Interaction of Rifabutin with Model Membranes

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Liposomal and free rifabutin were separated by the method of gel filtration. The percents of rifabutin bound to liposomes of different phospholipid composition were measured. The presence of negatively charged phospholipids increased the degree of binding. Binding decreased with increasing the ionic strength. Incubation of rifabutin with liposomes containing anthryl phosphatidylcholine was accompanied by fluorescence quenching. Activity of rifabutin depended on the phospholipid composition of liposomes. Our results indicate that binding of rifabutin is associated with electrostatic and hydrophobic interactions.

Key Words: *phospholipids; liposomes; rifabutin; anthryl phosphatidylcholine; fluorescence technique*

Rifabutin (RB) is a semisynthetic derivative of the rifamycin group. RB was used in chemotherapy of tuberculosis only for a relatively short time. Comparative study of RB and rifampicin (another synthetic derivative of rifamycin) showed that RB exhibits lower toxicity, has a longer half-life (about 30 h), and is characterized by partial cross-resistance relative to rifampicin-resistant mycobacteria [5]. The minimum inhibitory concentration of RB is one order of magnitude lower compared to that of rifampicin (0.03-0.06 and 0.1-1.0 mg/ml, respectively) [9,11].

Previous studies showed that RB suppresses division of mycobacteria in macrophages [10]. The development and use of colloidal RB formulations that should be engulfed by macrophages hold much promise. Only one study was performed to evaluate the effect of liposomal RB on mice infected with *Mycobacterium avium complex*. It was shown that liposomal RB is less effective compared to free RB [7].

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Here we studied the interaction of RB with model membranes. Experiments were performed with liposomes of different composition to estimate the nature of forces that determine binding of RB to membranes of animal cells and microorganisms.

MATERIALS AND METHODS

We used RB (Upjohn), soybean phosphatidylcholine (PC, Lipoid S-100, Lipoid), cardiolipin (Biolek), cholesterol (Sigma), and chemically pure solvents (Khimmed).

Multilamellar vesicles and liposomes were obtained as described elsewhere [3]. The size of liposomes was determined by means of turbidimetry [4]. Liposomal RB was separated from free RB by the method of gel filtration [3]. RB concentration in the liposomal fraction was measured spectrophotometrically at 500 nm. The extinction coefficient was 2.27×10^3 $M^{-1}\times cm^{-1}$. Anthryl phosphatidylcholine was obtained as described previously [1]. Fluorescence of anthryl phosphatidylcholine-containing liposomes was recorded at room temperature and pH 6.3 on a Jasco FP-777 spectrofluorometer. The total concentration of lipids and concentration of anthryl phosphatidylcholine in

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physiological saline were 1×10^{-3}-1×10^{-5} M and 1×10^{-6} M, respectively. The emission spectrum was recorded at an excitation wavelength of 260 nm (range 390-450 nm). The measurements were performed at an emission and excitation slots of 3 nm.

Liposomes (diameter 200 nm) were obtained from multilamellar vesicles by the method of extrusion.

The size of RB and cholesterol molecules was estimated after geometrical optimization with Parametric method 3 (Hyperchem 7.01 software).

RESULTS

We measured the concentration of RB bound to model membranes (liposomes) of different lipid composition (Fig. 1). PC was the major component of liposomes. The minor components were presented by cholesterol and cardiolipin. The cardiolipin molecule carries 2 negative charges. Therefore, the surface of liposomes from the mixture of PC and cardiolipin had a negative charge. Cardiolipin is capable of forming a bilayer. Cardiolipin in a mixture with PC has no effect on the state of hydrophobic region of PC membrane [2]. The introduction of cardiolipin into liposomes significantly increases the degree of RB binding. The percentage of bound RB increased with increasing the ratio of cardiolipin in liposomes. These data are consistent with the results of a comparative study on binding of RB to liposomes of PC and phosphatidylglycerol [7]. It was shown that the interaction of model membranes with RB is associated with electrostatic forces.

To test this hypothesis we studied whether the ionic strength affects binding of RB to liposomes of PC and cardiolipin (ratio 1:4, Table 1). An increase in the ionic strength of solution was followed by a progressive decrease in RB binding to liposomes. The system lost homogeneity at an ionic strength of 0.7, which resulted in the formation of precipitate. Our findings support the hypothesis that adsorption of this antibiotic on the surface of the model membrane is related to electrostatic interactions. These interactions are blocked at ionic strength >0.6. RB in a concentration of 7 mg/ml is poorly soluble in water and precipitates.

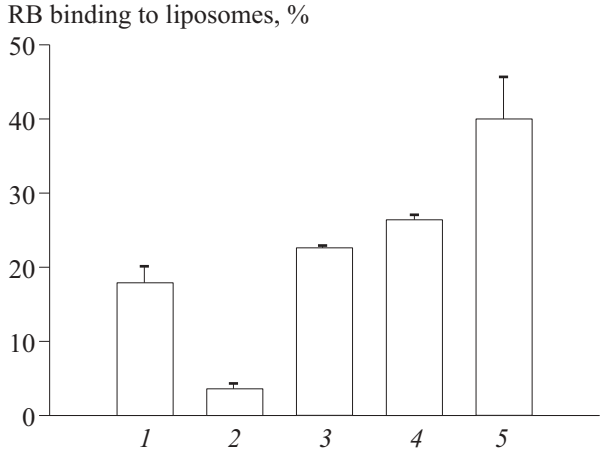

Fig. 1. Binding of rifabutin (RB, 7 mg/ml) to liposomes of different lipid composition (40 mg/ml) in physiological saline (ionic strength 0.15, pH 6.3±0.5). PC: phosphatidylcholine; CS: cholesterol; CL: cardiolipin. Lipid composition of liposomes: PC (*1*); PC:CS (4:1, *2*); PC:CL (4:1, *3*); PC:CL (1:1, *4*); PC:CL (1:4, *5*).

Introduction of cholesterol into liposomes significantly decreased binding of RB, which is consistent with published data [7]. It can be hypothesized that RB is adsorbed on the membrane surface and then migrates into the hydrophobic membrane region, while cholesterol makes the structure of phospholipid molecules more compact and inhibits this process [8].

To confirm the existence of hydrophobic interactions between RB and model membranes, we studied the effect of RB on fluorescence of anthryl phosphatidylcholine introduced into liposomes of different lipid composition. Addition of RB to liposomes containing a fluorescent label was followed by dose-dependent quenching of anthryl phospha-

TABLE 1. Dependence of RB Binding (7 mg/ml) to Liposomes of PC and Cardiolipin (40 mg/ml) in Physiological Saline on the Ionic Strength of the Incubation Medium*

Ionic strength of solution	Concentration of RB bound to liposomes, mol RB/mol phospholipid	Incorporation of RB into liposomes, % of the initial concentration of RB
0.07	0.137±0.003	50.0±0.7
0.15	0.110±0.017	40.0±5.7
0.3	0.073±0.020	27.1±7.1
0.7	Samples are unstable due to formation of a precipitate	
1		

Note. *PC/cardiolipin ratio 1:4 (40 mg/ml), pH 6.3±0.5, liposome diameter 200 nm.

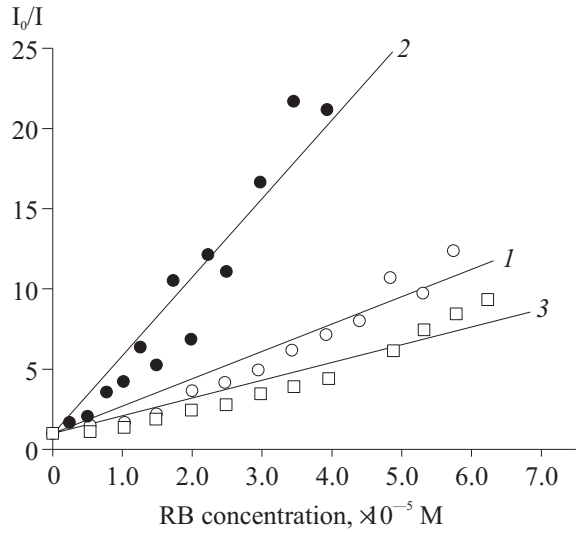

Fig. 2. I_0/I ratio for anthryl phosphatidylcholine (1×10^{-6} M) in liposomes (lipid concentration 1×10^{-4} M) in the presence and absence of RB. PC (*1*); PC:CL (1:4, *2*); PC:CS (4:1, *3*).

tidylcholine fluorescence (Fig. 2). In the molecule of anthryl phosphatidylcholine synthesized from soybean PC anthracene was introduced by double bonds. Most fatty acid residues in soybean PC are presented by unsaturated oleic (12%) and linoleic acids (65%). Molecules of these acids have double bonds at C_9 and C_9,C_{12} atoms of fatty acid chains [6]. The anthryl group in anthryl phosphatidylcholine is localized near the terminal end of the fatty acid residue and embedded deeply into the hydrophobic membrane region. Fluorescence quenching after addition of RB reflects its direct interaction with the label. These data provide direct evidence that RB is localized in the hydrophobic region of the model membrane.

The effectiveness of RB (ratio between fluorescence in the absence [I_0] and presence [I] of RB in various concentrations) depended on the nature of phospholipids and decreased in the following order: PC:cardiolipin>PH>PC:cholesterol. Our results are consistent with the results of a quantitative study on RB binding to liposomes of different lipid composition. In these experiments the degree of binding decreased in the same order.

We conclude that the interaction of RB with model membranes is realized via electrostatic forces and hydrophobic interactions. Adsorption of this antibiotic on the membrane surface is related to electrostatic interactions with polar groups of phospholipids. In the follow-up period RB migrates into the hydrophobic membrane region. The length of the RB molecule is similar to that of the cholesterol molecule (18×12×10 Å), which does not impair the bilayer structure. However, the volume of the RB molecule is 2-fold higher compared to that of the cholesterol molecule (18×7×5 Å). Therefore, incorporation of RB into the membrane can modify the phospholipid bilayer.

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