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In vitro biological properties and predicted DNA-BSA interaction of dicyanidoargentate(I)-based three new complexes: Synthesis and characterization

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Supplementary data

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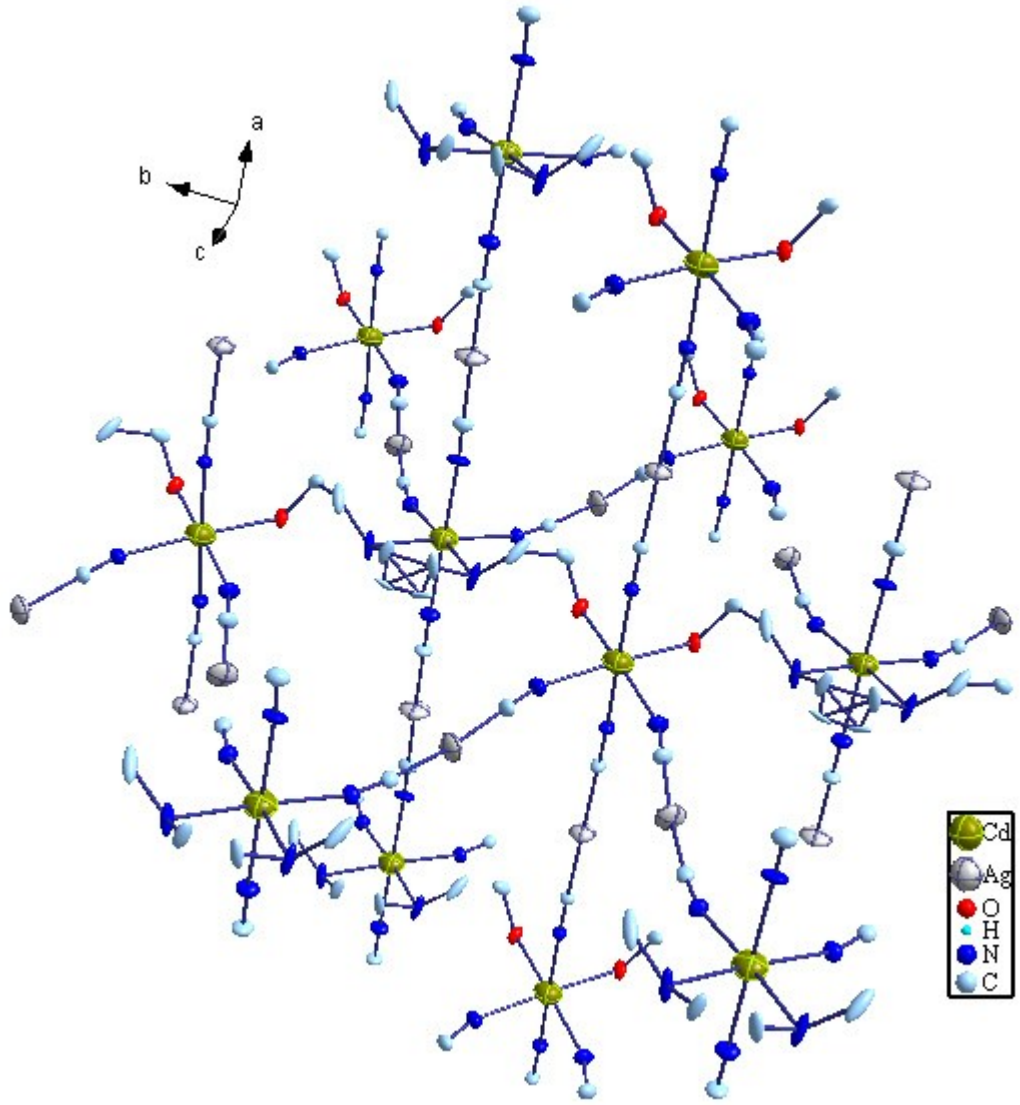

Figure S1. The *3D* structure of ***C3***. The structure drawing omitted the lattice waters and H atoms

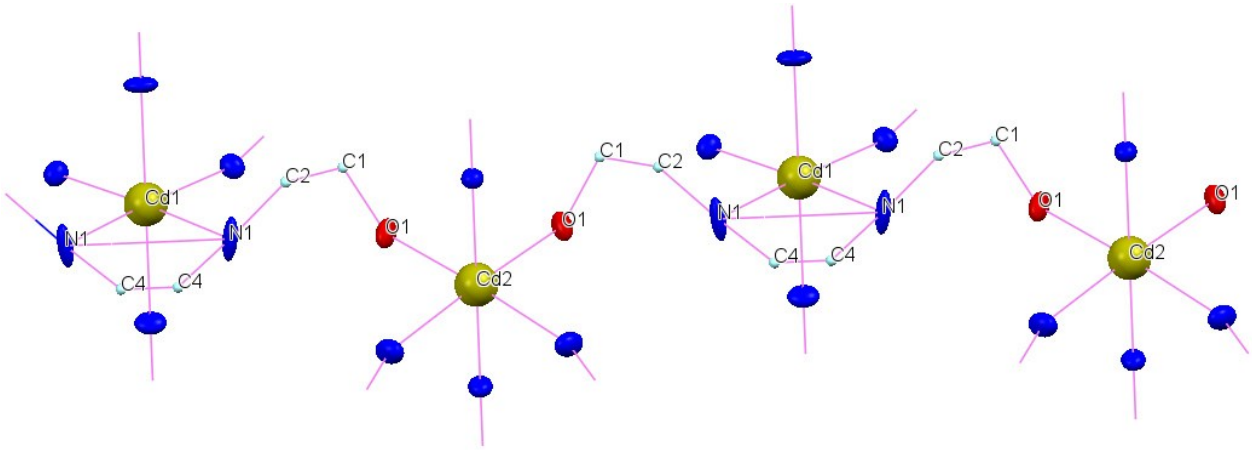

Figure S2. Polymeric chain structure formed by N-N′-bis(2-hydroxyethyl)ethylenediamine between Cd1 and Cd2. All other atoms were omitted except for cadmium and donor atoms

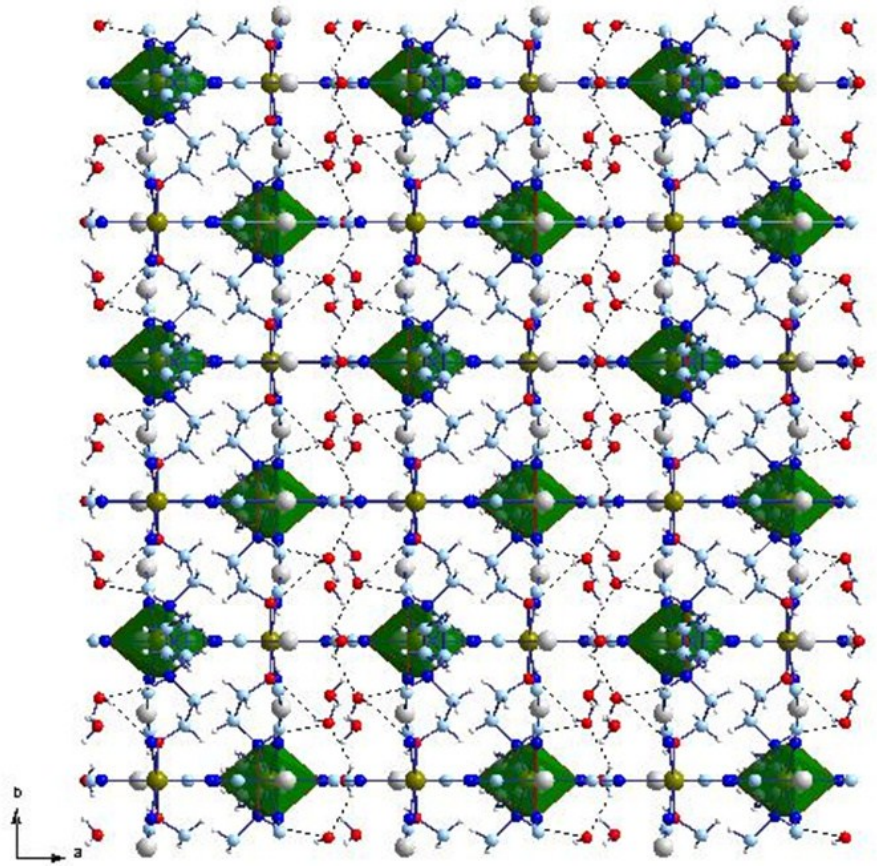

Figure S3. The two-dimensional structure formed in a-b plane of ***C3***

Table S1. Thermoanalytical data for ***C1***, ***C2*** and ***C3***

Complex	Stage	Temperature Range (°C)	DTG_{max} (°C)	ΔH (J/g)	Mass loss, Δm (%)		Total mass loss, Δm (%)		Removed group
					Found.	Calc.	Found.	Calc.	
$C_{16}H_{34}N_8O_5Ag_2Ni$ (***C1***) MA: 692.92	1	35-129	76	-30.77	7.08		7.08		
	2	129-258	241	527.10	11.84	45.37	18.92	45.37	H_2O + *2bishydeten*
	3	258-650	297	-60.89	25.79		44.51		
	4	650-964	784	-181.56	14.95	15.42	59.66	60.79	4CN
$C_{16}H_{32}N_8O_4Ag_2Cu$ (***C2***) MA: 679.76	1	103-169	147	107.61	6.45		6.45		
	2	169-284	262	86.64	17.64	47.43	24.09	47.43	*2bishydeten*+ *CN*
	3	284-459	317	-8.21	24.11		48.20		
	4	459-795	518	-3352.58	5.69		53.89		
	5	795-928	893	246.10	2.04	11.48	55.93	58.91	3CN
	6	928-1140	1086	50.85	2.29		58.22		
$C_{14}H_{25}Ag_4Cd_2N_{10}O_7$ (***C3***) MA: 1101.72	1	35-144	62	-29.71	2.76	3.37	2.76	3.37	
	2	144-315	254	-576.70	11.04	20.46	13.80	23.83	$3H_2O$ + *bishydeten 2CN*
	3	315-590	406	-574.47	9.42		23.22		
	4	590-910	735	970.02	15.41	14.64	38.63	38.47	6CN
	5	910-1177	989	-1475.93	17.18	16.86	55.81	55.33	1.6Cd

Scheme S1. Estimated thermal decomposition schemes for ***C1-C3***

$$[Ni(bishydeten)_2][Ag(CN)_2]_2.H_2O \xrightarrow[-H_2O \; -2bishydeten]{35\text{-}650^oC} [Ni[Ag(CN)_2]_2] \xrightarrow[-4\ CN]{650\text{-}964^oC} Ni + 2Ag$$

$$[Cu(bishydeten)_2Ag_2(CN)_4] \xrightarrow[-2bishydeten \; -CN]{103\text{-}459\ ^oC} [CuAg_2(CN)_3] \xrightarrow[-3CN]{459\text{-}1140\ ^oC} Cu + 2Ag$$

$$[Cd(bishydeten)_{0.5}]_2[Ag(CN)_2]_4.3H_2O \xrightarrow[-3\ H_2O \; -bishydeten \; -2CN]{35\text{-}590^oC} [Cd_2 + Ag_4(CN)_6] \xrightarrow[-6CN]{590\text{-}910^oC} 2Cd + 4Ag$$

$$\xrightarrow[-1,6Cd]{910\text{-}1177^oC} 0,4Cd+4Ag$$

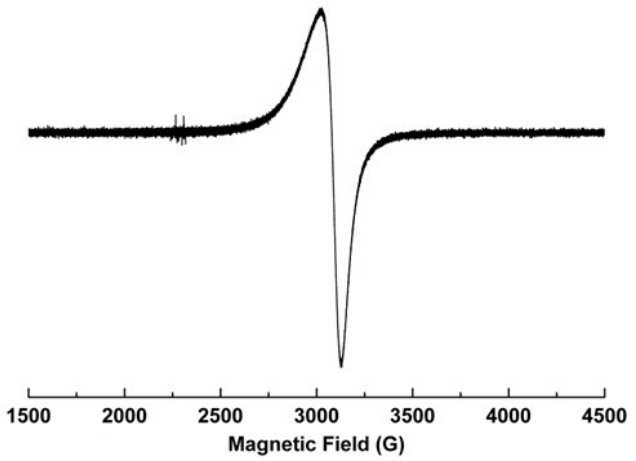

Figure S4. The powder EPR spectrum of ***C2*** at room temperature

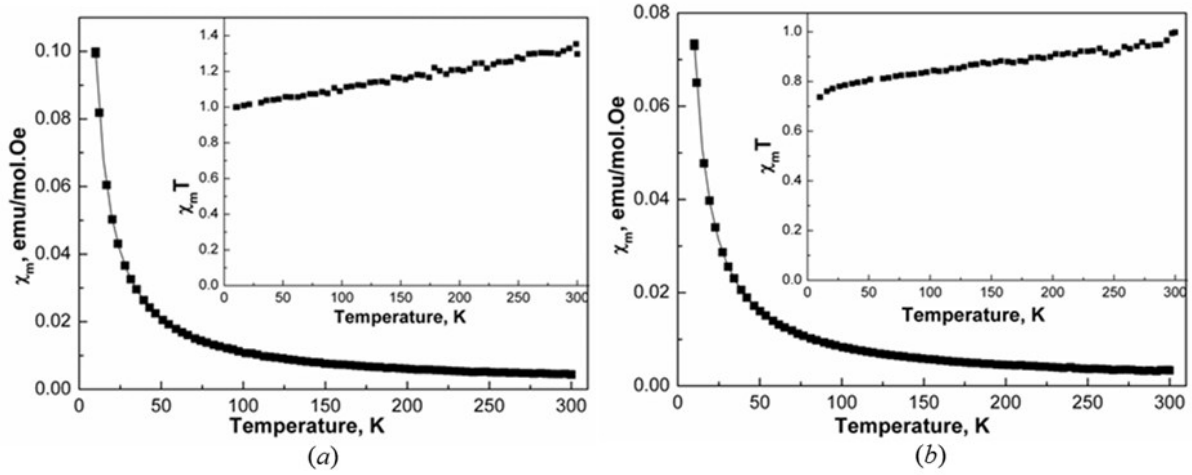

Figure S5. The temperature dependence of the molar magnetic susceptibility χ_m for ***C1*** (a) and ***C2*** (b). The solid line represents a fit by the Curie–Weiss law. Inset: The temperature dependence of $\chi_m T$

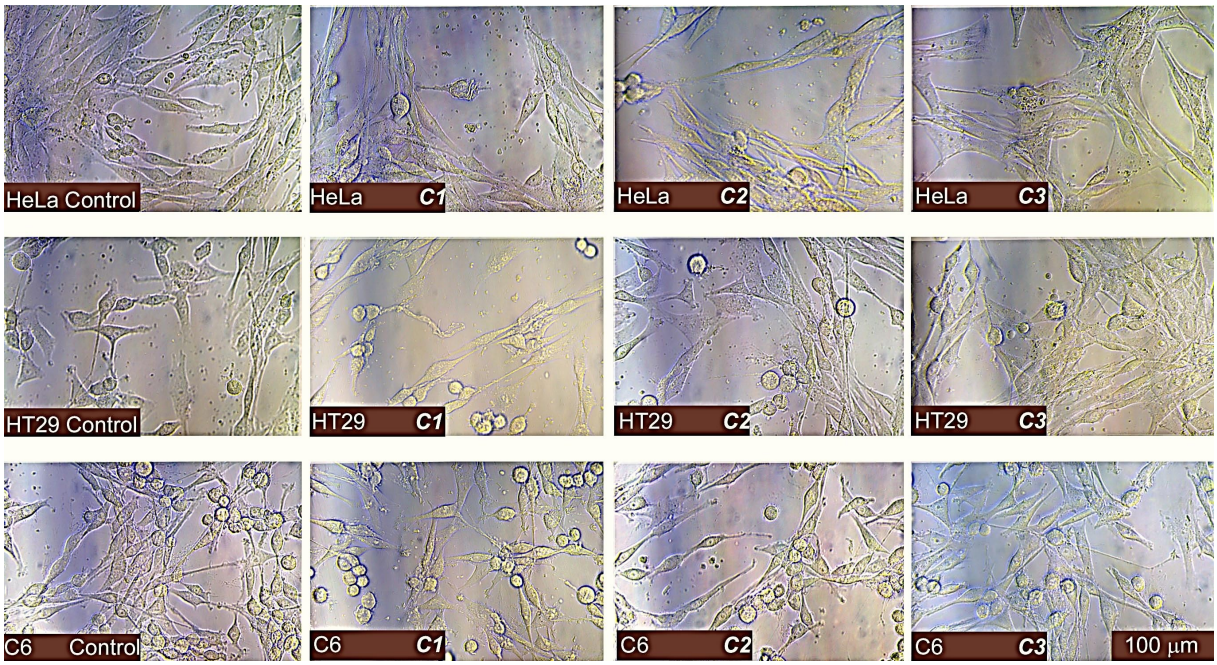

Figure S6. The effect of ***C1***, ***C2*** and ***C3*** on the morphology of HeLa, HT29 and C6 cells. Exponentially growing cells were incubated with IC_{50} concentrations of ***C1***, ***C2*** and ***C3*** at 37 °C for overnight and visualized by digital camera attached inverted microscope (Leica IL10, Germany). DMSO treated cells as controls. All scales are 100 µm

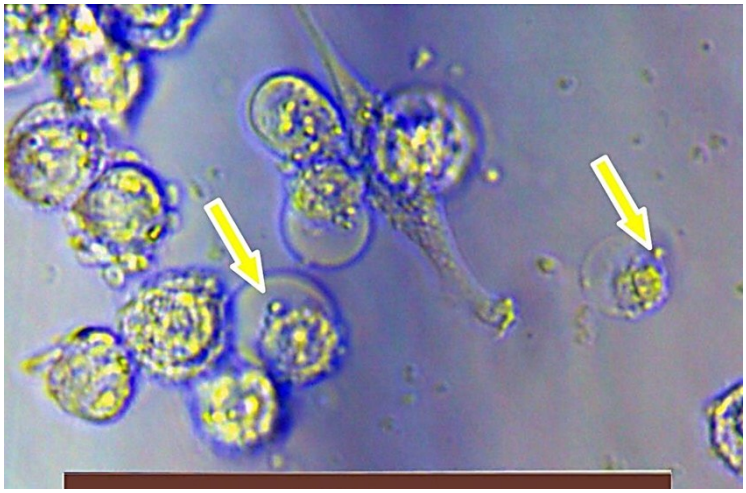

Figure S7. The effect of ***C1*** on the nuclear morphology of C6 cells. Exponentially growing cells were incubated with IC_{50} concentrations of ***C1*** at 37 °C for overnight and visualized by digital camera attached inverted microscope (Leica IL10, Germany). The scale is 100 µm

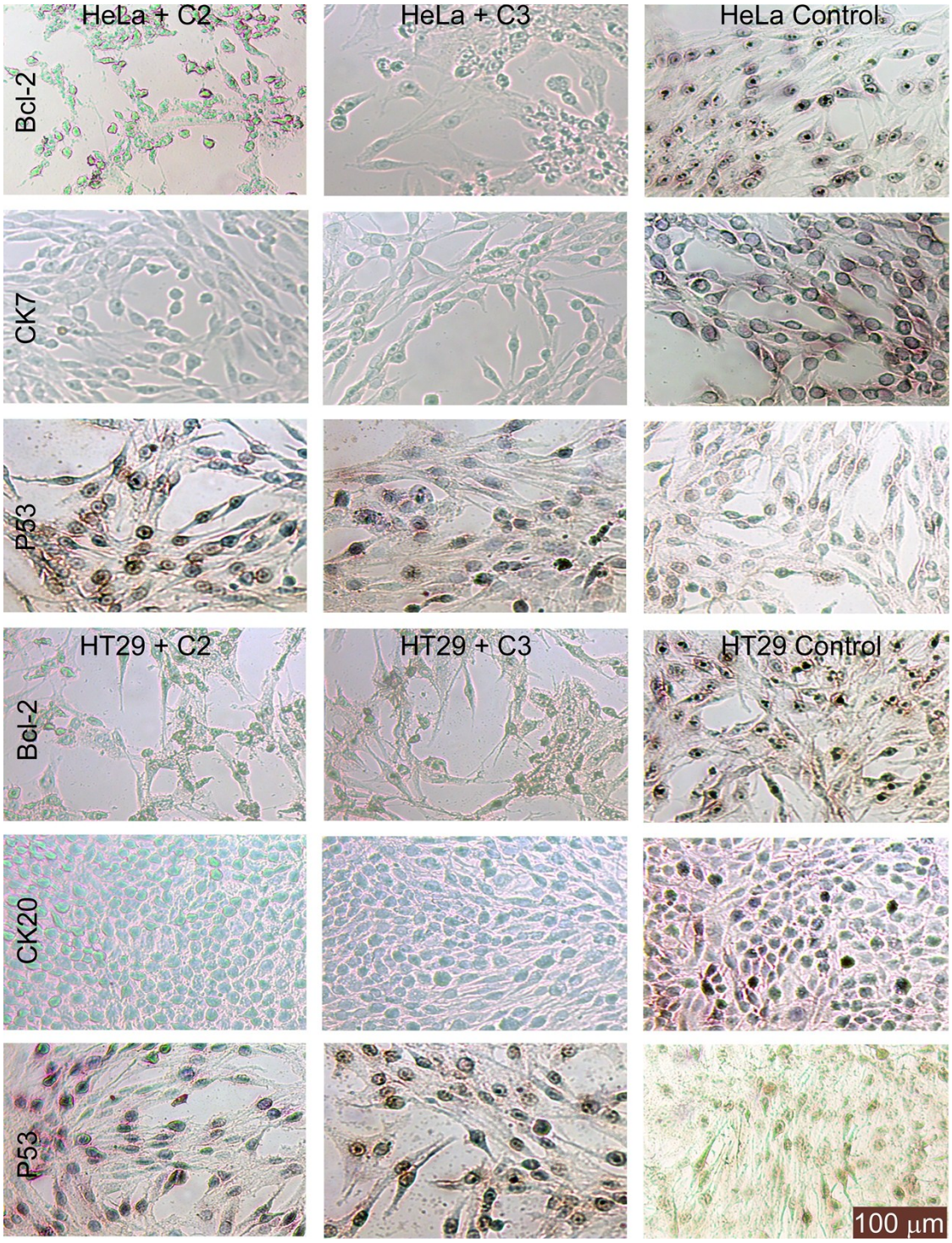

Figure S8. Representative images of the cells treated ***C2*** and ***C3*** examined by immunohistochemical staining for functional protein group (Bcl-2 and P53), and for marker protein group (CK7 and CK20). The specific signals are shown as brown staining. Bar is 100 μm.

Biological Studies

Cell Culture

C6 (Rat brain glioma, ATCC® CCL-107™), HT29 (Human colorectal adenocarcinoma, ATCC® HTB-38™), HeLa (Human cervix adenocarcinoma, ATCC® CCL-2™), and Vero (African green monkey kidney normal epithelial, ATCC® CCL-81™). cell lines were maintained in Dulbecco's modified eagle's medium (DMEM, Sigma), supplemented with 10 % (v/v) fetal bovine serum (Sigma, Germany) and PenStrep solution (Sigma, Germany). At confluence, cells were detached from the flasks using 4 mL of trypsin–EDTA (Sigma, Germany), centrifuged and cell pellet were resuspended with 4mL supplemented DMEM.

BrdU Cell Proliferation Assay (BCPA)

A Cell suspension containing $5x10^3$ cells in 100 µL was pipeted into wells of 96-well cell culture plates (COSTAR, Corning, USA). Test compounds (***C1***, ***C2***, ***C3*** and $[Ag(CN)_2]^-$) and control compound (5 fluorouracil, 5FU) was dissolved in sterile DMSO. DMSO amount was adjusted to 0.5 %. The cells were treated with ***C1***, ***C2***, ***C3***, $[Ag(CN)_2]^-$ and 5FU at final concentrations of 0.25, 0.50, 1.00, 1.50, 2.00, 2.50, 3.75, and 5.00 µg/mL. Cell controls and solvent controls were treated with supplemented DMEM and sterile DMSO respectively. The final volume of the wells was adjusted to 200 µL by supplemented DMEM. The cells then were incubated at 37 °C with 5 % CO_2 for overnight. The antiproliferative activity of the compounds was determined using BrdU Cell proliferation ELISA Kit (Roche, USA), a calorimetric immunoassay based on BrdU incorporation into the cellular DNA, according to manufacturer's protocol. Briefly, cells were exposed to BrdU labeling reagent for 4 h followed by fixation in FixDenat solution for 30 min at room temperature. Then, cells were cultured with 1:100 diluted anti-BrdU-POD for 1.30 h at room temperature, substrate solution was added to each well and BrdU incorporation was measured at 450-650 nm using a microplate reader (Rayto, China). Each experiment was repeated at least three times for each cell line.

Calculation of % inhibition and IC_{50}

IC_{50} represents the concentration of an agent that is required for 50 % inhibition in vitro. In BCPA assay results were reported as percent inhibition of test and control substances. The percent inhibition was calculated according to the formula: % inhabitations [1-(Absorbance of Treatments/ Absorbance of DMSO)

× 100]. The half maximal inhibitory concentration (IC_{50}) of the test and control compounds was calculated using XLfit5 software (IDBS) and expressed in µg/mL at 95 % confidence intervals.

Lactate Dehydrogenase (LDH) Cytotoxicity Assay

The cytotoxicity of ***C1***, ***C2***, ***C3***, $[Ag(CN)_2]^-$ and 5FU on the C6, HT29, HeLa, and Vero cells was determined through the calorimetric LDH Cytotoxicity Detection Kit (Roche, USA) based on the measurement of LDH activity released from the cytosol of damaged cells into the supernatant according to manufacturer's instructions. $5x10^3$ cells in 100 µL were seeded into 96-well microtiter plates as triplicates and treated with IC_{50} concentrations of ***C1***, ***C2***, ***C3*** and $[Ag(CN)_2]^-$ as described above at 37 °C with 5 % CO_2 for overnight. LDH activity was determined by measuring absorbance at 492-630 nm using a microplate reader. The percentage cytotoxicity was obtained by using the equation, experimental value - low control / high control - low control x 100, where experimental value is the test-compound treated cells, high control (maximum LDH release) is Triton X-100 treated cells, low control (spontaneous LDH release) is the untreated cells.

Apoptotic potential by DNA laddering Assay

DNA fragmentation effect of the test compounds was measured according to the method of Gong with some modifications.[65] Briefly, $7.5x10^5$ cells were seeded into 25 cm^2 culture flasks, and treated with IC_{50} concentrations of ***C1***, ***C2*** and ***C3*** at 37 °C with 5 % CO_2 for overnight. Treated cells were harvested using a sterile plastic scraper, transferred to a 15 mL sterile Falcon tube, washed with 1 mL sterile DPBS, and pelleted by spinning at 1500 xg for 5 min. The cell pellet resuspended with 200 µL ice cold DPBS by gently pipeting, fixed with 5 mL ice cold 70 % ethanol, vortexed shortly and incubated at -20 °C for 24 hours. The cells were centrifuged at 1500 xg for 5 min, the supernatant was removed and the remaining ethanol removed by air drying. The cell pellet was resuspended in 50 µL phosphate-citrate buffer (consisting of 192 parts of 0.2 M Na_2HPO_4 and 8 parts of 0.1 M citric acid, pH 7.8), incubated at 37 °C for 30 min in a shaker incubator, and centrifuged at 1500 xg for 5 min. A 40 µL of supernatant was transferred to a 1.5 mL microsantrifuge tube, mixed with 5 µL Tween20 solution (0.25 % in ddH_2O) and 5 µL RNase A solution and incubated at 37 °C for 30 min in a shaker incubator. Then, 5 µL proteinase K was added to each tube and incubated at 37 °C for 10 min. Finally, the entire content of the microcentrifuge tube was mixed with 4 µL of 6x loading buffer, loaded to 1.5% agarose gel containing 0.5 µg/mL ethidiumbromide and

electrophoresed at 200 mA for 40 min. DNA fragmentation in the gels was visualized using gel documentation system (UVP, England).

Apoptotic potential by TUNEL assay

In vitro detection of apoptosis was assessed using a TUNEL assay kit (Roche, Germany) according to the manufacturer's protocol. HT29 cell lines (30.000 cells/well) were placed in poly-L-lysine covered chamber slide. The cells were treated with IC_{50} concentration of ***C1***, ***C2*** and ***C3*** left for 24 hours incubation. There were two controls for this assay, one was positive control that had DNase-1 treatment and the other was negative control that had no terminal deoxynucleotidyltransferase (TdT). When the incubation time was over, the chamber was removed from slide and washed with DPBS to get rid of the medium and unattached cells. All the incubation and washing steps were done in a plastic jar. Slides were gently washed with DPBS and for fixation 4 % paraformaldehyde in DPBS, pH 7.4, were prepared freshly and added to slides for 60 min at room temperature. Following incubation, the slides were washed twice with DPBS. The cells were blocked with freshly prepared 3 % H_2O_2 in methanol for 10 min at room temperature. Following incubation, the slides were washed twice with DPBS. The cells were permeabilized by prechilled 0.1 % Triton X-100 and 0.1 % sodium citrate in water, freshly prepared, and incubated for 2 minutes on ice. All the slides were washed with DPBS twice for 5 minutes each. At this point in order to prepare a DNase I enzyme treated positive control, 100 µl of DNase-1 Buffer was added to slide and incubated at room temperature for 10 minutes. Fixative cells were transferred into TUNEL reaction mixture (50 µL/section) containing a TdT and fluorescein-dUTP. Intracellular DNA fragments were then labeled by exposing the cells to TUNEL reaction mixture for 1 h at 37 °C, in a humidified atmosphere and protected from light. After washing with DPBS twice and cells positive for apoptosis showed a green fluorescent signal and were visualized by a Leica fluorescent microscope (Leica DMIL LED fluo, Germany).

Cell migration assay

The migration inhibitory capability of the compounds was measured using the cell migration assay. Briefly, a culture insert (ibidi GmbH, Germany) consists of two reservoirs separated by a 500 µm thick wall, was placed on a 35-mm petri dish, and an equal number of HeLa cells (3.5×10^4 HeLa cells in 70 µL DMEM medium) were seeded into the two reservoirs of the same insert and allowed to grow to 90–95 % confluence, in order to generate a 500 µm gap between two cell populations. Subsequent to cell growth, the insert was gently removed and 2 mL of cell culture medium was

added and treated with IC_{50} concentrations of ***C1***, ***C2*** and ***C3*** shortly after an incubated overnight at 37 °C with 5 % CO_2. The closure of the gap by the cells was photographed 0, 1 and 2 days after incubation by using a phase contrast inverted microscope (Leica DMIL, Germany).

DNA topoisomerase I inhibition assay

DNA topoisomerase I inhibitory activities of ***C1***, ***C2*** and ***C3*** were evaluated by using a cell-free topoisomerase I assay kit (TopoGen, USA). The principle of the assay is to measure the conversion of supercoiled pHOT1 plasmid DNA to its relaxed form in the presence of DNA topoisomerase I alone and with test compounds. The supercoiled substrate (pHOT1 plasmid DNA) and its relaxed product can easily be distinguished in agarose gel because the relaxed isomers migrate more slowly than the supercoiled isomer. In brief, 20 µL of reaction mixture containing 1 µL plasmid pHOT1 DNA in relaxation buffer was incubated with 2 U recombinant human topoisomerase I in the presence of IC_{50} concentrations of ***C1***, ***C2***, ***C3*** or camptothecin as positive control. The reactions were carried out at 37 °C for 30 min and then terminated by the addition of stop solution. After the termination, the sample was analyzed using a 1 % agarose gel at 4 V/cm for 60 min. After electrophoresis, DNA bands were stained with ethidium bromide (1 mg/ml) solution and photographed through a gel imaging system (UVP BioSpectrum, Germany).

Immunohistochemistry

Immunohistochemistry (IHC) techniques used for to localize antigens changing expression level following ***C1***, ***C2***, and ***C3*** treatment. Accordingly, HT29 and HeLa cell lines (15.000 cells/well) were placed in a poly-L-lysine covered chamber slide. The cells were treated with IC_{50} concentration of test compounds and left for 24 h of incubation. There was a negative control that had no test compounds. When the incubation time was over, the chamber was removed from the slide and washed with DPBS to remove the medium and unattached cells. All of the incubation and washing steps were done in a plastic jar. The slides were gently washed with DPBS, and for fixation 4% paraformaldehyde in DPBS at pH 7.4 was freshly prepared and added to the slides for 60 min at room temperature. Following incubation, the slides were washed twice with DPBS. Heat-induced epitope retrieval (HIER) was performed using Cell Conditioning 1 (CC1), and visualization was achieved with the Universal DAB Detection Kit, according to manufacturer's instructions. IHC was performed using Bcl-2 (mouse monoclonal, clone 124; Ventana), CK7 (mouse monoclonal, clone OV-TL 12/30; Ventana), CK20 (mouse monoclonal, clone Ks20.8; Ventana), and P53 (mouse monoclonal, clone D07; Ventana) on the VENTANA Bench-Mark

XT System. Briefly, sections were newly pretreated with CC1 Ventana reagent for 30 min at 95 °C. After pretreatment, sections were incubated with above mentioned primary antibody for 32 min at 37 °C. Reactions were revealed with an ultraView Universal DAB Detection Kit (Ventana, USA). The slides were counterstained with Hematoxylin II (Ventana) for 4 min and Bluing Reagent (Ventana) for 4 min and coverslips were applied by an automated coverslipper (Leica CV5030). For the HeLa and the HT29 cell lines, the number of positive and negative cells was counted in five zones. This procedure was repeated 3x for each protein stained slide. The slides were scored staining intensity score rated as follows: no staining (0, no stained cells or <5% positive cells), weak staining (1+, 5–24% positive cells), moderate staining (2+, 25-49% positive cells), and strong staining (3+, >50% positive cells). A score of 2+ or 3+ was considered positive for relevant expression while a score of 0 or 1+ was considered negative.

Preparation of microorganisms and Disc diffusion assay

A total of 10 microbial cultures belonging to ten bacterial species was used in this study. The cultures were grown in Mueller–Hinton Broth (Merck) for all the bacterial strains for 24 h of incubation at 36 °C. Antimicrobial tests were carried out by disc-diffusion method[66] using 100 µL of suspension containing 10^8 CFU/mL of bacteria and 10^6 CFU/mL of yeast spread on Nutrient Agar (NA) and Potato Dextrose Agar (PDA) medium, respectively. The blank discs (Oxoid = 6 mm in diameter) were impregnated with 20 µL of the each substance (105 µg/disc) and placed on the inoculated agar. Negative control (KCN) was prepared using the same solvent (water) employed to dissolve each substance. Sulbactam (30 µg) + Cefoperazona (75 µg) (105 µg/disc) was used as positive reference standard to determine the sensitivity of a strain of each microbial species tested. The inoculated plates were incubated at 36 °C for 24 h for clinical bacterial strains. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms.

Microdilution assay

The minimal inhibitory concentration (MIC) values were also studied for the microorganisms, which were determined to be sensitive to the substances tested in the disc-diffusion assay. İnocula of microorganisms were prepared using 12 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. Each substance dissolved in 10% dimethyl sulfoxide (DMSO) was first diluted to the highest concentration (1000 µg/mL) to be tested. Then, serial twofold dilutions were made in a concentration range from 3.9–1000 µg/mL in 10-mL sterile test tubes containing nutrient broth. A prepared suspension of the standard microorganisms was added to each dilution in a 1:1

ratio. Growth (or its lack) of microorganisms was determined visually after incubation for 24 h at 36 °C. The lowest concentration at which there was no visible growth (turbidity) was taken as the MIC. This process was also repeated for the antibiotic. MIC values of the compounds against bacterial strains were determined on the basis of a micro-well dilution method.[67]

Test fungi

Four plant pathogenic fungi, namely *Alternaria solani*, *Rhizoctonia solani*, *Fusarium oxysporum* and *Sclerotinia sclerotiorum* were obtained from a culture collection of Department of Plant Protection, Agricultural Faculty, Gaziosmanpasa University. They were maintained on potato dextrose agar (PDA) medium and then stored at 4 °C. All the fungi were activated and then subcultured for 5-7 days in darkness at 25 ± 2 °C by transferring from the stock cultures to PDA medium in petri dishes before use.

Agar well diffusion bioassay

The antimicrobial test was performed according to the method of Boyanova et al., with some modification.[68] Briefly, a 10 mL of PDA medium was poured into a 6-cm diameter sterile petri dish before solidification. Discs (5-mm diameter) of the mycelial plugs from the edge of the cultured fungal colony were cut and placed mycelial surface down on the one side of the dishes and the wells (5 mm holes) were produced in the agar with sterile cork borer at 30 mm apart from mycelial discs of the fungus on opposite sides of Petri dishes. The chemical ***C1***, ***C2*** and ***C3*** were diluted with 50 % dimethyl sulfoxide (DMSO) solution to the final concentrations of 2.5, 5, 7.5, 10, 15, and 20 µg/mL. Of these, 20 µL of the diluted samples were pipetted into the well. All plates were then incubated in the dark at 25 ± 2 °C for 7 days. Reading the results was carried out by measuring the diameters of the zones of inhibition and clear growth (in mm). Three replicates were used for each treatment. 20 of the distilled water containing 50 % DMSO was also loaded in a control plate as a negative control along with Maneb (2.4 mg/mL) and copper hydroxide (1.25 mg/mL) at the manufacturer recommended rate in distilled water, as a positive control. The diameter of the inhibition zone was measured when the hyphae of the control extended to well in where 20 µl 50 % DMSO solution was loaded in a control plate. The rates of mycelial growth inhibition (GI %) were calculated by the following formula: GI % = dc-dt/dc*100 Where does is mean colony diameter of control sets and dt is the mean colony diameter of treatment sets.[69] Each test was performed in triplicate. Inhibition zone data were analyzed using POLO-PC Probit to estimate lethal concentration 50, and 90 values (LC50, and 90) and the regression line slope.[70]

DNA/BSA binding and gel electrophoresis studies

To find the interaction of the compounds with CT-DNA and to calculate the binding constants (Kb) were used UV spectroscopy. A CT-DNA solution was prepared by dissolving 2.5 mg CT-DNA in 10.0 mL Tris–HCl buffer (20 mM Tris–HCl, 20 mM NaCl at pH 7.0) and stored in the refrigerator. The concentration of CT-DNA was determined spectrophotometrically using the known Ɛ value of 6600 M^{-1} cm^{-1} at 260 nm. After dissolving the CT-DNA fibers in Tris–HCl buffer, the purity of this solution was checked from the absorbance ratio A_{260}/A_{280}. The CT-DNA solution in the buffer displayed an A_{260}/A_{280} ratio of 1.89, indicating that the DNA was sufficiently pure. These compounds were dissolved in DMSO and diluted with Tris–HCl buffer to obtain 25 μM concentrations. Test compounds in the solutions were incubated at 25 °C for about 30 min before measurements. The UV-visible spectral studies were performed in mixed solvent system (1/9 DMSO/Tris–HCl buffer) using eight points that the fine structure is observed for these compounds in this system by UV-visible absorption. The UV absorption titrations were conducted by keeping the concentration of these compounds fixed while varying the CT-DNA concentrations (6.5-800 μM). Absorption spectra were recorded by using 1-cm-path quartz cuvettes at room temperature.

To evaluate the interaction of the compounds with BSA was used UV spectroscopy. A BSA solution was prepared by dissolving 2.5 mg BSA in 10.0 mL in Tris–HCl buffer (5 mM Tris–HCl, 10 mM NaCl at pH 7.4) and stored in the refrigerator. The UV spectra of the BSA solutions (6.5-800 μM) in the presence of a fixed concentration of the complexes (25 μM) were scanned against the Tris–HCl buffer in the wavelength range from 250 to 320 nm.

Ethidium bromide (EB) displacement experiments were performed by tracking alters in the fluorescence intensities of the EB-DNA solutions in the presence of increasing amounts of the test compound. The fluorescence spectra of EB were measured using an excitation wavelength of 295 nm and the emission range was set between 200 and 600 nm. The spectra were analyzed according to the Stern–Volmer equation, $I_0/I = 1 + K_{SV}$ [Q], where I_0 is the fluorescence intensity in the absence of quencher, I is the fluorescence intensity in the presence of quencher, K_{SV} is the Stern–Volmer quenching constant, [Q] is the quencher concentration. K_{SV} can be calculated from the slope of the plot of I_0/I vs. *[DNA]*.

The restriction enzyme inhibition assay was conducted to evaluate both specific or nonspecific binding and enzyme inhibition by complexes ***C1-C3***. Supercoiled

pTOLT (10 μM) plasmid DNA was incubated with the complexes ***C1-C3*** (25 μM) and restriction enzymes *Kpn*I and *Bam*HI (2 units) at 37 °C in 50 mM Tris–HCl/18 mM NaCl buffer (pH 7.2) for 4 h. The digestion products were resolved by using 1.5% (wt/vol) agarose gels with ethidium bromide.