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THE BINDING OF VIOLAMYCIN B1 TO DNA

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This paper presents the interaction between an antracycline antibiotic violmycin B1 (V B1) and DNA double helix. We put into evidence the interaction of the aglycone (the antracyclinic skeleton) with the base pairs, inner side of the helix, by specific interactions. By non specific interactions V B1 forms stacked complex on DNA surface, outside of the helix, involving its two glycosidic residues. The binding parameters of both complexes were determined spectrofluorimetrically. The high value of the stability constant K_{int} of the intercalated complex recommended it as antitumoral drug, but due to its high cardiotoxicity it was rejected from the clinical therapy. We proposed a plausible mechanism to explain the cardiotoxicity, namely the electron transfer from the bases to the intercalated aglycone which leads to the appearance of $O_2^{\bullet-}$ radical, a known oxidative species.

INTRODUCTION

DNA may interact with small molecules forming different types of complexes. The nature of these complexes depends on the ligand structure and its geometry. As it is known, three types of complexes[1-5] can result from specific or non specific interactions: **1)** if the ligand has a planar structure (the aromatic nuclei), it may penetrate between the base pairs forming the intercalated complex, by specific interactions; **2)** if the ligand geometry is non planar it may interact with the base pairs, but only at the minor or major grooves level, depending on the ligand volume, generally by specific interactions and **3)** if the ligand carries charges it may form the surface complex by non specific interactions (electrostatic forces).

These complexes require as first stage the nucleation process forming a ligand monomeric complex on the DNA surface, although Dourlent[6] considers that for the intercalated complex formation this stage is not absolutely necessary.

We underline that only the complexes resulted from specific interactions are responsible for the biological effect.

We remember that in the period 1970-1995, the study of the interactions of the antracycline antibiotics with DNA has seen a strong progress in order to find a suitable drug for cancer therapy. But after it was shown that these antibiotics (from the first antracycline generation) present cardiotoxicity (V B1 having the highest one), the interest for these compounds decreased dramatically. Literature shows very well this actual fact.

This paper refers to the stacked and intercalated complexes resulted from the interactions of V B1 with DNA; an interaction mechanism in the intercalated complex formation was suggested explaining the V B1 cardiotoxic effect.

RESULTS

Violamycin B1 is an equimolecular mixture of three isomers and has the following structure:

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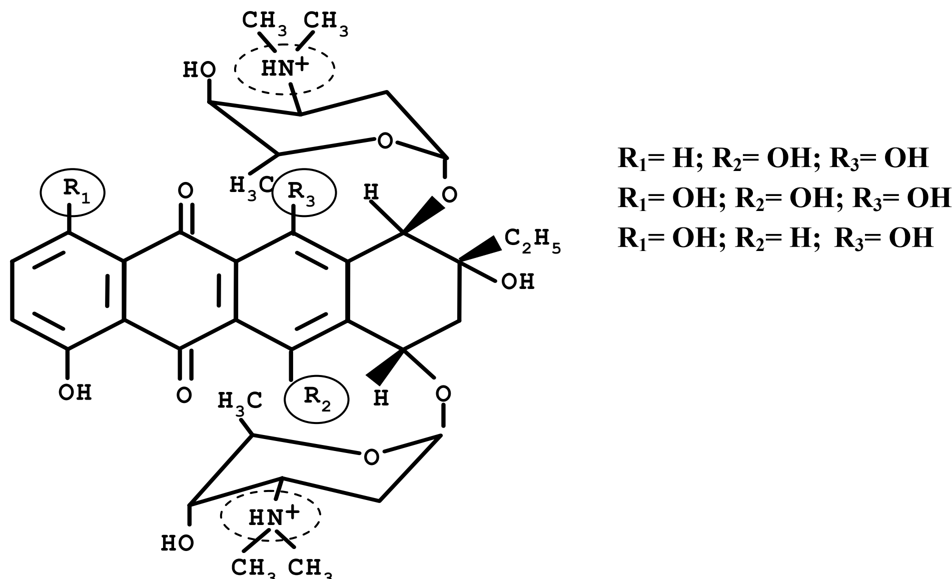


R_1= H; R_2= OH; R_3= OH
R_1= OH; R_2= OH; R_3= OH
R_1= OH; R_2= H; R_3= OH

One remarks an antracyclinic skeleton, the chromophore of the molecule, at which are attached two glycosidic residues, each one with a positive charge on the nitrogen. Our experimental data showed that these two parts of V B1 play different roles in the interaction with DNA: the aglycone with its planar structure is involved in the intercalated complex, whereas the glycosidic moieties in the surface complex, due to their positive charges, attracted electrostatically by the negative phosphates along the helix chain.

We studied their formation spectrophotometrically as well as spectrofluorimetrically as is shown in Fig. 1.

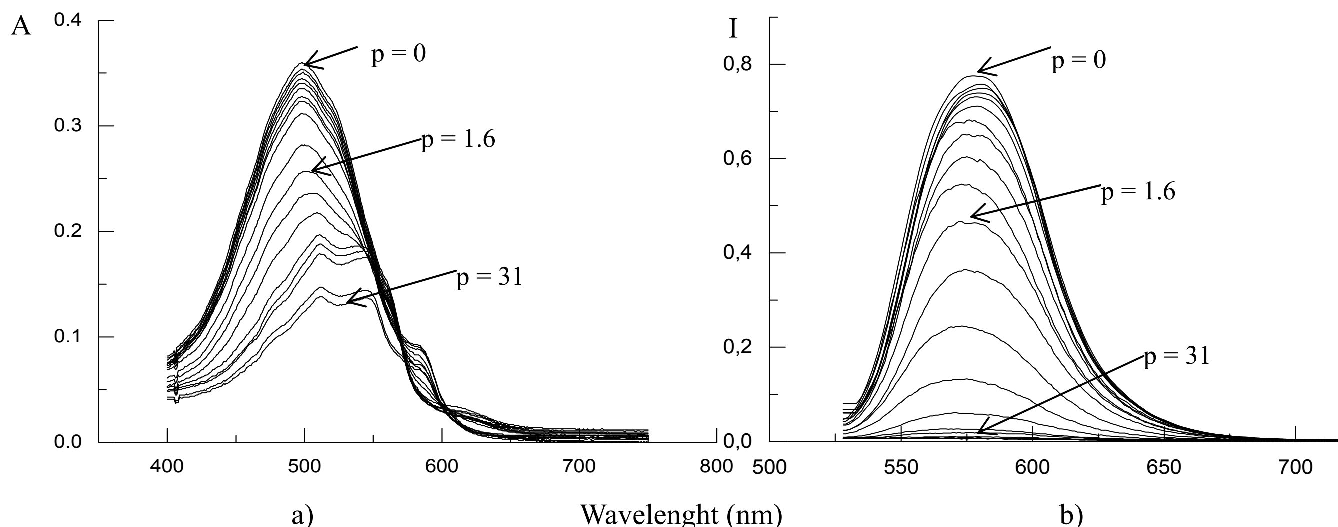


Fig. 1 – The evolution of a) absorption and b) fluorescence spectra during the titration with DNA.

Fig. 1a) presents the absorption spectrum of V B1 (3.33 x 10^{-5} M) and its evolution (from p=0 towards p=31, p being the ratio between DNA and V B1 concentrations c_{DNA} / c_{VB}) by adding DNA of 1.97 x 10^{-3} MP in a phosphate-EDTA buffer of pH 7. At lower values than p=1.6, we observed a pronounced hypochromic effect with the appearance of two isosbestic points at λ=560 nm and λ=600 nm which attests the presence of the equilibrium between the free and bound V B1 in the surface complex. When p exceeds 1.6, by adding continuously DNA in the spectrophotometric cell, we remarked also a pronounced shifting of the absorption maximum towards the long wavelengths accompanied by its splitting which reveals the presence of a new equilibrium between the free and bound V B1 in the intercalated complex. Based on the observed spectral

behaviour, we consider that at the p=1.6 the formation of the surface complex is finished and starts the intercalated complex one.

Fig. 1b) presents the evolution of the fluorescence spectrum during this titration. The solutions of V B1 were excited with λ=550 nm. The spectra are characterized by the decrease of the fluorescence intensity and the shifting of its emission maximum towards the short wavelengths due to the formation of these complexes.

Fig. 2 presents our experimental data from both types of spectral titration, by plotting γ (A/A_0 in absorption and I/I_0 in emission) versus p. The two curves have similar shape, namely a deep decrease at the beginning of the titration (low values of p), followed by a moderate one with the tendency to reach a plateau at high p values. However, there is also an important difference: whereas the fluorescence emission is practically quenched at the finish of the titration, the absorbance has still measurable values indicating that the formed complexes absorb in the same spectral domain with the free violamycin.

For this reason we preferred the spectrofluorimetric data to calculate the binding parameters preventing the overlapping of the absorbance measurements.

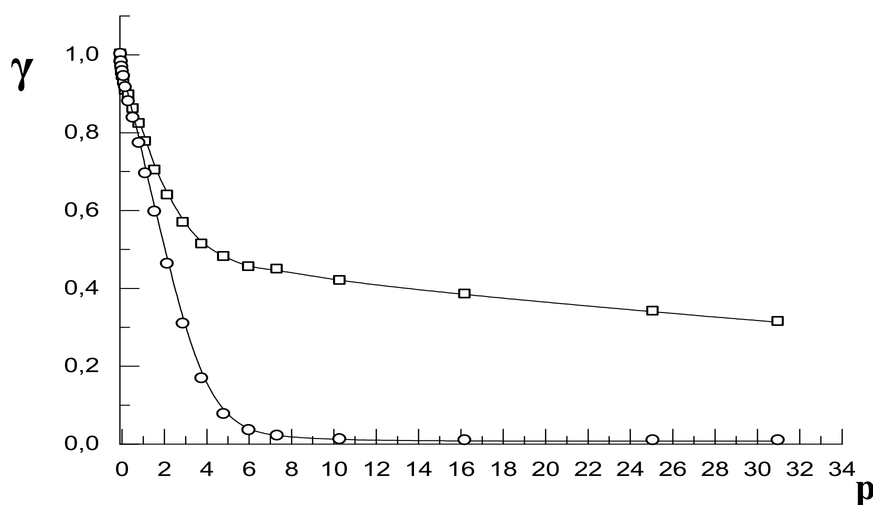


Fig. 2 – γ versus p (A/A_0 (□) and I/I_0 (○).

The surface complex

We adopted Schwarz model[7-10] to calculate the binding parameters at low values of p. Fig. 3 shows γ* (the total fraction of free V B1 in its monomeric and possible dimeric form) depending on the V B1 initial concentration, plotted versus p, accordingly to this model.

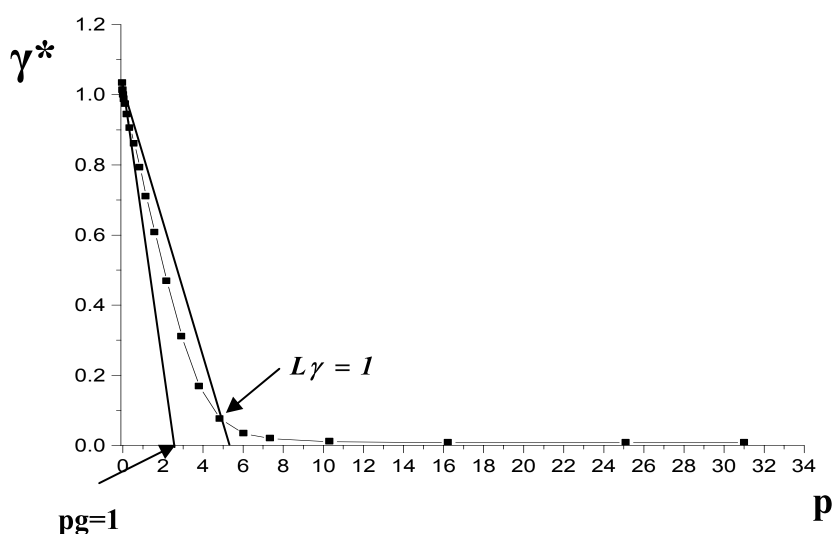


Fig. 3 – γ* versus p (Schwarz model).

The value of γ^* is given by equation (1):

$$\gamma^* = \gamma\left(1 + 2K_D c^0_{VB}\gamma\right) \quad (1)$$

where K_D is the dimerization constant of free monomeric violamycin in solution, with a value of 730 M^{-1} determined by us in a previous study.[11]

At the beginning of the titration, when in the spectral cell there is only V B1, by adding small amounts of DNA, all the binding sites of the biopolymer surface are occupied by violamycin; the degree of saturation equals 1 and γ^* is proportional to p. By extrapolating this linear part, it intersects the abscissa in a point for which pg=1. The parameter g is the number of the binding sites occupied by a single V B1 molecule in the nucleation process, in our case g = 0.38.

The stability constant K_{st} which characterizes the stacked complex was calculated by Schwarz model drawing a straight line with the tangent equals –g/2, as Fig. 3 shows. This line intersects the curve in a point for which $L\gamma=1$, L representing the binding strength. We calculated in this manner a value L = 8.32. Then, using equation (2), we obtained the value of $K_{st} = 2.50 \times 10^{-5}$ M^{-1}.

$$L = K_{st} c^0_{VB} \quad (2)$$

Our experimental data concerning the binding parameters of V B1 stacked complex for four different V B1 initial concentrations are summarized in Table 1.[12]

Table 1

The binding parameters of V B1 stacked complex

c^0_{VB} x 10^5, M	g	L	K_{st} x 10^{-5}, M^{-1}
1.44	0.40	1.91	1.33
3.33	0.38	8.32	2.50
5.35	0.34	14.28	2.67
7.87	0.30	25.02	3.18

The intercalated complex

If one continues the titration starting from p=1.6 when the formation of staked complex is practically finished, the value of p increases by adding DNA in the spectral cell.

The added DNA chains offer continuously free sites to V B1, which penetrates directly between the base pairs forming the intercalated complex. We applied Scatchard method[13] to calculate its binding parameters by plotting the equation (3), in which r is the ratio between the concentration of bound V B1 (c_B) and of DNA (c_B / c_{DNA}), K_{int} the equilibrium constant of the intercalated complex, B_{ap} the apparent number of binding sites occupied by the ligand per monomeric unit and c_F the free V B1 concentration.

$$r/c_F = K_{int}(B_{ap} - r) = K_{int}B_{ap} - K_{int}r \quad (3)$$

The parameter r was evaluated by the intermedium of the bound V B1 concentration (c_B) calculated by using equation (4):

$$c_B = \frac{(c^0_{VB})_{corr}(I_0 - I)}{I_0} \quad (4)$$

$(c^0_{VB})_{corr}$ is the V B1 concentration corrected with dilution resulted by DNA addition, I_0 is the fluorescence intensity of free V B1 (first spectrum) and I the fluorescence intensity at each addition of the nucleic acid.

The concentration of violamycin $(c^0_{VB})_{corr}$ is giving by equation (5):

$$(c^0_{VB})_{corr} = \frac{V_{VB} \times c^0_{VB}}{V_{VB} + V_{DNA}} \quad (5)$$

where V_{VB} represents the initial volume of V B1 introduced in the cell (2 ml) at c^0_{VB} initial concentration and V_{DNA} the total added DNA volume. Therefore, the free concentration of violamycin c_F was determined as the following difference: $(c^0_{VB})_{corr} - c_B$.

In Fig.4, which refers to Scatchard equation plot, one notices two linear segments: the first one with a positive slope we attributed to the stacked complex formation discussed before for r>0.5 that is for p<1.6, taking into account their definitions. When the formation of the stacked complex is practically finished, there is still 90% free V B1 in solution.

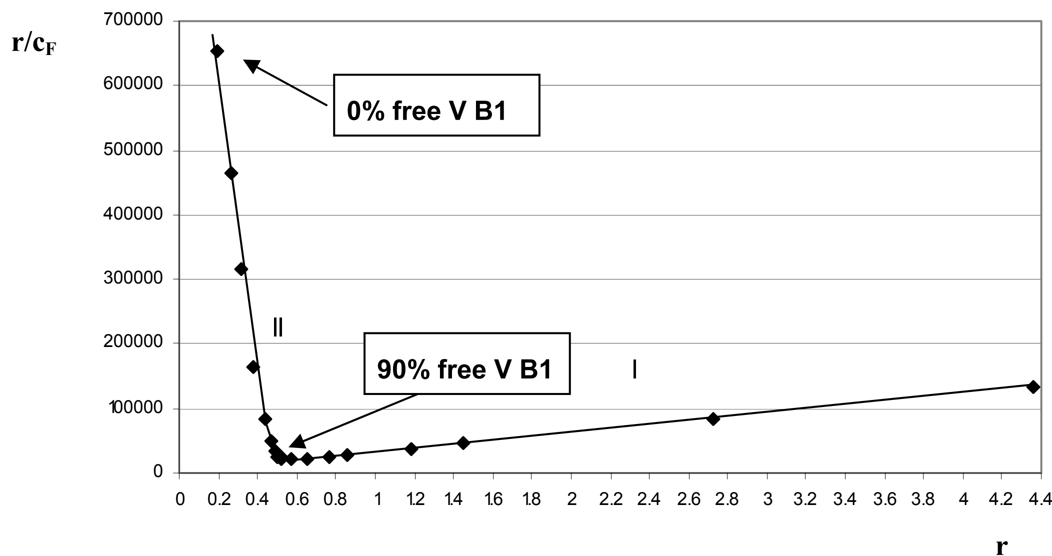


Fig. 4 – Scatchard plot of r/c_F vs r .

The segment II ranging $0.2<r<0.5$ refers to the intercalated complex formation, characterized by a negative slope according to Scatchard equation. From the slope we calculated the stability constant of this complex K_{int}. The negative slope shows that the complex formation may be a noncooperative or anticooperative process. Because in the stacked complex formation only 10% of the free V B1 was consumed and 90% is involved in the intercalated complex formation, Scatchard ecuation is obeyed within a very large range of r and this could be an argument in favour of a noncooperative interaction.

The same initial concentrations of V B1 used at the stacked complex formation were investigated to calculate the binding parameters of the intercalated complex. We present in Table 2 the results obtained at low values of r (high values of p).

Table 2

The binding parameters of the intercalated complex

c_{VB}^{0} x 10^5, M	K_{int} x 10^{-6}, M^{-1}	B_{ap}	n	
			$1/B_{ap}$	I/I_0 vs p
1.44	6.30	0.34	2.9	2.7
3.33	2.40	0.48	2.1	2.0
5.35	2.16	0.44	2.3	2.3
7.87	1.46	0.32	3.1	3.3

The number n of binding sites occupied by one ligand molecule was calculated in two different modes and the results are in very good agreement, as the two last columns of the Table 2 show.

DISCUSSION

The surface complex

The values of g, presented in Table 1, varies between 0.4-0.3 which shows that the number of binding sites offered by a nucleotide to violamycin, ranges between 0.2-0.15, that is a ligand molecule occupies 5-6 nucleotides when it forms the nucleation complex on the surface of biopolymer. This number is the double of the value we obtained when violamycin binds to single (poly A, poly G , poly dT and poly dGC) and double stranded (poly $[d(G-C)]_2$, poly $[d(A-T)]_2$, polydG·polidC and polydA·polydT) synthetic nucleic acids[14,15], because of DNA secondary structure preventing the binding of V B1 to more sites.

The binding strength L increases with the initial violamycin concentration, as was expected from its definition: $L=K_{st}\,c_{VB1}^{0}$.

At the beginning of the titration, because of the excess of violamycin, it forms immediately stack aggregates on the monomeric bound V B1 to DNA surface. The formation of the simplest aggregate -

the dimer - is favoured because in the nucleation process, the positive charges of V B1 are reduced by the attraction of the negative phosphates, so that the repulsion between the two violamycin molecules is substantially attenuated in comparison to the formation of the free dimer in solution. Then, the binding of the next V B1 molecules on the dimer aggregate is facilitated by the cooperativity of the violamycin molecules. Of course, the hydrophobic forces have to play also an important role in the formation of the stackes. In this manner, it forms stacked complex of different lengths with their aglycones superposed, characterized by the stability constant K_{st}.

The stability constant K_{st} increases with the initial V B1 concentration due to the cooperativity of violamycin molecules. It is interesting to mention that the value of $K_{st}=3.18 \times 10^5 M^{-1}$ for the highest initial V B1 concentration (7.87×10^{-5} M) is near 3.05×10^5 M^{-1} obtained by us[13] from the study of V B1 binding to the synthetic double stranded poly $[d(G\text{-}C)]_2$ and very far from 3.40×10^4 M^{-1} for poly $[d(A\text{-}T)]_2$. This result confirms that the strongest stacked complex is formed in the vicinity of G-C rather than of A-T; in other words, we can say that V B1 affinity is greater for G-C than for A-T base pairs.

The intercalated complex

The stability constant K_{int} presented in Table 2 decreases with the violamycin concentration increasing. This finding agrees with Löber[16], who worked also with V B1 but in a smaller concentration than 1.44×10^{-5} M, and calculated a stability constant higher than the value of 6.30×10^6 M^{-1} obtained in this study.

At low V B1 concentrations (higher values of p) the intercalated complex formation is favoured (K_{int} has higher values), whereas at high initial V B1 concentration (lower p), it forms preferentially the stacked complex. This behaviour shows that at each violamycin concentration an equilibrium between the two complexes is established, shifted towards the one or the other depending on the initial V B1 concentration.

The n values in Table 2 varies between 2 and 3 which indicates that violamycin may intercalate only after 2-3 base pairs, not immediately in its vicinity according to the nearest neighbour exclusion model. Because during the titration the quenching of fluorescence was noticed, due to the formation of the two complexes, we considered interesting to prove if Stern Volmer (equation (6)) is obeyed. In Fig. 5 we plotted this equation.

$$I_0/I = 1 + k_{SV}\,[c_{DNA}] \quad (6)$$

One observes again the two distinct linear segments of the stacked and intercalated complex formation. Then, the curve reaches a plateau, because the free violamycin was completely consumed and there is no emissive species in the solution.

From the segment II which refers to the intercalated complex we calculated the Stern Volmer constant k_{SV}. Because we determined experimentally also the mean life time of violamycin in its singlet excited state as 4.1×10^{-9} s, we were able to calculate the quenching rate constant $k_q = k_{SV}/\tau_{V\,B1}$ presented in Table 3.

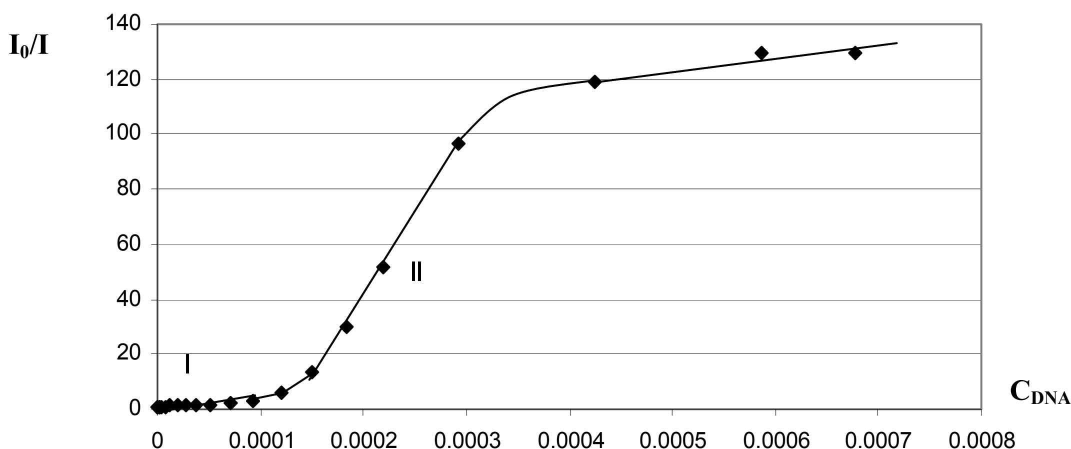


Fig. 5 – Stern Volmer equation plotting.

Table 3

The binding parameters of the intercalated complex

c^0_{VB} x 10^5, M	k_{SV} x 10^{-5}, M^{-1}	k_q x 10^{-14}, $M^{-1}s^{-1}$
1.44	9.00	2.19
3.33	5.88	1.43
5.35	4.92	1.20
7.87	2.18	0.53

It is interesting to remark that the Stern Volmer constant is of the same order of magnitude as the intercalation equilibrium constant K_{int}.

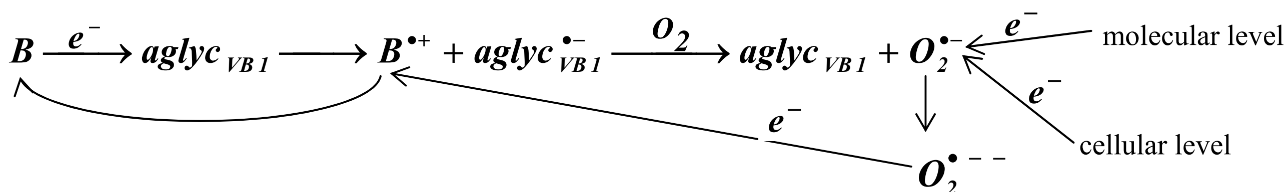
From the Table 3 one notices that k_q decreases with V B1 concentration increasing. Why? Two papers of Löber et al[16,17] inspired us to find the answer at this question. They studied the fluorescence quenching of some dyes and violamycin too, with G and A bases and assigned it to the electron transfer from the bases to the dye in its excited singlet state. They consider that the same mechanism may be valid also in the interaction of dyes with DNA. Their argument was the oxidation potentials of the bases, smaller than the reduction potential of the dyes, but they do not calculate any rate constant. Indeed, the series of the base oxidation potential is[17]:

$$E_G^{ox} < E_T^{ox} < E_A^{ox} < E_C^{ox} \quad \text{and} \quad E_{VB1}^{red} > E_C$$

Taking into account this series, we propose the following mechanism: concomitantly with V B1 aglycon intercalation, the electron transfer from the bases to violamycin takes place and the rate constant k_q is a measure of this fast process. The order of magnitude of 10^{14} $M^{-1}s^{-1}$ of k_q suggests the presence of a very fast process, such as the electron transfer one. Now it is easy to understand why the quenching rate k_q decreases with the violamycin concentration increasing: **a)** at low V B1 concentration the biopolymer presents many free sites so that violamycin may choose to intercalate between G-C pairs for which it has the highest affinity.[12] In this case also the potential difference between the donor-acceptor couple is the highest, so that also k_q has the highest value; **b)** at high V B1 concentration, G-C pairs are occupied so that violamycin has to intercalate between the other base pairs, but as the donor-acceptor potential difference is smaller, the electron is transferred with a lower rate constant k_q.

Although the stability constant of the intercalated complex K_{int} is high enough to be used as antitumoral drugs, it was rejected from the clinical therapy due to its cardiotoxicity. What is the cause of this cardiotoxicity?

We propose the following reaction scheme to explain it, where B is the DNA base:



The base B transfers the electron to V B1 aglycone when it forms the cation radical $B^{\bullet +}$ and the anion one $aglyc_{VB1}^{\bullet -}$. This last radical is a very unstable species, transferring the accepted electron to the surrounding O_2 molecules with the appearance of the superoxide $O_2^{\bullet -}$, a known oxidative species. It oxidizes many substances at molecular and cellular level by forming $O_2^{\bullet --}$ radical. This species reduces $B^{\bullet +}$ radical which re-enter in the above sequence. In turn, the oxidated substances at molecular and cellular level are responsible for the cardiotoxicity of the antracycline antibiotics.

EXPERIMENTAL MATERIALS AND METHODS

Violamycin IMET-JA 6844 was prepared for the first time from *Streptomyces violaceus*[18] and offered us by Dr. D. G. Strauss. V BI solutions were prepared in a phosphate-EDTA buffer pH 7 and kept in the dark before measurements to avoid

its photobleaching. Its concentration was determined by measuring the absorbance at λ=500nm, the molar absorption coefficient being known[19] to be ε_{500}=10250 $M^{-1}cm^{-1}$.

DNA calf thymus from SIGMA was used as sodium salts. Their solutions were prepared in the same buffer as V BI and the concentrations determined by measuring the absorbance at: λ=258 nm (ε=6600 $M^{-1}cm^{-1}$)[20].

The absorption spectra were measured at a Unicam α Helios spectrophotometer and the fluorescence spectra at an Aminco Bowman spectrofluorimeter. For the determination of binding parameters we carried out the titration of V BI with DNA into the spectral cell. The addition of the DNA into V BI solution in the measure cell was accompanied each time by the addition of an equal volume of acid in the reference cell to prevent matrix differences between the two cells. The experiments were carried out at 25^0C.

The pH was controlled using a Radiometer pH-meter.

CONCLUSIONS

We showed that V B1 interacts with DNA in two modes: **1)** by non specific interactions (electrostatic attraction, cooperativity and hydrophobic forces) forming stacked aggregates outside of the helix, due to its glycosidic moieties with positive charges on nitrogen, characterized by the formation equilibrium constant K_{st} and **2)** by specific interactions, when the aglycone intercalates between the base pairs inner side of the helix forming the intercalated complex, characterized by the stability constant K_{int}.

We put into evidence the fluorescence quenching of V B1 which we assigned to the electron transfer from the bases to the intercalated aglycone with the appereance of $O_2^{\bullet -}$ species. This oxidative radical acts at molecular and cellular level being responsible for the cardiotoxicity of the drug.

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