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Research article

Free radical induced activity of an anthracycline analogue and its Mn^{II} complex on biological targets through *in situ* electrochemical generation of semiquinone

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ABSTRACT

Cytotoxicity by anthracycline antibiotics is attributed to several pathways. Important among them are formation of free-radical intermediates. However, their generation makes anthracyclines cardiotoxic which is a concern on their use as anticancer agents. Hence, any change in redox behavior that address cardiotoxicity is welcome. Modulation of redox behavior raises the fear that cytotoxicity could be compromised. Regarding the generation of free radical intermediates on anthracyclines, a lot depends on the surrounding environment (oxic or anoxic), polarity and pH of the medium. In case of anthracyclines, one-electron reduction to semiquinone or two-electron reduction to quinone-dianion are crucial both for cytotoxicity and for cardiotoxic side effects. The disproportion-comproportionation equilibria at play between quinone-dianion, free quinone and semiquinone control biological activity. Whatever is the form of reduction, semiquinones are generated as a consequence of the presence of anthracyclines and these interact with a biological target. Alizarin, a simpler anthracycline analogue and its Mn^{II} complex were subjected to electrochemical reduction to realize what happens when anthracyclines are reduced by compounds present in cells as members of the electron transport chain. Glassy carbon electrode maintained at the pre-determined reduction potential of a compound was used for reduction of the compounds. Nucleobases and calf thymus DNA that were maintained in immediate vicinity of such radical generation were used as biological targets. Changes due to the generated species under aerated/de-aerated conditions on nucleobases and on DNA helps one to realize the process by which alizarin and its Mn^{II} complex might affect DNA. The study reveals alizarin was more effective on nucleobases than the complex in the free radical pathway. Difference in damage caused by alizarin and the Mn^{II} complex on DNA is comparatively less than that observed on nucleobases; the complex makes up for any inefficacy in the free radical pathway by its other attributes.

1. Introduction

In the present day context of cancer chemotherapy, anthracyclines are an important class of molecules used as effective anticancer agents in different forms of the disease [1, 2, 3, 4, 5, 6]. However, a disturbing aspect related to anthracyclines is their associated toxic side effects, an almost inseparable phenomenon affecting drug efficacy [4, 5, 6, 7, 8, 9, 10]. What is realized till now is that, both efficacy and toxic side effects (cardiotoxicity) involves a common intermediate [5, 7, 8, 9, 10, 11, 12]. Hence, while using anthracyclines, extreme care is necessary during drug administration, particularly in case of children, who even if cured of cancer, run the risk of living the rest of their lives with different forms of cardiac problems [7, 13, 14, 15]. In fact, during administration of most anthracyclines, functioning of the heart of the patient is monitored continuously and if complications arise treatment is discontinued. Dose-related heart problems have been reported to occur as late as 7–10 years after treatment [4, 5, 6, 7, 8, 9, 10, 13, 14, 15].

Hence, effort is now underway to modify anthracyclines in a manner that address such toxic side effects or search for analogues having less toxicity or administer drugs in presence of compounds that help to reduce toxic side effects or bring about changes in methodology of drug administration so that some improvement is achieved. Needless to say, they are to be done without compromising efficacy [16, 17, 18, 19, 20, 21, 22, 23, 24]. However, often, in trying to achieve that, a compromise is made with drug action, which is another area of concern [10, 11, 21, 23, 24].

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One approach to modifying anthracyclines is through complex formation using bio-friendly metal ions [25, 26, 27]. Simpler analogues seeking to decrease the cost of such drugs have also been tried [17, 18, 19, 20, 28, 29, 30, 31, 32, 33, 34, 35]. While results vary, there are several issues before such simpler analogues could become drugs [17, 18, 19, 20, 28, 29, 30, 33, 34, 35]. Whether it be anthracyclines or its hydroxy-9,10-anthraquinone analogues, studies reveal intermediates like semiquinone radical anion [36, 37] are an important component of drug action that are either moderately or significantly decreased following complex formation [25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35]. Therefore, while complex formation could address cardiotoxic side effects, efficacy could be affected as well [28, 29, 30, 31, 32, 33, 34, 35]. In majority of cases however, it is seen that complexes are more effective (*in vitro*) than the parent molecule [26, 28, 30, 32, 33, 34, 35]. This means in case of complexes, loss in efficacy owing to decreased formation of semiquinone is compensated by other attributes of complex formation [25, 26, 28, 29, 30, 31, 32, 33, 34, 35]. Although this is widely accepted as a logical explanation on the performance of metal complexes of anthracyclines or their analogues, there are only a few studies that show a comparative investigation of anthracyclines, its analogues and their respective complexes that involve the free radical pathway. We made attempts to explore aspects related to free radical pathways of some drugs or their analogues when they are either on their own or complexed with metal ions [38, 39, 40]. For anthracyclines, accumulating evidence suggest there is *in vivo* formation of semiquinone either due to one-electron reduction or by comproportionation when they undergo two-electron reduction to form quinone-dianion [28, 33, 41, 42, 43]. The reactive intermediates that are formed, damage DNA, serving as important signal-transduction networks either promoting cell cycle arrest or causing cell death in order to repair DNA lesions [44, 45, 46, 47]. DNA damage response leads to initiation of tumor growth and a somewhat defective damage response generates genomic instability [48]. An up-regulated response of DNA damage is known to cause resistance to treatment [44, 45, 46, 47]. Redox reactions of drugs also affect response of damaged DNA since reactive oxygen species (ROS) either activate or inhibit cellular proteins/enzymes related to response in healthy or cancer cells [49]. Hence, changes in response of damaged DNA by proper modulation of ROS is of interest and has an impact on several parameters [49]. ROS affects DNA in cancer patients differently either during progress of cancer or during treatment [50]. Through this study, we tried to identify the contribution of the free radical pathway to cell damage by an anthracycline analogue and its Mn^{II} complex.

To realize how anthracyclines or its analogues interact with DNA in the free radical pathway it is important to analyze the interactions between them [7, 8, 9, 10, 11, 12, 13, 14, 15]. Herein, investigation of such pathways is reported. The study was performed using a potentiostat as the source of electrons. *In situ* reactivity of electrochemically generated quinone di-anion or semiquinone formed either on alizarin or its Mn^{II} complex with different nucleobases and calf thymus DNA that were maintained in the immediate vicinity of their generation is discussed. The study could help to realize the role of semiquinone or superoxide radical anion [51] in causing DNA damage and to see if damage is initiated through modification of nucleobases or is a consequence of aspects like DNA binding or the abstraction of hydrogen from sugar units. The study helps to realize interactions of anthracyclines or similar compounds with a biological target suggesting reasons for their efficacy [29, 30, 31, 32, 33]. As is known from previous studies, complex formation of anthracyclines lead to decreased semiquinone formation [28, 31, 32, 33, 34, 35] and hence the risk of cardiotoxicity decreases, but complexes could be at a loss regarding efficacy in the free radical pathway when compared with anthracyclines or their analogues [15, 16, 18, 20, 21, 22, 23, 34, 35, 36].

Since Mn^{II} complexes show significant SOD like activity and because Mn^{II} is able to acquire higher oxidation states in presence of peroxides that are generated as part of ROS, we wanted to see if the Mn^{II} complex would be beneficial for cell killing [52, 53]. It is reported that almost all mitochondria contain a form of Mn-SOD where Mn could be in +2 or +3 oxidation states [54]. Our complex could then be a mimic of Mn-SOD found in human mitochondria since anthracyclines, its analogues or metal ion complexes on entering cells eventually interact with the mitochondrial electron transport system to show drug action [54].

2. Experimental

2.1. Materials and methods

Alizarin was procured from Sigma and purified by re-crystallization using ethanol. The Mn^{II} complex of alizarin $[Mn^{II}(alz)_2(H_2O)_2]$

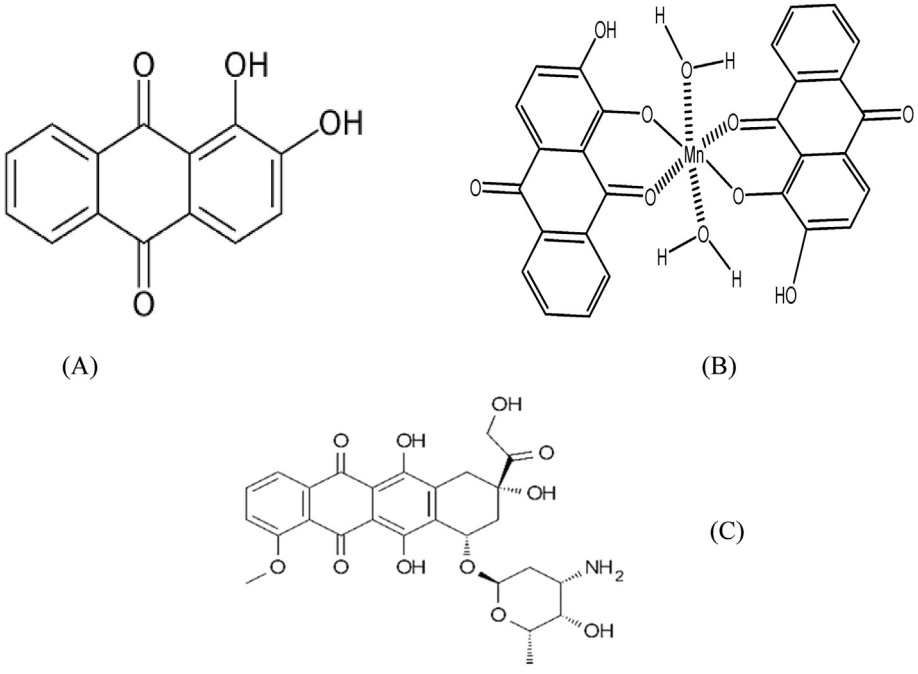

Figure 1. Structure of (A) Alizarin, (B) $[Mn^{II}(alz)_2(H_2O)_2]$ and (C) an anthracycline anticancer drug.

(Figure 1) was prepared and characterized earlier [55]. KCl (AR), purchased from Merck India, was used as an electrolyte for electrochemical experiments in aqueous medium. Nucleobases uracil, thymine and adenine were obtained from Sisco Research Laboratories, India; cytosine was obtained from Sigma. Calf thymus DNA was obtained from Sisco Research Laboratories, India. Tetrabutyl ammonium bromide (TBAB) (AR) and Ethidium bromide (EtBr) were purchased from Merck, India. Triple distilled water was used for preparing solutions. Phosphate buffer (pH ~ 7.4) was prepared in triple distilled water using sodium dihydrogen phosphate (AR) and disodium hydrogen phosphate (AR) procured from Merck, Germany.

2.2. Electrochemical measurements

Electrochemical experiments on alizarin and its Mn^{II} complex were carried out in an air-tight 50 ml electrochemical cell. Voltammograms were obtained on a Metrohm−Autolab model PGSTAT 101 potentiostat. Analysis of data was done using NOVA 1.10.1.9 program. A conventional three-electrode system, glassy carbon as the working electrode, platinum wire as the counter electrode and Ag/AgCl, satd. KCl as the reference electrode were used.

Before each electrochemical experiment, solutions were degassed for ~30 min using high purity argon. Reduction of the quinone moiety in compounds was followed in aqueous, aqueous-dimethyl formamide (DMF) and in pure DMF using cyclic voltammetry. In DMF, a two step one-electron reduction (first to semiquinone and then to quinone dianion) was observed [41, 42]. With increase in percentage of water, two reduction peaks approach each other and a single step two electron reduction occurs [56, 57]. Quinone-dianion and free quinone upon comproportionation form semiquinone radical anion that undergoes disproportionation as well [28, 33, 41, 42, 43, 58, 59, 60]. This was again verified as a part of this study. Voltammograms were analyzed by the Randles-Sevcik equation [Eq. 1, Figure 2A and B] to check that the process was diffusion controlled, which was extremely essential for experiments related to this study [61, 62].

$$i_{pc} = (2.69 \times 10^5).n^{3/2}.\ D_0^{1/2}.A.C.\ \nu^{1/2} \quad (1)$$

i_{pc} refers to current in amperes at the cathodic peak potential, n, the total number of electrons, D_0, diffusion coefficient of species, A, area of the electrode in cm^2, C, concentration of compounds in moles/cm^3 and ν, scan rate in Vs^{-1}. Figure 3 is a typical voltammogram of alizarin in aqueous solution from where its reduction potential (-0.65V) was obtained. Under exactly similar conditions, reduction potential of Mn^{II} complex (-0.75 V) was ascertained.

These reduction potentials were subsequently used for reducing the compounds using the same glassy carbon electrode, by maintaining

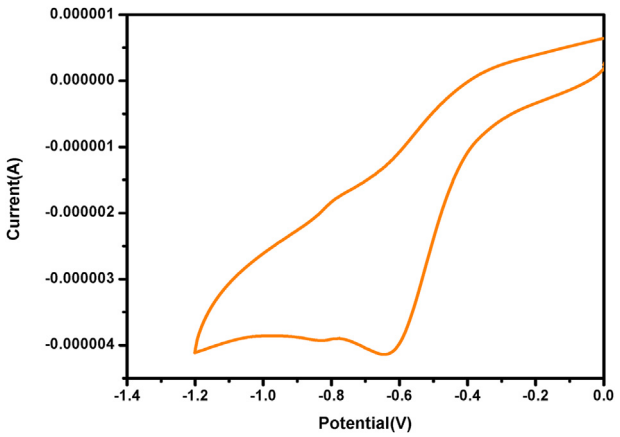

Figure 3. Cyclic voltammogram of 0.01 mM alizarin at pH ~ 7.4 showing single step two electron reduction of the quinone in aqueous solution containing 0.12 M KCl on a glassy carbon electrode (surface area 0.1256 cm^2); Scan rate 100 mV/s.

either a nucleo base (one at a time) or double stranded calf thymus DNA in the vicinity of the reduced products, under aerated/de-aerated (Ar saturated) conditions at constant pH (~7.4). Since biological targets were present in the immediate vicinity of *in situ* electrochemically generated quinone-dianion or semiquinone they get an opportunity to interact with reduced species. In aerated medium, there is a possibility for the formation of superoxide radical anion and that here the Mn^{II} complex could show SOD activity [53]. The time for *in situ* electrochemical generation of species was kept a constant so that similar charge was made available to each compound in all the sets of the experiment and that there is similar experimental error associated with the stimuli (here, the potentiostat) that helps to generate the reactive species. Constancy of charge transferred to compounds was checked by chrono-amperometry. This enabled a proper comparison of results obtained the following interaction of species (radical anions or radicals) generated in solution with a target maintained in the vicinity of such generation [38, 39, 40]. A semiquinone radical anion or protonated semiquinone under de-aerated condition or superoxide radical-anion alongwith the semiquinone radical anion (under aerated condition) interact with nucleobases or DNA, that was maintained in the electrochemical cell (Figure 4). In case of control experiments, no compound was used but respective potentials (i.e. -0.65 V for alizarin and -0.75 V for the complex) were applied using the same glassy carbon electrode with either a chosen nucleobase or calf thymus DNA in solution. Needless to say, all potentials were maintained and applied accurately since this

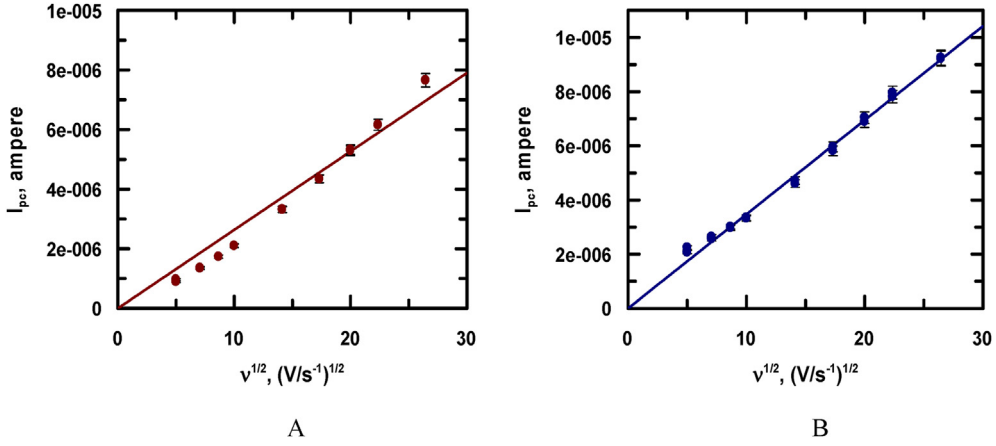

Figure 2. Plot of cathodic peak current (I_{pc}) vs. square root of scan rate (ν) for two-electron reduction of alizarin (A) and Mn(II)-alizarin complex (B) in aqueous solution at a potential of -0.65 V and -0.75 V respectively at pH ~ 7.4.

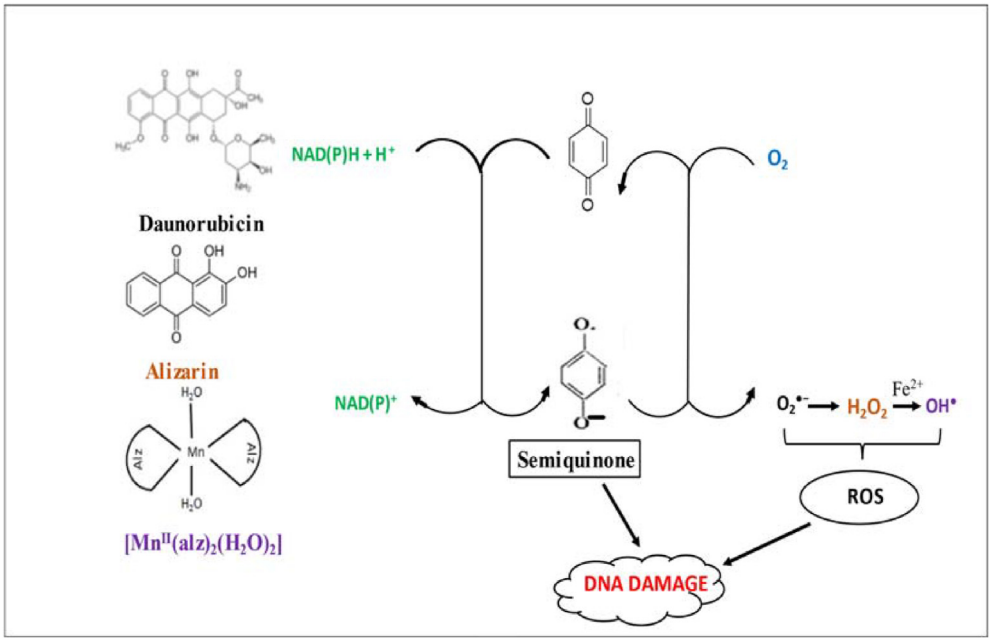

Figure 4. A scheme showing the basis for this study.

formed the basis of our experiments [38, 39, 40]. Concentration of nucleobases present in solution was approximately ten times that of the compounds used. pH was maintained at 7.4 with the help of phosphate buffer.

Aliquots were taken from an experimental solution, following the application of a constant pre-determined potential for a definite period of time, during which each nucleobase was subjected to interaction with *in situ* electrochemically generated species in solution. Then, HPLC was performed using a C-18 column as the stationary phase and 5 % aqueous-methanol as the mobile phase. From HPLC chromatograms, amount of each nucleobase remaining unaltered, was calculated. Control experiments were performed for each set (aerated or de-aerated) and for all targets using the same glassy carbon electrode maintained for the same time in solutions containing same amount of nucleobases but in the absence of a compound.

In experiments, where calf thymus DNA was the target, aliquots were taken from the reaction vessel and treated with EtBr. The DNA-EtBr adduct was excited at 510 nm and fluorescence was recorded at 602 nm [63, 64, 65]. From the loss in fluorescence of the DNA-EtBr adduct, amount of calf thymus DNA that underwent modification following interaction with the radicals generated under aerated and de-aerated (Ar saturated) conditions was ascertained [63]. For control experiments with DNA, the solution contained the same amount of calf thymus DNA but no compound. Solutions were subjected to a constant potential of -0.65 V to serve as the control for alizarin and at -0.75 V to serve as control for the complex using the same glassy carbon electrode for similar times. Aliquots from these solutions were subsequently treated with similar concentrations of EtBr and fluorescence was recorded at 602 nm.

3. Results and discussion

3.1. Interaction of electrochemically generated species with nucleobases

After interaction of semiquinone radical anions and/or protonated semiquinones under de-aerated condition or superoxide radical anions or semiquinone radical anions in aerated medium (pH ~7.4), aliquots collected from experimental solutions were subjected to HPLC to ascertain the amount of nucleobases remaining (Figure 5).

Damage caused to each nucleobase was plotted against time for which the electrochemical generation of species was attempted. Figures 6 (A), 4 (B), 4 (C) and 4 (D) are plots showing degradation of uracil, thymine, cytosine and adenine respectively in the absence and presence of compounds under similar experimental conditions. Slopes of degradation plots are an estimate of the amount of damage caused to each nucleobase under different conditions. Different extents of base damage were observed under different conditions that indicate selectivity owing to the type and the amount of radicals generated. Extent of damage depends on the ability of species formed in solution to interact with a target (Figure 6, Table 1).

Table 1 shows in the absence of alizarin or its Mn^{II} complex, if glassy carbon electrode was held at constant potentials of -0.65 V and -0.75 V respectively, there was no significant damage on any nucleobase. However, when a compound was present, application of a constant potential (-0.65 V for alizarin or -0.75 V for Mn^{II} complex) caused damage to nucleobases suggesting free radicals formed on a compound react with a target maintained in the vicinity of their generation. Extent of base damage was different for different targets and for different conditions suggesting selectivity of radicals during interaction. Base damage in Table 1 is expressed as EER, where EER denotes the electrochemical enhancement ratio, calculated by dividing the slope of any degradation plot achieved for a certain nucleobase in the presence of a compound under a specified condition by the slope obtained for the damage of the same nucleobase in the absence of that compound under aerated condition. The value of the slope for the plot obtained under aerated condition in the absence of a compound was considered fundamental since for these experiments when potential was held constant either at -0.65 V or -0.75 V there would be reduction of molecular oxygen to different species that might also interact with nucleobases; hence damage achieved over and above that value was considered.

In the presence of alizarin, damage on cytosine in the absence of O_2 (de-aerated condition) was maximum, significantly higher than on any other nucleobase used. For the complex however, damage caused to thymine under de-aerated condition was the highest. Baring the result on thymine in presence of alizarin (EER = 1.61), in aerated medium, no other nucleobase showed such significant damage under these conditions. An interesting aspect is that the Mn^{II} complex was less effective in

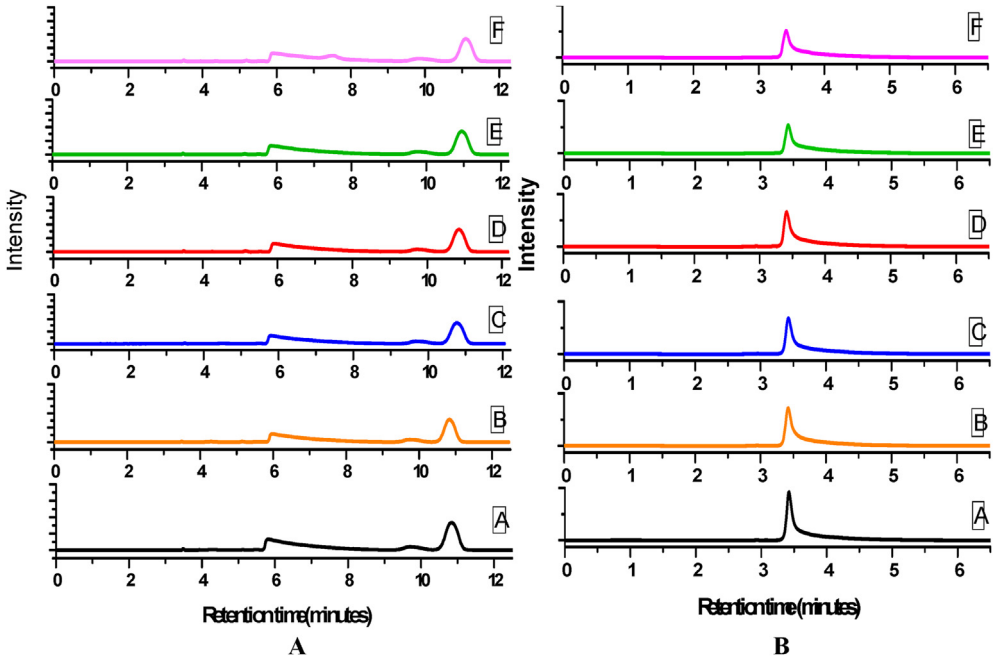

Figure 5. HPLC chromatograms obtained at 254 nm for 1×10^{-3} mol dm^{-3} of (A) thymine, (B) cytosine solutions that were subjected to a constant potential of -0.65 V in the presence of 1×10^{-5} mol dm^{-3} alizarin under de-aerated (Ar saturated) conditions. A to F indicates time in minutes for which such constant potential was applied to the solution; A: 0 min, B: 2 min, C: 4 min, D: 6 min, E: 8 min, F: 10 min.

the free radical pathway, a fact discussed in previous reports with regard to efficacy of complexes prepared, using anthracyclines, or with hydroxy-9,10-anthraquinones, owing to decrease in semiquinone formation [28, 34, 35]. It has been mentioned earlier on different occasions that complexes of anthracyclines or of their analogues are at a disadvantage with regard to activity in the free radical pathway [28, 34, 35] but very few studies have shown it by working on this aspect exclusively. Decrease in activity observed for the Mn^{II} complex in the free radical pathway in comparison to alizarin is expected, since reports on anthracyclines and on its analogues indicate decreased generation of semiquinone by the complexes [28, 31, 32, 33, 34, 35].

In this study, using alizarin, and its Mn^{II} complex, this got manifested by way of a maximum difference in damage observed for adenine and minimum for thymine suggesting under conditions of similar electrochemical generation of intermediates, there is either a difference in generation of intermediates on each compound or in their tendency to interact in the free radical pathway. If this isn't true, then base damage should have been similar. Table 1 indicates a difference in reactivity of the two compounds on different targets under identical conditions suggesting compounds might affect different types of DNA differently. Therefore, the general apprehension, that a complex prepared with anthracyclines, or with its analogues, compromise on aspects related to cytotoxicity in the free radical pathway, is true to a considerable extent. Yet, complexes of anthracyclines or of its analogues are prepared because they modulate the generation of semiquinone helping to decrease cardiotoxicity [28, 31, 32, 33, 34, 35]. In a previous study, we compared the performance of a Cu^{II} and a Mn^{II} complex of emodin against that of emodin itself, under similar experimental conditions and found that the emodin-Mn^{II} complex was almost similar in activity to emodin [39]. The Cu^{II} complex, performed much better, attributed to the presence of a stable lower oxidation state (Cu^{I}) enabling Cu^{II} complexes to participate in Fenton reactions generating ·OH, that in turn enhance modification of nucleobases [36, 37, 39, 40, 64, 65]. In that study, base damage due to Cu^{II}–emodin was higher in aerated medium than in de-aerated medium [39].

From the EER values in Table 1, on the performance of a compound on nucleobases it might become possible to predict the damage that a compound might cause on a particular DNA whose base composition is known. In fact, this aspect could have been an important outcome of this study itself. However, the difficulty in correlating nucleobase damage to that observed in case of calf thymus DNA lay in the fact we could not generate the data where guanine was a possible target. This was because preparing an aqueous solution of guanine having similar concentration as that of the others was difficult owing to its poor solubility in buffer.

Using the information from a previous study that was performed by our group with a dimeric Cu(II) complex of tinidazole, where base degradation was followed using γ radiation, products formed in this study were identified [66]. Products were characterized following the degradation of thymine, cytosine and uracil [66]. Since HPLC profiles of degraded products of thymine were saved as method files in our system as a part of that study [66], they were utilised for this one to identify products related to degradation of thymine, cytosine and uracil in the absence and presence of alizarin and its Mn^{II} complex. Results indicate in presence of alizarin and its Mn^{II} complex, 5,6-dihydroxy-5,6-dihydrothymine (thymine glycol) and 5-hydroxymethyl uracil were formed when thymine was the target, although amounts were extremely small as the charge provided at constant potential was not very high. The peak for 5, 6-dihydrothymine was not detected even when thymine was subjected to an electrochemical generation of intermediates using compounds (alizarin and Mn^{II} complex) at longer times. Products were identified based on retention times using authentic samples [66].

Cytosine differs from uracil at the fourth position (carbon) of the pyrimidine ring where there is an $–NH_2$ instead of –OH (if enol form of uracil be considered). Since 5,6-dihydroxy-5,6-dihydrocytosine (cytosine glycol) is unstable and converts to 5,6-dihydroxy-5,6-dihydrouracil (uracil glycol) by deamination [67], we used our existing HPLC method files on uracil to identify degraded products when cytosine or uracil were maintained as targets during experiments performed as a part of this study [67]; however peaks for the degraded products were very small. Observations suggest pyrimidine based nucleobases experience an initial free-radical attack by species generated as a consequence of the application of current at a constant potential on the C_5–C_6 double bond [66, 67]. For the nucleobase adenine, HPLC chromatograms did not show any new peak in the time frame of the application of constant potential.

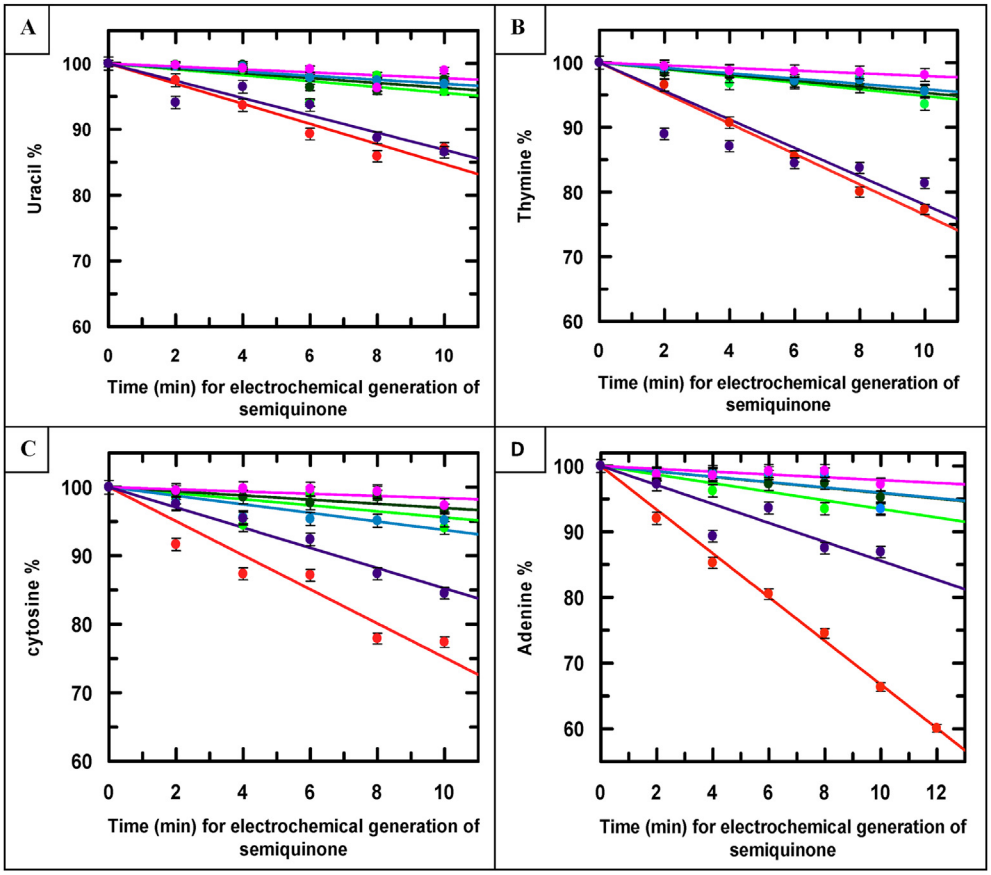

Figure 6. Plots shown in (A), (B), (C) and (D) indicate percentage of uracil, thymine, cytosine and adenine remaining following interaction of in situ electrochemically generated species on alizarin and its Mn^{II} complex. Different experimental conditions were: nucleobase alone in absence of O_2 (black circles); nucleo base alone in presence of O_2 (green circles); nucleobase in presence of alizarin but absence of O_2 (red circles); nucleobase in presence of alizarin and presence of O_2 (blue circles); nucleobase in presence of $Mn^{II}(alz)_2(H_2O)_2$ but absence of O_2 (magenta circles); nucleobase in presence of $Mn^{II}(alz)_2(H_2O)_2$ and presence of O_2 (pink circles).

Table 1. Degradation of nucleobases due to electrochemically generated species (semiquinone or quinone dianion or oxygen based radicals) followed by HPLC.

Compound	Target																
	Uracil				Thymine				Cytosine				Adenine				Calf thymus DNA
	Slope of plot in aerated Medium	EER	Slope of plot in de-aerated medium	EER	Slope of plot in aerated medium	EER	Slope of plot in de-aerated medium	EER	Slope of plot in aerated medium	EER	Slope of plot in de-aerated medium	EER	Slope of plot in aerated medium	EER	Slope of plot in de-aerated medium	EER	EER
–	-0.46	–	-0.37	–	-0.52	–	-0.48	–	-0.41	–	-0.30	–	-0.78	–	-0.42	–	-0.045	–
Alizarin	-0.36	–	-1.61	3.50	-0.41	–	-2.37	4.56	-0.66	1.61	-2.35	5.73	-0.46	–	-3.15	4.03	-0.16	3.55
Mn(II) complex of Alizarin	-0.28	–	-1.31	2.85	-0.22	–	-2.23	4.29	-0.24	–	-1.49	3.63	-0.31	–	-1.48	1.90	-0.10	2.22

3.2. Interaction of electrochemically generated species with calf thymus DNA

In experiments with calf thymus DNA, samples had similar concentrations as that for studies with nucleobases, subjected to interaction with *in situ* electrochemically generated species and other products formed thereof at different time intervals. Double strand modification was ascertained by the change in fluorescence of the DNA-EtBr adduct, considered a measure of the extent of modification caused to DNA by free radicals (Figure 7) [63-65].

Figure 8 shows the amount of DNA remaining intact following interaction with intermediates (semiquinone/protonated semiquinone, superoxide radical anion/its protonated form, quinone dianion and its corresponding protonated forms etc.) at pH 7.4. Using Figure 8, it may be said double strand modification of DNA was higher for alizarin than for $[Mn^{II}(alz)_2(H_2O)_2]$. Interestingly, the data shown in Figure 8 has the same trend as that of interaction of species (generated electrochemically) with the four nucleobases. It was reported earlier DNA and oligonucleotides tend to stabilize semiquinone radical anions through delocalization of electrons in a π-stacking framework, that result in a radical intercalated situation rather than forming covalent bonds [43]. This was further supported by some semi-empirical calculations showing there is a gain in energy by ~ 9.8 kcal mol^{-1} [43]. Insertion of semiquinone radical anions into a DNA strand was reported and believed to result in DNA strand breaks leading to disruption in DNA replication or activate radical mediated reactions. pH as expected, was reported to shift the equilibrium of dianions of hydroquinones, thereby having an influence on the formation of semiquinone radical anion which in turn influences interactions with DNA [43, 54]. The fact that our results show modification of nucleobases only adds to previous information following intercalation

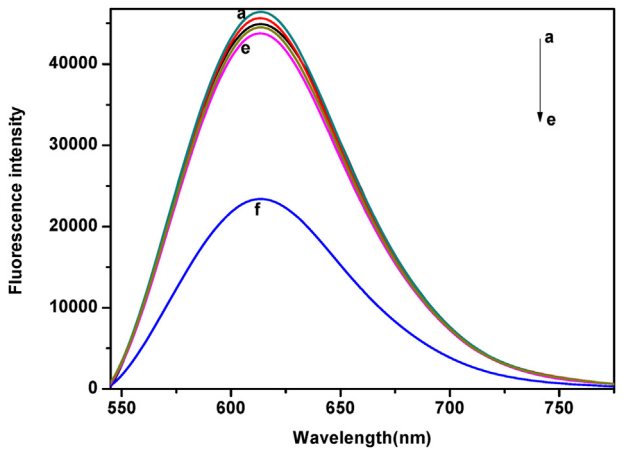

Figure 7. Fluorescence spectra of 1×10^{-3} mol dm^{-3} calf thymus DNA after treatment with EtBr following interaction with the products generated on the Mn(II) complex of alizarin that was subjected to reduction at constant potential (-0.75 V) in de-aerated (Argon saturated) conditions. $[Mn^{II}(alz)_2(H_2O)_2] = 3 \times 10^{-5}$ mol dm^{-3}."a" to "e" indicates time in minutes for which the potential was applied to the solution; a: 0 min, b: 15 min, c: 30 min, d: 45 min, e: 60 min "f" denotes the spectrum of EtBr when it was excited alone i.e. in the absence of DNA at 510 nm.

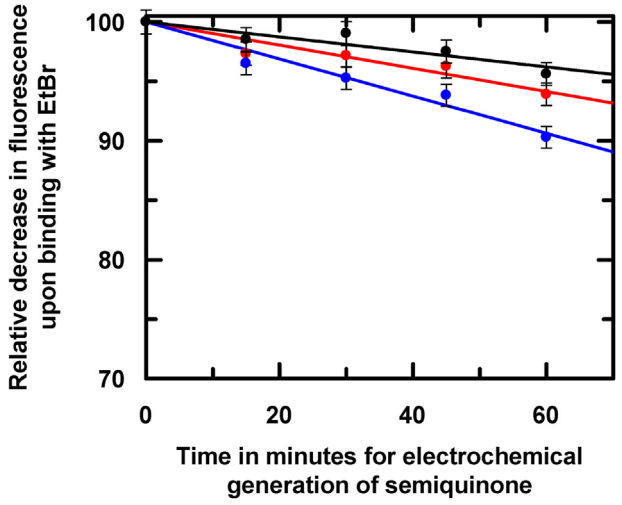

Figure 8. Plots obtained for semiquinone radical anion induced modification of calf thymus DNA in the absence and presence of alizarin and the Mn^{II} complex in an argon saturated medium at pH 7.4; in absence of a compound (black circles); in the presence of alizarin (blue circles) and $[Mn^{II}(alz)_2(H_2O)_2]$ (red circles).

leading to unwinding of DNA, the exposed nucleobases become vulnerable to further damage that might permanently prevent them from regenerating the double strands again, should a favourable situation arise.

Another interesting aspect of the study is that the difference between damage caused to DNA by species generated on alizarin and on $[Mn^{II}(alz)_2(H_2O)_2]$ was not as large as that observed for nucleobases. This suggests in case of DNA, some attribute of complex formation comes into play causing substantial modification to DNA by other pathways that are also detected by the loss of fluorescence due to the formation of the DNA-EtBr adduct. Therefore, excess modification due to a greater amount of free radicals formed on alizarin over that formed on the complex (the gap) is somewhat realized. Besides, Mn^{II} having stable higher oxidation states might get oxidized by ROS, by substances like H_2O_2 (if formed in the medium) to generate transient Mn^{III} that could then show its oxidative role [52, 53], leading to a damage of DNA that would also be detected by the EtBr-DNA fluorescence technique that we used. Therefore, although formation of semiquinone on alizarin is greater than that formed on the complex as realized from damage caused to nucleobases (Table 1), other aspects related to complex formation might bridge the gap between the performance of alizarin and its Mn^{II} complex on calf thymus DNA. Although binding of the complex to calf thymus DNA is better than alizarin [55], here it was probably not a major contributor towards any significant damage to DNA as one would have expected because in this study the concentration of the compounds were 0.03 mM (i. e. almost 100 times less than the concentration of DNA used) [65]. Therefore, although Mn^{II}-alizarin has a strong affinity for DNA, in case of this study there should not be much of an influence due to its binding to DNA [55] since the concentration of compounds used were too small compared to the substrate. Since the technique of decreasing fluorescence of a DNA-EtBr adduct detects double strand modification in general (i.e. however it may be caused), all modifications are actually detected [63, 64, 65]. Thus this technique provides overall double strand modification i.e. caused by the action of free radicals as well as by other pathways [63, 64, 65]. Hence, for the study with calf thymus DNA, a "leveling effect" might have been observed in case of the interaction of the compounds, following an *in situ* electrochemical generation of reactive species under the conditions of the experiment (Figure 8, Table 1). Even then, alizarin was better than the complex indicating the extent of compromise complexes of this class of compounds make in the free radical pathway.

4. Conclusion

The study demonstrates the manner in which nucleobases that constitute DNA might be affected by semiquinone-radical anion and other species generated in solution following the reduction of alizarin (an anthracycline analogue) and its Mn^{II} complex. It clearly demonstrates each compound's ability to initiate radical induced damage on different nucleobases, considered a significant pathway by which anthracyclines and its analogues (emodin or carminic acid) show cytotoxic activity on cancer cells [17, 18, 19, 20, 33]. The study provides evidence why complex formation of anthracyclines or its analogues, although beneficial with regard to decrease in cardiotoxic side effects, compromise with cytotoxicity in the free radical pathway. The study shows electrochemically generated species on alizarin are able to cause greater damage to nucleobases than those that are generated on the complex. In case of calf thymus DNA, results indicate a better performance by alizarin than by the complex, however the difference in performance is not as large as that observed for the nucleobases. This indicates the complex is able to make up lost ground substantially owing to several attributes of complex formation like being able to derive the benefits of the redox behavior of Mn^{II}. The study is also able to explain the performance of various hydroxy-9,10-anthraquinones and their metal complexes on different cancer cells and on normal cells [28, 29, 30, 31, 32, 33, 34, 35].

Declarations

Author contribution statement

Mouli Saha: Performed the experiments; Analyzed and interpreted the data.

Saurabh Das: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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