Orientation of Anthracyclines in Lipid Monolayers and Planar Asymmetrical Bilayers: A Surface-Enhanced Resonance Raman Scattering Study

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ABSTRACT The interaction of anthracyclines (daunorubicin and idarubicin) with monolayers of zwitterionic palmitoyloleoylphosphatidylcholine (POPC) and anionic dipalmitoylphosphatidic acid (POPC-DPPA 80–20 mol%) was studied by surface pressure measurements and compared with previous results obtained with other anthracyclines (pirarubicin and adriamycin). These anthracycline/phospholipid monolayers were next transferred by a Langmuir-Blodgett technique onto planar supports and studied by surface-enhanced resonance Raman scattering (SERRS), which gave information about the orientation of anthracycline in the monolayers. On the whole, the adsorption of anthracyclines in zwitterionic monolayers increases with the anthracycline hydrophobic/hydrophilic balance, which underlines the role of the hydrophobic component of the interaction. On the contrary, the anthracyclines remain adsorbed on the polar headgroups of the phospholipids in the presence of DPPA and form a screen that limits a deeper penetration of other anthracycline molecules. To study by SERRS measurements the crossing of pirarubicin through a phospholipid bilayer used as a membrane model, asymmetrical POPC-DPPA/POPC or POPC/POPC-DPPA bilayers were transferred by the Langmuir-Schäfer method, thanks to a laboratory-built set-up, and put in contact with a pirarubicin aqueous solution. It has been shown that the presence of anionic DPPA in the first monolayer in contact with pirarubicin would limit its crossing. This limiting effet is not observed if the first monolayer is zwitterionic.

INTRODUCTION

Anthracyclines are antibiotics used in antitumor chemotherapy. After the crossing of the cellular membrane by passive diffusion, anthracyclines intercalate between the base pairs of DNA, inhibiting the replication process of cells (Yunyu et al., 1993). Anthracyclines are also able to interact with the topoisomerase-DNA complex (Capranico et al., 1995).

A major problem appearing during treatment is the development of cells that are resistant to the drug used and to other molecules without any structural relation to the drug. This is multidrug resistance (MDR), related to the overexpression in the membrane of a glycoprotein, the P-gp (Bradley et al., 1988). This protein is an ATP-dependent pump expelling the drug out of the cell: thus the intracellular amount of anthracycline is not sufficient to inhibit cellular growth. Under these conditions, there are two possible solutions. The first consists of inhibiting the P-gp with specific blockers, but the use of a second active molecule during the treatment can be difficult. The second solution consists of finding new derivatives of anthracyclines able to cross the membrane more quickly: even if a part of the anthracycline is expelled from the cell, the intracellular amount would be sufficient to inhibit cellular growth. This second solution implies a better understanding of anthracycline-membrane interaction.

It has been shown that two types of interaction (electrostatic and hydrophobic) are involved in anthracycline-membrane interaction. The interaction of anthracyclines with zwitterionic phospholipids is limited, whereas the fixation is more important in the presence of anionic lipids such as cardiolipin (Goormaghtigh et al., 1980; Speelmans et al., 1994). This latter specific and strong interaction could be responsible for the cardiotoxicity of anthracyclines (Goormaghtigh et al., 1987). These results have also been observed with adriamycin in contact with phospholipid monolayers, although the use of monolayers as a simplified membrane model is not very common in this kind of study (Goormaghtigh et al., 1980; Nicolay et al., 1988; Dupou-Cézanne et al., 1989). The amount of anthracyclines in contact with lipids also depends on the anthracycline/lipid ratio. For instance, Ferrer-Montiel et al. (1988) have shown that the localization of daunorubicin in cellular membranes is deeper when the daunorubicin/lipid ratio is high; this result is also obtained by circular dichroism with adriamycin in interaction with liposomes containing egg-phosphatidic acid, egg-phosphatidylglycerol, or cardiolipin (Henry et al., 1985). The presence of anionic phospholipids can also change the permeability coefficient of anthracyclines: Speelmans et al. (1994) observed, for instance, that the permeability coefficient of adriamycin in DNA-containing liposomes was decreased in the presence of anionic phospholipids. They assigned this result to a tightening of the phospholipid polar headgroups due to the presence of adri-

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amycin-anionic phospholipid complexes and proposed that adriamycin would thus be adsorbed on the polar headgroups.

The work reported here includes two parts:

1. We first studied by surface pressure measurements (Langmuir method) the interaction of two anthracyclines, daunorubicin and idarubicin, with zwitterionic POPC (palmitoyloleoylphosphatidylcholine) monolayers and anionic POPC-DPPA (dipalmitoylphosphatidic acid) 80–20 mol% monolayers. This method enabled us to determine the percentage of anthracycline molecules adsorbed to and/or penetrating the phospholipid monolayers. In a second step, we transferred these monolayers onto planar supports, to study the orientation of anthracyclines by surface-enhanced resonance Raman spectroscopy (SERRS). Indeed, this spectroscopy enables us to increase the Raman signal by six orders of magnitude because of the presence of a rough silver layer and to study the orientation of exogenous molecules in contact with lipid monolayers deposited on planar supports, because of its short range (Kovacs et al., 1986; Cotton, 1988; Aroca et al., 1989). Under these conditions, the anthracycline molecules are thus integrated in the phospholipid monolayers before the transfer.

We have already applied these two complementary methods to the study of the interaction of pirarubicin and adriamycin with monolayers of zwitterionic phospholipids (POPC) and anionic phospholipids (POPC-DPPA 80–20 mol%) (Heywang et al., 1996, 1997). In particular, we have shown that the orientations of these two anthracyclines in the bilayer depend on different parameters: the nature of the anthracycline, the charge of the phospholipid, and the mode of preparation of the bilayer (Heywang et al., 1996, 1997).

In this work we have compared the results obtained in the case of daunorubicin and idarubicin with those obtained in the case of pirarubicin and adriamycin.

2. The second aspect of this work is to study the crossing of pirarubicin through phospholipid bilayers. In this case, we first transferred a phospholipid bilayer onto the planar support, and then put it in contact with an aqueous solution of pirarubicin; thus the anthracycline is initially only in contact with the monolayer, which is far from silver. Under these conditions, SERRS enables us, by using a particular laboratory-built set-up, to determine if these molecules are able to cross a phospholipid bilayer (Saint-Pierre Chazalet et al., 1994). As a matter of fact, an aspect of the Langmuir-Blodgett method is the possibility of preparing asymmetrical preformed bilayers; the compositions of the two monolayers constituting the bilayer are different, as in a biological membrane. Two types of asymmetrical bilayers have been transferred:

POPC/POPC-DPPA bilayers: The first monolayer in contact with the aqueous solution is composed of zwitterionic POPC, and the second monolayer in contact with silver is composed of a anionic POPC-DPPA mixture.

POPC-DPPA/POPC bilayers: The first monolayer in contact with the aqueous solution of pirarubicin is composed of a POPC-DPPA 80–20 mol% mixture, whereas the other one (in contact with silver) is composed of POPC.

We have compared, then, these results with the behavior of pirarubicin in contact with symmetrical POPC and POPC-DPPA bilayers studied previously (Heywang et al., 1996, 1997).

MATERIALS AND METHODS

Materials

Anthracyclines

Four anthracyclines were tested, adriamycin (or doxorubicin), pirarubicin (or 4′-*O*-tetrahydropyranyl-doxorubicin), daunorubicin, and idarubicin (Fig. 1). They were kindly provided by Farmitalia Laboratory (Italy) and Laboratoire Roger Bellon (France), in powder form mixed with lactose (as for hospital use). They were dissolved in Millipore water (pH = 5.5, R = 18 MΩ) or in an organic polar solvent (dimethyl sulfoxide, DMSO) at an initial concentration of 10^{-3} or 2×10^{-3} M. It has been checked that lactose does not adsorb at the air-water interface.

These anthracyclines present various chemical groups on the anthraquinone part and the sugar, and thus the resulting hydrophobicities are different. A possible parameter that can be used to define these differences is the partition coefficient P of the molecules in an octanol-water system and their resulting lipophilicity (Gallois et al., 1996). It has been shown that the order of increasing hydrophobicity is the following: adriamycin < pirarubicin < daunorubicin < idarubicin.

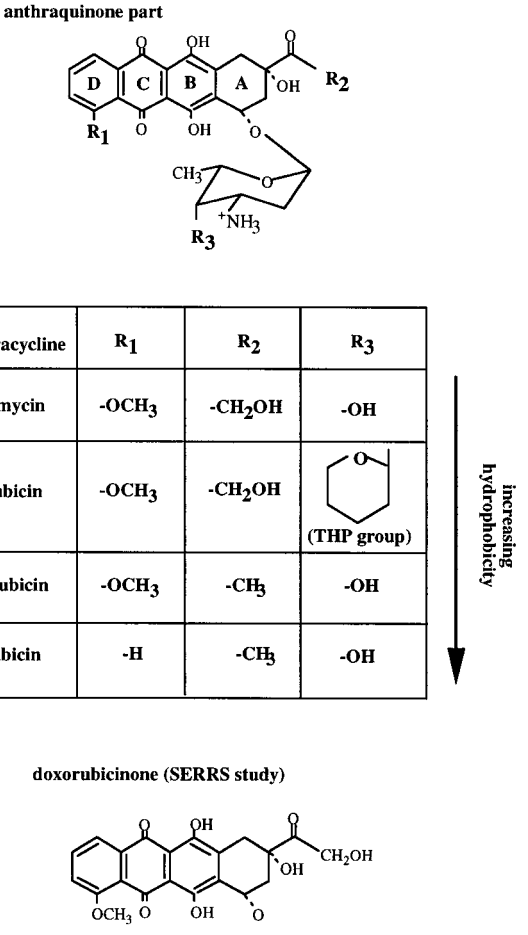

FIGURE 1 Chemical formulas of adriamycin, pirarubicin, daunorubicin, and idarubicin, and formula of doxorubicinone (SERRS study).

All of the anthracyclines used in this study are positively charged at pH 5.5, the pK_a of the amino function on the sugar being 8.6 for adriamycin, daunorubicin and idarubicin, and 7.7 in the case of pirarubicin (Frezard et al., 1990).

Lipids

Palmitoyloleoylphosphatidylcholine (POPC, a zwitterionic phospholipid), dipalmitoylphosphatidic acid (DPPA, an anionic phospholipid bearing one negative charge at pH 5.5), and cholesterol were purchased from Sigma (St. Louis, MO) and were 99% pure. They were used without further purification and dissolved in an ethanol/chloroform 1/1 (v/v) mixture at a concentration of 10^{-3} M.

Methods

Monolayers

A laboratory-built Langmuir trough was used for the preparation of the anthracycline/lipid monolayers, using a displacement force transducer (Kaman Sciences Corporation, Colorado Springs, CO). This set-up has been described in detail in previous works (Saint-Pierre Chazalet et al., 1994; Heywang et al., 1996, 1997). The subphase was Millipore water (pH 5.5). All experiments were performed at 21 ± 1°C. The trough was equipped with an electronic device that made it possible to keep the surface pressure of the monolayer constant by monitoring the displacement of the moving barrier. It was used during the penetration experiments and layer tranfers.

We first studied the adsorption and/or the penetration of aqueous solutions of anthracycline (idarubicin and daunorubicin) into POPC and POPC-DPPA (80–20 mol%) monolayers. The principle of this method is the following. A phospholipid monolayer is spread at the interface and compressed to 2 mN/m. This pressure is kept constant, and an aqueous solution of anthracycline is injected under the monolayer at a final concentration of 1.6 μM. If an interaction occurs between the anthracycline and the monolayer, the barrier moves back to keep the pressure at 2 mN/m. After the equilibrium (the barrier is stopped), the anthracycline-phospholipid monolayer is compressed. The P/A isotherm is recorded after the adsorption is shifted to the higher areas as compared to the pure phospholipid monolayer. The difference in the mean molecular areas measured between the two isotherms at the same pressure enables us to determine the percentage of anthracycline in contact with lipids, by taking into account the molecular area of the anthracycline in vacuum determined by a previous modeling study with Hyperchem software (Heywang et al., 1996).

Next, these monolayers can also be transferred by a Langmuir-Blodgett technique onto planar supports for the SERRS study.

Preparation of planar supported symmetrical and asymmetrical phospholipid bilayers by the Langmuir-Schäfer technique

The planar supports that we used in the SERRS study are prisms made of rutile of a high index ($n = 2.7$). After cleaning, they are coated by vacuum thermal evaporation with a 15-nm-thick silver layer.

The technique of transfer of planar bilayers in contact with water has been developed in the laboratory (Saint-Pierre Chazalet et al., 1994) and proceeds from the technique described by Tamm et al. (1985). We recall it briefly: the prism (Fig. 2) is previously dipped into the subphase, with the silver face perpendicular to the air-water interface. Molecules are spread at the interface and compressed at a surface pressure of 25 mN/m. This pressure is next kept constant at this value by the electronic device. The prism is brought out of the subphase at 5 mm/min, and a first monolayer is deposited on silver, with the polar headgroups of lipids in contact with the silver layer (Saint-Pierre Chazalet et al., 1994). We can thus transfer only a planar monolayer (see previous section, Monolayers). In the case of a bilayer, the prism is next rotated by 90° to have the first monolayer parallel to the interface. It is then carefully put in contact with the monolayer remaining at the interface (Langmuir-Schäfer technique) and finally fixed in a Teflon box previously put in the trough, to keep the polar headgroups of the second layer in contact with water (Fig. 2). It is also possible at this level to put the bilayer in contact with an aqueous solution of the exogenous molecule.

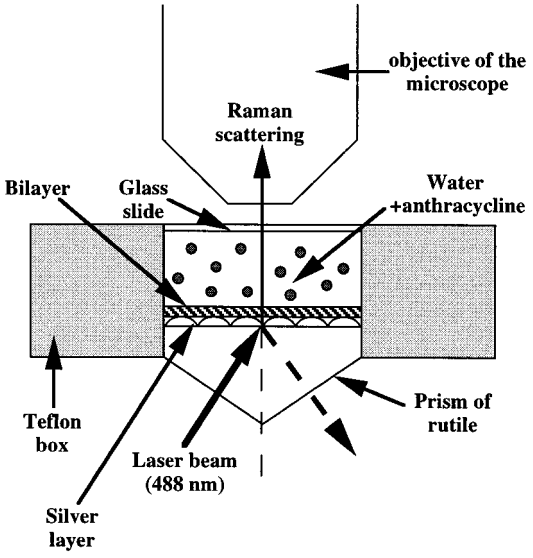

FIGURE 2 Experimental set-up used for the SERRS study of the planar anthracycline/lipid bilayers in contact with water.

Recording of the SERRS spectra of the planar mono- and bilayers

Surface-enhanced Raman spectroscopy (SERS) needs the presence of a thin silver layer (15–20 nm thick) deposited on the planar support before the transfer of the planar mono- or bilayer (Kovacs et al., 1986). Under these conditions, silver forms islands, which make it possible to enhance the Raman signal of deposited molecules, which otherwise could not be detected because of the small number of molecules and the weakness of the signal. Electrical and chemical phenomena are responsible for the increase in the signal at a very short distance, estimated to a few nanometers (Cotton, 1988; Aroca et al., 1989; Saint-Pierre Chazalet et al., 1994). Indeed, the decrease in the signal intensity is expected to follow a $(a/r)^{12}$ rule, where a is the radius of the silver particle and r is the distance of the observed point to the center of the silver particle (Cotton, 1988). Under our experimental conditions (by taking into account the dimensions of our silver islands), this means that the enhancement of the Raman signal decreases by 50% at ~2 nm from the silver surface. This spectroscopy is thus powerful for studying the behavior of anthracyclines directly in contact with a lipid bilayer transferred to silver.

The prism was illuminated with an Ar^+ laser beam, under an angle greater than the limit angle of reflection (Fig. 2). Because of the short range of SERS, the observed Raman spectrum is mainly due to the monolayer in contact with silver. The SERS effect is still increased, because the used laser wavelength is in the absorption band of the anthracyclines (surface-enhanced resonance Raman scattering, SERRS). The scattered light is then collected in a direction perpendicular to the surface of the sample at the level of a microscope objective and analyzed with a Dilor (Lille, France) triple monochromator associated with a data acquisition system. Because the signal is weak, an accumulation of several spectra is usually performed.

SURFACE PRESSURE AND SERRS STUDY OF THE DAUNORUBICIN-PHOSPHOLIPID AND IDARUBICIN-PHOSPHOLIPID INTERACTION

Results obtained by surface pressure measurements

Behavior of idarubicin and daunorubicin at the air-water interface

These molecules are amphiphilic and water soluble and thus can be studied by surface pressure measurements. Therefore, two techniques were applied to study their behavior at the air-water interface in the absence of phospholipid (Heywang et al., 1996). The first one consists of dissolving an aqueous solution of anthracycline in the subphase (the final concentration was 1.6 μM) and observing its adsorption at the interface. *P*/*A* isotherms are regularly recorded to monitor possible adsorption. The second method consists of spreading the anthracycline dissolved in an organic polar solvent (DMSO) at the interface; the total number of spread molecules is the same as the number injected into the subphase with the first method.

Adsorption of idarubicin and daunorubicin. In the two cases, no compression isotherm was observed after a 4- or 5-h experiment. Idarubicin and daunorubicin are thus unable to adsorb at the air-water interface, in contrast to pirarubicin, which presents a weak adsorption even after 1 h (Heywang et al., 1996).

Spreading of idarubicin and daunorubicin dissolved in DMSO. In contrast to the previous results, idarubicin and daunorubicin are able to remain partly at the interface after spreading. Fig. 3 shows the isotherms obtained after the compression (1 mm/s) of the molecules remaining at the interface. In the case of daunorubicin, the isotherm is similar to that with pirarubicin under the same conditions (Heywang et al., 1996). The mean molecular areas reported on this figure were estimated according to the total number of molecules initially spread at the air-water interface. Their weak values show that the number of molecules remaining at the interface after their spreading is also very weak: an important fraction of the spread molecules do not remain at the interface and dissolve into the subphase before the compression. The weak surface pressures observed on the isotherm confirm this dissolution.

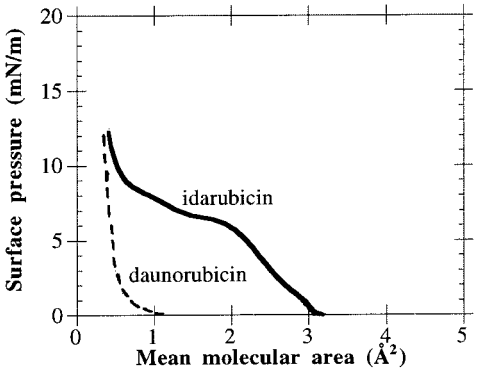

FIGURE 3 Compression isotherms of daunorubicin and idarubicin after the spreading of the two anthracyclines dissolved in DMSO (pH = 5.5, $T = 21 \pm 1$°C).

It is possible to determine the percentage of daunorubicin at the interface from the molecular area measured on the isotherm and an estimation of the cross section of the anthracycline in vacuum, performed by a molecular modeling. This mean cross section has thus been estimated to ~75 Å^2. As in the case of pirarubicin, the percentage of daunorubicin remaining at the interface is ~0.6% at 5 mN/m and can be neglected compared to the percentages that we observe in the presence of phospholipid monolayers (Heywang et al., 1996, 1997 and the next section).

The isotherm of idarubicin is very different from the daunorubicin isotherm. It presents a plateau at a surface pressure around 6–7 mN/m. To check that this plateau was not an artefact due to the presence of DMSO, the same experiment was performed with idarubicin dissolved in dimethylformamide and ethanol: the isotherms present a plateau again. Thus we can conclude that the solvent is not responsible for the presence of this plateau. Moreover, the decompression isotherm (1 mm/s) shows the same plateau at the same surface pressure.

We observe thus that 1) daunorubicin and pirarubicin have the same behavior after their spreading at the air-water interface, whereas daunorubicin is unable to adsorb at the interface, in contrast to pirarubicin; 2) only the isotherm of idarubicin spread at the interface presents a plateau at 6–7 mN/m. It has been shown that idarubicin presents aggregation properties different from those of other anthracyclines such as daunorubicin in an hydrophobic surrounding (Gallois et al., 1997). In solution, idarubicin is mainly in monomeric form, but it penetrates in lipid bilayers under a dimeric form, whereas other anthracyclines interact with lipids under the monomeric form. The air-water interface can be considered as a "hydrophobic" surrounding. After the spreading of the solution, the fraction of idarubicin molecules remaining at the interface could thus be under a dimer and/or aggregate form, responsible for the plateau observed on the isotherm. That the monomers are more water-soluble than the aggregates could also explain that no molecule adsorbs at the air-water interface when they are dissolved in the subphase (adsorption experiments).

Adsorption of aqueous solutions of idarubicin and daunorubicin in phospholipid POPC and POPC-DPPA (80–20 mol%) monolayers

The adsorption and/or penetration of idarubicin and daunorubicin into phospholipid monolayers were studied next. We have reported, as an example, in Fig. 4 the isotherms of POPC before and after the adsorption and/or the penetration of idarubicin (1.6 μM) at 2 mN/m and determined the resulting percentage of idarubicin kept at the interface at 25 mN/m (see Materials and Methods). The same estimation was performed with daunorubicin under the same experi-

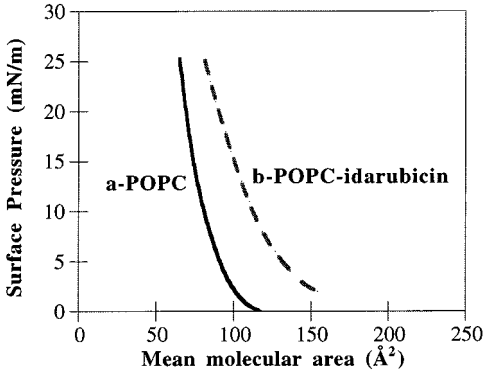

FIGURE 4 POPC compression isotherms before (*a*) and after (*b*) penetration of idarubicin ($C = 1.6$ μM, pH = 5.5, $T = 21 \pm 1$°C; the pressure during the compression was kept constant at 2 mN/m).

mental conditions (data not shown). We summarized these results in Table 1, where we also report the previous results obtained with pirarubicin and adriamycin (Heywang et al., 1996, 1997). We observe, in particular, that the adsorption and/or penetration of daunorubicin and idarubicin are very different in POPC monolayers (respectively, 7% and 17%), whereas the percentages are all rather close in the presence of POPC-DPPA (80–20 mol%) monolayers (respectively, 14% and 13%).

Goormaghtigh et al. (1980) also studied the interaction of adriamycin with monolayers of zwitterionic DPPC phospholipids and did not observe any adsorption of adriamycin at the air-water interface, because of its weak hydrophobic/hydrophilic balance. On the contrary, under our experimental conditions, we observed a weak adsorption of adriamycin in zwitterionic POPC monolayers. This difference is likely related to the different experimental conditions between the two experiments: we studied the adsorption of adriamycin in fluid monolayers kept at a surface pressure of 2 mN/m, whereas Goormaghtigh et al. performed it in a more condensed phase.

To complete this surface pressure study, these monolayers were also tranferred onto planar supports and studied by SERRS.

TABLE 1 Estimated percentages of anthracyclines at the interface ($P = 25$ mN/m) after adsorption and/or penetration in the different lipid monolayers

	Adsorption experiments			
	Adriamycin	Pirarubicin	Daunorubicin	Idarubicin
POPC	4%**	11%*	7%	17%
POPC-DPPA (80–20 mol%)	11%**	13%**	14%	13%

$C = 1.6$ μM, pH = 5.5, $T = 21 \pm 1$°C. The surface pressure was kept constant at 2 mN/m.
*Heywang et al. (1996).
**Heywang et al. (1997).

Results

Results obtained with SERRS

The previous monolayers of daunorubicin/phospholipid and idarubicin/phospholipid were transferred by the Langmuir-Blodgett technique onto rutile planar supports coated with a thin silver layer, and their SERRS spectra were recorded to estimate the orientation of the anthracyclines.

Anthracyclines present very complex Raman spectra between 400 and 1800 cm^{-1}. One of the most important problems is the assignment of their bands. Therefore different studies have already focused on this point (Smulevich et al., 1986, 1988; Nonaka et al., 1990; Nabiev et al., 1991, 1994). They have been determined by

1. Comparison between the resonant Raman (RR) and SERRS spectra of different anthracyclines and their chromophore models such as anthraquinones (in all of the SERRS studies, the SERRS spectra of the anthracyclines were recorded in aqueous solution with colloids prepared according to the procedures of Creighton or Lee-Meisel);
2. Changes in the experimental conditions (pH, ionic strength. . .);
3. Comparison with results of other methods such as fluorescence, resonant Raman, and infrared spectroscopies (Angeloni et al., 1982; Smulevich et al., 1982; Dutta et al., 1986).

Some of the major observations and results are the following. The Raman spectra of anthracyclines such as adriamycin are modified when the pH of the aqueous solution is increased (Dutta et al., 1986). Some bands are less intense or disappear and are thus assigned to δ(C-O-H) deformations (band at ~1210 cm^{-1}, for instance). The frequency of some other bands is shifted as the result of the appearance of phenolate functions that increase the electronic density on the chromophore. They are assigned to cycle vibrations. At last, some bands are not sensitive to pH modifications and are assigned, for instance, to the methoxy function $-OCH_3$.

In the case of adriamycin and aclacinomycin, some bands between 1200 and 1300 cm^{-1} also disappear after deuteration; this confirms the importance of the hydroxyl groups (-OH) in this region of the Raman spectrum. Finally, the Raman spectra of the chromophore models are very close to the spectra of anthracyclines, which suggests that the spectra are mainly related to the dihydroxyanthraquinone part of anthracyclines. Table 2 presents the frequencies and the assignment of some of these bands.

In our previous work (Heywang et al., 1996), this spectroscopy was first applied to the study of pirarubicin, adriamycin, and doxorubicinone molecules (doxorubicinone corresponding to the anthraquinone part of pirarubicin and adriamycin without the sugar and the THP group; Fig. 1). The SERRS spectra of pirarubicin and doxorubicinone are reported in the inset of Fig. 5. We observed, for instance, in the case of the aqueous solution of pirarubicin, that the aromatic rings are close to silver, whereas the tetrahydro-

TABLE 2 Principal assignments of the vibrational modes observed in the different SERRS spectra of anthracyclines and anthracycline/lipid mono- and bilayers

Frequency (cm^{-1})	980	1206	1240	1260	1400–1410	1450
Assignments	Ring breath	δ(C-O-H) in plane	δ(C-O-H) in plane	Ring stretch	Ring stretch	Ring stretch δ(CH_2), THP group in the case of pirarubicin
SERRS spectrum of an aqueous solution of pirarubicin (1 μM), (*)	+++	+++	+++	s	+?	+
SERRS spectrum of doxorubicinone (*)	+	+	++	+++	+++	s
SERRS spectrum of an aqueous solution of daunorubicin (10 μM)	+++	+++	+++	?	+++	s+
SERRS spectrum of "adsorbed" daunorubicin/POPC monolayers	+	+	?	+++	+++	s
SERRS spectrum of "adsorbed" daunorubicin/POPC-DPPA monolayers	+	+	++	+++	+++	s+
SERRS spectrum of an aqueous solution of idarubicin (10 μM)	+	?	+++	+++	+	+
SERRS spectrum of "adsorbed" idarubicin/POPC monolayers	++	+++	+++	+++	+++	+
SERRS spectrum of "adsorbed" idarubicin/POPC-DPPA monolayers	++?	+	+++?	+++	+++	+
SERRS spectrum of asymmetrical POPC/POPC-DPPA bilayers in contact with an aqueous solution of pirarubicin (0.4 μM)	++	+++	+++	s	+++	+++
SERRS spectrum of asymmetrical POPC-DPPA/POPC bilayers in contact with an aqueous solution of pirarubicin (0.4 μM)	−	−	−	−	−	−
SERRS spectrum of asymmetrical POPC-DPPA/POPC bilayers in contact with an aqueous solution of pirarubicin (2 μM)	++	++	+++	s	+++	+++

+++/++/+/− symbols indicate whether the band is very intense, intense, visible, or invisible. "s" indicates that the band appears as a shoulder. The question mark (?) indicates the ambiguous cases.
*Heywang et al. (1996).

pyren (THP) group is far from it. A possible orientation could thus be the following. The long axis of the anthraquinone part would be perpendicular or tilted to the silver coating. In the case of doxorubicinone, its SERRS spectrum suggests that the aromatic rings would be in contact with silver and parallel to the silver surface ("lying" configuration). The observed differences could be induced by the fact that doxorubicinone is "cleared" of the sugar and THP group. Finally, the SERRS spectrum of adriamycin also demonstrated that the anthraquinone part of this molecule would be lying on silver as doxorubicinone (Heywang et al., 1997).

Because the SERRS spectrum of anthracyclines is related mainly to the anthraquinone part, we used this characteristic to estimate, in a second step, the orientation of this anthraquinone part and thus the orientation of the whole molecule in lipid planar monolayers (Heywang et al., 1996, 1997). This orientation depends on 1) the nature of the anthracyclines, 2) the charge of phospholipids, and 3) the mode of preparation of the bilayers.

In the 900-1800 cm^{-1} region, two parasite broad bands at 1375 and 1590 cm^{-1} are always superimposed on the anthracycline spectrum (Saint-Pierre et al., 1994). These bands are assigned to the graphitized carbon resulting from a photochemical reaction between CO_2 present in air or water and the silver layer (Nabiev et al., 1988). They are always subtracted from the subsequent SERRS spectra. We will not take into account the 1500–1800 cm^{-1} region in our following interpretations because of this subtraction (the band at 1590 cm^{-1} is the more intense).

Finally, the integrity of the planar phospholipid monolayers and bilayers was checked for each transfer by observing the 2750–3050 cm^{-1} region. In this region, the vibration modes of phospholipids are mainly observed. In particular, the presence of an intense band at 2960 cm^{-1} (assigned to the asymmetrical stretching vibration of the methyl groups) is intense in the case of phospholipid bilayers (Saint-Pierre Chazalet et al., 1994).

SERRS study of planar daunorubicin/phospholipid monolayers

We began this study by the recording of the SERRS spectrum of a daunorubicin aqueous solution and then per-

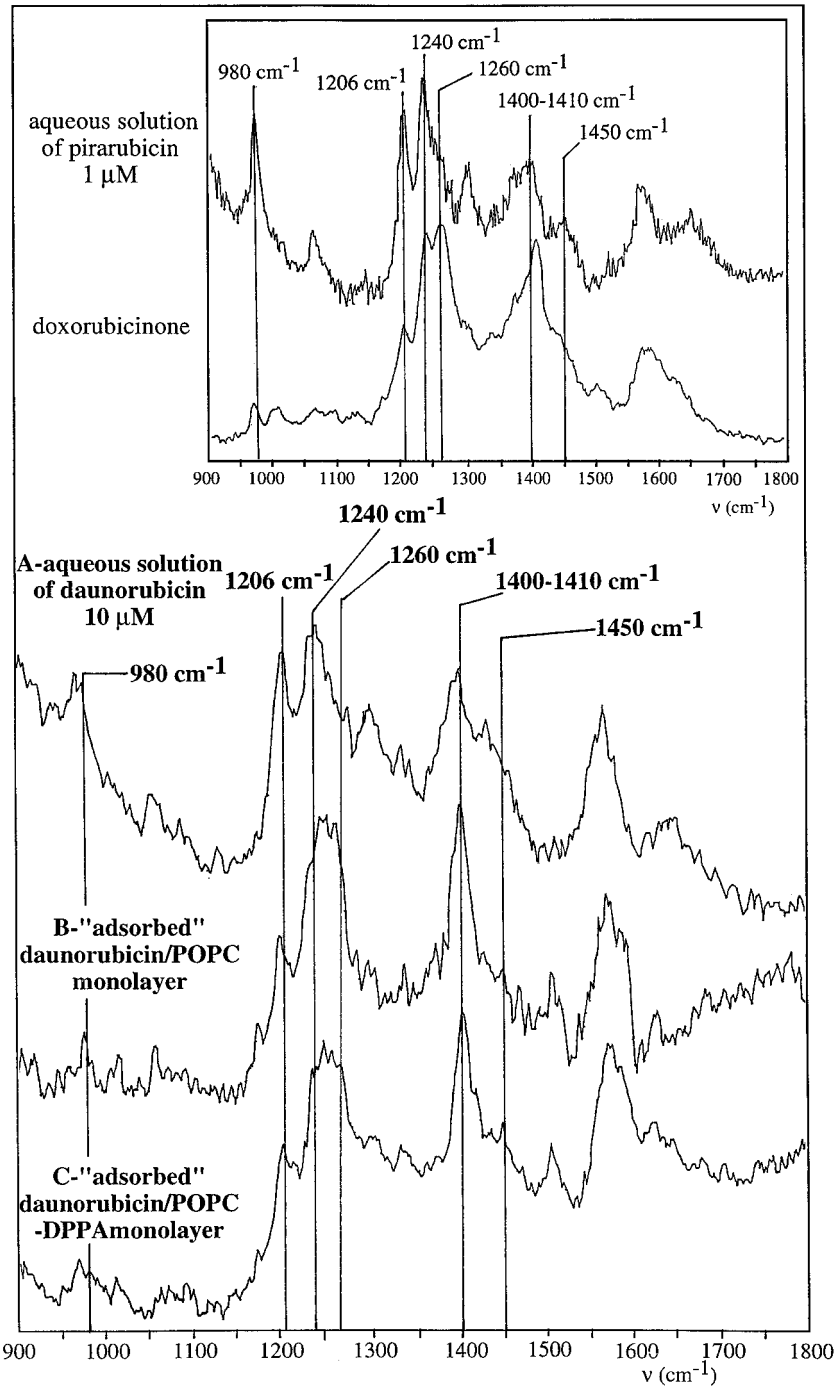

FIGURE 5 (*a*) SERRS spectrum of an aqueous solution of daunorubicin (C = 10 μM, pH = 5.5). (*b*) SERRS spectrum of an "adsorbed" daunorubicin/POPC monolayer after penetration of an aqueous solution of daunorubicin in a POPC monolayer. (*c*) SERRS spectrum of an "adsorbed" daunorubicin/POPC-DPPA 80–20 mol% monolayer after penetration of an aqueous solution of daunorubicin in a POPC-DPPA 80–20 mol% monolayer. (*Inset*) SERRS spectrum of an aqueous solution of pirarubicin (C = 1 μM, pH = 5.5) and SERRS spectrum of doxorubicinone powder. Experimental conditions for the preparation of the monolayers: C = 1.6 μM, pH = 5.5, T = 21 ± 1°C; the surface pressure was kept at 2 mN/m during the penetration, and the penetration time was limited to 15 min.

formed the spectra of planar daunorubicin/phospholipid monolayers. It has to be noted that the SERRS spectrum of the aqueous solutions of anthracyclines corresponds mainly to molecules that are close to the silver coating: molecules dissolved in the solution and far from silver are not observed because of the short range of SERRS (see Recording of the SERRS spectra of the planar mono- and bilayers, above). It was also checked in the case of pirarubicin that the anthracycline does not interact with silver. A prism previously coated with silver and dipped into an aqueous solution of pirarubicin was brought out after 30 min and observed in SERRS. The SERRS spectrum of pirarubicin was not observed, showing that pirarubicin molecules do not remain adsorbed on silver (data not shown). Thus if an interaction occurs between the anthracycline and silver, it is too weak as compared to the solubilization of the anthracycline into the subphase.

SERRS spectrum of an aqueous solution of daunorubicin. Fig. 5 *a* shows the SERRS spectrum of an aqueous solution of daunorubicin at 10 μM deposited on a prism. We observe the bands at 1206 and 1240 cm^{-1} (δ(C-O-H)) and 1400 cm^{-1} (ring stretch). No band is observed at 1260 cm^{-1} (or

it is so weak that it disappears in the base of the intense band at 1240 cm^{-1}). The band at 1450 cm^{-1} appears as a shoulder.

On the whole, this spectrum is rather close to the SERRS spectrum of the aqueous solution of pirarubicin, even if the intensity of the band at 980 cm^{-1} is weak. This suggests that the anthraquinone part of daunorubicin thus has the same orientation as the pirarubicin part. The major part of the molecules would be perpendicular to the silver coating.

SERRS spectra of daunorubicin/POPC planar monolayers. Daunorubicin/POPC planar monolayers were transferred to prisms after the adsorption of an aqueous solution of daunorubicin (1.6 μM) in a POPC monolayer ("adsorbed" monolayers). Under these conditions, the percentage of daunorubicin remaining in contact with POPC is estimated to be ~7%. The SERRS spectrum of these monolayers is presented in Fig. 5 *b*.

This spectrum is different from the spectrum of the aqueous daunorubicin solution: in particular, we observe a band at 1260 cm^{-1} that is intense and more or less overlaps the band at 1240 cm^{-1}. This last band is also intense (its intensity is similar in this spectrum and in the solution spectrum), even if it seems to be weaker; this is related to the overlapping with the band at 1260 cm^{-1}. The band at 1206 cm^{-1} is very weak (the broad base of the band at 1240–1260 cm^{-1} has to be taken into account). At last the band at 1400 cm^{-1} is thin and very intense, as compared to the same band in the SERRS spectrum of the daunorubicin solution.

This second spectrum is close to that of doxorubicinone; therefore, it could be suggested that in the "adsorbed" daunorubicin/POPC monolayers, the major part of the daunorubicin molecules is lying on silver and thus is adsorbed to the polar headgroups of phospholipids. The small differences between the spectra of doxorubicinone and daunorubicin/POPC monolayers could be assigned to the presence of phospholipids and some daunorubicin molecules embedded in phospholipid chains. However, the molecules mainly observed are very close to the silver coating.

SERRS spectra of daunorubicin/POPC-DPPA (80–20 mol%) planar monolayers. As previously noted, we recorded the SERRS spectrum of "adsorbed" daunorubicin/POPC-DPPA 80–20 mol% monolayers, under the same experimental conditions. The percentage of daunorubicin in contact with phospholipid is estimated to be ~14%. This spectrum (Fig. 6 *c*) is similar to the SERRS spectrum of "adsorbed" daunorubicin/POPC monolayers and suggests that the major part of the

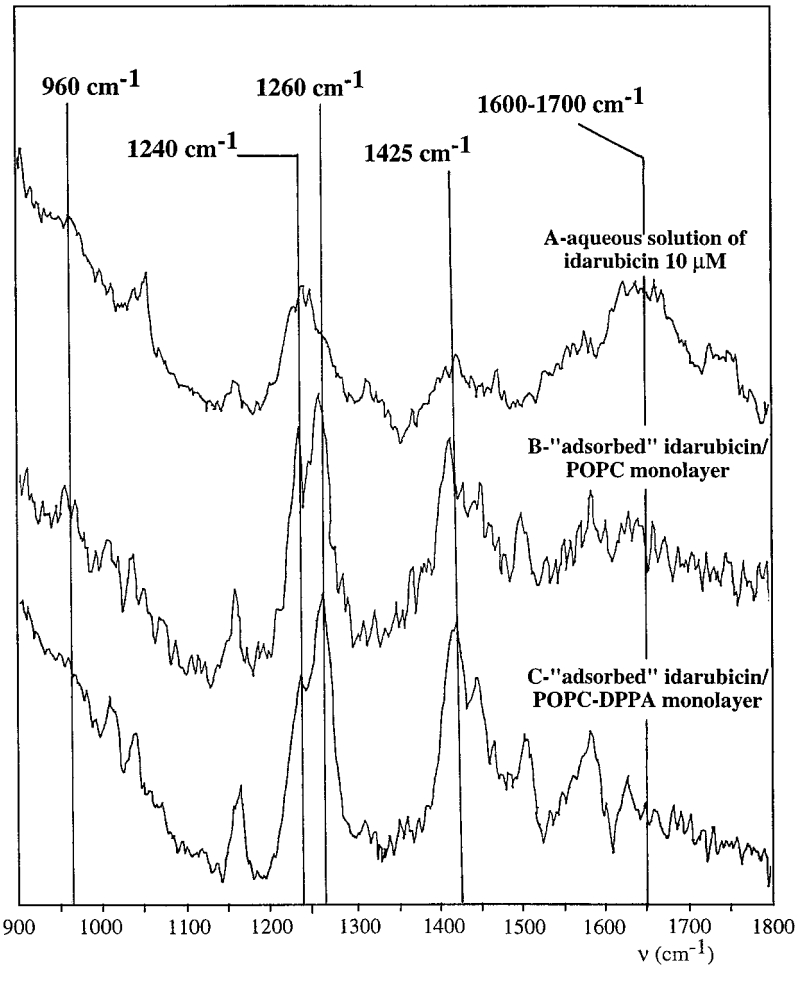

FIGURE 6 (*a*) SERRS spectrum of an aqueous solution of idarubicin (C = 10 μM, pH = 5.5). (*b*) SERRS spectrum of an "adsorbed" idarubicin/POPC monolayer after penetration of an aqueous solution of idarubicin in a POPC monolayer. (*c*) SERRS spectrum of an "adsorbed" idarubicin/POPC-DPPA 80–20 mol% monolayer, after penetration of an aqueous solution of idarubicin in a POPC-DPPA (80–20 mol%) monolayer. Experimental conditions for the preparation of the monolayers: C = 1.6 μM, pH = 5.5, T = 21 ± 1°C; the surface pressure was kept at 2 mN/m during the penetration, and the penetration time was limited to 15 min.

daunorubicin molecules would also be lying on silver and adsorbed to the polar headgroups of phospholipids.

SERRS study of planar idarubicin/phospholipid monolayers

As previously, the SERRS spectrum of an aqueous solution of idarubicin was recorded before the SERRS spectra of idarubicin/phospholipid planar monolayers.

SERRS spectrum of an aqueous solution of idarubicin. The SERRS spectrum of an aqueous solution of idarubicin (10 μM) is presented in Fig. 6 *a*. First we can observe that this spectrum is not as well-resolved as the spectrum of daunorubicin at the same concentration. However, we can observe broad bands between 1240 and 1260, 1425 or 1600–1700 cm^{-1}, and a weaker band at 960 cm^{-1}.

Moreover, the band at 1206 cm^{-1} assigned to the δ(C-O-H) vibration is missing. This can be explained by the chemical structure of the anthraquinone part of idarubicin: the Rs group is ($-$H) instead of ($-OCH_3$) in the other three anthracyclines (Fig. 1). This anthraquinone part is thus symmetrical along the long axis of the anthraquinone and the frequencies of the angular deformations of the two (C-O-H) groups are similar, with the presence of the cyclohexyl A (Fig. 1) condensed to the anthraquinone part inducing only small perturbations in the symmetry of the chromophore. A single band around 1240 cm^{-1} appears, instead of two bands at 1206 and 1240 cm^{-1}.

Finally, the bad quality of the spectrum is probably related to the absence of the (-OCH_3) group; indeed, Smulevich et al. studied adriamycin, epirubicin, and idarubicin and proposed that the methoxy group at the end of the anthraquinone part likely promotes the bonding of the anthracycline to their silver colloid by increasing the polarity of the C═O group (Smulevich et al., 1988).

The relative intensities of the two bands at 1206 and 1240 cm^{-1} were a parameter used in the previous studies to determine the orientation of pirarubicin and adriamycin (Heywang et al., 1996, 1997). Therefore, it is difficult to determine the orientation of idarubicin on silver in the absence of these two bands.

SERRS spectra of idarubicin/POPC and idarubicin/POPC-DPPA (80–20 mol%) planar monolayers. "Adsorbed" idarubicin/POPC and idarubicin/POPC-DPPA monolayers (containing, respectively, 17% and 12.6% of idarubicin, as shown by surface pressure measurements) were transferred, and their spectra were next compared to the idarubicin aqueous solution. Fig. 6, *b* and *c*, presents the SERRS spectra of these monolayers. In the two cases, the spectra are better than the spectrum of the aqueous solution of idarubicin, because the bands are narrow and more well resolved, in particular the band at 1260 cm^{-1}. The better quality of these spectra could be related to the presence of the phospholipid monolayers, which would maintain the idarubicin molecules close to the silver coating. Moreover, these two spectra are rather close. This suggests that idarubicin has a similar orientation in the two types of monolayers, and that this orientation is not modified by the presence of anionic DPPA in the monolayer. However, it is not possible to precisely describe this orientation.

DISCUSSION

The major results of this study can be summarized as follows.

1. In the case of the adsorption of the anthracycline molecules to a POPC monolayer, the percentages of anthracycline remaining at the interface at 25 mN/m increase from 4% to 17% in the following order: adriamycin $<$ daunorubicin $<$ pirarubicin $<$ idarubicin (Table 1). Except in the case of pirarubicin, the percentage increases with the hydrophobicity of the anthracycline (Gallois et al., 1996); in the presence of zwitterionic phospholipids it seems that the hydrophobicity is the major component of the interaction. The previous SERRS studies and the new results also confirm the importance of this hydrophobic/hydrophilic balance of the anthracycline to its location in the membrane; as in the case of the "adsorbed" adriamycin/POPC monolayers, the SERRS spectrum of the "adsorbed" daunorubicin/POPC monolayer suggests that the major part of the daunorubicin molecules would not penetrate deep into the POPC monolayer and would remain adsorbed on the polar headgroups.

Although our results are obtained with monolayers as a membrane model, they are consistent with previous observations of Burke and Tritton (1985a,b), who studied the interaction of eight daunorubicin analogs with fluid-phase dimyristoylphosphatidylcholine and solid-phase DPPC vesicles. These analogs all differed by a chemical modification of the chromophore, changing the overall hydrophobicity of the molecules. Burke and Tritton have shown that the decrease in the anthracycline hydrophobicity strongly reduces the membrane affinity of the anthracycline. In particular, the affinity for fluid membranes was higher in the case of daunorubicin than in the case of adriamycin. Later, they performed another study with a positively charged anthracycline presenting a hydrophobic valerate group on the chromophore (Burke et al., 1989). This hydrophobic modified antibiotic presents a comparable selectivity for liposomes containing zwitterionic (dimyristoylphosphatidylcholine) and anionic (dimyristoylphosphatidylglycerol) phospholipids, in contrast to other, less hydrophobic anthracyclines devoid of this valerate group, which have a strong affinity for anionic liposomes only.

In the case of pirarubicin, it seems that this anthracycline presents a particular behavior: the percentage of drug remaining at the interface after the adsorption in a POPC monolayer is higher than that of daunorubicin, whereas the hydrophobicities of these two compounds are inverted. This particular behavior is likely to be related to the presence of the additional hydrophobic THP group. This group also seems to play an important role in the incorporation of pirarubicin into POPC bilayers: our previous SERRS study suggested that pirarubicin was embedded in the phospho-

lipid chains in the "adsorbed" POPC monolayers (Heywang et al., 1996).

Finally, the surface pressure study shows that the percentage of idarubicin at the interface is higher than that obtained with the three other antibiotics (Table 1), suggesting that the hydrophobic interactions are at maximum. Unfortunately, it is not possible to estimate the orientation of idarubicin on the silver coating: some bands, which are usually used to determine the orientation, are missing, and the SERRS spectrum of the aqueous solution is not well resolved. Under these conditions, it is not possible to compare the solution and monolayer spectra.

2. In the case of the adsorption of the anthracycline molecules to a POPC/DPPA (80–20 mol%) monolayer, from the results reported on Table 1, the anthracyclines can be separated in two groups. The first group concerns adriamycin and daunorubicin. For these molecules, the percentages kept at the interface are higher in the presence of anionic DPPA in the monolayer; the percentages of adriamycin and daunorubicin are, respectively, 4% and 7.2% in the presence of a POPC zwitterionic monolayer, and 11% and 14.3% in the presence of a POPC-DPPA anionic monolayer.

Thus the presence of negative charges at the interface increases these percentages: the electrostatic component of the interaction is favored as compared to the hydrophobic component. This higher selectivity for the negatively charged phospholipids, observed in the case of monolayers, is in agreement with previous studies performed with adriamycin and daunorubicin. For instance, de Wolf et al. (1993) studied the affinity of adriamycin for mixed DOPC/DOPG anionic liposomes and observed that the overall fixation of adriamycin to liposomes is determined by the percentage of anionic phospholipids in the bilayer. Escriba et al. (1990) observed by fluorescence anisotropy the same increase in the daunorubicin affinity for liposomes in the presence of phosphatidylserine or other anionic phospholipids. Finally, Constantinides et al. (1986) showed by differential scanning calorimetry that adriamycin had the same selectivity for anionic vesicles. The presence of adriamycin does not affect the phase transition properties of multilamellar vesicles of DPPC, whereas the thermogram of DPPG vesicles is highly perturbed at the same concentration of adriamycin. This difference in behavior is related to a higher electrostatic interaction between adriamycin and DPPG. This strong electrostatic interaction could also be responsible for the orientation of adriamycin and daunorubicin in phospholipid monolayers observed in SERRS: indeed the SERRS results suggest that only some molecules could penetrate into the phospholipid chains and that the major part would remain adsorbed on the polar headgroups. This adsorption at the water-phospholipid polar headgroup interface has also been described in the case of adriamycin in interaction with anionic phospholipids (de Wolf et al., 1991) or with cardiolipin. Nicolay et al. (1988) proposed that the orientation of adriamycin in interaction with cardiolipin monolayers depends on the surface pressure and the adriamycin/cardiolipin ratio. In particular, in the 2/1 complex, the chromophore would be at the water-polar headgroup interface, whereas it would be more embedded at a weaker ratio.

The second group concerns pirarubicin and idarubicin. The percentages of pirarubicin and idarubicin are, respectively, 11% and 17% in the presence of a POPC zwitterionic monolayer, and 13% and 12.6% in the presence of a POPC-DPPA anionic monolayer. Thus, in contrast to the two previous molecules, the percentage of pirarubicin is not drastically increased in the presence of anionic DPPA, as compared to the percentage in the presence of pure POPC monolayers. In the case of idarubicin, the percentage remaining at the interface in the presence of DPPA is even weaker than in the presence of POPC.

The previous surface pressure and SERRS studies demonstrated that pirarubicin, despite its higher hydrophobicity, does not penetrate the phospholipid chains of the POPC-DPPA monolayers and is adsorbed to the polar headgroups of phospholipids, forming a screen. This screen would hinder a deeper penetration of the other molecules dissolved in the subphase, limiting the final percentage of pirarubicin at the interface (Heywang et al., 1997). Such a screen could also be formed by idarubicin adsorbed to the polar headgroups: the good resolution of the SERRS spectrum of "adsorbed" idarubicin/POPC-DPPA suggests that the major part of idarubicin could be out of the monolayer. However, it is not possible to check this hypothesis.

Finally, these results are partly in agreement with previous results obtained by Gallois et al., who estimated by fluorescence the depth of the chromophore of 12 anthracycline derivatives in phosphatidylcholine liposomes containing variable percentages of phosphatidic acid and cholesterol (Gallois et al., 1996). They observed that the chromophore of adriamycin and daunorubicin is localized in the middle of the polar headgroup region, whereas the pirarubicin chromophore would be slightly deeper in the polar headgroup region, perhaps partially in the hydrocarbon phase. In our case it is not possible to observe such differences. However, Gallois et al. performed their study on liposomes, whereas we used monolayers as a simplified membrane model. In the case of monolayers, there is no aqueous phase to dissolve the anthracyclines after their crossing. This certainly limits the incorporation of anthracycline deeper in the monolayer.

SERRS STUDY OF PLANAR ASYMMETRICAL BILAYERS IN CONTACT WITH AN AQUEOUS SOLUTION OF PIRARUBICIN

The previous study enabled us to precisely describe the interaction of anthracyclines with phospholipid monolayers. Another aspect of this work is to determine whether the anthracyclines, put in the presence of a phospholipid planar bilayer, are able to cross this bilayer. Under these conditions, SERS is a powerful method. Indeed, in the case of planar bilayers put in contact with an aqueous solution of anthracycline, the bilayer separates the anthracycline from

the silver layer. If the anthracycline does not cross the bilayer, it remains too far from the silver coating to give a usable spectrum. On the contrary, if the anthracycline crosses the bilayer, we can observe its SERRS spectrum and determine the orientation of the anthraquinone part of the anthracycline and thus the orientation of the whole molecule. By using the experimental set-up built in our laboratory (Saint-Pierre Chazalet et al., 1994), it is thus possible to observe whether the anthracycline crossed the bilayer. Finally, one of the characteristics of the Langmuir-Blodgett method is the ability to transfer asymmetrical phospholipid bilayers kept in contact with water, to study the interaction of anthracycline with a membrane model taking into account the asymmetry of biological membranes.

In our previous work, we studied the behavior of pirarubicin in contact with symmetrical phospholipid bilayers, the composition of the two monolayers being the same. Pirarubicin was first put in contact with symmetrical POPC bilayers. (Heywang et al., 1996). Two concentrations were studied: 0.4 and 2 μM. In the two cases, we observed the SERRS spectrum of pirarubicin, showing that the molecule, initially separated from silver by the bilayer, is able to cross the POPC bilayer. Moreover, its orientation depends on the concentration. At weak concentration, the anthraquinone part of pirarubicin would be perpendicular or tilted on silver and thus parallel to the phospholipid chains. A possible interpretation is the following: pirarubicin would remain embedded in phospholipids and would not go completely out of the bilayer. At higher concentration, pirarubicin would be lying on silver; in this case, the major part of the pirarubicin molecules would go out completely and would remain in contact with the polar headgroups of phospholipids. Because of the short range of SERS, we observe mainly the molecules that completely crossed the phospholipid bilayer and are thus localized between the polar headgroups and the silver coating.

Symmetrical bilayers of POPC-DPPA (80–20 mol%) were also put in contact with an aqueous solution of pirarubicin. In all cases, pirarubicin would be lying on silver in contact with the polar headgroups of phospholipids; the major part of pirarubicin molecules would go completely out of the bilayer (Heywang et al., 1997). This result is correlated with the results obtained by surface pressure measurements and SERRS; in the case of pirarubicin interacting with anionic phospholipid monolayers, the anthracycline would remain adsorbed on polar headgroups.

However, the composition of a biological membrane is not the same in the two monolayers, because the inner layer contains, for instance, more anionic phospholipids than the outer layer (Devaux et al., 1991). To get closer to the conditions of the biological membranes, two types of asymmetrical bilayers have been transferred; these are presented in Fig. 7:

POPC/POPC-DPPA bilayers: The first monolayer in contact with the aqueous solution is composed of POPC, and the second monolayer in contact with silver is composed of a POPC-DPPA mixture.

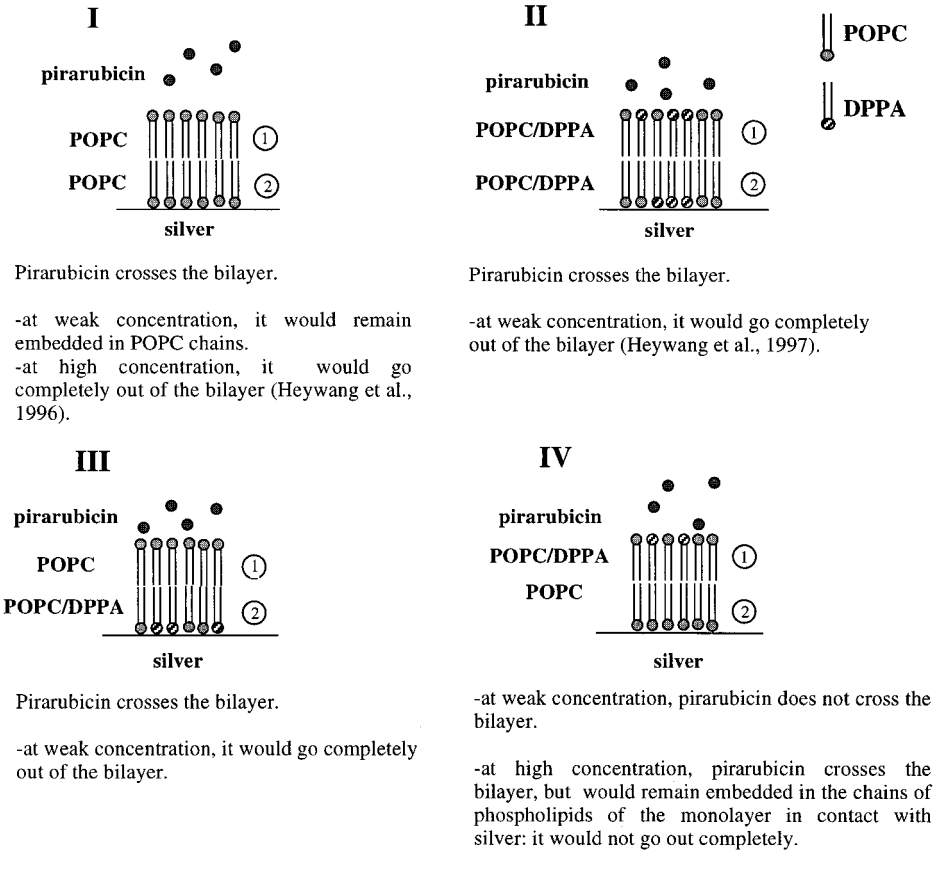

FIGURE 7 Summary of the different SERRS results obtained in the case of pirarubicin in contact with symmetrical and asymmetrical phospholipid bilayers.

POPC-DPPA/POPC bilayers: The first monolayer in contact with the aqueous solution of pirarubicin is composed of a POPC-DPPA 80–20 mol% mixture, whereas the other one (in contact with silver) is composed of POPC. Their SERRS spectra are presented in Fig. 8.

SERRS spectrum of asymmetrical preformed bilayers of POPC/POPC-DPPA in contact with an aqueous solution of pirarubicin 0.4 μM (Fig. 7 III). The spectrum (Fig. 8 *a*) shows the presence of pirarubicin molecules close to the silver coating: these molecules, initially separated from silver by the presence of the bilayer, have crossed the bilayer to come close to the metal. However, the spectrum is not very intense, which suggests that only some molecules crossed the bilayer.

Despite the rather bad quality of this spectrum, it is possible to observe a broad band between 1240 and 1260 cm^{-1} corresponding to the overlapping of these two peaks. Moreover, the band at 1206 cm^{-1} is weak, and the band at 1400–1410 cm^{-1} is broad and not well resolved. Even if this spectrum is rather difficult to analyze, it seems to be closer to the doxorubicinone spectrum; that could be an indication that the few molecules of pirarubicin that crossed the bilayer are lying on silver.

SERRS spectrum of asymmetrical preformed bilayers of POPC-DPPA/POPC in contact with an aqueous solution of pirarubicin (0.4 or 2 μM) (Fig. 7 IV). At weak concentration (0.4 μM), the spectrum (Fig. 8 *b*) does not show the presence of pirarubicin close to silver: only the two broad bands of graphitized carbon are observed. The range of SERRS being short, this means that pirarubicin does not cross the bilayer under these conditions.

When the concentration is increased at 2 μM, the spectrum shows the presence of pirarubicin molecules close to the silver coating (Fig. 8 *c*). On the whole, the spectrum is rather similar to the SERRS spectrum of pirarubicin in aqueous solution, even if the band at 980 cm^{-1} is strongly diminished. In this last case, the major part of the pirarubicin molecules would be perpendicular or tilted on the silver layer and would remain embedded in the phospholipid chains of the monolayer in contact with silver; under these conditions, pirarubicin does not completely cross the bilayer.

DISCUSSION

The results obtained with the asymmetrical bilayers are reported in Fig. 7 and compared with the previous results obtained with the symmetrical bilayers (Heywang et al., 1996, 1997).

We observe that pirarubicin at weak concentration in contact with a first monolayer of POPC (POPC/POPC-

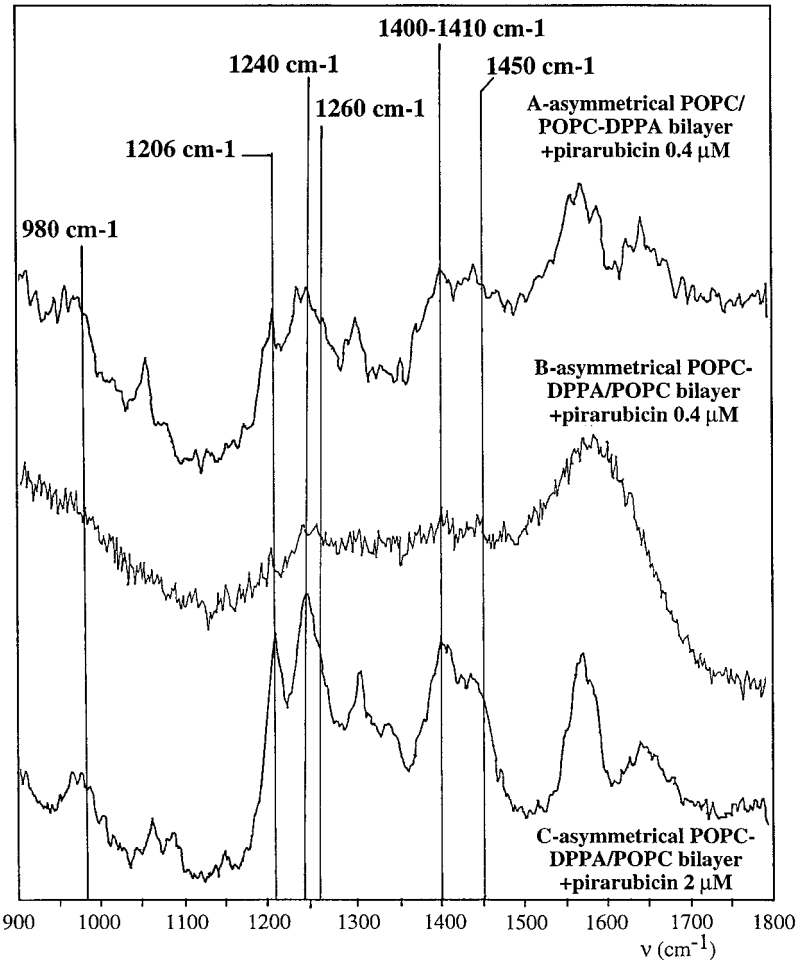

FIGURE 8 (*a*) SERRS spectrum of an asymmetrical bilayer of POPC (monolayer in contact with water)/ POPC-DPPA 80–20 mol% (monolayer in contact with silver) put in contact with an aqueous solution of pirarubicin (C = 0.4 μM, pH = 5.5). (*b*) SERRS spectrum of an asymmetrical bilayer of POPC-DPPA 80–20 mol%/POPC put in contact with an aqueous solution of pirarubicin (C = 0.4 μM, pH = 5.5). The spectrum of graphitized carbon has not been subtracted. (*c*) SERRS spectrum of an asymmetrical bilayer of POPC-DPPA 80–20 mol%/POPC put in contact with an aqueous solution of pirarubicin (C = 2 μM, pH = 5.5).

DPPA bilayers, Fig. 7 *III*) is able to cross the bilayer and to go out completely. In contrast, if the first monolayer is negatively charged (POPC-DPPA/POPC bilayers, Fig. 7 *IV*), pirarubicin at the same concentration is unable to cross the bilayer. The surface pressure study has shown that pirarubicin molecules adsorbed to the polar headgroups of DPPA form a screen limiting the deeper penetration of other molecules (Heywang et al., 1997).

The SERRS results suggested that the pirarubicin molecules, transferred after their adsorption to a anionic monolayer, remained in contact with the polar headgroups of phospholipids. This could explain why the pirarubicin molecules are kept at the interface in the case where pirarubicin is initially in contact with a POPC-DPPA/POPC bilayer. However, pirarubicin is able to cross the bilayer if the concentration of the solution is increased to 2 μM; only a weak fraction of the molecules penetrates the bilayer. This higher concentration makes it possible to remove the obstacle of the adsorbed molecules because of a higher concentration gradient.

The crossing of pirarubicin through the bilayer is also favored by the presence of negative charges in the second monolayer. At weak concentration (Fig. 7 *I*), pirarubicin is able to cross a symmetrical POPC bilayer but remains embedded in the POPC chains without going out completely. In the presence of DPPA in the second monolayer (Fig. 7 *III*), we observe, despite the bad quality of the spectrum, that a few molecules of pirarubicin cross the bilayer, go out completely, and thus are parallel to the silver coating. This is likely to be related to the presence of anionic DPPA, which favors the crossing of molecules by the electrostatic interaction. This aspect is confirmed by the comparison of bilayers II and IV: when the first monolayer is negatively charged (POPC-DPPA/POPC bilayers), pirarubicin cannot cross the bilayer, probably because of the screen formed by the adsorbed molecules. However, if DPPA is also present in the second monolayer (Fig. 7 *II*), pirarubicin crosses the bilayer completely because of the presence of the negative charges in the second monolayer.

These results suggest that the behavior of pirarubicin is different when the molecule is in contact with the outer or the inner monolayer of a cellular membrane; the penetration of pirarubicin into the cell from the surrounding medium by passive diffusion (our membrane model is devoid of any energy system) would be easier than its exit, because this exit would be limited by the adsorption of molecules to the negative polar headgroups. This behavior has also been proposed in the case of adriamycin by Speelmans et al. (1994), who analyzed the kinetics of passive transport of this anthracycline in DNA-containing liposomes. They observed that the presence of anionic phospholipids decreases the permeability coefficient of adriamycin. This inhibitory effect is likely to be related to a tightening of the polar headgroups induced by the presence of anionic phospholid-adriamycin complexes at the solvent-polar headgroup interface.

This conclusion is different from the conclusion proposed by Frezard et al. (1997), who studied the incorporation of three anthracyclines (adriamycin, daunorubicin, and pirarubicin) in DNA-containing vesicles of different compositions. They observed, as did Speelmans et al., a decrease in the permeability coefficient of anthracycline through the membrane in the presence of anionic phospholipids. However, they assigned it to a decrease of the amount of the anthracycline neutral form embedded in the membrane resulting from the presence of anionic phospholipids, the neutral form being the only form that is able to cross the membrane by passive diffusion.

CONCLUSION

The design of new derivatives of anthracyclines is a major challenge in chemotherapy. It implies a better understanding of the anthracycline-membrane interaction. In this work, it has been shown that surface pressure measurements and SERS are two powerful and complementary methods for the study of anthracycline-membrane interactions. In particular, the comparison of the behavior of four different anthracyclines (adriamycin, daunorubicin, pirarubicin, and idarubicin) underlined the importance of the hydrophobic component of anthracycline in its interaction with phospholipid monolayers.

However, phospholipid monolayers are not a completely realistic membrane model, because they can give information about the interaction of anthracyclines with only one side of the membrane. Therefore it is important to complete this kind of work with the study of the diffusion of anthracyclines through phospholipid bilayers. At this level, a significant aspect of the Langmuir-Blodgett technique is the ability to transfer symmetrical and asymmetrical phospholipid bilayers. The SERS study was thus divided into two complementary parts: the first one, coupled with the surface pressure study, made it possible to estimate the orientation of anthracyclines previously integrated into a phospholipid monolayer before the transfer. The second one consisted of observing the possible passive diffusion of pirarubicin through asymmetrical phospholipid bilayers. The results observed in this second part were interpretated on the basis of the hypotheses proposed after the first study. In particular, the study of the interaction of pirarubicin with asymmetrical DPPA-containing bilayers suggests that this antibiotic would easily penetrate the cells, whereas its exit by passive diffusion in the extracellular medium would be limited by the presence of anionic phospholipids in the inner layer of the membrane. However, two opposite phenomena could occur at the same time: after the penetration of the cells 1) pirarubicin molecules would dissolve in the cytoplasm, but 2) some pirarubicin molecules could remain adsorbed to the inner layer of the membrane because of the electrostatic interaction with anionic phospholipids; the uptake by the P-gp would be easier, and these molecules would be ejected from the cells (Speelmans et al., 1994). However, it is difficult to be more precise, because our membrane model is not complete; the motions of the anthracycline adsorbed to polar headgroups are limited because of the presence of the planar support.

Finally, we also do not take into account the difference in fluidity between the two leaflets of the bilayer. This parameter probably plays an important role in the incorporation of anthracyclines into membrane, because the presence of cholesterol or sphingomyeline, which decreases the membrane fluidity, limits the penetration of anthracyclines (Speelmans et al., 1994). This aspect of the anthracycline-membrane interaction will be treated in a later study.

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