5] It is one of the metals most widely used as chemical nucleases, as it can undergo redox reactions to produce reactive oxygen species (ROS), which are able to split nucleic acid chains. In recent years, copper complexes with aromatic heterocyclic compounds have drawn remarkable attention due to their DNA binding, nuclease and anti-cancer activities.[6,7] $[Cu(1,10\text{-phen})_2]^{2+}$ is a well-studied chemical nuclease reagent with two aromatic phenanthroline ligands that randomly cleaves nucleic

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acids in the presence of molecular oxygen. The DNA cleavage activity of $[Cu(1,10\text{-phen})_2]^{2+}$ was first reported by Sigman in 1979.[8] This agent oxidizes ds-DNA by binding at the minor groove and produces targeted ROS in the vicinity of the minor groove of DNA.

In this study, one of the 1,10-phenanthroline ligands of $[Cu(1,10\text{-phen})_2]^{2+}$ was replaced by a novel difluoromethoxy and sulphur containing ligand (Fig. 1). The aim was to enhance the DNA minor groove binding ability through the high H-bond capacity of the difluoromethoxy group (Fig. 1). An increase in the DNA binding constant was shown by the UV titration method and minor groove binding ability was compared with that of the well-known minor groove binder DAPI.

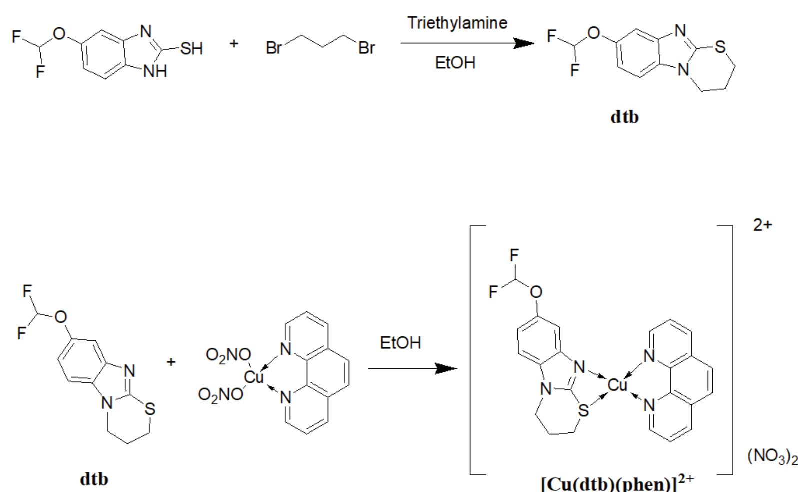

Fig. 1. Synthetic route for dtb and its Cu(II) complex.

EXPERIMENTAL

Materials and characterizations

All reagents and solvents were of commercial origin and used without further purification unless otherwise noted. Solutions of calf thymus DNA (CT-DNA; purchased from Sigma) in 100 mM KCl, 10 mM Tris (pH 7.0) had a UV–Vis absorbance ratio of 1.8–1.9 at 260 and 280 nm (A_{260}/A_{280} = 1.9), indicating that the DNA was sufficiently free of protein.[9] The concentration of DNA was determined spectrophotometrically using a molar absorptivity of 6600 M^{-1} cm^{-1} (260 nm). Solutions of the complex were prepared by dissolving a weighed amount in 0.5 mL DMSO for solubility reasons and were then diluted with 100 mM KCl, 10 mM Tris (pH 7.0) to the required concentration.

Microanalyses (C, H, and N) were performed with a Leco 932 CHNS analyzer. FT-IR spectra were recorded on a PerkinElmer Spectrum 100 instrument. ^{1}H-NMR spectra were recorded at 600 MHz on a Bruker Ultra Shield Plus ultra-long-hold-time spectrometer using $CDCl_3$ as the solvent for the ligand and tetramethylsilane as an internal standard at room temperature. All chemical shifts are given relative to tetramethylsilane. UV–Vis spectra were rec-

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orded using a Varian Cary 100 spectrophotometer and the emission spectra were recorded on a PerkinElmer LS 55 spectrofluorophotometer at room temperature.

DNA binding experiments

Absorption titrations. UV–Vis spectra for DNA titration were recorded on a Varian Cary 100 spectrophotometer. For the absorption titrations, 20 μM of complex was prepared by dissolving in DMSO and diluting with buffer (pH 7.0). Thereafter, 1 μL of 1.25 mM stock DNA solution was added to the 2.5 mL of complex solution and the solutions were incubated for 10 min at 25±0.1 °C and spectra were recorded for each addition.

Emission titrations. The competitive behaviour of $[Cu(dtb)(phen)]^{2+}$ with an intercalative molecule ethidium bromide (EB) and a groove binder molecule DAPI was studied by fluorescence spectroscopy in order to understand whether the complex is able to displace DAPI molecules from DNA minor groove or EB molecule from DNA base pairs. The displacing effect of the Cu(II) complex with the DNA–EB complex was studied by adding increasing amount of the Cu(II) complex solution to the DNA–EB mixture. Similarly, groove binding effect was studied by adding Cu(II) complex to DNA–DAPI solution.

The DNA was pre-treated with EB or DAPI for 30 min at 27 °C to prepare the initial complexes in the ratio of $c_{DNA}/c_{EB} = 5$ (c_{DNA} = 100 μM, c_{EB} = 20 μM) for EB and in the ratio of $c_{DAPI}/c_{DNA} = 6$ (c_{DNA} = 1 μM, c_{DAPI} = 6 μM) for DAPI. The kick-out effect of the complex was studied by adding 5-μL portions of solutions (1 mM) of the complex step by step into the 2.5 mL solution of the DNA–EB or DNA–DAPI complex. To study the competitive binding of complex with EB and DAPI, DNA-EB and DNA-DAPI solutions were excited at 427 and 350 nm respectively in the presence of DNA alone as well as in the presence of the complex.

Viscosity studies. Viscosity measurements were performed in an Ubbelohde viscometer maintained at a constant temperature of 30.0±0.1 °C (in a thermostatic bath). The DNA concentration was 100 μM and the flow time was measured with a digital stopwatch. Every sample was measured three times and an average flow time was calculated. The data are presented as $(\eta/\eta^0)^{1/3}$ *vs*. binding ratio[10], where η is the viscosity of DNA in the presence of complex and η^0 is the viscosity of DNA alone.

DNA cleavage experiments

Plasmid DNA, pBR322, was loaded onto 1 % agarose gels together with TBE (Tris/borate/EDTA, pH 8.3) as running buffer solution. Reaction mixtures (10 μL) containing 200 ng pBR322 and increasing amounts of Cu(II) complex (0, 5, 10, 15, 25 and 50 μM) in 100 mM KCl, 10 mM Tris–HCl, pH 7.5 buffer and 1 mM ascorbic acid (A.A) were prepared at 0 °C. For photo-induced cleavage experiments, reaction mixtures not including A.A were incubated under UV irradiation at 254 nm for 15 min. The cleavage of pBR322 DNA in the presence of common radical scavengers and reaction inhibitors were also studied. In these experiments, 1.0 M DMSO (as a hydroxyl radical scavenger), 100 mM NaN_3 (as a singlet oxygen scavenger) or 100 mM *t*-BuOH (as a hydroxyl radical scavenger) or 100 mM KI (as hydrogen peroxide scavenger) were added into the reaction mixtures, respectively.

Before loading the samples onto the gel, 2.5 μL of loading dye was added into the reaction mixtures. Gels were run by applying a potential of 35 V for 2 h, ethidium bromide (EB) stained and visualized[11] under UV light and photographed.

Preparation of the ligand

8-(Difluoromethoxy)-3,4-dihydro-2*H*-[1,3]thiazino[3,2-*a*]benzimidazole (dtb) was prepared by modification of a literature procedure[12] as follows: 1,3-dibromopropane (1.1 mmol, 0,112 mL) in 5 mL ethanol was added slowly to a well-stirred mixture of 5-(difluorometh-

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oxy)-1*H*-benzimidazole-2-thiol (1.0 mmol, 0.216 g) and triethylamine (1.1 mmol, 0.154 mL), in dry ethanol (10 mL), and stirring was continued overnight at 120 °C. After cooling to room temperature, the precipitate was collected by filtration. The crude product was washed with cold water, ethanol and ether. The product was purified by crystallization from water/ethanol mixture. Yield: 72 %.

Preparation of the complex

(8-(Difluoromethoxy)-3,4-dihydro-2*H*-[1,3]thiazino[3,2-*a*]benzimidazole)(1,10-phenanthroline)copper(II) nitrate complex, $[Cu(dtb)(phen)](NO_3)_2$, was obtained by adding a solution of dtb (0.5 mmol, 0.128 g) in ethanol to a solution of monophenanthroline complex $[Cu(Phen)(NO_3)_2]$ (0.5 mmol 0.184 g) in ethanol at 90 °C. Upon cooling to room temperature, a dark green precipitate formed. After filtration, the solution was washed well with acetone, ethanol and ether. Yield: 62 %.

RESULTS AND DISCUSSION

Synthesis of the compounds

^{1}H-NMR spectroscopy, ESI-MS, CHN analyses and FT-IR spectroscopy techniques were used to characterize the structures of the compounds. The obtained data are given in the Supplementary material to this paper. The appearance of an IR peak of an aliphatic group at 2934 cm^{-1} showed that synthesis of the ligand had been achieved. The elemental analyses results were found to be in good agreement with the calculated values, which confirmed the formation of the ligand and complex. The GC–MS results clearly confirmed the ligand structure showing a molar mass of 256.1 g mol^{-1} as calculated. The ^{1}H-NMR spectrum of dtb exactly supported the formation of the dtb structure. The singlet observed at 7.36 ppm and doublets observed at 7.16 and 6.98 ppm are clearly due to aromatic protons of the benzene ring. A triplet centered at 6.49 ppm was due to the fluorine coupling proton. The number of protons observed in ^{1}H-NMR in the spectra totally matched the number of protons in the compound. The ESI-MS of the complex displayed a peak at $m/z = 500.8$ in methanol, matching exactly with $[Cu(dtb)(phen)]^{2+}$. The electronic absorption bands of complex at 275 and 290 nm were assigned to the intraligand (π→π*) transitions of ligand. The broad band at 612 resulted from d→d transitions of the copper(II) complex.

DNA binding experiments

UV absorption titrations. UV–Vis spectroscopy is a useful way to investigate the interactions between DNA and complexes. Binding modes such as electrostatic interactions, groove binding and intercalation are major modes of binding. While small molecules interact with DNA by the groove binding mode, hydrogen bonding or van der Waals interactions are formed and hyperchromism is observed by interaction between a nucleic acid base and a compound.[12] A complex bound to DNA through groove binding usually results in a smaller (6–8 nm) bathochromism than that of intercalating agents or no bathochromism is

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usually observed. The intrinsic binding constant (K_b) is calculated according to the Eq. (1) to quantitatively compare the DNA-binding affinity of a molecule:[13]

$$c_{DNA}/(\varepsilon_A - \varepsilon_f) = c_{DNA}/(\varepsilon_B - \varepsilon_f) + 1/K_b(\varepsilon_B - \varepsilon_f) \quad (1)$$

where c_{DNA} is the concentration of DNA in base pairs, ε_A is the apparent absorbance, and ε_f and ε_B are the absorbances of the free and the fully bound complex, respectively.

A major peak at 272 nm and a shoulder at 295 nm (Fig. 2) were observed in the UV–Vis spectrum of the complex. With the addition of 1 µL portions of 1.25 mM DNA to this solution, 24 % hyperchromism was detected and the calculated DNA binding constant was 4.6×10^4 M^{-1}.

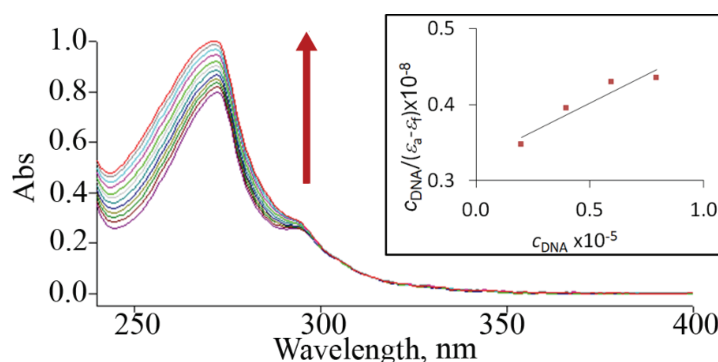

Fig. 2. UV–Vis spectra of $[Cu(dtb)(phen)]^{2+}$ with increasing amounts of DNA; $c_{DNA}/(\varepsilon_A - \varepsilon_f)$ in mol dm^{-3} cm, c_{DNA} in mol dm^{-3}.

The K_b value is about 10 times higher than some other intercalator copper complexes such as $[Cu(dipica)(diimine)]^{2+}$ (K_b, 2.7–6.5×10^3 M^{-1})[14] and $[Cu(L\text{-tyrosine})(5,6\text{-dmp})]^{2+}$ (K_b, 2.7×10^3 M^{-1}).[15] It is also higher than a series of [Cu(imda)(diimine)] complexes with high hydrogen bond capacities (K_b, 0.6––17.0×10^3 M^{-1}).[16] The K_b value is not only higher than those of some intercalators but also of the minor groove binder copper(II) complex with 3,5-bis(2′-pyridyl)-1,2,4-oxadiazole (K_b, 2.2×10^4 M^{-1}).[17]

It is reported in the literature that a high hydrogen bond capacity usually enhances ligand–DNA and ligand–protein interactions.[18–21] In accordance with the literature, the UV–Vis titration results indicate that the estimated high hydrogen bond capacity of difluoromethoxy group increased the DNA minor groove binding ability of complex.

Competitive emission titrations with EB and DAPI. To further understand the binding mode of the complex to ds-DNA, competitive fluorescence titration assays against EB and DAPI were performed. A complex that interacts with DNA by intercalation mode would force the intercalated EB molecules from the DNA base pairs and decrease the EB–DNA emission.[22]

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The change in the emission spectra of EB–DNA with the addition of increasing amounts of the copper complex is shown in Fig. 3. The addition of the complex to the DNA–EB solution did not cause any decrease in the emission intensity, indicating that $[Cu(dtb)(phen)]^{2+}$ does not interact with ct-DNA by the intercalation mode.

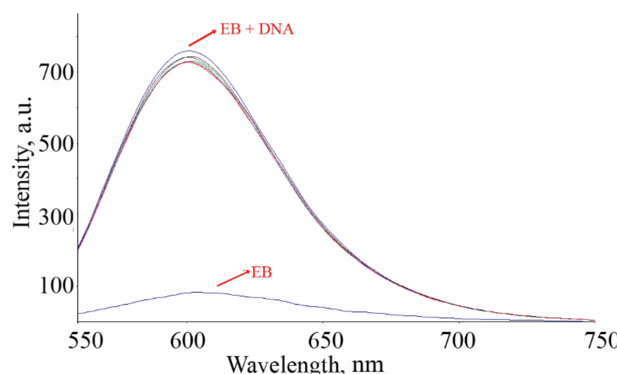

Fig. 3. Emission spectra of EB bound to DNA in the presence of $[Cu(dtb)(phen)]^{2+}$ with increasing concentration.

DAPI is a very well-known fluorogenic probe the fluorescence intensity of which increases upon binding DNA through the minor groove.[23] In the presence of another minor groove binding molecule, the DAPI molecule would be replaced in DNA and the emission of the solution would be greatly quenched.

As shown in Fig. 4, on addition of increasing amounts of $[Cu(dtb)(phen)]^{2+}$ to the DAPI–DNA complex, a significant decrease in the fluorescence intensity of DAPI bound to DNA was observed, thereby clearly suggesting that $[Cu(dtb)(phen)]^{2+}$ could be able to bind to the minor groove of DNA.

To compare the binding ability of $[Cu(dtb)(phen)]^{2+}$, the Stern–Volmer (SV) equation was employed:[24]

$$F_0/F = 1 + K_{SV}c_Q \quad (2)$$

where F_0 and F are the steady state fluorescence emission intensity in the absence and presence of quencher, respectively, and c_Q is the concentration of DNA.

The Stern–Volmer quenching constant (K_{SV}) for the interaction of the complex against DAPI–DNA was calculated as 5.3×10^4 M^{-1}, which is similar to the K_b value calculated from UV titrations. Furthermore, the K_{SV} value is higher than those of other DNA minor groove binder copper complexes with K_{SV} values of 0.94×10^4 and 1.34×10^4 mol^{-1} dm^3.[25]

Viscosity studies. Hydrodynamic studies, such as viscosity measurements, being sensitive to length change can give further information on the nature of the binding of molecules to DNA.[26] The classical intercalation model results in

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lengthening of DNA helix due to the separation of base-pairs by intercalative molecules, which leads to an increase in DNA viscosity.[27] Minor groove binders could induce a decrease in viscosity of a DNA solution due to twisting and shortening of the helix.[28]

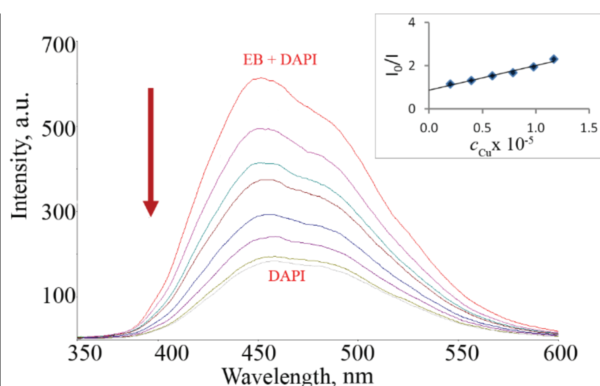

Fig. 4. Emission spectra of DAPI bound to DNA in the presence of increasing concentrations of $[Cu(dtb)(phen)]^{2+}$; c_{Cu} in mol dm^{-3}.

The effects of EB (intercalator), Hoechst (groove binder) and $[Cu(dtb)(phen)]^{2+}$ on the viscosity of 100 μM CT-DNA solution are shown in Fig. 5.

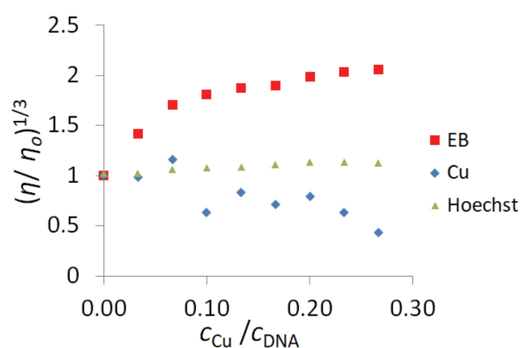

Fig. 5. Effects of increasing amounts of $[Cu(dtb)(phen)]^{2+}$ (♦), EB (■) and Hoechst (▲) on the viscosity of CT-DNA solutions at 30(±0.1) °C.

With increasing amounts of $[Cu(dtb)(phen)]^{2+}$, a slight decrease was observed for the relative viscosity of DNA. This result is similar to the previously reported $[Ru(dmp)_2(MCMIP)](ClO_4)_2$[29] and $[Ru(dmb)_2(pdpt)](ClO_4)_2$.[30] The partial intercalation from the minor/major groove may act as a ''wedge'' to pry apart one side of a base-pair stack, as observed for Δ-$[Ru(phen)_3]^{2+}$,[31] but does not fully separate the stack as required by the classical intercalation mode. This would cause a static bend or kink in the helix and a decrease in the viscosity of DNA. Considering the results of absorption titration, competitive emission tit-

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rations and viscosity measurements, it can be concluded that the complex partially intercalates to DNA through the minor groove.

DNA cleavage results

Chemical nuclease activity. The DNA cleavage activity of the complex under different conditions, including in the dark, under UV irradiation and oxidative media, was studied by gel electrophoresis using pBR322 DNA in 100 mM KCl, 10 mM Tris–HCl buffer (pH 7.5). When pBR322 plasmid DNA is subjected to gel electrophoresis, the supercoiled form pBR322 (form I) migrates the fastest. In the case of scission on one strand, form I begins to convert to the open circular form (form II) that migrates slower on the agarose gel. If both strands are cleaved, a linear form (form III) that migrates between forms I and II will be generated.

As shown in Fig. 6, $[Cu(dtb)(phen)]^{2+}$ is not able to cleave pBR322 DNA when incubated in the dark at 38 °C.

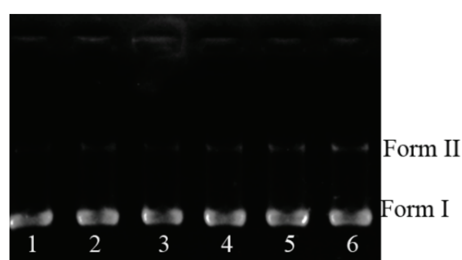

Fig. 6. Effect of compound 2 on DNA cleavage. Lane 1, DNA alone; lanes 2–6, DNA with increasing concentrations in dark (5, 10, 15, 25 and 50 μM) of $[Cu(dtb)(phen)]^2$.

The oxidative cleavage of supercoiled pBR322 DNA on addition of $[Cu(dtb)(phen)]^{2+}$ in the presence of ascorbic acid is shown in Fig. 7. For comparative purposes, the reactions were also performed with Cu(II) sulphate and the ligand.

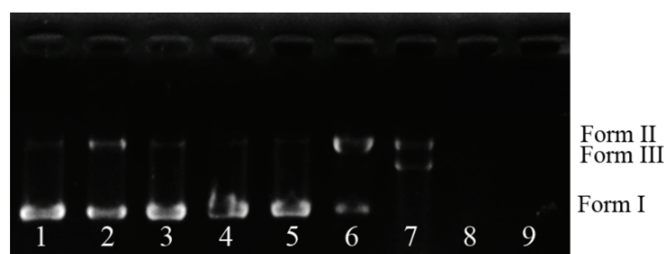

Fig. 7. Effect of compound **2** on DNA cleavage in the presence of 1 mM of ascorbic acid (A.A). Lane 1, DNA alone; Lane 2, DNA + A.A; Lane 3, DNA + A.A + Ligand; Lane 4, DNA + A.A + Cu(II) sulphate; lanes 5–9, DNA + A.A + $[Cu(dtb)(phen)]^{2+}$ (5, 10, 15, 25 and 50 μM).

As shown in Fig. 7, $[Cu(dtb)(phen)]^{2+}$ showed cleavage activity only at concentrations ≤10 μM and cleaved the supercoiled DNA to the nicked DNA (form

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II). At 15 μM concentration (Lane 7), $[Cu(dtb)(phen)]^{2+}$ could cleave the supercoiled DNA to both nicked DNA (form II) and linear DNA (form III) in the presence of ascorbic acid. As seen in lanes 3 and 4, the ligand and Cu(II) sulphate alone showed no cleavage activity. In lanes 8 and 9, the complex cuts DNA into pieces so small that they could not be visualized in the agarose gel.[32–34]

Photo-induced nuclease activity The photo-induced nuclease activity experiments were performed under UV (254 nm, 12 W) light using increasing concentrations of $[Cu(dtb)(phen)]^{2+}$ in the absence of ascorbic acid. The photo-induced DNA damage by the complex is shown in Fig. 8.

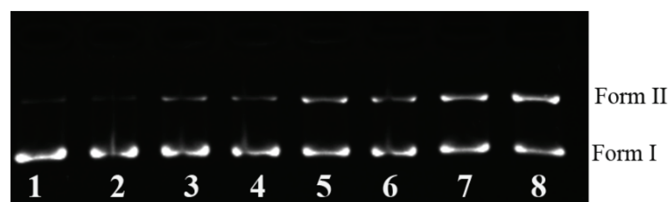

Fig. 8. Effect of increasing concentrations of compound **2** on DNA cleavage under UV irradiation without ascorbic acid. Lane 1, DNA alone; Lane 2, DNA + UV; lanes 3–8, DNA + UV + $[Cu(dtb)(phen)]^{2+}$ (5, 10, 15, 25, 50 and 100 μM).

When irradiated with UV light of 254 nm for 15 min, $[Cu(dtb)(phen)]^{2+}$ resulted in significant concentration-dependent photo-induced cleavage of supercoiled DNA. The complex could convert form I DNA to form II even at low concentrations. The amount of converted form II DNA significantly increased with increasing concentration of the complex. It can be seen in lane 2 that 254 nm UV irradiation did not cause any cleavage due to the short irradiation time.

These cleavage results indicated that $[Cu(dtb)(phen)]^{2+}$ has cleavage activity under UV light and in the presence of ascorbic acid. However it could not cleave DNA even at high concentrations without an oxidative agent as planned.

DNA oxidation with ROS scavengers. An oxidative mechanism is the major way for DNA cleavage by copper complexes.[35–37] In an oxidative DNA cleavage mechanism, the formation of a reactive hydroxyl radical ($^{\bullet}OH$), a superoxide anion radical ($^{\bullet}O_2^{-}$), and/or a singlet oxygen species ($^1O_2$), or a peroxide radical ($^{\bullet}O_2^{-2}$) may lead to damage to the base or/and sugar of DNA. To specify which reactive oxygen species (ROS) was responsible for the DNA cleavage, the DNA cleavage reactions of the complex were performed in the presence of ROS scavengers, such as DMSO, NaN_3, KI, and *t*-BuOH (D_2O, Fig. 9).

In the presence of the hydroxyl radical (·OH) scavenger DMSO, the cleavage of DNA was inhibited slightly (lane 3). When the hydrogen peroxide scavenger KI was added to the reaction mixture, inhibition of DNA cleavage increased significantly (lane 4), suggesting both hydroxyl and hydrogen peroxide radicals are involved in the scission of DNA.

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Fig. 9. DNA cleavage in the presence of increasing concentration of compound **2**, 1 mM of ascorbic acid (A.A) and ROS scavengers. Lane 1, DNA alone; lane 2, DNA + A.A + 10 µM [Cu(dtb)(phen)]2+; lane 3, DNA + A.A + 10 µM $[Cu(dtb)(phen)]^{2+}$ + DMSO; lane 4, DNA + A.A + 10 µM $[Cu(dtb)(phen)]^{2+}$ + KI; lane 5, DNA + A.A + 10 µM $[Cu(dtb)(phen)]^{2+}$ + NaN_3; lane 6, DNA + A.A + 10 µM $[Cu(dtb)(phen)]^{2+}$ + *t*-BuOH.

The presence of the singlet oxygen scavenger NaN_3 caused no inhibition of chemical nuclease activity of the tested complex (lane 5). However, it is noteworthy that the other $^{\bullet}OH$-scavenger *t*-BuOH had no impact on the cleavage activity of the complex (lane 6) while DMSO had a slight impact. This result suggests that peroxide radicals are major radical oxygen species in the cleavage mechanism of the present copper complex.

CONCLUSIONS

In conclusion, the DNA binding, photo-induced and chemical nuclease activity of a new type of copper(II) metal complex containing 1,10-phenanthroline (phen) and 8-(difluoromethoxy)-3,4-dihydro-2*H*-[1,3]thiazino[3,2-*a*]benzimidazole (dtb) ligands were studied in detail. UV titration, competitive emission titrations and viscosity study clearly indicated that the DNA binding mode of the complex is a non-classical intercalation through the minor groove. As expected, the H-bond donor difluoromethoxy group promoted a stronger DNA binding affinity. Thus, the UV binding constant was much higher than those of the copper complexes presented in the literature. The chemical and photo-induced nuclease activities of the complex were also studied. In the absence of ascorbic acid, no detectable change in the DNA was observed by electrophoresis experiments with different concentrations of the complex. However, in the presence of ascorbic acid, single- and double-strand cleavages leading to form II and linear DNA form III were observed, which indicates ROS produced in the vicinity of DNA. The studies of the DNA cleavage mechanism revealed that peroxide radicals play a major role in the oxidative cleavage of DNA.

SUPPLEMENTARY MATERIAL

Characterisation data are available electronically at the pages of journal website: http://www.shd.org.rs/JSCS/, or from the corresponding author on request.

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ИЗВОД

АКТИВНОСТ Cu(II) КОМПЛЕКСА, НОВОГ АГЕНСА ВЕЗАНОГ ЗА МАЛУ БРАЗДУ ДНК, КАО ХЕМИЈСКЕ И ФОТОИНДУКОВАНЕ НУКЛЕАЗЕ

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Синтетисани су и окарактерисани нови бакар(II) комплекси који садрже 1,10-фенантролин (phen) и 8-(дифлуорометокси)-3,4-дихидро-2*H*-[1,3]тиазино[3,2-*a*]бензимидазол (dtb) као лиганде. Испитиване су интеракције ових комплекса са ds-DNA применом различитих метода, као што су UV–Vis спектрофотометрија, флуорометријска титрација са етидијум-бромидом и 4',6-диамидино-2-фенилиндолом (DAPI), мерењем вискозитета и гел електрофорезом. Резултати ових испитивања су показали да испитивани бакар(II) комплекси интереагују са ds-DNA супституишући DAPI молекуле. На основу ових испитивања нађено је да водоник-пероксид узрокује оксидативни механизам раскидања DNA.

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