

## Article

# Copper(II) Complexes with Tetradentate Piperazine-Based Ligands: DNA Cleavage and Cytotoxicity

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**Abstract:** Five-coordinate Cu(II) complexes, [Cu(L<sup>n</sup>)X]ClO<sub>4</sub>/PF<sub>6</sub>, where L<sup>n</sup> = piperazine ligands bearing two pyridyl arms and X = ClO<sub>4</sub><sup>−</sup> for L<sup>1</sup> (1-ClO<sub>4</sub>), L<sup>2</sup> (2-ClO<sub>4</sub>), L<sup>3</sup> (3-ClO<sub>4</sub>), and L<sup>6</sup> (6-ClO<sub>4</sub>) as well as [Cu(L<sup>n</sup>)Cl]PF<sub>6</sub> for L<sup>1</sup> (1-Cl), L<sup>4</sup> (4-Cl), and L<sup>5</sup> (5-Cl) have been synthesized and characterized by spectroscopic techniques. The molecular structures of the last two complexes were determined by X-ray crystallography. In aqueous acetonitrile solutions, molar conductivity measurements and UV-VIS spectrophotometric titrations of the complexes revealed the hydrolysis of the complexes to [Cu(L<sup>n</sup>)(H<sub>2</sub>O)]<sup>2+</sup> species. The biological activity of the Cu(II) complexes with respect to DNA cleavage and cytotoxicity was investigated. At micromolar concentration within 2 h and pH 7.4, DNA cleavage rate decreased in the order: 1-Cl ≈ 1-ClO<sub>4</sub> > 3-ClO<sub>4</sub> ≥ 2-ClO<sub>4</sub> with cleavage enhancements of up to 23 million. Complexes 4-Cl, 5-Cl, and 6-ClO<sub>4</sub> were inactive. In order to elucidate the cleavage mechanism, the cleavage of bis(4-nitrophenyl)phosphate (BNPP) and reactive oxygen species (ROS) quenching studies were conducted. The mechanistic pathway of DNA cleavage depends on the ligand's skeleton: while an oxidative pathway was preferable for 1-Cl/1-ClO<sub>4</sub>, DNA cleavage by 2-ClO<sub>4</sub> and 3-ClO<sub>4</sub> predominantly proceeds via a hydrolytic mechanism. Complexes 1-ClO<sub>4</sub>, 3-ClO<sub>4</sub>, and 5-Cl were found to be cytotoxic against A2780 cells (IC<sub>50</sub> 30–40 μM). In fibroblasts, the IC<sub>50</sub> value was much higher for 3-ClO<sub>4</sub> with no toxic effect.

**Keywords:** copper; DNA cleavage; phosphodiester; cytotoxicity; piperazine

## 1. Introduction

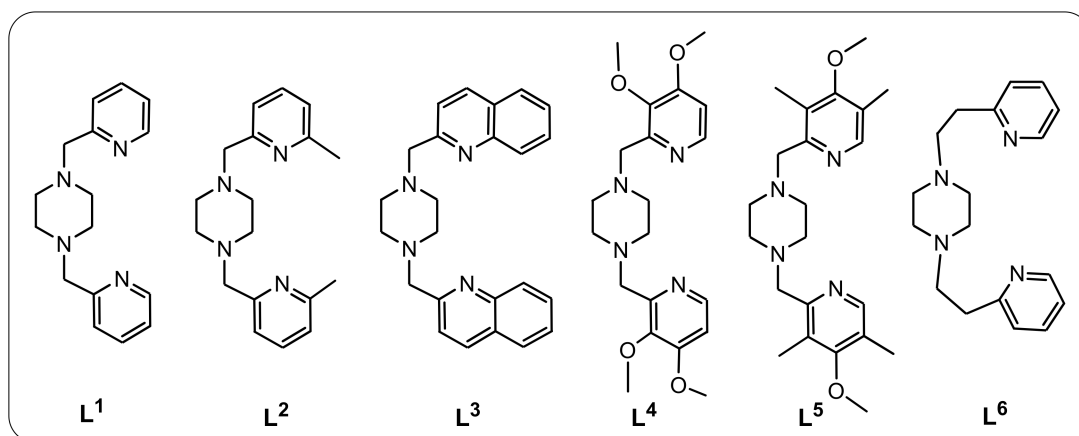
Deoxyribonucleic acid (DNA) is known to play an important role in the survival and maintenance of life on earth as it stores the genetic information for cellular functions. The stability and resistance of the phosphodiester bonds in DNA towards hydrolytic cleavage under physiological conditions is remarkable, considering that the half-life time of this process was estimated to be about 130,000 years [1,2]. However, nature developed its own enzymes, which efficiently and rapidly catalyze the hydrolytic cleavage of P–O bonds in DNA. To mimic the biological function of these enzymes, many artificial nucleases based on transition metal complexes with different metal ions, oxidation states, ligand skeletons, geometrical structures, and solubility in aqueous medium have been developed to study

the P–O bond cleavage in DNA. The metal ions include Co(II) and Co(III), Ru(II) and Ru(III), Mo(V), Zn(II), and Cu(II) [3–26]. Some Co(II) and Cu(II) compounds revealed dramatic enhancement for the catalytic cleavage of DNA under mild conditions by a factor greater than 100 million times compared to the noncatalyzed cleavage with mechanistic pathways proceeding through hydrolytic, oxidative, or both mechanisms [3,4,22–26].

On the other hand, metal-based pharmaceuticals are of considerable interest due to their potential application as anticancer agents. Aside from the tremendous success of platinum-based drugs in the treatment of different carcinogenic tumors [27–29], the serious side effects and drug resistance phenomena have limited the use of these compounds [27,30,31]. Therefore, there is a continuous and acute need in cancer therapy for the design of alternative chemotherapeutic agents based on metals other than platinum, which show improved pharmacological properties of high selective cytotoxicity. Recently, it was demonstrated in our laboratory and by other groups that copper complexes with various ligand skeletal and geometrical structures showed significantly higher cytotoxicity as anticancer agents than platinum compounds like cisplatin [32–42].

In general, anticancer agents, which damage DNA or block its replication, are often taken as preliminary indicators for its potential clinical suitability [43]. Therefore, studying the interaction between metal-based drugs and DNA is considered to be the crucial key step in understanding the mechanism of anticancer drugs. Several studies have suggested that DNA is the primary intracellular target of antitumor drugs because the interaction between small drug molecules and base pairs of DNA can cause DNA damage in cancer as this interferes with normal functioning of the enzyme topoisomerase II, which is involved in the breaking and releasing of DNA [27,29,37].

Herein, we report the synthesis and characterization of five-coordinate copper(II) complexes with piperazine bearing two pendant symmetrical pyridyl arms ( $L = L^1, L^2, L^4, L^5$  and  $L^6$ ) or quinolyl groups ( $L^3$ ),  $[Cu(L)X]ClO_4/ PF_6$  ( $X = Cl, ClO_4$ ). Structures of the piperazine derivatives used in this study and their abbreviations are illustrated in Scheme 1. The DNA cleavage activity by the copper(II) complexes under near-physiological conditions and their cytotoxic activity against some cancer cell lines were the target of this study.



**Scheme 1.** Structures and abbreviations of piperazine ligands used in this study.

## 2. Results and Discussion

### 2.1. Syntheses

A novel series of symmetrical tetradentate *N*-donor ligands (*L*) based on piperazine bearing different pyridyl substituents ( $L^2, L^4$ , and  $L^5$ ) or quinolyl group ( $L^3$ ) were synthesized. The design of these ligands depends on increasing the steric environment around the piperazine base compared to the parent unsubstituted 1,4-bis[(2-pyridinyl)methyl]piperazine ( $L^1$ ) compound (Scheme 1) [6]. The compounds were synthesized using a general method by refluxing a well-stirred mixture of piperazine and the hydrochloride salts of

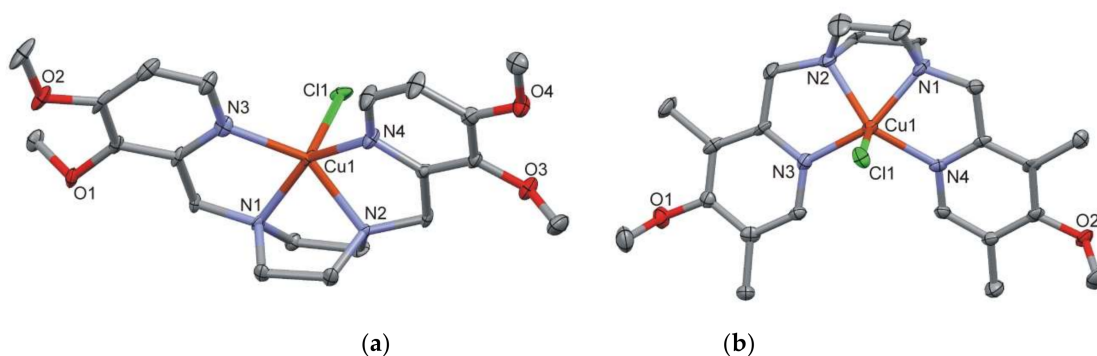
2-(chloromethyl)-pyridine derivatives or 2-(chloromethyl)-quinoline, in the stoichiometric ratio of 1:2, and a slight excess of anhydrous  $K_2CO_3$  (or  $Et_3N$ ) in dry  $CH_3CN$  (or THF) under  $N_2$  gas. The compounds were extracted by  $CH_2Cl_2$  and 10% NaOH solution and recrystallized with the appropriate solvent with the aid of activated charcoal. While the yield of  $L^2$  was good (84%), lower yields were obtained (42–54%) for  $L^3$ – $L^5$ . The isolated compounds were characterized by elemental microanalyses, IR,  $^1H$ , and  $^{13}C$  NMR (Figures S1–S4 are given in the Supplementary), whereas in case of  $L^2$  and  $L^3$  (Figure S5), also by ESI-MS.

Although the syntheses of the Cu(II) complexes under investigation,  $[Cu(L)X]ClO_4/PF_6$  where  $X = ClO_4$  for  $L = L^1$  (**1-ClO<sub>4</sub>**) [6],  $L^2$  (**2-ClO<sub>4</sub>**),  $L^3$  (**3-ClO<sub>4</sub>**),  $L^6$  (**6-ClO<sub>4</sub>**) [6], and  $X = Cl$  for  $[Cu(L^1)Cl]PF_6$  (**1-Cl**),  $[Cu(L^4)Cl]ClO_4 \cdot \frac{1}{2}H_2O$  (**4-Cl**), and  $[Cu(L^5)Cl]PF_6$  (**5-Cl**) were straightforward, attempts to synthesize each pair of complexes with  $X = Cl^-$  and  $ClO_4^-$  failed. Reactions of equimolar amounts of a methanolic solution containing  $Cu(ClO_4)_2 \cdot 6H_2O$  or  $CuCl_2 \cdot 2H_2O$  and the corresponding appropriate ligand followed by the addition of  $NaClO_4$  or  $NH_4PF_6$  afforded the desired products, which were further recrystallized from  $CH_3OH$ . Single crystals suitable for X-ray crystallography were obtained from dilute methanolic solutions. The synthesized complexes have limited solubility in  $H_2O$ , good solubility in  $CH_3OH$ , and are freely soluble in  $CH_3CN$ . The resulting Cu(II) complexes were characterized by IR and UV-VIS spectroscopy, elemental microanalyses, ESI-MS (Figures S6–S9), and by single crystal X-ray crystallography for  $[Cu(L^4)Cl]ClO_4 \cdot H_2O$  (**4-Cl**) and  $[Cu(L^5)Cl]PF_6$  (**5-Cl**).

## 2.2. Characterization of the Complexes

### 2.2.1. Crystal Structures of Complexes 4-Cl and 5-Cl

It was possible to analyze single crystal X-ray crystallographic data for the complexes  $[Cu(L^4)Cl]ClO_4 \cdot H_2O$  (**4-Cl**) and  $[Cu(L^5)Cl]PF_6$  (**5-Cl**). Perspective views of the molecular structures of the  $[Cu(L)Cl]^+$  complex cations of **4-Cl** and **5-Cl** (details of crystal structure determinations and crystallographic data and processing parameters (Table S1) are given in the Supplementary Material section) are depicted in Figure 1. The copper(II) centers are pentacoordinated by 4 N-donor atoms of the substituted pyridyl piperazine molecule (Cu–N bond distances are in the range of 1.991 to 2.064 Å) and one  $Cl^-$  anion (Cu–Cl bond lengths of 2.405 and 2.359 Å for **4-Cl** and **5-Cl**, respectively). The  $CuLCl$  polyhedrons in **4-Cl** and **5-Cl** form square pyramidal (SP) geometries, with almost perfect SP in the former complex ( $\tau = 0.02$ ) and somewhat more distorted in **5-Cl** ( $\tau = 0.19$ ) [44].



**Figure 1.** Molecular structures of complex cations of (a) **4-Cl** and (b) **5-Cl**.

The X-ray crystallography of the previously determined chlorido complex,  $[Cu(L^1)Cl]ClO_4$ , and its corresponding acetonitrile complex,  $[Cu(L^1)(CH_3CN)](PF_6)_2$  revealed that these molecules adopt square-pyramidal geometries [6,45].

## 2.2.2. Spectroscopic and Molar Conductivity Characterization

The IR spectra of the complexes under investigation display some general characteristic features, which include a series of weak to very weak bands over the frequency regions 3090–3000 and 2990–2830  $\text{cm}^{-1}$  attributable to C–H stretching frequencies of the pyridyl and aliphatic C–H groups, respectively. In some cases, the former series of bands were not observed because of their very weak intensities. Another series of medium intensity bands appeared in the range of 1610–1440  $\text{cm}^{-1}$  due to the C=C and C=N stretching of the pyridyl rings. The spectra of complexes  $[\text{Cu}(\text{L}^4)\text{Cl}]\text{ClO}_4 \cdot \frac{1}{2}\text{H}_2\text{O}$  (**4-Cl**) and  $[\text{Cu}(\text{L}^5)\text{Cl}]\text{PF}_6$  (**5-Cl**) displayed two bands of medium intensity in the range of 1300–1240  $\text{cm}^{-1}$  due to  $\nu_{\text{as}}(\text{C}-\text{O}-\text{CH}_3)$  stretching vibration of the methoxy groups. Complex **4-Cl** exhibits a medium broad intense band over the region of 3540–3420  $\text{cm}^{-1}$  due to the  $\nu_{\text{as}}(\text{H}-\text{O})$  stretching vibration of lattice water. The perchlorato complexes  $[\text{Cu}(\text{L})\text{ClO}_4]\text{ClO}_4$  (**1-ClO<sub>4</sub>**: L = L<sup>1</sup>, **2-ClO<sub>4</sub>**: L = L<sup>2</sup>, **3-ClO<sub>4</sub>**: L = L<sup>3</sup>, and **6-ClO<sub>4</sub>**: L = L<sup>6</sup>) displayed two strong split bands around 1100 and 1050  $\text{cm}^{-1}$  attributable to  $\nu_{\text{as}}(\text{O}-\text{Cl})$  stretching vibrations of the coordinated and counter  $\text{ClO}_4^-$  ions, whereas the bands observed at 1084 and 1052  $\text{cm}^{-1}$  in  $[\text{Cu}(\text{L}^4)\text{Cl}]\text{ClO}_4 \cdot \frac{1}{2}\text{H}_2\text{O}$  (**4-Cl**) most likely result from the reduction of the symmetry of the  $\text{ClO}_4^-$  counter ion from  $T_d$  to  $C_{3v}$  or  $C_{2v}$ . The hexafluorophosphate complexes  $[\text{Cu}(\text{L}^1)\text{Cl}]\text{PF}_6$  (**1-Cl**) and  $[\text{Cu}(\text{L}^5)\text{Cl}]\text{PF}_6$  (**5-Cl**) revealed a single very strong band around 830  $\text{cm}^{-1}$  attributable to  $\nu(\text{P}-\text{F})$  stretching frequency of the  $\text{PF}_6^-$  counter ion. The medium intense band observed at 3547  $\text{cm}^{-1}$  in  $[\text{Cu}(\text{L}^4)\text{Cl}]\text{ClO}_4 \cdot \frac{1}{2}\text{H}_2\text{O}$  (**4-Cl**) results from the  $\nu(\text{H}-\text{O})$  stretching frequencies of lattice  $\text{H}_2\text{O}$ .

The UV-VIS spectra of the synthesized Cu(II) complexes recorded in  $\text{CH}_3\text{CN}$  displayed a single broad band over the wavelength region of 645–770 nm for  $[\text{Cu}(\text{L})\text{X}]^+$  (L = L<sup>1</sup>–L<sup>5</sup>, X =  $\text{ClO}_4^-$  or  $\text{Cl}^-$ ) and at 583 nm for  $[\text{Cu}(\text{L}^6)\text{ClO}_4]^+$ . The position of this band depends entirely on the ligand field strength of the tetraamine. The electronic spectra of the complexes in  $\text{CH}_3\text{CN}$  are summarized in Table 1 together with their molar conductivity. Inspection of this data reveals that the complexes have similar spectral features. In general, the observed band is due to d–d transition in five-coordinate Cu(II)-d<sup>9</sup> complexes. The presence of this band, which is associated with or without low-energy shoulder indicates a square pyramidal (SP) geometry, whereas the single d–d band at  $\lambda_{\text{max}} \geq 850$  nm with a high-energy shoulder is typical for trigonal bipyramidal (TBP) stereochemistry [46–49]. Thus, on the basis of this criterion, the spectral data of the complexes are consistent with distorted SP, where the absorption maximum band observed in the visible region results from  ${}^2\text{B}_1 \leftarrow {}^2\text{E}$  transition [46].

**Table 1.** Visible spectral and molar conductivity data for the chlorido and perchlorato Cu(II)-based piperazine pyridyl complexes,  $[\text{Cu}(\text{L})\text{X}]\text{ClO}_4/\text{PF}_6$  in solution.

$[\text{Cu}(\text{L})\text{X}]\text{ClO}_4/\text{PF}_6$	$\lambda_{\text{max}}$ ( $\epsilon_{\text{max}}$ , $\text{M}^{-1}\text{cm}^{-1}$ )	$\Lambda_{\text{M}}$ ( $\Omega^{-1}\cdot\text{cm}^2\cdot\text{mol}^{-1}$ )	$\Lambda_{\text{M}}$ ( $\Omega^{-1}\cdot\text{cm}^2\cdot\text{mol}^{-1}$ )
	$\text{CH}_3\text{CN}$	$\text{CH}_3\text{CN}$	( $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ 1:1 v/v)
$[\text{Cu}(\text{L}^1)\text{ClO}_4]\text{ClO}_4$ ( <b>1-ClO<sub>4</sub></b> )	645 (310)	300	213
$[\text{Cu}(\text{L}^2)\text{ClO}_4]\text{ClO}_4$ ( <b>2-ClO<sub>4</sub></b> )	~752 (299)	297	189
$[\text{Cu}(\text{L}^3)\text{ClO}_4]\text{ClO}_4$ ( <b>3-ClO<sub>4</sub></b> )	~565 (sh), ~618 (sh), 745 (226)	301	188
$[\text{Cu}(\text{L}^6)\text{ClO}_4]\text{ClO}_4$ ( <b>6-ClO<sub>4</sub></b> )	583 (237)	304	184
$[\text{Cu}(\text{L}^1)\text{Cl}]\text{PF}_6$ ( <b>1-Cl</b> )	770 (304)	160	171
$[\text{Cu}(\text{L}^4)\text{Cl}]\text{ClO}_4 \cdot \frac{1}{2}\text{H}_2\text{O}$ ( <b>4-Cl</b> )	771 (324)	145	168
$[\text{Cu}(\text{L}^5)\text{Cl}]\text{PF}_6$ ( <b>5-Cl</b> )	765 (352)	162	177

The molar conductivity of the chlorido and perchlorato complexes, measured in acetonitrile, provided interesting results. The molar conductivities of  $[\text{Cu}(\text{L})\text{Cl}]\text{ClO}_4/\text{PF}_6$  (**1-Cl**, **4-Cl**, and **5-Cl**), in the range of  $\Lambda_{\text{M}} = 145\text{--}162$  ( $\Omega^{-1}\cdot\text{cm}^2\cdot\text{mol}^{-1}$ ) (Table 1) are in complete agreement with their predicted 1:1 electrolytic nature [50]. Unlike the chlorido compounds,

the corresponding perchlorato complexes,  $[\text{Cu}(\text{L})\text{ClO}_4]\text{ClO}_4$  (**1-ClO<sub>4</sub>-3-ClO<sub>4</sub>** and **6-ClO<sub>4</sub>**) in  $\text{CH}_3\text{CN}$  revealed conductivity values of  $\Lambda_M \sim 300 \Omega^{-1}\cdot\text{cm}^2\cdot\text{mol}^{-1}$ , which are fully consistent with the formation of 1:2 electrolytes [50]. This result evidently shows that the perchlorato compounds undergo solvolysis in  $\text{CH}_3\text{CN}$  with the release of  $\text{ClO}_4^-$  ions and the formation of  $[\text{Cu}(\text{L})(\text{CH}_3\text{CN})]^{2+}$  species in solution. This finding was confirmed by the isolation and characterization of  $[\text{Cu}(\text{L}^1)(\text{CH}_3\text{CN})](\text{PF}_6)_2$  [45].

### 2.2.3. Solution Study and Stability of Complexes

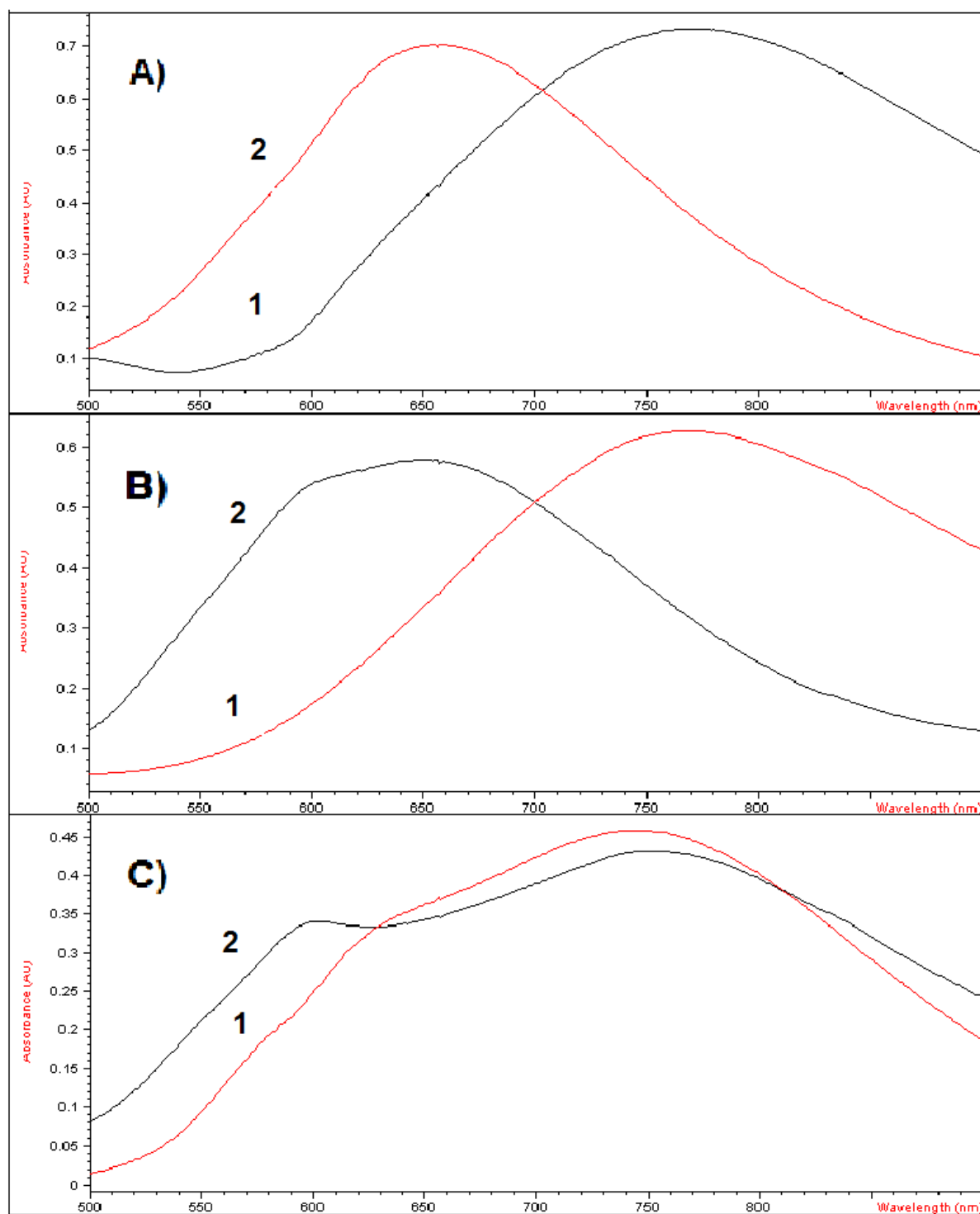
The visible spectra of the complexes under investigation and their molar conductivity,  $\Lambda_M$ , were examined in  $\text{CH}_3\text{CN}$  and aqueous acetonitrile solutions, and measurements were conducted over a period of 1 week. As indicated above, the electronic spectra of the complexes in  $\text{CH}_3\text{CN}$  solution suggest distorted square pyramidal geometry as all produce similar spectral patterns with a single broad band over the wavelength range of 645–770 nm. This band did not show any sign of spectral changes over a period of 1 week. The molar conductivities of the perchlorato complexes **1-ClO<sub>4</sub>-3-ClO<sub>4</sub>** ( $\Lambda_M = 300\text{--}310 \Omega^{-1}\cdot\text{cm}^2\cdot\text{mol}^{-1}$ ) and the corresponding chlorido complexes **1-Cl**, **4-Cl**, and **5-Cl** ( $\Lambda_M = 145\text{--}171 \Omega^{-1}\cdot\text{cm}^2\cdot\text{mol}^{-1}$ ;  $[\text{Cu}(\text{L})\text{Cl}]^+ + \text{ClO}_4^-/\text{PF}_6^-$ ) (Table 1) were in agreement with 1:2 and 1:1 electrolytes, respectively. The conductivity behavior observed in the former case can be explained on the basis of displacement of the coordinated  $\text{ClO}_4^-$  ion by  $\text{CH}_3\text{CN}$  and the formation of  $[\text{Cu}(\text{L})(\text{CH}_3\text{CN})]^{2+}$  species (Scheme 1); the chlorido complexes retained their identities in pure  $\text{CH}_3\text{CN}$  solution.

In contrast, the dissolution of the perchlorato or the chlorido complexes,  $[\text{Cu}(\text{L})\text{X}]\text{ClO}_4/\text{PF}_6$  ( $\text{X} = \text{ClO}_4^-$  or  $\text{Cl}^-$ ) in aqueous acetonitrile solutions is instantaneously associated with color change from teal or blue to aqua blue or cyan, and this process is accompanied by a blue shift in the visible region. Some examples are represented in Figure 2 in  $\text{CH}_3\text{CN}$  and  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  (1:1 by volume) solutions. Interestingly, the original molar conductivity values of the perchlorato complexes,  $[\text{Cu}(\text{L})\text{ClO}_4]^+$ , which were measured in pure  $\text{CH}_3\text{CN}$  ( $300\text{--}310 \Omega^{-1}\cdot\text{cm}^2\cdot\text{mol}^{-1}$ ), decrease in aqueous acetonitrile solutions to  $180\text{--}213 \Omega^{-1}\cdot\text{cm}^2\cdot\text{mol}^{-1}$  with limiting values; slightly bigger than typical values for 1:1 electrolytes and much smaller than predicted for 1:2 electrolytes. In addition, the chlorido complexes,  $[\text{Cu}(\text{L})\text{Cl}]^+$ , in aqueous acetonitrile behave in a similar way, except their molar conductivity values were a little bit higher (Table 1).

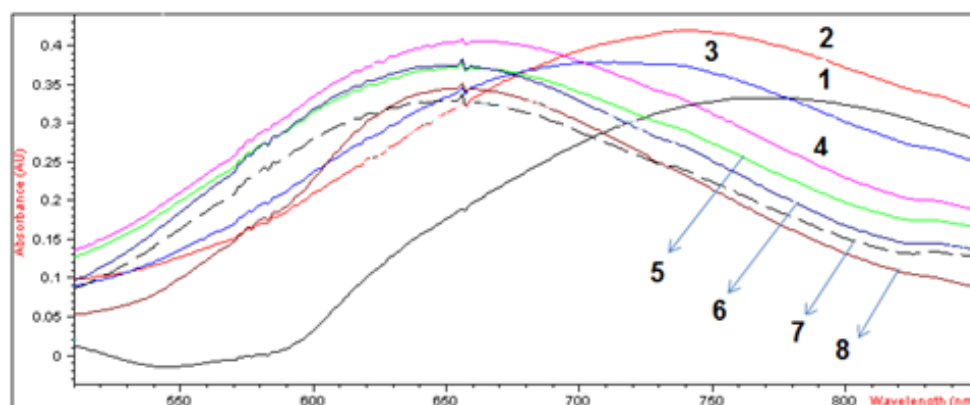
On the other hand, the spectrophotometric titration of  $[\text{Cu}(\text{L})\text{X}]\text{ClO}_4/\text{PF}_6$  dissolved in  $\text{CH}_3\text{CN}$  with  $\text{H}_2\text{O}$  showed a gradual shift of the band, which originally appeared at 645–770 nm region, to a shorter wavelength (ligand field strength of  $\text{H}_2\text{O}$  ligand is stronger than  $\text{CH}_3\text{CN}$ ). A typical example for the spectrophotometric titration of  $[\text{Cu}(\text{L}^1)\text{Cl}]\text{PF}_6$  (**1-Cl**) is illustrated in Figure 3. These results can be interpreted in terms of the formation of the six-coordinate aqua species,  $[\text{Cu}(\text{L})(\text{H}_2\text{O})\text{X}]^+$ , which presumably exists in equilibrium with the  $[\text{Cu}(\text{L})(\text{H}_2\text{O})]^{2+}$  ion. The behavior of these complexes in  $\text{CH}_3\text{CN}$  and  $\text{CH}_3\text{CN}\text{--}\text{H}_2\text{O}$  solutions can be represented in Scheme 2. The extent of this equilibrium and the molar conductivity of the equilibrated solution depend on the  $\text{H}_2\text{O}/\text{CH}_3\text{CN}$  volume ratios. A similar trend was observed in other Cu(II) chlorido and perchlorato complexes and recently in Co(II) complexes,  $[\text{Co}(\text{L})\text{Cl}]\text{ClO}_4/\text{PF}_6$  ( $\text{L} = \text{tripod tetraamine}$ ) in similar media [3,4].

In this scheme, we must emphasize on the fact that in pure  $\text{CH}_3\text{CN}$  solution, while the complex ions  $[\text{Cu}(\text{L})\text{Cl}]^+$  do not react with  $\text{CH}_3\text{CN}$ , their corresponding perchlorato species,  $[\text{Cu}(\text{L})\text{ClO}_4]^+$ , react leading to the formation of  $[\text{Cu}(\text{L})(\text{CH}_3\text{CN})]^{2+}$  as it has been supported by the molar conductivity measurements, which were in agreement with 1:2 electrolyte in the latter case (Table 1). In aqueous acetonitrile solutions, the complexes  $[\text{Cu}(\text{L})\text{X}]\text{ClO}_4/\text{PF}_6$  ( $\text{X} = \text{Cl}^-$  or  $\text{ClO}_4^-$ ) react instantaneously with  $\text{H}_2\text{O}$  with color change, which was accompanied with a blue shift in the visible spectral region and increased molar conductivity values to the limit that is somewhere between 1:1 and 1:2 electrolytes as the water contents increases. Attempts made to estimate the  $\text{p}K_a$  values of the coordinated  $\text{H}_2\text{O}$  in the aqua species  $[\text{Cu}(\text{L})(\text{H}_2\text{O})]^{2+}$  were unsuccessful. Probably due to its presence in equilibrium with the corresponding hydroxido species  $[\text{Cu}(\text{L})(\text{OH})]^+$ , which was proved to

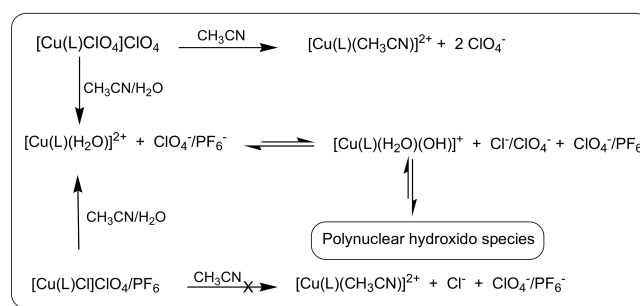
produce a dinuclear with bridged hydroxido derivatives (Scheme 2). This was supported by the isolation and structural characterization of  $[(L^1)Cu_2(\mu-OH)(H_2O)(OAc)_2Cl]$  [51].



**Figure 2.** Visible spectra of (A)  $1.14 \times 10^{-3}$  M of  $[Cu(L^1)Cl]PF_6$  (1-Cl), (B)  $8.87 \times 10^{-4}$  M of  $[Cu(L^4)Cl]ClO_4 \cdot H_2O$  (4-Cl), and (C)  $8.00 \times 10^{-4}$  M of  $[Cu(L^3)ClO_4]ClO_4$  (3-ClO<sub>4</sub>) in  $CH_3CN$  (1) and  $CH_3CN/H_2O$  (1:1 by volume) (2) solutions.



**Figure 3.** Spectrophotometric titration of  $[\text{Cu}(\text{L}^1)\text{Cl}]\text{PF}_6$  (**1-Cl**) ( $5.06 \times 10^{-4}$  M) in aqueous acetonitrile solutions at room temperature. The  $\text{H}_2\text{O}/\text{CH}_3\text{CN}$  volume ratios were as follows: (1) 0/10, (2) 0.3/9.7, (3) 0.6/9.4 (4) 1.5/8.5, (5) 2.0/8.0, (6) 2.6/7.4, (7) 3.3/6.7, and (8) 4.0/6.0.



**Scheme 2.** Proposed solvolysis and aquation reactions of  $[\text{Cu}(\text{L})\text{X}]\text{ClO}_4/\text{PF}_6$  complexes ( $\text{X} = \text{Cl}^-$  or  $\text{ClO}_4^-$ ;  $\text{L} = \text{L}^1\text{--L}^5$ ) in acetonitrile and aqueous acetonitrile solutions.

### 2.3. DNA Cleavage Studies

#### 2.3.1. Concentration Dependence

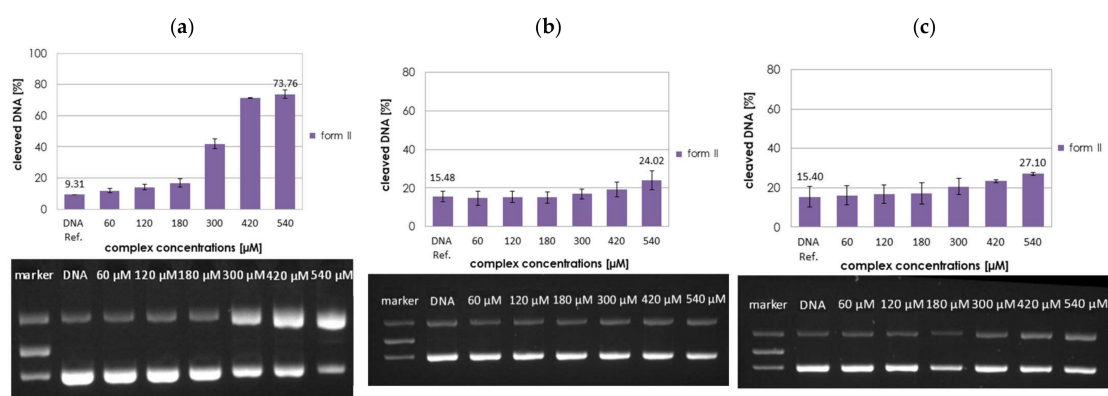
The cleavage activity of complexes  $[\text{Cu}(\text{L})(\text{ClO}_4)/\text{Cl}]\text{ClO}_4/\text{PF}_6$  was investigated towards plasmid DNA pBR322 at near-physiological conditions (10 mM MOPS (3-morpholino propane-1-sulfonic acid) buffer pH 7.4, 37 °C). The reactivity was monitored at 0, 60, 120, 180, 300, 420, and 540  $\mu\text{M}$  complex concentrations after 2 h of incubation (Figure 4 and Figure S10), by quantifying the conversion of supercoiled plasmid DNA (form I) into open circular (form II) and linear DNA (form III). These figures demonstrate that the nucleolytic activity of the investigated complexes is decreasing in the order: **1-Cl**  $\approx$  **1-ClO<sub>4</sub>** > **3-ClO<sub>4</sub>**  $\geq$  **2-ClO<sub>4</sub>**, with no discernible cleavage activity by **4-Cl**, **5-Cl**, and **6-ClO<sub>4</sub>** when compared with noncatalyzed plasmid DNA cleavage (background control).

A comparison between  $[\text{Cu}(\text{L}^1)(\text{ClO}_4)]\text{ClO}_4$ , **1-ClO<sub>4</sub>**, and  $[\text{Cu}(\text{L}^1)\text{Cl}]\text{PF}_6$ , **1-Cl** shows that the identity of the coordinated anionic ligand ( $\text{ClO}_4^-$  vs.  $\text{Cl}^-$  in the inner coordination sphere) and the counter ion ( $\text{ClO}_4^-$  vs.  $\text{PF}_6^-$ ) of the complexes have no influence on the cleavage activity. This is a common observation, which might be explained by the formation of the same reactive species,  $[\text{Cu}(\text{L})(\text{H}_2\text{O})]^{2+}$  in aqueous solutions in both complexes **1-ClO<sub>4</sub>** and **1-Cl** [3]. As expected, in comparison with the complexes, the corresponding ligands did not show any significant DNA cleavage activity, even at 540  $\mu\text{M}$  concentration (Figure S11).

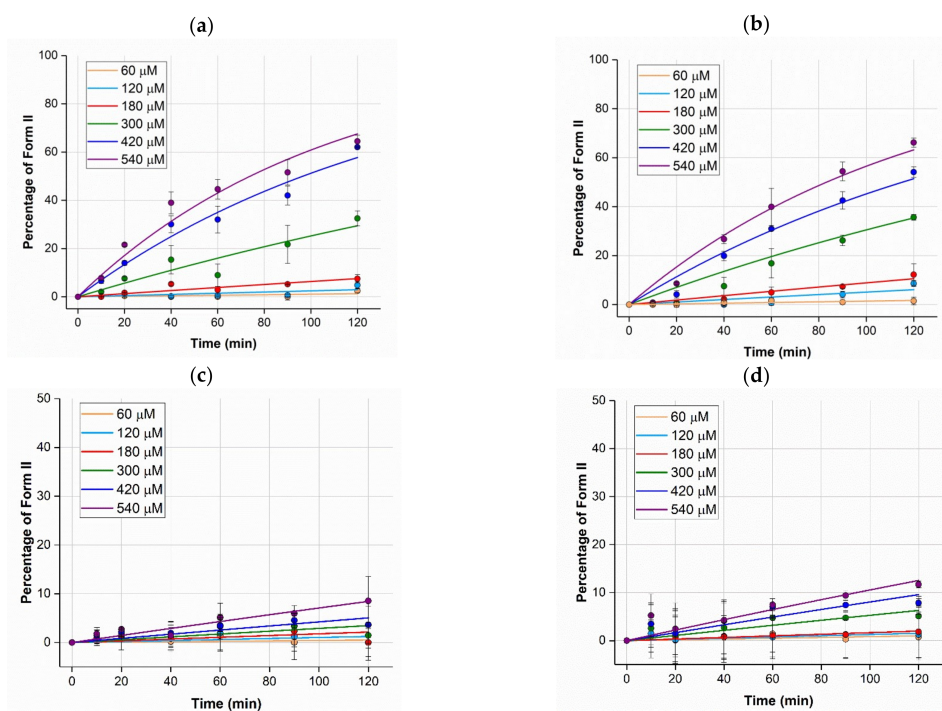
#### 2.3.2. Kinetics of DNA Cleavage

The conversion of DNA form I to form II was analyzed kinetically for the complexes **1-ClO<sub>4</sub>**–**3-ClO<sub>4</sub>** and **1-Cl** at concentrations of 0, 60, 120, 180, 300, 420, and 540  $\mu\text{M}$  after 10, 20, 40, 60, 90, and 120 min incubation time (Figure 5). Rates of reactions were performed under pseudo-first-order Michaelis–Menten kinetics, and the data were fitted to an exponential decay function (Equation (1), experimental section). The kinetic parameters, i.e., the

catalytic rate constant,  $k_{\text{cat}}$ , and the affinity constant,  $K_M$ , collected in Table 2, were derived from Lineweaver Burk plots ( $1/k_{\text{obs}}$  vs.  $1/[\text{Cu}]$ ). The values of  $k_{\text{cat}}$  decrease in the order:  $1\text{-Cl} \approx 1\text{-ClO}_4 > 3\text{-ClO}_4 \geq 2\text{-ClO}_4$ , corroborating the activity trend of the complexes based on concentration-dependent DNA cleavage data (*vide supra*). For complexes  $1\text{-ClO}_4$ – $3\text{-ClO}_4$  and  $1\text{-Cl}$  values in the range of  $0.1\text{--}0.8\text{ h}^{-1}$  were obtained. Rate enhancements over the uncatalyzed cleavage reaction ( $k = 3.6 \times 10^{-8}\text{ h}^{-1}$ ) [1] were in the range of 20 million for complexes **1**, but only around 2–4 million for the other tested complexes. This means that substituted pyridyl arms in the Cu(II)-piperazine complexes did not result in any significant higher reactivity compared to the unsubstituted pyridyl piperazine ligand system (complexes **1** with  $L^1$ ).



**Figure 4.** Cleavage of pBR322 plasmid DNA ( $0.025\text{ }\mu\text{g}/\mu\text{L}$ ) into form II by the complexes  $[\text{Cu}(\text{L})(\text{ClO}_4)]\text{ClO}_4$  at concentrations in the range of  $60\text{--}540\text{ }\mu\text{M}$  after an incubation time of 2 h, at pH 7.4 (10 mM MOPS) and  $37\text{ }^\circ\text{C}$ . (a)  $1\text{-ClO}_4$ , (b)  $2\text{-ClO}_4$ , and (c)  $3\text{-ClO}_4$ , Lane 1: marker and Lane 2: control without added complex.



**Figure 5.** Plot of the percentage of form II vs. incubation time for the cleavage of pBR322 plasmid DNA ( $0.025\text{ }\mu\text{g}/\mu\text{L}$ ) by complexes: (a)  $1\text{-ClO}_4$ , (b)  $1\text{-Cl}$ , (c)  $2\text{-ClO}_4$ , and (d)  $3\text{-ClO}_4$ , at varying concentrations ( $0\text{--}540\text{ }\mu\text{M}$ ), pH 7.4 (10 mM MOPS buffer), and  $37\text{ }^\circ\text{C}$ . The plots show the fitted function (Equation (1)) used to determine the observed rate constant,  $k_{\text{obs}}$  at each complex concentration.



**Table 2.** Pseudo-first-order Michaelis–Menten kinetic data for the cleavage of pBR322 plasmid DNA (0.025 µg/µL) by the complexes 1-ClO<sub>4</sub>-3-ClO<sub>4</sub> and 1-Cl at 37 °C and pH 7.4.

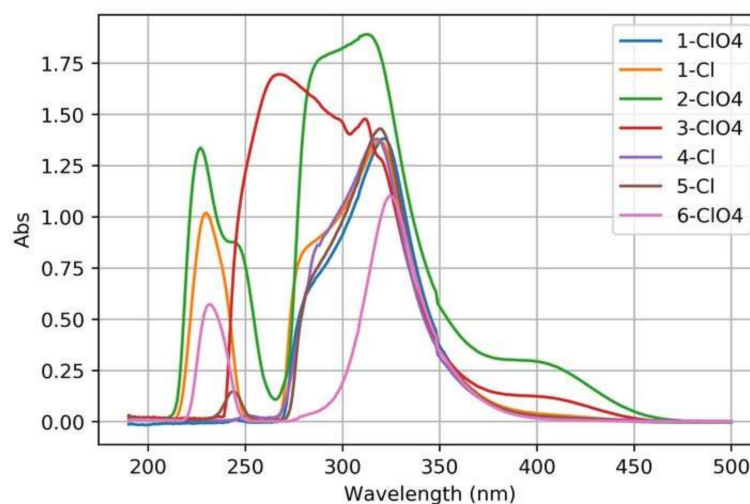
Complex	c (µM)	$k_{\text{obs}}$ (min <sup>-1</sup> )	$k_{\text{cat}}$ (h <sup>-1</sup> )	$K_{\text{M}}$ (M)	$k_{\text{cat}}/K_{\text{M}}$	Rate Enhancement <sup>(b)</sup>
1-ClO <sub>4</sub>	60	$2.65 \times 10^{-5}$	$7.79 \times 10^{-1}$	$5.03 \times 10^{-4}$	$5.58 \times 10^6$ <sup>(a)</sup>	$2.2 \times 10^7$
	120	$3.05 \times 10^{-5}$				
	180	$3.91 \times 10^{-5}$				
	300	$8.99 \times 10^{-5}$				
	420	$2.02 \times 10^{-4}$				
	540	$2.47 \times 10^{-4}$				
1-Cl	60	$2.44 \times 10^{-5}$	$8.23 \times 10^{-1}$	$5.89 \times 10^{-4}$	$5.03 \times 10^6$	$2.3 \times 10^7$
	120	$2.62 \times 10^{-5}$				
	180	$4.11 \times 10^{-5}$				
	300	$9.36 \times 10^{-5}$				
	420	$1.63 \times 10^{-4}$				
	540	$2.34 \times 10^{-4}$				
2-ClO <sub>4</sub>	60	$1.62 \times 10^{-5}$	$9.75 \times 10^{-2}$	$4.41 \times 10^{-5}$	$7.95 \times 10^6$	$2.7 \times 10^6$
	120	$1.86 \times 10^{-5}$				
	180	$2.02 \times 10^{-5}$				
	300	$2.25 \times 10^{-5}$				
	420	$2.58 \times 10^{-5}$				
	540	$2.81 \times 10^{-5}$				
3-ClO <sub>4</sub>	60	$1.84 \times 10^{-5}$	$1.55 \times 10^{-1}$	$9.72 \times 10^{-5}$	$5.72 \times 10^6$	$4.3 \times 10^6$
	120	$1.96 \times 10^{-5}$				
	180	$2.05 \times 10^{-5}$				
	300	$3.44 \times 10^{-5}$				
	420	$4.57 \times 10^{-5}$				
	540	$4.77 \times 10^{-5}$				

<sup>(a)</sup> The previously found values were  $k_{\text{cat}} = 8.89 \times 10^{-1} \text{ h}^{-1}$  and  $K_{\text{M}} = 3.35 \times 10^{-4} \text{ M}$  at pH 7.0 with pUC19 DNA (Reference [6]).

<sup>(b)</sup> Uncatalyzed DNA cleavage reaction  $k = 3.6 \times 10^{-8} \text{ h}^{-1}$  at 37 °C and pH 7.0 (Reference [1]).

#### 2.4. BNPP Assay

In order to further investigate the hydrolytic cleavage activity, BNPP (bis(*p*-nitrophenyl) phosphate) was used as a substrate to mimic the phosphate ester backbone of DNA [52]. Through cleavage of BNPP, the released *p*-nitrophenolate anion was monitored by UV-VIS spectroscopy, following the absorption increase at 400 nm [53]. As a positive control, phosphodiesterase was incubated for 2 h with BNPP (Figure S12). None of the studied complexes (1-ClO<sub>4</sub>-3-ClO<sub>4</sub>, 6-ClO<sub>4</sub>, 1-Cl, 4-Cl, and 5-Cl) was able to cleave BNPP measurably within 2 h (Figure S13), however, while 2-ClO<sub>4</sub> showed significant cleavage activity after 48 h incubation, some cleavage was observed for 3-ClO<sub>4</sub> and very little for 1-Cl (reactivity decreases: 2-ClO<sub>4</sub> > 3-ClO<sub>4</sub> >> 1-Cl) (Figure 6). These effects are more pronounced after 96 h (Figures S13 and S14). Thus, a hydrolytic cleavage mechanism can now be assumed for complexes 2 and 3.

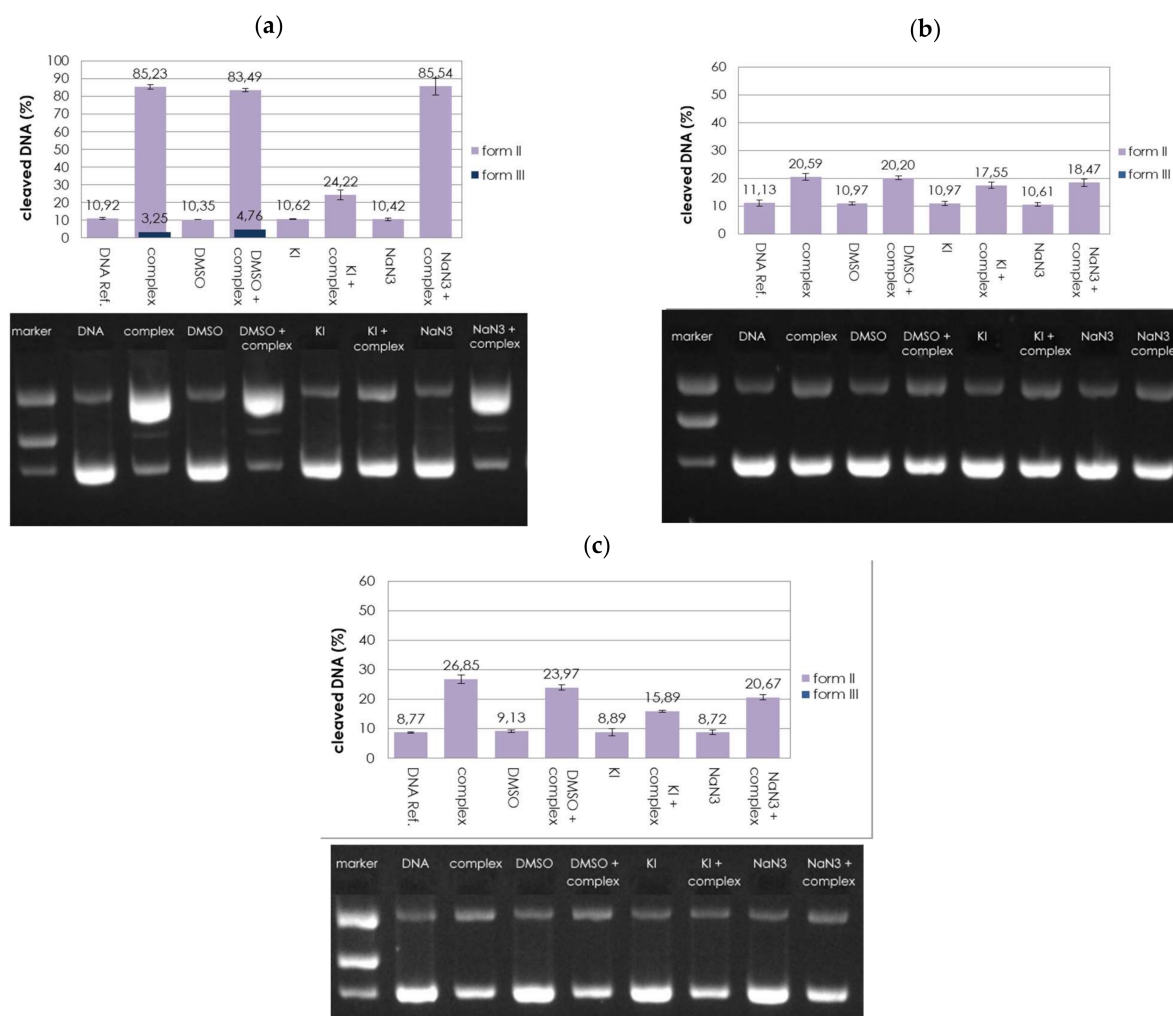


**Figure 6.** UV-VIS spectra of bis(*p*-nitrophenyl)phosphate (BNPP) (270  $\mu$ M) in MOPS buffer (10 mM, pH 7.4) in the presence of Cu(II) complexes (540  $\mu$ M) after 48 h incubation at 37  $^{\circ}$ C.

### 2.5. Reactive Oxygen Species (ROS)

In order to reveal the mechanistic pathway of the DNA cleavage (hydrolytic vs. redox) by the complexes under investigation **1-ClO<sub>4</sub>**, **2-ClO<sub>4</sub>**, and **3-ClO<sub>4</sub>** (540  $\mu$ M), it was necessary to study the role of redox species on the DNA cleavage process. This was done using quenchers for reactive oxygen species (DMSO for hydroxyl radicals, KI for peroxide, and NaN<sub>3</sub> for reactive singlet oxygen). The results are illustrated in Figure 7. These results indicate that DMSO did not reduce the cleavage activity in any of the complexes, which exclude the hydroxyl radicals as reactive species. The DNA cleavage activity was slightly affected by NaN<sub>3</sub> scavenger, which may reflect the minor contribution of singlet oxygen in the DNA cleavage mechanism. However, for some of the complexes, hydrogen peroxide was identified as the only ROS involved in DNA cleavage (Figure 7). Since such a quenching assay is sensitive regarding specific reaction conditions, the outcome of this experiment was verified exemplarily for **1-ClO<sub>4</sub>** with a fluorescence assay, where hydroxyl radicals and hydrogen peroxide are supposed to activate fluorogenic compounds (Figure S15) [54]. This test was positive for hydrogen peroxide and negative for hydroxyl radicals (singlet oxygen could not be tested).

Through inspection of the BNPP cleavage and ROS scavenging experiments one can get some insights into the mechanistic pathway of DNA cleavage by the title complexes. The DNA cleavage activity of **2-ClO<sub>4</sub>**, which efficiently cleaved BNPP and was slightly reduced when quenchers were added, leads us to exclude the oxidative mechanism and support the hydrolysis as the dominant mechanism. The same applies also for **3-ClO<sub>4</sub>** even though the situation is less clear and both mechanisms might contribute to the activity. In addition, it should be considered that BNPP and DNA are only conditionally comparable, i.e., BNPP is a DNA mimic only with respect to the phosphate diester, other properties like the secondary structure cannot be represented by BNPP. In case of **1-ClO<sub>4</sub>**, which was much less active for BNPP cleavage, an oxidative cleavage mechanism with hydrogen peroxide as a dominant ROS can be considered, since DNA cleavage was almost completely reduced in the presence of KI. This mechanistic detail had not been revealed in previous work [6]. Since no reducing agent was present for DNA cleavage, and the buffer improbably represents reducing agent (e.g., MOPS is inefficiently oxidized by H<sub>2</sub>O<sub>2</sub> [55]), the generation of ROS is not obvious, since they are commonly formed by reduction of Cu(II) and then reaction of Cu(I) with O<sub>2</sub>. Thus, self-activated DNA cleavage is to be assumed. This usually implies the ligand scaffold as the reducing species [10,56], however, also a redox reaction with the DNA itself (especially the guanine nucleobase) is conceivable [9].



**Figure 7.** Cleavage of pBR322 plasmid DNA (0.025  $\mu\text{g}/\mu\text{L}$ ) by complexes (540  $\mu\text{M}$ ): (a) 1-ClO<sub>4</sub>, (b) 2-ClO<sub>4</sub>, and (c) 3-ClO<sub>4</sub> after an incubation time of 2 h at 37 °C in the presence and absence of ROS scavengers (0.4 M DMSO, 500  $\mu\text{M}$  KI, 500  $\mu\text{M}$  NaN<sub>3</sub>) in MOPS buffer (10 mM, pH 7.4).

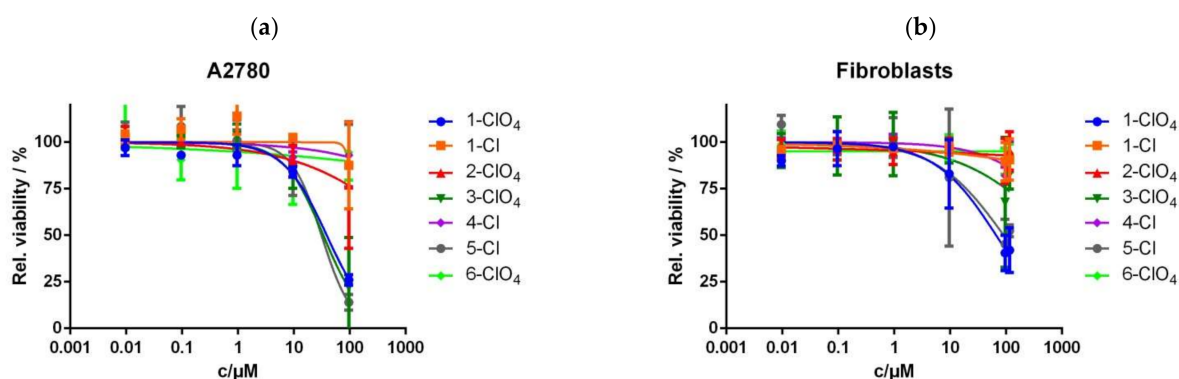
Thus, it was demonstrated that under pseudo-first-order Michaelis–Menten conditions, the DNA cleavage activity by the chlorido or perchlorato complexes 1–6 is sensitive to the ligand framework, which have pronounced influence not only on the rate of reactions but also on the preferred reaction mechanism; the catalytic rate constant,  $k_{\text{cat}}$ , decreases in the series  $1 > 3 \geq 2$ , whereas complexes 4–6 were found inactive. As it can be derived from the molecular structures of the inactive complexes 4-Cl and 5-Cl (Figure 1), the methoxy substituents at the pyridine moieties might hinder and/or suppress the intercalation of the complexes with DNA and hence their DNA cleavage activity. Previous results showed that increasing number of methoxy substituents at the pyridine moieties of pyridyl-derived ligand systems improves the hydrolytic DNA cleavage activity, but this trend could not be confirmed with the current ligands [3,11]. In addition, the molecular structure of complex 6-ClO<sub>4</sub>, which does not show DNA cleavage activity, indicates that the Cu(II) center is sterically shielded [47] and hence not easily accessible for neither hydrolytic (cleavage of the phosphate ester backbone) nor oxidative (reaction with O<sub>2</sub> for ROS generation) DNA cleavage reactions.

In this study, results of BNPP and ROS quenching examinations indicate that the most reactive complexes 1-ClO<sub>4</sub>/Cl seem to favor an oxidative pathway mechanism, whereas 2 and 3 dominantly cleave DNA in a hydrolytic fashion. Rate enhancements are 5–10 times higher for complexes 1 than for 2 and 3. Among the hydrolytically cleaving nucleases,

**3-ClO<sub>4</sub>** can intercalate into DNA more easily in comparison to **2-ClO<sub>4</sub>** due to the more extended quinoline-derived ligand system of **L<sup>3</sup>** compared to the pyridine-derived ligand of **L<sup>2</sup>** and this explains the reactivity trend **3-ClO<sub>4</sub>** > **2-ClO<sub>4</sub>**.

## 2.6. Cytotoxicity Studies

Cytotoxicity of the Cu(II) complexes was analyzed by means of the MTT assay in vitro in human ovarian carcinoma cells A2780 and human fibroblasts. The cells were incubated with different concentrations of the compounds (0.0096, 0.096, 0.96, 9.6, 96, and 115 μM). Results of the MTT assay in A2780 cells and the calculated IC<sub>50</sub> values are depicted in Figure 8, and other results in human fibroblast cells are shown in Figure S16. The IC<sub>50</sub> values for the two studied cell lines A2780 and fibroblasts are tabulated in Table 3. Inspection of the data in Table 3 reveals that the IC<sub>50</sub> values of 30–40 μM were found for **1-ClO<sub>4</sub>**, **3-ClO<sub>4</sub>**, and **5-Cl**, whereas the other complexes did not show cytotoxic activity. Even though IC<sub>50</sub> values are not that low for complexes **1-ClO<sub>4</sub>**, **3-ClO<sub>4</sub>**, and **5-Cl**, i.e., these compounds are not that cytotoxic, an important finding is that in fibroblasts, these values were much higher for **1-ClO<sub>4</sub>** (66.3 μM) and **5-Cl** (90.7 μM). For **3-ClO<sub>4</sub>** complex, even no effect on the fibroblasts was found.



**Figure 8.** Results of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in A2780 cells (a) and fibroblasts (b). Corresponding bar diagrams can be found in the Supplementary (Figure S16).

**Table 3.** The IC<sub>50</sub> values (95% confidence interval) in A2780 and fibroblasts cells.

Complex	IC <sub>50</sub> Value (μM) in A2780 Cells	IC <sub>50</sub> Value (μM) in Fibroblasts
<b>1-ClO<sub>4</sub></b>	38.7 (27.7–53.9)	66.4 (44.1–99.1)
1-Cl	>100	>100
<b>2-ClO<sub>4</sub></b>	>100	>100
<b>3-ClO<sub>4</sub></b>	33.6 (18.1–62.6)	>100
4-Cl	>100	>100
5-Cl	31.2 (18.1–53.9)	90.7 (51.0–161.5)
6-ClO <sub>4</sub>	>100	>100

Ligand **L<sup>3</sup>**, which shows slight DNA cleavage, was the only ligand to exhibit cytotoxicity—but only in the cancer cells (77.3 μM, in fibroblasts > 100 μM). This suggests that the ligand might also be involved in the high cytotoxicity of complex **3-ClO<sub>4</sub>** in A2780 cells. Cell uptake of metal complexes, which is an important prerequisite for their cytotoxicity, is usually dependent on their lipophilicity [57], and the quinoline-based ligand **L<sup>3</sup>** is supposed to be more lipophilic than the pyridine-based ligands. Selectivity for certain cell lines has been observed before for quinoline-derived compounds [58].

### 3. Materials and Methods

#### 3.1. General

Piperazine and the hydrochloride salts of 2-chloromethylquinoline, 2-chloromethylpyridine, 3,4-dimethoxy-2-chloromethylpyridine, and 3,5-dimethyl-4-methoxy-2-chloromethylpyridine were purchased from TCI-America. Further, 6-Methyl-2-pyridinemethanol was purchased from Alfa Aesar, whereas 2-vinylpyridine was obtained from Aldrich Chem. Comp., and purified by column chromatography using alumina eluted with Et<sub>2</sub>O before use. All other reagents were used without further purification. Infrared spectra were recorded on a Cary 630 (ATR-IR) spectrometer. NMR (<sup>1</sup>H and <sup>13</sup>C) spectra were obtained at room temperature on a Varian 400 NMR spectrometer operating at 400 MHz (<sup>1</sup>H) and 100 MHz (<sup>13</sup>C). Moreover, <sup>1</sup>H and <sup>13</sup>C NMR chemical shifts (δ) are reported in ppm and were referenced internally to residual solvent resonances (DMSO-*d*<sub>6</sub>: δ<sub>H</sub> = 2.49, δ<sub>C</sub> = 39.4 ppm). ESI-MS were measured on LC-MS Varian Saturn 2200 Spectrometer and electronic spectra were recorded using an Agilent 8453 HP diode UV-VIS spectrophotometer. Elemental analyses were carried out by the Atlantic Microlab, Norcross, GA, USA.

The molar conductivity of a solution sample was determined from  $\Lambda_M = (1.0 \times 10^3 \kappa)/[\text{Cu}]$ , where  $\kappa$  = specific conductance and [Cu] is the molar concentration of the complex. The measurements were performed using Mettler Toledo SevenEasy conductometer and calibrated with 1413 μS/cm conductivity standard.

Caution: Salts of perchlorate and their metal complexes are potentially explosive and should be handled with great care and in small quantities.

#### 3.2. Syntheses

In brief, 6-methyl-2-pyridinemethanol was converted into 6-methyl-2-chloromethylpyridine hydrochloride with the aid of SOCl<sub>2</sub> in CHCl<sub>3</sub>, followed by recrystallization from ethanol. Further, 1,4-bis[(2-pyridinyl)methyl]piperazine (L<sup>1</sup>) and 1,4-bis[(2-pyridinyl)-(2-ethyl)]piperazine (L<sup>6</sup>) were prepared according to the published literature [6,47]. The compounds L<sup>2</sup>–L<sup>5</sup> were synthesized as follows:

##### 3.2.1. 1,4-Bis[(6-methyl)-(2-pyridinyl)methyl]piperazine (L<sup>2</sup>· $\frac{1}{4}$ H<sub>2</sub>O)

To a mixture containing piperazine (0.431 g, 5 mmol) and 6-methyl-2-chloromethylpyridine hydrochloride (1.78 g, 10 mmol) suspended in anhydrous CH<sub>3</sub>CN (40 mL), excess K<sub>2</sub>CO<sub>3</sub> (2.08 g, 15 mmol) was added. Nitrogen gas was purged through the mixture, which was magnetically stirred under gentle reflux for 3 days, during which color turned bright yellow. The resulting mixture was cooled in the refrigerator and filtered off to remove KCl and unreacted K<sub>2</sub>CO<sub>3</sub>. The solvent was removed with a rotary evaporator under reduced pressure and the resulting crude product was recrystallized from Et<sub>2</sub>O with aid of activated charcoal. The solvent was evaporated and the desired product was obtained as a light-yellow solid upon drying in a desiccator containing P<sub>4</sub>O<sub>10</sub> under vacuum (overall yield: 1.25 g, 84%). m.p. = 108–112 °C. Found: C, 71.56; H, 8.10; N, 18.26%. Calcd for C<sub>18</sub>H<sub>24</sub>N<sub>4</sub>· $\frac{1}{4}$ H<sub>2</sub>O (MM = 296.410 g/mol): C, 71.85; H, 8.21; N, 18.62%. Selected IR (ATR-IR, cm<sup>-1</sup>): 2953 (vw), 2919 (vw), 2881 (vw), 2802 (m) (aliphatic C–H stretching), 1592 (s), 1576 (s), 1458 (s), 1446 (s) (C=C, C=N stretching of pyridyl rings), 1009 (s), 933 (m), 840 (s), 788 (vs), 757 (s) (C–H out of plane bending). ESI-MS in MeOH: *m/z* = 297.206 (100%) (Calcd for [M + H]<sup>+</sup> = 297.208) (Figure S5). <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 400 MHz, δ in ppm): δ = 2.42 (6H, s, –CH<sub>3</sub>), 3.32 (s, –CH<sub>2</sub>-pipz), 3.53 (s, 2H, –CH<sub>2</sub> of substituted py), 7.08, 7.10 and 7.19, 7.21 (d, 1H, each corresponds to one CH-py), 7.60, 7.62, 7.64 (t, 1H, CH-py) (Figure S1). <sup>13</sup>C NMR: (DMSO-*d*<sub>6</sub>, 100 MHz) δ = 23.92 (CH<sub>3</sub>-py), 52.82, 63.78 (CH<sub>2</sub>-py and CH<sub>2</sub>-pipz), 119.49, 121.20, 136.61, 156.92, 157.70 (pyridyl carbons) (Figure S1).

##### 3.2.2. 1,4-Bis[(2-quinolyl)methyl]piperazine (L<sup>3</sup>· $\frac{1}{4}$ H<sub>2</sub>O)

To a mixture containing piperazine (0.431 g, 5 mmol) and 2-chloromethylquinoline hydrochloride (2.15 g, 10 mmol) suspended in anhydrous THF (40 mL), an excess of anhydrous K<sub>2</sub>CO<sub>3</sub> (2.074 g, 15 mmol) was added. Nitrogen gas was purged through the

mixture, which was magnetically stirred under gentle reflux for 3 days, during which color turned to orange-brown. The resulting mixture was cooled in the refrigerator and filtered off to remove KCl and unreacted  $K_2CO_3$ , and the solvent was removed with a rotary evaporator under reduced pressure. The resulting residue was extracted with  $CH_2Cl_2$  (15 mL  $\times$  3) in 10% NaOH (20 mL) and saturated NaCl (20 mL), and this was then washed with  $H_2O$  (20 mL  $\times$  3). The combined organic layers were collected and solvent was evaporated by rotary evaporator. The resulting solid was recrystallized from benzene with the aid of activated charcoal, followed by the addition of anhydrous  $MgSO_4$ . The desired product was obtained as off-white to pale yellow color and dried in a desiccator containing  $P_4O_{10}$  under vacuum (yield 0.90 g, 46%). m.p. = 154–158 °C. Found: C, 61.13; H, 7.17; N, 14.28%. Calcd for  $C_{24}H_{28}N_4 \cdot \frac{1}{2}H_2O$  (MM = 389.22 g/mol): C, 61.13; H, 7.31; N, 14.26%. Selected IR (ATR-IR,  $cm^{-1}$ ): 3394 (m,b) (H–O water of crystallization), 3043 (w), 3011 (vw) (quinoliny C–H stretching), 2931 (w), 2864 (w), 2803 (m) (aliphatic C–H stretching), 1618 (s), 1601 (s), 1560 (m), 1503 (m), 1450 (m), 1423 (s) (C=C, C=N stretching of quinolyl rings), 837 (vs), 815 (vs), 785 (vs), 751 (vs) (C–H out of plane bending). ESI-MS in MeOH:  $m/z$  = 369.207 (100%) (Calcd for  $[M + H]^+$  = 369.208),  $m/z$  = 391.190 (100%) (Calcd for  $[M + Na]^+$  = 391.189) (Figure S5).  $^1H$  NMR (DMSO- $d_6$ , 400 MHz,  $\delta$  in ppm):  $\delta$  = 3.32 (2H, s,  $CH_2$ -pipz), 3.76 (2H, s,  $CH_2$ -py), 7.54 (1H, t), 7.62 (1H, d), 7.73 (1H, t), 7.94 (2H, t), 8.30 (1H, d) (quinolyl protons).  $^{13}C$  NMR: (DMSO- $d_6$ , 100 MHz)  $\delta$  = 52.96 ( $CH_2$ -pipz), 64.35 ( $CH_2$ -quinoline); 120.96, 126.07, 126.94, 127.73, 128.43, 129.35, 136.27, 146.97, 159.44 (quinolyl carbons) (Figure S2).

### 3.2.3. 1,4-Bis[(3,4-dimethoxy-2-pyridinyl)methyl]piperazine ( $L^4 \cdot \frac{1}{4}H_2O$ )

To a mixture containing piperazine (0.431 g, 5 mmol) and 3,4-dimethoxy-2-chloromethylpyridine hydrochloride (2.24 g, 10 mmol) suspended in anhydrous  $CH_3CN$  (40 mL), triethylamine (2.024 g, 20 mmol) was added and nitrogen gas was purged through the mixture, which was magnetically stirred under gentle reflux for 3 days, during which color turned brown. The resulting mixture was cooled in the refrigerator and filtered off to remove  $Et_3NHCl$ , and the solvent was removed under reduced pressure. The resulting residue was extracted with  $CH_2Cl_2$  (15 mL  $\times$  3) in 10% NaOH (20 mL) and saturated NaCl (20 mL), and then, it was washed with  $H_2O$  (20 mL  $\times$  3). The combined organic layers were collected and solvent was evaporated by rotary evaporator. The crude product was recrystallized from EtOAc with the aid of activated charcoal, followed by the addition of anhydrous  $MgSO_4$ . The product was obtained as off-white solid after drying over  $P_4O_{10}$  under vacuum (yield 1.0 g, 54%). m.p. = 135–138 °C. Found: C, 61.13; H, 7.17; N, 14.19%. Calcd for  $C_{24}H_{28}N_4O_4 \cdot \frac{1}{2}H_2O$  (MM = 392.96 g/mol): C, 61.13; H, 7.31; N, 14.26%. Selected IR (ATR-IR,  $cm^{-1}$ ): 3393 (w, b) (H–O water of crystallization), 3089 (vw) (pyridyl C–H stretching), 2965 (vw), 2932 (w), 2811 (m) (aliphatic C–H stretching), 1624 (w), 1580 (s), 1484 (s), 1444 (s), 1421 (s) (C=C, C=N stretching of pyridyl rings), 1296 (vs); 1256 (m), 1227 (m) (asymmetric C–O–C stretching), 1077 (vs), 994 (vs) (symmetric C–O–C stretching), 808 (s), 732 (s) (C–H out of plane bending). ESI-MS in MeOH:  $m/z$  = 389.219 (100%) (Calcd for  $[M + H]^+$  = 389.218),  $m/z$  = 411.200 (100%) (Calcd for  $[M + Na]^+$  = 411.200).  $^1H$  NMR (DMSO- $d_6$ , 400 MHz,  $\delta$  in ppm):  $\delta$  = 3.50 (4H, s,  $CH_2$ -pipz), 3.76 (6H, s), 3.86 (6H, s) ( $CH_3O$ -py), 7.02 (2H, d), 8.12 (2H, d) (substituted pyridyl protons).  $^{13}C$  NMR: (DMSO- $d_6$ , 100 MHz)  $\delta$  = 52.98, 55.74 ( $CH_2$ -pipz); 68.37, 60.61 ( $CH_2$ -py); 107.74, 143.96, 144.92, 151.65, 158.23 (pyridyl carbons) (Figure S3).

### 3.2.4. 1,4-Bis[(3,5-dimethyl-4-methoxy-2-pyridinyl)methyl]piperazine ( $L^5$ )

To a mixture containing piperazine (0.431 g, 5 mmol) and 3,5-dimethyl-4-methoxy-2-chloromethylpyridine hydrochloride (2.22 g, 10 mmol) suspended in anhydrous  $CH_3CN$  (40 mL), anhydrous  $K_2CO_3$  (2.07 g, 15 mmol) was added. Nitrogen gas was purged through the reaction mixture, which was magnetically stirred under gentle reflux for 3 days, during which color turned brown. The mixture was cooled in the refrigerator and filtered off, and then, the solvent was removed under reduced pressure. The resulting residue was extracted with  $CH_2Cl_2$  (15 mL  $\times$  3) in 10% NaOH (20 mL) and saturated NaCl (20 mL),

and then it washed with H<sub>2</sub>O (20 mL × 3). The combined organic layers were collected and solvent was evaporated by rotary evaporator. The crude product was recrystallized from Et<sub>2</sub>O with the aid of activated charcoal, followed by the addition of anhydrous MgSO<sub>4</sub>. The product was obtained as off-white solid after drying over P<sub>4</sub>O<sub>10</sub> under vacuum (yield 0.8 g, 42%). m.p. = 140–143 °C. Found: C, 68.85; H, 8.52; N, 14.22%. Calcd for C<sub>22</sub>H<sub>32</sub>N<sub>4</sub>O<sub>2</sub> (MM = 384.25 g/mol): C, 68.72; H, 8.39; N, 14.57%. Selected IR (ATR-IR, cm<sup>-1</sup>): 2999 (vw), (pyridyl C–H stretching), 2924 (w), 2810 (aliphatic C–H stretching), 1640 (w), 1560 (s), 1454 (s), 1397 (s), 1340 (m), 1289 (s) (C=C, C=N stretching of pyridyl rings), 1252 (s); 1147 (s) (asymmetric C–O–C stretching), 1094 (s), 1004 (vs) (symmetric C–O–C stretching), 769 (s) (C–H out of plane bending). ESI-MS in MeOH: *m/z* = 385.261 (100%) (Calcd for [M + H]<sup>+</sup> = 385.260), *m/z* = 407.242 (Calcd for [M + Na]<sup>+</sup> = 407.242). <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 400 MHz, δ in ppm): δ = 2.17 (3H, s), 2.25 (3H, s) (CH<sub>3</sub>-py); 3.71 (2H, s), 3.49 (2H, s) CH<sub>2</sub>-pipz; 3.32 (3H, s, CH<sub>3</sub>O); 8.09 (1H, s, pyridyl proton). <sup>13</sup>C NMR: (DMSO-*d*<sub>6</sub>, 100 MHz) δ = 10.63, 12.87 (CH<sub>3</sub>-py); 52.91 (CH<sub>2</sub>-pipz); 59.61, 62.68 (CH<sub>2</sub>-py); 125.10, 126.11, 148.35, 156.76, 163.84 (pyridyl carbons) (Figure S4).

### 3.2.5. Synthesis of Cu(II) Complexes

The two complexes [Cu(L<sup>1</sup>)ClO<sub>4</sub>]ClO<sub>4</sub> (**1-ClO<sub>4</sub>**) and [Cu(L<sup>6</sup>)ClO<sub>4</sub>]ClO<sub>4</sub> (**6-ClO<sub>4</sub>**) were prepared and characterized according to the published procedure [6,47]. Characterization of **1-ClO<sub>4</sub>**: selected IR bands (ATR-IR, cm<sup>-1</sup>): 2955 (vw), 2932 (vw), 2884 (vw) (aliphatic C–H stretching), 1612 (s), 1570 (w), 1488 (m), 1437 (s) (C=C, C=N stretching of pyridyl rings), 1082 (vs), 1052 (vs) (ν<sub>as</sub>(Cl–O) stretching of ClO<sub>4</sub><sup>-</sup>). UV-VIS spectrum (λ<sub>max</sub>, nm (ε, M<sup>-1</sup>cm<sup>-1</sup>)) in CH<sub>3</sub>CN: 645 (310). Molar conductivity, Λ<sub>M</sub> (CH<sub>3</sub>CN) = 300 and Λ<sub>M</sub> (H<sub>2</sub>O) = 188 Ω<sup>-1</sup> cm<sup>2</sup> mol<sup>-1</sup>. Characterization of **6-ClO<sub>4</sub>**: Selected IR bands (KBr, cm<sup>-1</sup>): 1112 (vs), 1091 (vs) (ν<sub>a</sub>(Cl–O) stretching of ClO<sub>4</sub><sup>-</sup>). UV-VIS spectrum (λ<sub>max</sub>, nm (ε, M<sup>-1</sup>cm<sup>-1</sup>)) in CH<sub>3</sub>CN 645 (310) [6,47]. Molar conductivity, Λ<sub>M</sub> (CH<sub>3</sub>CN) = 300 Ω<sup>-1</sup> cm<sup>2</sup> mol<sup>-1</sup>.

### 3.2.6. [Cu(L<sup>2</sup>)ClO<sub>4</sub>]ClO<sub>4</sub> (**2-ClO<sub>4</sub>**)

A mixture of 1,4-bis[(6-methyl)-(2-pyridinyl)methyl]piperazine (L<sup>2</sup>·¼H<sub>2</sub>O) (0.075 g, 0.250 mmol) and Cu(ClO<sub>4</sub>)<sub>2</sub>·6H<sub>2</sub>O (0.095 g, 0.256 mmol) were dissolved in MeOH (15 mL). The intense blue solution was heated on steam bath for 5 min, filtered through celite, and then allowed to crystallize at room temperature. The blue crystalline compound, which separated after 2 days, was collected by filtration, washed with propan-2-ol, Et<sub>2</sub>O, and air dried (yield: 0.121 g, 87%). Found: C, 38.64; H, 4.40; N, 9.85%. Calcd for C<sub>18</sub>H<sub>24</sub>Cl<sub>2</sub>CuN<sub>4</sub>O<sub>8</sub> (MM = 558.86 g/mol): C, 38.68; H, 4.33; N, 10.03%. Selected IR bands (ATR-IR, cm<sup>-1</sup>): 3069 (vw) (pyridyl C–H stretching), 2930 (w), 2869 (vw) (aliphatic C–H stretching), 1610 (m), 1578 (w), 1465 (m), 1443 (m) (C=C, C=N stretching of pyridyl rings), 1095 (vs), 1065 (vs) (ν<sub>as</sub>(Cl–O) stretching of ClO<sub>4</sub><sup>-</sup>). UV-VIS spectrum (λ<sub>max</sub>, nm (ε, M<sup>-1</sup>cm<sup>-1</sup>)) in CH<sub>3</sub>CN: ~752 (299). ESI-MS in CH<sub>3</sub>CN: *m/z* = 458.035 (Calcd for [Cu(L<sup>2</sup>)ClO<sub>4</sub>]<sup>+</sup> = 458.088) (Figure S7); *m/z* = 98.949 (Calcd for [ClO<sub>4</sub>]<sup>-</sup> = 98.95 (100%). Λ<sub>M</sub> (CH<sub>3</sub>CN) = 297 Ω<sup>-1</sup> cm<sup>2</sup> mol<sup>-1</sup>.

### 3.2.7. [Cu(L<sup>3</sup>)ClO<sub>4</sub>]ClO<sub>4</sub> (**3-ClO<sub>4</sub>**)

This complex was synthesized using a procedure similar to that described above except 1,4-bis[(2-quinolyl)methyl]piperazine (L<sup>3</sup>) was used instead of L<sup>2</sup>. The product was further recrystallized from MeOH to afford green crystalline compound (yield: 87%). Found: C, 45.60; H, 3.84; N, 8.81%. Calcd for C<sub>24</sub>H<sub>24</sub>Cl<sub>2</sub>CuN<sub>4</sub>O<sub>8</sub> (MM = 630.92 g/mol): C, 45.69; H, 3.83; N, 8.88%. Selected IR bands (ATR-IR, cm<sup>-1</sup>): 3071 (vw), 3032 (vw) (pyridyl C–H stretching), 2971 (vw), 2888 (vw) (aliphatic C–H stretching), 1617 (m), 1604 (m), 1567 (w), 1517 (m), 1465 (m), 1435 (m) (C=C, C=N stretching of quinolyl rings), 1076 (vs), 1063 (vs) (ν<sub>a</sub>(Cl–O) stretching of ClO<sub>4</sub><sup>-</sup>). UV-VIS spectrum (λ<sub>max</sub>, nm (ε, M<sup>-1</sup>cm<sup>-1</sup>)) in CH<sub>3</sub>CN: ~565 (sh), ~618 (sh), ~745 (226). ESI-MS in CH<sub>3</sub>CN: *m/z* = 530.033 (Calcd for [Cu(L<sup>3</sup>)ClO<sub>4</sub>]<sup>+</sup> = 530.088) (Figure S8); *m/z* = 98.949 (Calcd for [ClO<sub>4</sub>]<sup>-</sup> = 98.95 (100%). Λ<sub>M</sub> (CH<sub>3</sub>CN) = 301 Ω<sup>-1</sup> cm<sup>2</sup> mol<sup>-1</sup>.

### 3.2.8. [Cu(L<sup>4</sup>)Cl]ClO<sub>4</sub>·½H<sub>2</sub>O (**4-Cl**)

To a well-stirred warm solution containing 1,4-bis[(3,4-dimethoxy-2-pyridinyl)methyl]-piperazine (L<sup>4</sup>) (0.0971 g 0.25 mmol) and CuCl<sub>2</sub>·2H<sub>2</sub>O (0.043 g, 0.25 mmol) dissolved in MeOH (15 mL), NaClO<sub>4</sub> (0.032 g, 0.26 mmol) was added, and the solution was allowed to stand at room temperature. After 1 h, the well-shaped blue long needles were collected by filtration. These were washed with propan-2-ol and Et<sub>2</sub>O and then were air dried (yield: 81%). Found: C, 40.31; H, 4.91; N, 9.40%. Calcd for C<sub>20</sub>H<sub>28</sub>Cl<sub>2</sub>CuN<sub>4</sub>O<sub>12</sub>·½H<sub>2</sub>O (MM = 595.92 g/mol): C, 40.64; H, 5.06; N, 9.00%. Selected IR bands (ATR-IR, cm<sup>-1</sup>): 3547 (m) (ν<sub>as</sub>(H–O) stretching of H<sub>2</sub>O); 3090 (vw) (pyridyl C–H stretching), 2947 (w), 2883 (vw), 2837 (vw) (aliphatic C–H stretching); 1597 (s), 1497 (s), 1432 (m), 1436 (m) (C=C, C=N stretching of pyridyl rings), 1304 (s), 1232 (s) (asymmetric C–O–C stretching), 1084 (s), 1052 (vs) (ν<sub>as</sub>(Cl–O) stretching of ClO<sub>4</sub><sup>-</sup>). UV-VIS spectrum (λ<sub>max</sub>, nm (ε, M<sup>-1</sup>cm<sup>-1</sup>)) in CH<sub>3</sub>CN: 771 (324). ESI-MS (MeCN): *m/z* = 486.109 (Calcd for [Cu(L<sup>4</sup>)Cl]<sup>+</sup> = 486.109), *m/z* = 98.949 (Calcd for [ClO<sub>4</sub>]<sup>-</sup> = 98.95) (100%). Λ<sub>M</sub> (CH<sub>3</sub>CN) = 145 Ω<sup>-1</sup> cm<sup>2</sup> mol<sup>-1</sup>.

### 3.2.9. [Cu(L<sup>5</sup>)Cl]PF<sub>6</sub> (**5-Cl**)

To mixture of 1,4-bis[(3,5-dimethyl-4-methoxy-2-pyridinyl)methyl]-piperazine (L<sup>5</sup>) (0.105 g 0.25 mmol) and CuCl<sub>2</sub>·2H<sub>2</sub>O (0.043 g, 0.25 mmol) dissolved in MeOH (15 mL), (NH<sub>4</sub>)PF<sub>6</sub> (0.041 g, 0.25 mmol) was added. The resulting blue solution was warmed for 5 min on a steam bath and then allowed to stand at room temperature. On the following day, the separated precipitate was collected by filtration. Recrystallization of the crude product from warm MeOH resulted in unidentified green precipitate, which was discarded, and evaporation of the blue solution led to the isolation of shiny blue well-shaped crystals suitable for X-ray. These were washed with propan-2-ol and Et<sub>2</sub>O and then were air dried (yield: 70%). Found: C, 41.72; H, 5.25; N, 8.83%. Calcd for C<sub>22</sub>H<sub>32</sub>ClCuF<sub>6</sub>N<sub>4</sub>O<sub>2</sub>P (MM = 628.48 g/mol): C, 42.04; H, 5.13; N, 8.91%. Selected IR bands (ATR-IR, cm<sup>-1</sup>): 2926 (vw), 2880 (vw) (aliphatic C–H stretching); 1599 (m), 1496 (w), 1477 (m), 1461 (m), 1403 (m) (C=C, C=N stretching of pyridyl rings), 1287 (m), 1245 (m) (asymmetric C–O–C stretching), 1084 (s), 832 (vs) (ν<sub>as</sub>(F–P) stretching of PF<sub>6</sub><sup>-</sup>). UV-VIS spectrum (λ<sub>max</sub>, nm (ε, M<sup>-1</sup>cm<sup>-1</sup>)) in CH<sub>3</sub>CN: 765 (352). ESI-MS (MeCN): *m/z* = 482.150 (Calcd for [Cu(L<sup>5</sup>)Cl]<sup>+</sup> = 482.159) (100%) (Figure S9). Λ<sub>M</sub> (CH<sub>3</sub>CN) = 162 Ω<sup>-1</sup> cm<sup>2</sup> mol<sup>-1</sup>.

### 3.2.10. [Cu(L<sup>1</sup>)Cl]PF<sub>6</sub> (**1-Cl**)

This complex was synthesized using a similar procedure as that described above for [Cu(L<sup>5</sup>)Cl]PF<sub>6</sub> (**5-Cl**), except 1,4-bis[(2-pyridinyl)methyl]piperazine (L<sup>1</sup>) was used instead of L<sup>5</sup> (Yield: 68%): Found: C, 37.55; H, 3.93; N, 10.84%. Calcd for C<sub>16</sub>H<sub>20</sub>ClCuF<sub>6</sub>N<sub>4</sub>P (MM = 512.32 g/mol): C, 37.51; H, 3.93; N, 10.94%. Selected IR bands (ATR-IR, cm<sup>-1</sup>): 2927 (vw), 2890 (vw) (aliphatic C–H stretching), 1607 (s), 1485 (m), 1444 (s), (C=C, C=N stretching of pyridyl rings), 1269 (s) (asymmetric C–O–C stretching), 1085 (s) (symmetric C–O–C stretching), 829 (vs) (ν<sub>as</sub>(P–F) stretching of PF<sub>6</sub><sup>-</sup>). UV-VIS spectrum (λ<sub>max</sub>, nm (ε, M<sup>-1</sup>cm<sup>-1</sup>)) in CH<sub>3</sub>CN: 770 (304). ESI-MS (CH<sub>3</sub>CN): *m/z* = 366.066 (Calcd for [Cu(L<sup>1</sup>)Cl]<sup>+</sup> = 366.077) (Figure S6). Λ<sub>M</sub> (CH<sub>3</sub>CN) = 160 Ω<sup>-1</sup> cm<sup>2</sup> mol<sup>-1</sup>.

## 3.3. DNA Cleavage

The cleavage activity of the complexes towards pBR322 plasmid DNA (Carl Roth) was studied using gel electrophoresis. In a typical experiment, 1% agarose (Lonza) gels in 0.5× TBE buffer (Fisher Scientific, 50 mM Tris, 45 mM boric acid, 0.5 mM EDTA) containing ethidium bromide (0.2 µg/mL, Fisher Scientific) were used to monitor the conversion of supercoiled (form I) pBR322 into its nicked form (form II) and linear form (form III). If not stated otherwise, the experiments were carried out at least three times in order to assure reproducibility of the data and generate a reliable standard deviation. All experiments were carried out under normal air atmosphere without exclusion of light.

Plasmid DNA (0.025 µg/µL, Carl Roth) in a buffer solution, 10 mM MOPS (Fisher Scientific) pH 7.4, was treated with different concentrations of the complexes or ligands



(60, 120, 180, 300, 420, and 540  $\mu\text{M}$ , stock solutions of the complexes in acetonitrile were diluted to the final concentrations in 0.25–12% acetonitrile/deionized water). Deionized water (Millipore system) was added up to a final volume of 8  $\mu\text{L}$  before the samples were incubated for given time at 37  $^{\circ}\text{C}$  and 300 rpm in a thermomixer (Eppendorf).

For analysis, 1.5  $\mu\text{L}$  of loading buffer (3.7 mM bromophenol blue and 1.2 M saccharose in deionized water), was added and the sample was loaded onto the gel. Electrophoresis was carried out at 40 V for 2 h at room temperature using an electrophoresis unit (Carl Roth) in 0.5 $\times$  TBE buffer. Bands were visualized using fluorescence emission of ethidium bromide and photographed using a gel documentation system (Bio-Rad, Gel Doc<sup>TM</sup> EZ imager). DNA bands were quantified using Bio-Rad Laboratories Image Lab Software 5.2.1. In all assays, the intensity of the bands was measured using a reference DNA sample (no complex added) as standard. Taking into account that the supercoiled form I of plasmid DNA has a smaller affinity to bind ethidium bromide, its intensity was multiplied with a correction factor of 1.22 [59]. The average of results for each concentration was related to the DNA reference in percent. The statistical analysis was carried out with Microsoft Excel 2010. The DNA marker consisted of 50% purified, linearized (by EcoRI nuclease) and 50% nonlinearized plasmid pBR322 DNA.

### 3.3.1. Kinetics of DNA Cleavage

Cleavage of pBR322 supercoiled plasmid DNA by the Cu(II) complexes **1-Cl**, **1-ClO<sub>4</sub>**, **2-ClO<sub>4</sub>**, and **3-ClO<sub>4</sub>** was studied at 37  $^{\circ}\text{C}$  in MOPS buffer (10 mM) with a pH value of 7.4, over varying incubation times (10, 20, 40, 60, 90, and 120 min) and using different concentrations of the complexes (0  $\mu\text{M}$  as a reference, 60, 120, 180, 300, 420, and 540  $\mu\text{M}$ ). In accordance with previously published procedures [3,4,6], the percentages of form **II** were quantified using Bio-Rad Laboratories Image Lab Software 5.2.1 and were plotted against time for each complex concentration and fitted in Origin. To take into consideration the varying starting amount of form **II** when comparing experiments with different incubation times, the results were normalized (percentage of form **II** at 0 min = 0%) through subtraction of the reference DNA sample (no complex added). The observed rate constants ( $k_{\text{obs}}$ ) were determined based on equation (1) (pseudo first-order kinetics) using Microsoft Excel 2010.

$$y = (100 - y_0)(1 - \exp(-k_{\text{obs}}t)) \quad (1)$$

Herein,  $y$  represents the percentage of a specific form of DNA at a time  $t$ ,  $y_0$  is the percentage of that form at  $t = 0$  (for form **II**  $y_0 = 0$ ), and  $k_{\text{obs}}$  is the hydrolysis rate. The calculated values of  $1/k_{\text{obs}}$  were then plotted against the complex concentrations  $1/[\text{complex}]$  and fitted to equation (3) (pseudo first-order Michaelis–Menten analysis, linear fit rearranged from equation (2)) to determine the catalytic rate constant  $k_{\text{cat}}$ , which is interpreted as the maximum rate of cleavage at complex saturation ( $k_{\text{cat}} = v_{\text{max}}$ ), and the affinity constant  $K_M$ :

$$k_{\text{obs}} = k_{\text{cat}} \cdot [\text{complex}] / (K_M + [\text{complex}]) \quad (2)$$

$$1/k_{\text{obs}} = (K_M/k_{\text{cat}}) \cdot (1/[\text{complex}]) + 1/k_{\text{cat}} \quad (3)$$

The analysis of data was carried out with Microsoft Excel 2010.

### 3.3.2. ROS Scavenger Study

Cleavage of pBR322 plasmid DNA (0.025  $\mu\text{g}/\mu\text{L}$ ) in MOPS buffer (10 mM, pH 7.0) by complexes **1-ClO<sub>4</sub>–3-ClO<sub>4</sub>** (540  $\mu\text{M}$ ) in the presence of ROS scavengers for 2 h at 37  $^{\circ}\text{C}$  was studied. Experiments with ROS scavengers were carried out as described above using 400 mM DMSO, 0.5 mM potassium iodide, or 0.5 mM sodium azide. Illustrated is the average of three experiments, the standard deviation is shown as error bars. As a control experiment, DNA was incubated with each ROS scavenger in absence of the complex, and

one sample without the addition of any ROS showing the original activity. Analysis of data was done with Microsoft Excel 2010.

### 3.3.3. Cleavage of BNPP

For the cleavage of BNPP (270  $\mu\text{M}$ , Sigma-Aldrich), the Cu(II) complexes were used at a concentration of 540  $\mu\text{M}$  in 10 mM MOPS (pH 7.4) in the same cuvette with a volume of 1 mL. Before each measurement, the background was recorded with 540  $\mu\text{M}$  complex, 10 mM MOPS buffer (pH 7.4) in Millipore water. As a positive control, a sample containing 0.05 units  $\text{mL}^{-1}$  phosphodiesterase I from *Crotalus adamanteus* venom (Sigma-Aldrich) was used. As a negative control, a sample just containing BNPP in buffer was used. The samples were incubated for 2, 48, and 96 h at 37 °C and 300 rpm in a thermomixer (Eppendorf). UV/VIS spectra were recorded with a UV/VIS spectrometer Cary 100 (Agilent Technologies) at 25 °C in a region of 190–900 nm. The analysis of the data was carried out with Microsoft Excel 2010, the graphical representation with Julia 0.6.3.

### 3.3.4. Cell Culture and Cell Viability Assay

A2780 cells (human ovarian carcinoma cells from ECACC) were purchased from Merck. Human dermal fibroblasts isolated from neonate foreskin biopsies [60] after ethical approval (EA1/081/13, Ethics Committee from the Charité Campus Mitte, Berlin) and with informed parental consent, were kindly provided by the Institute of Pharmacy (Freie Universität Berlin). Cells were routinely maintained in DMEM with GlutaMAX (Dulbecco's minimal essential medium, Thermo Fisher Scientific), 15% FCS (Biochrom AG), and 1% penicillin/streptomycin (Thermo Fisher Scientific) at 37 °C and 5%  $\text{CO}_2$ .

For MTT assay,  $1 \times 10^5$  cells/mL were seeded into microtest plates 96 well, F (Sarstedt) and incubated overnight at 37 °C and 5%  $\text{CO}_2$ . The next day, medium was replaced with 100  $\mu\text{L}$  per well fresh medium containing dilutions of the test compounds (in duplicates). After 48 h of incubation, 100  $\mu\text{L}$  fresh medium, 10  $\mu\text{L}$  MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), and 5 mg/mL stock solution in PBS (Sigma) were added in each well. Incubation was then pursued for 4 h at 37 °C and 5%  $\text{CO}_2$ . The supernatant was then discarded and formazan crystals were solubilized in 100  $\mu\text{L}$ /well isopropanol with 0.04 M HCl (15 min on a plate shaker). Absorbance was read in a microplate reader (Tecan Infinite M200 PRO) at 550 nm. Assays were repeated at least three times independently. Relative cell viabilities were calculated by dividing the average absorbance values of duplicates by the absorbance value of untreated cells. The averages of at least three experiments were then plotted with their SEM against concentration.  $\text{IC}_{50}$  values were calculated from nonlinear dose–response curves (log inhibitor vs. normalized response—variable slope) fitted with Microsoft Excel 2010.

## 4. Conclusions

Two series of five-coordinate perchlorato and chlorido Cu(II)-based piperazine complexes:  $[\text{Cu}(\text{L})\text{ClO}_4]\text{ClO}_4$   $\text{L} = \text{L}^1$  (1- $\text{ClO}_4$ ),  $\text{L}^2$  (2- $\text{ClO}_4$ ),  $\text{L}^3$  (3- $\text{ClO}_4$ ) and  $\text{L}^6$  (6- $\text{ClO}_4$ ), and  $[\text{Cu}(\text{L}^1)\text{Cl}]\text{PF}_6$  (1-Cl),  $[\text{Cu}(\text{L}^4)\text{Cl}]\text{ClO}_4 \cdot \frac{1}{2}\text{H}_2\text{O}$  (4-Cl), and  $[\text{Cu}(\text{L}^5)\text{Cl}]\text{PF}_6$  (5-Cl) where  $\text{L}^1$ – $\text{L}^6$  refer to 1,4-bis[(2-pyridinyl)methyl]piperazine, 1,4-bis[(6-methyl)-2-pyridinyl)methyl]piperazine, 1,4-bis[(2-quinolyl)methyl]piperazine, 1,4-bis[(3,4-dimethoxy-2-pyridinyl)methyl]piperazine, 1,4-bis[(3,5-dimethyl-4-methoxy-2-pyridinyl)methyl]piperazine, and 1,4-bis[(2-pyridinyl)-(2-ethyl)]piperazine were synthesized and characterized. In these complexes the coordinated chloride or perchlorate ligands are weakly bound to the Cu(II) center, and as a result, they are hydrolyzed in aqueous acetonitrile solutions to the corresponding aqua species  $[\text{Cu}(\text{L})(\text{H}_2\text{O})]^{2+}$ . Similar species were also formed in five-coordinate complexes,  $[\text{M}(\text{L}')\text{X}]\text{ClO}_4/\text{PF}_6$  ( $\text{M} = \text{Cu}(\text{II})$  or  $\text{Co}(\text{II})$ ,  $\text{L}' =$  tripod-pyridyl tetraamine ligands) under comparable conditions [3,4,6,61].

The nucleolytic activity of the synthesized Cu(II) complexes towards plasmid DNA cleavage at near-physiological conditions (pH = 7.4, 37 °C) was studied and their cytotoxicity was investigated. Under this condition, the catalytic rate constant,  $k_{\text{cat}}$ , for DNA cleavage

activity by the complexes decreases in the order: **1-Cl**  $\approx$  **1-ClO<sub>4</sub>** > **3-ClO<sub>4</sub>**  $\geq$  **2-ClO<sub>4</sub>**, with rate enhancement up to 23 million over the uncatalyzed DNA for complex **1-Cl/1-ClO<sub>4</sub>**. Complexes **4-Cl**, **5-Cl**, and **6-ClO<sub>4</sub>** as well as all ligands were inactive. The mechanistic pathway for DNA cleavage by these complexes revealed that while complex **1** prefers the oxidative mechanism, complexes **2-ClO<sub>4</sub>** and **3-ClO<sub>4</sub>** proceed predominantly via a hydrolytic mechanism. Obviously, this depends on the ligand skeleton and steric hindrance encountered by the ligands, as demonstrated by the X-ray structures of complex **5-Cl** in this work and in the previously structurally characterized **6-ClO<sub>4</sub>** [47]. It is not clear at the moment why the mechanism is different in the two indicated pairs of compounds. Interestingly, under comparable conditions, very high DNA catalytic cleavage enhancements by a factor of 100–1100 million have been reported for some mononuclear Cu(II) complexes [17,62–64].

Under pseudo-Michaelis–Menten first-order kinetics at pH 7.0 and 37 °C, the catalytic rate constants  $k_{\text{cat}}$  for the DNA cleavage by a series of five-coordinate chlorido-Co(II)-tripod amine complexes were reported to decrease in the order: [Co(BPQA)Cl]<sup>+</sup> > [Co(6-MeTPA)Cl]<sup>+</sup> > [Co(BQPA)Cl]<sup>+</sup>  $\geq$  [Co(TPA)Cl]<sup>+</sup>  $\geq$  [Co(6-Me<sub>2</sub>TPA)Cl]<sup>+</sup> (TPA = tris(2-pyridylmethyl)amine, 6-MeTPA = [(6-methyl-2-pyridyl)methyl]bis(2-pyridylmethyl)amine, 6-Me<sub>2</sub>TPA = [(bis(6-methyl-2-pyridyl)methyl)-(2-pyridylmethyl)]amine, BPQA = [bis(2-pyridylmethyl)-(2-quinolylmethyl)]amine, and BQPA = [bis(2-quinolylmethyl)-(2-pyridylmethyl)]amine) [4]. The mechanistic pathway in this series was mainly hydrolytic, where the steric skeletal environment around the central Co(II) enhances the release of the coordinated chloride ligand but also indicates that large increase in the steric environment at the 6th position of the pyridyl groups suppresses the approach of DNA to the metal center [4]. A parallel trend was also observed in another series of compounds, where methyl and methoxy groups were incorporated at the 3–5 positions of the pyridyl groups with no substituents at the 6th position [3]. Under comparable conditions, the linear tetradentate-based piperazine Cu(II) complexes under investigations revealed the reactivity order: **1-Cl/ClO<sub>4</sub>** > **3-ClO<sub>4</sub>**  $\geq$  **2-ClO<sub>4</sub>** with lack of reactivity for **4-Cl**, **5-Cl** and **6-ClO<sub>4</sub>**. The interpretation of the reactivity order in the former three complexes cannot be attributed to the steric effect only but the change in the mechanistic DNA cleavage also must be taken into account that complexes **2** and **3** proceed via hydrolytic mechanism, which is different from that determined in **1** as predominantly through oxidative mechanism.

The cytotoxic properties of the title Cu(II) complexes and their corresponding ligands were investigated in vitro in human ovarian carcinoma cells A2780 and human fibroblasts. Only the complexes **1-ClO<sub>4</sub>**, **3-ClO<sub>4</sub>**, and **5-Cl** were found to be cytotoxic against cancer cells (IC<sub>50</sub> 30–40 μM), whereas the other complexes did not show cytotoxic activity. The cytotoxicity value of **3-ClO<sub>4</sub>** in fibroblasts was the highest (lowest IC<sub>50</sub>) among the studied complexes, and the complex exhibited no toxicity towards human fibroblasts, a property which suggests its selectivity for cancer cells over healthy cells. The elevated lipophilicity of the quinolyl-containing compound is most likely responsible for the observed increase of cytotoxicity for **3-ClO<sub>4</sub>**. A similar trend was also observed in [Cu(BQPA)Cl]ClO<sub>4</sub>/PF<sub>6</sub>, where BQPA = [bis(2-quinolylmethyl)-(2-pyridylmethyl)]amine [61]. There is no correlation between DNA cleavage activity and cytotoxicity. For example, **5-Cl**, which did not show any DNA cleavage activity, was cytotoxic, whereas **1-Cl** and **1-ClO<sub>4</sub>**, which revealed DNA cleavage of comparable activity surprisingly showed different cytotoxic behavior. The latter one is toxic in cancer cells and fibroblasts but the first one is not active in either cell line. Variations of the counter ions of the complexes or differences in their DNA cleavage mechanisms (oxidative/hydrolytic) do not clearly correlate with the differences in the cytotoxic activities indicating that interactions with other biological targets within the cells might be responsible for the cytotoxic effects.

**Supplementary Materials:** The following are available online at <https://www.mdpi.com/2304-6740/9/2/12/s1>, the CIF and the checkCIF output files are included in the Supplementary Materials. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via [www.ccdc.cam.ac.uk/data\\_request/cif](http://www.ccdc.cam.ac.uk/data_request/cif). Supplementary associated with crystal structure determinations and Figure S1, Figure S2, Figure S3, and Figure S4 show the  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of ligands  $\text{L}^1\text{-L}^4$ ; Figure S5: ESI-MS of  $\text{L}^2$  and  $\text{L}^3$ ; Figure S6, Figure S7, Figure S8, and Figure S9: ESI mass spectra of **1-Cl**, **2-ClO<sub>4</sub>**, **3-ClO<sub>4</sub>**, and **5-Cl**, respectively; Figure S10: Cleavage of pBR322 plasmid DNA by complexes; Figure S11: Cleavage of pBR322 plasmid DNA by complexes and ligands; Figure S12: UV-VIS spectra of BNPP (50  $\mu\text{M}$ ) at pH = 7.4 in the absence and presence of phosphodiesterase; Figure S13 and Figure S14: UV-VIS spectra of BNPP (270  $\mu\text{M}$ ) at pH 7.4 in the presence of Cu(II) complexes after 2 and 96 h, incubation; Figure S15: Fluorescence spectra of TPA and PBSF after incubation with/without  $[\text{Cu}(\text{L}^1)\text{ClO}_4]\text{ClO}_4$  (**1-ClO<sub>4</sub>**); and Figure S16: Results of the MTT assay in A2780 cells and human fibroblasts of Cu(II) complexes and their ligands of this article can be found in the online version.

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