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Interaction of rifampicin and isoniazid with large unilamellar liposomes: spectroscopic location studies

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Abstract

The location of isoniazid and rifampicin, two tuberculostatics commonly used for the treatment of *Mycobacterium tuberculosis* and *Mycobacterium avium* complex infectious diseases, in bilayers of dimyristoyl-L-α-phosphatidylcholine (DMPC) and dimyristoyl-L-a-phosphatidylglycerol (DMPG) have been studied by ^{1}H NMR and fluorimetric methods. Steady-state fluorescence intensity and fluorescence energy transfer studies between rifampicin and a set of functionalized probes {*n*-(9-anthroyloxy)stearic acids, $n = 2, 12$} reveal that, in both systems, isoniazid is located at the membrane surface whereas rifampicin is deeply buried inside the lipid bilayers. Steady-state fluorescence anisotropy studies performed with the probes 1,6-diphenyl-1,3,5-hexatriene (DPH) and trimethylammonium-diphenylhexa-triene (TMA-DPH), not only corroborate the above results, but also show that no changes in membrane fluidity were detected in either liposome. The ^{1}H NMR results, in DMPC liposomes, confirm the location of rifampicin near the methylene group of the acyl chains of the lipid bilayers.

Keywords: Tuberculostatic; Liposome; Drug delivery; ^{1}H NMR; Fluorescence method

1. Introduction

Most of the available tuberculostatics have toxic side effects, inducing hepatoxixity; consequently, attempts are being made either to develop alternative drugs or to reduce toxicity of already existing drugs. The prospect of finding newer and more effective drugs similar to the existing ones is small; therefore, tuberculostatics are being used as a liposomal form for the therapeutic treatment of *Mycobacterium tuberculosis* and *Mycobacterium avium* complex infectious diseases [1,2].

Liposomes, since their discovery, have not only been recognized as useful models of biological membranes but also as a unique biocompatible vehicle for drug delivery [3,4]. However, liposomes suffer certain limitations, mostly of a technical nature, such as the lack of reproducibility of the preparations, the limited stability of the drug–carrier complex during storage and potential changes in the structural organization of the lipid bilayer induced by certain drug molecules [5]. Studies performed with liposomal forms of tuberculostatics show that the bilayer composition is critical for targeting liposomes and for obtaining drug–liposome stable formulations [1,2,5–7].

The understanding of drug–liposome interactions at molecular level is not an easy task because, depending on their hydrolipophilic characteristics, drugs can interact with either the polar headgroups, the hydrophobic hydrocarbon chains of the lipid bilayer constituents or both parts of the membrane and specific changes in liposomes morphology or drug conformation can occur [5,8,9]. Moreover, the targeting of drugs by lipid vesicles requires a complete understanding of the physicochemical characteristics of the drug–liposome system in order to predict their behavior and stability in vivo [5,10–12].

In this work the location of isoniazid and rifampicin in two types of lipid bilayers with different hydrophobic/electrostatic characteristics, those of dimyristoyl-L-α-phosphatidylcholine (DMPC) and dimyristoyl-L-α-phosphatidylglycerol (DMPG), has been studied by nuclear magnetic resonance (^{1}H NMR) and fluorescence spectroscopy.

The determination of the partition coefficients of isoniazid and rifampicin in DMPC and DMPG liposomes have

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been reported in previous work [13,14]. By combining the values of the partition values with the results of the location studies, it became possible to estimate the extent of interactions of isoniazid and rifampicin with the liposomes, and to stress the role that electrostatic/hydrophobic properties of liposomes play on the incorporation and location of drugs in liposomal formulations.

2. Materials and methods

2.1. Reagents and sample preparation

Rifampicin, isoniazid, praseodymium chloride, *N*-(2-hydroxyethyl)piperazine-*N*′-ethane-sulfonic acid (HEPES), 1,6-diphenyl-1,3,5-hexatriene (DPH) and trimethylammonium-diphenylhexa-triene (TMA-DPH) were from Sigma; L-α-dimyristoylphos-phatidylcholine (DMPC) and L-α-dimyristoylphosphatidylglycerol (DMPG) were from Avanti Polar Lipids; and all other chemicals from Merck (grade: pro analysis). The *n*-(9-anthroyloxy)stearic acids probes (*n*-AS) (*n* = 2 and 12) were obtained from Molecular Probes.

All lipid suspensions were prepared with aqueous 10 mM HEPES buffer solution (I = 0.1 M NaCl; pH 7.4) and extruded on a Lipex Biomembranes extruder attached to a circulating water bath.

2.2. Physical measurements

Absorption spectra were recorded with a UNICAM UV-300 spectrophotometer equipped with a constant-temperature cell holder. All spectra were recorded at 37 ± 0.1 °C in 1-cm cuvettes, using a slit width of 2 nm and a spectral window from 225 to 600 nm. Fluorescence measurements were carried out at 37 ± 0.1 °C in 1-cm cuvettes using a Perkin Elmer LS 50B equipped with a thermostated cell holder. ^{1}H NMR spectra were record at 37.0 ± 0.1 °C in a Bruker AC 200, spectrometer. Size distribution of extruded DMPC and DMPG liposomes, with and without added drug, were determined by quasi-elastic light scattering analysis using a Malvern Instruments Zeta Sizer 5000, and the mean particle sizes were found to be 103 ± 4 nm for DMPC and 98 ± 2 nm for DMPG.

Lipid concentration in vesicle suspensions was determined by phosphate analysis, using a modified version of the Fiske and Subbarow method [15].

2.3. Liposome and drug–liposome preparation

Liposomes were prepared by evaporation to dryness, with a stream of argon, of a lipid solution in chloroform (DMPC) or in chloroform/methanol (1:1) (DMPG). The films were left under vacuum overnight to remove all traces of the organic solvent. The resulting dried lipid films were dispersed with 10 mM HEPES buffer (0.1 M NaCl, pH 7.4) and the mixture was vortexed above the phase transition temperature (37 ± 0.1 °C) to produce multilamellar liposomes (MLVs). Frozen and thawed MLVs were obtained by repeating five times the following cycle: freezing the vesicles in liquid nitrogen and thawing the sample in a water bath at 37 ± 0.1 °C. Suspensions of MLVs were then equilibrated at 37 ± 0.1 °C for 30 min and extruded 10 times through polycarbonate filters (100 nm) to produce large unilamellar vesicles (LUVs).

Drug samples were prepared by mixing a known volume of drug and a suitable aliquot of vesicle suspension in HEPES, whereas the correspondent reference solutions were prepared identically, but without drug. All suspensions were then vortexed for 5 min and incubated at 37 ± 0.1 °C for 30 min, and typically two sets of 10 vials (1.5 ml) were used in each experiment.

2.4. ^{1}H NMR experiments

The dried lipid films, prepared as mentioned above, were dispersed with 3.5 ml of deuterium oxide (D_2O 99,8%). Stock solutions of rifampicin, isoniazid and praseodymium chloride (0.2 M) were also prepared in D_2O. Solutions were obtained by addition of different aliquots of each drugs or praseodymium chloride to DMPC suspensions (14 mM). All the spectra were record at 37.0 ± 0.1 °C and the HDO band was used as internal reference.

2.5. Location studies with 2-AS and 12-AS probes

LUVs prepared as above, the *n*-AS probes were added from a stock solution in ethanol and after an incubation time of 1 h, the solution was divided into aliquots to which different concentrations of drugs were added. The probe/phospholipid ratio was always 1:100 and the concentrations of ethanol was always less than 2% v/v, to ensure that no alteration in the bilayer structure takes place [16,17]. Fluorescence measurements were performed 2 h after addition of drugs. Excitation wavelength was set to 390 nm and the emission wavelengths were 452 and 446 nm, respectively, for 2-AS and 12-AS probes. The quantum yields of the 2-AS and 12-AS in DMPC and DMPG vesicles were taken as 0.15 and 0.55, respectively [17]. Isoniazid concentrations were in the range 0–1200 μM and those of rifampicin in the range 0–80 μM. Five different concentration of lipids were used for DMPC and DMPG (100, 250, 350, 500 and 700 μM) and for each lipid concentration at least two independent experiments were performed. For DMPG, experiments were also performed with the probes co-solubilised with the lipid (500 μM DMPG). DMPG was dissolved in chloroform/methanol (1:1) and mixed with stock solutions of 2-AS and 12-AS in the same solvent and the corresponding LUVs were prepared by the method described above. Rifampicin solutions, in the range 0–80 μM (in HEPES buffer), were then added to the LUVs.

As the drugs partition in liposomes, their effective concentration in the LUVs, $[Q]_m$, must be determined. The effective concentration is given by [18]:

$$[Q]_m = [Q]_t\left(1 - \frac{K_p\gamma_L L}{1 - \gamma_L L + K_p\gamma_L L}\right)\frac{K_p}{1 - \gamma_L L} \quad (1)$$

where $[Q]_T$ is total drug concentration, K_p the partition coefficient of the drug, γ_L the molar volume of the lipid and L the lipid concentration.

The fluorescence intensities observed in the presence of rifampicin had to be corrected, as this drug absorbs at the wavelengths used for excitation and emission. The effect of rifampicin absorption at the excitation wavelength was corrected using the following expression:

$$I_{corr} = I_{exp}\frac{A_T}{A_{ex}}\frac{(1 - 10^{-A_{ex}})}{(1 - 10^{-A_T})} \quad (2)$$

where I_{exp} is the experimental fluorescence intensity, A_{ex} is the absorbance of the acceptor (rifampicin) and A_T is the absorbance of the donor and acceptor solution (rifampicin and *n*-AS probes) [19]. The correction for the absorption of rifampicin at the emission wavelength (trivial process or reabsorption) was carried out by:

$$I_{corr} = I_{exp}\frac{2.303A_{em}}{1 - 10^{-A_{em}}} \quad (3)$$

where A_{em} is the absorption of rifampicin at the emission wavelength [20].

2.6. Steady-state anisotropy experiments

Lipids were dissolved in chloroform or chloroform/methanol (1:1) and mixed with stock solutions of DPH or TMA-DPH in the same solvent. The molar ratio DPH/lipid and TMA-DPH/lipid was 1:300 and LUVs were obtained as described before. Drug solutions (in HEPES buffer) were added to liposome suspensions to obtain a final concentration of 264 μM for isoniazid and 15 μM for rifampicin. Reference solutions were prepared identically but without drugs.

The anisotropy was recorded between 15 and 40 °C, with an accuracy of ± 0.1 °C. The excitation wavelength for DPH was set at 360 nm and the emission wavelength at 427 nm, whereas for TMA-DPH the corresponding values were 365 and 427 nm.

3. Results

3.1. ^{1}H NMR measurements

The ^{1}H-RMN spectra of DMPG did not exhibit any observable changes upon addition of isoniazid or rifampicin and, on the other hand, addition of praseodymium (III) led to precipitation of the negatively charged liposomes, thus precluding any ^{1}H-RMN solution study.

For DMPC systems, no changes in the ^{1}H-RMN spectra could also be observed after addition of drugs, but addition of Pr^{3+} causes the initial single peak of $N^+(CH_3)_3$ to split into resolved downfield and upfield components. The downfield signal comes from the extra-vesicular (E) and the upfield resonance from intra-vesicular (I) choline headgroups (Fig. 1) [21]. The displacements of the external and internal peaks and their splitting (Δppm), vary linearly with the Pr^{3+} concentration, as expected [22,23].

Addition of rifampicin and isoniazid to Pr^{3+}-bound liposomes causes shifts in the E and I peaks and Δppm decreases as drug concentration increases. A single peak is again obtained when the concentration of rifampicin reaches 210 μM, a situation that is also observed for isoniazid but only for concentrations higher than 290 mM. A slight downfield shift of the methylene group signal of the acyl chains of DMPC was also observed in the presence of rifampicin.

3.2. Location measurements with n-AS probes

Insights about location of isoniazid and rifampicin could be gathered from the quenching of the membrane soluble probes 2-AS and 12-AS. Rifampicin was found to induce quenching of the probe's fluorescence in both liposomes, whereas isoniazid does not affect the fluorescence of the probes.

As the K_p values of rifampicin are known, the effective concentrations of rifampicin in the two liposomes systems were determined and in Fig. 2 are depicted the fluorescence intensities of the liposomes suspension vs. quencher concentration (rifampicin), both for total rifampicin concentration (A) and for membrane concentration of rifampicin (B). These results show that the quenching is higher for the 12-AS probe in both systems.

As the emission spectra of the *n*-AS probes and the absorption spectrum of rifampicin overlap (Fig. 3), the quenching observed for both probes must be a consequence of a fluorescence resonance energy transfer (RET), between the *n*-AS excited donors and rifampicin. The rate of energy transfer depends upon the extent of spectral overlap, the quantum yield of the donor, the relative orientation of the donor and acceptor dipoles, and the distance between the donor and acceptor molecules [24].

To obtain useful structural information from fluorescence energy transfer data the theory developed by Föster for a two-dimension system was used. Briefly, the efficiency of energy transfer (E) is related to de rate of energy transfer, which is defined as:

$$k_T = (1/\tau_D)(R_0/R)^6 \quad (4)$$

The lifetime of the donor is τ_D, and R_0, the critical transfer radius (Föster radius), is the characteristic distance for the donor–acceptor pair, which corresponds to the radius at which energy transfer and spontaneous decay of excited

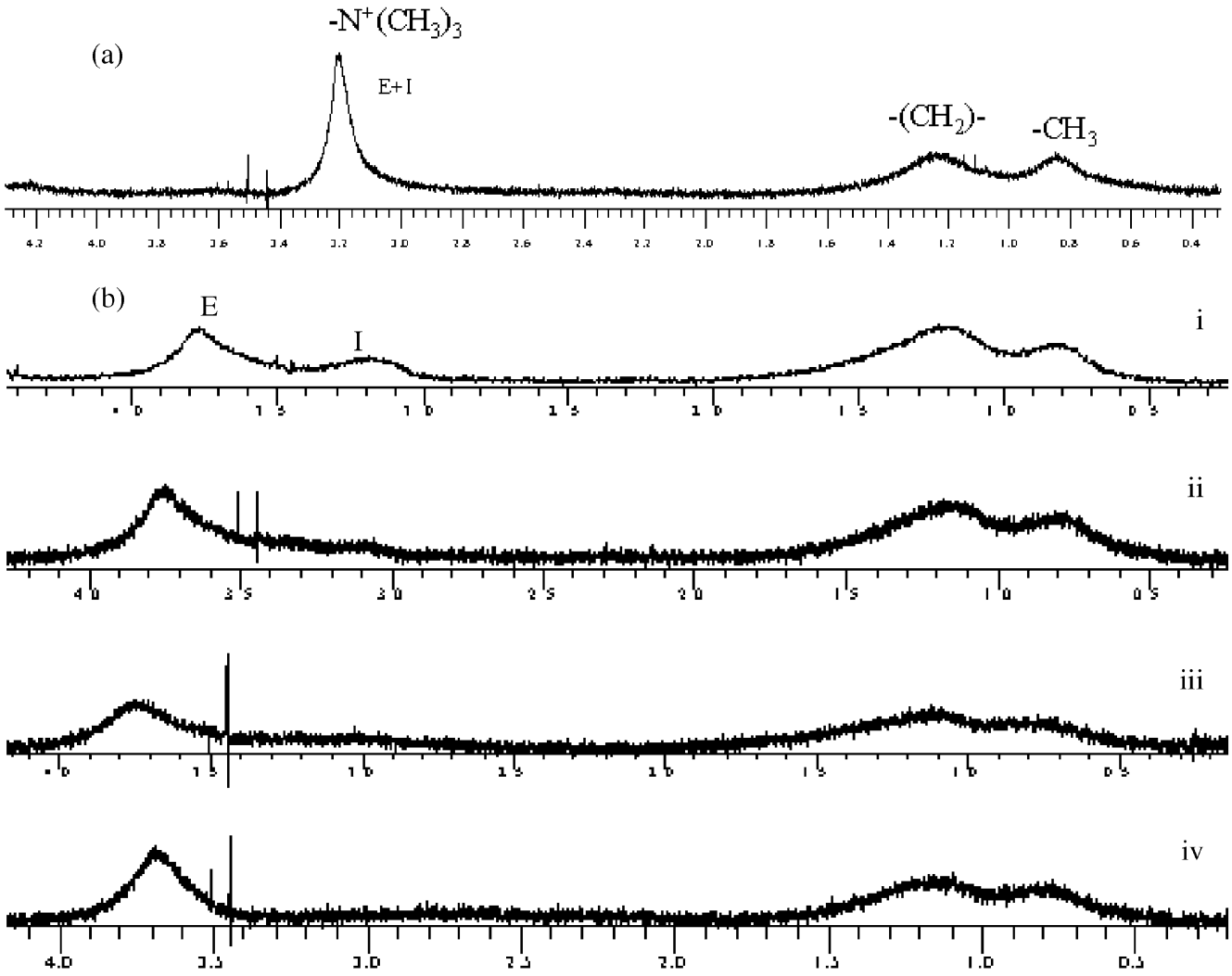

Fig. 1. ^{1}H-RMN spectra of (a) DMPC liposomes (14 mM); (b) DMPC liposomes (14 mM) and Pr^{3+} (10 mM) and rifampicin (i) 16 μM, (ii) 60 μM, (iii) 120 μM and (iv) 210 μM.

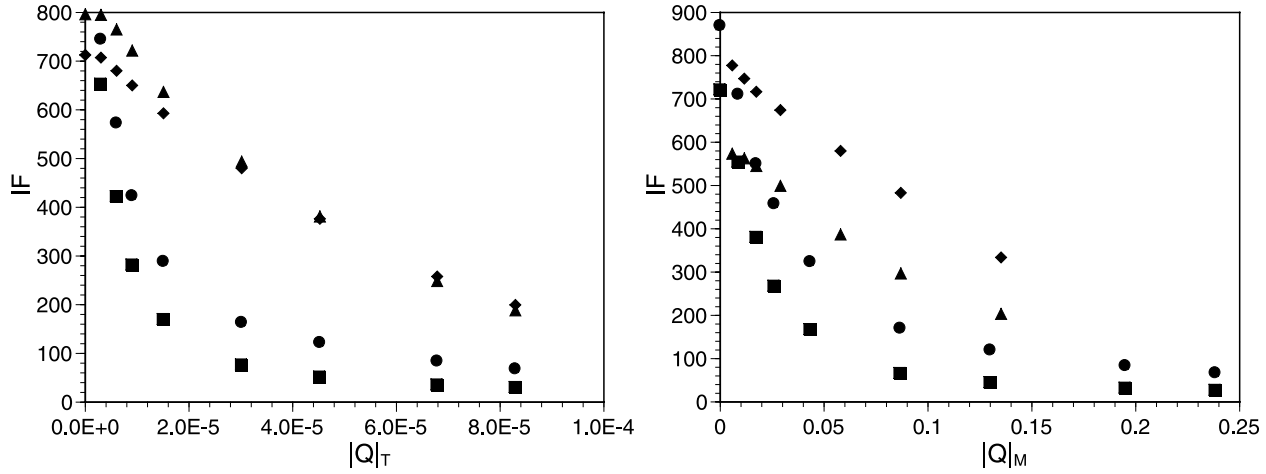

Fig. 2. Fluorescence intensities vs. quencher concentration (rifampicin) in DMPC and DMPG liposomes for total rifampicin concentration, $|Q|_T$ (A) and for the membrane concentration of rifampicin, $|Q|_M$ (B): (♦) 2-AS in DMPG; (▲) 12-AS in DMPG; (●) 2-AS in DMPG; and (■) 12-AS in DMPC.

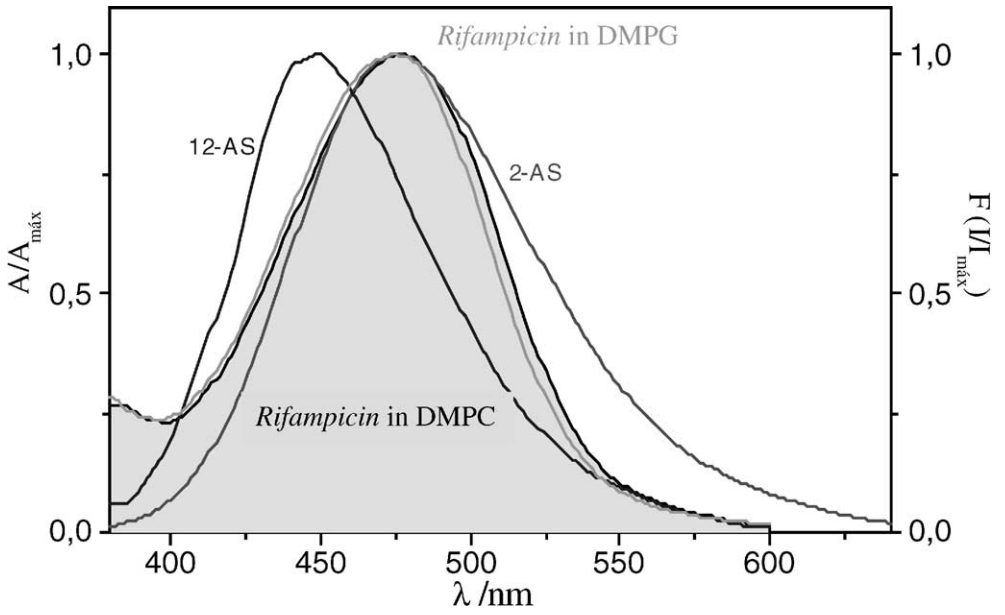

Fig. 3. Overlap between the emission spectra of the *n*-AS probes and the absorption spectrum of rifampicin.

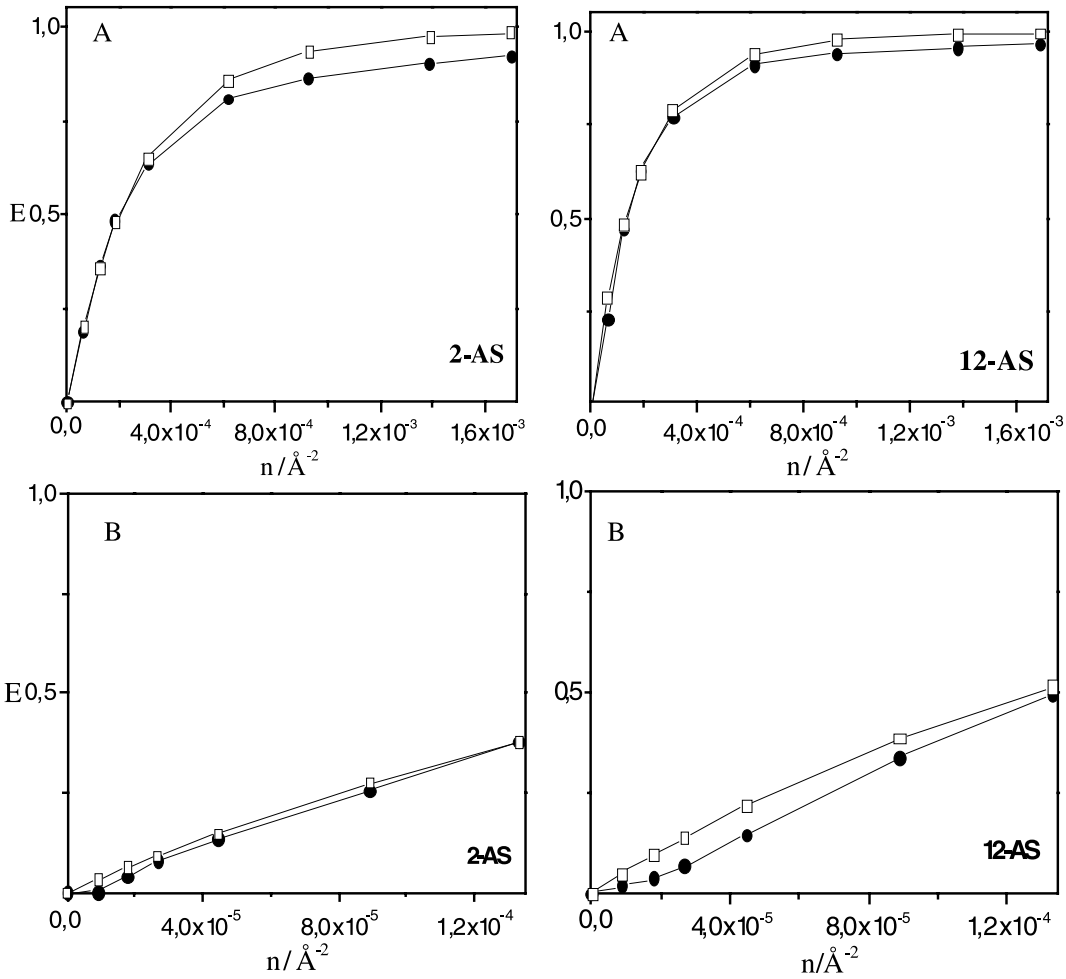

Fig. 4. (A) Theoretical energy transfer efficiency (□) and experimental (●) between 2-AS and 12-AS probes and rifampicin in DMPC; (B) theoretical energy transfer efficiency (□) and experimental (●) between 2-AS and 12-AS probes and rifampicin in DMPG.

state are equally probable. The values of R_0 (in nm) are given by

$$R_0 = 0.0108\left[k^2\phi_D n^{-4}\int_0^\infty I(\lambda)\varepsilon(\lambda)\lambda^4 d\lambda\right]^{1/6} \quad (5)$$

where k^2 is the orientation factor, ϕ_D the donor quantum yield in the absence of the acceptor, n the refractive index of the medium, $I(\lambda)$ is the normalized fluorescence spectrum, $\varepsilon(\lambda)$ the molar absorption coefficient (M^{-1} cm^{-1}); the wavelength of the radiation must be expressed in nanometers. The efficiency of energy transfer can thus be calculated by [25–28]

$$E = \frac{R_0^6}{R_0^6 + R^6} \quad (6)$$

For evaluation of the critical transfer radius (Eq. (5)), the following assumptions were made: (i) $k^2 = 2/3$ which corresponds to a dynamic isotropic (or pseudo-isotropic) regime of transfer, and (ii) the refractive index of the bilayer interior was 1.4 [29,30].

To obtain the theoretical expectation for energy transfer efficiency, the acceptor surface concentration was determined assuming (i) an area of 62.2 $Å^2$ for the phospholipid headgroup, (ii) that half of the phospholipid molecules are in the external membrane of the vesicles, and (iii) that there are no drug molecules in the high curvature inner surface of the vesicles, with the energy transfer being restricted to the outer leaflet [30]. With the above assumptions the critical transfer radius, R_0, was calculated from Eq. (5), for a random array of donors and acceptors [25], and was found to be 31.2 ± 0.2 Å for 2-AS and 38.7 ± 0.1 Å for 12-AS for DMPC and DMPG.

Experimental values for energy transfer efficiency were determined from the following relationship:

$$E = 1 - I/I_0 \quad (7)$$

where I_0 and I are the fluorescence intensity of the probes in the absence and presence of rifampicin, respectively.

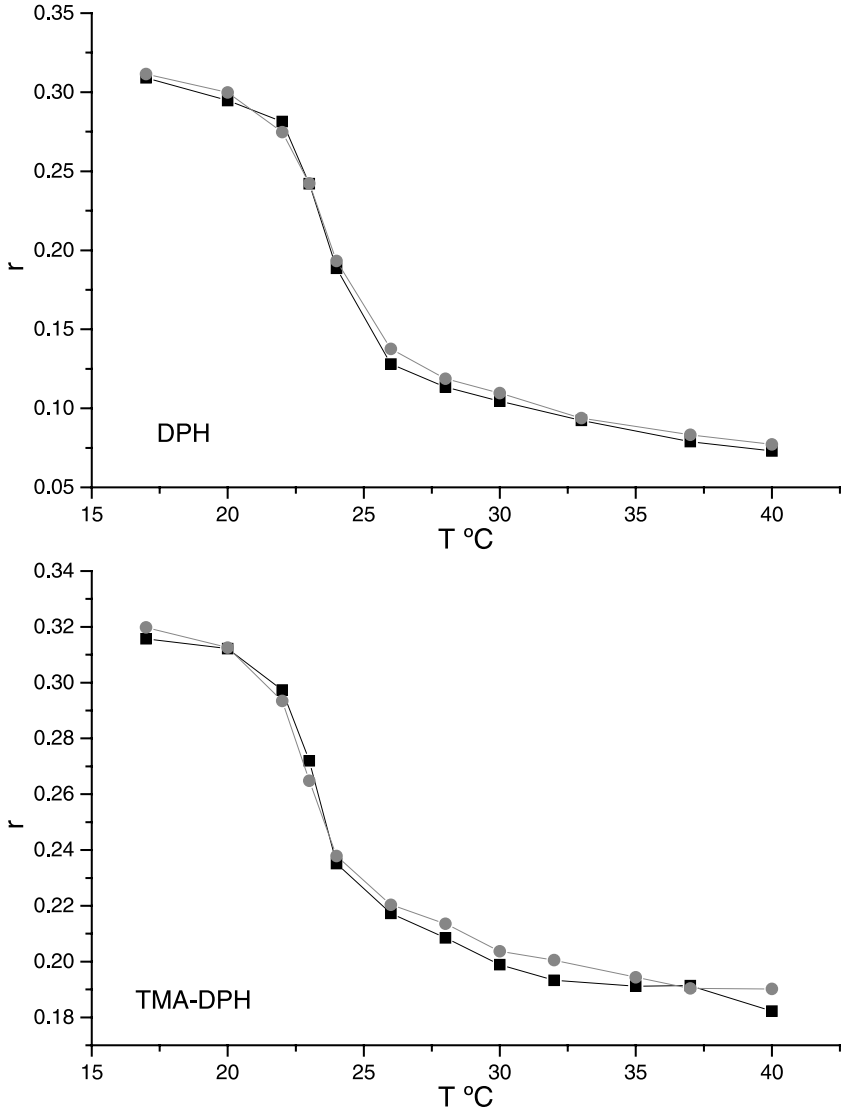

Fig. 5. Temperature dependence of the anisotropy of DPH and TMA-DPH in DMPG liposomes in absence (■) and presence (●) of rifampicin (20 μM).

The broad agreement between the experimental values of energy transfer and theoretical values, obtained from the Föster model, corroborates the fact that the observed quenching is a consequence of RET between *n*-AS probes and rifampicin (Fig. 4). The larger efficiency observed for the DMPC system results from the larger fraction of the drug that is incorporated in this system, as the K_p values of rifampicin are much larger in DMPC than DMPG.

It is important to stress that to obtain reliable results in DMPG liposomes it was necessary to co-solubilised the probes with the lipid (see Section 2 for details). The *n*-AS probes are negatively charged at physiological pH as DMPG is a negatively charged lipid, and the electrostatic repulsions that occur when the probes are added to already prepared liposomes led to the segregation of probe aggregates in the aqueous phase. These aggregates act as donors for the rifampicin in this phase, thus deceiving the real efficiency of energy transfer between donors and acceptors in the bilayer.

3.3. Steady state anisotropy measurements

The degree of fluorescence anisotropy (r) is defined by the following equation

$$r = \frac{I_{VV} - I_{VH}G}{I_{VV} + 2I_{VH}G} \quad (8)$$

where I_{VV} and I_{VH} are the intensities measured in directions parallel and perpendicular to the excitation beam. The correction factor G is the ratio of the detection system sensitivity for vertically and horizontally polarized light, which is given by the ratio of vertical to horizontal components when the excitation light is polarized in the horizontal direction, $G = I_{HV}/I_{HH}$ [28].

Changes in anisotropy of the two fluorescent probes, DPH and TMA-DPH, were used to assess membrane fluidity and drug location in DMPC and DMPG liposomes [31,32]. Temperature dependence of DPH and TMA-DPH anisotropy in negatively charged liposomes is shown in Fig. 5 in the absence and presence of rifampicin.

The anisotropy values for DPH and TMA-DPH, in both liposomes, were insensitive to the presence of isoniazid. For rifampicin it was not possible to obtain results in DMPC, since a complete quenching of the probes fluorescence occurred, even for very small concentrations of rifampicin (8 μM), but in DMPG the anisotropy values for DPH changed slightly.

4. Discussion

Rifampicin and isoniazid, drugs commonly used as tuberculostatics, have very different physiochemical characteristics: isoniazid is neutral at the physiological pH (7.4), whereas rifampicin is partially ionized at this pH, being ≈ 40% in the anionic form [14]. Furthermore, their partition coefficients in the two liposomes, used in this work, are also very different: in DMPC the K_p values are $5.09 \pm 0.52 \times 10^4$ for rifampicin and $0.84 \pm 0.12 \times 10^4$ for isoniazid; in DMPG the values are $0.54^4 \pm 0.25 \times 10^4$ for rifampicin and $0.59 \pm 0.12 \times 10^4$ for isoniazid [13,14]. These results are very important to know whether or how these drugs interact electrostatically with bilayers. It could be concluded that electrostatic/hydrophobic interactions were very important, especially when drugs exist in ionized forms at physiological pH.

In this work we have studied the location and interaction of rifampicin and isoniazid when incorporated into zwitterionic and negatively charged liposomes by ^{1}H NMR and fluorescence techniques.

^{1}NMR data studies corroborate the results already determined for the partition coefficient: rifampicin interacts more strongly than isoniazid with DMPC liposomes. Furthermore, the slight downfield shift of the methylene group signal of the DMPC acyl chains in the presence of rifampicin is indicative that this drug penetrates the liposome bilayers [21,23].

Further information on drug location was obtained from the quenching studies with the *n*-AS probes. The fluorophore group of 2-AS probe is located near the polar region of the bilayer (16.8 Å from the bilayer center) and that of 12-AS probe is deeply buried in the bilayer (7.5 Å from the bilayer center) [32,33]. For rifampicin, as the quenching efficiency of the 12-AS is higher than those of 2-AS, in both liposomes, this drug must be located deeply buried in the bilayer. For isoniazid the quenching data with *n*-AS probes show conclusively that this drug must be located in a very hydrophilic region, at the membrane surface, incapable to interact with any of the probes in both.

Furthermore, the results obtained from the efficiency of resonance energy transfer corroborates the results obtained for rifampicin. Location of rifampicin in the two systems can be inferred from the comparison between theoretical and experimental data: for the DMPC liposomes rifampicin is located halfway between the two probes, since theoretical and experimental data are in good agreement for both probes; for the DMPG liposomes rifampicin must also be located halfway between the two probes, but as there is a small divergence between the data obtained for 12-AS probe, it is likely that rifampicin prefers a more superficial region more near the membrane interface. The last result can be explained by the electrostatic repulsion that is expected between the negatively charged headgroup of DMPG and the ionized form of rifampicin [14].

Further information was gathered by the fluorescence anisotropy studies performed to determined the influence of drugs incorporation on membrane fluidity of DMPC and DMPG liposomes. As DPH is deeply buried (7.8 Å from the bilayer center), as expected from its hydrophobic nature, and pack well with fatty acyl chains than TMA-DPH (10.9 Å from the bilayer center), with a cationic group attached to DPH phenyl ring, which is located more shallowly than free

DPH, TMA-DPH is expected to monitor lipid order changes closer to the water/membrane interface than DPH [31–33].

For rifampicin, the complete quenching of both probe fluorescence in DMPC and the nondependence on the anisotropy of TMA-DPH and slight dependence on the anisotropy of DPH in the presence of drug in DMPG make it clear that rifampicin must be located deeper in the membrane as already predicted by RET studies. For isoniazid the anisotropy of DPH and TMA-DPH in both liposomes is insensitive to its presence; consequently, these results once more confirm that this drug must be located, in both systems, at the membrane surface completely inaccessible to both probes.

In summary, the results obtained for isoniazid clearly show that this drug is located at the membrane surface and the extent of its interaction is independent of the electrostatic/hydrophobic characteristics of the liposomes. In contrast, rifampicin, which also interacts with both liposome systems, is deeply buried in the membrane and has a degree of incorporation that depends on the electrostatic/hydrophobic characteristics of the liposomes.

From these results it is possible to conclude that an effective interaction between isoniazid and rifampicin and the lipid bilayer is observed and that this interaction does not induce significant changes in the structure of the membrane. Consequently, the physicochemical data suggest that it is possible to obtain stable preparation of liposomes containing isoniazid and rifampicin for use in tuberculosis therapy.

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References

[1] P. Deol, G.K. Khuller, K. Joshi, Therapeutic efficacies of isoniazid and rifampin encapsulated in lung-specific stealth liposomes against *mycobacterium tuberculosis* infection induce in mice, Antimicrob. Agents Chemother. 41 (1997) 1211–1214.

[2] D.C. Quenelle, J.K. Staas, G.A. Winchester, E.L.W. Barrow, W.W. Barrow, Efficacy of microencapsulated rifampicin in *Mycobacterium tuberculosis*-infected mice, Antimicrob. Agents Chemother. 43 (1999) 1144–1151.

[3] W.R. Oerkins, S.R. Minchey, P.L. Ahl, A.S. Janoff, The determination of liposome capture volume, Chem. Phys. Lipids 64 (1993) 197–217.

[4] D.D. Lasic, Liposomes—From Physics to Application, Elsevier, Amsterdam, 1993.

[5] M. Bermúdez, E. Martinez, M. Mora, M.L. Sagristá, M.A. Madariaga, Molecular and physicochemical aspects of the interactions of the tuberculostatics ofloxacin and rifampicin with liposomal bilayers: a ^{31}P-NMR and DSC study, Colloids Surf., A Physicochem. Eng. Asp. 158 (1999) 59–66.

[6] B.S. Rao, K.V.R. Murthy, Studies on rifampicin release from ethylcellulose coated nonpareil beads, Int. J. Pharm. 231 (2002) 97–106.

[7] L. Constatino, R.T. Mehta, M.E. Cruz, Formulation and toxicity of liposomes containing rifampicin, J. Liposome Res. 3 (1993) 275–301.

[8] J. Montenez, F. Bambeke, J. Piret, A. Schanck, R. Brasseur, P.M. Tulkens, M. Mingeot-Leclercq, Interaction of the macrolide azithromycin with phospholipids: II. Biophysical and computer-aided conformational studies, Eur. J. Pharmacol. 314 (1996) 215.

[9] F. Castelli, M.T. Cambria, P. Mazzone, R. Pignatello, Interaction of monoamine oxidase inhibitors with dipalmitoyl phosphatidylcholine liposomes. A comparison between structure and calorimetric data, Thermochim. Acta 302 (1997) 143–150.

[10] R.P. Mason, D.G. Rhodes, L.G. Herbette, Reevaluating equilibrium and kinetic binding parameters for lipophillic drugs based on a structural model for drug interaction with biological membranes, J. Med. Chem. 34 (1991) 869–877.

[11] M.T. Montero, J.H. Borrell, K.M.W. Keough, Fluoroquinolone–biomembrane interactions: monolayer and calorimetric studies, Langmuir 14 (1998) 2451–2454.

[12] N. Maurer, K.F. Wong, M.J. Hope, P.R. Cullis, Anomalous solubility behavior of the antibiotic ciprofloxacin encapsulated in liposomes: a ^{1}H-NMR study, Biochim. Biophys. Acta 1374 (1998) 9–20.

[13] C. Rodrigues, P. Gameiro, S. Reis, J.L.F.C. Lima, B. Castro, Spectrophotometric determination of drug partition coefficients in dimiristoyl-L-α-phosphatidylcholine/water: a comparative study using phase separation and liposome suspensions, Anal. Chim. Acta 428 (2001) 103–109.

[14] C. Rodrigues, P. Gameiro, S. Reis, J.L.F.C. Lima, B. Castro, Derivative spectrophotometry as a tool for the determination of drug partition coefficients in water/dimyristoyl-L-α-phosphatidylglycerol (DMPG) liposomes, Biophys. Chemist. 94 (2001) 97–106.

[15] R.R.C. New, in: D. Rickwood, B.D. Hames (Eds.), Liposomes—A Practical Approach, IRL Press, Oxford, 1990, pp. 106–107.

[16] M.J. Moreno, M. Prieto, Interaction of the peptide hormone adrenocorticotropin, ACTH(1–24), with a membrane model: fluorescence study, Photochem. Photobiol. 57 (1993) 431–437.

[17] D.B. Chalpin, A.M. Kleinfeld, Interaction of fluorescence quenchers with the *n*-(9-anthroyloxy) fatty acid membrane probes, Biochim. Biophys. Acta 731 (1983) 465–474.

[18] A. Coutinho, M. Prieto, Self-association of the polyene antibiotic nystatin in dipalmitoylphosphatidylcholine vesicles: a time-resolved fluorescence study, Biophys. J. 60 (1995) 2541–2557.

[19] A. Coutinho, M. Prieto, Ribonuclease T1 and alcohol dehydrogenase fluorescence quenching by acrilamide, J. Chem. Educ. 70 (1993) 425–428.

[20] C.R. Mateo, M. Prieto, V. Micol, S. Shapiro, J. Villalain, A fluorescence study of the interaction and location of (+)-totarol, a diterpenoid bioactive molecule, in model membranes, Biochim. Biophys. Acta 1509 (2000) 167–175.

[21] G.V. Betageri, Y. Theriault, J.A. Rogers, NMR study of the interaction of beta-blockers with sonicated dimyristoylphosphatidylcholine liposomes in presence of praseodymium cation, Membr. Biochem. 8 (1989) 197–206.

[22] L. Michaelis, P. Schlieper, 500 MHz ^{1}H NMR of phospholipid liposomes, Lanthanide shift on glycerol-γ and acyl-chain C2 resonance, FEBS Lett. 147 (1982) 40–44.

[23] J. Gabrielska, W.I. Gruszecki, Zeaxanthin (dihydroxy-β-carotene) but not β-carotene rigidifies lipid membranes: a ^{1}H-NMR study of carotenoid-egg phosphatidylcholine liposomes, Biochim. Biophys. Acta 1285 (1996) 167–174.

[24] C. Schimmel, Biophysical Chemistry, Part II, Freeman, New York, 1980.

[25] P.K. Wolber, B.S. Hudson, An analytic solution to the Förster energy transfer problem in two dimensions, Biophys. J. 28 (1979) 197–210.

[26] P. Wu, L. Brand, Resonance energy transfer: methods and applications, Anal. Biochem. 218 (1994) 1–13.

[27] M.N. Berberan-Santos, M.J.E. Prieto, Energy transfer in spherical geometry, J. Chem. Soc., Faraday Trans. 83 (1987) 1391–1409.

[28] J.R. Lackowicz, Principles of Fluorescence Spectroscopy, Kluwer Academic/Plenum Publishers, New York, 1999.

[29] L.M.S. Loura, A. Fedorov, M. Prieto, Partition of membrane probes in

a gel/fluid two-components lipid system: a fluorescence resonance energy transfer study, Biochim. Biophys. Acta 1467 (2000) 101–112.
[30] L.M.S. Loura, A. Fedorov, M. Prieto, Fluid–fluid membrane microheterogeneity: a fluorescence resonance energy transfer study, Biophys. J. 80 (2001) 776–788.
[31] M.R. Moya-Quiles, E.D. Munoz, C.J. Vidal, Effects of the pyrethoid insecticide permethrin on membrane fluidity, Chem. Phys. Lipids 79 (1996) 21–28.
[32] J. Slavic, Fluorescent Probes in Cellular and Molecular Biology, Prague, Czech Republic, 1994.
[33] R.D. Kaiser, E. London, Location of diphenylhexatriene (DPH) and its derivatives within membranes: comparison of different fluorescence quenching analyses of membrane depth, Biochemistry 37 (1998) 8180–8190.