The Secondary Multidrug Transporter LmrP Contains Multiple Drug Interaction Sites†

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ABSTRACT: The secondary multidrug transporter LmrP of *Lactococcus lactis* mediates the efflux of Hoechst 33342 from the cytoplasmic leaflet of the membrane. Kinetic analysis of Hoechst 33342 transport in inside-out membrane vesicles of *L. lactis* showed that the LmrP-mediated H^+/Hoechst 33342 antiport reaction obeyed Michaelis−Menten kinetics, with a low apparent affinity constant of 0.63 μM Hoechst 33342 (= 0.5 mmol Hoechst 33342/mol phospholipid). Several drugs significantly inhibited LmrP-mediated Hoechst 33342 transport through a direct interaction with the protein rather than through dissipation of the proton motive force or reduction of the membrane partitioning of Hoechst 33342. The characterization of the mechanism of inhibition of LmrP-mediated Hoechst 33342 transport indicated competitive inhibition by quinine and verapamil, noncompetitive inhibition by nicardipin and vinblastin, and uncompetitive inhibition by TPP^+. The three types of inhibition of LmrP-mediated Hoechst 33342 transport in inside-out membrane vesicles indicate for the first time the presence of multiple drug interaction sites in a secondary multidrug transporter.

The development of microbial resistance to antibiotics is a growing concern in the control of infectious diseases. The presence of multidrug resistance (MDR[1]) transport proteins in pathogenic microorganisms is a serious problem, since one such efflux system can confer resistance to a broad spectrum of toxic compounds (*1*). Most known bacterial MDR transporters are secondary transporters that use the proton motive force (pmf) to drive the excretion of drugs. Secondary MDR transporters belong to one of three distinct families of transport proteins: the major facilitator superfamily (MFS), the resistance nodulation division family (RND), and the small multidrug resistance family (SMR) (*2*). The MFS family includes multidrug transporters such as Bmr (*3*), LmrP (*4*), QacA and QacB (*5*). These proteins consist of either 12 or 14 transmembrane segments (TMS). RND proteins, such as AcrB and MexB, contain 12 TMS and interact with a membrane fusion protein and an outer membrane porin to enable drug transport across both the inner and outer membrane of Gram-negative bacteria (*6*, *7*). The smallest multidrug transporters belong to the SMR family. These proteins contain only four putative transmembrane helices and are thought to function as homotrimers (*8*, *9*).

Detailed knowledge about the molecular basis of drug recognition by MDR systems will help in the battle against pathogens. Drugs may be designed that inhibit the activity of drug transporters, or antibiotics can be developed that are not recognized by MDR systems. Although the drug specificity of pmf dependent multidrug transporters is extensively studied (*10*−*13*), little is known about the number and molecular properties of drug binding sites in secondary MDR transporters (*14*, *15*). Lalande et al. (*16*) suggested the potential application of the dye Hoechst 33342 in studies on drug resistance. Recently, this compound was successfully used to study the activity of the lactococcal multidrug transporter LmrP (*17*) and the human multidrug resistance P-glycoprotein (*18*, *19*). The strong decrease in fluorescence upon the movement of Hoechst 33342 from the membrane to the aqueous phase enabled us to study the drug specificity of the lactococcal multidrug transporter LmrP in greater detail. Here, it is shown for the first time that multiple drug interaction sites are involved in drug recognition by LmrP.

MATERIALS AND METHODS

Preparation of Inside-Out Membrane Vesicles. L. lactis NZ9000 harboring the plasmid pHLP5 (*17*) was grown at 30 °C to an A_{660} of about 0.5. LmrP expression was triggered by the addition of approximately 10 ng nisin A/mL (a 1:1000 dilution of the supernatant of the nisinA-producing *L. lactis* strain NZ9700 (*17*)), followed by an incubation for 1 h at 30 °C. The cells were lysed with a French press, as described in Putman et al. (*17*). The inside-out membrane vesicles were resuspended in 50 mM potassium phosphate (pH 7.0) containing 10% (v/v) glycerol and stored in liquid nitrogen until use. The protein concentration was determined accord-

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[1] The abbreviations used are: MDR, multidrug resistance; MFS, major facilitator superfamily; RND, resistance/nodulation/cell division; SMR, small multidrug resistance; TMS, transmembrane segment; pmf, proton motive force; $\Delta\psi$, transmembrane potential; ΔpH, transmembrane proton gradient.

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ing to Lowry et al. (*20*) in the presence of 0.5% SDS, using bovine serum albumin as the standard.

Hoechst 33342 Transport. To study transport of Hoechst 33342 (2-[2-(4-ethoxyphenyl)-6-benzimidazolyl]-6-(1-methyl)-4-piperazil)-benzimidazole; Molecular Probes Inc.) by LmrP, inside-out membrane vesicles (0.5 mg protein/mL) were resuspended in 50 mM potassium Hepes (pH 7.0) containing 2 mM $MgSO_4$, 8.5 mM NaCl, 0.1 mg/mL creatine kinase, plus 5 mM phosphocreatine. After 30 s of incubation at 30 °C, Hoechst 33342 was added at a final concentration of 1 μM. LmrP activity was initiated by the generation of a pmf by the F_0F_1 H^+-ATPase through the addition of 0.5 mM Mg^{2+}-ATP. The amount of membrane-associated Hoechst 33342 was measured fluorimetrically (Perkin-Elmer LS-50B fluorometer), using excitation and emission wavelengths of 355 and 457 nm, respectively, and slit widths of 5 nm each. The initial rate of Hoechst 33342 transport was determined by linear regression of the fluorescence data obtained in the first 15 s after Mg^{2+}-ATP addition. The data from the kinetic experiments were fitted using the Michaelis−Menten equation.

Partitioning of Hoechst 33342 in the Phospholipid Bilayer of Membrane Vesicles. To determine the partitioning of Hoechst 33342 between the water and phospholipid phase, inside-out membrane vesicles (0.5 mg protein/mL) were resuspended in 50 mM potassium Hepes (pH 7.0) containing 2 mM $MgSO_4$, and 8.5 mM NaCl. Hoechst 33342 was added at final concentrations ranging from 0 to 6 μM. After 30 min incubation at room temperature, the membrane vesicles were removed by centrifugation at 235000*g* for 15 min at 4 °C. Polypropylene microfuge tubes were used rather than polyallomer, polycarbonate, or Ultra-Clear tubes (Beckman Instruments Inc.), to minimize Hoechst 33342 binding to the centrifuge tube. The supernatant was collected and the amount of Hoechst 33342 in the aqueous phase was estimated by fluorimetry using standard solutions of Hoechst 33342 for calibration. An aliquot of 300 μL of the supernatant was added to 1.7 mL of 50 mM potassium Hepes (pH 7.0), containing 2 mM $MgSO_4$, 8.5 mM NaCl, and inside-out membrane vesicles at a concentration of 1.0 mg protein/mL. The fluorescence was measured as described under "Hoechst 33342 transport". The membrane−water partition coefficient was calculated on the basis of the respective weights of the membrane and buffer fractions present. The lipid/protein ratio of the *L. lactis* inside-out membrane vesicles was determined to be 1.43:1 (w/w) using the method of Rouser et al. (*21*) for estimations of the amount of lipid, and the method of Lowry et al. (*20*) for protein determinations.

Measurement of the Transmembrane H^+ Gradient (ΔpH) in Membrane Vesicles. The ΔpH (inside acid) in inside-out membrane vesicles was monitored by fluorescence quenching of Acridine orange as described previously (*17*). The transmembrane potential ($\Delta\psi$, inside positive) in inside-out membrane vesicles was estimated from the increase in ΔpH upon dissipation of the $\Delta\psi$ by the addition of the K^+ ionophore valinomycin. To study the effect of several compounds on the pmf, the drugs were added to a final concentration of 1 and 4 times the IC_{50} for inhibition of LmrP-mediated Hoechst 33342 transport.

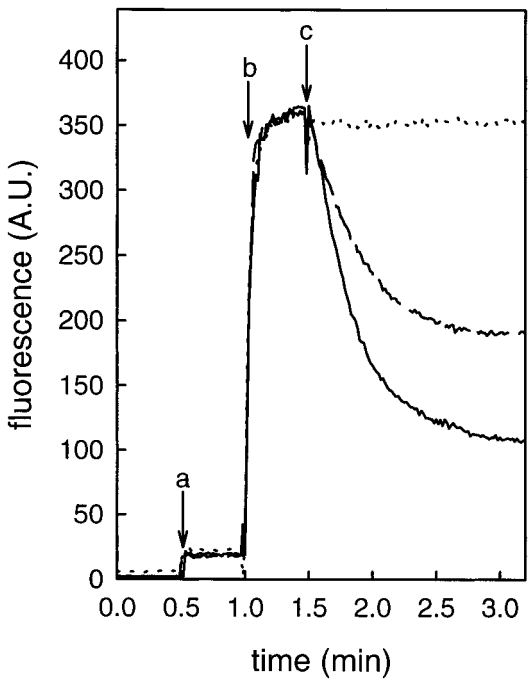

FIGURE 1: LmrP-mediated Hoechst 33342 transport in inside-out membrane vesicles. Inside-out membrane vesicles prepared from LmrP-expressing *L. lactis* cells were diluted to a concentration of 0.5 mg protein/ml in 50 mM potassium Hepes (pH 7.0) containing 2 mM $MgSO_4$, 8.5 mM NaCl, 0.1 mg/mL creatine kinase and 5 mM phosphocreatine. After a preincubation of 30 s (a), 1 μM Hoechst 33342 was added (b). Transport was initiated upon addition of 0.5 mM Mg^{2+}-ATP (c). The rate of Hoechst 33342 transport was measured in the absence of ionophores (solid line) and in the presence of 1 μM valinomycin (dashed line) or 1 μM valinomycin plus 1 μM nigericin (dotted line).

RESULTS

Hoechst 33342 Transport in Inside-Out Membrane Vesicles. In a previous study (*17*), the positively charged bisbenzimide dye Hoechst 33342 proved to be an excellent probe to study LmrP activity. Since Hoechst 33342 is fluorescent when bound to lipid membranes, but essentially nonfluorescent in an aqueous environment, the transport of Hoechst 33342 from the membrane to the aqueous phase can be followed by a decrease of Hoechst 33342 fluorescence in time. Figure 1 shows that, similar to observations on the human multidrug transporter P-glycoprotein (*19*), Hoechst 33342 was efficiently transported from the membrane into the lumen of inside-out membrane vesicles prepared from *Lactococcus lactis* cells overexpressing LmrP, when a pmf (inside acid and positive) was generated through proton pumping by the F_0F_1 H^+-ATPase. The involvement of both the $\Delta\psi$ and the ΔpH as a driving force for LmrP-mediated transport was demonstrated by the inhibition of Hoechst 33342 transport upon dissipation of the $\Delta\psi$, and the $\Delta\psi$ plus the ΔpH by the addition of the ionophores valinomycin, and valinomycin plus nigericin, respectively (Figure 1, and data not shown).

Partitioning of Hoechst in the Phospholipid Bilayer. Since Hoechst 33342 is only fluorescent in a hydrophobic environment, a decrease in fluorescence intensity represents a decrease in the amount of membrane-associated Hoechst 33342. The concentration of Hoechst 33342 in the membrane can be calculated from the total amount of Hoechst 33342 added, and the membrane−water partition coefficient of the drug. The membrane−water partition coefficient is often

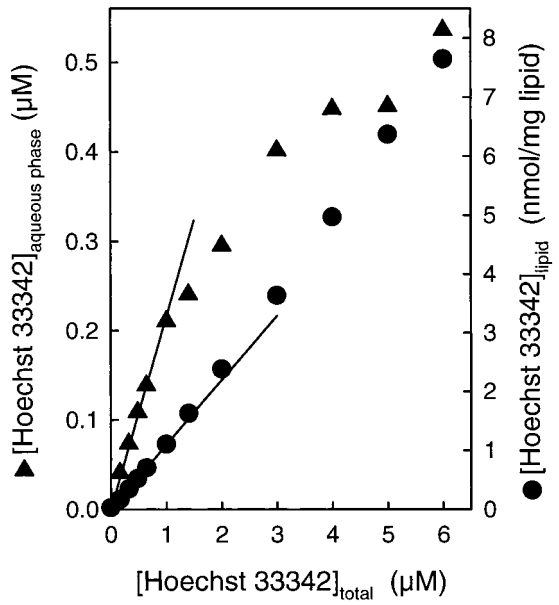

FIGURE 2: Partitioning of Hoechst 33342 in inside-out membrane vesicles. Concentration of Hoechst 33342 in the aqueous phase (▲) and in the phospholipid bilayer of inside-out membrane vesicles of *L. lactis* (0.72 mg of lipid/mL) (●) as a function of the total Hoechst 33342 concentration in the assay. A membrane-water partition coefficient of 5100 was determined for Hoechst 33342 from the linear part of the curves.

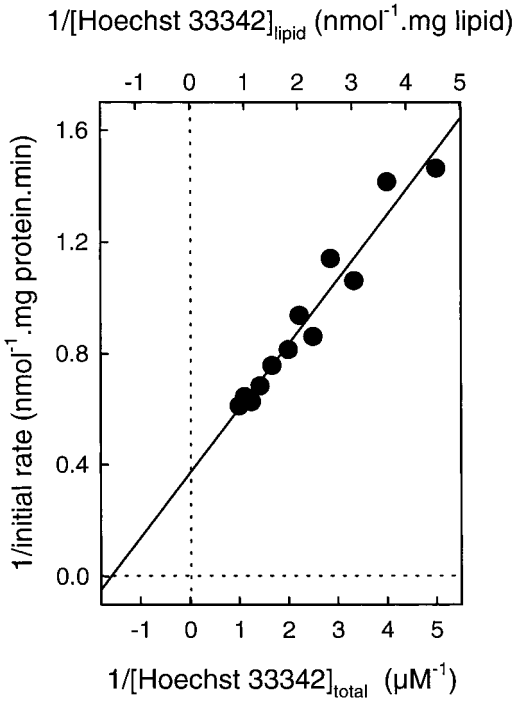

FIGURE 3: Kinetic characterization of LmrP-mediated Hoechst 33342 transport in inside-out membrane vesicles. Lineweaver−Burk plot of the initial rate of Hoechst 33342 transport in inside-out vesicles of *L. lactis* NZ9000/ pHLP5, in which a proton motive force (inside acid and positive) was generated by the F_0F_1 H^+-ATPase. The initial rate of Hoechst 33342 transport was determined over the first 15 s after addition of Mg^{2+}-ATP. Kinetic parameters were derived by linear regression analysis of the plotted data.

assumed to resemble the oil−water partition coefficient. However, De Young and Dill (*22*) showed that the partitioning of solutes into phospholipid bilayers is not well-represented by measurements of the partitioning of solutes into oil−water or octanol−water phases. To obtain a partition coefficient of physiological significance, the true partitioning of Hoechst 33342 in inside-out membrane vesicles of *L. lactis* was determined in this work. Figure 2 shows that the concentration of Hoechst 33342 in the lactococcal membrane increased linearly up to 1.1 nmol/mg of phospholipid when the total concentration of Hoechst 33342 in the assay was increased from 0 to 1 μM. From the slope of the curves in Figure 2, a membrane−water partition coefficient of 5100 was calculated for Hoechst 33342.

Kinetic Characterization of Hoechst 33342 Transport. The rate of LmrP-mediated Hoechst 33342 transport in inside-out membrane vesicles was measured as a function of the total Hoechst 33342 concentration. Since both the amount and the fluorescence of membrane-associated Hoechst 33342 increased linearly with the total concentration of Hoechst 33342 up to 1 μM (Figure 2 and data not shown), Hoechst 33342 concentrations between 0 and 1 μM were used in transport assays. Figure 3 shows the linear relationship between the inverse of the transport rate and the inverse of the Hoechst 33342 concentration, suggesting that the LmrP-mediated Hoechst 33342 transport obeyes Michaelis−Menten kinetics. From the Lineweaver−Burk plot an apparent affinity constant (K_{app}) of LmrP for Hoechst 33342 of 0.63 μM, and an apparent V_{max} of 2.71 nmol mg protein^{-1} min^{-1} were derived.

Inhibition of Hoechst 33342 Transport. The strong decrease in Hoechst 33342 fluorescence during LmrP-mediated transport in inside-out membrane vesicles of *L. lactis* offers a useful assay for the study of the drug specificity of LmrP. Several known substrates and modulators of the human multidrug transporter P-glycoprotein significantly affected the LmrP-mediated Hoechst 33342 transport: (i) the vinca-alkaloids vinblastin and vincristin, (ii) the 1,4-dihydropyridine nicardipin, (iii) the antimalarials chloroquine, quinine and quinidine, (iv) the phenylalkylamine verapamil, (v) the toxic compounds ethidium, rhodamine 6G, colchicine, and tetraphenyl phosphonium (TPP^+), and (vi) the adrenoreceptor antagonist prazosin. A typical result, obtained for verapamil, is shown in Figure 4. In this type of experiment, the half-maximal inhibitory concentration (IC_{50}) was determined for most of these drugs (Table 1).

These drugs can be subdivided in three categories, (i) drugs that directly interfere with the intrinsic Hoechst 33342 fluorescence and/or with Hoechst 33342 partitioning into the membrane, (ii) drugs that dissipate the driving force for LmrP-mediated transport, and (iii) drugs that inhibit LmrP by interacting with the protein. A number of experiments were performed to determine to which of these three categories the drugs belong. Colchicine, ethidium, prasozin, and rhodamine 6G interfered significantly with the fluorescence of Hoechst 33342, at concentrations below 2 μM (Table 1). These compounds were omitted from further studies. On the other hand, the presence of chloroquine, nicardipin, quinidine, quinine, TPP^+, verapamil, vinblastin, and vincristin did not affect the fluorescence of Hoechst 33342, at concentrations that exceeded their IC_{50} value by a factor of up to 4 (data not shown). The latter compounds did also not significantly affect the membrane partitioning of Hoechst 33342 under these conditions (data not shown).

The fluorescent probe Acridine orange was used to analyze the effects of the different drugs on the pmf in LmrP-

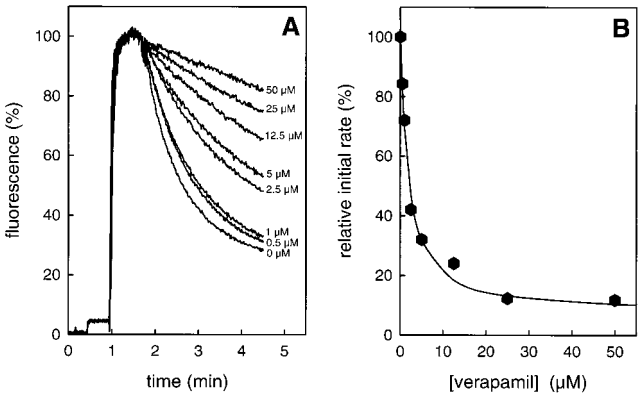

FIGURE 4: Inhibition by verapamil of LmrP-mediated Hoechst 33342 transport. (A) The rate of Hoechst 33342 transport in inside-out membrane vesicles of *L. lactis* was measured as described in the legend to Figure 1, in the absence or presence of increasing amounts of verapamil (0.5 to 50 μM). (B) Determination of the verapamil concentration giving 50% inhibition of the initial rate of LmrP-mediated Hoechst 33342 transport (IC_{50}). The initial rate of Hoechst 33342 transport was determined over the first 15 s after the addition of 0.5 mM Mg^{2+}-ATP. The IC_{50} was determined by nonlinear regression analysis using the general dose−response equation.

Table 1: The Effect of Inhibitors on the LmrP-Mediated Hoechst 33342 Transport in Inside-Out Membrane Vesicles

drug	IC_{50} (μM)[a]	effect on Hoechst 33342 fluorescence	effect on pmf	type of inhibition
colchicine	n.d.[b]	+	n.d.	
ethidium	n.d.	+	n.d.	
prazosin	n.d.	+	n.d.	
rhodamine 6G	n.d.	+	n.d.	
chloroquine	3.3	−	+[c]	
quinidine	22.2	−	+[c]	
nicardipin	1.7	−	−	noncompetitive
TPP^+	6.8	−	−	uncompetitive
quinine	4.8	−	−	competitive
verapamil	2.3	−	−	competitive
vinblastin	7.4	−	−	noncompetitive
vincristin	7.7	−	−	n.d.

[a] The IC_{50} is the concentration of the drug that inhibits LmrP-mediated Hoechst 33342 transport in inside-out membrane vesicles by 50% at a Hoechst 33342 concentration of 1 μM. [b] Not determined. [c] Tested at a concentration equal to the IC_{50}.

containing inside-out membrane vesicles. Generation of a ΔpH (inside acid) in inside-out membrane vesicles caused quenching of Acridine orange fluorescence (Figure 5, trace 1). Upon addition of valinomycin, the fluorescence decreased further due to the interconversion of the $\Delta\psi$ into the ΔpH. The subsequent dissipation of the pH gradient by the addition of nigericin resulted in the release of Acridine orange from the membrane vesicles with a concomitant increase in fluorescence (*22*). This change in Acridine orange fluorescence is indicative for the magnitude of the pmf that was generated. Figure 5 (trace 2 and 3) shows that verapamil and TPP^+, at concentrations of up to 4 times their IC_{50}, did not significantly affect the magnitude and composition of the pmf. Similar results were obtained for nicardipin, quinine, vinblastin, and vincristin (Table 1). However, quinidine (Figure 5, trace 4) and chloroquine already inhibited the generation of the pmf in inside-out membrane vesicles at a concentration that equalled the IC_{50} of both compounds.

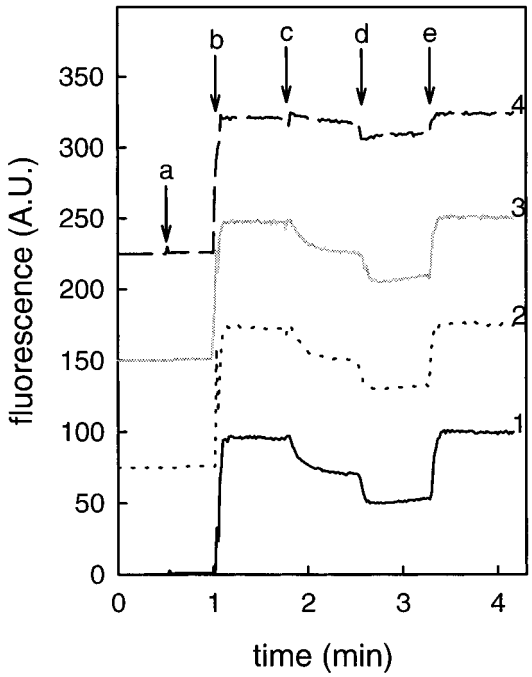

FIGURE 5: Effect of drugs on the proton motive force in inside-out membrane vesicles. Measurement of the ΔpH-dependent fluorescence of Acridine orange in inside-out membrane vesicles of *L. lactis*, in the absence (trace 1) or presence of 9.2 μM verapamil (trace 2), 27.2 μM TPP^+ (trace 3), or 22.2 μM quinidine (trace 4). For clarity of presentation, the traces are offset 75, 150 and 225 units from the solid black trace, respectively. Inside-out membrane vesicles were diluted to a concentration of 0.5 mg protein/ml in 50 mM potassium Hepes (pH 7.0) containing 2 mM $MgSO_4$, 8.5 mM NaCl, 0.1 mg/mL creatine kinase and 5 mM phosphocreatine (a). Acridine orange was added to a final concentration of 1.25 μM (b). A proton motive force (inside acid and positive) was generated by the F_1F_0 H^+-ATPase upon the addition of 0.5 mM Mg^{2+}-ATP (c). Valinomycin (d) and nigericin (e) were added to a final concentration of 1 μM each, to interconvert $\Delta\psi$ into ΔpH, and to dissipate the ΔpH, respectively.

Taken together, TPP^+, quinine, verapamil, vinblastin, vincristin, and nicardipin at micromolar concentrations did not affect the magnitude or composition of the pmf in inside-out membrane vesicles of *L. lactis*, the partitioning of Hoechst 33342 in the phospholipid bilayer of these membrane vesicles, or the fluorescence of Hoechst 33342. Therefore, the observed inhibition of LmrP-mediated Hoechst 33342 transport by these compounds must be due to direct drug−protein interactions.

Competitive, Noncompetitive, and Uncompetitive Inhibition. The mechanism of inhibition of LmrP-mediated Hoechst 33342 transport by TPP^+, quinine, verapamil, vinblastin, vincristin, and nicardipin was further characterized in experiments in which the concentration of both Hoechst 33342 and the inhibitor were varied. The Lineweaver−Burk plots obtained at different concentrations of quinine (Figure 6A) and verapamil (data nor shown) are characteristic for competitive inhibition. The K_{app} for LmrP-mediated Hoechst 33342 transport increased at increasing inhibitor concentrations, whereas the V_{max} for LmrP-mediated Hoechst 33342 transport remained unaltered. These results indicate that quinine and verapamil compete with Hoechst 33342 for binding to the same drug interaction site on LmrP (Figure 7, competitive inhibition) with K_i values of 2.1 and 2.3 μM, respectively. A different result was obtained for the inhibitors

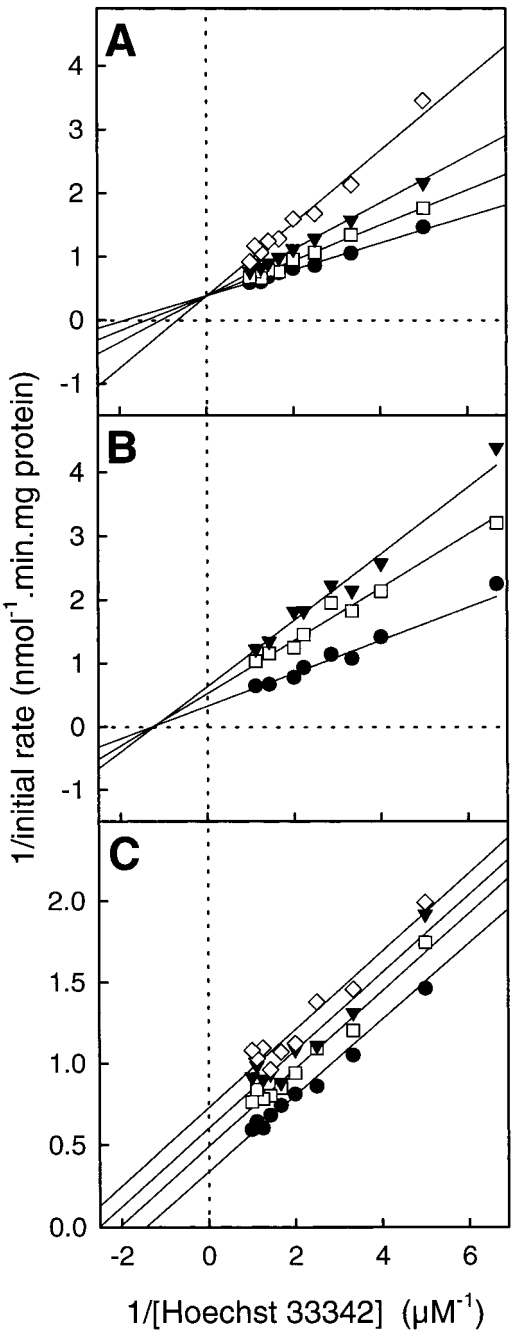

FIGURE 6: Kinetic characterization of the inhibition of LmrP-mediated Hoechst 33342 transport by quinine, nicardipin, and tetraphenyl phosphonium. A, competitive inhibition by quinine (●, no addition; □, 1 μM; ▼, 2 μM; 4 μM quinine). B, noncompetitive inhibition by nicardipin (●, no addition; □, 0.5 μM; ▼, 1 μM nicardipin). C, uncompetitive inhibition by TPP^+ (●, no addition; □, 2 μM; ▼, 4 μM;, 8 μM TPP^+).

nicardipin and vinblastin. A simple noncompetitive inhibition of Hoechst 33342 transport was observed for nicardipin (Figure 6B), whereas a mixed noncompetitive inhibition was found for vinblastin (data not shown). These results indicate that nicardipin binds to both the unliganded LmrP and the binary Hoechst 33342−LmrP complex with a K_i of 1.1 μM (Figure 7, noncompetitive inhibition). Furthermore, the affinity of the unliganded form of LmrP for vinblastin is higher ($K_i = 2.9$ μM) than that of the Hoechst 33342-bound form of LmrP ($K_i = 9.9$ μM). Finally, the parallel lines in the Lineweaver−Burk plot (Figure 6C) demonstrate uncompetitive inhibition of LmrP-mediated Hoechst 33342 transport by TPP^+. This type of inhibition suggests that TPP^+ is able to bind to the binary Hoechst 33342−LmrP complex with a K_i of 7.9 μM, but that TPP^+ is not able to bind to unliganded LmrP (Figure 7, uncompetitive inhibition). The observed noncompetitive and uncompetitive inhibition of LmrP-mediated Hoechst 33342 transport in inside-out membrane vesicles implies that LmrP must contain at least two drug interaction sites.

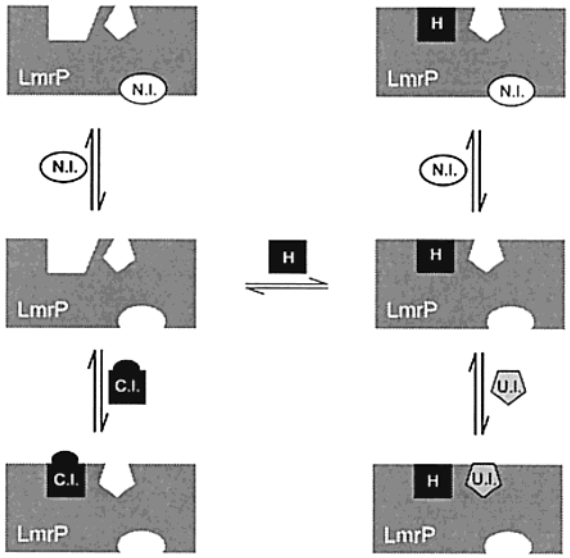

FIGURE 7: Competitive, noncompetitive, and uncompetitive inhibition of the binding of Hoechst 33342 to LmrP. Competitive inhibition: the inhibitor (C. I.) and Hoechst 33342 (H) compete for the same binding site on LmrP. Noncompetitive inhibition: the inhibitor (N. I.) can bind to both the unliganded form and the Hoechst 33342-bound form of LmrP. In the case that both forms of LmrP have an equal affinity for the inhibitor a classic noncompetitive inhibition is observed. If the affinities of the unliganded and Hoechst 33342-bound form of LmrP for the inhibitor are not equal, the inhibition is referred to as mixed. Uncompetitive inhibition: the inhibitor (U. I.) binds to the binary LmrP−Hoechst 33342 complex, but not to unliganded LmrP.

DISCUSSION

Hoechst 33342 is an excellent probe to study the transport properties of the multidrug transporter LmrP (*17*). To quantify the transport of Hoechst 33342 from the plasma membrane of *L. lactis*, its membrane−water partition coefficient was determined. The high partition coefficient of 5100 demonstrates that Hoechst 33342 strongly prefers the hydrophobic environment of the phospholipid bilayer.

Bolhuis et al. (*24*) provided evidence for the LmrP-mediated transport of the fluorescent substrate TMA-DPH from the cytoplasmic leaflet of the plasma membrane to the extracellular aqueous medium. Recently, a similar conclusion was reached for the transport of Hoechst 33342 by the human multidrug transporter P-glycoprotein (*19*). In view of the transport of drugs from the cytoplasmic leaflet of the plasma membrane, inside-out membrane vesicles provide an excellent model system to analyze the kinetic properties of LmrP. In inside-out membrane vesicles, the cytoplasmic leaflet is readily accessible to Hoechst 33342 from the external side. Therefore, the rate of LmrP-mediated drug transport is not limited by the slow flip-flop movement of Hoechst 33342 between the two leaflets of the plasma membrane.

Continuous fluorescence monitoring of LmrP-mediated Hoechst 33342 transport in inside-out membrane vesicles allowed the accurate measurement of initial rates of transport. LmrP-mediated Hoechst 33342 transport in inside-out membrane vesicles obeyed Michaelis−Menten kinetics with a K_{app} of 0.63 μM Hoechst 33342. In the transport assays, the total Hoechst 33342 concentration of 1 μM corresponds with a Hoechst 33342 concentration in the membrane of 1.1 nmol/mg lipid. Using this conversion factor, the K_{app} for Hoechst 33342 transport can also be expressed as 0.7 nmol Hoechst 33342/mg lipid or 0.5 mmol Hoechst 33342/mol lipid. Thus, LmrP is able to efficiently pump Hoechst 33342 from the plasma membrane against the high membrane−buffer partition coefficient of Hoechst 33342, with a low K_{app} of 1 Hoechst 33342 molecule per 2000 lipid molecules.

The Hoechst 33342 transport assay was used to study the drug specificity of LmrP in more detail. Nicardipin, quinine, TPP^+, verapamil, vinblastin, and vincristin inhibited LmrP activity at the protein level. The IC_{50} values for the inhibition of LmrP-mediated Hoechst 33342 transport, as determined from dose−response curves, correspond well to the K_i values for this inhibition, as determined from Lineweaver−Burk plots. Interestingly, the IC_{50} values for inhibition of LmrP-mediated Hoechst 33342 transport by these drugs are in the same range as the reported IC_{50} values for inhibition of the human multidrug resistance P-glycoprotein-mediated transport of vinblastin (*25*), and the concentrations of these drugs required for half-maximal activation of the ATPase activity of P-glycoprotein (*26*−*28*). The analysis of the mechanism of inhibition of LmrP-mediated transport of Hoechst 33342 by verapamil, quinine, nicardipin, vinblastin, and TPP^+ revealed competitive inhibition by verapamil and quinine, noncompetitive inhibition by nicardipin and vinblastin, and uncompetitive inhibition by TPP^+. These results suggest the presence of at least two drug interaction sites in LmrP, which may represent distinct drug binding sites on the protein or may represent drug binding regions within a common hydrophobic binding pocket.

Studies on the drug-stimulated ATPase activity, drug binding, and drug transport by the human P-glycoprotein (*19*, *28*−*31*) and on rhodamine 123 transport by the yeast multidrug transporter Pdr5p (*32*), support a model in which ABC-type MDR transporters have more than one drug interaction site. To our knowledge this is the first paper reporting the presence of multiple drug interaction sites in a secondary multidrug transporter.

Recently, 3D-structure analysis of the *Bacillus subtilis* transcriptional regulator BmrR (*33*) and site-directed mutagenesis studies on the *Escherichia coli* multidrug resistance protein MdfA (*34*) have revealed that a negatively charged glutamate residue in a hydrophobic environment plays a key role in the cation selectivity of these proteins. Similarly, the three negatively charged residues in putative transmembrane segments of LmrP (D142 in TM5, E327 in TM10, and E388 in TM12) may play a role in the binding of cationic drugs by LmrP. The molecular basis of drug−protein interaction in LmrP and other MDR transporters will be an intriguing area of research which may allow the rational development of new antibiotics and cytotoxic drugs that are not extruded from the cell.

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