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Interaction of 5-iminodaunorubicin with Fe(III) and with cardiolipin-containing vesicles

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5-Iminodaunorubicin is an anthracycline derivative exhibiting promising antitumor activity. Using potentiometric and spectroscopic measurements we have shown that 5-iminodaunorubicin forms with Fe(III) a complex in which three molecules of drug are bound to one Fe(III) ion. Each molecule is chelated through the C-12-carbonyl and the C-11-phenolate oxygen atoms. The stability constant is $1.6 \cdot 10^{34}$. Using circular dichroism measurements we have studied the interactions of 5-iminodaunorubicin with cardiolipin-containing vesicles. We have shown that cardiolipin could bind one molecule of drug without penetration of the dihydroanthraquinone moiety into the bilayer.

Introduction

The anthracyclines adriamycin and daunorubicin are antineoplastic antibiotics with substantial therapeutic activity against a wide variety of human malignancies [1]. Unfortunately, these agents produce cardiotoxic side-effects that are dose-dependent and cumulative. Thus the hope of finding a non-cardiotoxic but yet active anthracycline antibiotic has prompted the search for new naturally occurring anthracyclines and the development of a large number of semisynthetic analogues. Thus several hundred analogues of adriamycin and daunorubicin have been synthesized for evaluation of antitumor properties [2].

Several studies point to the quinone unit as a key site of biological action that might involve the redox properties of the quinone function [3–5] and it has been shown that the modification of the number and of the position of the hydroxyl groups on the aglycone moiety appear to greatly modify the redox chemistry of these compounds [6]. This is also true when the anthracycline is modified at the quinone but, surprisingly, 5-iminodaunorubicin (Scheme I) is the only anthracycline thus far to be modified at the quinone [7]. Thus, 5-iminodaunorubicin is relatively inactive, i.e. it cannot be reduced by a component of mitochondrial NADH dehydrogenase [8]. 5-Iminodaunorubicin exhibits promising antitumor activity [9,10] and shows significantly diminished cardiotoxic potency [11,12].

We have recently shown that complexation of anthracycline with metal ions appears to be one possible route to obtain new derivatives modified at the quinone and hydroxyl group [13,14]. The

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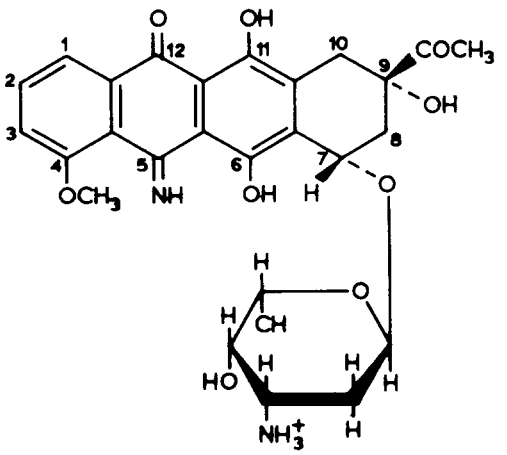
Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.



Scheme I. Iminodaunorubicin.

redox properties of these complexes are modified with regard to those of the drugs; for instance, these complexes, unlike the free drugs, do not catalyze the flow of electrons from NADH to molecular oxygen through NADH dehydrogenase. It was thus interesting to determine whether an anthracycline such as 5-iminodaunorubicin, which is modified at the quinone, is still able to bind Fe(III), and which kind complex is formed.

On the other hand, many reports suggest that adriamycin cardiotoxicity is related to disturbances of the biological functions of the inner mitochondrial membrane [15,16] possibly resulting from a specific adriamycin-cardiolipin binding [17,18]. We have thus studied the interaction of 5-iminodaunorubicin with cardiolipin-containing large and small unilamellar vesicles (LUV and SUV). These data were compared with those previously obtained with adriamycin [19].

Materials and Methods

Purified 5-iminodaunorubicin was kindly provided by Laboratoire Rhône Poulenc. Since anthracycline solutions are sensitive to light and oxygen, stock solutions were prepared just before use. The molar absorption coefficient was determined by dissolving a weighed amount of 5-iminodaunorubicin in water and diluting the solutions thus obtained to about 10 μM. Standard Fe(II) and Fe(III) solutions were prepared from reagent-grade $FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$ and $Fe(SO_4)_2 \cdot NH_4 \cdot 12H_2O$, respectively. L-$\alpha$-Phosphatidylcholine from egg yolk, type V-E, and cardiolipin from bovine heart were purchased from Sigma Chemical Co. All other reagents were of the highest quality available and deionized bi-distilled water was used throughout the experiments. Unless otherwise stated, buffer solutions were 0.01 M Hepes, 0.1 M KCl (pH 7.2).

Absorption spectra were recorded on a Cary 219 spectrophotometer and circular dichroism (CD) spectra on a Jobin Yvon Mark V dichrograph. Results are expressed in terms of ϵ (molar absorption coefficient) and $\Delta\epsilon = \epsilon_L - \epsilon_R$ (molar CD coefficient). The values of ϵ and $\Delta\epsilon$ are expressed in terms of [IMD], the molar concentration of 5-iminodaunorubicin.

Unilamellar phospholipid vesicles. SUV were prepared according to Newman and Huang [20]. Sonication was performed above the transition temperature of the phospholipids and under nitrogen. LUV were obtained by the phase-reversion method [21].

Results and Discussion

Spectroscopic titration of 5-iminodaunorubicin

Potentiometric and spectroscopic titrations of adriamycin and daunorubicin are now well-documented [14,22–24]; however, to our knowledge, these titrations have not been reported for iminodaunorubicin. In a first set of experiments we thus performed an investigation of the behavior of iminodaunorubicin as a function of pH. It is well-known that anthracyclines tend to form aggregates influencing spectroscopic data [25,26]; therefore the spectroscopic titration of iminodaunorubicin was performed at a low enough concentration to avoid self-association (i.e., about 10 μM). At physiological pH an aqueous solution of iminodaunorubicin is violet-colored. From acid pH value up to about 7 its absorption spectrum does not depend on pH; it exhibits, in the visible region, maxima at 550 and 585 nm and shoulders at 510 and 380 nm (Fig. 1). For comparison, the absorption spectra of 10 μM adriamycin at pH 7 and 12 are shown in the same figure. As can be seen, adriamycin at pH 12 and iminodaunorubicin at pH 7 exhibit exactly the same spectral pattern. It is well-documented that the shift of the visible

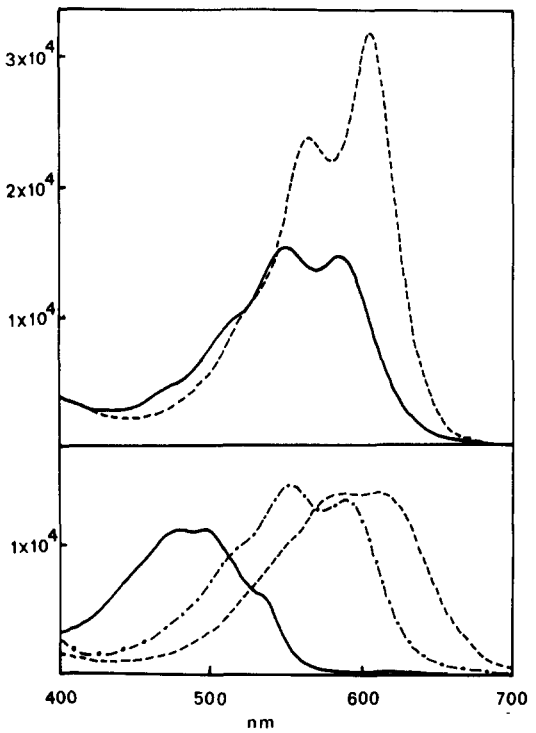


Fig. 1. Absorption spectrum of 5-iminodaunorubicin (upper) and adriamycin (lower) in aqueous solution at different pH values. ([iminodaunorubicin] = 8 μM, [adriamycin] = 8μM): pH 7.6 (————), 12.4 (– · –), 14 (- - - - - -). For more detailed experimental conditions, see the text.

absorption band to higher wavelengths, when the pH increased, is due to phenolic deprotonation; in the case of adriamycin one of the two hydroxyl groups on C-6 and C-12 is fully deprotonated at pH 12 [14,22]. Given the strong resemblance between the spectrum of adriamycin at pH 12 and that of iminodaunorubicin at acid pH value it appears reasonable to assume that one of the two hydroxyl group of iminodaunorubicin is deprotonated and one may suggest that the proton on the C-6-hydroxyl group lies in fact on the C-5-imino group.

At more basic pH, iminodaunorubicin is very unstable. This is shown by the decrease of absorbance as time elapses. In order to obtain reliable results, the pH of the solution was quickly raised from about 6 to the desired value, and the spectrum was recorded within 2 min. A new solution was used for each spectrum. An increase of the pH of a solution of iminodaunorubicin up to 12.5 gives rise to a shift of the absorption band to higher wavelengths (Fig. 1). The color of the solutions changes to blue and the absorption spectrum exhibits maxima at 605 and 564 nm. When the pH is increased from 12.5 to 14, no further modification is noticeable. Here again, the absorption spectrum of iminodaunorubicin at pH 12.5 (or higher) compares with that of adriamycin at pH 14 when adriamycin is deprotonated at both C-11- and C-6-hydroxyl groups [14,22]. We have used absorptivity at 600 nm to monitor the deprotonation of the C-11-hydroxyl group: the plot of ϵ at 600 nm as a function of pH gave a pK value of 11.1.

Circular dichroism of iminodaunorubicin as a function of concentration

As for others anthracyclines, the association state of iminodaunorubicin is highly dependent on the concentration. This can be checked using CD spectroscopy, and Fig. 2 shows the CD spectra of iminodaunorubicin in Hepes buffer (pH 7.2) at different concentrations. At 10^{-5} M, it is fully in the monomoeric form and the visible CD spectrum consists of one positive band at 535 nm. When the concentration is incrased, this band splits into a doublet characteristic of the associated dimeric form, with a positive band at 520 nm and two negative ones at 580 and 620 nm. An isodichroic

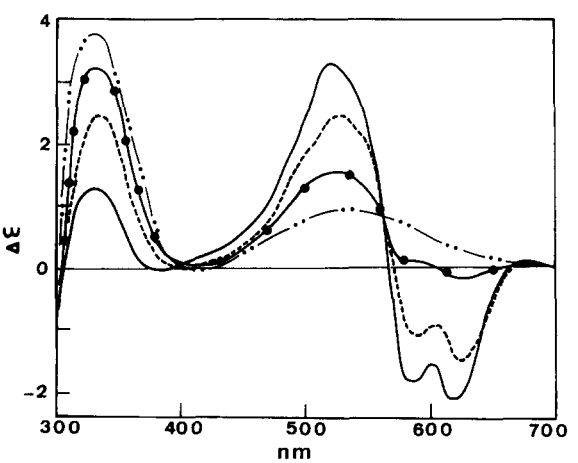


Fig. 2. Circular dichroism spectrum of 5-iminodaunorubicin at different concentrations (0.01 M Hepes, 0.2 M KCl, pH 7.2); [iminodaunorubicin] = $2.5 \cdot 10^{-3}$ (————), $3 \cdot 10^{-4}$ (- - - - - -), $5 \cdot 10^{-5}$ (●————●), 10^{-5} (– · · –) M.

point is seen at 560 nm indicating two species. The amplitude of the negative bands is indicative of the number of molecules in the associated state. Assuming a simple dimerization model and using the variation of $\Delta\epsilon$ at 580 nm as a function of concentration, a constant of dimerization was calculated for 0.1 M ionic strength. A value $K_d = (1.0 \pm 0.2) \cdot 10^4$ was obtained.

Fe(III)-iminodaunorubicin complex: spectroscopic and potentiometric titrations

The addition of Fe(II) to an aqueous iminodaunorubicin solution at pH 7.2 (0.1 M Hepes buffer) in the presence of oxygen yields a brownvilet complex. The CD spectrum exhibits positive bands at 350 and 650 nm and a shoulder at 580 nm. The visible absorption spectrum is characterized by the appearance of shoulder at 600 nm and the decrease of the absorption band at 550 nm. Two isosbestic points are at 600 and 495 nm. Increasing quantities of Fe(II) were added to iminodaunorubicin solution, at molar ratios of Fe to iminodaunorubicin varying from 0 : 1 to 3 : 1. The values of the $\Delta\epsilon$ and ϵ of the bands characteristic of the complex increase as the molar ratio of Fe(II) to iminodaunorubicin increases, reaching a maximum at [Fe]/[iminodaunorubicin] equal to 1 : 3. Thus an iron complex is formed in which one metal is bound to three iminodaunorubicin molecules. Analogous results are obtained when Fe(III) is added instead of Fe(II).

In order to accurately characterize the above species potentiometric and spectroscopic titrations of the Fe(III)-iminodaunorubicin complexes at molar ratios of 1 : 3 were performed in $1 \cdot 10^{-3}$ M antibiotic solution in the presence of KCl 0.05 M. The pH of the drug solution was first adjusted to 2 by the addition of HCl. We first checked that even at pH 2 the hydrolysis of the imino group back to the quinone [9] is very slow and that during the time necessary to perform our experiments the concentration of daunorubicin which could be formed is negligible compared to that of iminodaunorubicin. Fe(III) was subsequently added, and the solution was very slowly titrated with 0.1 M NaOH. Fig. 3 shows the absorption and CD spectra and in Fig. 4 the variations of ϵ at 550 and 660 nm and $\Delta\epsilon$ at 660 nm have been plotted as a function of pH together with $\bar{n}$, the number of

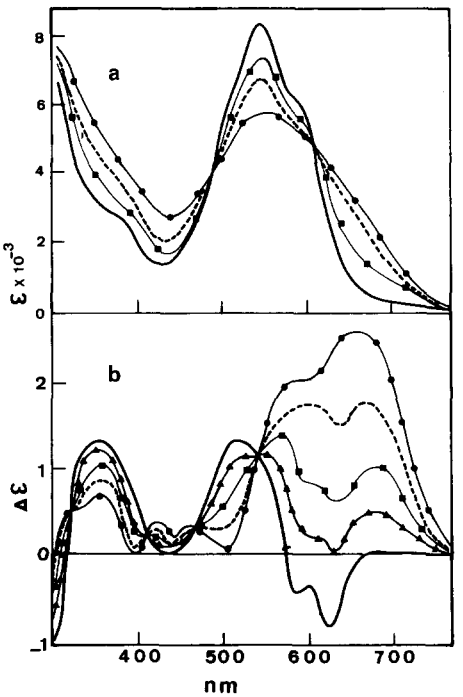


Fig. 3. Absorption (upper) and CD (lower) spectrum of the 1:3 Fe(III)-5-iminodaunorubicin system at different pH values ([iminodaunorubicin] = 10^{-3} M, [KCl] = 0.05 M); pH 2 (————), 2.3 (▲————▲), 2.6 (■————■), 3.2 (- - - - -), 3.6 (●————●).

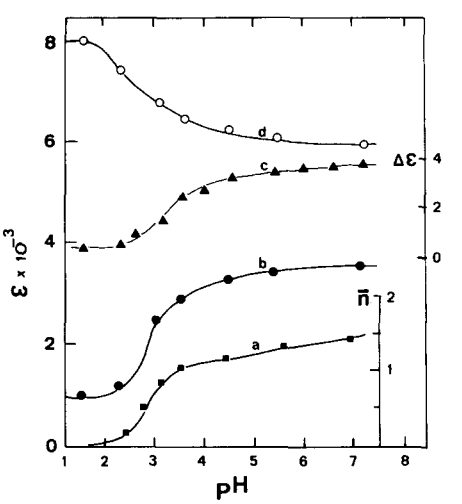


Fig. 4. Potentiometric and spectroscopic titrations of the 1:3 Fe(III) : 5-iminodaunorubicin system ([iminodaunorubicin] = 10^{-3} M, [KCl] = 0.05 M). Curve a: $\bar{n}$, the number of proton released per 5-iminodaunorubicin, as a function of pH. Curves b, c and d: ϵ at 660 nm, $\Delta\epsilon$ at 660 nm and ϵ at 550 nm as a function of pH, respectively.

protons released per iminodaunorubicin molecule. From our data we can state that the Fe(III)-iminodaunorubicin (1 : 3) complex is formed at pH 4 with the release of one proton per anthracycline and a pK equal to 3.0 ± 0.2.

All these data compare with those previously obtained for the Fe(adriamycin)$_3$ complex, i.e. the release of one proton per molecule of drug with a pK of 3 and the shift of the absorption band to higher wavelength [13]. This strongly suggests that complexation of iminodaunorubicin to Fe(III) occurs through the C-12-carbonyl and the C-11-phenolate oxygen atoms.

Magnetic susceptibility measurements give additional evidence for this type of complexation. The magnetic susceptibility of a lyophilized sample of Fe(iminodaunorubicin)$_3$ has been determined by the Faraday method at 292 K giving $\mu_{eff} = 4.8\ \mu_B$ per Fe(III). The data were correctd for the diamagnetism of all constituents. The Fe(iminodaunorubicin)$_3$ complex is thus essentially in the high-spin form. This result corroborates our previous assumption that iminodaunorubicin is chelated to Fe(III) through the oxygen atoms at C-11 and C-12 and that the nitrogen at C-5 is not involved in the coordination site.

It was then possible to calculate the stability constant of the complex. This was done using the potentiometric and spectroscopic titration data. The formation constants β_1 and β_1^* are defined by the following equilibrium:

$$Fe^{3+} + 3\ IMD \rightleftharpoons Fe(IMD)_3$$

$$Fe^{3+} + 3HIMD \rightleftharpoons Fe(IMD)_3 + 3\ H^+$$

where IMD is iminodaunorubicin and HIMD stands for 5-iminodaunorubicin with the hydroxyl group on C-11 protonated. We obtained $\beta_1^* = 8$

$$\beta_1 = \frac{\beta_1^*}{(K_1)^3}$$

while using for pK_1 the value of 11.1 previously determined one obtains $\beta_1 = (1.6 \pm 0.2) \cdot 10^{34}$.

A final experiment was performed to check that iminodaunorubicin was actually present in the iron complex and that a transformation to daunorubicin did not occur through reaction with Fe(III). EDTA in excess was added to the complex in solution. Fe(III) was thus slowly removed from its coordiantion site to iminodaunorubicin and at the end of the reaction the CD spectrum typical of free iminodaunorubicin was fully recovered.

Interaction of iminodaunorubicin with cardiolipin-containing vesicles

The interaction of anthracycline with membrane has been widely studied (for a review see Goormaghtigh and Ruysschaert [27]). Using pure cardiolipin monolayers [28] these authors have shown that cardiolipin could bind 2 mol adriamycin without penetration of the drug into the monolayer. On the other hand, Karczmar and Tritton [29] have shown that the presence of small amounts of negative lipids in a phosphatidylcholine matrix creates two types of binding environment for the drug, one relatively exposed and the other more deeply buried in the membrane. We have recently demonstrated [19] the presence of two different binding sites (I and II) for adriamycin on the negatively charged vesicles. In both, the amino sugar is bound to the ionized phosphate of either cardiolipin or phosphatidylcholine. In site I the dihydroanthraquinone moiety lies outside the bilayer, whereas in site II this moiety is embedded in the bilayer. As we have shown, CD spectra can be used to distinguish adriamycin bound to SUV or LUV either through type I or type II, since each of them exhibits a distinct CD pattern. It was interesting to determine whether these two binding sites were present in the case of iminodaunorubicin.

CD has thus been used to monitor the interaction of iminodaunorubicin with CL-containing SUV or LUV. Fig. 5 shows the CD spectra of 270 μM iminodaunorubicin in the presence of increasing amounts of cardiolipin-containing LUV. The LUV were prepared with phosphatidylcholine, cardiolipin and cholesterol in the molar ratio 7 : 1 : 2; but the results were independent of the presence of cholesterol. At the concentration of iminodaunorubicin used the percentage of dimer is 75%. When the molar ratio of cardiolipin to iminodaunorubicin was varied from 0 to 2.3, only one different spectral pattern was obtained. As can be seen in Fig. 5, when the molar ratio of cardiolipin to iminodaunorubicin was varied from 0 to about

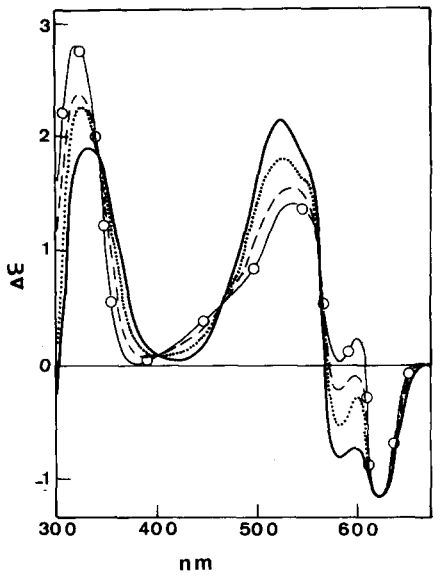


Fig. 5. Circular dichroism of 5-iminodaunorubicin in the presence of cardiolipin-containing LUV. ([iminodaunorubicin] = 2.7×10^{-4}, Hepes buffer pH 7.2, [KCl] = 0.1 M). The LUV were prepared with egg phosphatidylcholine, cardiolipin and cholesterol in the molar ratio 7:1:2. The molar ratio of cardiolipin to iminodaunorubicin was 0 (————); 0.5 (· · · · · ·); 1.3 (- - - - - -); 1.5–2.3 (○————○).

1 the CD spectrum changed from that typical of iminodaunorubicin in the dimeric form to another one which exhibited positive bands at 320 and 540 nm and a negative one at 620 nm. A further increase of the molar ratio of cardiolipin to iminodaunorubicin up to 2.3 did not give rise to further modification of the CD spectral pattern. Thus, at variance with what is observed in the case of adriamycin, only one spectral pattern was obtained when the molar ratio of cardiolipin to iminodaunorubicin was varied from 0 to 2.3. This strongly suggests the existence of only one type of binding site of iminodaunorubicin to cardiolipin-containing LUV. The variation of $\Delta\epsilon$ at 580 nm as a function of the cardiolipin-to-iminodaunorubicin molar ratio was used to monitor the fixation of iminodaunorubicin to this site. At this wavelength the values of $\Delta\epsilon$ are $+0.5$ and -1.5 for free iminodaunorubicin in the monomeric (M) and dimeric (D) forms, respectively. Thus, taking into account the equilibrium of dimerization of free iminodaunorubicin ($K_d = 1 \cdot 10^4$) and the data of Fig. 5 the concentrations of bound and free iminodaunorubicin (M plus D) as a function of the cardiolipin-to-iminodaunorubicin molar ratio were calculated. The binding data were analyzed by Scatchard plots. The slope gives the stability constant for the binding site $K = (5 \pm 2) \cdot 10^4$ and the intercept $n = 1.0 \pm 0.1$.

Similar results were obtained with cardiolipin-containing SUV, indicating that the surface curvature did not affect results.

Our data indicate that, at variance with what is observed in the case of adriamycin, only one type of binding occurs between iminodaunorubicin and the membrane of negatively charged vesicles. The observation that the CD band of iminodaunorubicin bound to this site lay at a wavelength which compares with that of free iminodaunorubicin suggested that the polarity is the same in both cases, i.e. that the dihydroanthraquinone moiety of iminodaunorubicin was not embedded in the phospholipid bilayer. We can thus suggest that the binding site for iminodaunorubicin to negatively charged vesicles compares with the binding site I in the case of adriamycin, i.e. an electrostatic interaction occurs between the negatively charged phosphate and the positively charged amino group of the sugar moiety. However, only one molecule of iminodaunorubicin binds per cardiolipin instead of two in the case of adriamycin. This is most probably due to the fact that iminodaunorubicin bears two positive charges, one at the amino group of the sugar moiety and the other at the imino group on C-6, which can interact with the two negatively charged phosphate group of a cardiolipin molecule.

Conclusion

We have shown that iminodaunorubicin forms with iron(III) a complex in which three molecules of drug are bound to one Fe(III) ion, each molecule being chelated through the C-12-carbonyl and the C-11-phenolate oxygen atoms. Thus, despite the presence of the C-6-imino group, iminodaunorubicin is able to form with Fe(III) the same kind of complex that adriamycin does. In addition, comparable stability constants have been determined for both systems.

Concerning the interaction of iminodaunorubicin with cardiolipin-containing vesicles we have

shown that cardiolipin could bind one molecule of iminodaunorubicin without penetration of the dihydroanthraquinone moiety into the bilayer. This point is important as it stresses that adriamycin and iminodaunorubicin have different behaviors towards cardiolipin-containing vesicles.

Acknowledgements

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