HEMIN *IN VITRO* INTERACTION WITH THE ANTICANCER DRUG DOXORUBICIN: ABSORPTION AND EMISSION MONITORING

Ana Maria TOADER,[a] Mihaela ȚONE[b] and Elena VOLANSCHI[b,*]

[a] "Ilie Murgulescu" Institute of Physical Chemistry of the Roumanian Academy, 202 Splaiul Independenței, 060021 Bucharest, Roumania
[b] Department of Physical Chemistry, University of Bucharest, 4-12 Elisabeta Bd., 030018 Bucharest, Roumania

The doxorubicin – hemin interaction was studied by absorption and emission spectroscopy. The absorption spectra outline two processes, in function of the concentration range of hemin. The fluorescence emission of doxorubicin shows a pronounced hypochromic effect in presence of hemin. The best fit was obtained using an (1:1) and (1:2) interaction for both methods. The doxorubicin – hematoporphyrin and doxorubicin – Fe^{III} systems were also investigated in similar experimental conditions, in order to outline the possible binding sites involved in the interaction. The quenching effect of hematoporphyrin is smaller than that of hemin, the binding parameters indicated an (1:1) interaction and are smaller than the corresponding values for hemin. For the doxorubicin – Fe^{III} system, the association constants for (1:1) and (1:2) complexes are in a reasonable agreement in both methods used. Our results are consistent with a two site binding model, where the Fe^{III} ions of hemin are involved to a higher extent than the planar porphyrin moiety in the hemin – doxorubicin interaction.
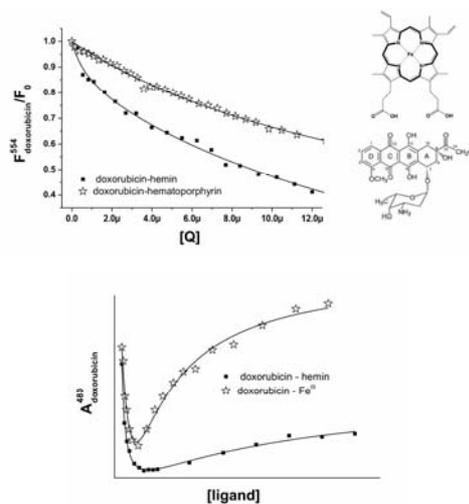
INTRODUCTION

Doxorubicin (DOX), an anthracycline drug (Fig. 1a), is a commercial and widely used antitumor agent that has substantial therapeutic activity against a broad variety of human cancers.[1-3] The clinical use of anthracycline antibiotics is limited due to important side effects, such as cardiotoxicity,[4,5] induction of multidrug resistance and cytotoxicity to normal tissues.[6,7] The enzymatic one electron reduction of anthracycline drugs, followed by electron transfer to molecular oxygen, known as the redox cycling of the drug, is responsible for the formation of reactive oxygen species (ROS) and for their cardiotoxicity.[8-11] The role of iron in DOX metabolism is essential, the iron (III) – DOX complex being reduced to an iron (II) complex and the semiquinone free radical which may reduce oxygen or hydrogen peroxide to the hydroxyl free radical.[12]

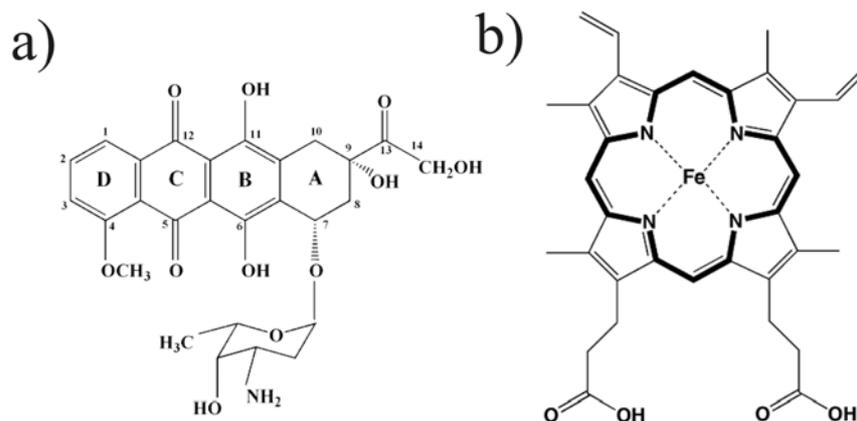* Corresponding author: elenavolanschi@gmail.com

Fig. 1 – The chemical structure of doxorubicin (a) and hemin (b).

Hemin – the oxidized state of heme (ferroprotoporphyrin IX) (Fig. 1b) – is a very active component in biologic media, being capable to perform multiple functions in free state or associated with proteins.[13,14]

It was discovered that hemin has clinical potential as result of its binding to DNA, producing fiber cleavage as anti-tumor agent in photodynamic therapy (PDT).[15] The hemin capacity to reduce the number of necrotic, apoptotic cells, from the infected cultures with Vvenv 1 virus[16] was also demonstrated.

In therapy of human leukemia, doxorubicin produce toxicity in K562 cells,[17] which affects the spinal cord. In this case, the hemin administration induced a selective decrease of toxicity by prevention of mitochondrial cytochrome *c* oxidase inhibition by Doxorubicin.[18] Moreover, these studies are showing that hemin could be a selective inhibitor for doxorubicin – inducer of apoptosis in human mioleucemia and in the same time conserve the cell structure of spinal cord for rabbits injected with doxorubicin.

The medical potential of hemin is also connected to the presence in certain diseases (hemolytic anemia) of unstable Hb tending to release hemin which, by interaction with the membrane proteins or intercalation into the membrane lipid bilayer may produce the premature red blood cell lysis.[19] Although the biological and potential clinical relevance of hemin interactions with peptides and different drugs[20-22] was outlined, the hemin – doxorubicin interaction was not investigated, after our knowledge.

In this context, the objective of this study was to characterize the *in-vitro* binding equilibrium of hemin to a widely used anti-tumoral drug doxorubicin, characterization based on the changes in the absorption and emission spectrum of doxorubicin, following the interaction with hemin. In order to outline the possible binding sites involved in the interaction, the related systems Doxorubicin–hematoporphyrin (same structure as hemin, not complexed with Fe^{III}) and Doxorubicin–Fe^{III} were also studied by means of fluorescence and spectrophotometric titration, in similar experimental conditions.

RESULTS AND DISCUSSION

1. Study of the Doxorubicin–hemin system

1.1. UV-VIS absorption spectroscopy

The doxorubicin – hemin interaction was followed by absorption spectroscopy in both doxorubicin and hemin absorption bands.

The absorption spectra of doxorubicin at pH = 7 in the absence and in the presence of different hemin concentrations are presented in Fig. 2a. The spectrum of doxorubicin in aqueous solution (spectrum 1 in Fig. 2a) was previously analysed by deconvolution[23] and presents an intense band centred at 480 – 500 nm and 535 nm (shoulder). The band at 480-500 nm was assigned to $\pi \rightarrow \pi^*$ transition,[24] with an intramolecular charge transfer character, due to the presence of >C=O and –OH groups in *C* and respectively *B* cycle (see Fig. 1a). The maxima at 480 nm and 500 nm are, most probably, vibrational components of HOMO – LUMO band, with a frequency difference $\Delta \nu = 831$ cm^{-1}. The dimerization equilibrium at pH = 7 was also investigated and the dimerization constant was determined to be 6.73×10^3 M^{-1}.[23]

On gradual addition of hemin to a doxorubicin solution, a hypochromic effect is observed on both bands at 480 nm and 500 nm (Fig. 2a), up to a P/D = 0.04 (c_{hemin} = 1.13x10^{-6} M). In addition, the isobestic points at 430 nm and 545 nm are possible indication of complex formation between hemin and doxorubicin. The plot of the absorbance at 480 nm in function of the hemin concentration is presented in Fig. 2b.

Two processes are observed, in function of the concentration range of hemin and Hemin/Doxorubicin molar ratio (P/D): for c_{hemin} < 1μM and P/D = 0 – 0.04, a decrease of absorbance is observed, followed by an increase at higher P/D ratios and hemin concentrations.

To obtain binding parameters, association constants, and stoichiometry from experimental absorption and fluorescence spectra, linear Scatchard[25] plots (equation (1)) are usually employed:

$$\frac{r}{C_F} = (n - r) \times K_S \qquad (1)$$

where: r = C_{Bound} / C_{Hemin}; C_F = C_{Drug} - C_{Bound}; n – the number of binding sites; K_S – the association constant.

In terms of absorption spectral data this equation becomes: [26]

$$\frac{\Delta A}{b*[L]} = \frac{K_S}{b} * \Delta A + S_t * K_S * \Delta \varepsilon_{11} \qquad (2)$$

where: b – the light path; ΔA = A - A_0 (A_0 and A are the absorbance in the absence and in the presence of hemin, respectively); S_t, [L] – the total concentration of drug, respectively ligand;

$\Delta \varepsilon_{11} = \varepsilon_{11} - \varepsilon_S - \varepsilon_L$ (ε_{11}, ε_S, ε_L – the molar absorption coefficients of the complex, substrate and ligand, respectively); K_S – the association constant for the (1:1) system.[26]

The negative slope of the linear Scatchard plot of this system is indication of an (1:1) interaction, with K_S = 1.40 x 10^7 M^{-1} (Table 1).

Considering a (1:1) stoichiometry of the binding process, the association constant K_{11} may be obtained by nonlinear fitting[27] using the following dependence of the absorbance in function of the total hemin concentration:

$$A = \frac{A_0 + \varepsilon_B c_D^0 K_{11} \times c_H^T}{1 + K_{11} \times c_H^T} \qquad (3)$$

where: A_0 and A are the absorbances in the absence, and in the presence of hemin, respectively, ε_b is the molar absorption coefficient of the bound drug, c_D^0 is the initial concentration of doxorubicin and c_H^T is the total hemin concentration.

Fitting with equation (3) the first process (Fig. 2b, insert) gives K_{11} = 1.04 x 10^7 M^{-1}. For small P/D ratios and hemin concentrations < 1 μM, *i.e.* in the range where hemin is predominant in monomer form (Dimerization constant of hemin[19] at pH ~ 7 is 3.92x 10^6 M^{-1}), close values are obtained by both methods for an (1:1) interaction (Table 1).

However, the data obtained for the hemin – doxorubicin system, for the whole range of P/D ratios (Fig. 2b) was better fitted with an (1:1) and (1:2) equation[28] (4):

$$A = \frac{A_0 + \Delta \varepsilon_1 c_D^0 * K_{11} * c_H^T + \Delta \varepsilon_2 c_D^0 * K_{11} * K_{12} * c_H^{T^2}}{1 + K_{11} * c_H^T + K_{11} * K_{12} * c_H^{T^2}} \qquad (4)$$

This result is consistent with the predominance of the dimeric form of hemin in aqueous solutions at pH = 7 and concentrations in the range 10^{-6} – 10^{-5} M.[19]

The interaction of hemin (c_{hemin} = 6.4x10^{-6} M) with doxorubicin at pH = 4.76 was followed also in the hemin absorption bands and the results are presented in Fig. 3 and Table 1.

The visible spectrum of hemin in absence of doxorubicin (spectrum 1 in Fig. 3a) presents the bands of the hemin monomer (*Soret* band) at 385 nm, the *Q* bands at 500 nm and 545 nm and

the charge transfer (*CT*) band at 635 nm. These bands indicated the presence of the diaqua-hemin species (Fe^{III}PP(H$_2$O)$_2$) in acid media.[29] In presence of increased concentrations of doxorubicin at constant concentration of hemin, a sharp hypochromic effect of the hemin *Soret* band and the same isobestic points at 430 nm and 545 nm to those in Fig. 2a are observed. The presence of these isobestic points was also reported[12] for the Adriamycin – Fe(III) complex and suggests the idea that the central ferric ion of hemin is involved in the interaction.
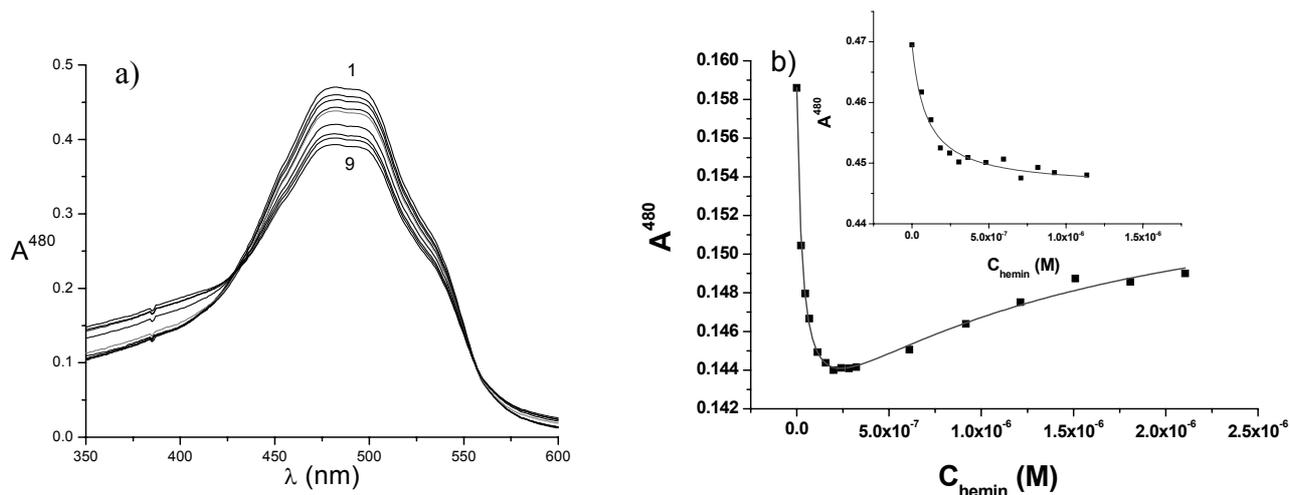
Fig. 2 – (a) Changes in the absorption spectra of doxorubicin upon titration with hemin, at constant doxorubicin concentration (c = 4x10^{-5} M, buffer solution pH = 7) at values of Hemin/Doxorubicin molar ratio, P/D: 0; 0.0015; 0.0031; 0.0061; 0.0092; 0.0214; 0.0306; 0.0337; 0.0398 (curves 1–9); (b) Binding curve of doxorubicin to hemin up to P/D = 0.2 (and up to P/D = 0.04 – *inset*); points represent experimental spectral data (A^{480nm}) and the solid curve represents the fit with eq. (3) and eq. (4).

Table 1

Binding parameters (association constants, stoichiometry, P/D range and pH)

for the doxorubicin–hemin interaction

		Absorption			Fluorescence				
pH	P/D range	K$_{11}$ or K$_S$* x10^{-7}/M^{-1}	K$_{12}$ x10^{-5}/ M^{-1}	Binding ratio	P/D range	K$_{SV}$ x10^{-5}/M^{-1}	K$_{11}$ or K$_s$* x10^{-7}/M^{-1}	K$_{12}$ x10^{-5}/ M^{-1}	Binding ratio
7	0-0.03	1.04±0.15 1.40±0.61*		1:1	0-35	1.15± 0.24	0.37±0.08*		1:1
7	0-0.4	3.26±0.28	7.11±0.21	1:1+1:2			0.14±0.03	0.40±0.06	1:1+1:2
4.76	0-4	0.08±0.01		1:1					

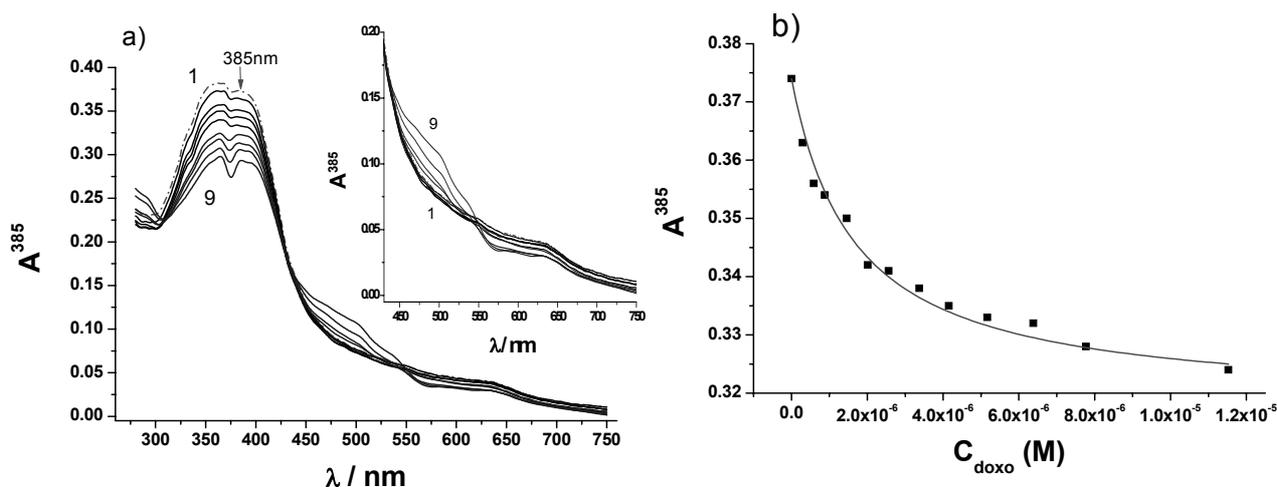* the binding constant calculated with the Scatchard model, K$_S$ (eq. (1), (2))

Fig. 3 – (a) Changes in the absorption spectra of hemin at pH=4.76 (c = 6.4x10^{-6} M) upon titration with doxorubicin, at values of P/D: 0; 0.046; 0.227; 0.314; 0.4; 0.996; 1.8; 2.228; 3.35; 4.03 (curves 1–9); (b) Binding curve of hemin to doxorubicin up to P/D = 1.8, points represent experimental spectral data (hemin monomer *Soret* band at 385 nm) and the solid curve represents the fit with eq. (3).

At pH values in the range 4–5, and μM range of concentration, both carboxyl groups of hemin are protonated[28] and doxorubicin has a positive charge due to the protonated glycoside ring nitrogen atom.[30] Therefore, due to electrostatic repulsion, a weaker drug – hemin interaction is to be expected in this case ($K_{11} = 0.8 \times 10^6 \, M^{-1}$, Table 1).

1.2. Fluorescence spectroscopy

The anthracycline anti-tumoral drug doxorubicin is an amphiphilic molecule that has a fluorescent 1, 4-hydroxyl-substituted antraquinone chromophore and a hydrophilic amino-glycosidic side chain.[31] This fact makes possible the study of her interaction with hemin by this technique.

The binding parameters for the Doxorubicin – hemin system from analysis of fluorescence data, including stoichiometry, association constants are also contained in Table 1.

The fluorescence measurements were performed at wavelength 554 nm (Λ excitation = 480 nm) on direct titration and the family of spectra is presented in Fig. 4a. The fluorescence titration shows a pronounced hypochromic effect on the band. The Stern – Volmer plot, according to equation (5) (Fig. 4a insert) is linear, attesting for the quenching of fluorescence by hemin, with $K_{SV} = 1.15 \times 10^5 \, M^{-1}$.

$$\frac{F_0}{F} = 1 + K_{SV}[Q] \qquad (5)$$

where: F_0 and F are the relative fluorescence intensities of the drug in the absence and presence of ligand, K_{SV} is the quenching constant, which is related to the bimolecular collision process and [Q] is the quencher concentration.[32]
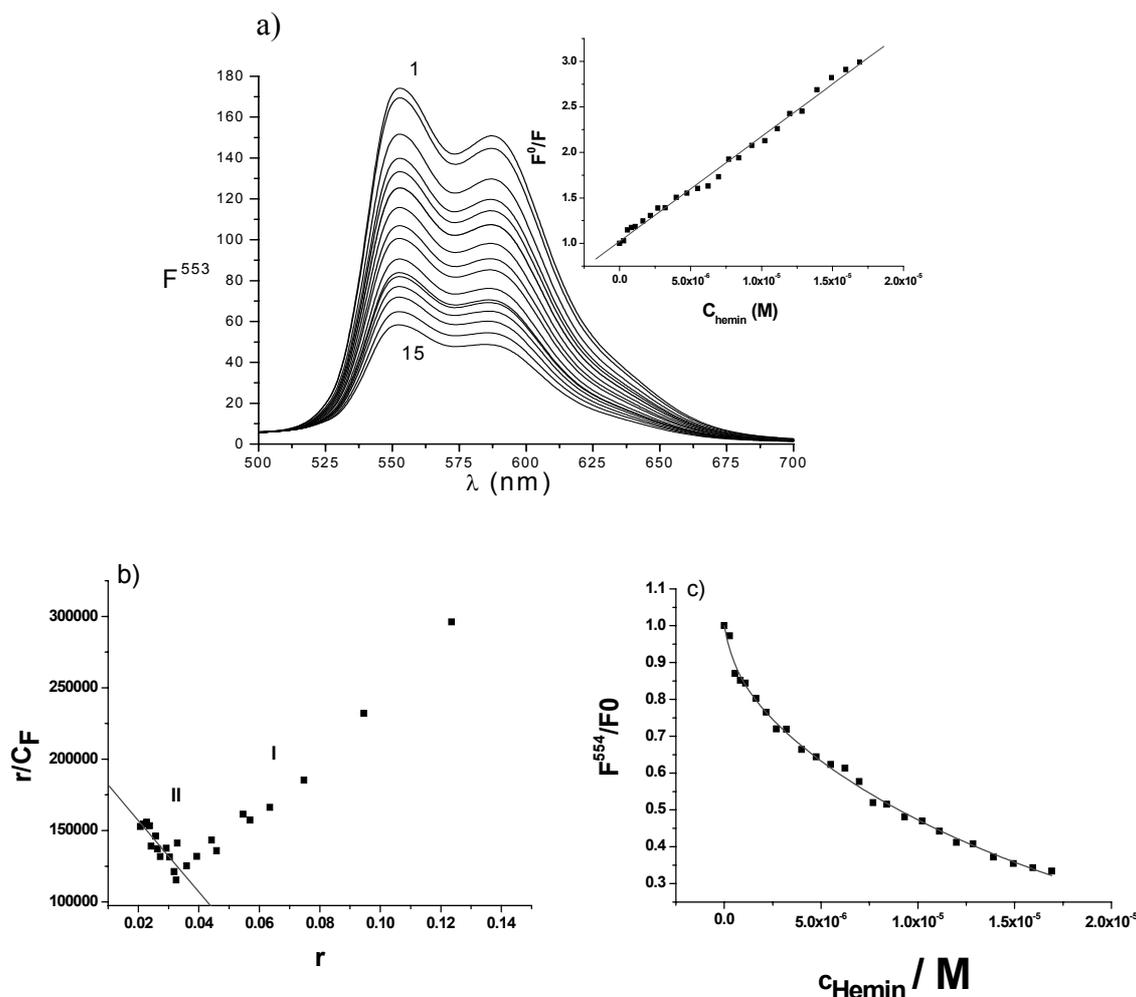

Fig. 4 – (a) – Changes in the emission spectra of doxorubicin upon titration with hemin, at values of P/D: 0; 0.57; 1.13; 3.37; 4.46; 5.52; 6.63; 11.34; 12.86; 15.84; 17.30; 19.21; 22.92; 24.73; 34.89 (curves 1–15), *inset* – Stern-Volmer fitting; (b) – Scatchard fitting; (c) – non-linear fitting with equation (4).

The lower concentrations of doxorubicin used in fluorescence experiments allow to obtain a larger P/D ratio range, 0–35, even for hemin concentrations up to $2 - 3 \times 10^{-5}$ M, *i.e.* in a range where the dimerization equilibrium of hemin is shifted toward dimers. The Scatchard plot outlines two processes: *i*) of positive slope attesting for a cooperative interaction and *ii*) of negative slope for small binding ratios, corresponding to an (1:1) interaction, and a $K_S = 0.37 \times 10^7$ M^{-1} (Table 1) *i.e.* in a reasonable agreement with the absorption results.

Moreover, analysis of fluorescence data according to the eq. (6),[33] gives n = 0.89, consistent with an (1:1) interaction.

$$\log\left[\frac{F_0 - F}{F}\right] = \log K + n \log[Q] \qquad (6)$$

where: *K* and *n* are the binding constant and the number of binding sites, respectively.

The plot of fluorescence versus hemin concentration over the whole P/D ratio range is presented in Fig. 4c. The best fit is obtained by

non-linear regression (eq. (4)), using a (1:1) and (1:2) binding model.

The binding constants, $K_{11} = 0.14 \times 10^7$ M^{-1} and $K_{12} = 0.4 \times 10^5$ M^{-1} are reasonably close to the values determined by absorption experiments, taking into account the different concentration range of the drug in both methods.

2. Study of doxorubicin–hematophyrin system

The binding of hematoporphyrin to doxorubicin has been evaluated also by means of fluorescence titration. The changes in the emission spectra occurring during titration of doxorubicin with hematoporphyrin are presented in Fig. 5a.

The hypochromic effect of hematoporphyrin, similar to that of hemin, is indication of the doxorubicin – hematoporphyrin interaction. The quenching of the doxorubicin fluorescence by hematoporphyrin is characterized by the binding parameters in Table 2.
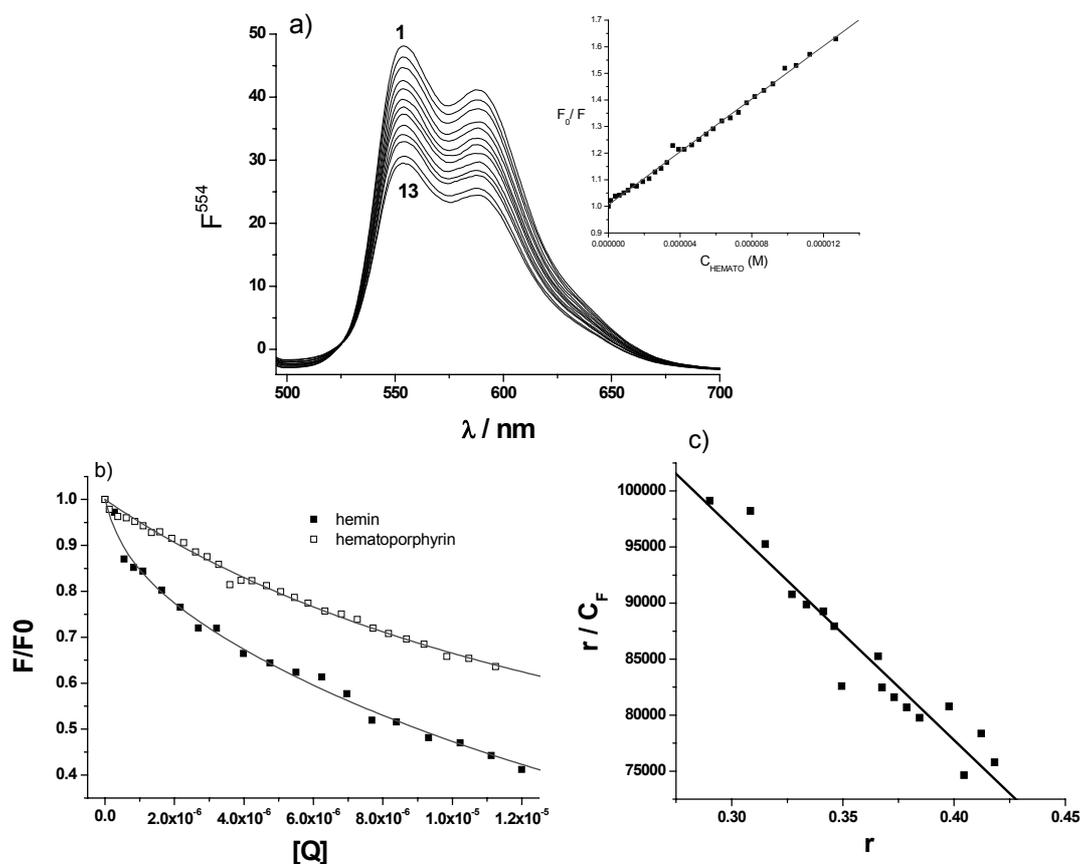

Fig. 5 – (a) – Changes in the emission spectra of doxorubicin ($c_{doxo} = 6.61 \times 10^{-6}$ M) at pH = 7 upon titration with hematoporphyrin, at values of P/D: 0 ÷ 1.92 (curves 1 ÷ 13) *inset* – Stern-Volmer plot; (b) – non-linear fitting for Doxorubicin – hematoporphyrin with eq. (3) and for Doxorubicin – hemin with eq. (4); (c) – Scatchard plot fitting with eq. (1), results in Table 2.

Table 2

Binding parameters (association constants, stoichiometry, P/D range and pH) for the doxorubicin – hematoporphyrin interaction

pH	P/D range	K_{SV} x10^{-5} / M^{-1}	K_{11} x10^{-5} / M^{-1}	K_S x10^{-5} / M^{-1}	Binding ratio
7	0-2	0.50±0.01	0.55±0.05	1.90±0.12	1:1

Comparison of the results in Table 2 and Table 1 allows for the following comments: *i)* the quenching effect of hematoporphyrin is smaller than that of hemin: for the same range of quencher concentrations (0 - 1.3 μM), the decrease of the relative fluorescence is about 60% for hemin and about 40% for hematoporphyrin; *ii)* the binding parameters of hematoporphyrin in Table 2, (Stern-Volmer constant, K_{SV}, binding constants calculated from the Scatchard plot, K_S, and non-linear fitting constant K_{11} for the (1:1) interaction) in the investigated P/D range, are smaller than the corresponding values for hemin (Table 1). This is indication that the drug – hematoporphyrin interaction is weaker than that with hemin.

As the single structural difference between these two ligands is the presence of Fe^{III} ions in hemin, this led us to investigate also, in our work conditions, the interaction of doxorubicin with Fe^{III} ions.

3. Study of the doxorubicin–Fe^{III} system

The interaction of doxorubicin with Fe ions is an important step in the drug metabolism, being one of the major causes of their toxicity by generating the semiquinone radical, capable of reducing molecular oxygen and hydrogen peroxide, leading to superoxide anion, hydroxyl radicals and other reactive intermediates (ROS).[33] Moreover, the complexes with metallic bivalent and trivalent metals of other structurally similar anticancer drugs[17,34] were proved to promote drug dimerization and the dimer complexes formed exhibit a higher cytotoxicity towards some cancer cell lines (K562) than the drug itself.

The UV-VIS absorption spectroscopy and fluorescence results are presented in Fig. 6 and Table 3.

The interaction of doxorubicin with Fe^{III} ions was previously investigated by Fiallo et al[17] from UV-VIS absorption and circular dichroism spectral data, in aqueous solution and alcohol – water mixtures. It was stated that, in aqueous solutions at pH ~ 7, although if Fe^{III} ions were generated *in situ* by oxidation of $(NH_4)_2Fe(SO_4)_2$ under aerobic conditions, no reliable results could be obtained because of Fe^{III} hydrolysis. Therefore both absorption and fluorescence experiments were performed at pH = 4.67.

The plot of the absorbance at 480 nm in function of the Fe^{III} / Doxorubicin ratio in Fig. 6 shows two processes: a decrease at small ratios, probably due to the known tendency to dimerization of this drug, reflected in a decrease of the apparent molar absorption coefficient[23] followed by an increase at higher Me/Ligand ratios. It was also shown that in other drugs structurally related to doxorubicin, chelation with metal ions promotes the dimerization of the drug.[34] The best fit was obtained with a (1:1) and (1:2) model, in agreement with literature data[17] which assign these two processes to the chelation of Fe^{III} ions at C=O...OH in site 11 and 6 respectively. The (1:1) complex, at the more accessible site 11 is favored at small Fe^{III}/ Drug ratio and low pH values, whereas (1:2) at high Fe^{III}/ Drug ratio.[17]

The lower drug concentration used in fluorescence experiments prevents the association of the drug at concentrations up to 10 μM [31] and allows a larger Fe^{III}/drug ratio range, and therefore the results obtained by this method are more reliable than those in absorption.

The fluorescence spectra at pH=4.67 in presence of different Fe^{III} ion concentrations in Fig. 6b attest for the enhancement of the doxorubicin fluorescence emission, assigned to the chelation of the metal ion at the C=O…OH sites. This phenomenon was observed for all 1,4-OH substituted anthraquinone derivatives with trivalent metal ions.[35] The change of slope in Fig. 6c corresponding to a (1:1) Fe/Doxo ratio is strong evidence for the complex formation. The association constants for (1:1) and (1:2) complexes are in a reasonable agreement in both methods used.
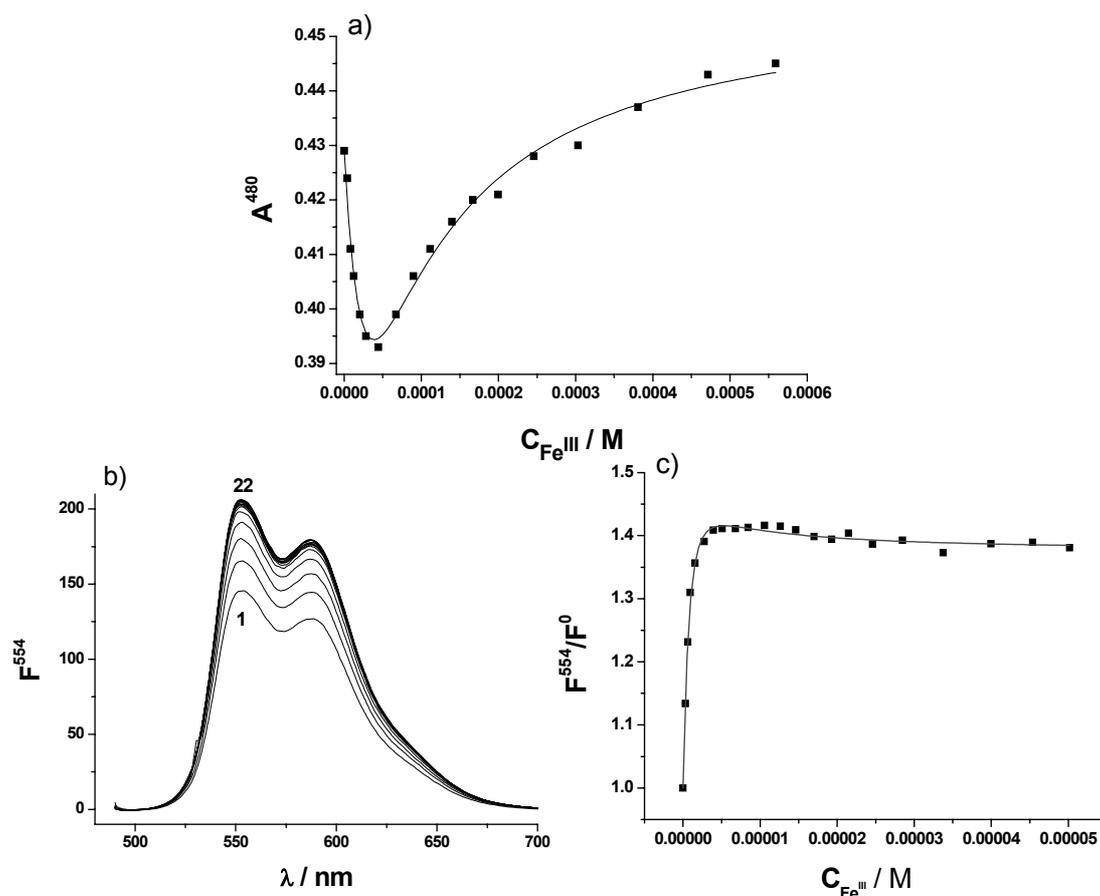
Fig. 6 – (a) – Binding curve for the UV-VIS absorption spectra of doxorubicin (c_{doxo} =3.73x10^{-5} M) at pH = 4.67 to Fe^{III} solution up to Fe^{III}/Doxorubicin = 10.2, points represent experimental spectral data and the solid curve represents the fit with eq. (4)); (b) – Changes in the emission spectra of doxorubicin (c_{doxo} =6.39x10^{-6} M) at pH = 4.67 upon titration with Fe^{III} solution at values of Fe^{III}/Doxorubicin up to 7.8 (curves 1–22); (c) – non-linear fitting with equation (4) for the emission spectra.

Table 3

Binding parameters (association constants, stoichiometry, P/D range and pH) for the doxorubicin-Fe^{III} interaction

	Absorption				Fluorescence			
pH	Me/ drug	K_{11} x10^{-5}/M^{-1}	K_{12} x10^{-5}/M^{-1}	Binding ratio	Me/ drug	K_{11} x10^{-6}/M^{-1}	K_{12} x10^{-5}/M^{-1}	Binding ratio
4.67	0-16	0.23±0.08	0.15±0.04	1:1+1:2	0-7.8	1.04±0.15	3.47±0.8	1:1+1:2

EXPERIMENTAL

Hemin chloride, doxorubicin and FeCl$_3$ were purchased from Sigma-Aldrich (Netherlands) and used without further purification. Dr. Carmen Diaconu from the Institute of Virology "St. Nicolau" of the Romanian Academy generously provided hematoporphyrin.

The stock solution of 10^{-4} M doxorubicin was prepared by dissolving doxorubicin with a few µl alcohol and subsequent addition of phosphate buffer solution to obtain a pH ≈ 7 values and subsequent addition of acid acetic / acetate buffer for a solution with pH ≈ 4.7. Experiments were carried out with diluted doxorubicin solutions in range 6.6x10^{-6} ÷ 4.85x10^{-5} M. Experiments were done with 10^{-3} M – 10^{-2} M stock solution of hemin, hematoporphyrin and FeCl$_3$ directly dissolving in buffer solution at pH ≈ 7 or pH ≈ 4.7. The concentration of the solutions was determined spectrophotometrically by using molar absorption coefficients ε_{480} = 11500M^{-1}cm^{-1} for doxorubicin and ε_{385} = 58400M^{-1}cm^{-1} for hemin.

Absorption spectra were measured with an UNICAM – UV HELIOS spectrophotometric system. Spectral titrations were carried out at 20÷25°C at constant concentration of doxorubicin, respectively hemin solution and a progressive addition of small aliquots of reactant solution.

Fluorescence measurements were performed with JASCO FP – 6300 spectrofluorimetric system and with Spectra Manager software. The fluorescence of doxorubicin was observed by exciting at 480 nm. Emission spectra were recorded in the range from 500 nm to 700 nm for each quencher addition. Emission and excitation bandwidth were

set at 5 nm. The solutions were maintained at constant mutagen concentration and were titrated with hemin / hematoporphyrin / FeCl₃ to obtain an increasing final ligand concentration from 10^{-7} to 10^{-5} M.

CONCLUSIONS

Comparing the binding constants of the three investigated systems, it may be inferred that the best fit for all three investigated systems over a larger P/D range is obtained with a (1:1 + 1:2) stoichiometry. The binding constants obtained by nonlinear fitting for K_{11} for the doxorubicin − hemin interaction, 0.14×10^7 M^{-1} (fluorescence) and 3.27×10^7 M^{-1} (absorption) (Table 1) are higher by about two orders of magnitude as against those obtained for the doxorubicin − hematoporphyrin system, 0.547×10^5 M^{-1} (Table 2), where only an (1:1) interaction gave the best fit of the experimental data. On the contrary, the interaction of doxorubicin with Fe^{III} ions outlines a better fit for the (1:1 + 1:2) model and gives $K_{11} = 0.104 \times 10^7$ M^{-1} and $K_{12} = 3.47 \times 10^5$ M^{-1} (Table 3), values close to those corresponding for the doxorubicin − hemin interaction. This observation seems to be consistent with a two site binding model, where the Fe^{III} ions of hemin are involved to a higher extent than the planar porphyrin moiety in the hemin − doxorubicin interaction. However, no further mechanistic suggestions can be given at the moment, theoretical modelling is necessary to understand at a molecular level the anthracycline drugs − hemin interaction, and is in progress in our laboratory.

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