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REGULAR ARTICLE

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Modification of the toxicity of an azo compound through complex formation help target bacterial strains

TATHAGATA DEB[a], DURBA GANGULY[b], SAURADIP SEN[c], PANKAJ GIRI[b], PUBALI DHAR[c] and SAURABH DAS[b,*]

[a]Department of Chemistry, RCC Institute of Information Technology, Kolkata, West Bengal 700 015, India
[b]Department of Chemistry (Inorganic Section), Jadavpur University, Kolkata, West Bengal 700 032, India
[c]Laboratory of Food Science and Technology, Food and Nutrition Division, University of Calcutta, Kolkata, West Bengal 700 027, India
E-mail: sdas@chemistry.jdvu.ac.in; dasrsv@yahoo.in

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Abstract. The antibacterial efficacy of a Cu(II) complex of 2-hydroxyphenyl-azo-2′-naphthol (HPAN) was studied on gram-positive *Bacillus subtilis* and gram-negative *Escherichia coli*. *In vitro* antimicrobial activity was determined by an agar-well diffusion assay and minimum inhibitory concentration. $Cu^{II}(HPAN)_2$ was found to be better than HPAN in antibacterial activity. Although both bacterial strains succumbed to high concentrations of each compound, at low concentrations only $Cu^{II}(HPAN)_2$ was active on *B. subtilis*. To explain the observations, reductive cleavage of the azo-bond to aromatic amines was followed by an *in vitro* enzyme assay using cell extracts of *E. coli* containing azo-reductase. Interaction of $Cu^{II}(HPAN)_2$ with calf thymus DNA was compared with HPAN for correlation with antibacterial activity. Enzyme-assay on the reductive degradation of azo bond and DNA interaction do not individually explain trends observed in antibacterial activity. Comparable binding of $Cu^{II}(HPAN)_2$ and HPAN with calf thymus DNA was attributed to the presence of anionic species of the complex in solution. Significant activity of the complex was probably due to effective cellular uptake of it by bacterial cells as shown by a fluorescence study. The activity of the complex resembled some established antimicrobial agents. Since the complex has a moiety, not common to most antibacterial agents, resistance towards it should be significantly less, hence an advantage.

Keywords. Cu(II) complex; *Bacillus subtilis*; *Escherichia coli*; azo-reductase; reductive-cleavage; fluorescence microscope.

Abbreviations

HPAN	2-Hydroxyphenyl azo-2′-naphthol
$[Cu^{(II)}(HPAN)_2]$	Complex of Cu(II) with 2-hydroxy phenyl azo-2′-naphthol
c t DNA	Calf thymus DNA
MIC	Minimum inhibitory concentration

1. Introduction

Search for new molecules as anti-microbial agents is an important aspect of research today.[1–3] There is a growing need to synthesize new molecules to address issues pertaining to drug resistance and to specifically target the pathogenic microbes.[4–7] The effectiveness of antibiotics along with an easy access has led to their overuse, enabling several bacteria to develop different levels of resistance.[8–10] Modifying compounds for which resistance has developed is therefore a way of extending the lifespan of many antimicrobial agents.[4] Although some success has been achieved through this approach, resistance to the modified forms developed after some time. Another major problem with most broad-spectrum antibiotics is that they destroy a lot of the 'useful and friendly microbes' present in our system in an effort to kill the pathogenic ones resulting in the development of new human disorders like obesity, asthma and cancer.[11] In this perspective, drug design needs to look at chemical moieties that have remained unexplored but for whom there is sufficient evidence to believe they could be active on disease-causing microbes.[12,13] Azo dyes are one such group of compounds that could be important in this regard.[12–16] They are cytotoxic, often correlated

*For correspondence
Durba Ganguly and Sauradip Sen have made an equal contribution to this work.

to the reductive cleavage of the azo bond that could be utilized for antimicrobial activity provided they do not adversely affect the host.[16–20] The fact that azo compounds are cytotoxic on microbes is well known but what is important is to prepare compounds for a specific cause choosing suitable starting materials so that the final product is an effective antimicrobial agent, not much toxic to the host.[16] Earlier, we demonstrated that an azo compound (AHPD) and its Cu^{II} complex show antibacterial activity on *Escherichia coli* and *Staphylococcus aureus* and that complex formation was able to modulate cytotoxicity.[21] Such modulation is important to restrict toxic side effects associated with drug action.[22]

The most discussed reason for azo-toxicity is the degradation of the azo bond forming primary amines and the ability of the compounds or of the amines to interact with cellular material.[14,23] It is also realized that factors important for one organism might not be important for another. Often action by the same compound is seen to depend on the organism's biochemical constitution and on its ability to resist action.[16,24,25] Therefore, it is not at all surprising that in response to such resistance, major pharmaceutical companies concentrate their effort on modifying an established agent rather than look for new molecules as substitutes. For a while, most researchers did try to understand alterations within a particular parent structure for eliminating drug resistance.[8] However, today there is greater endeavor to develop new drugs capable of working on different targets but more importantly to see to the fact they lack a history of microbial resistance.[12,26] In this study, 2-hydroxyphenyl azo-2′-naphthol (HPAN) and its Cu^{II} complex were tried on *Bacillus subtilis* and *Escherichia coli*. An earlier study on the complex revealed that Cu^{II} in combination with HPAN increased cellular uptake that helped to explain enhanced activity of the complex on MOLT-4 leukemia cells.[27]

2. Experimental

2.1 *Materials*

HPAN and $Cu^{II}(HPAN)_2$ were prepared from suitable starting materials.[27] Stock solutions of HPAN and $Cu^{II}(HPAN)_2$ were prepared in DMSO ($\sim 10^{-4}$ M). NADPH and calf thymus DNA were purchased from Sisco Research Laboratories (SRL), India. The DNA was dissolved in triple distilled water and absorbance was recorded at 260 and 280 nm respectively. Ratio of absorbance (A_{260}/A_{280}) was calculated and found to be in the range $1.8 < A_{260}/A_{280} < 1.9$ indicating that the DNA was sufficiently free of protein. Concentration was determined in terms of nucleotide taking molar extinction coefficient as 6,600 M^{-1} cm^{-1} at 260 nm. Quality of the DNA was also checked from the characteristic CD band at 260 nm. Solutions containing DNA had 120 mM NaCl, 35 mM KCl and 10 mM phosphate buffer ($\sim$ pH 7.4). All solutions were prepared in triple distilled water.

2.1a *Bacterial strains*: *Bacillus subtilis* (ATCC 6633) and *Escherichia coli* (ATCC 10536) were used. The strains were stored at −70 °C in 15% (v/v) glycerol until they were sub-cultured onto respective media. Bovine heart infusion (BHI) media used for culture of bacterial cells was purchased from High Media Laboratories, India. Agar powder and Trypticase soy broth (TSB) was procured from SRL, India. Cell extracts of *Escherichia coli* from which cells were removed by centrifugation was used in enzyme assay to follow the azo-reductase activity.[28,29]

2.2 *Methods*

2.2a *Physical measurements*: A CD spectropolarimeter J815, JASCO, Japan was used for determining the quality of calf thymus DNA. UV-Vis spectra were recorded on JASCO J-630 spectrophotometer, Japan.

2.2b *Sensitivity of test organisms against compounds by agar well diffusion method*: An agar well-diffusion method was used to evaluate inhibitory character of the Cu^{II} complex of HPAN on the test micro-organisms.[30] A freshly grown culture was serially diluted. 0.1 mL diluted inoculums (10^6 CFU/mL) of test organism was spread on agar plates. MHA (HIMEDIA) was used for gram-positive *Bacillus subtilis* (ATCC 6633) and gram-negative *Escherichia coli* (ATCC 10536). Wells (6 mm in diameter) were made on agar plates using a sterilized stainless steel borer. Wells were filled with 50 μL diluted solution. Concentrations employed were 100 μM, 200 μM and 400 μM, respectively. Streptomycin, tetracycline and ampicillin were used as positive control while DMSO was the negative control. The plates were left at room temperature for 30 min to allow diffusion of materials in media. Plates were then incubated at 37 °C for 24 h. Inhibition zones in cm (including well diameter) around wells were measured. Antimicrobial activity has been expressed as the diameter of inhibition zones created by the compounds against test microorganisms. The experiment was repeated four times. A zone size greater than 7 mm indicated that bacteria were susceptible to compounds.[31] Zone of inhibition were compared with the zone size interpretative chart supplied by HIMEDIA.[32] The simple character of the agar-diffusion assay, its wide range for effective concentrations and compatibility with many organisms allow it to be used extensively in drug discovery.

2.2c *Determination of minimum inhibitory concentration (MIC) of the compounds against test organisms by successive dilution method*: Minimum inhibitory concentration (MIC) was determined by the method of successive dilution. Compounds were added to Tryptic soya broth (TSB) and two-fold serially diluted to obtain concentrations in the

range of 0.001–3 mg/mL. 0.1 mL of bacterial suspension (10^6 CFU/mL) was inoculated into each of the broth dilution tubes. After incubation overnight at 37 °C, micro-dilution tubes were checked visually to detect growth inhibition of bacteria. Growth endpoints were determined by comparing the amount of growth in the tubes containing test samples with that in the negative control; MIC being defined as the lowest concentration of the compounds able to inhibit any visible growth.[33]

2.2d *Enzyme assay for in vitro reduction of the azo bond using bacterial cell extracts obtained from Escherichia coli*: Azo-reductase present in bacterial cells catalyze the reduction of the azo-bond forming primary amines.[17–19,24] Cell extract from *Escherichia coli* was taken as the source of azo-reductase and reduction was followed at the λ_{max} of each compound.[28,29] NADPH was the reducing substrate while compounds HPAN and $Cu^{II}(HPAN)_2$ were electron acceptors. 410 μL of phosphate buffer, 120 μL of 0.5 M NaCl, 100 μL of compound (10^{-3} M), 70 μL of NADPH were mixed in a 1.7 mL quartz cuvette. The reaction was initiated by adding 300 μL cell extract to the mixture in the cuvette. The final assay solution (1.0 mL) had 100 μM compound and 444 μM NADPH. Contents of the cuvette were mixed and UV/Vis spectra were recorded against a buffer-DMSO blank. The decrease in absorbance of the azo group was followed at 529 nm for HPAN and 522 nm for $Cu^{II}(HPAN)_2$ for 30 min with the spectrum being recorded every minute. The assay was done three times.

2.2e *Binding of $Cu^{II}(HPAN)_2$ with calf thymus DNA*: Interaction of $Cu^{II}(HPAN)_2$ with calf thymus DNA was studied by following the change in absorbance at 527 nm. Keeping concentration of $Cu^{II}(HPAN)_2$ constant at 100 μM, calf thymus DNA was gradually increased. Binding parameters were evaluated using different equations.[34–38]

$$L + DNA \rightleftharpoons L - DNA \quad K_d = \frac{[L][DNA]}{[L - DNA]} \tag{1}$$

L represents $Cu^{II}(HPAN)_2$ and K_d denotes the dissociation constant. Eq. 1 yields Eq. 2 where reciprocal of the change in absorbance was plotted against reciprocal of $(C_D - C_0)$. C_D is the concentration of calf thymus DNA and C_0 the concentration of $Cu^{II}(HPAN)_2$. Eq. 2 provides ΔA_{max} and K_d $(= 1/K_{app})$ as intercept and slope respectively.[35–37]

$$\frac{1}{\Delta A} = \frac{1}{\Delta A_{max}} + \frac{K_d}{\Delta A_{max}(C_D - C_0)} \tag{2}$$

ΔA indicates change in absorbance of $Cu^{II}(HPAN)_2$ at 527 nm while ΔA_{max} denotes the maximum change in absorbance.

$$K_d = \frac{\left[C_{0-}\left(\frac{\Delta A}{\Delta A_{max}}\right)C_0\right]\left[C_{D-}\left(\frac{\Delta A}{\Delta A_{max}}\right)C_0\right]}{\left(\frac{\Delta A}{\Delta A_{max}}\right)C_0} \tag{3}$$

$$C_0\left(\frac{\Delta A}{\Delta A_{max}}\right)^2 - (C_0 + C_D + K_d)\left(\frac{\Delta A}{\Delta A_{max}}\right) + C_D = 0 \tag{4}$$

$\Delta A/\Delta A_{max}$ was plotted against the concentration of calf thymus DNA and fitted to Eq. 4 yielding K_d $(= 1/K_{app})$.[34–38] Data obtained from the titration of the complex with calf thymus DNA was analyzed according to Scatchard.[39] Intrinsic binding constant (K′) and site size (n) were obtained [Eq. 5].

$$r/C_f = K'(n - r) \tag{5}$$

$r = C_b/C_D$; 'C_b' and 'C_f' being concentrations of the bound and free forms of $Cu^{II}(HPAN)_2$ respectively. 'n' denotes binding stoichiometry (number of bound molecules of $Cu^{II}(HPAN)_2$ per nucleotide while 'n_b', reciprocal of 'n' indicates the number of nucleotides bound to $Cu^{II}(HPAN)_2$. Intrinsic binding constant K′ is then related to K_{app} as $K' = K_{app} \times n_b$.[34–38]

2.2f *Cellular uptake of $Cu^{II}(HPAN)_2$ by bacterial cells*: Gram-positive *Bacillus subtilis* (ATCC 6633) and gram-negative *Escherichia coli* (ATCC 10536) were treated with $Cu^{II}(HPAN)_2$. Cell extracts of the lysed bacteria were obtained by centrifugation. Uptake of $Cu^{II}(HPAN)_2$ by bacterial cells was checked by treating cell extracts with potassium ferrocyanide. This is based on the understanding if $Cu^{II}(HPAN)_2$ is present in the cell extract it would interact with ferrocyanide ions $[Fe(CN)_6^{4-}]$ [Eq. 6] to form cupric ferrocyanide which would absorb at 478 nm.[27]

$$2Cu^{II}(HPAN)_2 + Fe(CN)_6^{4-} \rightleftharpoons Cu_2[Fe(CN)_6] + 4HPAN^- \tag{6}$$

A standard curve was prepared with Cu^{II} in the same concentration range as that present in the complex. It was used in experiments with bacterial cell extracts and ferrocyanide. It was prepared prior to the start of the actual experiment.[27] The absorbance for $Cu_2[Fe(CN)_6]$ was recorded at 478 nm.

2.2g *Cellular uptake of $Cu^{II}(HPAN)_2$ by bacterial cells using a fluorescence microscope*: Bacteria were cultured overnight for growth and 100 μL was taken in a test tube. HPAN and $Cu^{II}(HPAN)_2$ dissolved in DMSO were added in different test tubes and incubated in the dark for 2 h. The bacterial population in each test tube was 10^9 – 10^{10}. After incubation, pellets were formed by centrifugation and washed twice with PBS. Cells were fixed with 4% formaldehyde. Fluorescence was measured at DAPI range (358–461 nm) using a fluorescence microscope (Thermo Fisher model EVOS FL). Direct staining of bacterial samples with our complex $[Cu^{II}(HPAN)_2]$ was possible.

3. Results and Discussion

3.1 *Antimicrobial Activity of HPAN and $Cu^{II}(HPAN)_2$*

3.1a *Zone of inhibition*: Results suggest that both compounds possess moderate to good antibacterial

Table 1. Antibacterial activity of HPAN and $Cu^{II}(HPAN)_2$ by zone of inhibition.

Compounds	Concentration	Zone of inhibition (cm)	
		Gram positive *Bacillus Subtilis*	Gram negative *Eschereschia Coli*
HPAN	400.0 μM (105.6 μg/mL)	1.50 ± 0.02	1.50 ± 0.01
	200.0 μM (52.8 μg/mL)	1.15 ± 0.01	1.10 ± 0.02
	100.0 μM (26.4 μg/mL	No inhibition	No inhibition
$Cu^{II}(HPAN)_2$	400.0 μM (250.2 μg/mL)	1.60 ± 0.01	1.75 ± 0.01
	200.0 μM (125.1 μg/mL)	1.55 ± 0.01	1.15 ± 0.02
	100.0 μM (62.5 μg/mL)	1.50 ± 0.02	No inhibition
Tetracyclin	67.5 μM (30.0 μg/mL)	1.80 ± 0.01	1.80 ± 0.01
Ampicillin	28.6 μM (10.0 μg/mL)	1.70 ± 0.01	1.75 ± 0.01
Streptomycin	17.2 μM (10.0 μg/mL)	1.85 ± 0.01	1.80 ± 0.01

activity; the complex being superior at all concentrations (Table 1). Zone of inhibition increased with an increase in concentration. While HPAN could not inhibit the growth of either bacteria at low concentrations (< 100 μM), $Cu^{II}(HPAN)_2$ was effective on *Bacillus Subtilis*. An important observation with regard to the study on the zone of inhibition was that efficacy of $Cu^{II}(HPAN)_2$, although not exactly similar, was close to some standard antimicrobial agents in clinical use which is encouraging (Table 1).

3.1b *Minimum Inhibitory Concentration (MIC)*: MIC for HPAN and $Cu^{II}(HPAN)_2$ on bacterial strains further revealed the complex was more effective. While MIC for $Cu^{II}(HPAN)_2$ on *Bacillus subtilis* was 2.08 μM, on *Eschereschia coli* it was 4.32 μM. No value for HPAN was obtained in the concentration range 0.024 μM to 6.25 μM indicating HPAN did not inhibit growth of either bacteria in the concentration range mentioned. HPAN was tested using concentrations 64 μM and 128 μM; MIC was obtained at 128 μM. This clearly confirmed that complex formation of HPAN with copper(II) increased anti-bacterial activity considerably. $Cu^{II}(HPAN)_2$ was more effective on *Bacillus subtilis* than on *Escherichia coli* with MIC values being almost half of that found for *Escherichia coli*. Bacterial cells were treated with aqueous Cu^{2+} in the form of an aqueous solution of copper(II) nitrate as a control. They were also treated with an innocent complex of Cu^{II} namely Cu^{II}-EDTA [EDTA=ethylenediaminetetraacetic acid] considering it to be the other control. MIC for aq. Cu^{II} was > 162 μM while for Cu^{II}(EDTA) it was 162 μM. Thus, aq. Cu^{II} or Cu^{II}-EDTA (having an approximately similar molecular weight as $Cu^{II}(HPAN)_2$) were not effective on bacterial cells studied. It is worth mentioning here that earlier studies on complexes of HPAN showed they were not toxic to normal cells as well.[27–29]

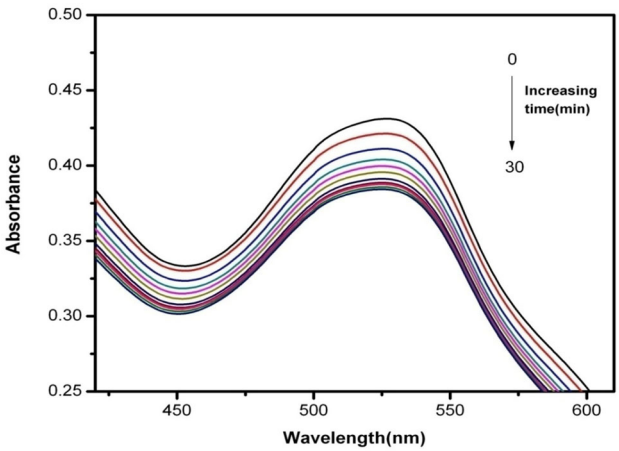

Figure 1. Plots showing the absorbance of HPAN in the presence of NADPH and azo-reductase (from *Escherichia coli*) in phosphate buffer (pH ∼ 7.4) containing 0.06 M NaCl for time t = 0 to t = 30 min at 310 K in an enzyme assay for a gradual reduction of an azo bond. The spectra indicate a gradual decrease in absorbance at 529 nm. Cell extract from *Escherichia coli* = 300 μL; [NADPH] = 3.2×10^{-4} gm/mL; [HPAN] = 100 μM.

3.2 *Assay for azo-reductase activity ofEschereschia colion HPAN and $Cu^{II}(HPAN)_2$*

Bacterial azo-reductase activity is responsible for the breakdown of azo bonds to primary amines.[16–19,24] Reductive fission of the azo bond occurs in the presence of NADPH, taken as an electron donor.[17–19,24] Enzyme assay was done using cell extracts from *Escherichia coli* [Figure 1: HPAN; Figure 2: $Cu^{II}(HPAN)_2$]. Figure 3 demonstrates the percentage of each compound remaining after the assay revealing reductive fission of the azo bond was slightly less for $Cu^{II}(HPAN)_2$ which is in line with earlier reports from our laboratory where the same compounds were used in a model azo-reductase assay.[27]

With only a very marginal decrease in the formation of azo-cleaved products in case of the complex, one

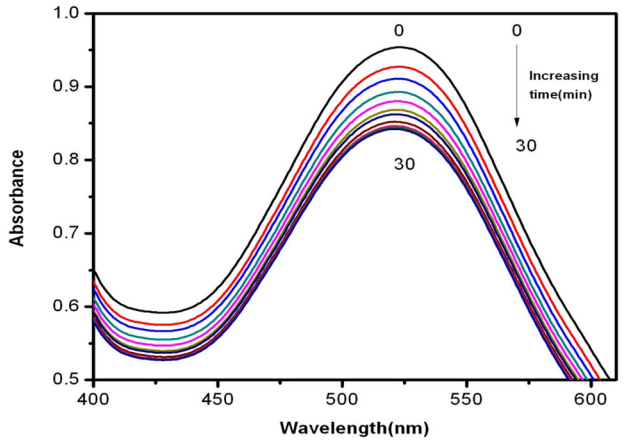

Figure 2. Plot of absorbance of $Cu^{II}(HPAN)_2$ in presence of NADPH and azo-reductase (from *Escherichia coli*) in phosphate buffer (pH $\sim$ 7.4) containing 0.06 M NaCl for time t = 0 to t = 30 min at 310 K in an enzyme assay showing a gradual reduction of an azo bond. Spectra indicate a gradual decrease in absorbance at 522 nm. Cell extract from *E. coli* = 300 µL; [NADPH] = 3.2×10^{-4} gm/mL; $[Cu^{II}(HPAN)_2] = 100$ µM.

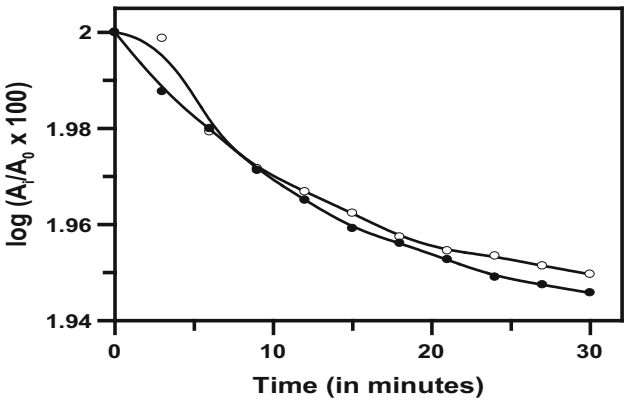

Figure 3. Comparison of the rate of reduction of HPAN (●) and $Cu^{II}(HPAN)_2$(○) [Cell extract from *Eschereschia coli* = 300 µL; HPAN = $Cu^{II}(HPAN)_2$ = 100 µM, NADPH = 444 µM].

would expect both compounds to have similar effects on bacterial cells, if the formation of azo-cleaved products (i.e., amines) were solely responsible for the cytotoxic action. However, studies on the interaction of the compounds with *Bacillus subtilis* and *Escherichia coli* indicate that $Cu^{II}(HPAN)_2$ was much more effective. This meant, besides formation of amines within bacterial cells, the presence of Cu^{II} is also important and probably helped the complex to show better antibacterial activity.[27,37,40]

3.3 *Binding of $Cu^{II}(HPAN)_2$ with calf thymus DNA using UV-Vis spectroscopy*

Interaction of $Cu^{II}(HPAN)_2$ with calf thymus DNA was studied to see if DNA binding was important and played

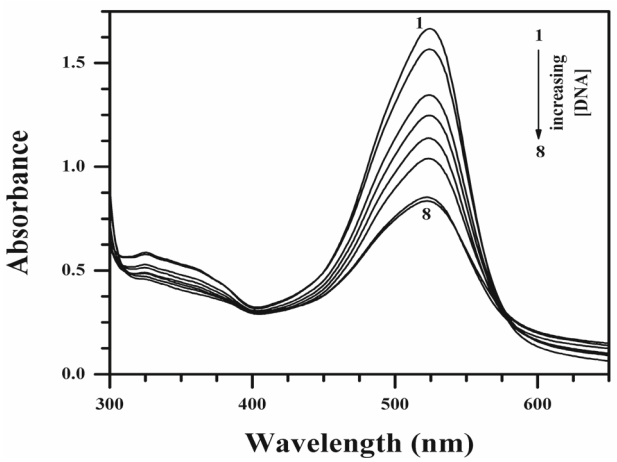

Figure 4. Absorbance spectra of 100 µM $Cu^{II}(HPAN)_2$ in 120 µM of NaCl and phosphate buffer in the absence (1) and in the presence of increasing ct DNA concentrations; T = 298 K.

a part in the observed antibacterial activity. Such model studies are often useful in understanding how compounds might interact when present within cells. Since the interaction of HPAN with calf thymus DNA was reported earlier, only that for $Cu^{II}(HPAN)_2$ is provided in this report.[27]

Addition of calf thymus DNA to 100 µM $Cu^{II}(HPAN)_2$ results in a gradual decrease in absorbance at 527 nm. We obtained a regular pattern till saturation was reached (Figure 4). Addition of DNA was continued well beyond saturation to be sure no further interaction occurred. Titration was repeated six times. Binding isotherms were analyzed using the double reciprocal plot (Eq. 2, Figure 5), non-linear curve fit analysis (Eqs. 3 and 4, Figure 6) and the Scatchard plot (Eq. 5, Figure 7) considering the equilibrium in Eq. 1.[34–38] Calculations were based on the assumption, absorbance at 527 nm is linearly proportional to the interaction of $Cu^{II}(HPAN)_2$ with calf thymus DNA.

Using Eq. 2 (Figure 5), the value of the apparent binding constant was $[(3.51 \pm 0.30) \times 10^3\ M^{-1}]$ obtained as the inverse of K_d. ΔA_{max} was calculated from the intercept of Figure 5. Using Eqs. 3 and 4 (Figure 6), K_{app} was obtained as $[(3.74 \pm 0.25) \times 10^3\ M^{-1}]$. Values for K_{app} were in good agreement with each other. Inset of Figure 6 shows a plot of $\Delta A/\Delta A_{max}$ against the ratio of nucleotide concentration to $Cu^{II}(HPAN)_2$. Two straight lines were obtained, the intersection of which provides n_b(= 13.0). Multiplying K_{app} obtained from the double reciprocal plot and non-linear fit by n_b, K′ was found to be $[(4.56 \pm 0.40) \times 10^4 M^{-1}]$ and $[(4.86 \pm 0.32) \times 10^4 M^{-1}]$, respectively.

Data for the titration was also treated according to the Scatchard equation and Figure 7 yields K′ = [(4.29 ±

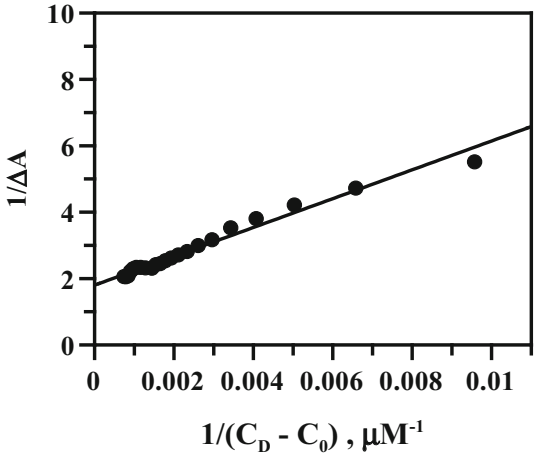

Figure 5. Double reciprocal plot obtained from the spectrophotometric titration of $Cu^{II}(HPAN)_2$ with calf thymus DNA. The dark line shows the fitted data according to Eq. 2. $[Cu^{II}(HPAN)_2]$ = 100 μM, [NaCl] = 120 mM, pH = 7.4, T = 298 K.

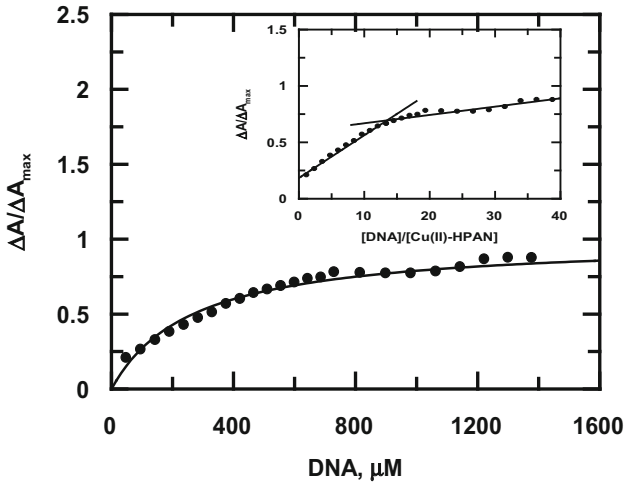

Figure 6. Binding isotherm of $Cu^{II}(HPAN)_2$ interacting with calf thymus DNA. The dark line shows a non-linear fit according to Eq. 4. $[Cu^{II}(HPAN)_2]$ = 100 μM, [NaCl] = 120 mM; pH = 7.4 and T = 298 K; Inset: Plot of normalized increase of absorbance as a function of mole-ratio of $Cu^{II}(HPAN)_2$ to calf thymus DNA. $[Cu^{II}(HPAN)_2]$ = 100 μM, [NaCl] = 120 mM; pH = 7.4; T = 298 K.

0.4) × 10^4 M^{-1}]. Values for intrinsic binding constant were not only in good agreement with each other but also with those obtained earlier using fluorescence spectroscopy.[27] The site size of interaction obtained from this study was in good agreement with values obtained earlier where change in fluorescence was used to follow the interaction with calf thymus DNA.[27] Interaction of HPAN with calf thymus DNA reported earlier had apparent binding constant values in the range (1.42 − 3.65) × 10^4 M^{-1}, while intrinsic binding constant values were in the range (5.4−9.0) × 10^4 M^{-1}.[27] Values

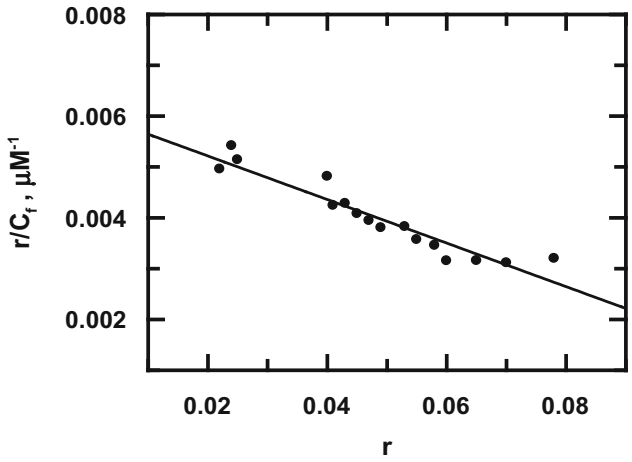

Figure 7. Scatchard plot for the spectrophotometric titration of $Cu^{II}(HPAN)_2$ with calf thymus DNA; $[Cu^{II}(HPAN)_2]$ = 100 μM; [NaCl] = 120 mM; pH = 7.4; Temp. = 298 K.

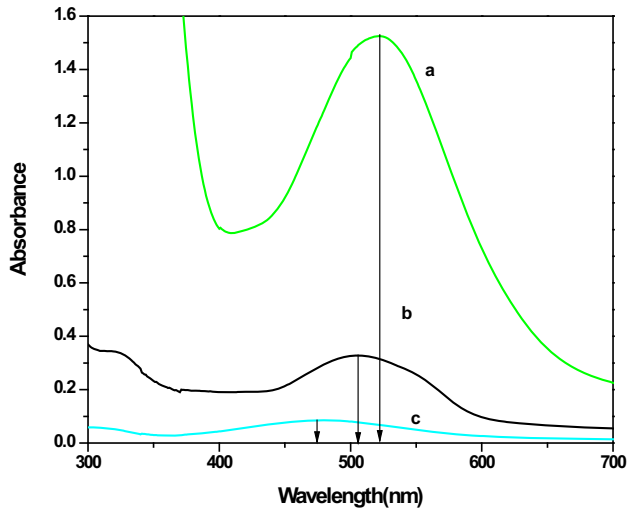

Figure 8. (a) Absorption spectrum of pure $Cu^{II}(HPAN)_2$ of concentration ∼ 100 μM; (b) absorption spectrum obtained after addition of potassium ferrocyanide to a bacterial cell lysate of $Cu^{II}(HPAN)_2$ treated *Escherichia coli;* (c) absorption spectrum of 100 μM Cu^{2+} treated with excess potassium ferrocyanide.

for $Cu^{II}(HPAN)_2$ with calf thymus DNA in this study as well as an earlier study (by fluorescence) were therefore comparable to HPAN.[27] It is usually seen that the binding of a complex to DNA is greater than the ligand used to prepare the complex.[41,42] However, in this study, we did not find any significant increase in the value for the binding constant of the complex which could be due to the presence of anionic forms of it in a solution that tends to decrease interaction with DNA.[36,37,43,44]

Since the pK_a of the dissociation of the second proton of HPAN in presence of Cu^{II} is 8.1, hence at physiological pH (∼ 7.4), a good amount of $Cu^{II}(HPAN)_2$ would remain dissociated due to deprotonation on

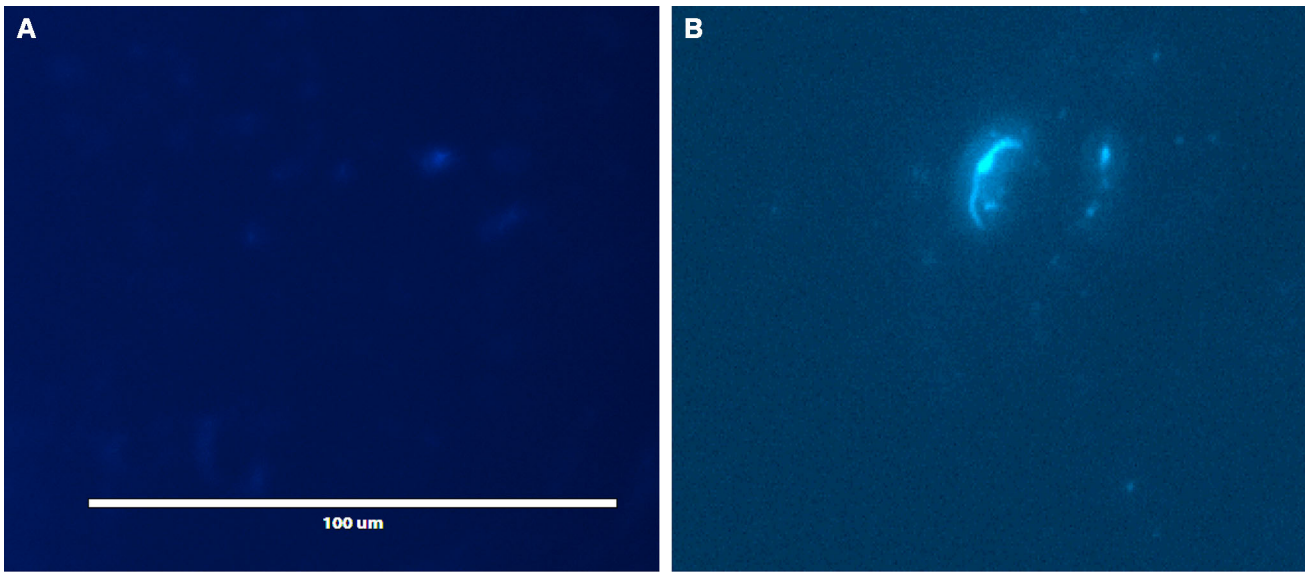

Figure 9. Preferential cellular uptake of $Cu^{II}(HPAN)_2$ in *Escherichia coli* (ATCC 25922) as evidenced by the fluorescence of the compound recorded in a fluorescence microscope. 'A' shows the presence of HPAN in bacterial cells following incubation and 'B' that of $Cu^{II}(HPAN)_2$ in the same bacterial cells under identical experimental conditions.

each ligand.[27] This results in di-anionic species of the complex in solution that would lower its ability to bind to DNA. Hence, in the light of results obtained with regard to DNA interaction, one should expect $Cu^{II}(HPAN)_2$ to have almost comparable activity as HPAN on bacterial cells; i.e., if DNA binding has a role in the efficacy of the molecules.

Hence, if we now consider the findings of the antimicrobial assay in the light of the two experiments performed, none really suggests conclusively why the complex is more effective. However, the fact remains, as demonstrated through experiments performed on two different bacterial cells that the complex is a better anti-bacterial agent. Hence, there is some reason that enabled $Cu^{II}(HPAN)_2$ to show such significant antibacterial activity. One reason could be an effective cellular uptake of $Cu^{II}(HPAN)_2$ by bacterial cells. This is suggested since we detected similar behavior for $Cu^{II}(HPAN)_2$ on MOLT-4 leukaemia cells earlier where the complex could enter cells much better and impart cytotoxic activity.[27]

3.4 *Detection of cellular uptake of $Cu^{II}(HPAN)_2$ by cells using potassium ferrocyanide*

Following incubation of the compounds with *Bacillus subtilis* (ATCC 6633) and *Escherichia coli* (ATCC 10536), cell lysates were treated with potassium ferrocyanide and the spectrum of the resulting solution was recorded. The spectrum of the lysate solution (Figure 8b) appeared as a broadband with its λ_{max} lying between pure $Cu^{II}(HPAN)_2$ at 522 nm (Figure 8a) and that of cupric ferrocyanide at 478 nm (Figure 8c). The appearance of a broadband is an evidence that Cu(II) is present in the cell lysate. The same experiment when performed on cell lysates obtained from bacteria not incubated with $Cu^{II}(HPAN)_2$ did not show spectrum in Figure 8b (i.e., neither the broadband nor any peak at 478 nm was obtained indicating an absence of Cu(II) in these cells. This experiment clearly supports the fact $Cu^{II}(HPAN)_2$ is capable of penetrating bacterial cells and was hence found present in bacterial cell extracts when the latter was treated with potassium ferrocyanide (Eq. 6). The appearance of the broadband for spectrum b in Figure 8 is also proof that both $Cu^{II}(HPAN)_2$ and $Cu_2[Fe(CN)_6]$ are present in equilibrium following treatment of the cell lysate with potassium ferrocyanide. The control experiment not containing $Cu^{II}(HPAN)_2$ serves as evidence of the fact bacterial cell extracts do not contain Cu(II) on their own. The molar absorptivity (ε) calculated for the spectrum in Figure 8b was seen to be in good agreement with that of $Cu^{II}(HPAN)_2$ (Figure 8a) and $Cu_2[Fe(CN)_6]$ (Figure 8c) with the value lying in between spectrum a and spectrum c (Figure 8).

3.5 *Cellular uptake of $Cu^{II}(HPAN)_2$ using fluorescence microscopy*

Escherichia coli (ATCC 25922) was used to study cellular uptake of $Cu^{II}(HPAN)_2$ in comparison to HPAN.

The comma-shaped cells were clearly visible under a fluorescence microscope after being stained with the complex (Figure 9). Cells showed blue fluorescence. A comparison reveals $Cu^{II}(HPAN)_2$ provides better resolution than HPAN that clearly demonstrate $Cu^{II}(HPAN)_2$ penetrated cells much better than the ligand.

Another important outcome of the present study was that it helped us realize that the Cu(II) complex of HPAN could be used as an important fluorescence probe in studies related to cell biology.

4. Conclusions

Some interesting observations were made in a study on the interaction of *Bacillus subtilis* and *Escherichia coli* with HPAN and $Cu^{II}(HPAN)_2$. Low concentrations of HPAN (< 100 μM) had no impact on any bacterial strain while 100 μM of $Cu^{II}(HPAN)_2$ showed some activity on *Bacillus subtilis* but not on *Escherichia coli*. At concentrations equal to or higher than 200 μM, both HPAN and $Cu^{II}(HPAN)_2$ showed inhibition of bacterial growth with the complex being more effective. MIC values reveal $Cu^{II}(HPAN)_2$ could induce cell death at low concentrations of 2.08 μM for *Bacillus subtilis* and 4.32 μM for *Escherichia coli*. MIC for HPAN was found to be 128 μM. An enzyme assay on the generation of amines and studies on DNA binding failed to explain the higher bactericidal effects of the complex with regard to HPAN. However, a combination of the two probes allowed us to predict better cellular activity for the complex. The idea of effective cellular uptake of the complex by bacterial cells was considered an important aspect. With the help of fluorescence microscopy, we could show the difference in cellular uptake between the copper complex and HPAN. Although there could be more reasons for the difference observed in the interaction of the compounds with bacterial cells than understood from the two probes we used, the involvement of Cu^{II} with HPAN was thought to be important. Since $Cu^{II}(HPAN)_2$ has a chemical moiety not very common in antimicrobial agents, microbial resistance should be less which was another important outcome of this work. While trying to design experiments to prove cellular uptake in the chosen bacterial cells using a fluorescence microscope, we realized that our complex could be potentially useful as a good staining agent for these experiments.

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References

1. Matos M J, Vazquez-Rodriguez S, Santana L, Uriarte E, Fuentes-Edfuf C, Santos Y and Muñoz-Crego A 2012 Looking for new targets: Simple coumarins as antibacterial agents *Med. Chem.* **8** 1140
2. Muegge I, Heald S L and Brittelli D 2001 Simple selection criteria for drug-like chemical matter *J. Med. Chem.* **44** 1841
3. Tenover F C 2006 Mechanisms of antimicrobial resistance in bacteria *Amer. J. Med.* **119** S3
4. Ames J R, Ryan M D and Kovacic P 1986 Mechanism of antibacterial action: Electron transfer and oxy radicals *J. Free Rad. Biol. Med.* **2** 377
5. Chait R, Craney A and Kishony R 2007 Antibiotic interactions that select against resistance *Nature* **446** 668
6. Chait R, Shrestha S, Shah A, Michel J-B and Kishony R 2010 A differential drug screen for compounds that select against antibiotic resistance *PLoS ONE* **5** e15179
7. Yeh P J, Hegreness M J, Aiden A P and Kishony R 2009 Drug interactions and the evolution of antibiotic resistance *Nat. Rev. Microbiol.* **7** 460
8. Taylor P W, Stapleton P D and Luzio J P 2002 New ways to treat bacterial infections *Drug Discov. Today* **7** 1086
9. Aminov R I 2010 A brief history of the antibiotic era: Lessons learned and challenges for the future *Front. Microbiol.* **1** 134
10. WHO's first global report on antibiotic resistance reveals serious, worldwide threat to public health (Press release 30 April 2014). The World Health Organization.
11. Blaser M J 2011 Antibiotic overuse: Stop the killing of beneficial bacteria *Nature* **476** 393
12. Mkpenie V, Ebong G, Obot I B and Abasiekong B 2008 Evaluation of the effect of azo group on the biological activity of 1-(4-methylphenylazo)-2-naphthol *E-J. Chem.* **5** 431
13. Gopalakrishnan S, Nevaditha N T and Mythili C V 2011 Antibacterial activity of azo compounds synthesized from the natural renewable source, cardanol *J. Chem. Pharma Res.* **3** 490

14. Puvaneswari N, Muthukrishnan J and Gunasekaran P 2006 Toxicity assessment and microbial degradation of azo dyes *Ind. J. Expt. Biol.* **44** 618
15. de Ventura-Camargo B C and Marin-Morales M A 2013 Azo dyes: Characterization and toxicity—A review *Text. Light Ind. Sci. Technol.* **2** 85
16. Mahmood S, Khalid A, Arshad M, Mahmood T and Crowley D E 2016 Detoxification of azo dyes by bacterial oxidoreductase enzymes *Crit. Rev. Biotechnol.* **36** 639
17. Hernandez P H, Gillette J R and Mazel P 1967 Studies on the mechanism of action of mammalian hepatic azoreductase. I. Azoreductase activity of reduced nicotinamide adenine dinucleotide phosphate-cytochrome c reductase *Biochem. Pharmacol.* **16** 1859
18. Rafii F and Cerniglia C E 1995 Reduction of azo dyes and nitroaromatic compounds by bacterial enzymes from the human intestinal tract *Environ. Health Perspect.* **103** 17
19. Chen H 2006 Recent advances in azo dye degrading enzyme research *Curr. Protein Pept. Sci.* **7** 101
20. Maria S G, Anca D, Elena G M, Germaine S B, Mihaiela A and Gianina B 2010 In 14th International Electronic Conference on Synthetic Organic Chemistry (ECSOC-14)
21. Ganguly D, Sarkar R, Santra R C, Deb T, Sen T and Das S 2014 Synthesis and characterization of 5-amino-2-((3-hydroxy-4-((3-hydroxyphenyl) phenyl) diazenyl) phenol and its Cu(II) complex—A strategy toward developing azo complexes for reduction of cytotoxicity *Complex Met.* **1** 13
22. Santra R C, Ganguly D, Singh J, Mukhopadhyay K and Das S 2015 A study on the formation of the nitro radical anion by ornidazole and its significant decrease in a structurally characterized binuclear Cu^{II}-complex: Impact in biology *Dalton Trans.* **44** 1992
23. Hartman C P, Fulk G E and Andrews A W 1978 Azo reduction of trypan blue to a known carcinogen by a cell-free extract of a human intestinal anaerobe *Mut. Res/Gen. Toxicol.* **58** 125
24. Stolz A 2001 Basic and applied aspects in the microbial degradation of azo dyes *Appl. Microb. Biotech.* **56** 69
25. Walsh C 2003 *Antibiotics: Actions, Origins, Resistance* (Washington, DC: American Society for Microbiology Press)
26. Cushnie T P T and Lamb A J 2005 Antimicrobial activity of flavonoids *Int. J. Antimicrob. Agents* **26** 343
27. Deb T, Gopal P, Ganguly D, Das P, Paul M, Saha M B, Paul S and Das S 2014 Enhancement of anti-leukemic potential of 2-hydroxyphenyl-azo-2′-naphthol (HPAN) on MOLT-4 cells through conjugation with Cu(II) *RSC Adv.* **4** 18419
28. Nakanishi M, Yatome C, Ishida N and Kitade Y 2001 Putative ACP phosphodiesterase gene (acpD) encodes an azoreductase *J. Biol. Chem.* **276** 46394
29. Ito K, Nakanishi M, Lee W C, Sasaki H, Zenno S, Saigo K, Kitade Y and Tanokura M 2006 Three-dimensional structure of *AzoR* from *Escherichia coli*. An oxidereductase conserved in microorganisms *J. Biol. Chem.* **281** 20567
30. Perez C, Paul M and Bazerque P 1990 Antibiotic assay by agar–well diffusion method *Acta Biol. Med. Exp.* **15** 113
31. Nascimento G G F, Locatelli J, Freitas P C and Silva G L 2000 Antibacterial activity of plant extracts and phytochemicals on antibiotic-resistant bacteria *Braz. J. Microbiol.* **31** 247
32. Bauer A W, Kirby W M M, Sherris J C and Turck M 1966 Antibiotic susceptibility testing by a standardized single disk method *Am. J. Clin. Pathol.* **45** 493
33. Vuotto M L, Basile A, Moscatiello V, Sole P De, Castaldo-Cobianchi R, Laqhi E and Lelpo M T 2000 Antimicrobial and antioxidant activities of Feijoaselowiana fruit *Int. J. Antimicrob. Agents* **13** 197
34. Guin P S, Mandal P C and Das S 2012 The binding of a hydroxy-9, 10-anthraquinone Cu^{II} complex to calf thymus DNA: Electrochemistry and UV/Vis spectroscopy *ChemPlusChem* **77** 361
35. Guin P S, Mandal P C and Das S 2012 A comparative study on the interaction with calf thymus DNA of a Ni(II) complex of the anticancer drug adriamycin and a Ni(II) complex of sodium 1,4-dihydroxy-9,10-anthraquinone-2-sulphonate *J. Coord. Chem.* **65** 705
36. Mukherjee S, Das P and Das S 2012 Exploration of small hydroxy-9,10-anthraquinones as anthracycline analogues: Physicochemical characteristics and DNA binding for comparison *J. Phys. Org. Chem.* **25** 385
37. Das P, Jain C K, Dey S K, Saha R, Choudhury A D, Roychowdhury S, Majumder H K, Kumar S and Das S 2014 Synthesis, crystal structure, DNA interaction and *in vitro* anticancer activity of a Cu(II) complex of purpurin: Dual poison for human DNA topoisomerase I and II *RSC Adv.* **4** 59344
38. Deb T, Chaudhury D, Guin P S, Saha M B, Chakrabarti G and Das S 2011 A complex of Co(II) with 2-hydroxyphenyl-azo-2′-naphthol (HPAN) is far less cytotoxic than the parent compound on A549-lung carcinoma and peripheral blood mononuclear cells: Reasons for reduction in cytotoxicity *Chem. Biol. Interact.* **189** 206
39. Scatchard G 1949 The attractions of proteins for small molecules and ions *Ann. N. Y. Acad. Sci.* **51** 660
40. Mandal B, Singha S, Dey S K, Mazumdar S, Kumar S, Karmakar P and Das S 2017 Cu^{II} complex of emodin with improved anticancer activity as demonstrated by its performance on HeLa and Hep G2 cells *RSC Adv.* **74** 1403
41. Eichhorn G L, Butzow J J, Clark P, Von Hahn H P, Rao G, Heim J M, Tarien E, Craper D R and Karlik S J 1980 in *Inorganic Chemistry in Biology and Medicine* ACA Symp. Ser. 140 A E Martell (Ed.) (Washington, DC: American Chemical Society) p. 75
42. Skov K A, Adomat H and Farrell N P 1987 In *Platinum Co-ordination Compounds in Cancer Chemotherapy* M Nicolini (Ed.) (The Hague: Martinus-Nijholf) p. 733
43. Mukherjee S, Gopal P K, Paul S and Das S 2014 Acetylation of 1,2,5,8-tetrahydroxy-9,10-anthraquinone improves binding to DNA and shows enhanced superoxide formation that explains better cytotoxicity on JURKAT T lymphocyte cells *J. Anal. Oncol.* **3** 122
44. Das P, Jain C K, Roychoudhury S, Majumder H K and Das S 2016 Design, synthesis and in vitro anticancer activity of a Cu(II) complex of carminic acid: A novel small molecule inhibitor of human DNA Topoisomerase I and Topoisomerase II *ChemistrySelect* **1** 6623