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European Journal of Pharmaceutics and Biopharmaceutics 68 (2008) 400–405
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Distribution coefficient of rifabutin in liposome/water system as measured by different methods

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Received 31 December 2006; accepted in revised form 22 May 2007
Available online 3 June 2007

Abstract

Distribution coefficient (D) of rifabutin in liposome/water system was measured by phase separation and fluorescence probe quenching techniques. D values were identical suggesting that rifabutin is fully immersed into lipid bilayer. Structural studies of phospholipid bilayer employing ^{31}P NMR spectroscopy demonstrated that introduction of rifabutin does not alter the bilayer structure. A scheme of the rifabutin position in lipid bilayer based on the calculated size of rifabutin molecule is proposed.

Keywords: Rifabutin; Distribution coefficient; ^{31}P NMR; Liposomes; Fluorescence

1. Introduction

It is believed that pharmaceutical properties of a drug to a great extent depend on its absorption, which in turn is defined mostly by two parameters, solubility and distribution coefficient of a drug [1]. Partition coefficient (P) of a non-ionizable drug is determined as the ratio of the drug concentrations in apolar (C_{Apolar}) and polar (C_{Polar}) phases:

$$P = \frac{C_{\text{Apolar}}}{C_{\text{Polar}}} = \frac{n_{\text{Apolar}} \cdot V_{\text{Polar}}}{n_{\text{Polar}} \cdot V_{\text{Apolar}}} \quad (1)$$

In the case of an ionizable drug that exists in several electrical species, the relation between distribution coefficient D and partition coefficient P for n electrical species of an ionizable drug is given by

$$D = \sum_{i}^{n} (\varphi_i \cdot P_i) \quad (2)$$

where φ_i is the molar fraction of charged species i.

The D values of an anti-tuberculosis drug rifabutin (RBT) in octanol/water and liposome/water systems have been determined at pH 6.8 only [2]. It should be noted that RBT interacts with lung tissues affected by tuberculosis, where pH is lower than in normal tissues.

RBT molecule contains several groups that can undergo protonation and deprotonation (Fig. 1). Hence, if the medium pH is altered, the contents of charged species and the D values will change, and consequently the amount of the drug that may penetrate into membrane would become different.

In order to better understand the interactions between this antibiotic and membranes in normal and pathological conditions, we have studied the distribution coefficient of RBT in liposome/water system as a function of medium pH as well as the partition coefficient of the RBT charged species. Phase separation and quenching of hydrophobic

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doi:10.1016/j.ejpb.2007.05.014

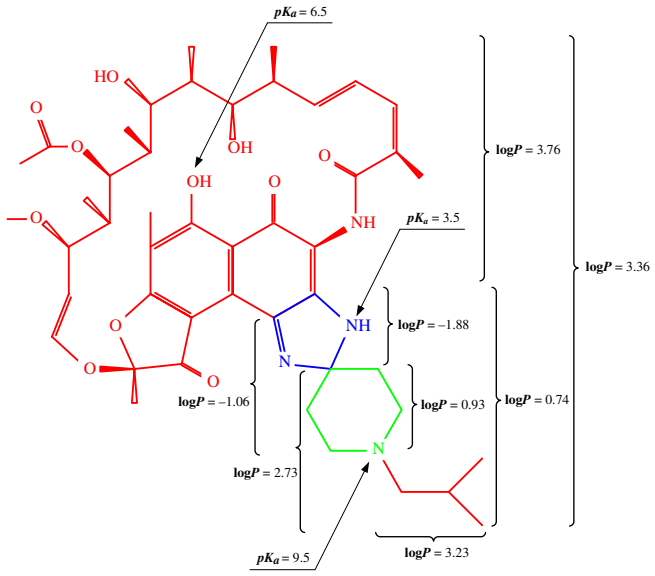

Fig. 1. Rifabutin structure, ionization constants and calculated partition coefficients for RBT fragments.

fluorescent probe anthrylphosphatidylcholine (anthryl-PC) were employed to obtain the D values.

2. Materials and methods

The following substances were used in experiments: rifabutin (Upjohn, USA), soybean phosphatidylcholine (PC) Lipoid S-100 94% pure (Lipoid, Germany), cardiolipin (CL) (Biolek, Ukraine). Anthryl-PC was synthesized as described elsewhere [3]. All other reagents and solvents used were of "chemically pure" grade (Khimmed, Russia).

Absorption spectra were recorded on Beckman DU-7 HS spectrophotometer (Beckman Instruments Inc., USA). Steady-state fluorescence spectra were recorded on Jasco FP-777 spectrofluorimeter (Jasco Corporation, Japan).

Multilamellar vesicles (MLV) prepared from PC in 0.15 M NaCl were obtained as described previously [4]. Medium pH was adjusted by the addition of 0.01 M Tris–HCl buffer solution. In order to measure D by phase separation, ethanol solution of RBT was added to MLV, with ethanol volume not exceeding 5%. The MLV emulsion was then incubated for several hours and centrifuged at 4000g. Supernatant was removed and pellet was dissolved in ethanol. RBT concentrations were determined by means of spectrophotometry at 500 nm both in the supernatant and the dissolved pellet.

Fluorescence quenching technique was performed on large unilamellar vesicles (LUV) containing fluorescent probe. Ethanol solution of PC was mixed with an appropriate amount of hexane solution of anthryl-PC. Solvent was removed on a rotary evaporator at 40 °C. Lipid film was dispersed in 0.15 M NaCl solution (pH 6.4 ± 0.1) or 0.01 M Tris–HCl buffer (pH 7.4) containing 0.15 M NaCl. MLV emulsion was freeze-thawed three times and then subjected to the extrusion through the 200-nm filter (Avestin Inc., USA). Steady-state fluorescence spectra were obtained using excitation wavelength 262 nm. Emission was recorded in the 350–500-nm range. Excitation and emission slits were set at 3 nm. Stern–Volmer constants were calculated from fluorescence intensities at 420 nm.

Concentration range of the components used is shown in Table 1.

log P values of RBT fragments were calculated employing ACD v. 8 software (Advanced Chemistry Developments, USA).

Three-dimensional structure of RBT was extracted from Protein Data Bank (structure id 2A68). Geometry optimization was performed in Hyperchem 7.01 software using the semi-empirical quantum-mechanical calculations with PM3 parameterizations. Calculations were performed within unrestricted Hartree–Fock formalism until root mean square deviation value of 1×10^{-4} kcal/Å mol was reached.

^{31}P NMR proton-decoupled spectra were recorded on the Bruker Avance 600 MHz spectrometer (242.95 MHz for ^{31}P). Typically 5000 scans were acquired for each spectrum. Prior to Fourier transformation, free induction decays were multiplied by an exponential weighting function with 100 Hz line broadening. Temperature was kept constant at 25 °C. Chemical shifts are given relative to phosphoric acid used as an external standard (0 ppm).

3. Results

The goal of this study was to determine the distribution coefficient of RBT as a function of pH in liposome/water system employing different methods. The complexity of this task arises from the fact that RBT contains several groups that are capable of undergoing protonation and deprotonation (Fig. 1). Balon et al. reported two pK_a values for RBT: 6.90 and 9.37 [2], but no assignment of these values to particular ionization groups has been made. Earlier we had employed potentiometric and spectrophotometric titration and determined three pK_a values of RBT. Analysis of titration data and computation in ACD software

Table 1
Concentrations of lipids and RBT used for the D measurements by different methods and in NMR experiments

Method	[PC], M	[anthryl-PC], M	[RBT], M	System
Phase separation	4.0×10^{-2}	–	6.0×10^{-4}–2.4×10^{-3}	MLV
Fluorescence spectroscopy	1.0×10^{-4}–2.5×10^{-3}	1.0×10^{-6}	2.5×10^{-6}–1.9×10^{-5}	LUV, 200 nm
^{31}P NMR spectroscopy	5.0×10^{-2}	–	1.8×10^{-2}	MLV

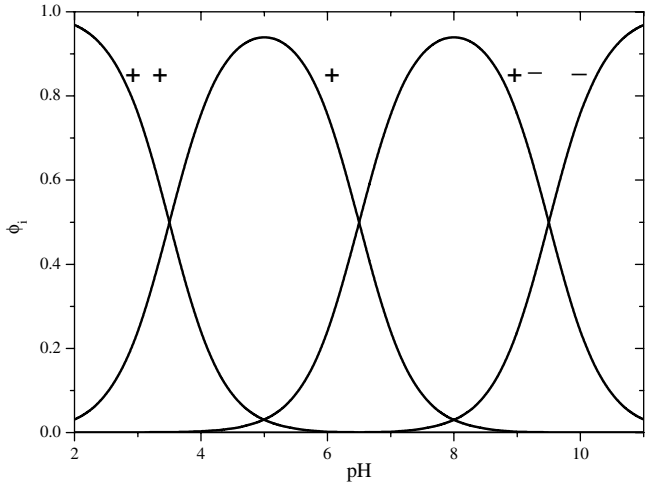

Fig. 2. Distribution of RBT charged species as a function of pH.

allowed assigning them to imidazole nitrogen (pK_a 3.5), naphthol oxygen (pK_a 6.5) and piperidine nitrogen (pK_a 9.5) (Fig. 1) [5]. Treatment of these data with Henderson–Hasselbach Eq. (3) [6] yields the distribution of the charged species over the whole pH range.

$$\mathrm{pH} = \mathrm{p}K_a + \log\frac{[X]}{[HX]} \tag{3}$$

where X and HX refer to deprotonated and protonated species, respectively (charges not shown).

Calculated distribution of the charged species is shown in Fig. 2. At pH 7.4 zwitterion species predominate (88%), while cations make up 11%. At pH 6.4, abundance of these species is nearly equal (44% and 56%, respectively), with a slight excess of the cation form. The pK_a values of RBT lie relatively close to each other, and RBT exists in at least two different ionization states over a wide pH range. Therefore, experimental techniques can provide only D value for RBT.

3.1. Measurement of D by phase separation techniques

Distribution coefficients of RBT calculated from Eq. (1) are shown in Table 2. RBT is highly hydrophobic over a wide pH range with D reaching maximum between pH 6.4 and 7.4. Lower D values were obtained only in basic (pH 10.0) and highly acidic (pH 2.5) media. In the second case, it is caused by electrostatic repulsion between dicationic RBT and protonated headgroups of PC (pK_a of phosphoric acid moiety in PC is reported to be 1.97 [1]). In highly basic media (pH over 11) [7] PC can hydrolyze to lyso-PC and free fatty acids, which carry a negative charge. After phase separation and measurement of D values lipid fraction was extracted with chloroform and analyzed by thin layer chromatography as reported elsewhere [8]. Analysis of rifabutin stability was performed according to the United States Pharmacopeia [9] and did not reveal any degradation products.

Table 2
Distribution coefficients of RBT

pH	log D	
	Phase separation	Fluorescence spectroscopy
2.5	1.20 ± 0.12	
6.4	2.45 ± 0.12	2.60 ± 0.02
7.1	2.58 ± 0.03	
7.4	2.60*	2.59 ± 0.06
9.0	2.16 ± 0.21	
10.0	1.96 ± 0.29	

* Value calculated from known log P.

Partition coefficients of the RBT species, as calculated from D values employing inversion matrix algorithm, yielded P values for zwitterionic and cationic forms 432 and 168, respectively ($\log P^{\pm} = 2.64$, $\log P^{+} = 2.23$). It is an anticipated result that correlates well with the statement that neutral species are more hydrophobic than the charged ones. These obtained P values refute an assertion that partitioning of cationic and zwitterionic charged species of the RBT in liposome/water system is identical [2]. Distribution coefficients at pH 6.4 and 7.4 as calculated from P values employing Eq. (2) are comparable and equal 285 and 400, respectively ($\log D^{6.4} = 2.45$, $\log D^{7.4} = 2.60$).

3.2. Measurement of D by fluorescence quenching

Distribution coefficient of rifabutin was also measured using the RBT-induced quenching of the anthryl-PC fluorescent probe incorporated into PC liposomes. The probe concentration was 1×10^{-6} M and the PC concentration range was between 1×10^{-6} and 2.5×10^{-3} M. The RBT concentration was varied from 2.5×10^{-6} to 2.0×10^{-5} M, so that it is at least an order of magnitude less than the PC concentration. Measurements were carried out at pH 6.4 and 7.4.

According to the Stern–Volmer (S–V) model, quenching of a fluorescent probe occurs due to a collision of a RBT molecule with an anthryl-PC molecule in excited state. It is a dynamic process described by

$$\frac{I_0}{I} = 1 + K_{SV} \cdot [\mathrm{RBT}] \tag{4}$$

where I_0 is the initial fluorescence intensity, I is the fluorescence intensity after the RBT addition, and K_{SV} is the Stern–Volmer constant that describes the interaction between fluorescence donor and acceptor. K_{SV} was calculated as a slope of the experimental curve in the $\frac{I_0}{I} - [\mathrm{RBT}]$ plot (Stern–Volmer plot). Experimental data demonstrated an upward deviation in these plots – an effect caused by optical properties of the antibiotic. Since RBT absorbs light at the anthryl-PC excitation and emission wavelengths, experimental data were corrected for the "inner filter effect" according to

$$I = I' \cdot 10^{\frac{1}{2}(A_{ex}+A_{em})} \tag{5}$$

where I is the corrected fluorescence intensity, I' is the experimental fluorescence intensity, A_{ex} and A_{em} are the

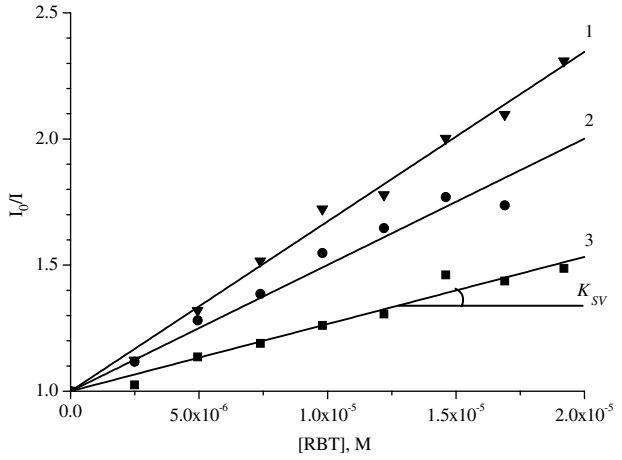

Fig. 3. Stern–Volmer plots of measured fluorescence intensity decay versus concentration of RBT. PC concentrations: 1–7.5×10^{-4} M; 2–1.25×10^{-3} M; 3–2.5×10^{-3} M.

optical densities of RBT at the excitation and emission wavelengths [10].

When experimental data were treated according to Eq. (5), neither upward nor downward deviations were observed in Stern–Volmer plots. It means that the Stern–Volmer model is applicable for the analysis of the RBT interaction with zwitterionic phospholipid membrane.

Series of experiments with various PC concentrations (Fig. 3) allowed calculating the RBT distribution coefficient at two pH of the medium as described in [11]. Results obtained are shown in Table 2. According to these data, D values of RBT in liposome/water system obtained by two different experimental methods are similar both at pH 6.4 and 7.4.

3.3. Hydrophobicity ($\log P$) of the RBT molecule and its fragments

In order to interpret the obtained results, hydrophobicity of certain fragments of the RBT molecule was evaluated employing ACD software. Fig. 1 provides the structural formula of RBT and calculated $\log P$ values of its fragments. It should be noted that as this calculation represents the partition coefficient between octanol and water, the absolute values can be different from those obtained in PC–water system. Octanol lacks the complexity of lipid bilayer and calculated $\log P$ values can be used for evaluation only. It can be inferred that RBT contains both hydrophilic moieties (piperidine and imidazole residues) and hydrophobic ones (naphthol cycle and aliphatic ansa-chain). Hydrophobicity of the residues decreases in the following order: naphthol > piperidine > imidazole.

3.4. Three-dimensional structure of RBT

Three-dimensional structure of RBT was obtained after geometry optimization by semi-empirical PM3 method. Initial structure of RBT molecule was extracted from the crystal structure of RBT – DNA-directed RNA polymerase complex (Brookhaven Protein Databank id 2A68) [12]. Hydrogen atoms were added by a standard procedure in Hyperchem software. According to the calculation results, naphthol, imidazole and piperidine moieties are mostly located in the same plane, that is nearly perpendicular to the plane of ansa-chain. The whole molecule can be confined to a $17\times9\times10$ Å box (Fig. 4). Among the longest axis of this box the moieties with different $\log P$ values are located. In other words, there is a hydrophobicity gradient in the RBT molecule.

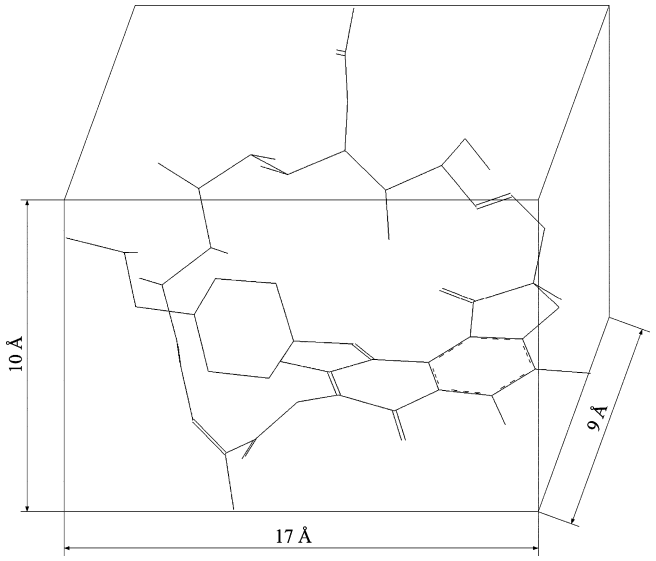

Fig. 4. Three-dimensional model of rifabutin.

3.5. Influence of RBT on the lipid bilayer structure

Influence of bulky hydrophobic RBT molecules on the lipid bilayer structure was studied by ^{31}P NMR spectroscopy. According to the data obtained earlier [4,10], introduction of negatively charged cardiolipin, a phospholipid abundant in mycobacteria [13], increases the binding of RBT to the lipid bilayer. This finding suggests that in this case the effect of RBT on the lipid bilayer structure can be more pronounced, and thus we have also performed ^{31}P NMR experiments on cardiolipin containing samples.

The samples were prepared at pH 7.4 (0.1 M Tris buffer). Ionic strength was adjusted to 0.15 M with NaCl. Phosphorous spectra were recorded for MLV samples that contained either PC or PC:cardiolipin (4:1 w/w). Zwitterionic phosphatidylcholine membrane served as a reference. Introduction of cardiolipin did not alter the bilayer packing but caused an excessive negative charge on the membrane surface.

The recorded NMR spectra are shown in Fig. 5. It can be seen that all the spectra exhibit features typical of a bilayer structure: peak at −14 to −16 ppm and chemical shift anisotropy of 50–55 ppm. These data suggest that incorporation of RBT into model membranes does not

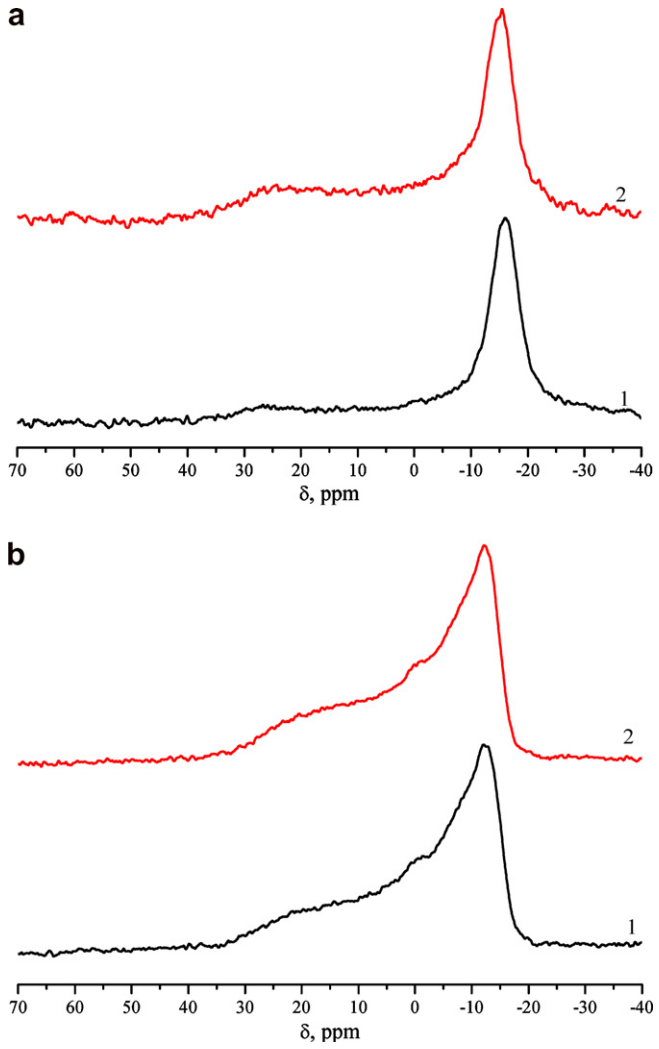

Fig. 5. (a) ^{31}P NMR spectra of lipid dispersions. PC. 1 – phospholipids, 40 mg/ml; 2 – phospholipids, 40 mg/ml with RBT, 15 mg/ml. (b) ^{31}P NMR spectra of lipid dispersions. PC:cardiolipin 4:1. 1 – phospholipids, 40 mg/ml; 2 – phospholipids, 40 mg/ml with RBT, 15 mg/ml.

modify the structural organization of phospholipids in a bilayer membrane.

4. Discussion

In this study, we have measured a distribution coefficient of RBT in liposome/water system. Such system is essentially anisotropic as the lipid phase (liposomes) is discretely distributed in an aqueous phase. In this system, a considerable lipid/water interfacial area containing polar headgroups allows adsorption of the partitioning drugs due to polar interactions.

Earlier we have demonstrated that the binding of RBT to large unilamellar vesicles increases upon the introduction of negatively charged phospholipids (CL) and decreases as the ionic strength goes up [4]. These data indicate the contribution of electrostatics in the binding process. It has also been shown that the introduction of cholesterol, a lipid localized in the hydrophobic region of the bilayer making it more rigid, conversely leads to a lesser binding. The latter observation indicates the hydrophobic interaction, that is in line with the results reported in this paper. First, it is RBT-induced quenching of the fluorescent probe, attached to an acyl chain of PC. Second, the D values of RBT reported in the Table 2 indicate that it is a hydrophobic compound.

The data presented here and reported earlier demonstrate the influence of different forces on binding hydrophobic antibiotic molecule with a membrane. This statement is elaborated as follows: (1) Using pK_a values calculated earlier [5] we calculated the percentage of RBT charged species at the wide pH range. (2) By employing phase separation method we obtained pH dependence of distribution coefficients of the antibiotic. Comparison of these data allows one to understand how RBT binding to a membrane is affected by the ionization of particular groups.

Two methods were used to measure the D values: a direct method – phase separation, and an indirect one – fluorescent probe quenching. Phase separation allows estimating the total amount of the drug bound to the membrane, including both the fraction adsorbed on the interface region and the fraction partitioned into the hydrophobic core of liposomes.

On the other hand, the method of fluorescent probe quenching takes into account only the drug fraction buried deeply in the membrane. The D values of RBT obtained by both methods are similar to each other. These data provide evidence that after the adsorption to the membrane interface via polar interactions [4], RBT molecules undergo full immersion into the hydrophobic core.

Several reports on rifampicin – a compound structurally similar to RBT – support this suggestion. For instance, it has been shown that ζ-potential of zwitterionic lipids containing a minor fraction of negatively charged lipids does not change after rifampicin introduction [14]. Neutron scattering technique has revealed that binding of rifampicin causes changes in the scattering profile mainly at the acyl chain region, while changes at the membrane interface were negligible [15].

Comparison of data obtained by phase separation and fluorescence quenching implies that RBT is primarily localized to the hydrophobic region of the membrane. Calculation of three-dimensional structure of RBT revealed that it is a relatively big and bulky molecule, suggesting that its incorporation into the lipid bilayer can alter the structural organization of the latter. However, ^{31}P NMR spectroscopy did not reveal any non-bilayer phases after the RBT introduction. In order to understand how this molecule can orient in the membrane without causing perturbations in the phospholipid structure, hydrophobicity of RBT fragments was calculated. Calculated log D values indicate that hydrophobicity of a molecule consisting of ansa-chain and naphthalene moiety is three orders of magnitude higher than the one of imidazole–piperidine part, despite the

presence of aliphatic isobutyl chain. This uneven distribution creates a hydrophobicity gradient in the RBT molecule. Obviously the calculations performed in vacuum cannot be readily applied to real world systems. Nevertheless the differences in hydrophobicity between these two parts of the RBT molecule are so large that they cannot be canceled out by mere ionization. We propose that in order to minimize the disturbance of lipid bilayer, the antibiotic molecule is oriented so that its hydrophobicity gradient coincides with the hydrophobicity profile of the membrane. Most of the hydrophobic moieties of RBT are located in the acyl chain region. For palmitic and oleic acids that predominate in soybean PC, the length of this region is 13–14 Å [16]. Hydrophilic part of RBT, namely imidazole and piperidine moieties, should therefore be localized in the glycerol backbone.

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