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Original article

Solvatochromism, DNA binding, antitumor activity and molecular modeling study of mixed-ligand copper(II) complexes containing the bulky ligand: Bis[*N*-(*p*-tolyl)imino]acenaphthene

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Abstract

Four mixed-ligand copper(II) complexes of the nitrogen ligand bis[*N*-(*p*-tolyl)imino]acenaphthene **1** (*p*-Tol-BIAN). These complexes, namely [Cu(*p*-Tol-BIAN)$_2$](ClO$_4$)$_2$ **2**, [Cu(*p*-Tol-BIAN)(acac)](ClO$_4$) **3**, [Cu(*p*-Tol-BIAN)Cl$_2$] **4** and [Cu(*p*-Tol-BIAN)(AcOH)$_2$](ClO$_4$)$_2$ **5**, were prepared and characterized. Solvatochromism of the novel copper complexes in various solvents has been studied. Molecular mechanics (MM+) and molecular dynamic simulations have been performed to learn more about the solvatochromic behaviour and the DNA binding affinity of these complexes.
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Keywords: Solvatochromism; Bis[*N*-(*p*-tolyl)imino]acenaphthene; DNA binding; Molecular modeling

1. Introduction

Organometallic chemistry has been developed, in the last four decades, to be the largest and important branch as a link connecting the fields of organic and inorganic chemistry [1]. One of the major applications of the transition metal complexes is its medical testing as antibacterial and antitumor agents aiming toward the discovery of an effective and safe therapeutic regimen for the treatment of bacterial infections and cancers [2]. In addition, a great many Schiff' base complexes with metals have also provoked wide interest because they possess a diverse spectrum of biological and pharmaceutical activities, including antitumor and antioxidative activities [3]. Moreover, mixed-ligand complexes are observed in biological systems or in the intermediate chemical reactions with metal ions, which are important to understand the respective chemistry. Investigations concerning diimine mixed-ligand chelate systems would provide toward understanding the driving forces that led to the formation of such mixed-ligand complexes [4–6].

The investigation of solvatochromic behaviour [7] of mixed-ligand metal complexes has been of importance, because it provides a quantitative approach to recognize the solvent behaviour and the role of the solvent in physico-chemical studies [8]. Moreover, it is very helpful for developing environmental sensor materials, which are chromotropic and exhibit color change when exposed to solvent or pollutant molecules [9].

In the present study, we report the solvatochromism, DNA binding affinity and cytotoxic activity of four mixed-ligand copper(II) complexes of the nitrogen ligand bis[*N*-(*p*-tolyl)imino]acenaphthene **1** (*p*-Tol-BIAN). These complexes are [Cu(*p*-Tol-BIAN)$_2$](ClO$_4$)$_2$ **2**, [Cu(*p*-Tol-BIAN)(acac)](ClO$_4$) **3**,

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[Cu(*p*-Tol-BIAN)Cl_2] **4** and [Cu(*p*-Tol-BIAN)$(AcOH)_2$]$(ClO_4)_2$ **5**. Molecular mechanics (MM+) and molecular dynamic simulation have been performed to learn more about the solvatochromic behaviour and DNA binding affinity of the described complexes.

2. Results and discussion

2.1. Electronic spectra of complexes in various solvents

The absorption spectra of complexes **2–5** were measured in different organic solvents with different donor numbers (DN). The characteristic properties of these complexes are (i) their high solubility in various organic solvents, and (ii) the change in the color of the solution on going from one solvent to another, that is, strong solvatochromism of their solutions [10]. The spectra show one broad band attributed to the promotions of the electron in the lower energy orbitals to the hole in $d_{x^2-y^2}$ orbital of the copper(II) ion (d^9). The position of this band is shifted to longer wavelength (red shift) as the donor number of solvent increases (Table 1). Conductivity data (Table 2) of studied complexes suggest that ClO_4^- acts only as a counter ion [11]. Therefore, the complex structure depends only on the donor properties of the solvent used (Gutmann's donor number DN) [12], and only solute–solvent interactions can be considered.

Solvatochromic behaviour was studied quantitatively by applying the linear solvation free energy relationship [13]. Linearity of the ν_{max} *vs*. DN, confirms the solvatochromic behaviour in perchlorate complexes. The slope value is the smallest in case of **2**, in which two diimine ligands are coordinated to the copper(II) centre imposing a large steric hindrance to the axial coordination of incoming solvent leading to less solvatochromic behaviour.

2.2. Biological activity

2.2.1. DNA as an affinity probe for evaluation of biologically active compounds

DNA is the pharmacologic target of many drugs currently in clinical use or in advanced clinical trials. Small molecules that bind genomic DNA have proven to be effective anticancer, antibiotic, and antiviral therapeutic agents. It is known that a small molecule can bind to DNA through various modes of interaction. They are (i) minor groove binding, (ii) major groove binding, (iii) intercalation (between two base pairs) and (iv) surface binding [14]. Metal complexes having a planar aromatic heterocyclic functionality (that can insert and stack between the base pairs of double helical DNA) have contributed to our understanding of fundamental nucleic acid recognition. These metal complexes have been applied in the development of DNA cleavage reagents, have assisted in the development of low molecular weight drugs, and as stand-alone metal complexes and have been used as agents to understand fundamental complex interactions with DNA [15]. A variety of methods have been utilized for the interaction of small molecular weight compounds with DNA, such as equilibrium dialysis [16].

Briefly [17], a fixed amount of ligand is spotted on the RP-18 TLC plates followed by addition of known amount of DNA on the same spot. The plate was then developed and the position of the DNA was determined by spraying the plates with anisaldehyde reagent. It is important to establish if the response of the test system is dependent on the dose of the test substance. In the presence of increasing quantities of DNA intercalators, a greater portion of DNA is bound to form a complex, and consequently, the free DNA was detected as a blue spot (MeOH–H_2O, 8:2) on RP-18 TLC after spraying with anisaldehyde reagent. On the other hand, compounds with high binding affinity to DNA retained on the base line. However, when the DNA was mixed with compounds with which it is known to interact (ethidium bromide), the complex was retained at the origin when MeOH–H_2O (8:2) was used for elution. On the other hand, inactive compounds did not cause the DNA to be retained at the origin.

Moreover, methyl green reversibly binds polymerized DNA forming a stable complex at neutral pH. The maximum absorption for the DNA–methyl green complex is 642–645 nm. This colorimetric assay [17b,18] was used to measure the displacement of methyl green from DNA by compounds having the ability to bind with DNA. The degree of displacement was determined spectrophotometrically by measuring the change in the initial absorbance of the DNA–methyl green solution in the presence of reference compound.

Results from DNA binding assay (Table 3) revealed that compounds **2** and **5** showed the highest affinity to DNA, which was demonstrated by retaining the complex at the origin or by migrating for very short distances, and by measuring IC_{50} (concentration required for 50% decrease in the initial absorbance of the DNA–methyl green solution). Compounds **3** and **4** showed moderate activity while the original ligand **1** showed weak activity.

2.2.2. Antitumor activity against Ehrlich ascite carcinoma cells in mice [19,20]

2.2.2.1. Effect on survival time. The first measure that can be used to compare the anti-neoplastic activities for the tested compounds is the increase in survival time for each treated group over the control group [19]. The mean survival time (MST) for each group was calculated by dividing the total

Table 1
Absorption maxima λ_{max} (nm) of complexes, **2–5**, in different solvents

Solvent	DN[a]	λ_{max} (ε l/mol cm)[b]			
		2	**3**	**4**	**5**
CH_2Cl_2	0	656 (429)	610 (338)	620 (215)	607 (429)
CH_3NO_2	2.7	661 (387)	630 (255)	N.d.[c]	N.d.[c]
CH_3CN	14.1	693 (195)	N.d.[c]	720 (200)	N.d.[c]
CH_3OH	19.1	714 (150)	720 (209)	750 (153)	745 (272)
$HCON(CH_3)_2$	26.6	740 (272)	763 (331)	769 (206)	763 (472)
CH_3SOCH_3	29.8	763 (65)	830 (44)	868 (56)	845 (67)

[a] Donor number of solvents, from Ref. [12].
[b] The extinction coefficient values are given in parenthesis.
[c] N.d., not detected.

Table 2
Analytical and physical data of complexes **2**–**5**

Compound, empirical formula	Λ_m cm^2/Ω mol			Found (calcd.) %			μ_{eff} B.M.
	F. wt	AN	MN	C	H	N	
[Cu(*p*-Tol-BIAN)$_2$](ClO$_4$)$_2$ (**2**) $C_{52}H_{40}Cl_2CuN_4O_8$	983.35	330	192	62.28 (63.51)	4.15 (4.10)	5.44 (5.69)	1.39
[Cu(*p*-Tol-BIAN)(acac)](ClO$_4$) (**3**) $C_{31}H_{28}ClCuN_2O_6$	623.56	180	100	60.07 (59.71)	4.54 (4.53)	4.27 (4.49)	1.56
[Cu(*p*-Tol-BIAN)Cl$_2$] (**4**) $C_{26}H_{20}Cl_2CuN_2$	494.9	25	5	62.47 (63.10)	4.24 (4.10)	5.27 (5.66)	1.88
[Cu(*p*-Tol-BIAN)(AcOH)$_2$](ClO$_4$)$_2$ (**5**) $C_{30}H_{28}Cl_2CuN_2O_{12}$	743.0	320	188	48.1 (48.5)	3.66 (3.80)	3.60 (3.77)	1.98

survival times for all the mice in that group by the number of mice in the same group, then the percent increase in lifespan for each group over the control group was calculated as follows [19]:

$$\%\ \text{Increase in lifespan over control} = \frac{\text{MST of treated group}}{\text{MST of control group}} \times 100 - 100.$$

Comparing the % increase in lifespan of the control group in each treated group (Table 4) revealed that the compound **3** has shown the same increase in lifespan produced by the standard drug 5-FU. For compound **5**, the increase in lifespan was even higher than that of 5-FU. The maximum increase in lifespan was obtained by compound **2**, which nearly cured the animals, expanding their lifespan to be approached to the normal non-diseased group.

2.2.2.2. Effect on biochemical and haematological parameters [19]. On the day 14, the biochemical and haematological parameters, as regard to haemoglobin level, erythrocytes and leucocytes counts, were compared in the group treated with the standard drug 5-FU and the group treated with the newly synthesized compound **2**, which has shown the highest % increase in lifespan over control, with values obtained from normal and control groups.

As shown in Table 5, the biochemical and haematological parameters in the group treated with the compound **2** have been nearly recovered completely to be within the normal values. The values are comparable to those obtained from the group treated with 5-FU, and they are even better in some of the aspects, as regard to leucocyte count.

2.2.2.3. Effect on viable cell count of Ehrlich ascite carcinoma cells [19]. After five days of treatment, 100 μl samples were taken from *Ehrlich ascite carcinoma cells* from three mice in each one of the treated groups and from the control group. A 20-fold dilution was made for the taken cells in saline. The cells in the final dilution were stained with Giemsa stain, and the number of the viable cells was counted under the microscope.

As shown in Table 6, both compounds **2** and **5** showed significant reduction in viable cell count even higher than that produced by 5-FU. However, the highest degree of reduction in viable cell count was demonstrated by compound **2**.

2.2.2.4. Effect on body weight [19]. Both compounds **2** and 5-FU showed minimal ascites and slight increase in body weight unlike the control group, in which large ascites were produced and a marked increase in body weight was observed.

2.3. Molecular modeling studies

2.3.1. Solvation energy calculations

An attempt to gain a better insight on the molecular structures of the solvated complex **4**, and the free ligand bis[*N*-(*p*-tolyl)imino]acenaphthene **1**, conformational analysis of the target compounds has been performed by MM+ [21] force field as implemented in HyperChem 5.1 [22]. The starting atomic coordinates of the target compounds were obtained from the X-ray data of the structure analogues [23]. The PM3 semi-empirical [24] calculations performed on free ligand bis[*N*-(*p*-tolyl)imino]acenaphthene showed that the diaryl groups were arranged itself perpendicular to the plane of acenaphthene with face to edge interaction and are not in the plane of the imine N-bond but make an angle ∠C2–N2–C6–C7 of 61.4° to it. This non-coplanar orientation of *p*-tolyl substituent is caused by the presence of naphthalene backbone, which prevents rotation of *p*-tolyl substituent in the plane formed by the imine function as we mentioned in our previous report [2a]. Moreover, it is noteworthy to say that the MM+ and PM3 calculations give results which are in good agreement with the X-ray data of the structure analogues [2a,23].

Table 3
DNA binding activity of compounds **1**–**5** using DNA–methyl green displacement assay

Compd. no.	IC_{50} (μg/ml)[a]
1	78 ± 2
2	36 ± 3
3	56 ± 4
4	63 ± 2
5	48 ± 1
Daunomycin	28 ± 3

[a] Values represent the concentration (mean ± SD, $n = 3 - 5$) required for 50% decrease in the initial absorbance of DNA–methyl green solution.

Table 4
The % increase in lifespan for each treated group over the control group in EAC test

Group no.	Treatment (I.P.)	% Increase in lifespan
1	Normal (untreated)	71.4
2	Control (Ehrlich only)	0.0
3	**2**	55.1
4	**3**	42.0
5	**5**	44.1
6	5-FU	42.8

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Table 5
Effect of the treatment on biochemical and haematological parameters

Group type	(Hb/g) %	RBCs/10^6 mm^3	Total WBCs/10^3 mm^3
Normal	13.4	5.1	4.35
Control (Ehrlich only)	5.7	3.15	47.4
2	9.1	4.96	12.45
5-FU	15.6	5.87	14.7

The correlation between the solvatochromism as expressed by the absorption spectra of compound **4** measured in different organic solvents and the calculated solvation energy was studied using molecular dynamic calculation as shown in Table 7 and Fig. 1. However, each of the solvated complexes has been subjected to molecular dynamic (MD) simulations followed by energy minimization of the snap shots collected at regular time intervals during the MD simulations. After energy minimization all the energy minima obtained have been compared with each other and the one with the lowest energy has been picked as the representative of the solvated complex (Fig. 1). The least energy complex thus picked up has been selected to calculate the solvation energy as a measure of solvatochromism. The solvation energies between compound **4** and solvents are given in Table 7. The complexes are characterized by the presence of a number of non-bonded interactions formed between compound **4** and the solvent molecules in addition to the covalent linkage formed between the metal centre and solvent molecules. The solvents (DMSO, DMF, and MeOH) offer more favourable non-bonded interactions as compared to the CH_2Cl_2 and MeCN, this is evident from their respective non-bonded energy terms (Table 7), which are about 3–20 kcal/mol less as compared to CH_2Cl_2. Among the studied solvated complex, **4**–DMSO and **4**–DMF complexes are associated with the lowest non-bonded energy term and are stabilized by the CH/π interaction of alkyl group with acenaphthene ring (∼2.9 Å) [2a,25]. This non-bonded interaction may be strong enough to compensate the steric repulsion between the solvent molecule and the compound **4**. The relative solvation energies calculated in HyperChem for complex **4** with DMSO, DMF, MeOH, MeCN and CH_2Cl_2 were found to be −6.0, −2.31, 2.9, 8.43 and 17.01 kcal/mol, respectively, correlating well with the experimental solvatochromism values. The more negative the solvation energy, the more stable the **4**-solvent complex.

2.3.2. DNA binding energy calculations

Docking studies were also performed to investigate the DNA binding differences of the most potent **2** and the least potent **4** as a representative example. Each of the DNA–target complexes has been subjected to molecular dynamic (MD) simulations followed by energy minimization of the snap shots collected at regular time intervals during the MD simulations. After energy minimization all the energy minima obtained have been compared with each other and the one with the lowest energy has been picked as the representative of the DNA–target complex (Fig. 2). The least energy complex thus picked up has been selected to calculate the interaction energy as a measure of stability of complex. The interaction energies between DNA (AT) base pairs and compounds **2** and **4** are given in Table 8. It can be seen from this table that the dimer DNA–**2B** renders more stability to the complex compared to the monomers DNA–**2A** and DNA–**4**. This property correlates well with the experimentally determined values of the DNA binding in these complexes. The complexes are characterized by the presence of a number of non-bonded interactions (Table 8) formed between DNA and copper complexes. The dimeric **2B** offers more favourable non-bonded interactions as compared to the monomeric **2A** or **4** and this is evident from their respective non-bonded energy terms (Table 8), which are about 18–25 kcal/mol less as compared to **2A** and **4**. The dimeric **2B** is associated with the lowest non-bonded energy term and is stabilized by π–π interaction among the *p*-tolyl groups, π-stacking interaction between each pair of *p*-tolyl aromatic ring and CH/π interaction among the *p*-tolyl groups and aliphatic frame of pair bases (AT) [2a,25]. The binding energies calculated in HyperChem for the three models, dimeric **2B**, monomeric **2A** and compound **4**, were found to be −70.9, −34.2 and −30.7 kcal/mol, respectively, correlating well with the experimental DNA binding values. The more negative the relative binding energy, the more potent the binding is between DNA and target molecules.

Table 6
Viable cells count of EAC after 5 days of treatment with tested compounds and 5-FU

Group no.	Group type	Count (cells)/100 μl
2	Control (Ehrlich only)	192.8×10^6
3	**2**	37.6×10^6
4	**3**	50.2×10^6
5	**5**	45.6×10^6
6	5-FU	83.6×10^6

Table 7
Experimental, calculated solvation energies, van der Waals energies and electrostatic energies of complex **4** with various solvents

Solvent	λ_{max}(ε l/mol cm)[a]	Esolvation[b], (kcal/mol)	Evan der Waals[c], (kcal/mol)	Eelectrostatic[d], (kcal/mol)
CH_2Cl_2	620	17.01	12.70	4.41
CH_3CN	720	8.43	12.77	0.81
CH_3OH	750	2.90	8.04	1.26
$HCON(CH_3)_2$	769	−2.31	11.48	−16.22
$(CH_3)_2SO$	868	−6.00	1.05	1.01

[a] Data were taken from Table 1.
[b] The energy of interactions between complex **4** and solvents.
[c] Evan der Waals term describes the repulsive forces keeping two non-bonded atoms apart at close range and the attractive force drawing them together at long range.
[d] Eelectrostatic describes the classical non-bonded electrostatic interactions of charge distributions.

2.4. Conclusion

The solvatochromic behaviour of four mixed-ligand copper(II) complexes containing the bidentate nitrogen ligand

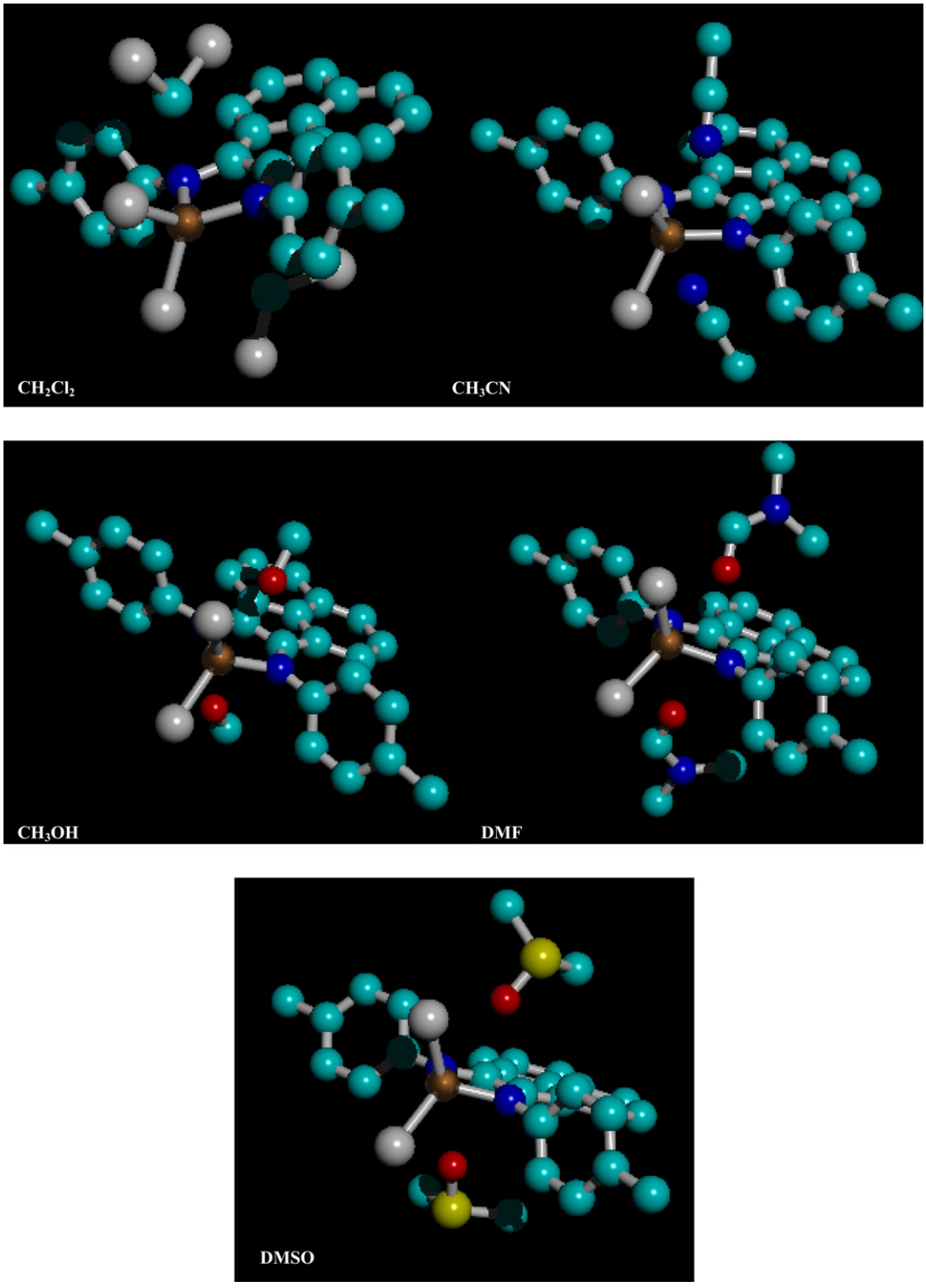

Fig. 1. Docking of various solvents with the energy minimized complex **4** with ball and cylinder rendering.

bis[*N*-(*p*-tolyl)imino]acenaphthene **1** was studied. These complexes are [Cu(*p*-Tol-BIAN)$_2$](ClO$_4$)$_2$ **2**, [Cu(*p*-Tol-BIAN)(acac)](ClO$_4$) **3**, [Cu(*p*-Tol-BIAN)Cl$_2$] **4** and [Cu(*p*-Tol-BIAN)(AcOH)$_2$](ClO$_4$)$_2$ **5**. However, the relative solvation energies calculated for complex **4** with DMSO, DMF, MeOH, MeCN and CH_2Cl_2 were found to be −6.0, −2.31, 2.9, 8.43 and 17.01 kcal/mol, respectively, which correlated well with the experimental solvatochromism values. *In vivo* antitumor and *in vitro* DNA binding activities were performed showing that complexes **2**, **3** and **5** gave the highest DNA binding affinity and antitumor activity which are more active than the parent ligand bis[*N*-(*p*-tolyl)imino]acenaphthene **1**. Molecular dynamics simulation has been performed to study the DNA binding affinity of the most and least active complexes

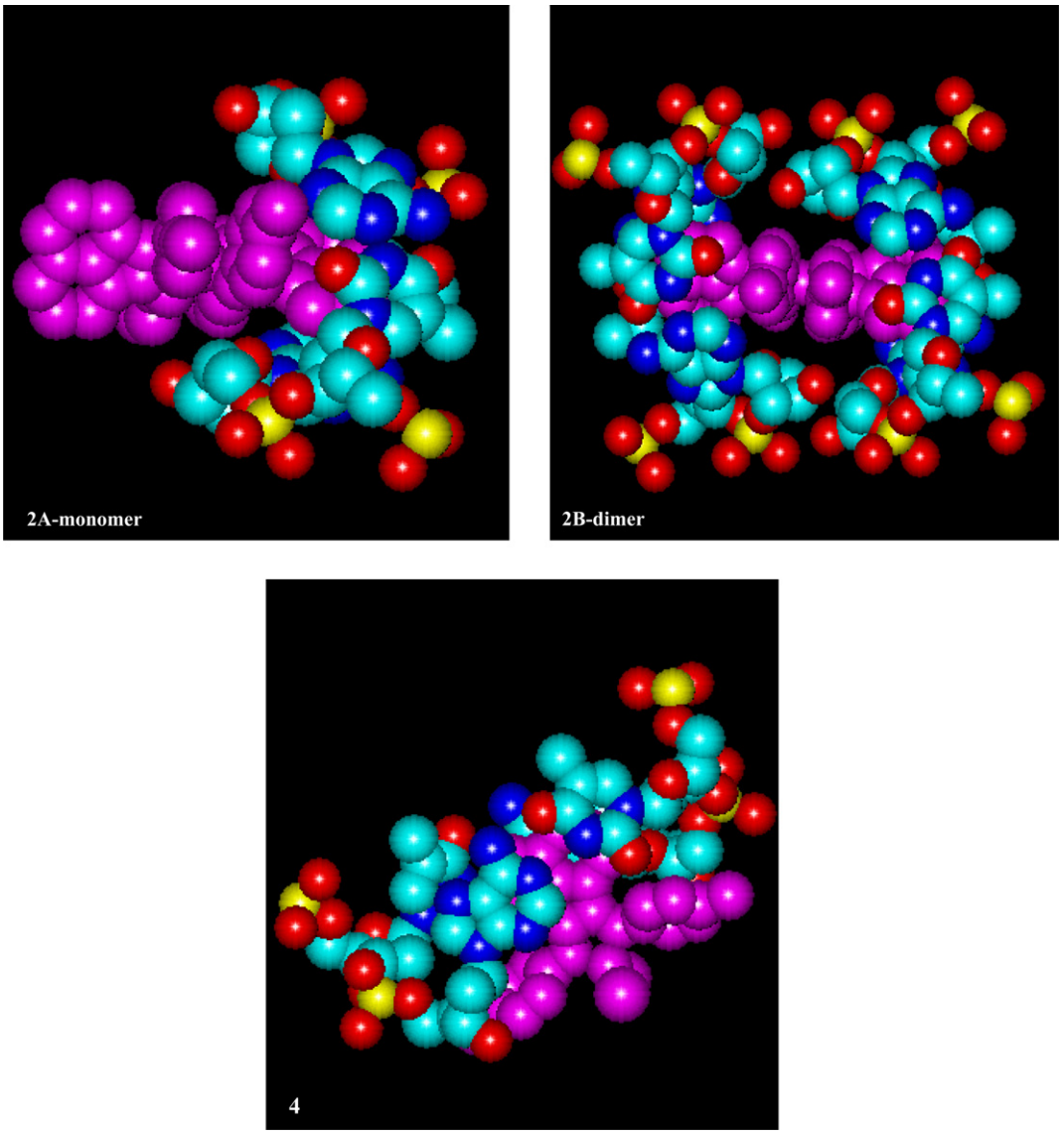

Fig. 2. Docking studies of the most potent DNA binding compound, **2** (A, B; upper panel), and least potent, **4** (lower panel), into DNA base pairs (AT) with CPK rendering. The compounds **2** and **4** are shown in violet, to distinguish them from the DNA base pairs and hydrogen atoms were omitted for clarity (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

2 and **4**, respectively. The relative binding energies calculated for the deduced models, dimeric **2B**, monomeric **2A** and compound **4**, were found to be −70.9, −34.2 and −30.7 kcal/mol, respectively, correlating well with the experimental DNA binding values.

Table 8
The values of energy of interaction calculated for the DNA-monomer and dimer complexes

Compd. No.	Binding energy (kcal/mol)	Evan der Waals (kcal/mol)	Eelectrostatic (kcal/mol)	IC_{50} (μg/ml)[a]
4	−30.7	−7.34	−22.38	63 ± 2
2A (monomer)	−34.2	−0.067	−17.57	36 ± 3
2B (dimer)	−70.8	−18.40	−42.14	36 ± 3

[a] Data were taken from Table 3.

3. Experimental

3.1. Instrumentation and materials

All starting materials were purchased from Sigma–Aldrich Co., USA, and used without further purification. Elemental analyses (C, H, N) were performed on a Perkin-Elmer 2400 Series II Analyzer. Electronic spectra were recorded on a UV-UNICAM 2001 spectrophotometer using 10 mm pass length quartz cells at room temperature. Magnetic susceptibility was measured with a Sherwood Scientific magnetic susceptibility balance at 297 K. Infrared spectra were recorded on a Perkin-Elmer FT-IR Spectrometer 2000 as KBr pellets and as Nujol mulls in the 4000–370 cm^{-1} spectral range. ^{1}H and ^{13}C NMR measurements at room temperature were obtained on a Jeol JNM

LA 300 WB spectrometer at 400 MHz, using a 5 mm probe head in $CDCl_3$. Chemical shifts are given in ppm relative to internal TMS (tetramethylsilane); Giemsa stain was from Fisher Scientific Co, Fair Lawn, NY, USA, TLC plates from RP-18F_{254}; 0.25 mm, Merck, DNA, calf thymus type I, Sigma, 100 μg/ml, and DNA–methyl green was from Sigma, St. Louis, MO, USA.

3.2. Synthesis of bis[N-(p-tolyl)imino]acenaphthene (p-Tol-BIAN), **1**

Preparation of the ligand was carried out in two steps as follow.

3.2.1. Step 1: preparation of (p-Tol-BIAN)$ZnCl_2$

A mixture of 3.0 g acenaphthenequinone (16.5 mmol), 2.5 g anhydrous $ZnCl_2$ (18.0 mmol) and 3.75 g *p*-toluidine (35.0 mmol) in 100 ml acetic acid was heated under reflux (80 °C) for 1 h. Then the mixture was cooled to room temperature and the solid product was filtered off to give an orange solid that was washed with acetic acid followed by diethyl ether and air dried, yield 7.5 g of (*p*-Tol-BIAN)$ZnCl_2$ (91%).

3.2.2. Step 2: removal of $ZnCl_2$

(*p*-Tol-BIAN)$ZnCl_2$ (6.7 g) was added to a solution of 4 g K_2CO_3 in 150 ml water and the mixture was heated under reflux with continuous stirring. The mixture was then filtered and the product was washed repeatedly with water. The product was dissolved in boiling ethanol while the solid zinc carbonate was removed by filtration. Ethanolic solution of the ligand was evaporated to the quarter and set aside. After one day the product was filtered and dried *in vacuo*.

Yield: 3.50 g (72%). Found: C, 86.60; H, 5.50; N, 7.66. Calc. for $C_{26}H_{20}N_2$ (360.45): C, 86.64; H, 5.59; N, 7.77%. ^{1}H NMR ($CDCl_3$, recorded at 400 MHz at 23.6 °C) $\delta = 2.44$ (s, *p*-Me), 6.92 (d, H3), 7.03 (d, H9), 7.26 (d, H10), 7.37 (pst, H4), 7.86 (d, H5). ^{13}C NMR ($CDCl_3$, 400 MHz, 24.5 °C): $\delta = 21.06$ (*p*-Me), 161.06 (C1), 128.49 (C2), 123.68 (C3), 127.38 (C4), 128.62 (C5), 131.0 (C6), 141.49 (C7), 149.0 (C8), 118.04 (C9, C13), 129.77 (C10, C12), 133.68 (C11).

3.3. Synthesis of copper(II) complexes

Mixed-ligand copper complexes (**2**–**5**) containing the rigid ligand bis[*N*-(*p*-tolyl)imino]acenaphthene were prepared by adding the appropriate amount of copper perchlorate or copper chloride (in case of **4**) to an ethanolic solution of the *p*-Tol-BIAN ligand. To this mixture acac (in case of **3**) or acetic acid (in case of **5**) was added. The mixtures were stirred (while heating) for 1–3 h. The resulting green solid product was filtered, washed with ethanol and air dried. The analytical and physical data of complexes are listed in Table 2.

3.4. Biological screening

3.4.1. Evaluation of the degree of DNA binding

3.4.1.1. DNA binding assay on TLC plates

3.4.1.1.1. Methods. The TLC plates used in the assay were pre-developed first using methanol–water (8:2). The tested compounds were then applied (5 mg/ml in methanol) at the origin, followed by the spotting of DNA (1 mg/ml in methanol–water mixture (8:2)) at the same positions at the origin. Ethidium bromide was used as a positive control. After complete spotting, the plates were developed with the same solvent system, and the positions of DNA were visualized by spraying the plates with anisaldehyde, which produces a blue color with DNA. The intensity of the color was proportional to the quantity of DNA added to the plate.

3.4.1.2. Colorimetric assay for the degree of DNA binding

3.4.1.2.1. Methods. DNA–methyl green complex (20 mg) was suspended in 100 ml of 0.05 M tris–HCl buffer (pH 7.5) containing 7.5 mM $MgSO_4$ and stirred at 37 °C with a magnetic stirrer for 24 h. The calculated amounts of samples were placed in Eppendorf tubes, and 200 μl of the DNA–methyl green solution was added to each tube. The samples were incubated in dark at ambient temperature and after 24 h, the final absorbance of each sample was determined at 642–645 nm. The results were recorded in form of the IC_{50} of each compound, which is the sample concentration required to produce 50% decrease in the initial absorbance of the DNA–methyl green complex. Daunomycin was used as a positive control.

3.4.2. In vivo antitumor activity against Ehrlich ascite carcinoma cells in mice

The prolongations of lifespan of Ehrlich ascite carcinoma cells (EAC) bearing hosts, the recovery of normal biochemical and haematological profiles and the reduction in viable tumor cell count are three important measures that have been used in this *in vivo* testing for the evaluation of the anti-neoplastic activity for three of the newly synthesized compounds, selected on the basis of the results obtained from the previous evaluation method, where they showed the highest DNA-affinity. These compounds are **2**, **3** and **5**.

3.4.2.1. Materials. Ehrlich ascite carcinoma cells (EACs): the cells of Ehrlich ascites tumor were obtained from National Cancer Institute, Cairo, Egypt. After harvesting and preparation of the cells, their total number and viability were determined by counting using Trypan blue. The desired concentration of tumor cells (2×10^6 cells/0.2 ml) was obtained by dilution with saline (0.9% sodium chloride solution). The viability of tumor cells obtained and used in this experiment was always higher than 90%. Below this percentage, the cells were discarded and the entire procedure was repeated. 5-Fluorouracil was obtained from Sigma–Aldrich Co., USA. Adult Swiss male albino mice (20–25 g) of both sexes were used in this experiment. They were purchased from

Pharmacology Department, Faculty of pharmacy, Mansoura University, Egypt. They were housed in microlon boxes in a controlled environment (temperature 25 ± 2 °C and 12 h dark/light cycle) with standard laboratory diet and water regimen.

3.4.2.2. Methods. Animals were divided into 6 groups with 10 animals for each including normal (non-treated), control group (Ehrlich only, non-treated), treated group with the target compounds (5 mg/kg) and positive control group treated with 5-fluorouracil (20 mg/kg). All the animals in the treated groups (from 2 to 6) were inoculated with 2×10^6 Ehrlich ascite carcinoma cells/mouse on the day zero. Treatment started 24 h after inoculation by intra-protenial injection of the drug. The animals in groups 3–5 were injected by the tested compound in a dose of 100 μg/mouse, which is nearly equivalent to 5 mg/kg of body weight, while the standard group (group 6) has received I.P. treatment with 20 mg/kg of body weight of 5-fluorouracil. The control group (group 2) was treated with the same volume of 0.9% sodium chloride solution. All the treatments were given for nine successive days.

3.5. Molecular modeling

3.5.1. Modeling of solvatochromism through solvation energy calculations

Initial structures for the complex **4** as a representative example and the solvents (Table 7) used in this study were constructed using the HyperChem version 5.1 program, which was also used for manipulation and interactive solvation energy calculation protocol. The conformational searching in torsional space was performed using the multi-conformer method [17b]. Energy minima for **1**, **2** and **4** were determined by a semi-empirical method PM3 (as implemented in HyperChem 5.1). The conformations thus obtained were confirmed as minima by vibrational analysis. Atom-centred charges for each molecule were computed from the PM3 wavefunctions (HyperChem 5.1) which provides derived charges that closely resemble those obtainable from *ab initio* 6-31G* calculations. The MM+ force field (as implemented in HyperChem 5.1) was used for all interactive docking of the host–guest molecular dynamic calculations. For reasons of computational expense, solvent effect was simulated using a simple distance-dependent dielectric constant and two molecules of a solvent were used for docking into vicinity of complex **4**. The distance between the metal core of complex and the docked solvent was restrained up to 4 Å. The complex–solvent molecules were initially regularized by conjugate-gradient molecular modeling to reduce poor intermolecular steric contacts so as to minimize the energy of the bound solvent alone and for minimization of the unrestrained complex to an energy gradient of <0.01 kcal. MD simulations of each complex were subsequently performed for 50 ps at 300 K. Atomic coordinates were sampled at 5 ps intervals during the simulation period. In each case, the averaged structure from the accumulated snap shots was subjected to final molecular mechanics relaxation. The Polak–Ribiere minimization method was applied with a gradient value of 0.01 kcal to test for convergence, to generate the refined complex. The energy of the complex–solvent ($E_{\text{complex-solvent}}$) and the energies of solvent (E_{solvent}) and complex (E_{complex}) individually after separating from the complex were calculated. Energy of solvation ($E_{\text{solvation}}$) between complex and solvent molecules was calculated using the following formula: $E_{\text{solv}} = E_{\text{complex-solvent}} - (E_{\text{complex}} + E_{\text{solvent}})$, where E_{solv} = total energy of interaction of complex and solvent, E_{complex} = total energy of the complex alone, E_{solvent} = total energy of the two molecules of solvent.

3.5.2. DNA binding calculation using molecular dynamic docking

Molecular dynamic studies were carried out using the following protocol: heating phase (equilibration) = 30 ps and sampling phase = 100 ps. Intermittent structures of the complex formed at every 10 ps of simulation were collected and subjected to EM. The minima of all the snap shots were examined in order to select the lowest energy conformation as a representative of DNA–target complex for further studies. The energy of the DNA–target complex (E_{complex}) and the energies of DNA (EAT) and target molecules (E_{target}) individually after separating from the complex were calculated. Energy of interaction (E_{int}) between DNA (AT) and target molecule was calculated using the following formula: $E_{\text{int}} = E_{\text{complex}} - (E_{\text{AT}} + E_{\text{target}})$, where E_{int} = energy of interaction of the complex, E_{complex} = total energy of the complex, E_{AT} and E_{target} are the individual total energies of the DNA (AT) and the target molecules calculated after they are separated from each other, respectively.

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