RESEARCH ARTICLE

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Selective Cytotoxicity of Novel Copper (II) Complex on Colon Cancer Cell Lines

Zuhal Gerçek, Ufuk Yıldız, Remzi Okan Akar, and Engin Ulukaya

ABSTRACT

The synthesis of a novel copper (II) complex, namely, $[Cu(phen)(NO_2\text{-impi})]^{2+}$ (where phen is 1,10-phenanthroline; NO_2-impi is 2-(2-(4-nitrophenyl)-5-(pyridin-2-yl)-1H-imidazol-4-yl) pyridine, ZU-3, was performed. UV titration and viscosity measurements were used to determine its interaction with ds-DNA. The binding constant of complex to ds-DNA was $1.3x10^5$ M^{-1}. The Stern-Volmer extinction constant (K_{SV}) for ZU-3 was calculated as 1.2×10^5 M^{-1}. The complex was screened against five human cell lines, namely A 549, PC-3, BEAS-2B, HCT-116 and MDZ-MB-231, and it was found that ZU-3 shows selective cytotoxicity on colon cancer cells. It has been found that ZU-3 reduced HCT-116 cell density and caused pyknotic morphology that are associated with cell death by apoptosis.

Keywords: Apoptosis, Copper (II) Complex, Cytotoxicity, DNA Binding, HCT-116 Cell Line.

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I. Introduction

Colon cancer (CRC) ranks third among the most common cancer types and fourth in mortality [1]. Diet, smoking, heavy alcohol use and heredity can be counted as certain risk factors which are strongly linked to the disease. Surgery, radiotherapy, and chemotherapy are used for the treatment of disease. Chemotherapy uses cytotoxic molecules (drugs) to kill cancer cells. 5-Fluorouracil (5-FU), Capecitabine (Xeloda), Irinotecan (Camptosar) and Oxaliplatin (Eloxatin) are US Food and Drug Administration (FDA) approved chemotherapeutic drugs used today. However some side effects such as, difficulty of controlling drug concentrations at the action side, non-specific drug distribution or less selectivity, limited ability to selectively bind to the target site, renal-, hepato-, and cardiac toxicities, neutropenia, or neuropathy, and decrease of the effectivity of drug have been reported for them [2]. Therefore, the realization of novel anti-CRC molecules with better efficacy and safety than existing drugs is an inevitable necessity.

The early evidence of the therapeutic use of metals was found in ancient China and India [3]-[5]. cis-$PtCl_2(NH_3)_2$, or cisplatin was the first metal-containing chemotrophic agent. After the success of cisplatin in cancer treatment, many other metal-containing compounds have been developed for cancer treatment [6-10]. Cisplatin can effectively bind DNA and induce apoptosis. Despite its activity, it causes kidney damage and hearing loss and an increase in tumor resistance to cisplatin treatment [11]-[14]. Numerous analogues of cisplatin such as oxaliplatin and carboplatin were synthesized to overcome these drawbacks. Oxaliplatin, one of the FDA approved drugs for colon cancer, can be used when a tumor shows resistance to cisplatin [15],[16].

Copper complexes are also included as potent alternatives to cisplatin [17]. The advantages of copper complexes can be listed as their lower toxicity to normal cells relative to cancer cells [18],[19], its redox

activity, and the link between copper metabolism with angiogenesis and metastasis [20,21]. Interaction with DNA, inhibition of enzymes and redox-mediated generation of ROS can lead to the anticancer activity of copper complexes [22],[23].

This study aims to synthesize novel copper (II) complex ZU-3, $[Cu(phen)(NO_2\text{-impi})]^{2+}$ and determine its interaction with DNA and cytotoxic activity. Its binding constant with pBR322 DNA was calculated. The ZU-3 compound has been tested on A549 (lung cancer), PC3 (prostate cancer), HCT-116 (colorectal cancer), MDA-MB-231 (breast cancer), and BEAS-2B (lung epithelium) cell lines. IC50, IC90, GI50, TGI, and LC50 values have been determined for HCT-116 cell line (Fig. 1).

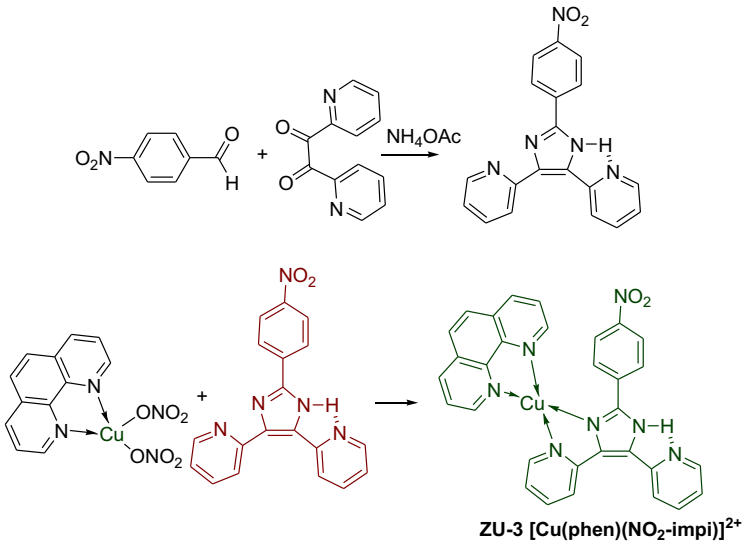

Fig. 1. Synthesis of ZU-3.

II. Results and Discussion

Synthesis of 2-(2-(4-nitrophenyl)-5-(pyridin-2-yl)-1H-imidazol-4-yl) pyridine (NO_2-impi) was achieved by the literature procedure [28]. Pure reddish crystals of ligand were obtained with 54 % yield. The specific signal for NH appears at 13.48 ppm as singlet, and -CH6 signals belong to pyridine rings can be seen at 8.66 and 8.53 ppm in ^{1} H NMR spectrum. The construction of the imidazole ring can be proofed with signals at in ^{13}C NMR spectrum.

The reaction of copper (II) phen with NO_2-impi in ethanol gave the novel copper complex, namely, ZU-3, $[Cu(phen)(NO_2\text{-impi})]^{2+}$. Characterization of the complex was achieved by FTIR, ESI-MS and UV-Vis spectroscopy. In order to show– C = N ——Cu bond formation, IR of complex was compared with that of ligand. Since the electron density decreases in the C=N linkage, all ligand bands shifted to lower frequencies in complex (Table I).

TABLE I: IR Frequencies of Ligand And Its CU(II) Complex

	No. IR frequency (cm^{-1})
Ligand	3466, 3088, 1682, 1604, 1589, 1238, 1142, 1048
Complex	3417; 3085; 1601; 1591 1513; 1235; 1173; 1026; 988

A. UV Absorption Titrations

UV absorption spectroscopy is a powerful method to investigate the interactions of small molecules with DNA. According to the mode of binding of molecule to DNA, UV spectrum bands change dramatically. If the binding is intercalation, hypochromism is observed, while the groove binding mode results in hyperchromism [25]. The UV spectra were recorded with 20 μM solutions of complex the absence and with increasing amount of CT-DNA. Kb (intrinsic binding constant) was calculated according to (1) to quantitatively compare the DNA-binding affinity of molecules [26]. (Fig. 2).

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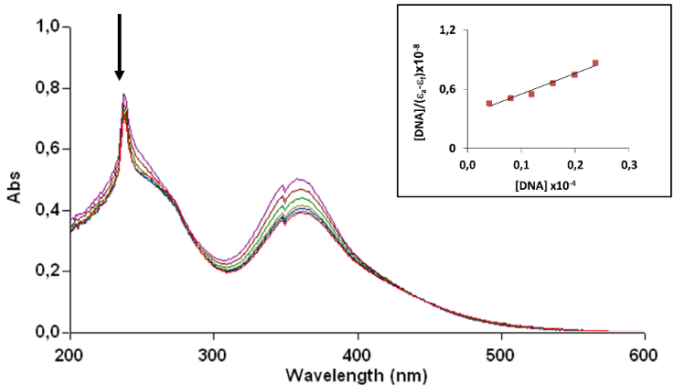

Fig. 2. UV-vis spectra of [Cu(phen)(NO_2-impi)] $^{2+}$ with increasing amounts of DNA.

$$[DNA]/(\varepsilon A - \varepsilon f) = [DNA]/(\varepsilon B - \varepsilon f) + 1/Kb(\varepsilon B - \varepsilon f) \quad (1)$$

Where
[DNA]: concentration of DNA in base pairs,
εA: apparent absorptivity and
εB: absorptivity of the free and the fully bound complex.

The binding constant of the complex which has an electron-withdrawing (-NO_2) group, was found to as 1.3x10^5 M^{-1}which is in agreement with the literature. In comparing the K_b of the synthesized complex with some DNA intercalative drugs, it can be dedicated that the [Cu(phen)(NO_2-impi)] $^{2+}$ binds strongly to CT-DNA via intercalation [27], [28].

The DNA binding mode of the ZU-3 was determined by competitive titration of EB-DNA with complex. The addition of the complex results in the reduction of the intensity of the emission peaks of the EB-DNA complex molecule (Fig. 3). The concentration required to halve of EB emission intensity is 91 µM. These data show that the complex could intercalate between DNA base pairs type of interaction is intercalation.

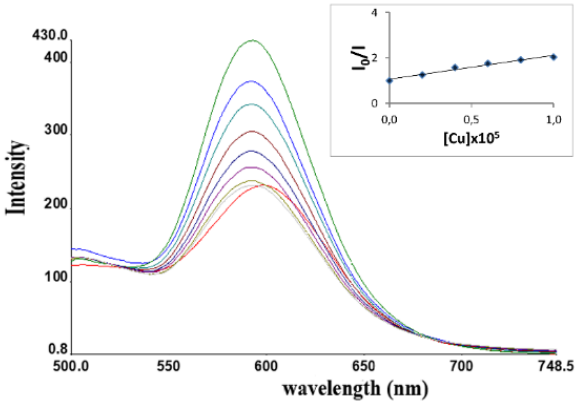

Fig. 3. Effect of [Cu(phen)(NO_2-impi)] $^{2+}$on the emission spectrum of the EB-DNA mixture at increasing concentrations. The Stern-Volmer extinction constant (K_{SV}) for the complex was calculated as 1.2 x 10^5 M^{-1}.

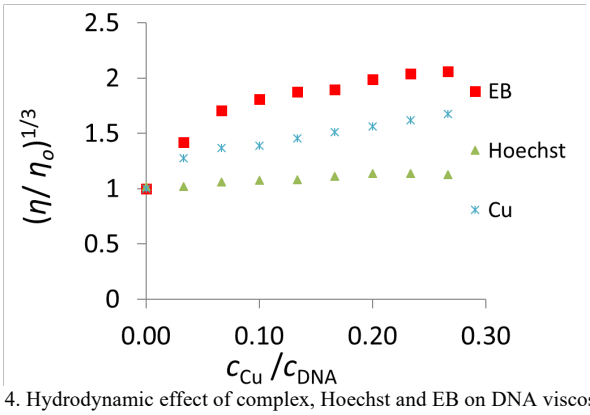

Fig. 4. Hydrodynamic effect of complex, Hoechst and EB on DNA viscosity.

A. Viscosity Studies

In order to be sure of the type of interaction between the novel complex and DNA viscosity studies were done. It is well known that intercalator molecules causes an increase in viscosity [29]. The viscosity of 100 µM of CT-DNA was studied with the presence of EB (intercalator), Hoechst (groove binder) and complex (Fig. 4).

The complex appears to have a similar effect on DNA viscosity as the EB molecule, which is known to intercalate with DNA. This result is in accordance with the previous data; ZU-3 bind to DNA through the classical intercalation mode.

B. Determination of Cytotoxic Activity

For the initial screening of ZU-3's cytotoxic activity at a dose of 10 µM for 48 hours was performed by SRB cell viability assay in four cancer cell lines (PC-3 prostate cancer; A549 lung cancer; MDA-MB-231 breast cancer; HCT 116 colon cancer) and in a non-cancer epithelial cell line (BEAS-2B lung epithelium). As it is shown on the Fig. 5, ZU-3 complex reduced viability approximately 50% in HCT 116 colorectal cell line while it did not show significant cytotoxic activity against the noncancerous BEAS-2B cell line. Hence, ZU-3 complex and HCT 116 cells were selected for further experiments.

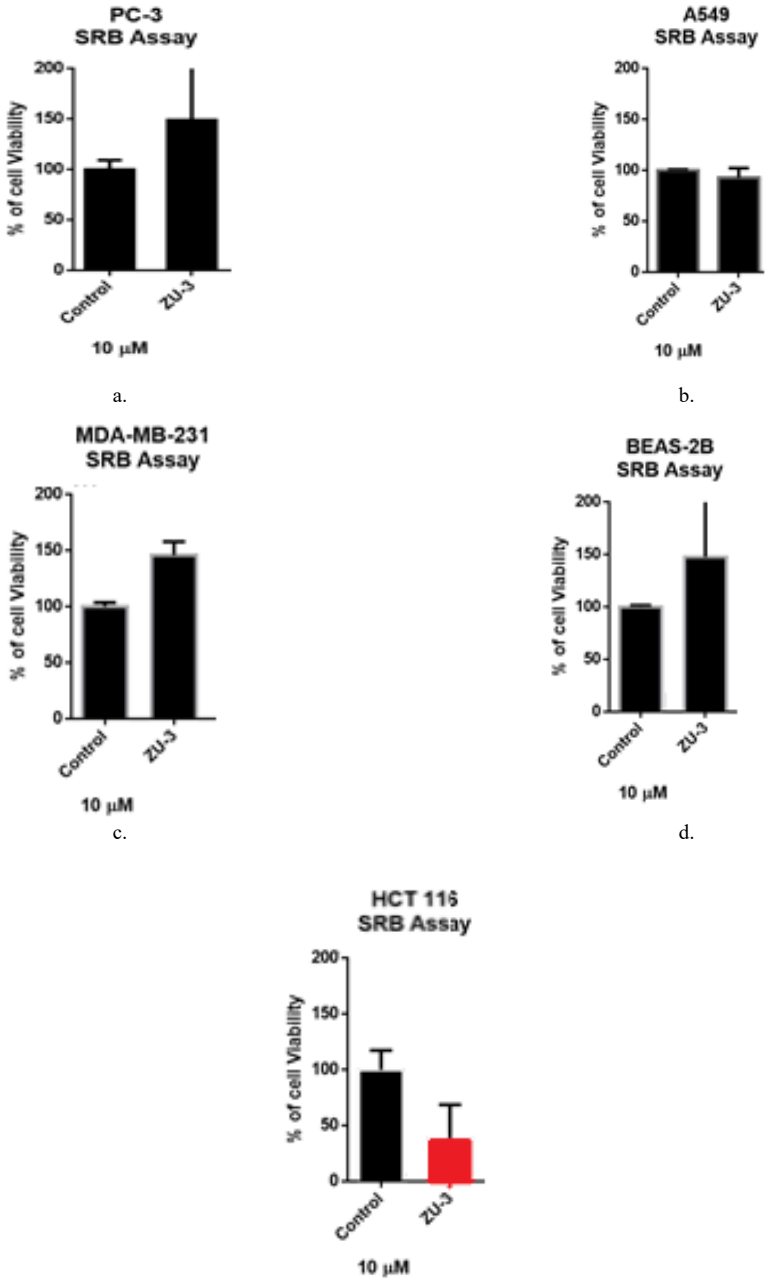

Fig. 5. a. Cell viability of PC-3 cell lines treated with ZU-3 for 48 h. b. Cell viability of A549cell lines treated with ZU-3 for 48 h. c. Cell viability of MDA-MB-231 cell lines treated with ZU-3 for 48 h. d. Cell viability of BEAS-2D cell lines treated with ZU-3 for 48 h. e. Cell viability of HCT 116 cell lines treated with ZU-3 for 48 h. Viability was measured by SRB assay.

HCT-116 cells were treated with ZU-3 and SRB assay was performed (Fig. 6a). It has been detected that cell viability decreased in a dose-dependent manner. In order to confirm the cell viability results, an ATP assay, which is more sensitive than SRB assay, was performed using low doses (0.3-20 µ) (Fig. 6b) and IC50 and IC90 doses were calculated (Table II). It has been found that 2,5 µM and higher doses were statistically decreased cell viability. Also, the effect of ZU-3 doses on cell morphology was evaluated under the phase-contrast microscope (Fig. 7). It has been detected that ZU-3 reduced cell density and caused pyknotic morphology that are associated with cell death. In addition, the growth rate was calculated with the ATP test in the 0.3-20 µM dose range (Fig. 8). Depending on Tz, the change in cell growth after ZU-3 application and GI50, TGI, and LC50 doses were determined (Table II). The GI50, TGI, and LC50 doses are terms that express cell death kinetics and are compared to the number of cells just prior to drug administration (Tz) [30].

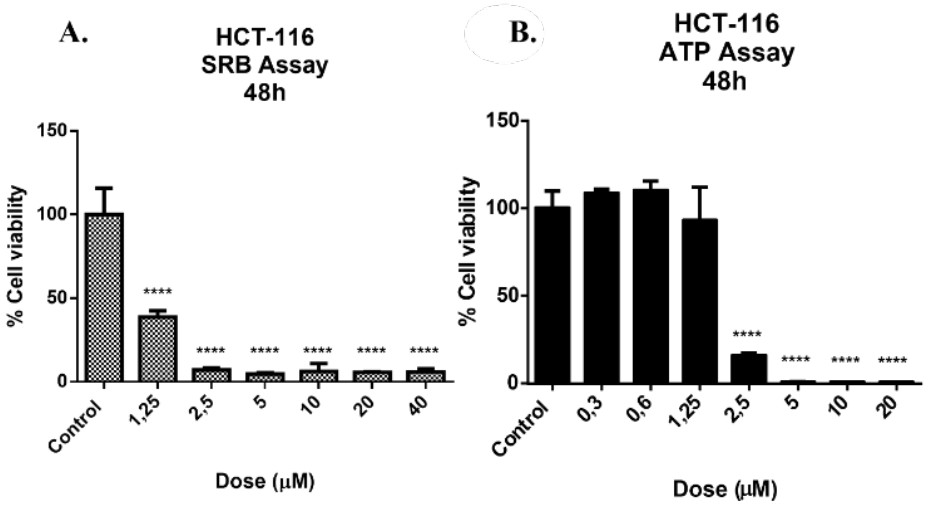

Fig. 6. a. The SRB assay b. ATP assay on ZU-3 treated HCT-116 cells in a dose range (1,25-40 µM and 0,3-20 µM).

TABLE II: GI50, TGI, AND LC50 WERE CALCULATED BY GROWTH RATE ATP ASSAY AND IC50, AND IC90 WERE CALCULATED BY ATP ASSAY FOR 48 H ZU-3 TREATMENT IN HCT116 CELLS

Growth rate-based features		Commonly used cytotoxicity features	
GI50	1,66 µM	IC50	1,95 µM
TGI	2,16 µM	IC90	3,47 µM
LC50	3,14 µM	–	–

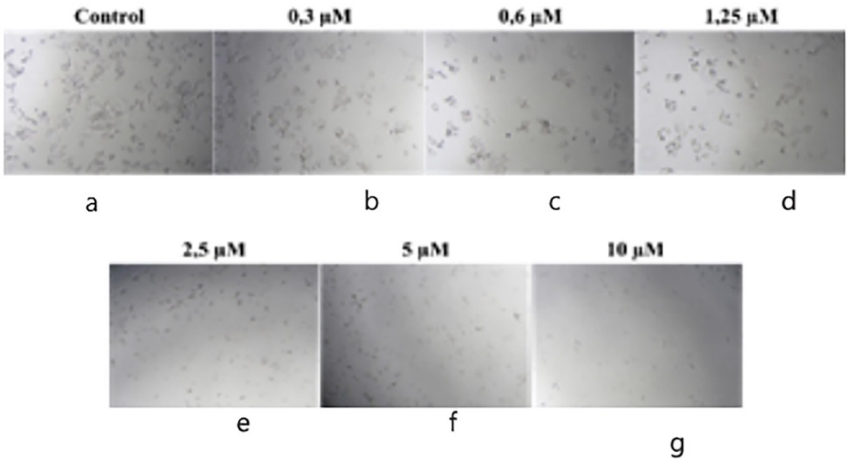

Fig. 7. a. The image of the effect of ZU-3 on control cells. b. The image of the effect of 0.3 µM ZU-3 on the HCT 116 cell line for 48 hours. c. The image of the effect of 0.6 µM ZU-3 on the HCT 116 cell line for 48 hours. d. The image of the effect of 1.25 µM ZU-3 on the HCT 116 cell line for 48 hours. e. The image of the effect of 2.5 µM ZU-3 on the HCT 116 cell line for 48 hours. f. The image of the effect of 5 µM ZU-3 on the HCT 116 cell line for 48 hours. g. The image of the effect of 10 µM ZU-3 on the HCT 116 cell line for 48 hours. Images were taken with 10X magnification under the phase contrast microscope.

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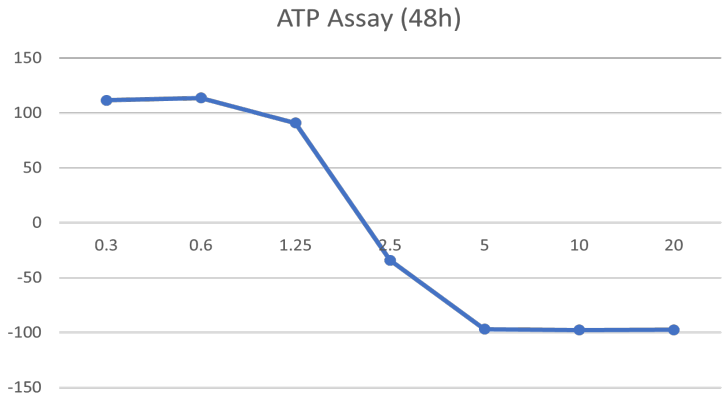

Fig. 8. The effect of dose-dependent application of ZU-3 on viability in HCT 116 colon cancer cell line by ATP assay. The growth rate of cells at respective doses after 48 hours compared to the viability of cells at the start of the test (Tz) were calculated based on the ATP test results.

To distinguish between living cells from dead ones, two dyes DAPI and propidium iodide (PI) were mixed. The basic principle behind this approach to determine viability is to see if the cellular plasma membrane is intact or not. The PI, which selectively stains cells with damaged membranes, is not able to stain the cells with intact cellular membranes. As a result, cells can be double stained with a DAPI, which stains all cells allowing to distinguish between dead and live cells [34]. It has been shown that ZU-3 increased cell death evidenced by pyknosis, cell shrinkage and reduction in cell density and PI-positivity in a dose-dependent manner, especially 2,5 µM and higher doses that is positively correlated with SRB assay (Fig. 9).

The apoptotic potential of ZU-3 (IC90 dose) was evaluated by using Annexin V/Dead Cell kit in the HCT-116 cell line (Fig. 10). The ZU-3 treatment increased apoptosis by approximately 41% and 50% for 12h and 24h, respectively. In order to see if caspase activation involves apoptosis, a flow cytometric study for Caspase 3/7 activation was performed. At 12 and 24 hours, ZU-3 therapy appeared to boost caspase 3/7 activity dramatically. It has been reported that similar copper(II) complexes showed apoptotic activity on colorectal cancer cells [35]. Also it has been showed that similar copper(II) complexes increased apoptosis by activating caspases in different cancer cells [31], [35].

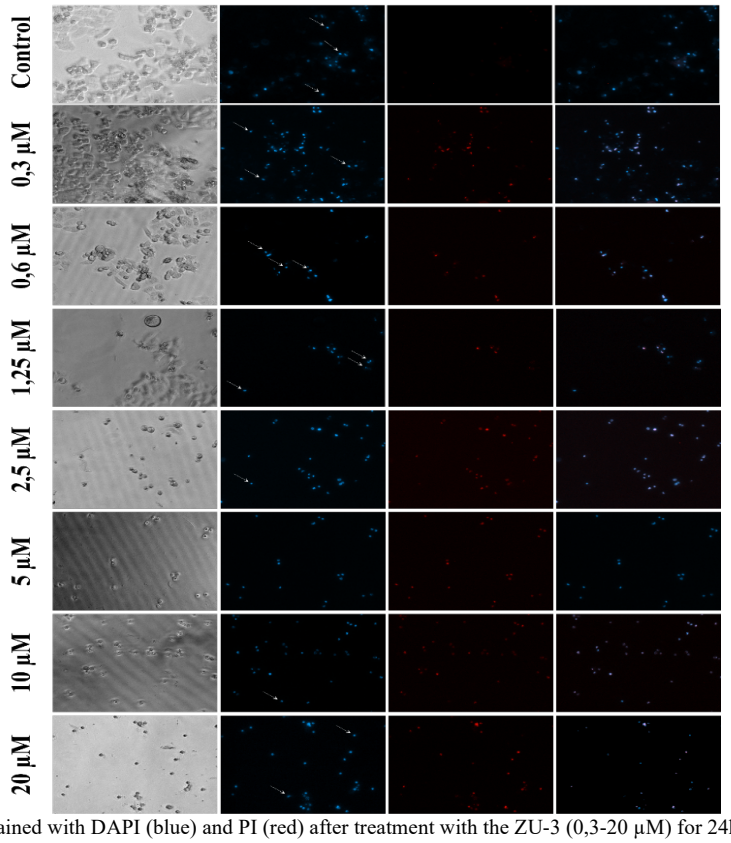

Fig. 9. Cells were stained with DAPI (blue) and PI (red) after treatment with the ZU-3 (0,3-20 µM) for 24h. The long arrows indicate DAPI positive cells that are early apoptotic cells.

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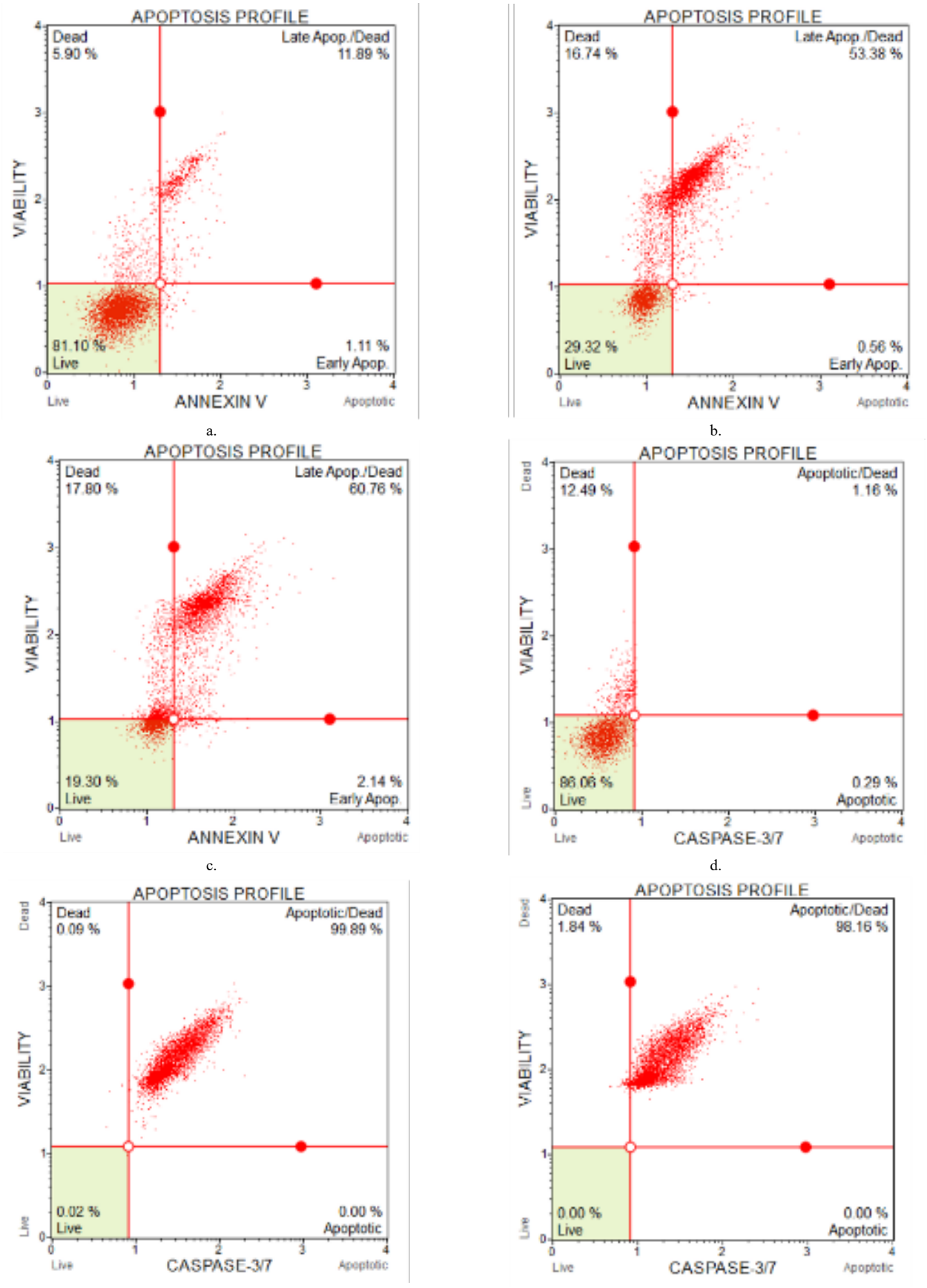

Fig. 10. a. Apoptosis of control cells assessed by Annexin-V positivity. b. Determination of apoptosis activation in HCT-116 cell line treated with ZU-3 (IC90 dose) for 12 h assessed by Annexin-V positivity. c. Determination of apoptosis activation in HCT-116 cell line treated with ZU-3 (IC90 dose) for 24 h assessed by Annexin-V positivity. d. Caspase 3/7 activation of control cells. e. Determination of Caspase 3/7 activation in HCT-116 cell line treated with ZU-3 (IC90 dose) for 12h assessed by caspase3/7 positivity. f. Determination of Caspase 3/7 activation in HCT-116 cell line treated with ZU-3 (IC90 dose) for 24h assessed by caspase3/7 positivity.

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III. Experimental Section

A. 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 7.5) had a UV–Vis absorbance ratio of 1.8–1.9 at 260 and 280 nm (A260/A280 = 1.9), indicating that the DNA was sufficiently free of protein [30]. The concentration of DNA was determined spectrophotometrically using a molar absorptivity of 6600 M^{-1} cm^{-1} (260 nm). FT-IR spectra were recorded with a PerkinElmer Spectrum 100 instrument. ^{1} H NMR spectra were recorded with a Bruker Ultra Shield Plus ultra-long-hold time spectrometer with DMSO-d_6 as the solvent. All chemical shifts are given relative to tetramethylsilane. UV–Vis spectra were recorded with a Varian Cary 100 spectrophotometer and emission spectra were recorded with a PerkinElmer LS 55 spectrofluorophotometer at room temperature.

Synthesis of 2-(2-(4-nitrophenyl)-5-(pyridin-2-yl)-1H-imidazol-4-yl) pyridine (NO_2-impi) [24]:

A mixture of 1 mmol 1,2-di(pyridin-2-yl) ethane-1,2-dione, 1 mmol 4-nitrobenzaldehyde and 8 mmol ammonium acetate in 25 ml acetic acid was refluxed at 118°C for 5 hours. After the reaction period, it was poured into 75 ml ice-water. The neutralization was done with 30 % NH_4OH solution. The filtration of the crude product was followed by recrystallization with with i-propyl alcohol and 4 N HCl solution. The color of the pure crystals was red. The yield of the reaction was 54 %. ^{1} H NMR (DMSO-d_6, 600 MHz) δ (ppm) 13.48 s, 1H, NH, 8.66 s, 1H, 8.53 s, (from H-bonding), 8.47 d, J=8.24 Hz, 1H, 8.33 d, 1H, J= 8.18 Hz, 8.10-8.04 s, 3H, 7.84, s, J=7.556 Hz, 2H. ^{13}C NMR (DMSO-d_6, 150MHz) δ (ppm) 155, 149, 147, 145, 144, 137, 136, 132, 131, 126, 124, 123. m.p. 166-168°C.

B. Preparation of $[Cu(phen)(NO_2\text{-impi})]^{2+}$

Monophenanthroline complex $[Cu(Phen)](NO_3)_2$ (0.5 mmol 0.184 g) was dissolved in ethanol. Than a solution of NO_2-impi (0.5 mmol, 0.377 g) in ethanol was added to this solution. After the reflux period a dark green precipitate was formed. The filtration of the precepitate was followed by the washing with acetone, ethanol, and ether. Yield: 61%. ESI-MS: m/z = 588.1 [M^+ H].

C. DNA Binding Experiments

Absorption titrations, emission titrations and viscosity studies were performed according to methods reported in our previous paper [19].

D. Determination of Cytotoxic Activities

Preparation of cell culture and media, and determination of viability by Sulforhodamine B (SRB) test were performed according to methods reported in our previous paper [19], [32].

E. Determination of Growth Rate by ATP Test

ATP viability assay was performed in order to determine the cytotoxicity of ZU-3 on HCT 116 cells. Cells were seeded at a density of 5×10^3 cells per well with 100 µl of culture medium in 96-well plates. The ATP level of control cells was detected right after seeding and marked as time zero (Tz). Different concentrations of ZU-3 (40-0,3 µM) were applied to other wells for 48 hours. After the treatment period, 50 µl of ATP-releasing reagent (a detergent-based reagent) was added to each well and incubated at room temperature for 20 min. 50 µl of this suspension from each well and 50 µl luciferin–luciferase mixture per well (FLAAM, Sigma Aldrich, MO, USA) were transferred into a white opaque 96-well plate. Luminescence from each well was measured. The viability of the treated cells was calculated with reference to the untreated controls (Tz). The GI50 (Growth Inhibition 50; The dose causes the 50% inhibition of total growth), TGI (Total Growth Inhibition), and LC50 (Lethal Concentration 50; The dose cause half of the seeded cells to be dead) values of ZU-3 were calculated.

F. The Evaluation of Cell Death by DAPI/PI Staining

To evaluate the cell death based on nuclear morphology and membrane integrity, two different fluorescent dyes (DAPI and propidium iodide) were used. HCT 116 cells were seeded at 5×10^3 cells per well in 96-well plates. The cells were treated with ZU-3 at different doses (20-0,3 µM) for 24 h. At the end of the treatment period, the cells were stained with DAPI (5 µg/ml) and PI (1 µg/ml) and incubated for 20 min at room temperature. Then the cells were monitored by fluorescence microscopy.

G. Determination of Apoptosis

The AnnexinV/PI staining was used to determine the apoptotic effect of ZU-3 on HCT 116 cells. Cells were seeded as 2×10^5 per well in 6-well plates and treated with IC90 dose of ZU-3 for 12 and 24 h. At the end of the treatment, cells were trypsinized, and cell death was determined by using Annexin V/Dead Cell Kit (MCH100105, Millipore, Darmstadt, Germany) according to the manufacturer's instructions.

H. Determination of Caspase 3/7

The activity of Caspase 3/7 levels was determined by flow cytometer on ZU-3 treated HCT 116 cells. Cells were seeded as 2×10^5 per well in 6-well plates and treated with IC90 dose of ZU-3 for 12 and 24 h. At the end of the treatment, cells were trypsinized, and cell death was determined by using Caspase-3/7 kit (MCH100108, Millipore, Darmstadt, Germany) according to the manufacturer's instructions.

IV. Conclusion

The novel copper (II) complex (ZU-3) was synthesized and characterized. It was found that the binding constant of ZU-3 to ds-DNA is $1.3x10^5$ M^{-1}. The Stern-Volmer extinction constant (KSV) for ZU-3 was calculated as 1.2×10^5 M^{-1}. Its cytotoxic activity was examined against A 549, PC-3, BEAS-2B, HCT-116 and MDZ-MB-231 cell lines. It was found that the complex shows selective activity on colon cancer cell lines with GI 50: 1,66µM, TGI: 2,16 µM, LC50: 3,14 µM, IC50: 1,95 µ and IC90: 3,47 µM. Therefore, it deserves further attention to be explored as a novel compound for the treatment of colon cancer.

Acknowledgment

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Conflict of Interest

Authors declare that they do not have any conflict of interest.

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