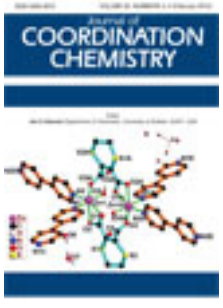

Journal of Coordination Chemistry
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/gcoo20

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

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Published online: 14 Feb 2012.

To cite this article: Partha Sarathi Guin , P.C. Mandal & Saurabh Das (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, Journal of Coordination Chemistry, 65:4, 705-721, DOI: 10.1080/00958972.2012.659730

To link to this article: http://dx.doi.org/10.1080/00958972.2012.659730

Journal of Coordination Chemistry
Vol. 65, No. 4, 20 February 2012, 705–721

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

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(*Received 28 October 2011; in final form 19 December 2011*)

The anthracycline drug adriamycin and its metal complexes are efficient in treating several forms of human cancers with recognized antineoplastic activity attributed to strong interactions with DNA within the target cells. The hydroxy-9,10-anthraquinone unit present in the molecule controls and regulates drug action. Metal ions when linked to adriamycin help to reduce the generation of radicals responsible for toxic side effects. A complex of adriamycin with Ni(II) was prepared and its physicochemical characteristics and DNA-binding ability were compared to a Ni(II) complex of sodium-1,4-dihydroxy-9,10-anthraquinone-2-sulphonate ($NaLH_2$), an analog of adriamycin. Interactions with calf thymus DNA of both complexes were studied by UV-Vis and fluorescence spectroscopy. Binding parameters determined for both complexes agree with each other. Binding of the Ni(II)-adriamycin complex to DNA was five to eight times stronger than for the Ni(II) complex of the hydroxy-9,10-anthraquinone analog, $Na_2[Ni(NaLH)_2Cl_2]\cdot 2H_2O$, i.e., $Ni(NaLH)_2$. The difference in binding was attributed to the presence of sugar units in adriamycin and to its absence in $NaLH_2$. Although the Ni(II) complex of the hydroxy-9,10-anthraquinone analog of adriamycin [$Ni(NaLH)_2$] was slightly weaker in binding DNA than the drug and its Ni(II) complex, a much lower cost of the former justifies its consideration as a substitute for the anthracycline drugs that are now in use.

Keywords: Adriamycin; Ni(II); $Ni(NaLH)_2$; Calf thymus DNA; Binding parameters

1. Introduction

The anthracycline drugs adriamycin (doxorubicin) and daunorubicin are anticancer agents [1] used in different forms of cancer. Anthracyclines contain a planar anthraquinone aglycone ring along with an amino-sugar moiety that exhibit several biological phenomena. The planar ring intercalates DNA base pairs and the amino-sugar moiety was reported to interact with the negatively charged phosphate groups in

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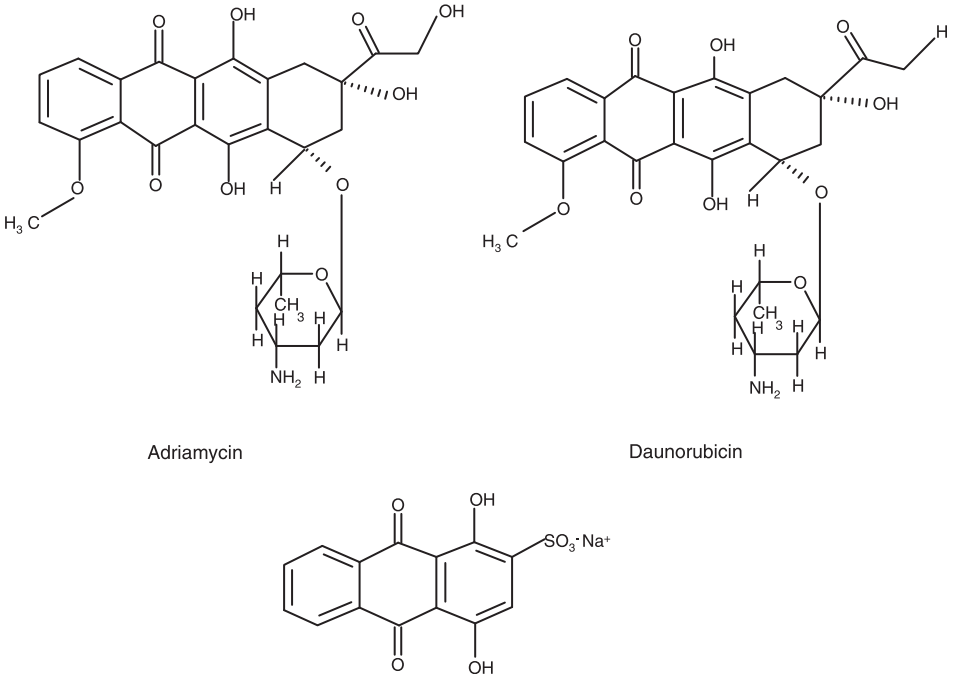
ISSN 0095-8972 print/ISSN 1029-0389 online
http://dx.doi.org/10.1080/00958972.2012.659730

the DNA major groove [2, 3]. Such intercalation causes a change in the shape of the DNA helix and the process hinders DNA replication and RNA transcription [4].

Clinical use of these drugs is limited owing to clinical and histopathologic evidence of dose-related cardiotoxicity [5]. Accumulating evidence indicates *in vivo* formation of semiquinone free radical intermediates by one-electron reduction of the quinone present in the drugs, undergo a sequence of reactions important for therapeutic efficacy but simultaneously being the reason for toxicity leading to severe side effects [6, 7]. Metal complex formation of anthracyclines provides a check on the formation of semiquinones and is a route to reduce toxic side effects [8–10]. Another important drawback of these drugs is their high cost resulting in a search for less costly alternatives. Adriamycin and daunomycin are natural products isolated from *Streptomyces peucetius* var. *caesius* [11, 12] and are highly potent active pharmaceutical ingredients that need special handling in production or preparation, making the entire process of isolation and purification costly. Hydroxy-9,10-anthraquinones that very closely resemble anthracyclines and mimic them through different chemical reactions either in their native state or as bound to metal ions are quite cheap [13–18]. The structures of sodium 1,4-dihydroxy-9,10-anthraquinone-2-sulphonate and the most widely used anthracycline anticancer drugs adriamycin and daunorubicin are shown in scheme 1 to indicate their similarity. Metal complexes of different anthracyclines [8–10, 19–24] were prepared and several studies were performed. Although some preliminary studies on a Ni(II)-adriamycin complex were reported earlier, details of its interaction with DNA were not mentioned [25]. The study qualitatively reported the ability of Ni^{2+} to interact with an adriamycin-DNA adduct [25]. Nickel ions have established roles



Scheme 1. Adriamycin, daunorubicin, and sodium 1,4-dihydroxy-9,10-anthraquinone-2-sulphonate.

in biology. For example, urease, an enzyme assisting in the hydrolysis of urea, contains nickel. Some of the Ni–Fe–hydrogenases contain nickel and such hydrogenases characteristically oxidize H_2. One of the carbon monoxide dehydrogenase enzymes contains an Fe–Ni–S cluster. Other nickel-containing enzymes include the class of superoxide dismutase and a glyoxalase. Very recent studies with some Ni(II) complexes showed their ability to intercalate and degrade DNA that could exhibit significant biological activity [26–30]. Since Ni^{2+} participates in various biological processes [31, 32] involving nucleic acids, the presence of a metal–anthracycline chelate inside the cell could serve as an important step in anticancer activity.

This study was performed to determine whether hydroxy-9,10-anthraquinones and their metal complexes mimic the chemical nature of an established anthracycline drug like adriamycin. If this could be shown to be the case, then one could argue in favor of hydroxy-9,10-anthraquinones as chemotherapeutic agents since they are easily prepared, readily available, and much cheaper. There has been some effort in this direction already [33–36]. We report the formation of a Ni(II) complex of adriamycin to compare it with a Ni(II) complex of sodium 1,4-dihydroxy-9-10-anthraquinone-2-sulphonate $Na_2[Ni(NaLH)_2Cl_2]\cdot 2H_2O$, i.e., $Ni(NaLH)_2$, that was prepared earlier [37, 38]. The study provides interesting similarities and differences on physicochemical attributes of Ni(II)-adriamycin (determined in this study) with that of $Ni(NaLH)_2$ determined earlier [37]. Biophysical interactions of both complexes with calf thymus DNA (ct DNA) were also compared. Interaction was correlated with reported biological activity of metal anthracyclines [9]. The difference in the binding parameters for the complexes was thought to have a manifestation for $Ni(NaLH)_2$ with regard to biological activity, arrived from a structure–function correlation.

2. Experimental

Adriamycin (doxorubicin hydrochloride) purchased from Sigma Aldrich Company, USA, was used without purification. $NiCl_2$ (AR Grade) (BDH, London) was used for the preparation of Ni(II) solutions. Sodium 1,4-dihydroxy-9,10-anthraquinone-2-sulphonate ($NaLH_2$) and its Ni(II) complex, $Ni(NaLH)_2$, were prepared by a method described earlier [37]. Hepes buffer (*N*-2-hydroxyethylpiperazine-*N*-2-ethane-sulphonic acid, 10 mmol L^{-1}), Spectrochem Pvt. Ltd., India, was used to maintain physiological pH (7.4) in all experiments. Sodium chloride (AR grade) from Merck, Germany, was used to maintain ionic strength. The quinone moiety being sensitive to light, solutions were prepared just before the experiment and kept in the dark. Ct DNA was purchased from Sisco Research Laboratories, India, and after dissolution of the fibers in buffer, the purity was checked from the absorbance ratio A_{260}/A_{280}. For all ct DNA solutions the absorption ratio was in the range $1.8 < A_{260}/A_{280} < 1.9$. Therefore, no further deproteinization of DNA was required. The concentration of DNA in terms of nucleotide was determined taking $\varepsilon_{260} = 6600\ (\text{mol L}^{-1})^{-1}\,\text{cm}^{-1}$ per base for ct DNA [14–18]. In all the experiments, the DNA concentration has been expressed in terms of bases. Stock solutions of adriamycin, $Ni(NaLH)_2$, $NiCl_2$, Hepes buffer, NaCl, EB, etc., were prepared by weighing the exact amount of the compounds. The error in weighing is less than 0.3%, indicating that the error in weighing is negligible or within

acceptable limits. Experimental solutions having appropriate strength were prepared by exact dilution of stock solutions using a calibrated micropipette (Gilson Pipetman, made in France). All solutions were prepared in triple distilled water. Absorption spectra were recorded using a spectrophotometer (model JASCO V-650). A pair of $10 \times 10\,mm^2$ quartz cuvettes was used for absorption experiments. Fluorescence measurements were carried out using a fluorescence spectrophotometer (model HITACHI S-7000). A $10 \times 10\,mm^2$ fluorescence cuvette was used. For fluorescence studies, the excitation wavelength was 518 nm for the Ni(II)–adriamycin complex and 482 nm for $Na_2[Ni(NaLH)_2Cl_2] \cdot 2H_2O$. Both spectrophotometers were equipped with a thermo electronic peltier element, with which a temperature of 25°C was maintained for experimental solutions.

3. Results and discussion

3.1. *Stoichiometry of the Ni(II) – adriamycin complex*

Stoichiometry of the Ni(II)-adriamycin complex was determined in solution by the mole ratio method. Keeping the concentration of the drug constant, the Ni(II) concentration was varied. Changes were observed in the absorption spectrum of adriamycin monitored at 480 nm at pH 7.4 and plotted against the mole ratio of Ni(II) to adriamycin. The plot (Supplementary figure S1) shows two straight lines, the intersection of which occurs at a ratio that is very close to 0.5, indicating the formation of a 1 : 2 complex of Ni(II) with adriamycin. This was similar to that reported earlier for Pd(II) [9]. Ni(II) and Pd(II) are quite similar with regard to their chemistry, but Ni(II) is friendlier to biological systems, a complex of it with adriamycin should be better for probing antitumor activity than a complex of Pd(II) [9].

3.2. *Determination of the formation constant of Ni(II)–adriamycin*

In order to determine the stability constant of the Ni(II)–adriamycin complex, Ni(II) and adriamycin were taken in the ratio 1 : 2 (determined by the mole ratio method) and a spectrophotometric titration was performed. As many as 20 volumetric flasks (10 mL each) were taken. Ni(II) and adriamycin were added to each volumetric flask in the ratio 1 : 2. Titration was done using 0.01 mol L^{-1} HCl and/or 0.01 mol L^{-1} NaOH such that the first pH adjusted for a volumetric flask was 3.5. Under this condition adriamycin was fully protonated [19]. As the pH was increased beyond 3.5, absorbance of the peak at 480 nm decreased. A further increase in pH recorded a decrease in absorbance at 480 nm without much shift in wavelength, indicating it was due to the ammonium group of the amino sugar [19]. However, for an increase in pH beyond 8.2, the peak at 480 nm decreased sharply and disappeared eventually. The absorption shifts to a higher wavelength forming a new peak at 560 nm. With further increase in pH there was a gradual increase in absorbance at this wavelength (560 nm) that became constant over the pH range 8.8–10.0 (Supplementary figure S2). These changes indicate adriamycin (H_2Adr) undergoes prominent changes in its structure, attributed to dissociation of a proton from a phenolic-OH. The absorbance (A_{obs}) for adriamycin at

560 nm in the presence of Ni(II) was fitted according to equation (1) (Supplementary figure S3).

$$A_{\text{obs}} = \frac{A_1}{1 + 10^{(pH-pK)}} + \frac{A_2}{1 + 10^{(pK-pH)}}, \tag{1}$$

where A_1 and A_2 refer to the absorbance of H_2Adr and $HAdr^-$, respectively, in the presence of Ni(II). Fitting the experimental data according to equation (1), the pK of adriamycin in the presence of Ni(II) was calculated and found to be 8.36 ± 0.05. The value being lower than that determined by Beraldo *et al*. [19] for free adriamycin (8.94) indicates complex formation between Ni(II) and adriamycin involving dissociation of a phenolic OH group.

The formation constants β^* and β for the 1 : 2 complex could then be described as

$$Ni^{2+} + 2H_2Adr \rightleftharpoons Ni(HAdr)_2 + 2H^+, \tag{2}$$

$$\beta^* = \frac{[Ni(HAdr)_2][H^+]^2}{[Ni^{2+}][H_2Adr]^2}, \tag{3}$$

$$Ni^{2+} + 2HAdr^- \rightleftharpoons Ni(HAdr)_2, \tag{4}$$

$$\beta = \frac{[Ni(HAdr)_2]}{[Ni^{2+}][HAdr^-]^2}, \tag{5}$$

$$\beta = \beta^*/K_1^2. \tag{6}$$

Equations (2)–(6) enable determination of the stability constant β. K_1 is the dissociation of adriamycin corresponding to the phenolic-OH as reported earlier [19].

By using equations (2)–(6) we calculated the formation constant (β) of Ni(II)–adriamycin and found it to be $(1.1 \pm 0.1) \times 10^{12}$. In an earlier study [37], a complex of Ni(II) was formed using sodium-1,4-dihydroxy-9,10-anthraquinone-2-sulphonate ($NaLH_2$), a molecule that resembles adriamycin at the anthraquinone portion. The complex had the formula $Ni(NaLH)_2$ [37, 38], with an effective formation constant of 2.46×10^{13}. Thus, the stability constant values of adriamycin with Ni(II) and the hydroxy-9,10-anthraquinone analog with Ni(II) are similar. The values of the stability constants of the 1 : 2 Cu(II)–adriamycin [19] and 1 : 2 Cu(II)–$NaLH_2$ complex [15] were also similar having values of $(4.6 \pm 1.1) \times 10^{16}$ and $(9.6 \pm 1.1) \times 10^{16}$, respectively. Therefore, the stability constant values of adriamycin and $NaLH_2$, the hydroxy-9,10-anthraquinone analog, with metal ions are similar indicating that $Ni(NaLH)_2$ could possibly resemble Ni(II)–adriamycin in biological interactions.

3.3. *Interaction of the two complexes with ct DNA*

Interaction with ct DNA was studied using absorption and fluorescence spectroscopy. Separate aliquots were made containing a constant concentration of each Ni(II) complex and different concentrations of ct DNA. Throughout the experiments the concentration of each Ni(II) complex was kept constant while the concentration of ct DNA was varied. The concentrations of $Ni(HAdr)_2$ and $Ni(NaLH)_2$ were 3.62 and

$10\,\mu mol\,L^{-1}$, respectively. The binding constant and site size of interaction of the two complexes with ct DNA were calculated by the methods described earlier [15].

The change in absorbance or fluorescence of both complexes were used to construct binding isotherms that were analyzed using non-linear curve fit analysis using the compound–DNA equilibrium shown by equation (7) [14, 15, 39].

$$L + D \rightleftharpoons LD, \tag{7}$$

$$K_d = \frac{[L][D]}{[LD]} = \frac{\{L_0 - [LD]\}\{D_0 - [LD]\}}{[LD]}, \tag{8}$$

L represents the two complexes of Ni(II) and D represents ct DNA. K_d was the apparent dissociation constant ($K_d = 1/K$, where K was the apparent binding constant).

$$K_d = \frac{\{C_0 - [LD]\}\{C_D - [LD]\}}{[LD]}, \tag{9}$$

$$K_d = \frac{\left[C_0 - \left(\frac{\Delta A}{\Delta A_{max}}\right)C_0\right]\left[C_D - \left(\frac{\Delta A}{\Delta A_{max}}\right)C_0\right]}{\left(\frac{\Delta A}{\Delta A_{max}}\right)C_0}, \tag{10}$$

$$C_0\left(\frac{\Delta A}{\Delta A_{max}}\right)^2 - (C_0 + C_D + K_d)\left(\frac{\Delta A}{\Delta A_{max}}\right) + C_D = 0, \tag{11}$$

D_0 or C_D indicates the concentration of ct DNA, while L_0 or C_0 was the initial concentration of each Ni(II) complex during the titration. The change in absorbance at a particular wavelength was represented as $\Delta A = (A_0 - A)$, A_0 and A being absorbance of the compound in the absence and presence of ct DNA. ΔA_{max} was the same parameter when the complexes were totally bound to ct DNA. Therefore, $(\Delta A/\Delta A_{max})$ denotes the fraction of each complex bound to DNA from which one gets $(\Delta A/\Delta A_{max}) \times C_0 = [LD]$.

All experimental points for binding were fitted to equation (11) by least-square analysis to obtain K_d. ΔA_{max} for equation (11) was determined with the help of a double reciprocal plot (figure not shown) of $1/\Delta A$ *versus* $1/(C_D - C_0)$ using equation (12) [14, 15, 39].

$$\frac{1}{\Delta A} = \frac{1}{\Delta A_{max}} + \frac{K_d}{\Delta A_{max}(C_D - C_0)}. \tag{12}$$

Similar equations were used to determine binding parameters using fluorescence.

3.3.1. Binding followed by UV-Vis spectroscopy. Prior to titration with ct DNA, $Ni(HAdr)_2$ was generated in a medium containing Hepes buffer (pH 7.4) and $120\,mmol\,L^{-1}$ NaCl. Adriamycin and Ni(II) were added to the medium in such a way that the concentration of adriamycin was $7.25\,\mu mol\,L^{-1}$, while Ni(II) was $50\,\mu mol\,L^{-1}$. Since the formation constant of $Ni(HAdr)_2$ was very high, as found through physicochemical experiments, once the complex was formed the equilibrium concentration of free adriamycin would be almost negligible. Moreover, owing to a very high presence of Ni(II) at equilibrium the chances of the complex dissociating into

adriamycin and Ni(II) was minimized. In order to ensure complete formation of $Ni(HAdr)_2$, a large concentration of Ni(II) was taken in comparison to adriamycin and an equilibration time of 90 min was allowed. Therefore, the chemical moiety interacting with ct DNA during titration was $Ni(HAdr)_2$. In the case of $Ni(NaLH)_2$, a solution was prepared by dissolving the solid complex [37, 38] in Hepes buffer (pH 7.4) containing 500 mmol L^{-1} NaCl. For $Ni(NaLH)_2$, a relatively high concentration of NaCl was used to suppress the high negative charge of the DNA backbone due to phosphates as also the negative charge on the complex itself.

The change in absorbance $[\Delta A = (A_0 - A)]$ for $Ni(HAdr)_2$ was followed at 583 nm, while for $Ni(NaLH)_2$ it was followed at 575 nm during titration with ct DNA. Absorption spectra of 3.62 μmol L^{-1} $Ni(HAdr)_2$ and 10 μmol L^{-1} $Ni(NaLH)_2$ at physiological pH in the absence and presence of different concentrations of ct DNA are shown in figures 1 and 2, respectively. It is important to mention here that in the case of the titration of $Ni(HAdr)_2$ with ct DNA absorbance due to free adriamycin or free Ni(II) was negligible at the monitoring wavelength of 583 nm.

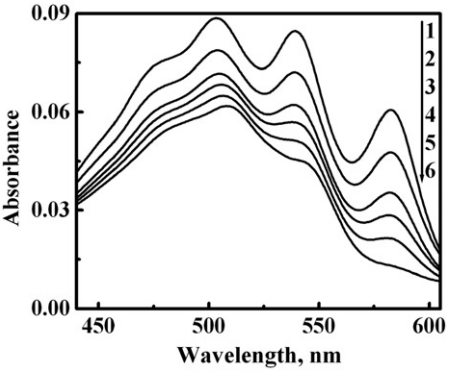


Figure 1. Absorption spectra of 3.62 μmol L^{-1} $Ni(HAdr)_2$ in the absence (1) and presence of different ct DNA concentrations: 12.03 μmol L^{-1} (2), 23.91 μmol L^{-1} (3), 34.18 μmol L^{-1} (4), 44.34 μmol L^{-1} (5), 73.33 μmol L^{-1} (6); pH = 7.4, [NaCl] = 120 mmol L^{-1}, $T = 25°C$.

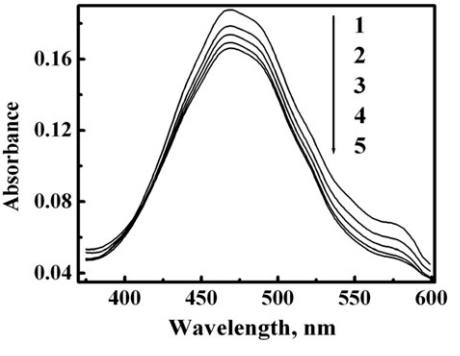


Figure 2. Absorption spectra of 10 μmol L^{-1} $Ni(NaLH)_2$ in the absence (1) and presence of different ct DNA concentrations: 147.45 μmol L^{-1} (2), 253.11 μmol L^{-1} (3), 452.77 μmol L^{-1} (4), 608.25 μmol L^{-1} (5); pH = 7.4, [NaCl] = 500 mmol L^{-1}, $T = 25°C$.

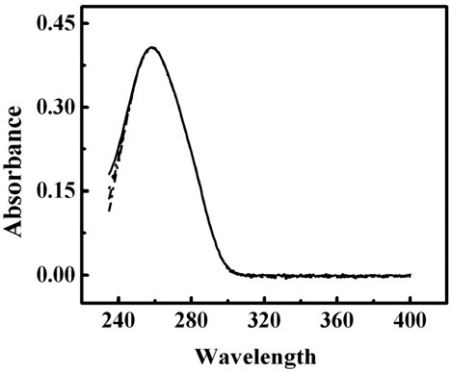


Figure 3. Absorption spectra of 61.52 μmol L^{-1} ct DNA (solid line) in 0.1 mmol L^{-1} Hepes buffer of pH 7.4 and 120 mmol L^{-1} NaCl with Ni(II) concentration: 50 μmol L^{-1} (dotted line). Two spectra are almost the same and therefore merge.

As mentioned above, for each titration by ct DNA, $Ni(HAdr)_2$ was prepared *in situ* by the method described above having a concentration 3.62 μmol L^{-1} with a large excess of free Ni(II) in its immediate vicinity. Therefore, each such solution used for titration contained an excess of Ni(II) with respect to the complex. For this reason a control study was done to be sure that free Ni(II) does not interfere with the binding of $Ni(HAdr)_2$ with ct DNA nor denatures the DNA used. In the control experiment (figure 3), a definite concentration of ct DNA was taken and its spectrum was recorded. To this solution Ni(II) was added from a stock solution such that the concentration of Ni(II) in solution was 50 μmol L^{-1}. From figure 3 it is very clear that there is no change in the DNA spectrum so that the solid line [without Ni(II)] and the dotted line [in the presence of Ni(II)] actually merge with each other making identification of the separate lines very difficult. In fact, there was no shift whatsoever at the λ_{max} (260 nm) for DNA considered as the signature peak for it which is used to calculate concentrations of DNA in solution [14–18]. This is evidence that in the actual experiment the free Ni(II) present alongside $Ni(HAdr)_2$ does not interact with or denature ct DNA.

Therefore, the decrease in the absorptions at 503, 539, and 583 nm (figure 1) upon increasing ct DNA concentration for a fixed concentration of $Ni(HAdr)_2$ may be attributed to interaction of the complex with DNA. In the case of $Ni(NaLH)_2$, two peaks were seen at 467 and 575 nm (figure 2). When ct DNA was added to a solution of the latter at physiological pH the intensity of both peaks at 467 and 575 nm decreased (figure 2). The hypochromic effect observed with both complexes was due to interaction between the electronic states of the compounds' chromophores with those of the DNA bases [40–43]. The strength of the electronic interaction was expected to decrease as the cube of the distance of separation of the compound chromophore and DNA. The hypochromism observed in this study indicates close proximity of the compounds to the DNA bases [42]. For $Ni(HAdr)_2$, along with the hypochromic shift, a hypsochromic shift of about 5 nm was observed for the two peaks at 503 and 539 nm. For $Ni(NaLH)_2$, in addition to a hypochromic shift, slight bathochromic shift was observed for both peaks, i.e., a shift of 3 nm for the peak at 467 nm and 5 nm for that at 575 nm. These spectral features are indicative of intercalation of the compound into the DNA helix [43] that results in an ordered stacking of the complex between aromatic heterocyclic

base pairs. The intercalating surface is sandwiched tightly between the base pairs and stabilized electronically in the helix by π–π stacking and dipole–dipole interactions [41].

Based on variations in the absorption spectra of $Ni(HAdr)_2$ and $Ni(NaLH)_2$ binding to ct DNA, the apparent binding constant, K, was calculated using equation (13) [44],

$$\frac{A_0}{A-A_0}=\frac{\varepsilon_G}{\varepsilon_{H-G}-\varepsilon_G}+\frac{\varepsilon_G}{\varepsilon_{H-G}-\varepsilon_G}\frac{1}{K[DNA]}, \tag{13}$$

where A_0 and A are the absorbance of the compound in the absence and presence of DNA at 583 nm for $Ni(HAdr)_2$ and 575 nm for $Ni(NaLH)_2$. ε_G and ε_{H-G} are the corresponding absorption coefficients of the complexes and its interacted form with DNA, respectively. Using equation (13), a plot of $A_0/(A-A_0)$ *versus* 1/[DNA] is shown in Supplementary figures S4 and S5 for $Ni(HAdr)_2$ and $Ni(NaLH)_2$, respectively. The double reciprocal plots were linear, generating an apparent binding constant value of $(3.4\pm0.1)\times10^4\,(mol\,L^{-1})^{-1}$ for $Ni(HAdr)_2$ and $(2.21\pm0.09)\times10^3\,(mol\,L^{-1})^{-1}$ for $Ni(NaLH)_2$ interacting with ct DNA. Reduced chi squares for the fit of experimental data (equation (13)) were 0.0451 and 0.0144 for Supplementary figures S4 and S5, respectively, using Grafit 3.0 software.

The apparent binding constant may be determined from a double reciprocal plot of the changes of the apparent extinction coefficient of either $Ni(HAdr)_2$ or $Ni(NaLH)_2$ *versus* the DNA concentration. Equation (14) represents such a relationship [45, 46].

$$\frac{1}{\Delta\varepsilon_{ap}}=\frac{1}{\Delta\varepsilon K[DNA]}+\frac{1}{\Delta\varepsilon}. \tag{14}$$

In equation (14) $\Delta\varepsilon_{ap}=|\varepsilon_a-\varepsilon_f|$ and $\Delta\varepsilon=|\varepsilon_b-\varepsilon_f|$. The apparent extinction coefficient (ε_a) at 583 nm for $Ni(HAdr)_2$ and at 575 nm for $Ni(NaLH)_2$ were calculated from A_{obs}/[compound], where ε_f and ε_b correspond to extinction coefficients for the free complex and fully bound form (to DNA) at such wavelengths, respectively. Multiplying both sides of equation (14) by [DNA] one obtains a half-reciprocal form (equation (15)).

$$\frac{[DNA]}{\Delta\varepsilon_{ap}}=\frac{1}{\Delta\varepsilon}[DNA]+\frac{1}{\Delta\varepsilon K}. \tag{15}$$

Using equation (15), the plot of $[DNA]/\Delta\varepsilon_{ap}$ *versus* [DNA] is shown as inset of Supplementary figures S4 and S5 for $Ni(HAdr)_2$ and $Ni(NaLH)_2$, respectively. From the half reciprocal plots the apparent binding constant values were obtained as $(4.3\pm0.1)\times10^4$ for $Ni(HAdr)_2$ and $(2.5\pm0.1)\times10^3$ for $Ni(NaLH)_2$, respectively. Reduced chi squares for the fit of the experimental data (equation (15)) were 0.0435 and 0.0028 for the inset of Supplementary figures S4 and S5, respectively, using Grafit 3.0 software.

Since a double reciprocal plot tends to give more importance to data points at low DNA concentration, half reciprocal plots were done as they should generally be more accurate.

All experimental points for binding were fitted to equation (11) by least-square analysis to obtain K_d. ΔA_{max} for equation (11) was determined with the help of a double reciprocal plot (figure not shown) of $1/\Delta A$ *versus* $1/(C_D-C_0)$ using equation (12) [14, 15, 39]. This approach was based on the basic assumption that absorbance was linearly proportional to the concentration of each complex. The binding isotherms

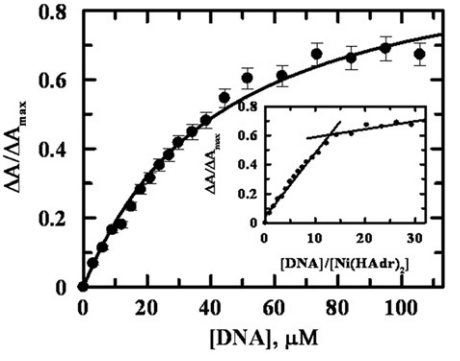


Figure 4. Binding isotherm of $Ni(HAdr)_2$ and ct DNA and corresponding non-linear fit using spectrophotometric method; $[Ni(HAdr)_2] = 3.62\,\mu mol\,L^{-1}$, pH = 7.4, $[NaCl] = 120\,mmol\,L^{-1}$, $T = 25^{\circ}C$. Inset: Plot of normalized increase of absorbance as a function of mole ratio of ct DNA to $Ni(HAdr)_2$.

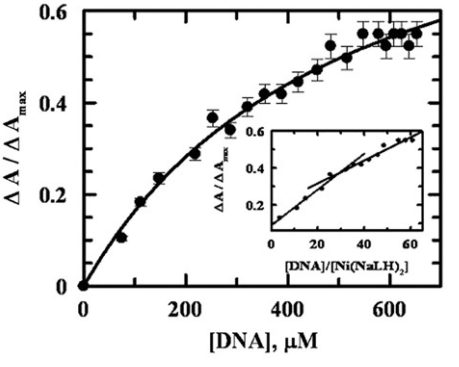


Figure 5. Binding isotherm of $Ni(NaLH)_2$ and ct DNA and corresponding non-linear fit using the spectrophotometric method; $[Ni(NaLH)_2] = 10\,\mu mol\,L^{-1}$, pH = 7.4, $[NaCl] = 500\,mmol\,L^{-1}$, $T = 25^{\circ}C$. Inset: Plot of normalized increase of absorbance as a function of mole ratio of ct DNA to $Ni(NaLH)_2$.

(figures 4 and 5) were analyzed by non-linear curve fitting using equations (10) and (11) to evaluate the apparent binding constant values that were found to be $(2.49 \pm 0.08) \times 10^4\,(mol\,L^{-1})^{-1}$ and $(2.03 \pm 0.09) \times 10^3\,(mol\,L^{-1})^{-1}$ for $Ni(HAdr)_2$ and $Ni(NaLH)_2$, respectively. The mole ratio plots (insets of figures 4 and 5) indicate the stoichiometry or the binding site-size (n) as the point of intersection of two straight lines drawn using points before and after saturation during titration [39]. For $Ni(HAdr)_2$ the value of "n" for binding to ct DNA was 12.9 ± 1.1 bases, i.e., 6.5 ± 0.5 base pairs, while for $Ni(NaLH)_2$ the value of "n" was 30 ± 2 bases, i.e., 15 ± 1 base pairs. Knowing n, the intrinsic binding constant K', i.e., $(K \times n)$ for $Ni(HAdr)_2 - ct\ DNA$ interaction was obtained as $(3.22 \pm 0.09) \times 10^5\,(mol\,L^{-1})^{-1}$, while that for $Ni(NaLH)_2$ was $(6.09 \pm 0.12) \times 10^4\,(mol\,L^{-1})^{-1}$ (summarized in table 1).

3.3.2. Binding followed by fluorescence spectroscopy. Interactions of $Ni(HAdr)_2$ and $Ni(NaLH)_2$ with ct DNA were also studied using fluorescence spectroscopy. $Ni(HAdr)_2$

Table 1. Comparison of binding parameters for $Ni(NaLH)_2$–ct DNA and $Ni(HAdr)_2$–ct DNA interactions obtained from different techniques and fitting analyses.

Compound	Monitoring techniques	Apparent binding constant (K) ($(mol\,L^{-1})^{-1}$)			Binding site size (n) (Bases)	Intrinsic binding constant $K' = (K \times n)$ ($(mol\,L^{-1})^{-1}$)
		Double reciprocal plot using equation (13)	Half reciprocal plot using equation (15)	Non-linear fit analysis using equations (10) and (11)		
Ni(II)–$NaQSH_2$ complex	UV-Vis spectroscopy	$(2.21 \pm 0.09) \times 10^3$	$(2.51 \pm 0.10) \times 10^3$	$(2.03 \pm 0.09) \times 10^3$	(30 ± 2.45)	$(6.09 \pm 0.12) \times 10^4$
	Fluorescence spectroscopy	–	–	$(1.85 \pm 0.08) \times 10^3$	(32 ± 1.87)	$(5.92 \pm 0.07) \times 10^4$
	Cyclic voltammetry [38]	–	–	$(2.03 \pm 0.10) \times 10^3$	(29.02 ± 1.70)	$(5.89 \pm 0.10) \times 10^4$
Ni(II)–H_2Adr complex	UV-Vis spectroscopy	$(3.44 \pm 0.12) \times 10^4$	$(4.26 \pm 0.11) \times 10^4$	$(2.49 \pm 0.08) \times 10^4$	(12.92 ± 1.10)	$(3.22 \pm 0.09) \times 10^5$
	Fluorescence spectroscopy	–	–	$(4.27 \pm 0.11) \times 10^4$	(10.48 ± 1.68)	$(4.48 \pm 0.12) \times 10^5$

has an emission maximum at 556 nm ($\lambda_{ex} = 518$ nm) while $Ni(NaLH)_2$ has an emission maximum at 548 nm ($\lambda_{ex} = 482$ nm). Keeping the concentration of each Ni(II) complex constant, solutions were titrated with increasing amounts of ct DNA. The fluorescence emission spectra of $Ni(HAdr)_2$ and $Ni(NaLH)_2$ in the absence and presence of different amounts of ct DNA are shown in figures 6 and 7, respectively. From these figures it is clear that fluorescence emission intensity gradually decreased with a slight blue shift of the emission peak with increasing amounts of ct DNA, indicating association of each compound with ct DNA. The binding isotherms obtained from the fluorescence study were also analyzed by the method of non-linear curve fitting as mentioned earlier (equations (8)–(12)). For studies using fluorescence, ΔF and ΔF_{max} were used instead of ΔA and ΔA_{max}. ΔF is the change in fluorescence emission intensity at 556 nm for $Ni(HAdr)_2$ and at 548 nm for $Ni(NaLH)_2$. ΔF_{max} is the same parameter when each Ni(II) complex was totally bound to ct DNA. This approach was also based on the assumption that fluorescence intensity was linearly proportional to concentration of

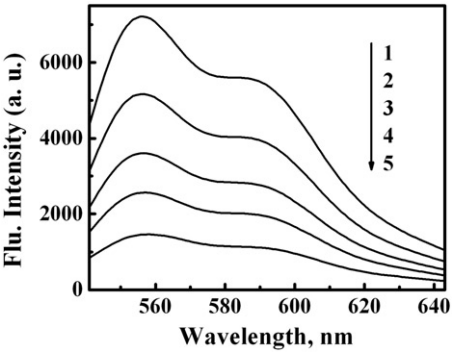


Figure 6. Fluorescence spectra of 3.62 μmol L^{-1} $Ni(HAdr)_2$ in the absence (1) and presence of different ct DNA concentrations: 12.03 μmol L^{-1} (2), 23.91 μmol L^{-1} (3), 34.18 μmol L^{-1} (4), 60.56 μmol L^{-1} (5); pH = 7.4, [NaCl] = 120 mmol L^{-1}, $T = 25°C$.

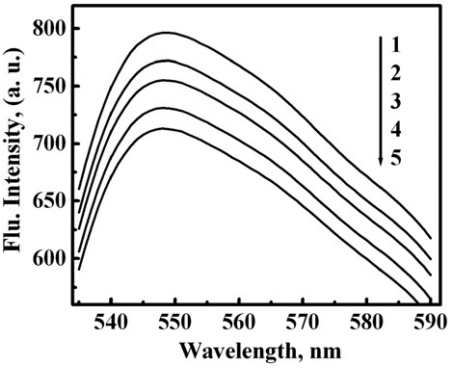


Figure 7. Fluorescence spectra of 10 μmol L^{-1} $Ni(NaLH)_2$ in the absence (1) and presence of different ct DNA concentrations: 147.45 μmol L^{-1} (2), 253.11 μmol L^{-1} (3), 452.77 μmol L^{-1} (4), 608.25 μmol L^{-1} (5); pH = 7.4, [NaCl] = 500 mmol L^{-1}, $T = 25°C$.

each compound. Concentrations of the Ni(II) complexes and ct DNA were the same as that used in absorbance studies.

Here also the binding isotherms (figures 8 and 9) were analyzed by non-linear curve fit (equations (10) and (11)) to evaluate the apparent binding constant values. These were found to be $(4.3 \pm 0.1) \times 10^4 (\text{mol L}^{-1})^{-1}$ and $(1.85 \pm 0.08) \times 10^3 (\text{mol L}^{-1})^{-1}$ for $Ni(HAdr)_2$ and $Ni(NaLH)_2$, respectively. The insets of figures 8 and 9 show the plot of normalized increase of $\Delta F/\Delta F_{max}$ as a function of mole ratio of DNA to Ni(II) complex and provides the stoichiometry or binding site-size (n) [39]. For $Ni(HAdr)_2$ the value of "n" binding to ct DNA was 10.5 ± 1.7 bases, or 5.2 ± 0.8 base pairs, and the intrinsic binding constant K', i.e., $(K \times n)$, was obtained as $(4.5 \pm 0.1) \times 10^5 (\text{mol L}^{-1})^{-1}$ (summarized in table 1). The value of "n" for the interaction of $Ni(NaLH)_2$ with ct DNA was 32 ± 2 bases or 16 ± 1 base pairs, and the intrinsic binding constant was found to be $(5.92 \pm 0.07) \times 10^4$ $(\text{mol L}^{-1})^{-1}$ (summarized in table 1). In a very recent study using cyclic voltammetry [38], the intrinsic binding constant (K') and the binding site size of interaction of $Ni(NaLH)_2$ with ct DNA were found to be

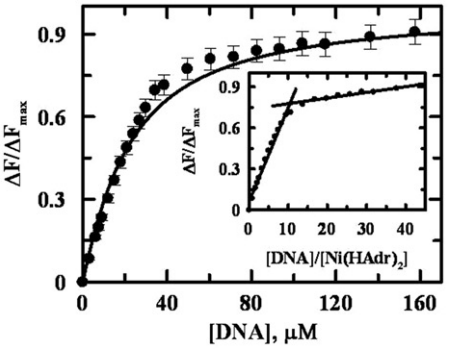


Figure 8. Binding isotherm of $Ni(HAdr)_2$ and ct DNA and corresponding non-linear fit using the fluorimetric method; $[Ni(HAdr)_2] = 3.62\,\mu\text{mol L}^{-1}$, pH = 7.4, $[NaCl] = 120\,\text{mmol L}^{-1}$, $T = 25^\circ$C. Inset: Plot of normalized increase of fluorescence as a function of mole ratio of ct DNA to $Ni(HAdr)_2$.

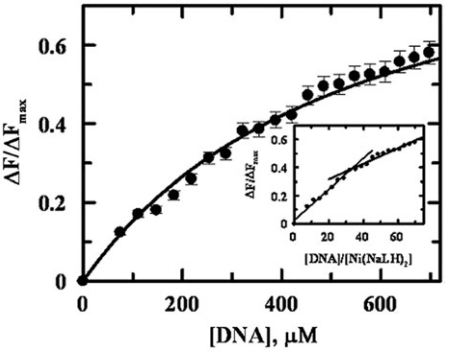


Figure 9. Binding isotherm of $Ni(NaLH)_2$ and CT DNA and corresponding non-linear fit using the fluorimetric method; $[Ni(NaLH)_2] = 10\,\mu\text{mol L}^{-1}$, pH = 7.4, $[NaCl] = 500\,\text{mmol L}^{-1}$, $T = 25^\circ$C. Inset: Plot of normalized increase of fluorescence as a function of mole ratio of ct DNA to $Ni(NaLH)_2$.

$(5.9 \pm 0.1) \times 10^4 (\text{mol L}^{-1})^{-1}$ and 29 ± 2 bases, respectively, that were similar to values obtained using fluorescence and UV-Vis spectroscopy (summarized in table 1). This further lends support to the methods of measurement.

In another recent investigation [14], we found that sodium 1,4-dihydroxy-9,10-anthraquinone-2-sulphonate ($NaLH_2$) intercalates ct DNA with an intrinsic binding constant of $3.44 \times 10^4 (\text{mol L}^{-1})^{-1}$, while binding site size was 16 bases, i.e., 8 base pairs. Therefore, the binding site size obtained for $Ni(NaLH)_2$ with ct DNA from spectrophotometric and fluorimetric methods was almost double that of the interaction of $NaLH_2$ with ct DNA. As Ni(II) is very small, it can be said that the size of the complex was approximately double that of $NaLH_2$ which supports such a ratio (16 : 8) for binding site size in base pairs. From this study, it is also evident that the intrinsic binding constant (K') for the Ni(II) complex of $NaLH_2$ was greater by ~1.7 times in comparison to free $NaLH_2$ as obtained from spectrophotometry and fluorimetric studies. Recently, in another investigation we observed that the 1 : 2 Cu(II) complex of $NaLH_2$ intercalates ct DNA with intrinsic binding constant and binding site size values of $4.58 \times 10^4 (\text{mol L}^{-1})^{-1}$ and 32 bases, respectively [15]. Therefore, both Cu(II) and Ni(II) complexes interact with ct DNA with an almost identical value for binding and site size of interaction, supporting an almost identical structure for the two complexes. A previous study reported that adriamycin has an intrinsic binding constant value of $2.7 \times 10^5 (\text{mol L}^{-1})^{-1}$ at pH 7.4 [47] and binding site size value of 3.1 ± 0.4 base pairs [48]. This study therefore shows that complex formation of adriamycin with Ni(II) increases its binding constant with ct DNA by ~1.2 times in experiments that were followed by spectrophotometry and by ~1.6 times through experiments using fluorescence. Recent studies showed that metal complex formation using Cu(II) and Ni(II) improved the DNA-binding ability of their respective ligands [27, 28]. The binding site size value for the $Ni(HAdr)_2$–ct DNA interaction was 6.5 base pairs from absorbance and 5.2 base pairs from fluorescence measurements. Therefore, in the case of $Ni(HAdr)_2$ also the binding site size for the complex was double that of adriamycin [48], showing remarkable similarity with regard to interactions of the compounds (Adr and $NaLH_2$) as well as their respective Ni(II) complexes [$Ni(HAdr)_2$ and $Ni(NaLH)_2$] with ct DNA.

Consistently, the binding constant values obtained for interaction of Ni(II) complexes with ct DNA were approximately five to eight times less for the sodium 1,4-dihydroxy-9,10-anthraquinone-2-sulphonate ligand than for adriamycin, which may be attributed to two reasons. The complex portion of $Ni(NaLH)_2$ has two negative charges on it owing to the presence of a sulfonate group on each LH and DNA being a negative polymer, probably repelling the compound as it interacts with DNA. A possible second reason could be that sugar moieties present in anthracyclines [15, 17, 18] play an important role in the binding process that is manifested in a higher binding constant value for $Ni(HAdr)_2$.

3.4. *Mode of interaction of the complexes with DNA*

That the interaction of the complexes with ct DNA was intercalation was further established by carrying out competitive binding using an established DNA intercalator ethidium bromide (EB) and monitoring the change in fluorescence under similar conditions of pH, ionic strength and temperature. EB, an intercalator, approaches the

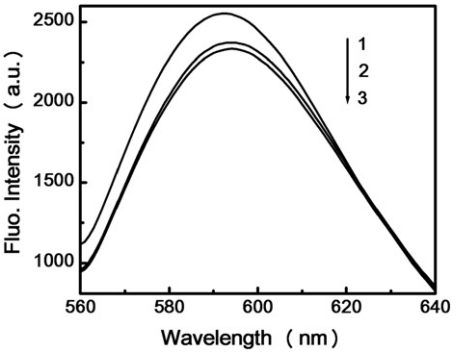


Figure 10. Fluorescence spectra of (1) 200 μmol L^{-1} DNA with saturating EB (4000 μmol L^{-1}) incubated for 1 h; (2) 200 μmol L^{-1} DNA with saturating EB (4000 μmol L^{-1}) incubated for 1 h followed by addition of 3 μmol L^{-1} $Ni(NaLH)_2$; (3) 200 μmol L^{-1} DNA with saturating EB (4000 μmol L^{-1}) incubated for 1 h followed by addition of 3 μmol L^{-1} $Ni(NaLH)_2$ with incubation time 30 min; pH = 7.4, [NaCl] = 500 mmol L^{-1}, $T = 25°C$.

DNA backbone *via* the minor groove [49]. A solution of EB shows a strong absorption band with λ_{max} at 470 nm which upon interaction with DNA shifts to 510 nm. When DNA-EB was excited at 510 nm, the fluorescence emission maximum was observed at 590 nm. A mixture containing 200 μmol L^{-1} ct DNA with saturating EB (4000 μmol L^{-1}) was incubated for 1 h and fluorescence was recorded (figure 10). To this mixture, $Ni(NaLH)_2$ was added and fluorescence of the resulting mixture was taken at different time intervals ranging from an incubation time $t = 0$ to $t = 30$ min (figure 10). It is evident from the figure that addition of $Ni(NaLH)_2$ causes a decrease in fluorescence intensity with increase in incubation time. Since EB is an intercalator and the fluorescence is due to intercalation of it into DNA, the decrease in fluorescence observed upon addition of $Ni(NaLH)_2$ clearly indicates that the complex also intercalates between the strands of DNA and replaces EB. The same study was carried out for $Ni(HAdr)_2$. As already reported earlier for adriamycin and its related complexes [21, 50], this Ni(II) complex of adriamycin also intercalates during its interaction with DNA.

4. Conclusion

The anthracycline anticancer drug adriamycin and its simpler analog sodium 1,4-dihydroxy-9,10-anthraquinone-2-sulphonate ($NaLH_2$) form 1 : 2 metal-ligand complexes with Ni(II) at physiological pH. The stability constants of the complexes were comparable, thus encouraging use of the much cheaper $NaLH_2$ as a substitute to the costly anthracyclines. The 1 : 2 Ni(II)–adriamycin complex, $Ni(HAdr)_2$ and the 1 : 2 Ni(II) complex of $NaLH_2$, $Ni(NaLH)_2$, interact with ct DNA at physiological pH by intercalation. The apparent binding constant and binding site size obtained for both complexes from non-linear curve fit analysis using spectrophotometry and fluorimetry provide almost identical values. The overall or intrinsic binding constant for $Ni(HAdr)_2$–ct DNA interaction was $(3.22 \pm 0.09) \times 10^5 (\text{mol L}^{-1})^{-1}$ using

spectrophotometry and $(4.5 \pm 0.1) \times 10^5 (\text{mol L}^{-1})^{-1}$ using fluorimetry, while for $Ni(NaLH)_2$ the values were $(6.1 \pm 0.1) \times 10^4 (\text{mol L}^{-1})^{-1}$ using spectrophotometry and $(5.92 \pm 0.07) \times 10^4 (\text{mol L}^{-1})^{-1}$ by fluorimetry. Since the interaction of the complexes with ct DNA at physiological pH shows very good agreement between spectrophotometric and fluorimetric methods of measurement, the data lend support to the validity of the methods used in the experiments. This study was done as part of an effort to see if hydroxy-9,10-anthraquinones and their metal complexes could serve as alternatives to the very costly anthracyclines in order to provide important clues to co-workers who wish to redesign and simplify anthracyclines to create simpler molecules capable of DNA targeting.

Acknowledgments

SD wishes to thank "Centre for Advanced Studies," and the DST PURSE program (Government of India) operating at the Department of Chemistry, Jadavpur University, for the financial support. PCM wishes to thank Intramural MMDDA project at Saha Institute of Nuclear Physics for the financial support.

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