

Novel Copper(II) Complex of *N*-Propyl-norfloxacin and 1,10-Phenanthroline with Enhanced Antileukemic and DNA Nuclease Activities

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We have synthesized and characterized a novel copper(II) complex of the fluoroquinolone antibacterial drug *N*-propyl-norfloxacin (Hpr-norf) in the presence of 1,10-phenanthroline (Phen) and studied its biological properties as antitumor antibiotic and antimicrobial agent. Human acute myeloid leukemia cell line HL-60, MTT assay, and Trypan blue assay were used to test the antileukemic, the cell viability, and the structural integrity of the cell membrane and cell proliferation properties of (chloro)(Phen)(*N*-propyl-norfloxacinato) copper(II) (complex **1**), respectively. We found that the proliferation rate and viability of HL-60 cells decreased after treatment with complex **1**, leading to cell death through apoptosis in a time-dependent manner. The antimicrobial activity of complex **1** has been tested, revealing an increased potency in comparison to the free Hpr-norf. Complex **1** proved to be capable of acting as an independent nuclease by inducing nicking of supercoiled pUC19 plasmid. Our results suggest that **1** may provide a valuable tool in cancer chemotherapy.

Introduction

Norfloxacin (Figure 1A)^{1,2} is an oral, broad-spectrum fluoroquinolone antibacterial agent used for the treatment of urinary tract infections.^{3–6} This agent was shown to inhibit specifically the bacterial DNA gyrase,^{7–9} an enzyme which is responsible for negatively supercoiling covalently closed, circular DNA, as well as for catenation and decatenation reactions.¹⁰ The antibacterial activity of fluoroquinolones is highly dependent on their bicyclic heteroaromatic pharmacophore, although the importance of the nature of the peripheral substituents and their spatial relationship has also been illustrated.^{11,12} Specifically, they provide additional affinity for the bacterial enzymes, they enhance cell penetration, and/or they alter the relative pharmacokinetics.^{9,11,12} Hpr-norf (Figure 1B) is the *N*-propyl form of norfloxacin and it can act as a bidentate ligand through the pyridone and one of the carboxylate oxygens.^{13,14}

Studies on the biological properties of quinolones include their interaction with DNA,^{15,16} their antibacterial activity on diverse microorganisms,^{17–19} their cytotoxicity,^{18–20} and their potential antitumor activity.^{21,22} Additionally, quinolone–metal complexes have been studied mainly for their antibacterial activity against diverse microorganisms^{23–28} and, in some cases, for their interaction with DNA,^{29,30} exemplifying the important role of the metal ions for the mechanism of actions of these drugs. Specifically, the *in vitro* cytotoxicity of the complex (chloro)(2,2′-bipyridine)(*N*-propyl-norfloxacinato)copper(II), Cu(pr-norf)(bipy)Cl, against two leukemia cell lines, HL-60 (human acute myeloid leukemia) and K562 (human chronic myeloid leukemia in blast crisis), revealed an increased antiproliferative and necrotic effect, in comparison to the free ligand Hpr-norf.³¹

Copper complexes of Phen (Figure 1C) and its derivatives are of great interest because they exhibit numerous biological activities such as antitumor,³² antibacterial,²⁴ and antimicro-

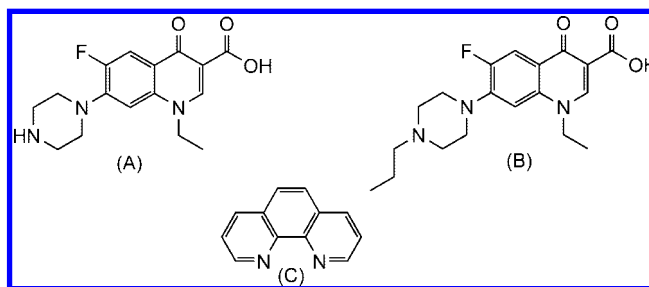


Figure 1. (A) Norfloxacin; (B) *N*-propyl-norfloxacin (Hpr-norf = 1-ethyl-6-fluoro-7-(4-propyl-piperazin-1-yl)-4-oxo-1,4-dihydro-quinoline-3-carboxylic acid); (C) 1,10-phenanthroline (Phen).

bial.³³ Their intercalating properties³⁴ along with their function as artificial nucleases have also been reported.^{35–37} Specifically for bis(Phen)–copper(II) complex [Cu(Phen)₂Cl]Cl, (Cu(OP)₂), it has been shown that in the presence of thiols and hydrogen peroxide,³⁹ through noncovalent binding to the minor groove of DNA, it generates active species that lead to DNA strand scission.^{40,41} Additionally, Cu(OP)₂ acts as an intercalator³⁸ as well as an artificial nuclease.³⁸

In this work, we have examined (a) the synthesis and the structural characterization of the novel Cu(II) complex of Hpr-norf and Phen, complex **1**, (b) the cytotoxic, antiproliferative, and apoptotic properties of complex **1** on human acute myeloid leukemia HL-60 cells, (c) its antimicrobial properties against three different microorganisms, and (d) its efficiency in performing DNA strand scission using calf thymus (CT^a) DNA and supercoiled pUC19 DNA. It is important to note that apoptosis, or programmed cell death (PCD), represents an important phenomenon in cancer chemotherapy, because the

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^a Abbreviations: MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; FBS, fetal bovine serum; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; DMF, dimethylformamide; MIC, minimum inhibitory concentration; LB, luria broth medium; MMS, minimal medium salts broth; HEPES, *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]; SDS, sodium dodecyl sulfate; CT, calf thymus; sh, shoulder; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; TBE, Tris-borate-EDTA; TE, Tris-EDTA.

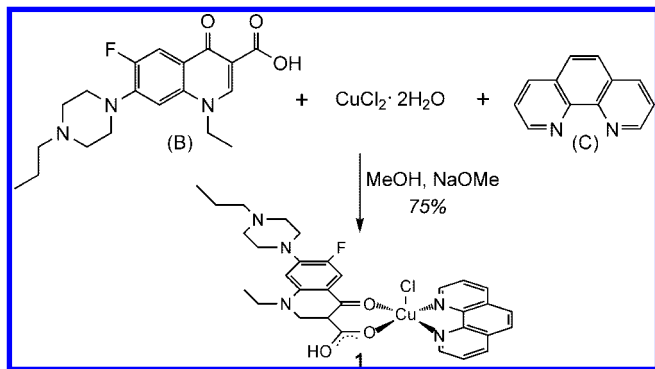


Figure 2. Synthesis of complex **1**.

main function of a potential anticancer agent is to exert its antitumor activity through the induction of apoptosis and is often correlated with the tumor response and the clinical outcome in cancer patients.^{42–44} The release of cytochrome *c* initiates a caspase cascade, which results in the activation of caspase-9 and caspase-3, thereby leading to PCD.⁴⁵ Normally, induction of apoptosis following chemotherapy is associated with the activation of pro-apoptotic genes of the BCL-2 family (BAX, BAK, BOK, BCL2L12) and the suppression of antiapoptotic genes (BCL2, BCLX_L).^{46,47} In our studies we have found that **1** has enhanced antiproliferative and cytotoxic activities when compared to the free Hpr-norf or Cu(OP)₂, as well as it exhibits the highest effect in the induction of apoptosis in HL-60 cells out of all the compounds tested, including Hpr-norf, Phen, and Cu(OP)₂, measured by flow cytometry.

Results

Preparation and Characterization of the Complex. Complex **1** has been synthesized in high yield (75%) via the reaction of equimolar quantities of copper(II) chloride dihydrate, deprotonated *N*-propyl-norfloroxacinato ligand, and Phen (Figure 2) and resulted as a blue crystalline solid, soluble in water, DMSO, and DMF, being a nonelectrolyte.

In the IR spectrum of complex **1**, the absorption of the $\nu(\text{C}=\text{O})_{\text{carb}}$ at 1731 cm^{-1} of the fluoroquinolone ligand has been replaced by two very strong characteristic bands at 1614 cm^{-1} and 1403 cm^{-1} , assigned as asymmetric ($\nu(\text{CO}_2)_{\text{asym}}$) and symmetric ($\nu(\text{CO}_2)_{\text{sym}}$) stretching $\nu(\text{O}-\text{C}-\text{O})$ vibrations, respectively. The difference, $\Delta = \nu(\text{CO}_2)_{\text{asym}} - \nu(\text{CO}_2)_{\text{sym}}$, a useful characteristic for determining the coordination mode of the carboxylate ligands, is 211 cm^{-1} , indicating a monodentate coordination mode of the carboxylate group.^{48,49} Finally, the pyridone stretch $\nu(\text{C}=\text{O})_{\text{p}}$ is shifted from 1618 to 1629 cm^{-1} upon bonding. The overall changes of the IR spectrum of the complex suggest that *N*-propyl-norfloroxacinato ligand (Figure 1B) is coordinated to the metal via the pyridone and one of the carboxylate oxygens.^{14,31,50}

The electronic spectra of the complex, recorded as nujol mulls and in aqueous solutions, appear to be very similar, suggesting that the complex retains its structure in solution. Complex **1** exhibits a d–d transition band at 650 nm ($\epsilon = 40\text{ M}^{-1}\text{cm}^{-1}$) and an additional weak broadband at 840 nm ($\epsilon = 10\text{ M}^{-1}\text{cm}^{-1}$), typical for distorted square pyramidal geometry.^{51,52} The complex also exhibits an absorption band at 410 nm ($\epsilon = 195\text{ M}^{-1}\text{cm}^{-1}$) that can be assigned to the ligand-to-metal charge-transfer transition for the fluoroquinolone ligand. This band is also present in the spectrum of other quinolone copper(II) complexes in aqueous solution.^{31,53–55} Finally, the UV spectrum of the complex is practically identical with that

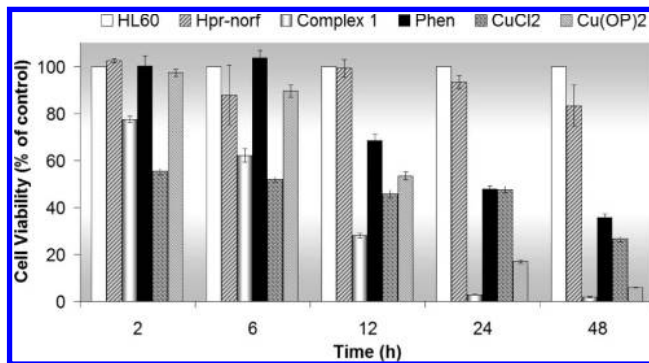


Figure 3. Treatment of HL-60 cells with complex **1** showed enhanced cytotoxic effect, in comparison to Hpr-norf, Phen, Cu(OP)₂, or copper chloride, in a time-dependent manner. HL-60 cells were treated in the absence (HL60) and in the presence of $100\text{ }\mu\text{g/mL}$ of complex **1**, Cu(OP)₂, Hpr-norf, Phen, and CuCl₂ for 2, 6, 12, 24, and 48 h, and cell viability was measured by the MTT assay. Each data point represents the mean for two separate experiments (mean \pm SD) performed in triplicate.

of the quinolone ligand, while differences due to Phen (Figure 1C) are not easily distinguished.

Complex **1** is expected to be isostructural to complex Cu(pr-norf)(bipy)Cl, the structure of which has already been reported.³¹ The copper atom is five-coordinate and could be described as having a distorted square pyramidal geometry. More specifically, for Cu(pr-norf)(bipy)Cl, the tetragonality T^5 ($= 0.775$) along with the trigonality index τ ($= 0.0878$) have shown slight distortion from the regular square-based pyramidal geometry.³¹ In complex **1**, the pyridone oxygen and one carboxylate oxygen of the *N*-propyl-norfloroxacinato ligand and the two nitrogen atoms of Phen are bound to copper ion and occupy the basal plane of the square pyramid, while the chlorine atom lies in the apical position (Figure 2).

An arrangement similar to that of the *N*-donor ligand has been observed in a series of neutral mononuclear complexes of the type Cu(L)₂(Phen)(H₂O) (L[−] = a monodentate phenoxy-alkanoato ligand)^{56–59} and in the quinolone complexes Cu(oxolinato)(Phen)Cl⁵⁵ and Cu(ciprofloxacinato)(Phen)Cl,²⁴ where the O_{water} and Cl, respectively, lie in the apical position.

Effect of **1 on HL-60 Cells: Cell Growth, Cell Viability, Necrosis, Induction of Apoptosis.** To determine whether complex **1** has increased antiproliferative, cytotoxic, or necrotic effects on HL-60 cells, in comparison to Cu(OP)₂, Hpr-norf, Phen, or CuCl₂, the cells were exposed to $100\text{ }\mu\text{g/mL}$ of **1**, Cu(OP)₂, Hpr-norf, Phen, and CuCl₂ individually for increasing periods of time (2, 6, 12, 24, and 48 h), and cell viability was measured by the MTT assay. As shown in Figure 3, leukemia HL-60 cells exhibited enhanced sensitivity to complex **1**, in a time-dependent manner, compared to Cu(OP)₂ and a minor sensitivity to the free antibacterial Hpr-norf, Phen, and CuCl₂, independently.

The antiproliferative as well as necrotic effects of **1** on the same cell line, compared to the growth arrest or necrosis induced by Hpr-norf, Phen, or CuCl₂ were measured as the concentration of trypan blue unstained cells (alive cells) and trypan blue +ve cells (necrotic cells). The cell growth curve presented in Figure 4 shows that complex **1** has increased antiproliferative effect on HL-60 cells, when compared to Hpr-norf, Phen or CuCl₂. The trypan blue staining revealed that **1** also induced some necrotic effect on the cells with time, whereas the necrosis induced by Hpr-norf, Phen, or CuCl₂ independently was insignificant (Figure 5). Despite the fact that necrosis was

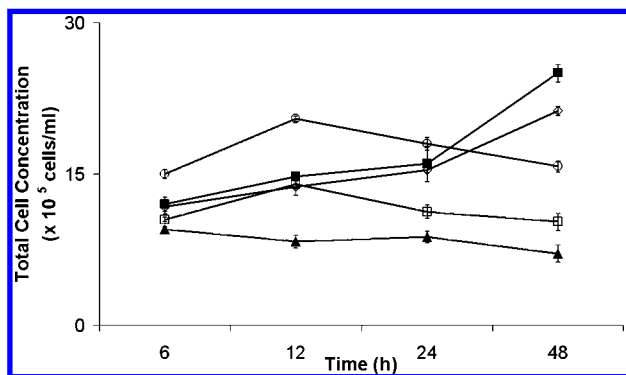


Figure 4. Treatment of HL-60 cells with complex **1** displayed enhanced antiproliferative effect, compared to Hpr-norf, Phen, or CuCl₂. The cells were treated in the absence (■) and in the presence (▲) of 100 μ g/mL of the complex, as well as the antibacterial Hpr-norf (◇), Phen (□), or CuCl₂ (○), as individual entities for 6, 12, 24, and 48 h. Total cell concentration was measured using a hemocytometer by observing under a microscope. Each data point represents the mean for three separate experiments (mean \pm SD).

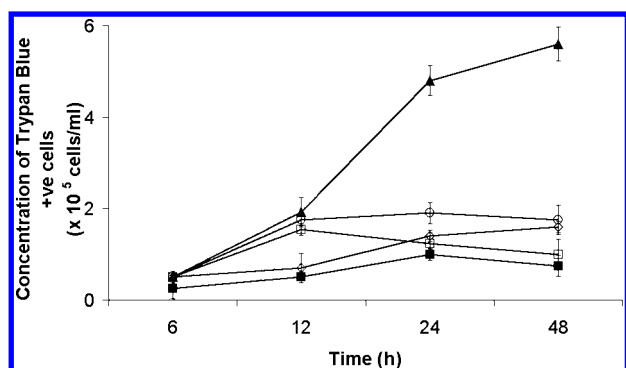


Figure 5. Treatment of HL-60 cells with complex **1** induced enhanced necrotic phenomena, compared to Hpr-norf, Phen, or CuCl₂. The cells were treated in the absence (■) and in the presence (▲) of 100 μ g/mL of the complex, as well as the antibacterial Hpr-norf (◇), Phen (□), or CuCl₂ (○), as individual entities for 6, 12, 24, and 48 h. The necrotic phenomena were observed using trypan blue staining. The results are expressed as the concentration of trypan blue +ve (stained) cells. Each data point represents the mean for three separate experiments (mean \pm SD).

observed with **1**, the overall percentage appears to be minimal, as it will be discussed later.

Apoptosis, as mentioned before, is a physiologically gene-directed programmed cell death mechanism that regulates cell homeostasis and plays a critical role in the proliferation and turnover in a variety of tumors and cancer cells. To investigate if complex **1** induces apoptosis in leukemia cells, we applied a gel-electrophoresis-based technique, where HL-60 cells were exposed to 100 μ g/mL of the complex in selected time periods (12, 24, and 48 h). The antibacterial Hpr-norf was also tested under the same conditions, as well as complex Cu(OP)₂. As shown in Figure 6, complex **1** and Cu(OP)₂ both induce DNA fragmentation (laddering) after 12 h of incubation, whereas Hpr-norf does not have any apoptotic effect.

Quantification of apoptosis for complex **1**, Cu(OP)₂, Hpr-norf, Phen, and CuCl₂ was performed by flow cytometry (FACS). Exponentially growing HL-60 cells were exposed to 100 μ g/mL of each compound for 12 h, and the percentage of cells in M1 phase was measured (Figure 7). The results demonstrate that complex **1** induces apoptosis of HL-60 cells in the highest percentage (57.97%) of all compounds tested,

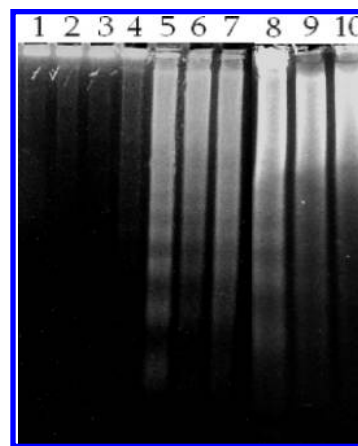


Figure 6. Treatment of HL-60 cells with complex **1** induced apoptosis in the cells (DNA fragmentation). The cells (10⁶ cells/sample) were treated in the absence (line 1) and in the presence (lines 5, 6, 7) of 100 μ g/mL of complex **1** for 12, 24, and 48 h, respectively. In addition, cells were treated with 100 μ g/mL of antibacterial Hpr-norf (lines 2, 3, 4) and 100 μ g/mL of complex Cu(OP)₂ (lines 8, 9, 10) for the same time periods. The characteristic endonucleosomal fragmentation of DNA can be clearly seen after 12 h incubation with complex **1** and Cu(OP)₂, whereas Hpr-norf did not have any apoptotic effect on HL-60 cells.

including complex Cu(OP)₂ (37.96%). Interestingly, Cu(OP)₂ exhibited the same apoptotic effect with CuCl₂.

Antibacterial Activity of the Complex. The antibacterial efficacies of complex **1** in comparison to the free ligands and other known antibacterial agents were tested against two Gram(−), *E. coli* and *P. aeruginosa*, and one Gram(+), *S. aureus*, microorganisms. The results are presented in Table 1.

Interaction of 1 with CT DNA. DNA can provide three distinctive binding sites for the quinolone complexes; namely, groove binding, binding to a phosphate group, and intercalation.⁶⁰ The absorption spectra of **1** individually, along with its complex with CT DNA after incubation for 24 h, has been recorded for a constant DNA concentration (3.125×10^{-4} M) in different **1**/CT DNA mixing ratios (*r*). The increase in the absorption intensity observed (Figure 8) indicates that the interaction takes place through the direct formation of a new complex between **1** and double-helical CT DNA.⁶¹

Additionally, the CD spectra of CT DNA in the presence of **1** for different *r* values are shown in Figure 9, and they provide us with useful qualitative information regarding the interaction between **1** and the nucleotide.

The CD spectra obtained consist of a strong positive band-I in the range 270–285 nm and an equally intense negative band-II in the range 240–245 nm. In all the CD spectra acquired, the intensity of both positive and negative bands is increased, in comparison to the free CT DNA bands, which is in agreement with previous observations⁶² of band-I indicating base stacking interactions through intercalation and band-II exemplifying stabilization of the right-handed B form of DNA. Both bands are extremely sensitive to the mode of DNA interaction with small molecules.⁶² The above data, along with our previous experience with other complexes of the type Cu(quinolonato)(N-donor)Cl,^{31,53,54} indicate that the observed phenomenon may be due to the intercalation of **1** through π – π stacking, which stabilizes the right-handed B form of DNA.

Effect of Complex 1 on Plasmid pUC19. The capacity of **1** to cleave DNA was examined by gel electrophoresis using

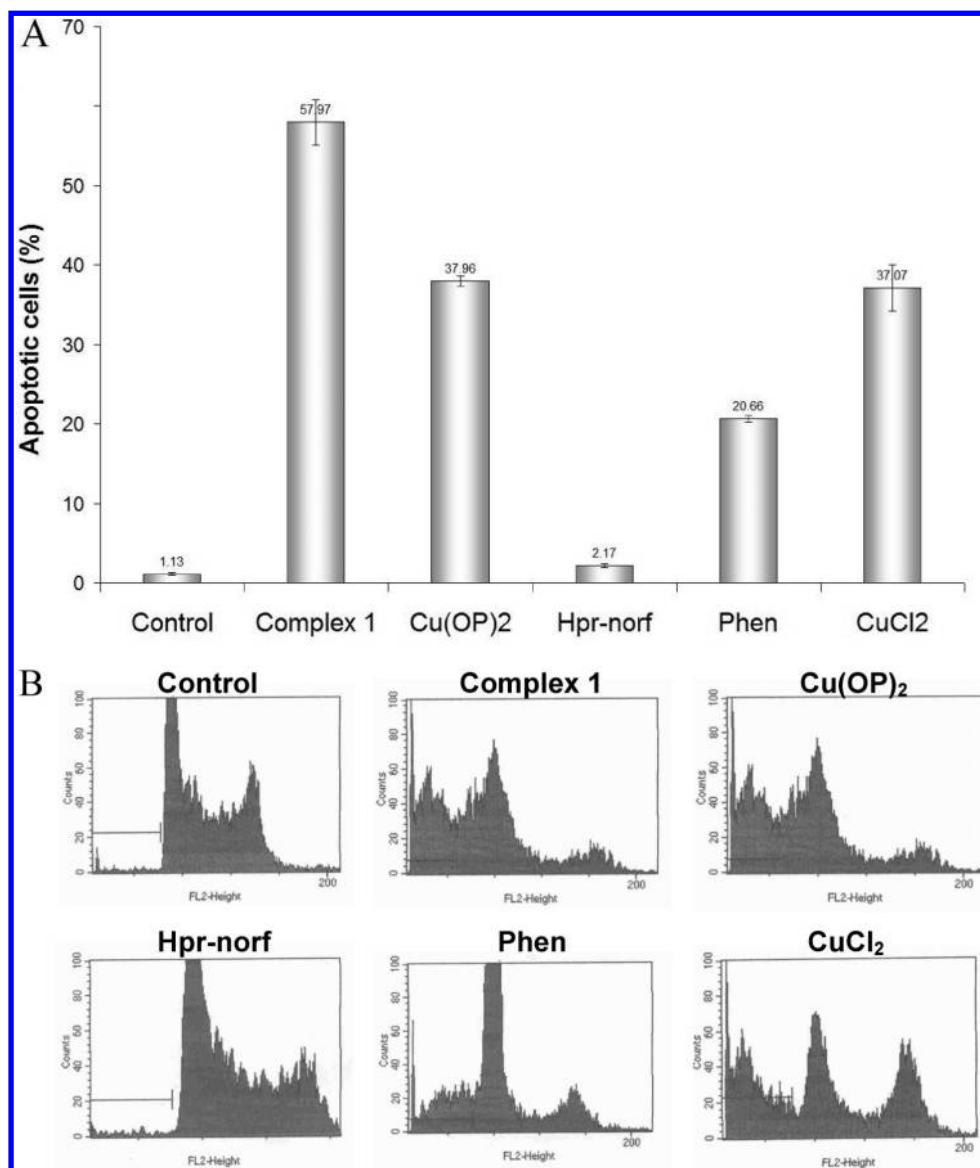


Figure 7. (A) Apoptotic rate of HL-60 cells induced by complex **1**, Cu(OP)₂, Hpr-norf, Phen, and CuCl₂. Cells were treated with 100 $\mu\text{g/mL}$ of each compound for 12 h, where DNA fragmentation is observed. Percentage of apoptotic cells was measured by flow cytometry. Results were obtained from two separate experiments. (B) Flow cytometry graphs for complex **1**, Cu(OP)₂, Hpr-norf, Phen, and CuCl₂.

Table 1. MIC in $\mu\text{g/mL}$

cmpd	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>
Hpr-norf	4.0	4.0	16.0
Phen	32.0	32.0	32.0
bipy ³¹	256.0	256.0	256.0
Cu(Hpr-norf)(Phen)Cl, 1	2.0	0.25	16.0
Cu(Hpr-norf)(bipy)Cl ³¹	0.25	8.0	8.0
oxolinic acid (Hoxo) ⁵⁵	1.0	16	16
Cu(oxo)(Phen)Cl ⁵⁵	64	64	32
enrofloxacin (Herx) ⁵³	1.0	1.0	8.0
Cu(oxo)(Phen)Cl ⁵³	2.0	1.0	4.0
Cu(oxo)(bipy)Cl ⁵⁵	64	32	64

plasmid pUC19. The concentration of pUC19 DNA in the reaction mixture was 0.5 $\mu\text{g/mL}$, while complex **1** samples were prepared in such a way as to have final metal/DNA base pair ratios (r') of 0.1, 0.3, and 0.5 (complex concentration was 5, 15, and 10 μM) in TE buffer. After the addition of plasmid pUC19 to the samples, they were incubated for 14 h at 37 $^{\circ}\text{C}$. Samples were loaded on an agarose gel and electrophorized under a constant voltage. Figure 10 presents that complex **1** causes nicking of supercoiled pUC19 at a concentration of 30

μM , indicating that, at this concentration, complex **1** behaves as a chemical nuclease, mediating the conversion of form I (supercoiled DNA) to form II (nicked circular DNA). Linearized DNA was not observed under these conditions, suggesting that cleavage may occur randomly. Similar DNAase activity of other copper–phen complexes has been previously reported.^{36,63–65}

Discussion

A novel copper(II) complex of antibacterial Hpr-norf and Phen has been prepared and characterized. Complex **1** was proven to be isostructural to Cu(pr-norf)(bipy)Cl,³¹ possessing a distorted square pyramidal geometry around the five-coordinate copper(II) atom (Figure 2).

In this study, we examined the biological properties of complex **1** in an attempt to investigate its in vitro antileukemic potential, as well as its activity as a nuclease. For that purpose, human acute myeloid leukemia cell line HL-60 was utilized, and two assays, one cell viability (MTT) and one inhibition of cell division, were performed. Many different concentrations of the compounds in the range of 10–300 $\mu\text{g/mL}$ were initially used in our studies, and 100 $\mu\text{g/mL}$ was identified as the ideal

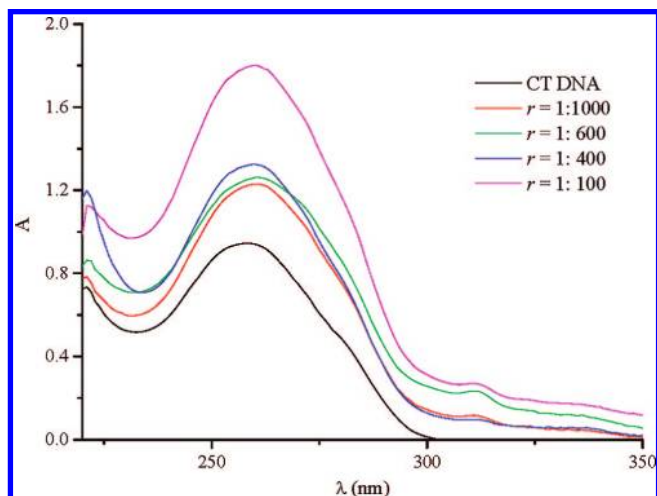


Figure 8. Absorption spectra of CT DNA in the presence of **1** in 5 mM buffer (containing 150 mM NaCl and 15 mM trisodium citrate at pH 7.0) in different r values ($r = [\text{I}]/[\text{CT DNA}]$). The spectra were recorded at 25 °C after samples had been incubated with CT DNA for 24 h at 37 °C.

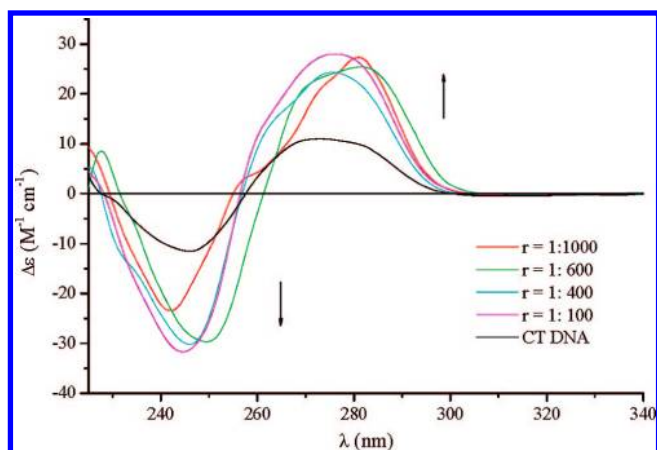


Figure 9. CD spectra of CT DNA in 5 mM buffer (containing 150 mM NaCl and 15 mM trisodium-citrate at pH 7.0) in the presence of **1** in different r values ($r = [\text{I}]/[\text{CT DNA}]$). The spectra were recorded at 25 °C after samples had been incubated with CT DNA for 24 h at 37 °C.

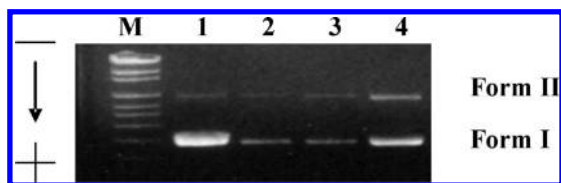


Figure 10. Electrophoresis of the plasmid DNA (1) pUC19 and with the addition of the complex **1** at ratios (2) $r' = 0.1$, (3) $r' = 0.3$, (4) $r' = 0.5$ ($r' = [\text{Cu}]/[\text{DNA}]$). The first lane (M) contains a pUC19 marker DNA from 19 329 bp–421 bp top to bottom.

one for performing the required experiments. Consequently, this concentration was utilized in all reported experiments. The results obtained from the MTT assay indicate that complex **1** dramatically decreased cell viability in a time-dependent manner when compared to Hpr-norf, Phen, $\text{Cu}(\text{OP})_2$, and CuCl_2 . Specifically, the antibacterial Hpr-norf did not have, as expected, a significant effect on the viability of the cells because it is devoid of antineoplastic activity. In addition, Phen, CuCl_2 , as well as the complex $\text{Cu}(\text{OP})_2$ exhibited a similar, but not as profound, effect. Interpretation of these results, along with

previously reported high in vitro cytotoxicity of $[\text{Cu}(\text{Phen})(\text{L-Thr})(\text{H}_2\text{O})](\text{ClO}_4)$,⁶⁵ and increased antiproliferative effect of $\text{Cu}(\text{pr-norf})(\text{bipy})\text{Cl}$ ³¹ leads to the conclusion that the enhanced effect of **1** on the viability of leukemia cells is due to the synergistic contribution of its components.

The loss of cell viability can be attributed to either necrosis or programmed cell death (apoptosis), although apoptosis is the preferred mode of action for a potent anticancer drug.^{66–75} We have shown that complex **1** induces DNA fragmentation on leukemia cells, characteristic of apoptosis, whereas the percentage of necrotic cells in the total cell population was insignificant in comparison.

Quantification of the apoptotic effect induced by **1**, $\text{Cu}(\text{OP})_2$, Hpr-norf, Phen, and CuCl_2 in HL-60 cells was performed using flow cytometry. It was found that complex **1** induces apoptosis in the highest percentage (57.97%) of all compounds tested. The percentage of apoptotic cells for $\text{Cu}(\text{OP})_2$ was 37.96%, for Hpr-norf was 2.17%, for Phen 20.66%, and for CuCl_2 was 37.07%. Specifically for complex $\text{Cu}(\text{OP})_2$, studies on its induction of apoptosis, as well as on its mechanism of action have been reported.^{76–79}

For metal complexes showing antibacterial activity, the following five principal factors^{57,58,80,81} should be considered: (i) The chelate effect, that is, ligands that are bound to metal ions in a bidentate fashion, such as the quinolones and Phen or bipy, show higher antimicrobial efficiency toward complexes with unidentate *O*- or *N*-donor ligands, for example, pyridine; (ii) the nature of the ligands; (iii) the total charge of the complex, generally the antimicrobial efficiency decreases in the order cationic > neutral > anionic complex; (iv) the nature of the ion neutralizing the ionic complex; and (v) the nuclearity of the metal center in the complex; dinuclear centers are usually more active than mononuclear ones.

Comparison between complex **1** and the free ligands, Hpr-norf and Phen (nature of the ligands effect) shows that the coordination of the *N*-propyl-norflaxacin (MIC = 4–16 $\mu\text{g}/\text{mL}$) with $\text{Cu}(\text{II})$ in the presence of Phen (chelate effect) results in an improvement of the biological activity against *E. coli* (MIC = 2 $\mu\text{g}/\text{mL}$) and *P. aeruginosa* (MIC = 0.25 $\mu\text{g}/\text{mL}$), while a similar antimicrobial effect is observed against *S. aureus* (MIC = 16 $\mu\text{g}/\text{mL}$). Complex **1** exhibits the best inhibition (MIC = 0.25 $\mu\text{g}/\text{mL}$) against *P. aeruginosa*.

When the antibacterial activity of complex **1** is compared to other $\text{Cu}(\text{quinolone})(\text{Phen})\text{Cl}$ complexes (Table 1), it is evident that **1** shows similar antimicrobial activity with $\text{Cu}(\text{enrofloxacin})(\text{Phen})\text{Cl}$, while it is much more active than $\text{Cu}(\text{oxolinato})(\text{Phen})\text{Cl}$. This fact can be attributed to the activity of the Hpr-norf itself, a second-generation quinolone with enhanced activity in comparison to a first-generation quinolone, such as oxolinic acid.³

A very interesting observation was made after comparison of the activity between complex **1** and $\text{Cu}(\text{pr-norf})(\text{bipy})\text{Cl}$, exemplified by a selective increase in potency of the antibacterial effect of Hpr-norf in the two microorganisms, *E. coli* and *P. aeruginosa* (Table 1). This behavior appears unique with the specific combinations of Hpr-norf and Phen or bipy, out of all the compounds tested, and is attributed to differences in the mechanism of action⁸² and permeability of cell membranes or efflux-pump capabilities between the two bacteria.^{82,83}

DNA enzymes are important targets for anticancer drugs. A large number of agents bind to duplex DNA in a covalent or noncovalent fashion, thereby interfering with its role as a template in replication and transcription.⁸⁴ In an attempt to exploit the possible mechanism of action of complex **1**, we

performed complex-DNA experiments, using CT DNA and supercoiled plasmid pUC19. From the observed increase of the intensity of both the negative and the positive bands in the CD spectra in response to the interaction of **1** with CT DNA, we can conclude that the complex might interact with DNA by the intercalative binding model, where Cu(II) plays both the intercalator and the mediator role.^{16,31,53,85,38,54,62} The addition of **1** to pUC19 DNA converted supercoiled plasmid (form I) into nicked circular plasmid (form II) in a dose-dependent manner.

The in vitro biological results obtained from this work show that complex **1** could be a valuable tool in cancer chemotherapy and, to further investigate this suggestion, experiments on its mode of interaction with DNA and its mechanism of action are currently under way.

Experimental Section

Chemistry. Hpr-norf, $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, CH_3ONa , Phen, and solvents were used as purchased from Sigma-Aldrich, unless indicated otherwise. Dimethylformamide (DMF) was distilled from calcium hydride (CaH_2) and CH_3OH from magnesium (Mg) and were stored over 3 Å molecular sieves. All the chemicals and solvents were reagent grade.

Synthesis of the Complex $\text{Cu}(\text{pr-norf})(\text{Phen})\text{Cl}$, **1.** Hpr-norf (0.4 mmol, 145 mg) was dissolved in CH_3OH (15 mL) and CH_3ONa (22 mg, 0.4 mmol) was added. After 30 min of stirring, $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (68 mg, 0.4 mmol) in CH_3OH (10 mL) was added, followed by the dropwise addition of Phen (72 mg, 0.4 mmol) in CH_3OH (10 mL). The reaction mixture was refluxed for 1 h. The blue solution was partially evaporated under reduced pressure and allowed to crystallize slowly. The blue-green crystalline product of **1** was deposited over one week. Yield: 190 mg, 75%. The complex is soluble in H_2O , DMSO, and DMF and is a nonelectrolyte.

IR Analysis. The infrared spectral data were recorded on a Nicolet Magna-IR 550 spectrophotometer. The solid was taken in a KBr pellet, with a frequency range of 400–4000 cm^{-1} . IR data: $\nu(\text{C}=\text{O})_{\text{p}}$, 1629 (vs); $\nu(\text{CO}_2)_{\text{asym}}$, 1614 (vs); $\nu(\text{CO}_2)_{\text{sym}}$, 1403 (vs); $\nu(\text{C-N})_{\text{pyr}}$, 750 (s).

Elemental Analysis. C, H, and N elemental analysis was performed on a Perkin-Elmer 240B elemental analyzer. Anal. Calcd for $\text{Cu}(\text{pr-norf})(\text{Phen})\text{Cl}$ ($\text{C}_{31}\text{H}_{31}\text{FN}_5\text{O}_3\text{ClCu}$; MW = 639.62): C, 58.21; H, 4.89; N, 10.95. Found: C, 58.05; H, 4.70; N, 10.85%.

UV-vis Analysis. UV-vis spectra of the complex were recorded independently as nujol mulls and in aqueous solution (concentration range 2×10^{-5} – 5×10^{-3} M) on a Thermo Electron Corporation Helios α dual beam spectrophotometer. UV-vis $\lambda(\text{nm})$ data (ϵ , $\text{M}^{-1}\text{cm}^{-1}$): as nujol mull, 845(sh), 665, 410(sh), 352, 330, 289; in H_2O , 840 (sh) (10), 650 (40), 410(sh) (195), 352(sh) (2550), 330 (3100), 289 (5700).

CD Spectroscopy. The CD spectra were recorded on a Jasco J-700 spectropolarimeter using a 1.0 cm path quartz cell and the following acquisition parameters: λ = 190–400 nm, scan speed 50 nm \times min⁻¹, resolution step 0.2 nm, sensitivity 50 mdeg, response 1 s, bandwidth 1.0 nm, accumulations 10. In some cases, the spectra were averaged over an appropriate number of scans.

Antibacterial Activity—Minimum Inhibitory Concentration. The antibacterial activity of the compounds (ligands and complex) was studied against *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), and *Pseudomonas aeruginosa* (*P. aeruginosa*). Screening was performed by determining the minimum inhibitory concentration (MIC). Two different media

[luria broth medium (LB, containing 1% w/v tryptone, 0.5% w/v NaCl, and 0.5% w/v yeast extract) and minimal medium salts broth (MMS, containing 1.5% w/v glucose, 0.5% w/v NH_4Cl , 0.5% w/v K_2HPO_4 , 0.1% w/v NaCl, 0.01% w/v $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.1% w/v yeast extract)] were used. The compounds were dissolved in distilled water with 2-fold serial dilutions from 256 to 0.125 $\mu\text{g/mL}$. All the cultures were incubated at 37 °C. Control tests with no active ingredients were also performed.

MIC was determined using 2-fold serial dilutions in liquid media containing 256–0.125 $\mu\text{g/mL}$ of the compound being tested. A preculture of bacteria was grown in LB overnight at the optimal temperature for each species, where 2 mL of MMS were inoculated with 20 mL of this preculture. This culture was used as a control to examine if the growth of the bacteria tested is normal. In a similar second culture, 20 μL of the bacteria as well as the tested compound at the desired concentration were added. A third sample containing 2 mL of MMS supplemented with the same compound concentration was used as a second control to check the effect of the compound on MMS. All samples were measured in triplicate. We monitored bacterial growth by measuring the turbidity of the culture after 12 and 24 h. If a certain concentration of a compound inhibited bacterial growth, half the concentration of the compound was tested. This procedure was carried on to a concentration where bacteria grow normally. The lowest concentration that inhibited bacterial growth was determined as the MIC value. All the equipment and culture media used were sterile.

Cell Culture. HL-60 (peripheral blood human promyelocytic leukemia) cell line was maintained in RPMI 1640 (PAA), supplemented with 10% FBS (PAA), 2 mM L-glutamine (PAA), 0.85 g/L NaHCO_3 (BioChrom), 25 mM HEPES (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]; PAA), 100 U/mL penicillin, and 500 $\mu\text{g/mL}$ streptomycin (PAA), at 37 °C, in a 5% CO_2 atmosphere. Cells were seeded at 4×10^5 cells/mL, incubated at 37 °C, and 24 h later, while in an exponential growth phase, they were treated with **1**, $\text{Cu}(\text{OP})_2$, Hpr-norf, Phen, and CuCl_2 , individually, for increasing time periods. All compounds used were diluted with water and were added to the cell medium, where they remained for the indicated time periods.

Cytotoxicity Assay by MTT. The viability of HL-60 cells upon treatment with complex **1**, $\text{Cu}(\text{OP})_2$, Hpr-norf, Phen, and CuCl_2 at different incubation periods were examined using MTT assay, as described previously.⁸⁶ MTT is absorbed by the mitochondria, where it is transformed into formazan by the enzyme succinic dehydrogenase. By assessing the activity of the mitochondrial dehydrogenases, the activity of viable cells in a cell population after treatment with each compound for different time periods is measured. As a result, the sensitivity of the cells to the concentration and the incubation period with the different compounds is determined. Briefly, exponentially growing HL-60 cells, initially seeded at 4×10^5 cells/mL, were treated with 100 $\mu\text{g/mL}$ of complex **1**, Hpr-norf, Phen, and CuCl_2 and incubated for 2, 6, 12, 24, and 48 h at 37 °C in an atmosphere supplemented with 5% CO_2 . Furthermore, MTT (Sigma Chemical Co, St. Louis, MO) was added at a final concentration of 0.5 mg/mL, and they were further incubated for 4 h at 37 °C, 5% CO_2 , for MTT (yellow) to be transformed into formazan crystals (purple) by the viable cells. The formazan crystals were solubilized for 14 h upon addition of a solution containing 12.5% SDS (Sigma Chemical Co, St. Louis, MO) and 45% (v/v) formamide (Acros Organics, Geel, Belgium) and incubation at 37 °C. The absorbance of each cell lysate solution

was measured at 550 nm. The results from the MTT assay are expressed as the means of the absorptions at 550 nm \pm SD, using data from two independent experiments that had each been repeated three times.

Cell Proliferation. The ability of complex **1**, Hpr-norf, Phen, and CuCl₂ to inhibit cell division and growth individually was assessed in leukemia cells HL-60. For this purpose, cells at an exponential growth phase were treated with 100 μ g/mL of complex **1**, Hpr-norf, Phen, and CuCl₂ and incubated at 37 °C in an atmosphere supplemented with 5% CO₂ for 6, 12, 24, and 48 h. Untreated HL-60 cells were used as control. Then 50 μ L from each sample were diluted 10 \times in PBS (1 \times), and the total cell number was counted on a hemocytometer under a microscope. The results were expressed as the total cell concentration of cells, with each data point representing the mean of three separate experiments (mean \pm SD).

Necrotic Effects by Trypan Blue Staining. Viable cells are characterized by a structurally integral cell membrane, in contrast to dead cells or secondary necrotic cells (cells which are in the final stage of apoptosis; secondary necrosis), which are characterized by the loss of integrity of their membrane. Trypan blue dye penetrates the cell membrane only in the case where it is damaged; it enters the internal and binds to several intracellular proteins, staining the cell blue. As a result, viable cells do not uptake the dye, whereas dead cells are stained blue. Trypan blue staining is used only to determine the dead cells in a population and not the apoptotic cells.⁸⁷

HL-60 cells were seeded at a concentration of 4×10^5 cells/mL, and after 24 h, while at an exponential growth phase, they were treated with 100 μ g/mL of complex **1**, Hpr-norf, Phen, and CuCl₂ and incubated at 37 °C in an atmosphere supplemented with 5% CO₂ for 6, 12, 24, and 48 h. Untreated HL-60 cells were used as control. Then 50 μ L from each sample was diluted 10 \times in PBS (1 \times) and 2 μ L of 0.4% (w/v) trypan blue stain (Sigma Chemical Co, St. Louis, MO) was added to 18 μ L of each cell suspension. The total number of cells and the number of blue-stained cells were counted on a hemocytometer under a microscope. The results are presented as the concentration of trypan blue +ve cells. Each data point represents the mean for three separate experiments (mean \pm SD).

Induction of Apoptosis by DNA Fragmentation Assay. DNA fragmentation is a nonreversible process that engages the cell into committing suicide (death by apoptosis) and is widely used as a technique to determine the induction of apoptosis. In a variety of cell systems, the internucleosomal DNA fragmentation has been shown to be due to the action of several endonucleases, depending on the cell type and the apoptotic pathway that is followed.⁸⁸ DNA fragmentation takes place in the area among nucleosomes (connective parts of DNA), and the result is the generation of mono- or oligo-nucleosomal parts of DNA. If the fragmented DNA is run on an agarose gel under constant voltage, then DNA laddering can be visualized as 180–185 base-pair DNA fragments.

Cell DNA fragmentation after treatment of HL-60 cells with complex **1** and Hpr-norf was monitored by a gel electrophoresis method, according to Eastman protocol.⁸⁹ Briefly, 10⁶ cells treated with 100 μ g/mL of complex **1**, Cu(OP)₂, and Hpr-norf for 12, 24, and 48 h at 37 °C, 5% CO₂, were transferred into 2% agarose gel wells, where they were lysed by use of SDS (Sigma Chemical Co, St. Louis, MO), proteinase K (Sigma Chemical Co, St. Louis, MO), and ribonuclease A (Sigma Chemical Co, St. Louis, MO). All treated cell lysates, including untreated HL-60 cells as control, were subjected to electrophoresis on a 2% agarose gel (GENAXYS). High molecular

weight (HMW) DNA fragments were trapped in or near the well, whereas DNA fragments of low molecular weight migrated and, thus, were separated through the gel. The gel was stained with 10% ethidium bromide (Sigma Chemical Co, St. Louis, MO), visualized in a UV transilluminator and photographed using a Polaroid camera.

Induction of Apoptosis by Flow Cytometry (FACS).⁹⁰ Exponentially grown HL-60 cells were treated with 100 μ g/mL of **1**, Cu(OP)₂, Hpr-norf, Phen, and CuCl₂. After incubation, cells were collected and washed three times with phosphate buffer saline (PBS) and then suspended in 1 mL PBS (1 \times) and 1 mL of frozen 70% ethanol that was added slowly to the cell suspension and remained at 4 °C overnight. Fixed cells were centrifuged and washed with PBS once. For detecting DNA content, cells were stained in the dark with PI (50 μ g/mL) and 0.1% RNaseA in PBS at 25 °C for 15 min. Stained cells were applied to a flow cytometer (Becton Dickinson). For each analysis, 5000 events were recorded.

Interaction with CT DNA. DNA stock solutions (5 mM) were prepared by dilution of CT DNA to buffer (containing 150 mM NaCl and 15 mM trisodium citrate at pH = 7.0), followed by exhaustive stirring at 4 °C for three days⁹¹ and kept at 4 °C for no longer than a week. The nucleotide concentrations were determined by their absorption at 260 nm using $\epsilon = 6600 \text{ M}^{-1}\text{cm}^{-1}$ (expressed as phosphate). The interaction of complex **1** with CT DNA has been investigated with UV and CD spectroscopies. In both techniques, the spectra of CT DNA in the presence of the complex have been recorded for a constant CT DNA concentration ($3.125 \times 10^{-4} \text{ M}$) in diverse complex/CT DNA mixing ratios (*r*). All the UV and CD spectra were recorded after incubation of the complex with CT DNA for 24 h at 37 °C.

Cleavage of pUC19 by Complex 1. A compound that unwinds the DNA duplex reduces the number of supercoils in closed circular DNA so that their number decreases. This decrease upon binding of unwinding agents causes a decrease in the rate of migration through agarose gel, which makes it possible to observe.⁹² Plasmid pUC19 (0.5 μ g/ μ L in 10 mM Tris-HCl and 1 mM EDTA, pH 7.6) was purchased from Fermentas, Life Sciences. All reactions were carried out in a total volume of 20 μ L. The resulting mixtures contained 5, 16, and 30 μ M of complex **1** and 1 μ g of supercoiled pUC19 (0.5 μ g/ μ L) in TE buffer (10 mM Tris/1 mM EDTA, pH 7.4). Samples were incubated at 37 °C overnight. A 3 μ L aliquot of a loading dye solution (0.25% bromophenol blue, 0.25% xylene cyanole, and 30% glycerol) was then added, and the samples were loaded on agarose gel (0.8%) and stained with 10% ethidium bromide (Sigma Chemical Co, St. Louis, MO). Electrophoresis was carried out at 50V for 1 h in 0.5 \times TBE buffer (0.045 M Tris, 0.045 M boric acid, and 1 mM EDTA), and the gel was visualized in a UV transilluminator and photographed using a Polaroid camera.

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References

- (1) Koga, H.; Itoh, A.; Murayama, S.; Suzue, S.; Irikura, T. Structure–activity relationships of antibacterial 6,7- and 7,8-disubstituted 1-alkyl-1,4-

- dihydro-4-oxoquinoline-3-carboxylic acids. *J. Med. Chem.* **1980**, *23*, 1358–1363.
- (2) Goldstein, E. J. Norfloxacin, a fluoroquinolone antibacterial agent. Classification, mechanism of action, and in vitro activity. *Am. J. Med.* **1987**, *82*, 3–17.
- (3) King, D. E.; Malone, R.; Lilley, S. H. New classification and update on the quinolone antibiotics. *Am. Fam. Physician* **2000**, *61*, 2741–2748.
- (4) Schaeffer, A. J. The expanding role of fluoroquinolones. *Am. J. Med.* **2002**, *113* (Suppl 1A), 45S–54S.
- (5) Hooper, D. C.; Wolfson, J. S. The fluoroquinolones: Pharmacology, clinical uses, and toxicities in humans. *Antimicrob. Agents Chemother.* **1985**, *28*, 716–721.
- (6) Koyle, M. A.; Barqawi, A.; Wild, J.; Passamanek, M.; Furness, P. D., 3rd. Pediatric urinary tract infections: The role of fluoroquinolones. *Pediatr. Infect. Dis. J.* **2003**, *22*, 1133–1137.
- (7) Shen, L. L.; Chu, D. T. W. Type II DNA topoisomerase as antibacterial targets. *Curr. Pharm. Des.* **1996**, *2*, 195–208.
- (8) Marians, K. J.; Hiasa, H. Mechanism of quinolone action. A drug-induced structural perturbation of the DNA precedes strand cleavage by topoisomerase IV. *J. Biol. Chem.* **1997**, *272*, 9401–9409.
- (9) Chu, D. T. W. Recent development in antibacterial research. *Annu. Rep. Med. Chem.* **1998**, *33*, 141–150.
- (10) Cozzarelli, N. R. DNA gyrase and the supercoiling of DNA. *Science* **1980**, *207*, 953–960.
- (11) Chu, D. T.; Fernandes, P. B.; Claiborne, A. K.; Pihuleac, E.; Nordeen, C. W.; Maleczka, R. E., Jr.; Pernet, A. G. Synthesis and structure–activity relationships of novel arylfluoroquinolone antibacterial agents. *J. Med. Chem.* **1985**, *28*, 1558–1564.
- (12) Domagala, J. M.; Heifetz, C. L.; Hutt, M. P.; Mich, T. F.; Nichols, J. B.; Solomon, M.; Worth, D. F. 1-Substituted 7-[3-[(ethylamino)methyl]-1-pyrrolidinyl]-6,8-difluoro-1,4-dihydro-4-oxo-3-quinolinecarboxylic acids. New quantitative structure–activity relationships at N1 for the quinolone antibacterials. *J. Med. Chem.* **1988**, *31*, 991–1001.
- (13) Hughes, M. N. *The Inorganic Chemistry of Biological Processes*, 2nd ed.; Wiley: New York, 1981.
- (14) Turel, I. The interactions of metal ions with quinolone antibacterial agents. *Coord. Chem. Rev.* **2002**, *232*, 27–47.
- (15) Sandstrom, K.; Warmlander, S.; Leijon, M.; Graslund, A. ¹H NMR studies of selective interactions of norfloxacin with double-stranded DNA. *Biochem. Biophys. Res. Commun.* **2003**, *304*, 55–59.
- (16) Fan, J. Y.; Sun, D.; Yu, H.; Kerwin, S. M.; Hurley, L. H. Self-assembly of a quinobenzoxazine-Mg²⁺ complex on DNA: A new paradigm for the structure of a drug–DNA complex and implications for the structure of the quinolone bacterial gyrase–DNA complex. *J. Med. Chem.* **1995**, *38*, 408–424.
- (17) Canton, E.; Peman, J.; Jimenez, M. T.; Ramon, M. S.; Gobernado, M. In vitro activity of sparfloxacin compared with those of five other quinolones. *Antimicrob. Agents Chemother.* **1992**, *36*, 558–565.
- (18) Wiles, J. A.; Wang, Q.; Lucien, E.; Hashimoto, A.; Song, Y.; Cheng, J.; Marlor, C. W.; Ou, Y.; Podos, S. D.; Thanassi, J. A.; Thoma, C. L.; Deshpande, M.; Pucci, M. J.; Bradbury, B. J. Isothiazoloquinolones containing functionalized aromatic hydrocarbons at the 7-position: Synthesis and in vitro activity of a series of potent antibacterial agents with diminished cytotoxicity in human cells. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 1272–1276.
- (19) Fang, K. C.; Chen, Y. L.; Sheu, J. Y.; Wang, T. C.; Tzeng, C. C. Synthesis, antibacterial, and cytotoxic evaluation of certain 7-substituted norfloxacin derivatives. *J. Med. Chem.* **2000**, *43*, 3809–3812.
- (20) Pfeiffer, E. S.; Hiasa, H. Replacement of ParC alpha4 helix with that of GyrA increases the stability and cytotoxicity of topoisomerase IV–quinolone–DNA ternary complexes. *Antimicrob. Agents Chemother.* **2004**, *48*, 608–611.
- (21) Xia, Y.; Yang, Z. Y.; Xia, P.; Bastow, K. F.; Tachibana, Y.; Kuo, S. C.; Hamel, E.; Hackl, T.; Lee, K. H. Antitumor agents. 181. Synthesis and biological evaluation of 6,7,2',3',4'-substituted-1,2,3,4-tetrahydro-2-phenyl-4-quinolones as a new class of antimitotic antitumor agents. *J. Med. Chem.* **1998**, *41*, 1155–1162.
- (22) Kamat, A. M.; DeHaven, J. I.; Lamm, D. L. Quinolone antibiotics: A potential adjunct to intravesical chemotherapy for bladder cancer. *Urology* **1999**, *54*, 56–61.
- (23) Turel, I.; Golobic, A.; Klavzar, A.; Pihlar, B.; Buglyo, P.; Tolis, E.; Rehder, D.; Sepcic, K. Interactions of oxovanadium(IV) and the quinolone family member ciprofloxacin. *J. Inorg. Biochem.* **2003**, *95*, 199–207.
- (24) Saha, D. K.; Sandbhor, U.; Shirisha, K.; Padhye, S.; Deobagkar, D.; Anson, C. E.; Powell, A. K. A novel mixed-ligand antimycobacterial dimeric copper complex of ciprofloxacin and phenanthroline. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 3027–3032.
- (25) Ruiz, M.; Perello, L.; Ortiz, R.; Castineiras, A.; Maichle-Mossmar, C.; Canton, E. Synthesis, characterization, and crystal structure of [Cu(cinoxacinatate)₂]·2H₂O complex: A square-planar CuO₄ chromophore. Antibacterial studies. *J. Inorg. Biochem.* **1995**, *59*, 801–810.
- (26) Lopez-Gresa, M. P.; Ortiz, R.; Perello, L.; Latorre, J.; Liu-Gonzalez, M.; Garcia-Granda, S.; Perez-Priede, M.; Canton, E. Interactions of metal ions with two quinolone antimicrobial agents (cinoxacin and ciprofloxacin). Spectroscopic and X-ray structural characterization. Antibacterial studies. *J. Inorg. Biochem.* **2002**, *92*, 65–74.
- (27) Ruiz, M.; Perello, L.; Server-Carrio, J.; Ortiz, R.; Garcia-Granda, S.; Diaz, M. R.; Canton, E. Cinoxacin complexes with divalent metal ions. Spectroscopic characterization. Crystal structure of a new dinuclear Cd(II) complex having two chelate-bridging carboxylate groups. Antibacterial studies. *J. Inorg. Biochem.* **1998**, *69*, 231–239.
- (28) Turel, I.; Golic, L.; Bukovec, P.; Gubina, M. Antibacterial tests of bismuth(III)-quinolone (ciprofloxacin, cf) compounds against *Helicobacter pylori* and some other bacteria. Crystal structure of (cfH₂)₂[Bi₂Cl₁₀]·4H₂O. *J. Inorg. Biochem.* **1998**, *71*, 53–60.
- (29) Jimenez-Garrido, N.; Perello, L.; Ortiz, R.; Alzuet, G.; Gonzalez-Alvarez, M.; Canton, E.; Liu-Gonzalez, M.; Garcia-Granda, S.; Perez-Priede, M. Antibacterial studies, DNA oxidative cleavage, and crystal structures of Cu(II) and Co(II) complexes with two quinolone family members, ciprofloxacin, and enoxacin. *J. Inorg. Biochem.* **2005**, *99*, 677–689.
- (30) Drevensek, P.; Turel, I.; Poklar Ulrih, N. Influence of copper(II) and magnesium(II) ions on the ciprofloxacin binding to DNA. *J. Inorg. Biochem.* **2003**, *96*, 407–415.
- (31) Efthimiadou, E. K.; Thomadaki, H.; Sanakis, Y.; Raptopoulou, C. P.; Katsaros, N.; Scorilas, A.; Karaliota, A.; Psomas, G. Structure and biological properties of the copper(II) complex with the quinolone antibacterial drug *N*-propyl-norfloxacin and 2,2'-bipyridine. *J. Inorg. Biochem.* **2007**, *101*, 64–73.
- (32) Ranford, J. D.; Sadler, P. J.; Tocher, D. A. Cytotoxicity and antiviral activity of transition-metal salicylate complexes and crystal structure of bis(diisopropylsalicylate)(1,10-phenanthroline)copper(II). *J. Chem. Soc., Dalton Trans.* **1993**, *22*, 3393–3399.
- (33) Zoroddu, M. A.; Zanetti, S.; Pogni, R.; Basosi, R. An electron spin resonance study and antimicrobial activity of copper(II)–phenanthroline complexes. *J. Inorg. Biochem.* **1996**, *63*, 291–300.
- (34) Erkkila, K. E.; Odom, D. T.; Barton, J. K. Recognition and reaction of metallointercalators with DNA. *Chem. Rev.* **1999**, *99*, 2777–2796.
- (35) Sigman, D. S. Chemical nucleases. *Biochemistry* **1990**, *29*, 9097–9105.
- (36) Sigman, D. S.; Mazumder, A.; Perrin, D. M. Chemical nucleases. *Chem. Rev.* **1993**, *93*, 2295–2316.
- (37) Pogozelski, W. K.; Tullius, T. D. Oxidative strand scission of nucleic acids: Routes initiated by hydrogen abstraction from the sugar moiety. *Chem. Rev.* **1998**, *98*, 1089–1108.
- (38) Lu, L. P.; Zhu, M. L.; Yang, P. Crystal structure and nuclease activity of mono(1,10-phenanthroline) copper complex. *J. Inorg. Biochem.* **2003**, *95*, 31–36.
- (39) Sigman, D. S.; Graham, D. R.; D'Aurora, V.; Stern, A. M. Oxygen-dependent cleavage of DNA by the 1,10-phenanthroline–cuprous complex. Inhibition of *Escherichia coli* DNA polymerase I. *J. Biol. Chem.* **1979**, *254*, 12269–12272.
- (40) Thederahn, T. B.; Kuwabara, T. A.; Larsen, D. S.; Sigman, D. S. Nuclease activity of 1,10-phenanthroline–copper: Kinetic mechanism. *J. Am. Chem. Soc.* **1989**, *111*, 4941–4946.
- (41) Zelencu, O.; Gallagher, J.; Sigman, D. S. Scission of DNA with bis(1,10-phenanthroline)copper without intramolecular hydrogen migration. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 2776–2778.
- (42) Salomons, G. S.; Smets, L. A.; Verwijns-Janssen, M.; Hart, A. A.; Haarman, E. G.; Kaspers, G. J.; Wering, E. V.; Der Does-Van Den Berg, A. V.; Kamps, W. A. Bcl-2 family members in childhood acute lymphoblastic leukemia: Relationships with features at presentation, in vitro and in vivo drug response, and long-term clinical outcome. *Leukemia* **1999**, *13*, 1574–1580.
- (43) Cassinelli, G.; Supino, R.; Perego, P.; Polizzi, D.; Lanzi, C.; Pratesi, G.; Zunino, F. A role for loss of p53 function in sensitivity of ovarian carcinoma cells to taxanes. *Int. J. Cancer* **2001**, *92*, 738–747.
- (44) Kim, R.; Nishimoto, N.; Inoue, H.; Yoshida, K.; Toge, T. An analysis of the therapeutic efficacy of protracted infusion of low-dose 5-fluorouracil and cisplatin in advanced gastric cancer. *J. Infect. Chemother.* **2000**, *6*, 222–228.
- (45) Kim, R.; Tanabe, K.; Uchida, Y.; Emi, M.; Inoue, H.; Toge, T. Current status of the molecular mechanisms of anticancer drug-induced apoptosis. The contribution of molecular-level analysis to cancer chemotherapy. *Cancer Chemother. Pharmacol.* **2002**, *50*, 343–352.
- (46) Thomadaki, H.; Scorilas, A. BCL2 family of apoptosis-related genes: Functions and clinical implications in cancer. *Crit. Rev. Clin. Lab. Sci.* **2006**, *43*, 1–67.
- (47) Stegh, A. H.; Kim, H.; Bachoo, R. M.; Forloney, K. L.; Zhang, J.; Schulze, H.; Park, K.; Hannon, G. J.; Yuan, J.; Louis, D. N.; DePinho, R. A.; Chin, L. Bcl2L12 inhibits post-mitochondrial apoptosis signaling in glioblastoma. *Genes Dev.* **2007**, *21*, 98–111.
- (48) Nakamoto, K. *Infrared and Raman Spectra of Inorganic and Coordination Compounds*, 4th ed.; Wiley: New York, 1986.

- (49) Deacon, G. B.; Phillips, R. J. Relationships between the carbon–oxygen stretching frequencies of carboxylato complexes and the type of carboxylate coordination. *Coord. Chem. Rev.* **1980**, *33*, 227–250.
- (50) Efthimiadou, E. K.; Psomas, G.; Sanakis, Y.; Katsaros, N.; Karaliota, A. Metal complexes with the quinolone antibacterial agent *N*-propyl-norfloxacin: Synthesis, structure, and bioactivity. *J. Inorg. Biochem.* **2007**, *101*, 525–535.
- (51) Hathaway, B. J. Copper. In *Comprehensive Coordination Chemistry*; Wilkinson, G., Ed.; Pergamon Press: Oxford, 1987; Vol. 5, pp 533–773, and references cited therein.
- (52) Mukherjee, R. Copper. In *Comprehensive Coordination Chemistry II*; McCleverty, J. A.; Meyer, T. J., Eds.; Elsevier: New York, 2003; Vol. 5, pp 403–553, and references cited therein.
- (53) Efthimiadou, E. K.; Sanakis, Y.; Katsarou, M.; Raptopoulou, C. P.; Karaliota, A.; Katsaros, N.; Psomas, G. Neutral and cationic mononuclear copper(II) complexes with enrofloxacin: Structure and biological activity. *J. Inorg. Biochem.* **2006**, *100*, 1378–1388.
- (54) Efthimiadou, E. K.; Sanakis, Y.; Raptopoulou, C. P.; Karaliota, A.; Katsaros, N.; Psomas, G. Crystal structure, spectroscopic, and biological study of the copper(II) complex with third-generation quinolone antibiotic sparfloxacin. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 3864–3867.
- (55) Psomas, G.; Tarushi, A.; Efthimiadou, E. K.; Sanakis, Y.; Raptopoulou, C. P.; Katsaros, N. Synthesis, structure, and biological activity of copper(II) complexes with oxolinic acid. *J. Inorg. Biochem.* **2006**, *100*, 1764–1773.
- (56) Dendrinou-Samara, C.; Psomas, G.; Christophorou, K.; Tangoulis, V.; Raptopoulou, C. P.; Terzis, A.; Kessissoglou, D. P. Structurally diverse of copper(II) herbicide complexes: Mono- and bi-nuclear neutral or cationic complexes. *J. Chem. Soc., Dalton Trans.* **1996**, 3737–3743.
- (57) Psomas, G.; Dendrinou-Samara, C.; Philippakopoulos, P.; Tangoulis, V.; Raptopoulou, C. P.; Samaras, E.; Kessissoglou, D. P. Cu(II)-herbicide complexes: Structure and bioactivity. *Inorg. Chim. Acta* **1998**, *272*, 24–32.
- (58) Dendrinou-Samara, C.; Psomas, G.; Raptopoulou, C. P.; Kessissoglou, D. P. Copper(II) complexes with phenoxyalkanoic acids and nitrogen donor heterocyclic ligands: Structure and bioactivity. *J. Inorg. Biochem.* **2001**, *83*, 7–16.
- (59) Psomas, G.; Raptopoulou, C. P.; Iordanidis, L.; Dendrinou-Samara, C.; Tangoulis, V.; Kessissoglou, D. P. Structurally diverse copper(II)-carboxylato complexes: Neutral and ionic mononuclear structures and a novel binuclear structure. *Inorg. Chem.* **2000**, *39*, 3042–3048.
- (60) Rodger, A.; Norden, B. Circular Dichroism and Linear Dichroism. *Circular Dichroism and Linear Dichroism*; Oxford University Press: Oxford, New York, Tokyo, 1997.
- (61) Son, G. S.; Yeo, J. A.; Kim, M. S.; Kim, S. K.; Holmen, A.; Akerman, B.; Norden, B. Binding mode of norfloxacin to calf thymus DNA. *J. Am. Chem. Soc.* **1998**, *120*, 6451–6457.
- (62) Maheswari, P. U.; Palaniandavar, M. DNA binding and cleavage properties of certain tetrammine ruthenium(II) complexes of modified 1,10 phenanthrolines-effect of hydrogen-bonding on DNA-binding affinity. *J. Inorg. Biochem.* **2004**, *98*, 219–230.
- (63) Schaeffer, P.; Mollet, J.; Aubert, J. P. Catabolic repression of bacterial sporulation. *Proc. Natl. Acad. Sci. U.S.A.* **1965**, *54*, 704–711.
- (64) Ruiz, P.; Ortiz, R.; Perello, L.; Alzueta, G.; Gonzalez-Alvarez, M.; Liu-Gonzalez, M.; Sanz-Ruiz, F. Synthesis, structure, and nuclease properties of several binary and ternary complexes of copper(II) with norfloxacin and 1,10-phenanthroline. *J. Inorg. Biochem.* **2007**, *101*, 831–840.
- (65) Zhang, S.; Zhu, Y.; Tu, C.; Wei, H.; Yang, Z.; Lin, L.; Ding, J.; Zhang, J.; Guo, Z. A novel cytotoxic ternary copper(II) complex of 1,10-phenanthroline and L-threonine with DNA nuclease activity. *J. Inorg. Biochem.* **2004**, *98*, 2099–2106.
- (66) Reed, J. C. Dysregulation of apoptosis in cancer. *J. Clin. Oncol.* **1999**, *17*, 2941–2953.
- (67) Sellers, W. R.; Fisher, D. E. Apoptosis and cancer drug targeting. *J. Clin. Invest.* **1999**, *104*, 1655–1661.
- (68) Fisher, D. E. Apoptosis in cancer therapy: Crossing the threshold. *Cell* **1994**, *78*, 539–542.
- (69) Kerr, J. F.; Winterford, C. M.; Harmon, B. V. Apoptosis. Its significance in cancer and cancer therapy. *Cancer* **1994**, *73*, 2013–2026.
- (70) Bellamy, C. O.; Malcomson, R. D.; Harrison, D. J.; Wyllie, A. H. Cell death in health and disease: The biology and regulation of apoptosis. *Semin. Cancer Biol.* **1995**, *6*, 3–16.
- (71) McDonnell, T. J.; Meyn, R. E.; Robertson, L. E. Implications of apoptotic cell death regulation in cancer therapy. *Semin. Cancer Biol.* **1995**, *6*, 53–60.
- (72) Eastman, A. Survival factors, intracellular signal transduction, and the activation of endonucleases in apoptosis. *Semin. Cancer Biol.* **1995**, *6*, 45–52.
- (73) Shah, A. M.; Schwartz, G. K. Cell-cycle mediated drug resistance: An emerging concept in cancer therapy. *Clin. Cancer Res.* **2001**, *7*, 2168–2181.
- (74) Wong, E.; Giandomenico, C. M. Current status of platinum-based antitumor drugs. *Chem. Rev.* **1999**, *99*, 2451–2466.
- (75) Kanduc, D.; Mittelman, A.; Serpico, R.; Sinigaglia, E.; Sinha, A. A.; Natale, C.; Santacroce, R.; Di Corcia, M. G.; Lucchese, A.; Dini, L.; Pani, P.; Santacroce, S.; Simone, S.; Bucci, R.; Farber, E. Cell death: Apoptosis versus necrosis (review). *Int. J. Oncol.* **2002**, *21*, 165–170.
- (76) Hill, I. E.; Murray, C.; Richard, J.; Rasquinha, I. Macmanus despite the internucleosomal cleavage of DNA, reactive oxygen species do not produce other markers of apoptosis in cultured neurons. *Exp. Neurol.* **2000**, *162*, 73–78.
- (77) Tsang, S. Y.; Tam, S. C.; Bremner, I.; Burkitt, M. J. Research communication copper-1,10-phenanthroline induces internucleosomal DNA fragmentation in HepG2 cells, resulting from direct oxidation by the hydroxyl radical. *Biochem. J.* **1996**, *317*, 13–16.
- (78) Zhou, H.; Zheng, C.; Zou, G.; Tao, D.; Gong, J. G1-phase specific apoptosis in liver carcinoma cell line induced by copper-1,10-phenanthroline. *Int. J. Biochem. Cell Biol.* **2002**, *34*, 678–684.
- (79) Cai, X.; Pan, N.; Zou, G. Copper-1,10-phenanthroline-induced apoptosis in liver carcinoma Bel-7402 cells associates with copper overload, reactive oxygen species production, glutathione depletion and oxidative DNA damage. *BioMetals* **2007**, *20*, 1–11.
- (80) Russel, A. D. Fundamental principles of activity principles of antimicrobial activity and resistance. In *Disinfection, Sterilization and Preservation*, 4th ed.; Block, S. S. Ed.; Lippincott Williams & Wilkins: Philadelphia, PA, 2000; pp 31–56.
- (81) Rossmore, H. W. Nitrogen compounds. *Disinfection, Sterilization and Preservation*, 5th ed.; Lippincott Williams & Wilkins: Philadelphia, PA, 2000; pp 383–414.
- (82) Soussy, C. J.; Wolfson, J. S.; Ng, E. Y.; Hooper, D. C. Limitations of plasmid complementation test for determination of quinolone resistance due to changes in the gyrase A protein and identification of conditional quinolone resistance locus. *Antimicrob. Agents Chemother.* **1993**, *37*, 2588–2592.
- (83) Mahamoud, A.; Chevalier, J.; Alibert-Franco, S.; Kern, W. V.; Pages, J. M. Antibiotic efflux pumps in Gram-negative bacteria: the inhibitor response strategy. *J. Antimicrob. Chemother.* **2007**, *59*, 1223–1229.
- (84) Gellert, M.; Mizuuchi, K.; O'Dea, M. H.; Nash, H. A. DNA gyrase: An enzyme that introduces superhelical turns into DNA. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 3872–3876.
- (85) Yeo, J. A.; Cho, T. S.; Kim, S. K.; Moon, H. R.; Jhon, G. H.; Nam, W. Interaction between norfloxacin and single-stranded DNA. *Bull. Korean Chem. Soc.* **1998**, *19*, 449–457.
- (86) Mosmann, T. Rapid colorimetric assay for the cellular growth and survival: application and cytotoxic assays. *J. Immunol.* **1983**, *65*, 55.
- (87) Freshney, R. In *Culture of Animal Cells: A Manual of Basic Technique*; Alan, R., Ed.; Liss: New York, 1987; p 117.
- (88) Barry, M. A.; Behnke, C. A.; Eastman, A. Activation of programmed cell death (apoptosis) by cisplatin, other anticancer drugs, toxins and hyperthermia. *Biochem. Pharmacol.* **1990**, *40*, 2353–2362.
- (89) Eastman, A.; Barry, M. A. Interaction of trans-diamminedichloroplatinum(II) with DNA: Formation of monofunctional adducts and their reaction with glutathione. *Biochemistry* **1987**, *26*, 3303–3307.
- (90) Chou, R. H.; Huang, H. Restoration of p53 tumor suppressor pathway in human cervical carcinoma cells by sodium arsenite. *Biochem. Biophys. Res. Commun.* **2002**, *293*, 298–306.
- (91) Tselepi-Kalouli, E.; Katsaros, N. The interaction of $[\text{Ru}(\text{NH}_3)_5\text{Cl}]^{2+}$ and $[\text{Ru}(\text{NH}_3)_6]^{3+}$ ions with DNA. *J. Inorg. Biochem.* **1989**, *37*, 271–282.
- (92) Keck, M. V.; Lippard, S. J. Unwinding of supercoiled DNA by platinum ethidium and related complexes. *J. Am. Chem. Soc.* **1992**, *114*, 3386–3390.