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# MODE OF INSERTION OF PRAZIQUANTEL AND DERIVATIVES INTO LIPID MEMBRANES

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**Abstract**—We report here about the relationship between the destabilization of the lipid organization induced by praziquantel and derivatives and their mode of insertion into the lipid matrix. Measurements of lipid transition temperature and efflux of 6-carboxyfluorescein encapsulated in liposomes establish the lipid destabilizing capacity of praziquantel as compared to praziquantel derivatives. IR spectroscopy (attenuated total reflection technique) applied to oriented lipid bilayers indicates that praziquantel or derivatives do not modify significantly the lipid structure. In order to give a molecular description of the position of the drug into the lipid bilayer, we applied a conformational analysis procedure making it possible to calculate the structure of amphiphilic molecules assembled in aggregates and the mode of insertion of amphiphilic drugs into a lipid layer. The praziquantel lipid destabilizing capacity is explained in terms of the high praziquantel–lipid interaction and the large area occupied per drug molecule in the lipid layer.

Praziquantel (PZ1, Biltricide, 2-cyclohexylcarbonyl-1, 2, 3, 6, 7, 11b-hexahydro-4*H*-pyrazino(2, 1-*a*)isoquinolin-4-one) is an anthelmintic with a very broad spectrum of activity against parasitic trematodes and cestodes [1]. It is now available for the treatment of human schistosome and for the treatment of cestodes infections in veterinary medicine and in man. As an anthelmintic, PZ1 has attracted the interest of many investigators who have helped to elucidate its pharmacokinetics and its toxicology. However, its mode of action is poorly understood [2].

Two striking phenomena can be observed in schistosomes exposed to PZ1: an almost instantaneous tetanic contraction of the parasite musculature and a rapid structural damage of the syncytial tegument. These two processes are obviously related to a modification of the host membrane properties and are the consequence of a massive PZ1-induced influx of calcium into the parasite [3]. Incubation with $^{45}Ca^{2+}$, in the presence of PZ1, causes a rapid increase of radioactivity inside the worms which corresponds to a massive influx of $^{45}Ca^{2+}$. The extracellular $Ca^{2+}$ concentration plays an essential role in the mode of action of PZ1 as demonstrated by *in vitro* experiments in a calcium-free medium or in the presence of an excess of magnesium. In both cases, the contraction of the parasite musculature is inhibited [4, 5]. The role of $Ca^{2+}$ influx is confirmed by experiments made with ionophores like A23187 which is able to transport $Ca^{2+}$ ions through parasite membranes and induces an increase in muscle tension.

The molecular mechanism of this $Ca^{2+}$ transport remains unknown. Most inhibitors of known neurotransmitters of *Schistosoma mansoni* do not antagonize the action of PZ1 [5–8]. PZ1 is not an ionophore like A23187 or ionomycine which act as carriers. Furthermore, PZ1 does not affect ATPase activities [9]. D-600, a specific blocker of calcium ion movement in vertebrate smooth muscle, does not inhibit the praziquantel induced influx of $Ca^{2+}$ into schistosome [10]. On the contrary, pre-treatment with fluoxetine or $La^{3+}$ prevents the influx of $Ca^{2+}$ and the muscular contraction [6]. Since several arguments suggest an interaction of the drug with sites not classically associated with the regulation of $Ca^{2+}$ transport it was tempting [11] to speculate that PZ1 might exert its mode of action by interaction with tegumental phospholipids.

It is our purpose to investigate here the mode of interaction of PZ1 and derivatives with lipids and more specifically with DL-α-dipalmitoylphosphatidylcholine, asolectine and a mixture made of the major lipids of *S. mansoni* membranes. Experimental techniques (differential scanning calorimetry, attenuated total reflection (ATR) infrared spectroscopy and fluorimetry) and theoretical approach (conformational analysis) were combined in order to get more insight into the lipid–drug interaction.

## MATERIALS AND METHODS

*Materials*. The lipid mixture used for small unilamellar vesicles formation (SUV) contained the major lipids of *Schistosoma mansoni* [12, 13], i.e. phospholipids 36%: phosphatidylcholine from egg yolk (28%), phosphatidylethanolamine from bovine brain (25%), phosphatidylserine from bovine brain (15%), phosphatidylglycerol from egg yolk lecithin (8%), phosphatidylinositol from soybean (5%), cardiolipin from bovine heart (3%) and phosphatidic acid from egg yolk lecithin (2%); cholesterol (28%), triolein (22%), cholesteryl acetate (6%) and oleic acid (7%). All these lipids, DL-α-dipalmitoylphos-

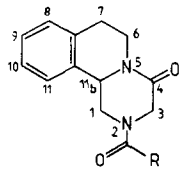
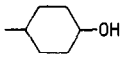
| Formulas of praziquantel and derivatives | | Anthelmintic activity against S. mansoni (1) | |
|---|---|---|---|
| | | in vivo | in vitro |
|  | PZ 1 praziquantel R=  | +++ | +++ |
| | PZ 2 R=  | ++ | ++ |
| | PZ 12 metabolite R=  | ++ | ++ |
| | PZ 11 R=  | ++ | ++ |
| | PZ 10 R=  | ++ | + |
| | PZ 7 R= $-CH(CH_3)_2$ | ++ | + |
| | PZ 9 R=  | + | 0 |
| | PZ 6 R= $-CH_3$ | 0 | + |
| | PZ 8 R= $-C(CH_3)_3$ | 0 | 0 |

Fig. 1. Formulas and *in vivo* and *in vitro* anthelmintic activity against *S. mansoni* of praziquantel and derivatives (listed by order of decreasing activity). The symbols used indicate *in vivo*: (+++) complete reduction of worms with 5 × 50 mg/kg or less; (++) complete reduction of worms with 5 × 500 mg/kg or less; (+) less than 90% worm reduction with 5 × 500 mg/kg; (○) no effect with 5 × 500 mg/kg. *In vitro*: (+++) full effect at $10^{-6}$ g/ml or less; (++) full effect at $10^{-4}$ g/ml or less; (+) less than full effect at $10^{-4}$ g/ml; (○) no effect at $10^{-4}$ g/ml [1].

phatidylcholine (DPPC) and asolectin were purchased from Sigma Chemical Company. 6-Carboxyfluorescein was obtained from Eastman Kodak. Praziquantel and derivatives (Fig. 1) were gifts from Dr P. Andrews (Bayer AG, Wuppertal).

*Preparation of lipids vesicles.* Multilamellar lipid vesicles (MLV) were obtained by stirring a lipid dry film (or a drug–lipid film) in 10 mM Tris–HCl (pH 7.4) buffer. Small unilamellar vesicles sonication was performed with a Branson Sonifier B12 (15 min; 60 W). The sonicated suspension was centrifuged at 600 *g* for 10 min to remove titanium and residual multilamellar vesicles.

*Differential scanning calorimetry measurements (DSC).* DPPC multilamellar vesicles containing praziquantel or derivatives were formed as described above at a final lipid concentration of 55 μmol/ml (DPPC-drug, 4/1 molar ratio). Aliquots (100 μl) of lipid suspension were placed in sealed inox sample pans. A reference sample was similarly prepared using 100 μl buffer. Measurements were carried out on a Setaram DSC III differential scanning calorimeter (Lyon, France) operating at a heating rate of 2°K/min.

*IR spectroscopy (attenuated total reflection technique).* DPPC and drugs were dissolved in $CHCl_3$ (DPPC-drug, 4/1 molar ratio) and a drop of this mixture (50 μl, 0.3 μmol) was placed on one side (10 $cm^2$) of a KRS-5 ATR internal reflection plate (50 × 20 × 2 mm, Harrick EU2121) with an aperture angle of 45°, yielding 25 internal reflections. Oriented multibilayers were obtained by evaporation of the solvent as described by Fringeli and Günthard [14, 15]. Spectra were recorded with a Perkin Elmer infrared spectrophotometer 983G equipped with a Perkin Elmer micro specular reflectance accessory

and a Polariser Mount Assembly equipped with a silver bromide element. The optical part of the spectrophotometer was purged with dry air. Spectra were encoded every $cm^{-1}$ and transferred at the end of the scan from the memory of the spectrophotometer to an Olivetti M40 computer through a RS232C interface [16]. The samples were scanned with an incident light polarized parallel and perpendicular with respect to a normal to the ATR plate. The absorption coefficient of an IR band is maximum if the electric field vector of the IR radiation is parallel to the transition dipole moment but tends to zero if the former is perpendicular to the latter. Bond orientation is determined via the measurement of the dichroic ratio defined as the ratio of the absorption coefficients of parallel and perpendicular polarized light as described by Fringeli and Günthard [14, 15]. Information about the orientation of the entire drug molecule was obtained from the orientation of individual bonds.

*Release of 6-carboxyfluorescein (6-CF).* SUV made of asolectin or *S. mansoni* lipids were prepared as described above. They were formed in 42 mM 6-CF/10 mM Tris–HCl pH 7.4 buffer; non-encapsulated material was removed by gel filtration through a Sephadex G-25 column equilibrated with buffer. Lipid concentration in the cuvette was 0.1 μmol/ml. Drugs (final concentration: 1 mM) were dissolved in DMSO and added to the liposome suspension. No CF release was observed with DMSO alone. 100% release corresponds to the fluorescence measured after addition of deoxycholate 1.3%. Excitation wavelength of 6-CF was 492 nm and fluorescence emission was measured at 520 nm in a Jobin Yvon JY3D spectrofluorimeter.

*Conformational analysis.* As described previously [17–20], a stepwise computation approach was used to predict the configuration of mixed monolayers made of praziquantel or derivatives and dipalmitoylphosphatidylcholine (DPPC). The values used for valence angles, bond lengths, atomic charges and torsional potentials were those currently used for conformational analysis. The conformation of the isolated molecule and its orientation at the lipid–water interface have been established as described elsewhere [16, 17]. The procedure of drug insertion can be summarized as follows. (a) Position of the drug was fixed and position of the lipid molecule was modified along the $x$-axis. Each distance was equal to 0.05 nm. For each separating distance a rotation was imposed to the lipid around its own $z$-axis and around the drug by steps of 30°. Among all possible orientations only the structure of minimum energy was considered. (b) The lipid molecule was allowed to move along the $z$-axis perpendicular to the lipid–water interface. Again, only the structure of minimum energy was considered. (c) The lipid molecule had the possibility of changing its orientation around the $z$-axis compared with the drug molecule. This procedure allowed the probable packing of drug and lipid molecules to be defined. Packing of these two molecules was maintained and the orientation of a third lipid molecule around them was considered. We limited our analysis to the number of lipid molecules sufficient to surround the drug. When the configuration of the cluster of m molecules was determined, both areas occupied by each molecule and the intermolecular area were estimated after projection on the $x$–$y$ plane, and the mean molecular area was calculated. This procedure has been followed to evaluate the structure of dipalmitoylphosphatidylcholine assembled in bilayers and an excellent agreement [21] was found between the predictions and the neutron diffraction data. Positions of atoms in lipid molecules were predicted with a precision which was in the limit of the experimental error. Calculations were made on a CDC Cyber 170 computer coupled to a Calcomp 1051 drawing table (Computing Center of Free University of Brussels) with the drawing program PLUTO (Motherwell and Clegg 1978).

## RESULTS AND DISCUSSION

### *Differential scanning calorimetry (DSC)*

Profiles of two typical DSC spectra corresponding to DPPC multilamellar vesicles containing increasing amounts of praziquantel (PZ1) or derivatives (PZ6) are shown in Fig. 2a, b. Values of lipid transition temperature (Ttr) shift and enthalpy associated to the gel–liquid crystalline lipid phase transition ($\Delta H$) are reported in Table 1.

The shift of the transition temperature ($\Delta T$) to lower temperature is maximal for PZ1 (5.1°) which has the highest anthelmintic activity against *S. mansoni*. No significant change of the enthalpy associated to the gel–liquid crystalline lipid phase transition ($\Delta H$ DPPC = 35.95 ± 4.18 kJ/mole) could be detected which suggests that PZ1 does not modify the lipid structure but is rather randomly distributed into the lipid matrix, acting as an inert spacer between lipid molecules.

### *Infrared spectroscopy (attenuated total reflection technique)*

The IR spectra of PZ1, DPPC and DPPC/PZ1 (4/1 molar ratio) are shown in Fig. 3a–c respectively.

The polarisation of five absorption bands [14, 15] was considered. Four of them are associated to DPPC: the C=O double bond stretching of the acyl chains at 1736 $cm^{-1}$ (dipole moment parallel to the C=O double bond); the $CH_2$ bending of the hydrocarbon chains at 1466 $cm^{-1}$ (dipole moment parallel

Table 1. Shift ($\Delta T$) of lipid transition temperature and enthalpy associated to the gel–liquid crystalline lipid phase transition determined by differential scanning calorimetry

| Drug | $\Delta T$ (°) | $\Delta H$ (kJ/mole) |
|---|---|---|
| DPPC | 0 | 35.95 ± 4.18 |
| PZ1 | 5.1 ± 0.1 | 33.44 ± 4.18 |
| PZ2 | 0.3 ± 0.1 | 41.80 ± 4.18 |
| PZ12 | 0.9 ± 0.1 | 29.96 ± 4.18 |
| PZ11 | 0.9 ± 0.1 | 39.71 ± 4.18 |
| PZ10 | 0.7 ± 0.1 | 39.71 ± 4.18 |
| PZ7 | 1.2 ± 0.1 | 37.62 ± 4.18 |
| PZ9 | 1.4 ± 0.1 | 37.62 ± 4.18 |
| PZ6 | 0.4 ± 0.1 | 39.71 ± 4.18 |
| PZ8 | 0.4 ± 0.1 | 41.80 ± 4.18 |

Experimental conditions are described in Materials and Methods.

Table 2. Bond orientation as compared to a perpendicular to the lipid–water interface measured by attenuated total reflection IR technique

| | 1736 $cm^{-1}$ $\nu$(C=O) | 1466 $cm^{-1}$ $\delta(CH_2)$ | 1256 $cm^{-1}$ $\nu_{as}(PO_2^-)$ | 1094 $cm^{-1}$ $\nu_s(PO_2^-)$ | 1650 $cm^{-1}$ $\nu$(C=O) |
|---|---|---|---|---|---|
| Drug alone | 59° ± 5° | 71° ± 5° | 59° ± 5° | 51° ± 5° | |
| Drug | | | | | |
| PZ1 | 57° ± 5° | 67° ± 5° | 57° ± 5° | 50° ± 5° | 53° ± 5° |
| PZ2 | 58° ± 5° | 63° ± 5° | 57° ± 5° | 49° ± 5° | 52° ± 5° |
| PZ12 | 57° ± 5° | 76° ± 5° | 58° ± 5° | 50° ± 5° | 55° ± 5° |
| PZ11 | 58° ± 5° | 72° ± 5° | 64° ± 5° | 48° ± 5° | 54° ± 5° |
| PZ10 | 60° ± 5° | 69° ± 5° | 59° ± 5° | 51° ± 5° | 57° ± 5° |
| PZ7 | 60° ± 5° | 69° ± 5° | 58° ± 5° | 49° ± 5° | 56° ± 5° |
| PZ9 | 58° ± 5° | 67° ± 5° | 58° ± 5° | 47° ± 5° | 53° ± 5° |
| PZ6 | 58° ± 5° | 68° ± 5° | 55° ± 5° | 49° ± 5° | 54° ± 5° |
| PZ8 | 60° ± 5° | 61° ± 5° | 62° ± 5° | 53° ± 5° | 58° ± 5° |

Experimental conditions are described in Materials and Methods.

to the bisector of the H–C–H angle); the antisymmetric $PO_2^-$ stretching at 1256 $cm^{-1}$ (dipole moment perpendicular to the bisector of the O . . . P . . . O angle) and symmetric $PO_2^-$ stretching at 1094 $cm^{-1}$ (dipole moment parallel to the bisector of the O . . . P . . . O angle). One band is associated to PZ1 and derivatives: the C=O double bond stretching of the amide groups at 1650 $cm^{-1}$ (parallel to the C=O double bond).

Since these bands present a high absorbance and are not significantly overlapped, they were studied for the DPPC–drug complex with no further treatment of the spectra. The orientations of the dipole moment associated to the previous bonds are reported in Table 2. This table indicates that praziquantel or derivatives do not modify significantly the orientation of the different groups associated to DPPC. Indeed, the values obtained remain closely

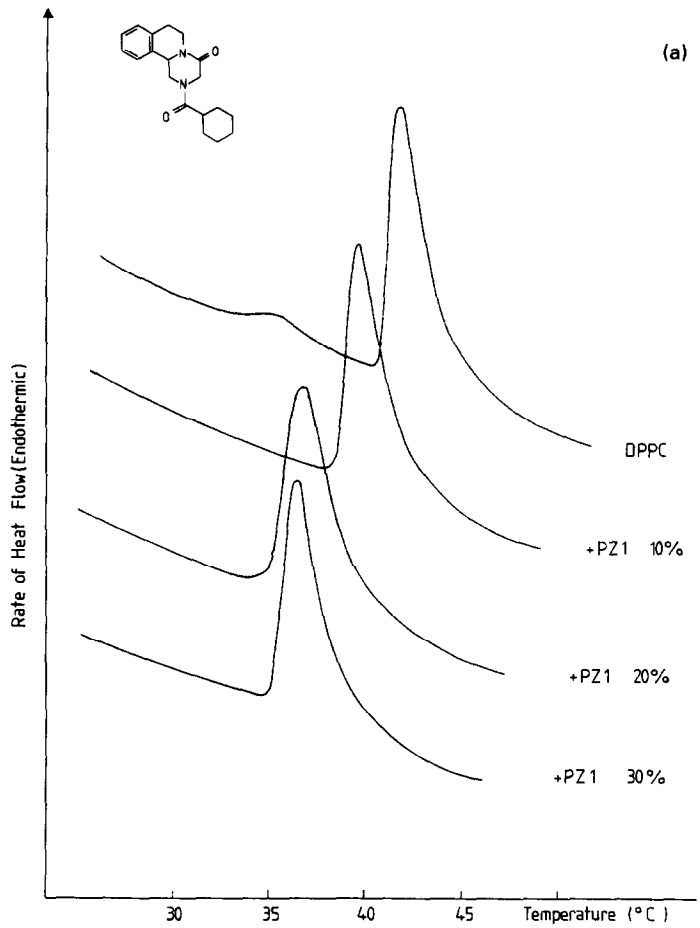



Fig. 2(a).

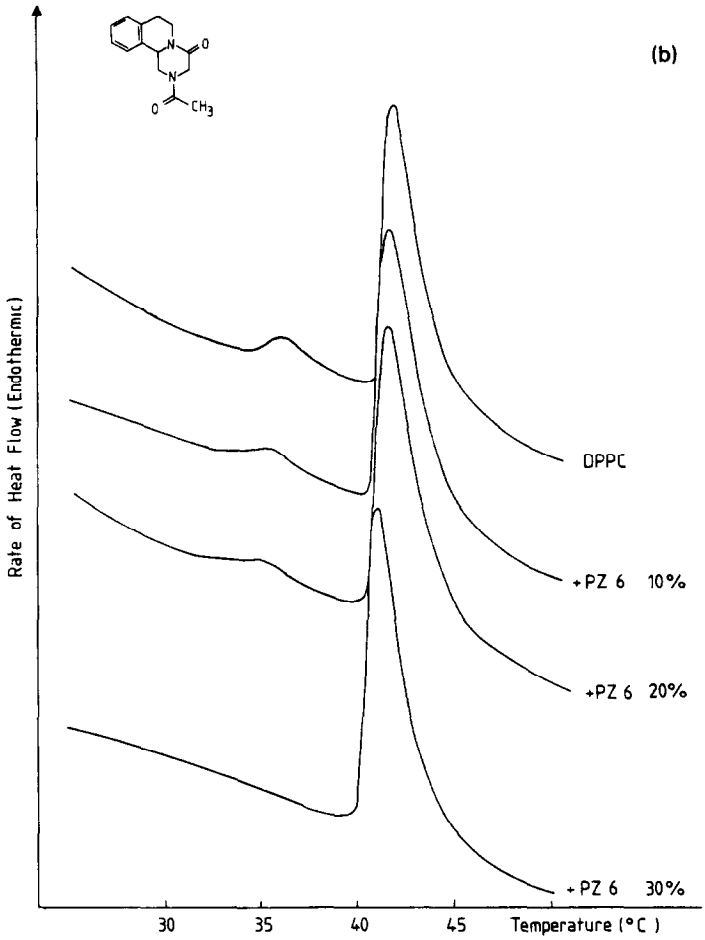



Fig. 2. Differential scanning calorimetry of DPPC multilamellar vesicles containing increasing molar amounts of PZ1 (a) and PZ6 (b). Lipid concentration: 55 μmole/ml. Liposomes were formed in Tris–HCl buffer (10 mM, pH = 7.4). Drug–lipid molar ratios are indicated in the figure.

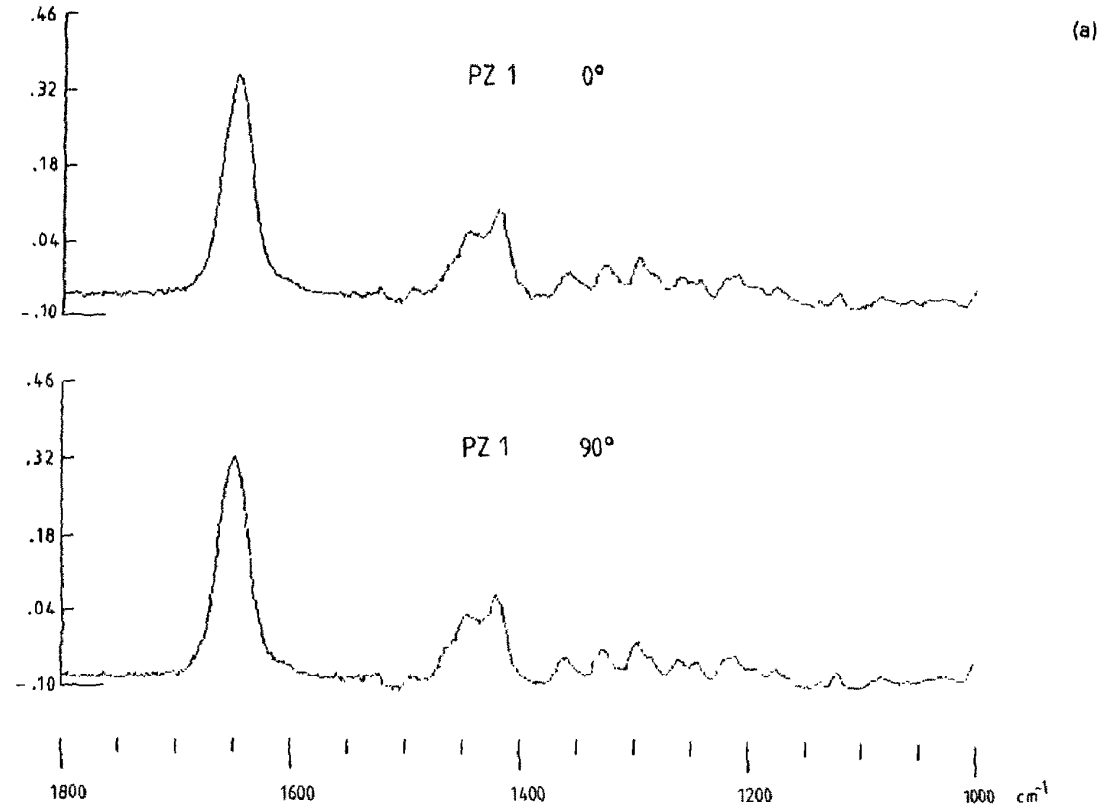
related to those of pure DPPC. Moreover, for all the derivatives, the $\gamma_W$ ($CH_2$) band of DPPC at 1200 $cm^{-1}$ does not decrease in intensity and preserves a high dichroic ratio (R > 2.5, dipole moment parallel to the hydrocarbon chain). Since this band results from a coupling of the $\gamma_W$ ($CH_2$) vibration with a vibration of the ester group, it vanishes when deviation of the hydrocarbon chain conformation from all-*trans* occurs [14, 15]. The insensitivity of this band and of its polarization towards addition of drugs demonstrates that the amount of all-*trans* conformation in the DPPC hydrocarbon chains is not modified upon interaction with the drugs and that the mean orientation of these chains is not modified either. In conclusion, it seems that PZ1 or derivatives modify neither the global orientation nor the conformation of the DPPC molecule and that their insertion does not modify the lipid matrix organization. As suggested by DSC, PZ1 and derivatives would act like inert spacers. On the other hand, the orientation of the carbonyl groups associated to the 9 drugs is similar, whatever the drug structure.

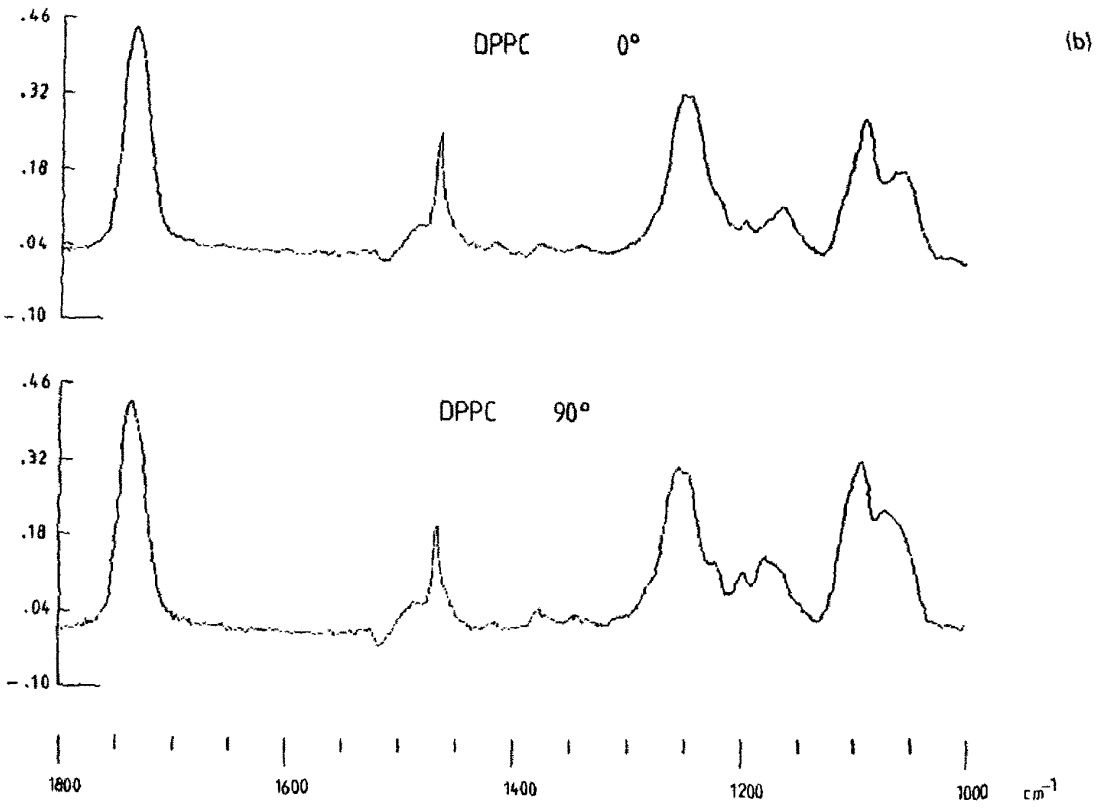
*Release of 6-carboxyfluorescein*

To confirm the previous observations, we investigated the PZ1-induced membrane destabilization on lipid mixtures, more representative of schistosomes membranes. A fluorescent probe, 6-carboxyfluorescein (6-CF), was encapsulated in small unilamellar vesicles at high concentration [22]. Fluorescence is selfquenched and any dilution of the probe enhances the fluorescence signal. PZ1 produces the most efficient destabilization of liposomes made of asolectine or *S. mansoni* lipids as already observed in calorimetric measurements (Table 3).

*Conformational analysis*

Structural formulas of PZ1 and some derivatives and numbering of the torsional angles are illustrated in Fig. 4. An initial systematic study was performed on the two torsional angles of each derivative which were given successive increments of 20°. This approach yielded 324 different conformations. Structures of maximum probabilities were minimized by


(a)
.46
.32
.18
.04
-.10
PZ 1 0°
PZ 1 90°
1800
1600
1400
1200
1000
cm⁻¹


(b)
.46
.32
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DPPC 0°
DPPC 90°
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Fig. 3(a) and (b).

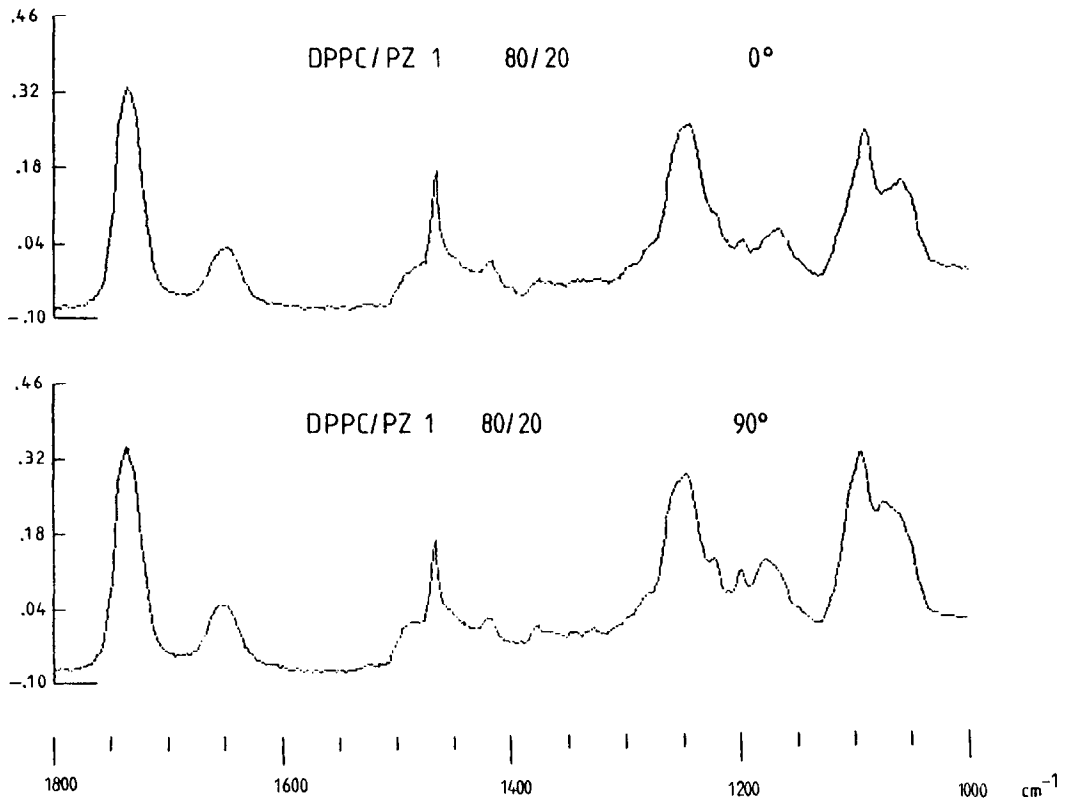


Fig. 3. Infrared spectra of oriented layers of PZ1 (a), DPPC (b) and DPPC/PZ1 (4/1 molar ratio) (c) on a KRS-5 ATR plate (0.3 μmole), polarized at 0° and 90°, T = 25°.

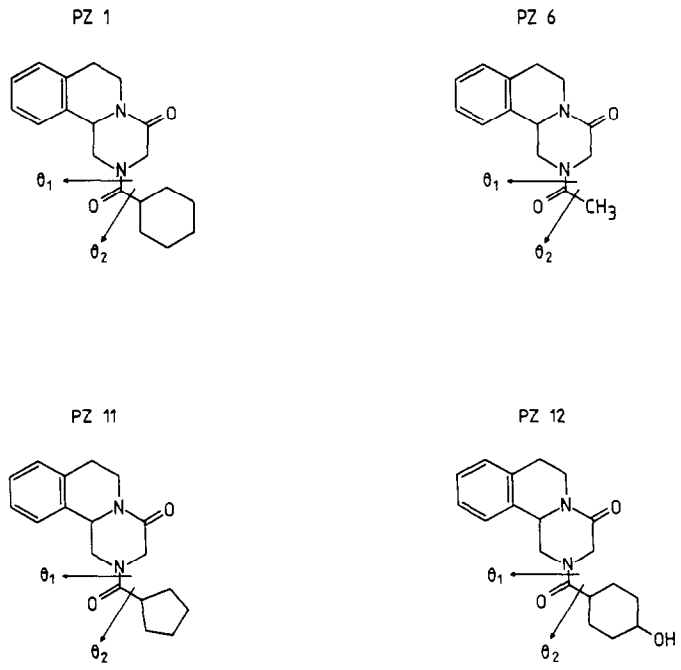


Fig. 4. Molecular structure and numbering of the torsional angles of PZ1 and several derivatives.

Table 3. Release of 6-carboxyfluorescein

| Drug | Asolectine SUV % of relative fluorescence 100% = deoxycholate 1.3% | *S. mansoni* lipids SUV |
|---|---|---|
| PZ1 | 39.8 ± 3% | 10.3 ± 3% |
| PZ2 | 8.0 ± 3% | 5.5 ± 3% |
| PZ12 | 2.3 ± 3% | 2.0 ± 3% |
| PZ11 | 13.7 ± 3% | 4.0 ± 3% |
| PZ10 | 5.2 ± 3% | 2.4 ± 3% |
| PZ7 | 6.3 ± 3% | 3.6 ± 3% |
| PZ9 | 2.2 ± 3% | 2.7 ± 3% |
| PZ6 | 2.1 ± 3% | 3.8 ± 3% |
| PZ8 | 5.0 ± 3% | 3.3 ± 3% |

Experimental conditions are described in Materials and Methods.

Table 4. Main characteristics of the isolated praziquantel molecules or derivatives at the simulated hydrophobic/hydrophilic interface

| Drug | $\theta 1$ (°) | $\theta 2$ (°) | Epho (kJ/mole) | Ephi (kJ/mole) | $\Delta$ (Å) |
|---|---|---|---|---|---|
| PZ1 | 240 | 240 | 162.18 | 48.9 | 1.88 |
| PZ12 | 240 | 220 | 162.1 | 64.8 | 2.01 |
| PZ11 | 240 | 160 | 152.1 | 48.9 | 2.07 |
| PZ6 | 240 | 180 | 111.2 | 48.9 | 2.53 |

$\theta 1$ and $\theta 2$ are torsional angles, Epho and Ephi are the hydrophobic and the hydrophilic transfer energy, $\Delta$ is the distance between hydrophobic and hydrophilic center. The conformational analysis procedure is described in Materials and Methods.

the simplex procedure [23] and oriented at the air–water interface [18]. The values of torsional angles are listed in Table 4, along with the distance ($\Delta$) between the hydrophobic and the hydrophilic centers and the hydrophobic–hydrophilic balance. Each molecule was inserted into a DL-$\alpha$-dipalmitoylphosphatidylcholine (DPPC) monolayer (Fig. 5). The

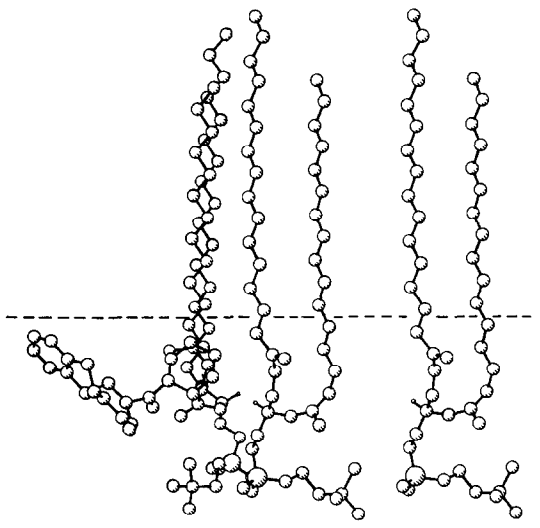

Fig. 5. Conformation of PZ1–DPPC mixed monolayer in a frontal view. Dotted line delineates the hydrophobic (upper) and hydrophilic medium (lower).

Table 5. Interaction parameters of praziquantel and derivatives with dipalmitoylphosphatidylcholine

| | $A$ (Å$^2$) | $E_{int}$ (kJ/mole) |
|---|---|---|
| PZ1 | 60 | −35.53 |
| PZ12 | 53 | −29.99 |
| PZ11 | 51 | −22.15 |
| PZ6 | 50 | −24.66 |

$A$, mean molecular area; $E_{int}$, drug–lipid energy of interaction. The conformational analysis is described in Materials and Methods.

conformation and orientation of DPPC at the air–water interface was computed previously [21] according to the procedure described in Materials and Methods. Recent work (manuscript in preparation) has shown that there is a linear relationship between the shift of the lipid transition temperature and the mean area occupied per drug molecule in the lipid layer. Below an area of 30 Å$^2$ per molecule, the drug volume is too small to affect significantly the lipid organization and no shift of temperature transition is observed. This area corresponds to the "free space" existing between assembled lipid molecules. A molecule occupying a much larger area strongly modifies the lipid dynamics and temperature transition is drastically shifted to lower temperatures. The higher area occupied per PZ1 molecule in the DPPC lipid layer could explain the lipid destabilization as compared to PZ derivatives. All praziquantel derivatives were less destabilizing than the parent compound. The total drug–DPPC interaction energy and the mean area occupied per drug molecule in a lipid layer are listed in Table 5. The higher PZ1-DPPC energy of interaction as compared to the PZ derivatives is in agreement with the DSC and 6-carboxyfluorescein release data.

## CONCLUSIONS

The combined use of a theoretical approach (conformational analysis) and experimental procedures made it possible to give a molecular description of the mode of insertion of praziquantel and derivatives into a lipid matrix.

The data demonstrated essentially that praziquantel which is the most active against *S. mansoni* is the most efficient in destabilizing the lipid organization. Since for the other derivatives, no evident relationship was observed between the destabilizing activity and the biological activity, it would be premature to consider that the mode of action of praziquantel would consist in an aspecific destabilization of the *S. mansoni* membrane rendering it permeable to $Ca^{2+}$ ions. It should, however, be kept in mind that the lipid destabilization is rather weak as observed for other $Ca^{2+}$ ionophores (ionomycine) [24]. Indeed a high destabilizing capacity would be responsible for an aspecific ion transport ($Na^+$, $K^+$, $Ca^{2+}$, . . .). The capability of praziquantel of transferring $Ca^{2+}$ ion across a lipid bilayer is presently under investigation. It is worthwhile to mention that praziquantel does not modify the lipid structure but acts as a spacer between lipid molecules. For this reason, the enthalpy of

melting does not vary significantly as a function of the drug concentration whereas the transition temperature is shifted to lower values.

We attempted to relate this lipid destabilization with the structure of the PZ1 molecule inserted into the lipid layer. Drugs can modify the lipid organization by acting as spacer between lipid molecules or by direct interaction with the lipid molecule. We calculated (submitted paper) that the lipid–drug interaction should be higher than 41.8 kJ/mole in order to induce a lipid conformational change. The calculated value of −35.53 kJ/mole in the DPPC–PZ1 system is in agreement with the DSC data. If we except the area occupied per PZ1 molecule, the conformational analysis indicated very similar structures and mode of insertion into the lipid layer for the different derivatives. It should, however, be kept in mind that, in the present stage of development of the conformational analysis, the procedure does not permit identification of new structures resulting from close interaction between adjacent molecules, and future refinements should resolve this limiting factor.

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