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Synthesis and spectroscopic calf thymus deoxyribonucleic acid binding investigations of luteolin – zinc(II) complex

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A luteolin–zinc(II) (lut–Zn) complex has been synthesized by the reaction of luteolin with copper acetate in alcohol. The binding mode of lut–Zn with calf thymus deoxyribonucleic acid (ctDNA) is studied by different spectroscopic methods in pH 7.4 tris(hydroxymethyl)aminomethane–HCl (Tris–HCl) buffer solution. Ultraviolet (UV)–visible absorption spectrophotometry and fluorescence spectroscopy as well as viscosity measurements have proved the formation of lut–Zn–ctDNA complex. Binding constant (K_a) of lut–Zn–ctDNA complex is 4.29×10^4 L mol^{-1} (310 K). Fluorophotometry measurements has proved that the quenching mechanism of fluorescence of acridine orange (AO)–ctDNA by lut–Zn is static quenching. The thermodynamic parameters entropy change (ΔS), enthalpy change (ΔH) and Gibbs free energy (ΔG) of binding reaction are calculated to be -20.87 J K^{-1} mol^{-1}, -3.39×10^4 J mol^{-1} and -2.74×10^4 J mol^{-1} at 310 K, respectively. Negative values of ΔH and ΔS have indicated that there are hydrogen bonds and van der Waals forces in the binding reaction of lut–Zn with ctDNA. The fluorescence results and UV–visible absorption together have revealed that the interaction mode of lut–Zn to ctDNA is an intercalation mode. This conclusion is further confirmed by viscosity measurements.

Keywords: Binding mode, Calf thymus DNA, Fluorescence spectroscopy, Luteolin–zinc(II) complex, UV–visible spectroscopy

Luteolin is one of the flavonoid family and is abundant in fruits, red wine, vegetables, medicinal herbs, and tea.[1] It has a lot of pharmacological activities, such as scavenging free radical, anti-inflammatory, anticancer, and neuroprotection for neurological diseases.[2-5] Further study proved that the complexes formed by metal ions and luteolin not only reduce the side effects, but also enhance their biological activity and even produce some novel biological effects.[6-8] Therefore, the studies on the complexes formed by luteolin and metal ions have become the research hot point of researcher. Xiao et al. have synthesized luteolin complexes of luteolin with Zn^{2+} and Mn^{2+}, and compared the inhibitory capacities on xanthine oxidase of luteolin and its complexes. The results indicated that compared to luteolin, its complexes exhibited a better inhibitory effect.[9] In order to locate the exact chelation site, Song et al. have studied the chelation between luteolin and Cd^{2+} using theoretical methods. Complexes, formed by natural and deprotonated luteolin chelating with Cd^{2+} and hydrated Cd^{2+}, were studied respectively, by using "Density Functional Theory" (DFT) method.[10] Teng et al. have synthesized luteolin-Zn(II) complex and compared the inhibitory activity against α-glucosidase of luteolin and luteolin-Zn(II) complex. The results indicated that inhibitory activity of luteolin-Zn(II) complex against α-glucosidase is higher than that of luteolin.[11]

Study on the binding mechanism of small molecules with DNA can provide useful information for understanding the influence of small molecules on the gene expression, constructing DNA biosensors for DNA binders detection, and developing new antivirus and antitumor drugs.[1] Therefore, many researchers have been focusing on binding mechanism of DNA with small molecules in recent years.[12-14]

It is generally accepted that the small molecules can interact with DNA in either covalent way or non covalent. The non covalent is classified into three categories: (1) Groove binding involves van der Waals or hydrogen bonding interactions with the nucleic acid bases in the minor or major groove of the DNA. (2) External binding interactions between negative charged DNA phosphate backbone and cationic species. (3) Intercalative binding involves stacking interactions between DNA and small molecules, which is stronger than other binding

modes.[15] Pharmacological activity of small molecules is mainly depends on the intensity and method of binding between compound and DNA. So, it is useful to study the binding mode between DNA and small molecule, which could lead to the invention of new DNA targeted drugs.[16]

Many methods have been used to study the interaction mechanism of drug molecules with DNA, such as infrared (IR), voltammetry, UV–visible spectrophotometry, fluorescence, Raman spectroscopies, circular dichroism (CD), dynamic viscosity measurements and etc. Among the above methods, fluorescence spectroscopy and UV–visible spectroscopy are considered to be effective methods as these methods are rapid, sensitive and simple. The interactions of compound with DNA can be monitored by the changes in the position and intensity of the spectroscopic peak.[15]

In this paper, a lut–Zn complex was synthesized and the interaction between lut–Zn and ctDNA in a pH 7.4 Tris–HCl buffer solution was studied by UV–visible spectroscopy, fluorescence spectroscopy and viscosity determination. The experiment results proved that the interaction mode between lut–Zn and DNA might be intercalation.

Materials and Methods

Chemicals and Reagents

Zinc acetate [$Zn(Ac)_2$, Sinopharm Chemical Reagent Company Ltd, Shanghai, China]. Luteolin (>98%, Shanghai Jingchun Reagent Ltd Company, Shanghai, China). ctDNA (Sigma biological Co.) was used without further purification and dissolved in doubly distilled water at concentration of 1.0×10^{-3} mol L^{-1} (stock solutions). The purity of DNA was checked by measuring the ratio of A_{260}/A_{280} = 1.80, and the concentration of DNA was calculated by using a molar absorption coefficient of ε_{260} = 6600 L mol^{-1} cm^{-1}.[17] Acridine orange (AO) (Sigma) solution was prepared by dissolving AO in doubly distilled water with a concentration of 1.0×10^{-4} mol L^{-1}. Tris–HCl solution (pH = 7.40, containing 0.1 mol L^{-1} NaCl) was used as buffer solution. The stock solution and the diluted solutions above were stored in a refrigerator at 4 °C. Other chemicals used were of analytical or higher grade.

Instrumentations

Spectroscopic measurements of lut–Zn were made on a CARY300 spectrophotometer (Varian, America) using 1 cm quartz cell. The fluorescence spectra of AO-DNA were recorded using a F–4600 spectrofluorophotometer (HITACHI, Japan) with a thermostat bath (Hengping Instrument Factory, Shanghai, China). The pH was measured on a pHSJ–4A acidometer (Shanghai Lei Ci Device Works, Shanghai, China).

Synthesis of luteolin –Zn(II) complex

Lut –Zn(II) complex was prepared as described in literature.[11] 25 mL 0.025 mol/L luteolin solution of ethanol and 25 mL 0.025 mol/L $Cu(Ac)_2$ ethanol solution were added to a 100 mL round bottom flask. The solution was adjusted to pH 9.0 with 1 mol/L NaOH solution. The mixed solution was heated with continuous stirring for 4 h at 80 °C. The complex was filtered in a vacuum system, washed with ethanol and dried in vacuum at 40 °C for 30 min. Yield: 70 %. Fig. 1 shows the chemical structure of the lut–Zn complex. A stock solution of lut–Zn (1.0×10^{-3} mol L^{-1}) was prepared by dissolving the corresponding lut–Zn in N–N dimethylformamide.

UV- spectroscopic measurements

UV–visible spectra of lut-Zn with a fixed concentration of 4.76×10^{-5} mol L^{-1} in Tris–HCl solution were recorded in the presence of different concentrations of the DNA (0 – 8.70×10^{-5} mol L^{-1}). Using Tris–HCl buffer solution as reference solution, UV–visible spectra were measured from 250 nm to 500 nm after the mixture was mixed thoroughly.

Fluorescence spectroscopic measurements

The fluorescence titration was carried out using the fixed concentration of AO (4.76×10^{-6} mol L^{-1}) and ctDNA (4.76×10^{-5} mol L^{-1}) and varying the concentration of lut-Zn (0 – 6.25×10^{-6} mol L^{-1}) in Tris–HCl solution. The emission spectra were

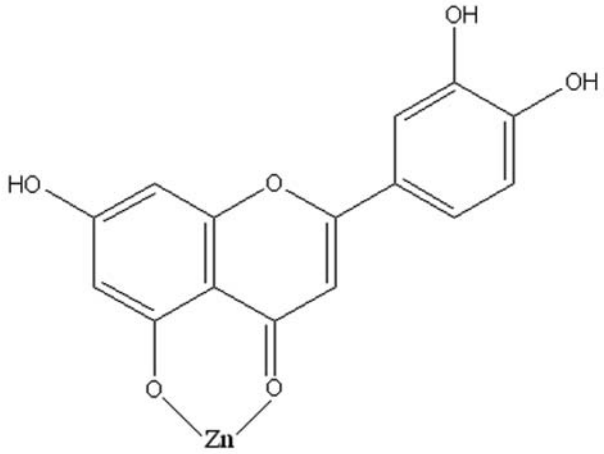

Fig. 1 — Chemical structure of the lut-Zn complex

recorded from 500 nm to 600 nm with an excitation wavelength at 480 nm. The widths of both the emission and the excitation slits were set at 5 nm.

Viscosity measurements

Viscosity experiments were carried out using an Ubbelohde viscometer suspended vertically in a thermostat water bath at 25 ± 0.1 °C. The viscosity of ctDNA was measured in the presence and absence of lut–Zn. The flow times of the solution through the capillary were the average of three measurements. The data were presented as $(\eta/\eta_0)^{1/3}$ versus the ratios of the concentration of lut–Zn to DNA ($c_{lut\text{-}Zn}$ / c_{DNA}),[18, 19] where η_0 and η are the viscosity of DNA solution in the absence and presence of lut–Zn, respectively. The values of η_0 and η were calculated from the flow time of DNA solutions (t) corrected for the flow time of Tris–HCl solution (t_0) by the following equations:

$$\eta_0 = t - t_0 \text{ and } \eta = \frac{t - t_0}{t_0}$$

Results and Discussion

Absorption studies

UV–visible spectrophotometry is a relatively effective and simple method to study the interaction of small molecules with biological macromolecules such as DNA and proteins. When small molecules bind to DNA, the absorption of small molecules and the absorption peak position will change. The absorption spectrum of lut–Zn displayed two absorption peak at 390 nm and 277 nm because of the transition of π-π* and n–π* conjugated system. When lut–Zn was titrated with increasing DNA concentrations, hypochromism at 390 nm (with a red shift from 390 to 392 nm) and hyperchromism at 277 nm (with a blue shift from 277 to 270 nm) could be observed (Fig. 2), indicating an interaction between lut–Zn and ctDNA. In addition, two isoabsorptive points were observed at 325 nm and 440 nm, which proved the formation of lut–Zn–DNA complex.[20]

Hypochromism with red shift at 390 nm can be interpreted that the π* orbital of lut–Zn coupled with the π orbital of the DNA base pairs, leading to a decrease of the π–π* transition energy, which resulted in a red shift of absorption peak. At the same time, the coupling π*–orbital was partially filled with electrons, leading to a reduction of transition probability, which resulted in the hypochromicity.[21] These various spectra changes such as a small red shift, decrease in intensity and isosbestic points are evidences of intercalation mode.[22, 23] The reason of hyperchromic effect with blue shift at 277 nm is that DNA has a absorption peak at 260 nm, which attributed to the chromophoric groups in pyrimidine (thymine and cytosine) and purine (guanine and adenine) moieties responsible for the electronic transitions.[24] These results indicated that there is binding interaction of lut–Zn with ctDNA, and the intercalation mode appeared to be more acceptable.

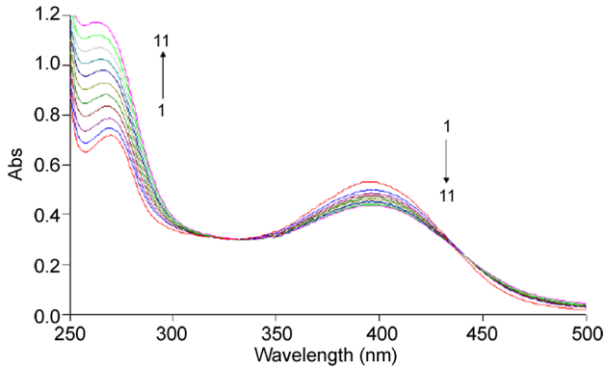

Fig. 2 —Absorption spectra of lut-Zn varying with concentrations of ctDNA (pH = 7.4, T = 298 K), $c_{lut\text{-}Zn}$ = 4.76 × 10^{-5} mol L^{-1}, c_{DNA} = 0, 0.94, 1.87, 2.78, 3.67, 4.55, 5.41, 6.25, 7.08, 7.89, and 8.70 × 10^{-5} mol L^{-1} for curves 1–11, respectively

Fluorescence spectroscopic measurements

Many organic dyes, such as ethidium bromide (EtBr) and AO have already proved to be sensitive probes of DNA.[25, 26] In our experiments, AO was used as the probe for its higher stability and lower toxicity. Due to its planar structure, AO is one of the most sensitive probes with a natural fluorescence. The fluorescence intensity of AO-DNA is obviously stronger than AO due to its strong intercalation mode. However, when other molecules, which can take the place of AO, bind to DNA by intercalation mode, the fluorescence intensity of AO-DNA is obviously decreased. If small molecules bind to DNA by electrostatic bonding or groove, the fluorescence intensity of AO-DNA has no significant change. Thus, AO can be used as a probe for the determination of binding mode between the small molecules and DNA. From Fig. 3, it can be seen that addition of lut–Zn to DNA–AO complex led to obvious decrease in the fluorescence intensity; meanwhile, lut–Zn gave no detectable emission under this condition. The results proved that the binding between lut–Zn and ctDNA might be through intercalation mode.

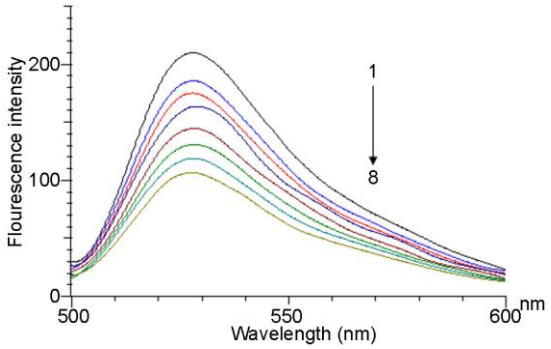

Fig. 3 — Fluorescence spectra of AO–ctDNA in the presence of lut-Zn at different concentrations (pH = 7.4, T = 298 K, λ_{ex} = 480 nm), $c_{AO} = 4.76 \times 10^{-6}$ mol L^{-1}, $c_{DNA} = 4.76 \times 10^{-5}$ mol L^{-1}, $c_{lut\text{-}Zn}$= 0, 0.94, 1.87, 2.78, 3.67, 4.55, 5.41, and 6.25×10^{-6} mol L^{-1} for curves 1–8, respectively

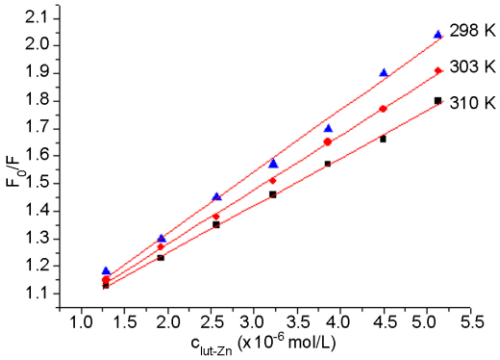

Fig. 4 — Stern–Volmer plots for the fluorescence quenching of AO–ctDNA by lut–Zn at different temperatures

Table 1 — Linear equations of F_0 / (F_0-F) versus $1/c_{lut\text{-}Zn}$ and K_a of lut–Zn with ctDNA at different temperatures

Temperature (K)	Linear equation	R	K_a (L mol^{-1})
298	Y = 0.6846 + 0.9460x	0.9997	7.24×10^4
303	Y = 0.4782 + 0.8379x	0.9970	5.71×10^4
310	Y = 0.3193 + 0.7450x	0.9976	4.29×10^4

The quenching mechanism

Generally, fluorescence quenching mechanisms can be divided into two types: (1) static quenching, the formation of a fluorophore–quencher complex and (2) dynamic quenching, the collision of the quencher and the fluorophore during the transient existence of the excited state.[21] The two different mechanisms can be distinguished by their quenching constants dependence on temperature, viscosity, and by lifetime measurements.[27] In our work, we used the quenching constants dependent on temperature to determine the quenching mechanism.

The fluorescence intensities of AO–ctDNA at 525 nm with different concentration of lut–Zn were determined at 298, 303 and 310 K. In order to elucidate the quenching mechanism, we employed the Stern–Volmer equation (Eqn (1)) to calculate the quenching constant.[28, 29]

$$F_0/F = 1 + K_{SV}[Q] \quad \ldots(1)$$

Here, [Q] is the concentration of quencher (lut–Zn). K_{sv} is Stern–Volmer quenching constant, which can be determined by linear regression of a plot of F_0/F against [Q]. F and F_0 are the fluorescence intensities of AO-DNA in the presence and absence of lut–Zn, respectively.

The plots of Stern–Volmer are shown in Fig. 4 and the K_{SV} values calculated from the Stern–Volmer equation are found to be 2.25×10^5, 1.98×10^5 and 1.72×10^5 L mol^{-1} at 298, 303 and 310 K, respectively. The results showed that the quenching constants value decreased with the increase of temperature, indicating that the quenching mechanism between AO–DNA and lut–Zn is static quenching.[30] The quenching was mainly a DNA–lut–Zn complex formation process. The result was consistent with that of absorption study above.

Determination of binding constants

The binding constants are usually used to measure the strength of small molecules binding to DNA. In this experiment, we used the modified Stern–Volmer equation (Eqn (2)) to calculate the binding constant K_a of the interaction between lut–Zn and ctDNA.[28, 31]

$$\frac{F_0}{\Delta F} = \frac{F_0}{F_0 - F} = \frac{1}{f_a K_a}\frac{1}{[Q]} + \frac{1}{f_a} \quad \ldots(2)$$

Where, K_a is the binding constant. F_0 and F represent the fluorescence intensities of the AO–ctDNA in the absence and the presence of lut–Zn (Q), respectively. f_a stands for the fraction of accessible fluorescence, and the value of $1/f_a$ is fixed on the ordinate. $F_0/\Delta F$ is linear to 1/[Q], with slope equal to $(f_a \cdot K_a)^{-1}$. The constant K_a is the quotient of an ordinate $1/f_a$ and slope $1/f_aK_a$. K_a values were listed in Table 1 and Fig. 5 showed the plots of the modified Stern–Volmer equation at different temperatures. The results indicated that there is a strong interaction force between lut–Zn and ctDNA.

The determination of the binding forces

The binding forces between small molecules and DNA mainly include van der Waals force, hydrogen bonds, electrostatic interactions, and hydrophobic force. The thermodynamic parameters (ΔS, ΔH, ΔG) of binding reaction are the main evidence for measuring the binding force.[32] Using the binding constants above, ΔS,

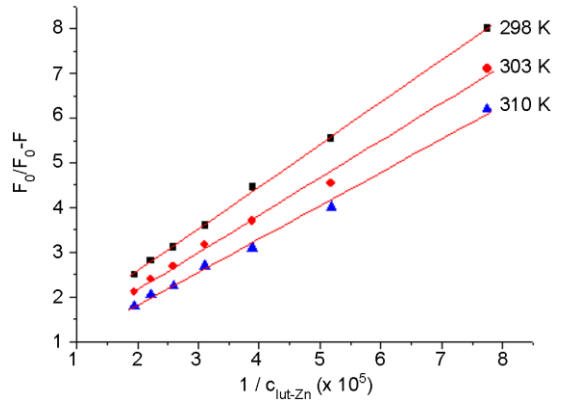

Fig. 5 — The modified Stern–Volmer plots of the lut–Zn–ctDNA system at different temperatures

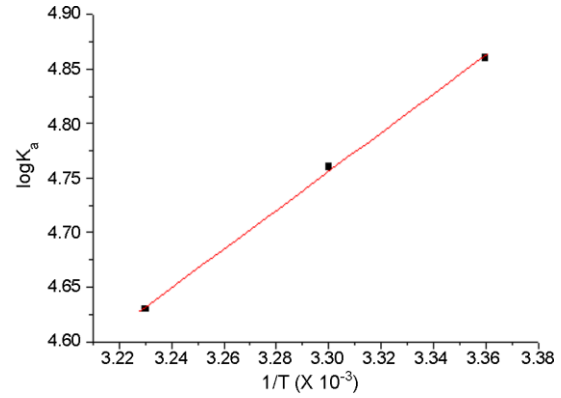

Fig. 6 — van't Hoff plot for lut–Zn –ctDNA complex at different temperatures

Table 2 — Thermodynamic parameters of the binding reaction

Temperature (K)	ΔH (J mol^{-1})	ΔS (J K^{-1} mol^{-1})	ΔG (J mol^{-1})
298	-3.39 × 10^4	-20.87	-2.77 × 10^4
303	-3.39 × 10^4	-20.87	-2.76 × 10^4
310	-3.39 × 10^4	-20.87	-2.74 × 10^4

ΔH, and ΔG of lut–Zn–ctDNA formation can be calculated via van't Hoff equation:[16]

$$\log K_a = -\frac{\Delta H}{2.303RT} + \frac{\Delta S}{2.303R} \quad \ldots(3)$$

Here, K_a is the binding constant of lut–Zn and DNA. From the intercept and slope of the linear van't Hoff plot based on logK_a versus 1/T, the values of ΔS and ΔH can be calculated. The van't Hoff plot for lut–Zn –ctDNA complex at different temperatures was showed in Fig. 6. The ΔG can be calculated by using the basic thermodynamic relationship as given in Eqn (4):

$$\Delta G = \Delta H - T\Delta S \quad \ldots(4)$$

The thermodynamic parameters for the interaction of lut-Zn with ctDNA at 298, 303, and 310 K were listed in Table 2. Negative values of ΔH and ΔS

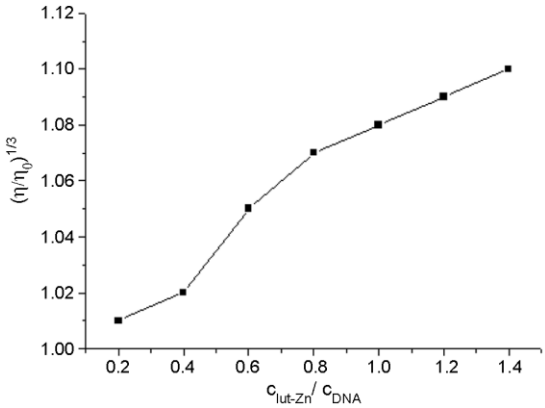

Fig. 7 — The effects of increasing amounts of lut–Zn on the viscosity of ctDNA (pH = 7.4, T = 298K), c_{DNA} = 4.0 × 10^{-4} mol L^{-1}, c_{lut-Zn} = 0, 0.4, 0.8, 1.2, 1.6, 2.0, 2.4 and 2.8 × 10^{-4} mol L^{-1}

indicated that van der Waals forces and hydrogen bonds played a major role in the binding of lut–Zn and ctDNA. While the negative value of ΔG suggested that the binding interaction between lut–Zn and DNA was spontaneous.[32] In addition, negative value of ΔS is an evidence of intercalation mode of lut–Zn to ctDNA.[33]

Viscosity measurements

The viscosity experiment was carried out in order to further verify the accuracy of intercalation mode. It is a fact that the relative viscosity of DNA solution will increase when interaction with the small molecules by intercalative binding, stay the same for classical groove binding, and decrease for partial intercalation.[32] Fig. 7 shows the changes of relative viscosity of DNA with increasing the concentrations of lut–Zn.

From Fig. 7, it can be seen that the viscosity of DNA increased with the increase of concentrations of lut–Zn. The results further proved that the interaction between lut–Zn and DNA may occurred by classical intercalation mode. The reason of increasing the viscosity of the DNA is that intercalation of lut–Zn with DNA causes the DNA double helix to lengthen.

Conclusions

In this paper, the interactions of lut–Zn with DNA have been studied by fluorescence spectroscopy and UV-visible spectrometry in pH 7.4 Tris–HCl buffer solution. The studies here proved that the interaction of lut–Zn with ctDNA was an intercalation mode, which was supported by the results of ctDNA viscosity determination. The results of absorption spectrometry showed that lut–Zn and ctDNA could interact with each other to form a complex.

Fluorophotometry experiment proved that lut–Zn could quench the fluorescence of AO–DNA by substituting AO probe in AO–DNA complex. The thermodynamic parameters (ΔS, ΔH, ΔG) and binding constants of lut–Zn–DNA formation were calculated. Moreover, it was found that the binding forces between lut–Zn and ctDNA mainly included van der Waals forces and hydrogen bonds. The viscosity measurements indicated that the viscosity of ctDNA was enhanced with increasing the concentrations of lut–Zn. In conclusion, the results provided from our work should be useful in understanding the interaction of lut–Zn with ctDNA, as well as designing the structure of new and efficient drug molecules targeted to DNA.

Acknowledgement

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