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Lipid-itraconazole Interaction in Lipid Model Membranes

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Abstract—Itraconazole, a lipophilic, fungal sterol-biosynthesis inhibitor, does not disturb membrane organization parameters measured by differential scanning calorimetry and infrared spectroscopy. Conformational analysis studies suggest that the molecular volume and the position of itraconazole in the lipid membrane is similar to that of dipalmitoyl phosphatidylcholine. The mean energy of interaction between itraconazole and the phospholipid is $-60{\cdot}6$ kJ mol^{-1} whereas this energy in the pure lipid matrix is $-54{\cdot}3$ kJ mol^{-1}. The mean molecular area of itraconazole calculated by projecting the molecule on the lipid–water interface is equal to that occupied by the pure lipid (60 Å^2/molecule).

The *N*-substituted triazole, itraconazole, is a lipophilic antifungal drug. Pharmacokinetic studies indicate that tissue levels of this compound far exceed those found in the blood. This suggests its preferential membrane localization (Heykants et al 1987). Both in-vivo and in-vitro pharmacological studies reveal that it possesses a broad spectrum of activity against yeasts and dimorphic and filamentous fungi (Van Cutsem et al 1987a, b). Itraconazole, however, differs from other azoles, such as ketoconazole, by its high efficacy against, for example, *Aspergillus fumigatus*, *Cryptococcus neoformans* and *Pityrosporum ovale* (Marichal et al 1985, 1986; Van Cutsem et al 1987a, b). The azole antifungal drugs belong to the group of sterolbiosynthesis inhibitors and interfere at nanomolar concentrations with the cytochrome $P450_{14DM}$ located in the endoplasmic reticulum of fungi (Vanden Bossche et al 1984; Vanden Bossche 1985). This enzyme catalyses the oxidative removal of the 14α-methyl group of the sterol precursors. Inhibition of this enzyme leads to depletion of ergosterol, the main sterol in fungi, together with an accumulation of 14α-methylated sterols which disturbs normal membrane functioning. Because of the possible role membranes could play in itraconazole's activity, we have investigated its interaction with phospholipid molecules.

Materials and Methods

Preparation of liposomes and differential scanning calorimetry
Multilamellar vesicles of DL-α-dipalmitoyl phosphatidylcholine (DPPC, Sigma) were prepared at a lipid concentration of 55 μmol mL^{-1} in Tris-HCl buffer (10 mM 0·15 M NaCl, pH 7·4). Azole derivatives (Janssen Pharmaceutica) were incorporated into the lipid film before liposome formation as described by Vanden Bossche et al (1984). Measurements were carried out on a Setaram DSC III calorimeter (Lyon, France) operating at a heating rate of 2°K min^{-1}. All experiments were performed with 100 μL inox cells. Tris-HCl buffer (100 μL) was used as a reference sample.

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Preparation of liposomes and radioactivity measurements
Multilamellar vesicles of DPPC, were prepared according to Vanden Bossche et al (1982). The lipid concentration was 6·6 μmol in one mL 0·1 M potassium phosphate buffer pH 7·4. Tritium-labelled itraconazole (71·3 μCi mmol^{-1}) was added to the lipid either (a) to the chloroform solution at a concentration of 2 μmol mL^{-1}, or (b) to the preformed liposome suspension at a concentration of 500 pmol mL^{-1}.

(a) Liposomes were centrifuged for 10 min at 10000 *g* and the radioactivity and the phospholipid content of the supernatant (s1) and the pellet (p1) were measured. The pellet was resuspended in 1 mL buffer and incubated for 1 h at room temperature (20°C). The liposomal preparation was then centrifuged again, resulting in a pellet (p2) and a supernatant (s2). To evaluate non-specific binding to the glass tubes, the recovered content, i.e. the sum of p1 and s1, was taken as reference. Results were expressed as the ratio of the content of p1 and p2 to the recovered content. Radioactivity was measured in a liquid scintillation counter. Phospholipid content was determined using an enzymatic diagnostic kit (Boehringer).
(b) The antimycotics dissolved in DMSO were added to the preformed liposomes and the suspension briefly vortexed. After 1 h incubation at room temperature the suspension was centrifuged and treated as described above.

IR spectroscopy (attenuated total reflection technique)
DPPC and drugs were dissolved in $CHCl_3$ (DPPC:drug, 4:1 mol/mol) 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 & Günthard (1981). Spectra were recorded with a Perkin Elmer infrared spectrophotometer 983G equipped with a Perkin Elmer microspecular reflectance accessory and a polarizer 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 (Goormaghtigh et al 1987). The mean scan rate was 174 cm^{-1} min^{-1}. The samples were scanned with an incident light polarized parallel and perpendicular with respect to the ATR plate. A baseline recorded with the same ATR element and the same polarization was subtracted from each spectrum. Polarization was expressed as the dichroic ratio $R = A_{90°}/A_{0°}$ where $A_{90°}$ and $A_{0°}$ are the absorbances measured with the parallel and the perpendicular incident light, respectively. The angle between the oscillating dipole moment of transition and the normal to the bilayer support was calculated according to Fringeli & Günthard (1981).

Conformational analysis

A stepwise computation approach was used to predict the configuration of mixed monolayers (Brasseur et al 1983; Brasseur & Ruysschaert 1986; Brasseur 1986). Values 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 were established as described elsewhere (Brasseur et al 1983; Brasseur & Ruysschaert 1986; Brasseur 1986). The procedure for drug insertion can be summarized as follows. The position of itraconazole was fixed. (a) The 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 angle of 30° was imposed on the lipid around its own z-axis and around the drug. Of 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 was allowed to change 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 characterize the structure of DPPC organized in bilayers and an excellent agreement (Brasseur et al 1981) was found between the predictions and the neutron diffraction data. The positions of atoms in the lipid molecules were predicted with a precision which was within the limit of the experimental deviation. Calculations were performed with PC-MSA+ (Molecular Structure Analysis) and PC-TAMMO+ (Theoretical Analysis of Model Membrane Organization) programs. All graphics were drawn with PC-MGM+ program (Molecular Graphics Manipulation).

Results

Differential scanning calorimetry (DSC) and radioactivity measurements

The DSC pattern for multilamellar DPPC liposomes (Fig. 1) containing increasing amounts of itraconazole shows that even at 30% drug:lipid molar ratio the lipid transition temperature (Tr) and the enthalpy of transition are not significantly modified (Table 1). Experiments performed with ^3H-labelled itraconazole indicate that 90% of itraconazole added before liposome formation is inserted at time 0 h. Eighty-five % is still incorporated after 1 h. If the itraconazole is added after liposome formation (see Materials and Methods) 78% of the drug is associated with the liposomes.

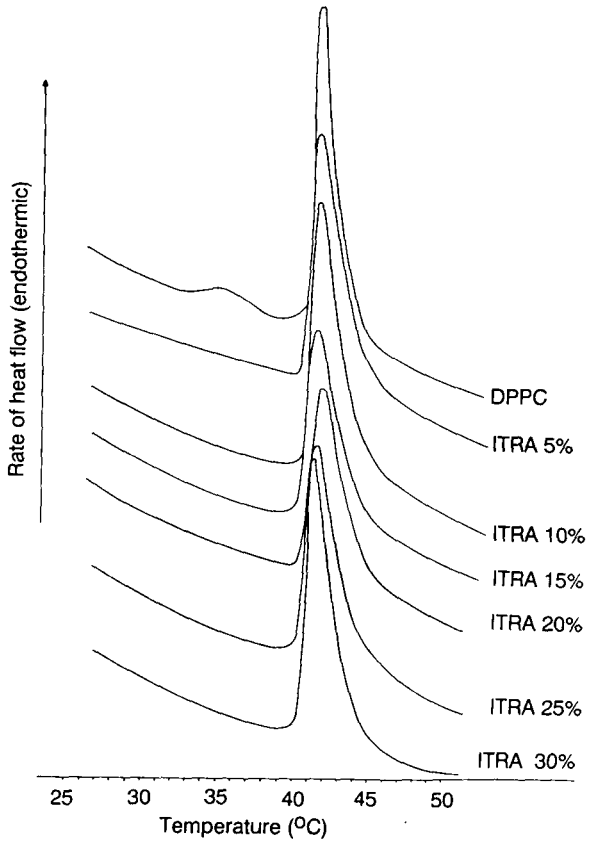

FIG. 1. Differential scanning calorimetry of DPPC multilamellar vesicles containing increasing molar concentrations of itraconazole. Lipid concentration: 55 μmol mL^{-1}. Liposomes were formed in Tris-HCL buffer (10 mM, pH 7·4, 0·15 M NaCl). Drug-lipid molar ratios are indicated in the figure.

Infrared spectroscopy

Effect of itraconazole on the conformation of DPPC (Fig. 2). The amount of hydrocarbon chains in the all-*trans*

Table 1. Enthalpy of transition measured by differential scanning calorimetry for different itraconazole:lipid molar ratios.

Itraconazole:lipid (Ratio)	Enthalpy of transition (kJ mol^{-1})
Pure DPPC	35·1 ± 2·5
5	34·2 ± 2·5
10	35·1 ± 2·5
15	36·0 ± 2·5
20	28·4 ± 2·5
25	30·5 ± 2·5
30	33·0 ± 2·5

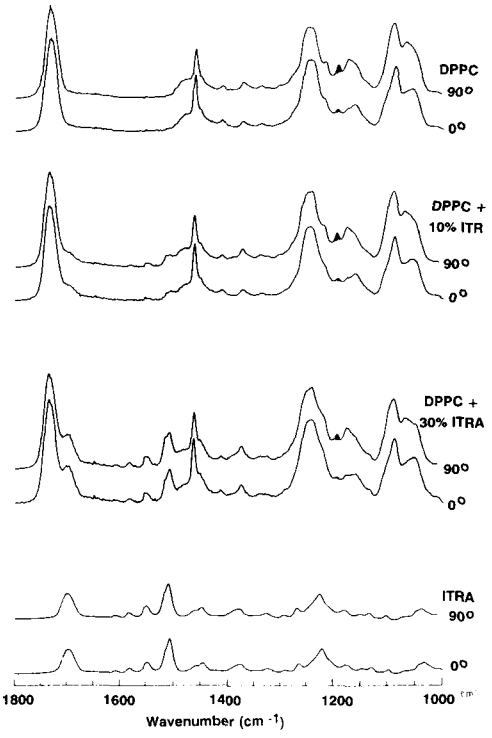

FIG. 2. Polarized IR spectra of DPPC, itraconazole and two mixtures of DPPC with 10 and 30% itraconazole. 0° and 90° refer to the polarization of the incident light as described in Materials and Methods. The hatched peak corresponds to the lipid $\gamma_w(CH_2)$ vibration at 1200 cm^{-1}.

configuration can be estimated by the intensity of the $\gamma_w(CH_2)$ vibration at 1200 cm^{-1}. This adsorption band results from a coupling between the (CH_2) vibration and a vibration of the ester group (Fringeli & Günthard 1981). The disappearance of the all-*trans* configuration is accompanied by an almost complete loss of intensity of this band. The orientation of its transition dipole moment, parallel to the all-*trans* hydrocarbon chain, also allows the determination of the mean orientation of the all-*trans* chains with respect to a normal to the interface (Fringeli & Günthard 1981). In the presence of 10% itraconazole, this band is not modified and shows a very high dichroism ($R > 3$) similar to the one measured for pure DPPC. In the presence of 30% itraconazole, the band is still present with a high dichroic ratio ($R > 3$) although quantitative comparison is made difficult because of a slight overlapping of the strong bands at 1229 and 1183 cm^{-1} arising from the itraconazole molecule. The 1180 and 1165 cm^{-1} bands were assigned respectively to the C-O of the planar fragment C-C-O-C of the α-fatty acyl group and of the non-planar fragment C-C-O-C of the β-fatty acyl group (Fringeli & Günthard 1981). Their respective ratios were not modified by addition of 10 or 30% itraconazole. Moreover, the band at 1180 cm^{-1} remains strongly polarized (90°) as in pure DPPC (i.e. the single bond C-O is close to a normal to the interface). Finally, the frequency and the polarization of the C-O(P) moiety at 1071 cm^{-1} remains unaffected by the presence of 10 or 30% itraconazole. This result indicates that the conformation of the C-O-P-O-C fragment is not significantly modified by the itraconazole (Fringeli & Günthard 1981). We did not attempt to analyse other vibrations because of the overlapping with strong itraconazole bands.

Effect of DPPC on the conformation of itraconazole. The assignment of the bands of itraconazole was considered to be beyond the scope of these experiments because of the complexity of the molecule and because of the presence of repetitive units in the molecule which would have prevented any orientation determination by means of dichroism measurements. However, a general analysis of the whole spectrum reveals that for pure itraconazole very little dichroism occurs demonstrating that no preferential organization exists in the sample (Fig. 2, bottom). The situation changes drastically when itraconazole is in contact with DPPC as illustrated by the strong 90° polarization of the peaks at 1613, 1585, 1555 and 1513 cm^{-1} which are not overlapped by DPPC bands. It is of special interest that the dichroic ratio of those bands varies between 1·3 and 2·3. These high values of R are only compatible with a high degree of organization of the itraconazole molecules generating those peaks. Calculations performed on the theoretical basis described by Fringeli & Günthard (1981), lead to the conclusion that the order parameter S ($0 < S < 1$) must be larger than 0·6.

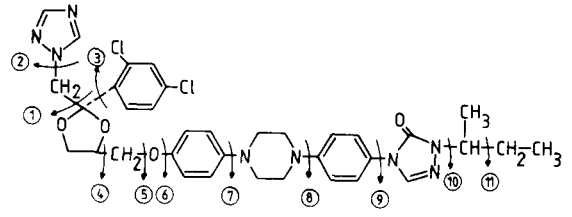

FIG. 3. Molecular structure and numbering of the torsional angles of itraconazole.

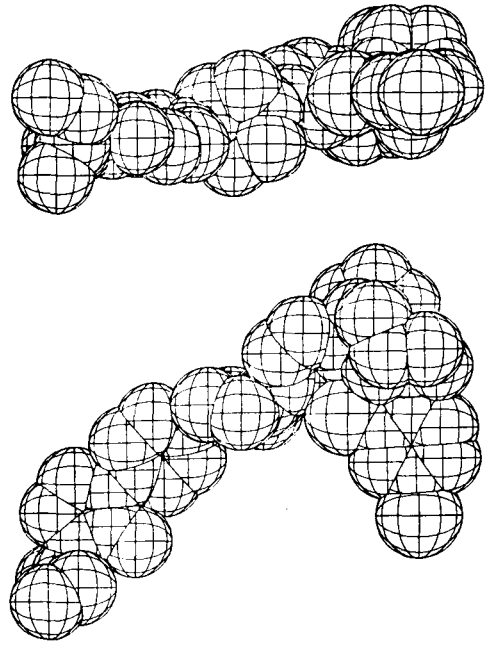

FIG. 4. Two orthogonal views of a space filling model (cross hatching) of the most probable structure of itraconazole.

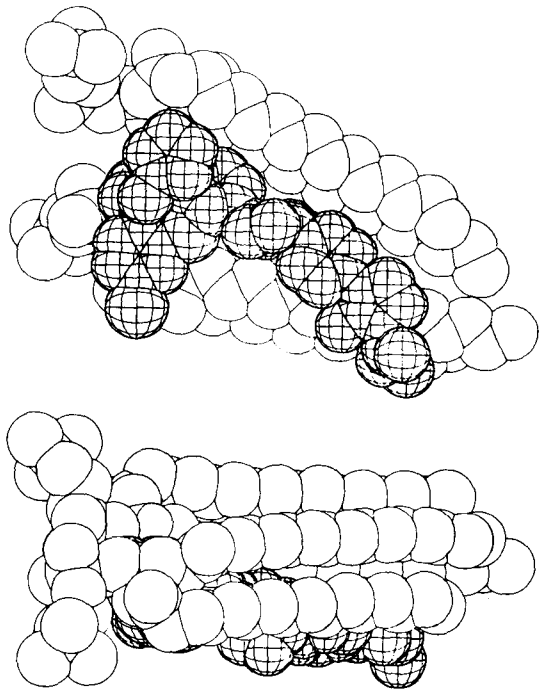

FIG. 5. Two orthogonal views of a space filling model of itraconazole (cross hatching) inserted in dipalmitoyl phosphatidylcholine. The lipid in front of the itraconazole has not been drawn.

Conformational analysis

Analysis of itraconazole was performed with the procedure developed previously for the azole derivatives. The definitions of the torsional angles are given in Fig. 3. Fig. 4 shows the most probable structure after systematic analysis and minimization at the lipid-water interface. Fig. 5 is a space filling view of the mode of insertion of itraconazole in the lipid matrix. Three DPPC molecules interact directly with itraconazole with a mean energy of interaction of $-60{\cdot}6$ kJ mol^{-1} as compared with that observed in the pure lipid matrix ($-54{\cdot}3$ kJ mol^{-1}) (Brasseur et al 1981). Moreover, the mean molecular area calculated by projecting the itraconazole molecule on the lipid-water interface is equal to that occupied by the pure lipid (60 $Å^2$/molecule). Fig. 5 underlines the surprising topological similarity between itraconazole and lipid when both are represented in real molecular volumes. This spatial organization and orientation of itraconazole into a lipid layer might explain the experimental observation that itraconazole does not perturb the lipid organization.

Discussion

Itraconazole does not induce significant changes (with concentrations up to 30 mol%) in the lipid membrane organization parameters, as measured by DSC, infrared spectroscopy and leakage of entrapped [^{14}C]glucose (Vanden Bossche 1985). A first possibility is that itraconazole is not in close contact with the membrane and, as such, cannot affect the lipid organization. Itraconazole's log P value of 5·66 strongly suggests a lipophilic positioning of the molecules. Secondly, the solubility of itraconazole in a 0·1 M phosphate buffer pH 7·4 is limited to 0·1 μg mL^{-1}. Above this concentration, precipitation occurs. The results of the entrapment studies with radioactive itraconazole indicate that the drug is at least strongly associated with the lipid membrane, both when the compound is mixed with the lipid in the organic phase and when the drug is added to preformed liposomes. Examination of the infrared spectra of phospholipid-itraconazole mixtures indicate that although itraconazole does not interfere with the phospholipid associated peaks, there is indeed a change in dichroic ratio of peaks assigned to itraconazole. The high values of this dichroic ratio are only compatible with a high degree of organization of the itraconazole molecules. The conformational analysis suggests, instead, an orientation similar to that of lipids in the bilayer as a consequence of the similarity between the area occupied per itraconazole molecule and the volume occupied in the lipid bilayer and the corresponding values of the DPPC. Due to the complex structure of itraconazole, we were unable to evaluate this orientation, but it is unlikely that a drug with such a high n-octanol/water partition coefficient would concentrate at the lipid–water interface and would adopt an orientation parallel to this interface. Our model could explain the preferential tissue distribution of the drug as measured in pharmacokinetic studies, the high activity of the drug against lipophilic fungi as, e.g. *Pityrosporum ovale*, and the higher affinity of the compound for its target, the cytochrome P450, which in eukaryotic systems is a membrane bound enzyme (Vanden Bossche 1987).

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