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Verapamil competes with doxorubicin for binding to anionic phospholipids resulting in increased internal concentrations and rates of passive transport of doxorubicin

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Abstract

It is well documented that the Ca^{2+} channel antagonist verapamil can reverse multidrug resistance in cancer cells by decreasing P-glycoprotein mediated drug efflux. However, less information is available about effects of verapamil on drug–phospholipid interactions and on passive diffusion of drugs across the membrane, which both may play an important role in resensitizing cells to anti-cancer drugs. Therefore we studied the binding of verapamil to model membranes (large unilamellar vesicles) composed of various phospholipids and biological membranes. An increase of the amount of anionic phospholipids resulted in an enhanced binding of verapamil. Competition between verapamil and the anti-cancer drug and P-glycoprotein substrate doxorubicin for binding to anionic phospholipids was observed in model membranes composed of synthetic lipids, or composed of native *Escherichia coli* phospholipid mixtures, and in cytoplasmic membrane vesicles of this organism. Furthermore, verapamil specifically increased the rate of passive diffusion of doxorubicin across model membranes containing anionic phospholipids. It can be concluded that besides the decrease of P-glycoprotein mediated efflux at least two other effects may account for an increase of the internal (free and DNA-bound) doxorubicin concentration in the presence of verapamil; (i) a decrease of binding to anionic phospholipids in plasma-and intracellular membranes and (ii) an increase of the rate of passive import of doxorubicin across the plasma membrane.

Keywords: Anionic phospholipid; Doxorubicin; Drug–membrane interaction; Multidrug resistance; Passive diffusion; P-glycoprotein; Verapamil

1. Introduction

Doxorubicin is a potent and widely applied anti-cancer drug, that interacts with DNA, DNA-associated proteins [1,2] and membranes. In membranes, anionic phospholipids are important targets [3–7], and membrane binding involves electrostatic interactions as well as penetration of the electrostatically-bound drug between the acyl chains [6,7]. The uncharged form of the drug is transported via passive diffusion across the membrane [8], which is thought to be the main route of entry into a cell. The driving force for this process is the concentration gradient, maintained by a pH gradient (inside acid) and initially also by accumulation in acidic organelles and DNA-binding [1–3,8–11]. Efflux of doxorubicin can occur via passive diffusion or by an active drug pump (for a review see [12]). Acquired resistance of cancer cells against drugs is frequently accompanied by the appearance of a 170 kDa plasma membrane protein, the P-glycoprotein (Pg-170), which utilizes ATP to actively extrude a variety of structurally non-related drugs, including anthracyclines such as doxorubicin [12]. Usually, these drugs are moderately hydrophobic. They have a planar geometry, and are often positively charged on a (protonable) nitrogen atom [12]. Multidrug resistance (mdr) is an undesirable phenomenon in anti-cancer treatment and many strategies developed to overcome this resistance include the use of so-called

Abbreviations: CF, 6-carboxyfluorescein; CL, cardiolipin; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOPS 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; LUVET, large unilamellar vesicles prepared by extrusion; mdr, multidrug resistance; Pipes, 1,4-piperazinediethanesulfonic acid; SUV, small unilamellar vesicles (prepared by sonication).

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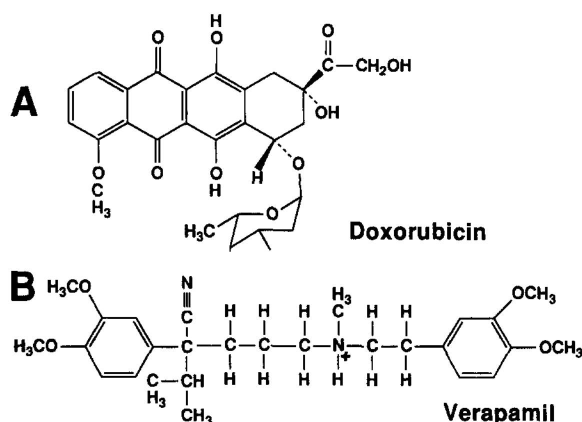


Fig. 1. Structures of (A) doxorubicin and (B) verapamil.

chemo-sensitizers or multidrug resistance reversal agents [12–14].

Verapamil is one of the best studied chemo-sensitizers [13,15]. It is a Ca^{2+} channel antagonist, which has in common with P-glycoprotein substrates that it is moderately hydrophobic and positively charged at physiological pH [14]. In Fig. 1 the structures of verapamil and doxorubicin are shown. It is assumed that verapamil reverses multidrug resistance by inhibiting P-glycoprotein mediated drug efflux. This occurs, depending on the drug investigated, by a direct competition for drug efflux or by binding at the enzyme at a different binding site [16–20]. In contrast to anti-cancer drugs such as doxorubicin, verapamil does not bind to DNA and is not (or much less) toxic to cells [14]. Upon addition of verapamil resistant cell lines reverse with respect to morphology [21], drug transport rates [22–24], steady-state intracellular drug concentrations [25], and intracellular distribution of drugs [26–29] and become comparable to the sensitive parental strains.

Studies on the mechanism of mdr reversal by verapamil have focused on the effect on active efflux. However, very little information is available about the binding of verapamil to membranes, its effect on the intrinsic properties of membranes, and the potential consequences of verapamil-phospholipid interactions for sensitivity to anti-cancer drugs [30]. Therefore, we first investigated the binding behavior of verapamil towards model- and biological membranes, paying special attention to negatively charged phospholipids. Subsequently, competition between doxorubicin and verapamil for binding was studied, using *Escherichia coli* inner membrane vesicles as a model for biological membranes. In previous studies this organism was used to study the influence of the lipid composition on drug interaction in biological membranes, since in contrast to mammalian cancer cells the phospholipid composition of this organism can easily be manipulated [7]. Finally, the effect of verapamil on the rate of passive transport of doxorubicin was studied. The implications of our findings for the effects of verapamil on intracellular doxorubicin concentrations and on drug pumping by the P-glycoprotein are discussed.

2. Materials and methods

2.1. Growth of Escherichia coli and preparation of inner membrane vesicles

Escherichia coli strain MRE600 was grown in Luria broth (10 g l^{-1} bacto-tryptone (Sigma), 5 g l^{-1} yeast extract (Sigma), and 10 g l^{-1} NaCl). Cells were grown at 37°C until late logarithmic phase (A_{660} 0.6–0.8), chilled on ice, and harvested by low-speed centrifugation at 4°C.

Spheroplasts and right-side out inner membrane vesicles were prepared and isolated as described [31]. Inside-out inner membrane vesicles were prepared with modifications as described [32,33]. Inside-out and right-side out inner membrane vesicles were washed in Pipes buffer (10 mM Pipes, 100 mM NaCl, 1 mM EGTA, pH 7.4), frozen in liquid nitrogen and stored at −80°C. The total P_i content was determined according to [34]. Under these circumstances the amount of DNA-P_i is 2–6% in inside-out and 6–9% in right-sideout inner membrane vesicles and the amount of 2-keto-3-deoxyoctonate 0.01–0.03 mol/mol total P_i [7].

2.2. Isolation and determination of the composition of Escherichia coli lipids

Total lipid extracts were prepared from washed *E. coli* cells [35] by extracting the lipids [36] and removing proteins and neutral lipids on a silica column. Lipids were separated by two dimensional high-performance thin-layer chromatography (Kieselgel 60, Merck, Germany) with chloroform/methanol/ammonia/water (68:28:2:2, v/v) in the first dimension and chloroform/methanol/acetic acid (65:25:10, v/v) in the second dimension. Spots were visualized with I_2 and excised for P_i quantification.

2.3. Preparation of small unilamellar vesicles (SUV) and large unilamellar vesicles (LUVET) with or without enclosed DNA

SUV composed of DOPC or DOPG were prepared in Pipes buffer by sonication as described [6]. LUVET used in the binding experiments and carboxyfluorescein efflux measurements were prepared in Pipes buffer by extrusion through polycarbonate filters with 400 nm pores (Costar-Nucleopore Europe, Badhoevedorp, the Netherlands) as described previously [7]. LUVET with (or as a control without) DNA enclosed used for transport experiments were prepared by the method described by Speelmans et al. [37].

2.4. Binding assays

Doxorubicin binding to ∅ 400 nm LUVET and *E. coli* inner membrane vesicles was assayed according to de Wolf et al. [7], by mixing 100 μl of membrane suspensions (10 mM P_i, which is about 7.5–8 g lipid per l) with 900 μl of doxorubicin solution in the range of 25 to 250 μM (final concentration) (both in Pipes buffer), incubating duplicate samples for 1 h in the dark and at room temperature, and pelleting the vesicles and bound drug (60 min at 436000 × *g* and 20°C, Beckman TLA 100.2 rotor). The top 800 μl was collected for determination of free drug. The amount of lipid-P_i in the supernatant was negligible (less than 0.5% of the total P_i). Blanks without lipids were used to determine the amount of total drug. Corrections were made for binding of doxorubicin to tubes (which was

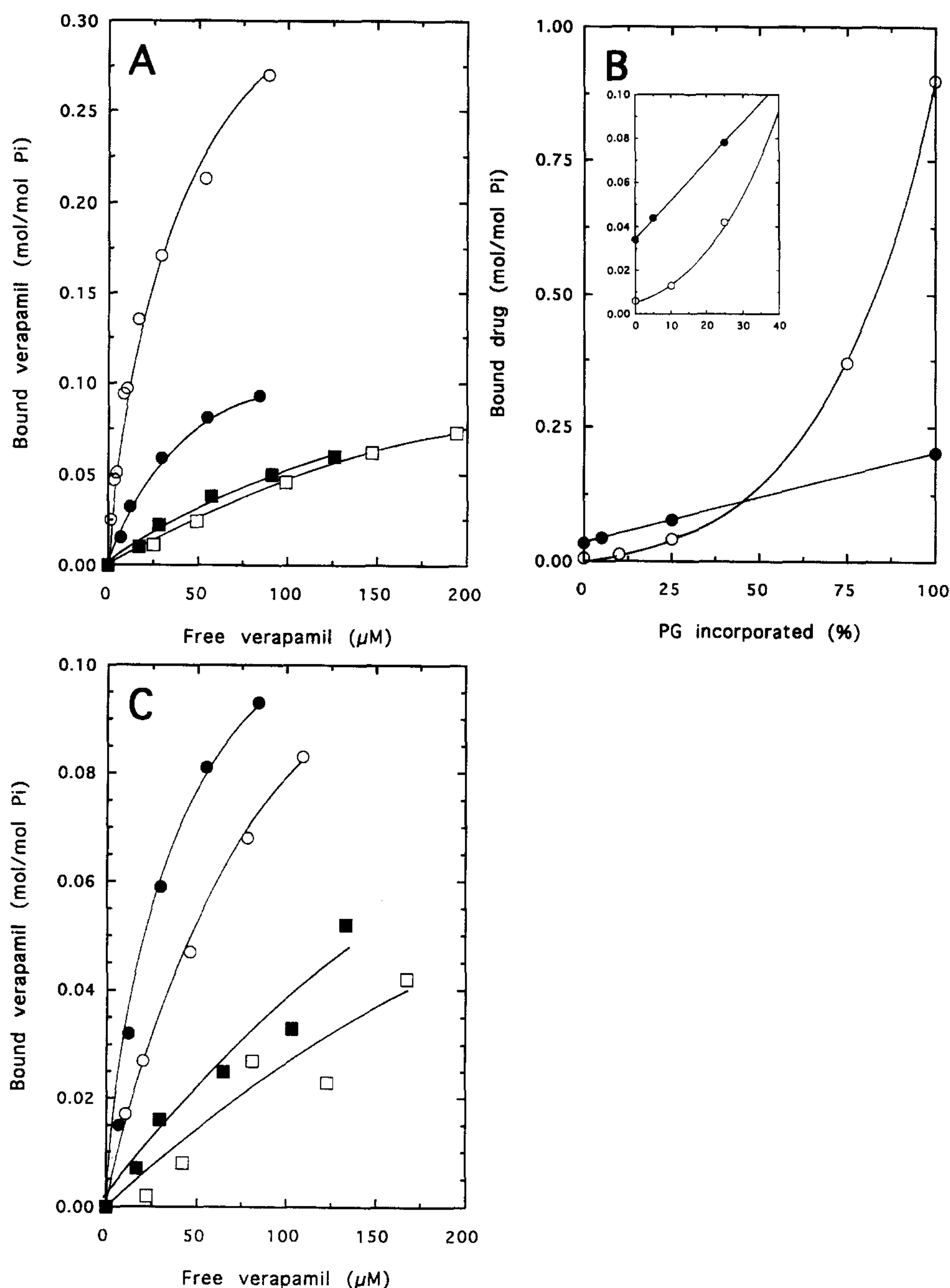


Fig. 2. Binding of verapamil to membranes. (A) Binding of verapamil to LUVET composed of DOPC containing 0% (□), 5% (■), 25% (●), or 100% (○) of the anionic phospholipid DOPG. (B) Binding of verapamil (●) or doxorubicin (○) to DOPC LUVET with an increasing amount of incorporated DOPG. Drug binding was compared at a free drug concentration of 50 μM. The doxorubicin binding data were obtained from [7]. The inset shows an enlargement at low % DOPG graph (C) Binding of verapamil to LUVET composed of DOPC/DOPG (3:1) (●), DOPE/DOPG (3:1) (○), LUVET composed of *E. coli* lipids (■), and to inside-out inner membrane vesicles of *E. coli* (□).

low). The concentration of doxorubicin was determined after dilution to 5–10 μM drug, by light absorption at 480 nm using an extinction coefficient of $1.06 \cdot 10^4$ M^{-1} cm^{-1}.

Verapamil binding experiments were performed as described above for the doxorubicin binding assay. Verapamil was solubilized in 96% ethanol to a concentration of about 15 mM and subsequently diluted in water to 5 mM final concentration and used within 4 h. Verapamil was added to the LUVET (approximately 1 mM lipid-P_i) to final concentrations of 50 to 250 μM. To the control LUVET the same volume of ethanol/water, 1:2 (v/v) was added. The concentration of verapamil was determined by light absorption at 279 nm using an extinction coefficient of $6.09 \cdot 10^3$ M^{-1} cm^{-1} [38].

Verapamil and doxorubicin binding competition experiments were performed as follows: After incubation of vesicles with doxorubicin for 1 h at room temperature in the dark verapamil was added, and incubation was prolonged for 1 h. Upon reversing the sequence of addition no differences were observed. The presence of verapamil did not influence the molar extinction coefficient of doxorubicin. Verapamil measurements in the presence of doxorubicin, on the other hand, had to be corrected for the presence of doxorubicin ($A_{279,dox} = 0.70$ $A_{480,dox}$).

2.5. Transport experiments

Transport of doxorubicin across model membranes in which DNA was enclosed, was determined from the rate of fluorescence decrease (Exc. 490 nm, Em. 594 nm) in an Amino fluorimeter as described [37]. The fraction of free doxorubicin was determined in separate equilibrium dialysis experiments and corrections were made for changes in quantum yield and the fraction of free doxorubicin by binding to membranes upon addition of DNA-containing LUVET [37]. The permeability coefficient was calculated according to [37] and an area of 31.5 Å^2 per lipid-P_i for two opposing phospholipid molecules (in a bilayer) was assumed [37].

2.6. Other methods

Carboxyfluorescein efflux experiments were performed as described previously [6].

2.7. Materials

Doxorubicin (Pharmachemie, the Netherlands) was shown to be pure by high-performance thin-layer liquid chromatography [3] and dissolved just before use. DNA (Type I from calf thymus), DNAse I (type DN-25 from bovine pancreas) and verapamil-hydrochloride were obtained from Sigma, USA. Phospholipids were obtained from Avanti Polar-Lipids, USA, and checked for purity by thin-layer chromatography. 6-Carboxyfluorescein was obtained from Kodak, USA, and purified according to [39].

3. Results

3.1. Binding of verapamil to model membranes containing various amounts of anionic phospholipids and biological membranes

From previous studies it is known that binding of doxorubicin to membranes is largely determined by electrostatic interactions, and that binding increases with the amount of anionic phospholipids [7]. Therefore, we studied binding of verapamil to LUVET composed of the zwitterionic phospholipid DOPC containing a variable amount of the negatively charged phospholipid DOPG. An enhanced binding of verapamil to the membranes was observed upon increasing the amount of incorporated DOPG, indicating the involvement of electrostatic interactions (Fig. 2A). Under the experimental conditions of Fig. 2A 72% of the added verapamil (250 μM) was bound to the 100% DOPG LUVET, 50% to the 25% DOPG LUVET, and 33% to the 100% DOPC LUVET. Compared to doxorubicin, binding of verapamil to LUVET composed of 100% DOPC was higher. At 50 μM free drug (and in the presence of 1 mM phospholipid) the amount of drug bound per mol DOPC was 0.006 and 0.034 mol for doxorubicin and verapamil, respectively (Fig. 2B). At DOPG concentrations below 55 to 60% the amount of verapamil bound to the membranes remained higher than doxorubicin. Above this concentration the situation was reversed and binding of doxorubicin was higher. The relationship between binding of verapamil and the concentration of incorporated DOPG was linear, whereas a cooperative binding behavior of doxorubicin was observed (Fig. 2B). It was not possible to determine binding of verapamil at saturation levels, due to the low solubility of verapamil in buffer. At pH 7.4 the solubility of verapamil is about 800 μM [38].

Next, we compared binding of verapamil to model and *E. coli* cytoplasmic membranes. The different membranes employed all contained approximately 25% negatively charged phospholipids. Since phosphatidylethanolamine is the predominant zwitterionic phospholipid in *E. coli*, LUVET composed of DOPE/DOPG (3:1) were included next to LUVET composed of native *E. coli* phospholipids. The phospholipid composition (on P_i basis) of these LUVET was 76% PE, 16% PG, and 6% of the negatively charged cardiolipin (CL). The inner membrane vesicles of *E. coli* contained 73% PE, 11% PG, and 14% CL, respectively. A decreased binding of verapamil to LUVET composed of native *E. coli* phospholipids and to inner membrane vesicles (with the latter displaying the lowest binding level) than to LUVET composed of DOPC/DOPG lipids was observed (Fig. 2C). This difference was not due to the presence of PE instead of PC, since binding of verapamil

to DOPE/DOPG vesicles was only slightly lower than to DOPC/DOPG LUVET (Fig. 2C). No differences were observed in verapamil binding when inside-out inner membrane vesicles or right-side out inner membrane vesicles of *E. coli* were used (data not shown). Note that, as opposed to verapamil, doxorubicin binding is higher in the *E. coli* derived membrane systems than in the model DOPC/DOPG systems [7].

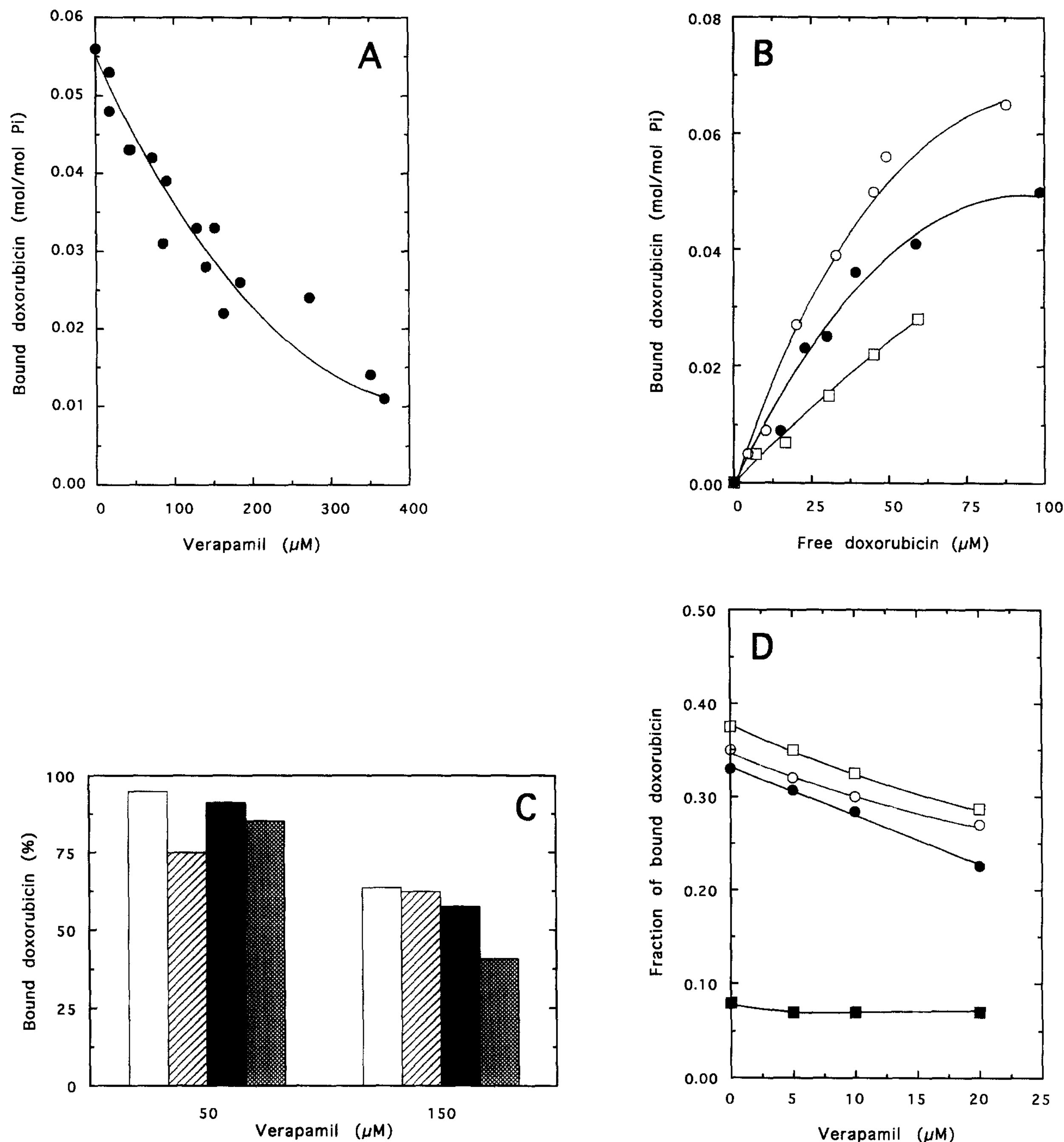


Fig. 3. Competition between binding of doxorubicin and verapamil. (A) Binding of doxorubicin (100 μM) in the presence of increasing concentration of verapamil in LUVET composed of DOPC/DOPG (3:1). (B) Doxorubicin binding to LUVET composed of DOPC/DOPG (3:1) in the presence of 0 (○), 50 (●), and 150 μM (□) verapamil. The data represent the average of four experiments, with a S.D. < 10%. (C) Binding of doxorubicin to LUVET composed of DOPC/DOPG (3:1), DOPE/DOPG (3:1), or *E. coli* lipid, and *E. coli* inside-out inner membrane vesicles, in the presence of 50, and 150 μM verapamil, relative to 0 μM verapamil (100%). The amount of bound doxorubicin is given at a free doxorubicin concentration of 50 μM. The data were obtained from graphs as represented under B. White bars: DOPC/DOPG (3:1), Arced bars: DOPE/DOPG (3:1), Black bars: *E. coli* lipid, double arced bars: *E. coli* membrane vesicles. (D) Fraction of bound doxorubicin (at a total concentration of 1 μM) in the presence of 106 μM DOPC (■), DOPG/DOPC (1:1) (●), DOPS/DOPC (1:1) (○), or DOPS/DOPE (1:1) (□).

3.2. Competition between doxorubicin and verapamil for binding to model and biological membranes

In the presence of 100 μM doxorubicin a decreased binding of verapamil was observed as compared to absence of doxorubicin (data not shown). This effect was observed in LUVET composed of DOPC containing various amounts of DOPG, LUVET composed of (3:1) DOPE/DOPG, or *E. coli* lipids, and in inner membrane vesicles of *E. coli*. Due to the interference of doxorubicin in the verapamil concentration determinations, no accurate quantitative data can be given. However, the decrease of verapamil bound to the membrane upon addition of 100 μM doxorubicin seemed to be largest when inner membrane vesicles were used and smallest in DOPC/DOPG LUVET, in accordance with the difference in binding affinities of verapamil and doxorubicin.

The reverse effect was also observed. Binding of doxorubicin (total concentration 100 μM) to membranes decreased in the presence of verapamil (Fig. 3A). The relative decrease of doxorubicin binding upon addition of verapamil is highest in LUVET composed of 25% DOPG and 75% DOPC. In LUVET composed of 100% DOPG the number of binding sites for doxorubicin present is so high, that the relative decrease of doxorubicin binding upon addition of verapamil is low. At low concentrations of PG (0 to 5 mol%) the binding of doxorubicin is intrinsically low and a significant decrease of binding is hard to observe (data not shown). Fig. 3B shows that verapamil efficiently competes with doxorubicin binding over a large concentration range. This concentration-dependent decrease of bound doxorubicin was also observed in other model and biological membranes and a summary of the results is shown in Fig. 3C. The largest effect was observed in DOPC/DOPG (3:1) LUVET, and the smallest in inner membrane vesicles of *E. coli*. This is in accordance with the binding behavior of verapamil and doxorubicin (see above). The concentration of verapamil at which the amount of bound doxorubicin (at 50 μM free doxorubicin and at 1 mM lipid-P_i) was 50% compared to that in the absence of verapamil appeared to be 130, 182, 189, and 206 μM for LUVET composed of DOPC/DOPG, DOPE/DOPC, *E. coli* lipids, and *E. coli* inner membrane vesicles, respectively.

Also under physiologically more relevant low doxorubicin concentrations, competition between verapamil and doxorubicin occurred. In the presence of 106 μM lipid-P_i 8% of the added doxorubicin (1 μM) was bound to DOPC vesicles, whereas 42% was bound to DOPG/DOPC LUVET. Also in LUVET containing the negatively charged phospholipid DOPS more doxorubicin was bound. PS and PE are phospholipids present in the inner leaflet of a mammalian plasma membrane and are included here because doxorubicin transport experiments were also performed with these preparations (see below). At increasing verapamil concentration the amount of free doxorubicin in the presence of DOPC LUVET remained the same, whereas the amount of free doxorubicin increased in the presence of LUVET containing negatively charged phospholipids (Fig. 3D).

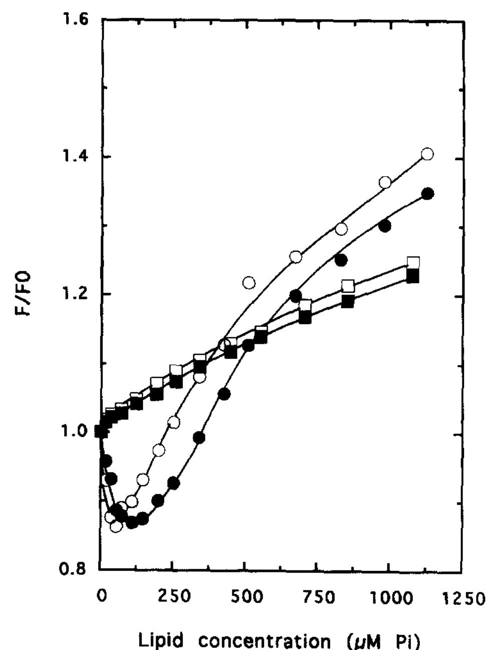
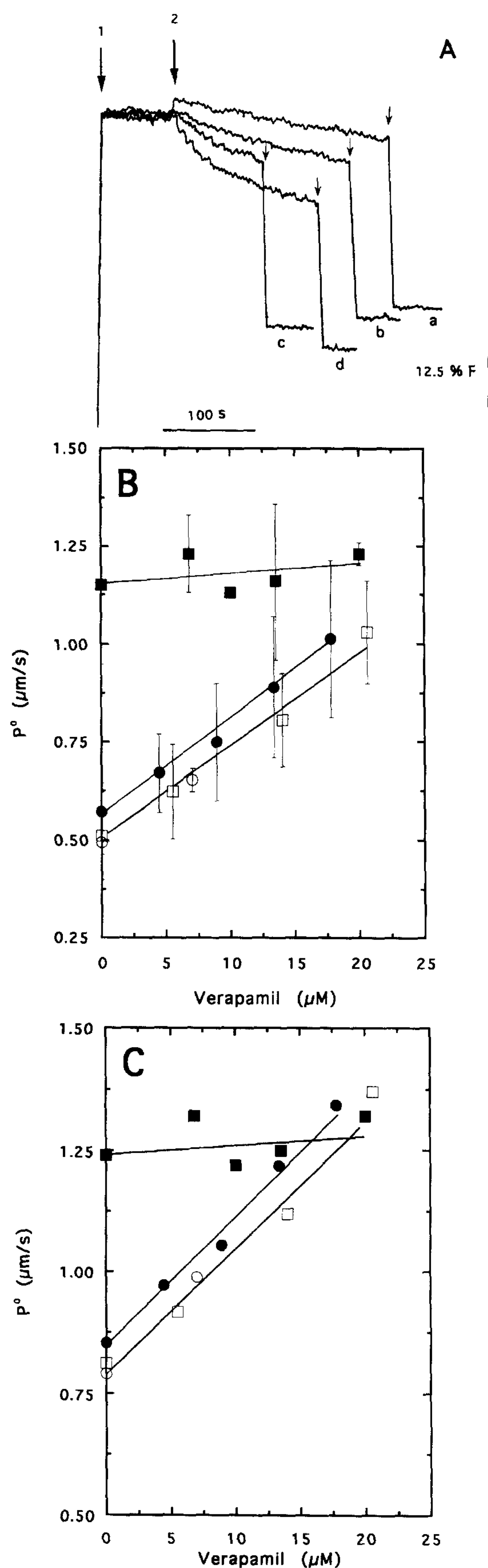


Fig. 4. Effect of verapamil on the fluorescence characteristics of doxorubicin in the absence or presence of membranes. Titration of 10 μM doxorubicin with an increasing amount of lipid-P_i of DOPG SUV (○,●) or DOPC SUV (□,■), in the presence (closed symbols) or absence (open symbols) of 30 μM verapamil.

3.3. Effect of verapamil on the fluorescence behavior of doxorubicin in the presence of phospholipids

The fluorescence behavior of doxorubicin can give information about the localization of the drug in the membrane [6] and so the effects of verapamil on this behavior should provide us with information about the location of binding competition. At high ratios of doxorubicin/negatively charged phospholipid self-association of doxorubicin occurs at the surface of small unilamellar vesicles (SUV) resulting in self quenching due to stacking of the fluorescent aglycon moiety (Fig. 4). Upon dilution of bound doxorubicin by titrating more lipid into the cuvette this self quenching is relieved and fluorescence becomes even higher than in the absence of SUV, due to the fact that the aglycon moiety is now residing in the apolar environment of the membrane [6,29]. This phenomenon is specific for SUV containing anionic phospholipids and is not observed for DOPC. The lower affinity of doxorubicin for DOPC results only in a gradual an less pronounced increase of fluorescence. Verapamil caused a pronounced shift in the titration curve (Fig. 4) for DOPG and a smaller shift in that for DOPC. At low drug/phospholipid ratios the increase of fluorescence was less in the presence of verapamil. At high drug/negatively charged phospholipid ratios, the maximal self quenching of 10 μM doxorubicin now oc-



curred at a DOPG concentration of 110 μM P_i instead of 56 μM. The emission spectrum and quantum yield of free doxorubicin was not influenced by verapamil (data not shown). It can be concluded from these experiments that verapamil competes with doxorubicin for (deep) hydrophobic binding sites and, since it is assumed that self quenching is due to drug stacking at the membrane surface [6,40], the present results also indicate that there is competition in the presence of anionic phospholipids for binding sites at the surface. In the presence of verapamil an increased lipid concentration is needed to induce the maximal stacking of doxorubicin.

Competition between verapamil and doxorubicin for hydrophobic binding sites is also observed at lower total doxorubicin concentrations. Upon addition of 200 μM SUV composed of DOPG to 1 μM free doxorubicin (high lipid/drug ratio) an increase of doxorubicin fluorescence was observed. However, this DOPG dependent increase of doxorubicin fluorescence was absent when 30 μM verapamil was present before DOPG was added, indicating that verapamil prevented the insertion of the apolar part of doxorubicin into the membrane (data not shown). When verapamil was added to doxorubicin after the addition of DOPG, i.e., when the doxorubicin was already inserted into and/or bound to DOPG SUV, a decrease of doxorubicin fluorescence up to the fluorescence level of free doxorubicin was observed, indicating that doxorubicin was removed from the (hydrophobic part of the) membrane, and/or shifted to the surface of the SUV (data not shown).

3.4. Increase of the rate of passive transport of doxorubicin by verapamil across model membranes containing anionic phospholipids

The rate of passive diffusion of doxorubicin across membranes containing anionic phospholipids is lower than the rate across membranes containing zwitterionic phospholipids [37]. This is due to (i) a decreased concen-

Fig. 5. Passive diffusion of doxorubicin across membranes of DNA containing LUVET. (A) Time-based fluorescence trace of doxorubicin upon addition of 200 μM DOPG/DOPC (1:1) LUVET-P_i containing DNA in the absence (trace a) or presence of 5 μM (trace b), 13 μM (trace c), and 20 μM verapamil (trace d). Experiments were performed as described under Materials and methods at 25°C. 1 μM doxorubicin was added to buffer containing verapamil or (as a control) 0.2% ethanol (timepoint 1), giving rise to an immediate increase in fluorescence. After the fluorescence signal was stable LUVET were added (timepoint 2). Finally, 0.05% Triton X-100 was added to permeabilize the LUVET (arrow). (B,C) Permeability coefficient of doxorubicin (μm s^{-1}) across DOPC (■), DOPG/DOPC (1:1) (●), DOPS/DOPC (1:1) (□), or DOPE/DOPS (1:1) LUVET (○), in the presence of an increasing amount of verapamil. The data represent an average of 4 to 12 experiments, and are not corrected for drug binding (B) or corrected for drug binding (C) by dividing the values of the permeability coefficients in (B) by the fraction of free doxorubicin. The fraction of free doxorubicin at increasing concentrations of verapamil and in the presence of 106 μM outer leaflet lipid can be deduced from Fig. 3D and can be determined accurately (S.D. < 3%).

tration of free doxorubicin in the presence of negatively charged phospholipids, and (ii) the intrinsic inhibitory effect of doxorubicin bound to the membrane on its passive diffusion [37]. Therefore, we were interested in the effect of verapamil on passive diffusion of doxorubicin. The decrease of fluorescence of doxorubicin upon addition of LUVET with enclosed DNA is a measure for passive diffusion of doxorubicin across the membrane. When LUVET containing 50% of DOPC and 50% of the negatively charged DOPG were used, an increase on the rate of transport was observed in the presence of an increasing concentration of verapamil (Fig. 5A). After calculation of the permeability coefficient for doxorubicin across the membranes without taking into account the change of fraction of transportable (free uncharged) doxorubicin, the rate of doxorubicin diffusion is much lower across membranes containing negatively charged phospholipids (DOPG or DOPS) than across membranes composed of the zwitterionic DOPC (Fig. 5B, [37]). Upon addition of an increasing amount of verapamil the transport rate was increased across membranes containing negatively charged phospholipids, but not influenced in membranes composed of zwitterionic lipids (Fig. 5B). After correction for differences in fraction of free drug at each different verapamil concentration a higher permeability coefficient in LUVET composed of anionic phospholipids was calculated, but the permeability coefficient was still lower in anionic phospholipid containing LUVET than in 100% DOPC LUVET (Fig. 5C, [37]). At increasing verapamil concentrations permeability coefficients for DOPG or DOPS containing LUVET now increased to values observed for DOPC containing LUVET (Fig. 5C). The enhancing effect of verapamil on passive doxorubicin transport was also observed for LUVET composed of DOPE/DOPS, as a model of the inner leaflet of the plasma membrane of mammalian cells.

Besides the changes in free doxorubicin, the presence of verapamil also had effects on the quantum yield of doxorubicin outside the LUVET and in the lumen of the DNA containing LUVET. (This is also reflected in the traces of Fig. 5A.) The external quantum yield was decreased slightly in the presence of verapamil, due to the fact that less doxorubicin was inserted in the hydrophobic part of the membrane (see also section 3.3). The internal quantum yield was decreased slightly, because a relatively larger amount of doxorubicin was now bound to the DNA instead of to the membrane. However, the effects of these changes in internal and external quantum yield were taken into account in the calculation of the permeability coefficient [37] for Fig. 5B and C and were low compared to the changes in the fraction of free doxorubicin.

4. Discussion

This paper describes the effects of the multidrug reversal agent verapamil on the binding of the anti-cancer drug doxorubicin to membranes and on the rate of passive diffusion of doxorubicin. We first studied the binding behavior of verapamil to model- and biological membranes and compared it with the binding behavior of doxorubicin. Both compounds are amphipathic and positively charged at physiological pH. The pK_a of verapamil is 8.45 [41], comparable with the pK_a of doxorubicin (8.3), so for both drugs the positively charged species is the predominant one at physiological pH. Both drugs showed an enhanced binding in the presence of an increased concentration of negatively charged phospholipids, indicating that electrostatic interactions are involved in membrane binding for the two drugs. For verapamil the relationship between membrane binding and concentration of negatively charged phospholipids appears to be linear, whereas for doxorubicin a cooperative binding behavior is observed [7] probably due to the ability to form stacks at the membrane surface [7,40]. Apparently verapamil does not form these stacks. Below 55–60% negatively charged phospholipids, verapamil bound more strongly to the membranes than doxorubicin, apparently because hydrophobic forces are relatively more important for membrane-binding of verapamil as compared to doxorubicin. This is reflected in the octanol/water partition coefficients, P, which are $5.5 \cdot 10^2$ [41] and $2.5 \cdot 10^{-1}$ [14], for verapamil and doxorubicin, respectively. When various membranes, containing approximately the same amount of negatively charged phospholipids, were used to study drug binding a difference between verapamil and doxorubicin was observed. An increase in binding of verapamil is observed in the sequence: *E. coli* inner membrane vesicles, LUVET composed of *E. coli* lipid, DOPE/DOPG, and DOPC/DOPG. At 50 μM free verapamil (and 1 mM phospholipid present) the amount of verapamil bound per mol phospholipid was 0.011, 0.020, 0.051, and 0.079, respectively. Doxorubicin exhibits the opposite behavior, namely an increase in binding in the sequence: LUVET composed of DOPC/DOPG, *E. coli* lipids, and *E. coli* inner membrane vesicles [7]. At 50 μM free doxorubicin (and 1 mM phospholipid present) the amount bound per mol phospholipid is 0.032, 0.22, and 0.93, respectively [7]. This difference could be due to a different sensitivity of verapamil binding to the presence of membrane proteins (in case of inner membrane vesicles) and/or to membrane order, which increases with the sequence DOPC/PG, DOPE/PG, lipids extracted from *E. coli* [35]. The latter proposal is in accordance with other studies in which an inhibiting effect of the presence of cholesterol was observed on Ca^{2+} channel antagonists binding to membranes [42]. In contrast to verapamil, doxorubicin binding is less sensitive to membrane order. A higher membrane order promotes stacking of doxorubicin at the membrane surface by decreasing the penetration of the drug [43]. Thus, high affinity binding sites could be formed at the surface instead of in the hydrophobic part of the membrane. In accordance, the binding of doxorubicin to the various membrane types is

equal at low doxorubicin concentrations (below 10 μM), even in membranes with a higher order.

In the presence of doxorubicin less verapamil was bound to membranes and visa versa. Competition between daunorubicin and verapamil for binding to model membranes has been observed elsewhere [30,44], but the effects were smaller, probably because no negatively charged phospholipids were used in those cases. The concentration of verapamil at which the amount of bound doxorubicin (at 50 μM free doxorubicin and at 1 mM lipid-P_i) was 50% compared to that in the absence of verapamil appeared to be 130, 182, 189, and 206 μM for LUVET composed of DOPC/DOPG, DOPE/DOPC, *E. coli* lipids, and *E. coli* inner membrane vesicles, respectively. The apparent affinity of doxorubicin for DOPC/DOPG (3:1) LUVET and LUVET composed of *E. coli* phospholipids, expressed as the concentration of free doxorubicin at which 50% of the maximal doxorubicin binding occurs (in the presence of 1 mM lipid-P_i), is 360 and 256 μM [6,7], which is in the same order of magnitude. Verapamil is thought to be located in the hydrophobic part of the membrane near the headgroup region [42] so it was expected to compete with doxorubicin for the hydrophobic binding sites and this seems to be the case. However, in the presence of anionic phospholipids verapamil had a stronger effect on binding of doxorubicin to both 'deep' and 'surfacial' binding sites, in both cases probably by neutralization of negative charges of the phospholipids.

Interestingly, verapamil showed an enhancing effect on the rate of passive diffusion of doxorubicin. This effect was specifically observed with LUVET composed of negatively charged phospholipids. Our explanation for this phenomenon is the following: binding of doxorubicin to the membrane decreases the rate of its passive diffusion [37], and since in the presence of verapamil doxorubicin binding is diminished, an increased diffusion rate of doxorubicin is the result. In previous work we showed that the rate of passive influx of doxorubicin across model membranes in which DNA was enclosed is decreased when anionic phospholipids are present. This effect is due to (i) a decrease of the amount of free, transportable drug, and (ii) an intrinsic effect, since even after correction for doxorubicin binding, a significantly lower influx rate was observed in the presence of anionic phospholipids [37]. Probably, the incorporation of doxorubicin close to the surface of the membrane induces a tightening of the interfacial region and hinders passive diffusion of doxorubicin, which is relieved upon the displacement with verapamil. So verapamil enhances passive diffusion of doxorubicin by (i) increasing the concentration of transportable doxorubicin and by (ii) diminishing the intrinsic membrane-disturbing, inhibitory effect of positively charged, membrane-bound doxorubicin. Although an increase in membrane fluidity has been described upon incorporation of a high amount of verapamil, which can also enhance passive diffusion, this effect was not observed under the conditions of our transport experiments [40]. Furthermore, we tested the effect of verapamil on carboxyfluorescein (CF) efflux from LUVET containing DOPG and observed no CF leakage under transport experiment conditions (data not shown), indicating that under transport experiment conditions the membrane barrier function was not disrupted by verapamil.

Our findings might have implications for drug transport studies in intact cancer cells. In previous studies the passive flow of anthracyclines was determined by blocking the active efflux pump, P-glycoprotein, by adding verapamil [45]. However, our studies on model systems indicate that passive transport and permeability coefficients are not the same in the presence and absence of verapamil and competition for binding between verapamil and doxorubicin will initially increase the amount of cytosolic non-membrane-bound drug in sensitive and multidrug resistant cancer cells. In addition, the enhancing effect of verapamil on passive influx of doxorubicin will have a sensitizing effect in multidrug resistant cancer cells by reducing the effect of the pump because of increased leak. Studies in cancer cells often show an effect of verapamil on the sensitive, parental strain or on multidrug resistance cells that do not contain P-glycoprotein (although the effects are usually smaller than in P-glycoprotein containing cells). These effects were thought to result from a residual or undetectable amount of P-glycoprotein or the presence of another active pump [22,28,45–49]. Direct effects of verapamil on phospholipids offer (at least partially) an alternative explanation for these phenomena, since at least in model membranes the ratio DNA-bound doxorubicin versus free plus membrane-bound doxorubicin is increased (see Fig. 5A). If this phenomenon can also be observed in more complex systems such as cancer cells we do not know. Taking into account the physico-chemical properties of mdr substrates and mdr reversal agents [14] we assume that the effects of verapamil on internal doxorubicin concentration is not specific and that these effects will be general for mdr reversal agents.

It can be concluded that verapamil can sensitize mdr cells to anti-cancer drugs such as doxorubicin by more than one mechanism, namely (i) by a direct effect of verapamil on the P-glycoprotein (i.e., occupying a different binding site on the protein or competing for the same binding site both resulting in a lower active efflux) [16–20], and (ii) by a competition between doxorubicin and verapamil for binding on the phospholipid part of the membrane including its subsequent enhancing effects on passive doxorubicin transport. Finally, an indirect effect of verapamil on the active efflux of doxorubicin by the P-glycoprotein or another mdr-conferring active pump can be speculated upon. If a direct pumping of the drug from the interphase of the inner leaflet is assumed [12,50], because the positively charged, moderately hydrophobic anti-cancer drugs accumulate at that position, our results indicate that verapamil lowers the substrate concentration

in the compartment from which the enzyme pumps, which might have kinetic effects on the pump activity.

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