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INTERACTIONS BETWEEN TRYPANOCIDAL DRUGS AND MEMBRANE PHOSPHOLIPIDS

A SURFACE PRESSURE, SURFACE POTENTIAL AND ELECTROPHORETIC MOBILITY STUDY

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Abstract—Amphiphilic diphenyl methane derivatives exhibiting both antiproliferative and trypanocidal effects were studied with respect to their interactions with phospholipids, in monolayers and bilayers. These compounds, namely (4-benzyl)-phenoxy-2 trimethylammonium ethane iodide (D_1), (4-tertiobutyl)-phenoxy-2 morpholinium ethane chloride (D_2), and (4-benzyl)-phenoxy-2 morpholinium ethane chloride (D_3), were shown to interact with phosphatidylcholine (PC) and phosphatidylserine (PS) in monolayers, as monitored by surface pressure and surface potential measurements. The film expansion of monolayers, on 10 mM NaCl subphase at pH 7.1, was more pronounced in the presence of D_2 and D_3 in the subphase before spreading of the lipids than with the injection of the drugs underneath a preformed film. Apparent binding constants of 10^4 M^{-1} were determined for both drugs from monolayer experiments. With D_2 in the presence of PS, results of monolayer compressions and electrophoretic mobility measurements indicate binding of the drug to the lipid molecules only when the molecular area was large. D_3 was shown to interact with PS, both in monolayers and bilayers, with a drug-to-lipid binding constant of about 2×10^4 M^{-1}, as evaluated from electrophoretic mobility measurements on PS liposomes. These results, which indicate binding of these drugs to phospholipids in the order $D_2 < D_3$, correlate with the biological activity of the drugs, and may account for the discrepancy observed between the drug concentrations required for biological and binding activities.

In the course of research concerning the design of biologically active compounds and after preliminary results obtained by Brandes [1], we became interested in the study of diphenyl methane derivatives by comparison with tamoxifen (Fig. 1). These compounds reveal a significant *in vitro* activity against the human breast cancer cell line MCF7 and this activity was shown to correlate linearly with their affinity constants for a receptor-like protein named the anti-oestrogen binding site [2]. The existence of this receptor has been recognized for some time [3, 4] and further characterized as being a membrane protein embedded in the endoplasmic reticulum [5]. The same compounds exhibit significant activity on cultures of trypanosomes in the concentration range of 1 to 5 μM [2]. This result can be rationalized on the basis of several features common to cancer cells and trypanosomes, in particular their mutual capacity for rapid cell division [6]. For the trypanosome system, it was shown on the grounds of trypsin digestion and photolabelling experiments that the target for the compounds of the diphenyl methane class (Fig. 1) is also a protein.‖

Both antiproliferative and trypanocidal effects were observed for concentrations of these compounds in the micromolar range. In contrast, the affinity constants of these molecules for their target protein when measured on purified fractions are in the nanomolar range. Tamoxifen and its analogues are lipophilic and cationic molecules at physiological pH with a marked amphiphilic character (Fig. 1). They possess all necessary attributes for strong interaction with lipids in membranes, mainly the acidic ones.

Accumulation of these compounds at the cell membrane level might explain the large differences between the effective (nanomolar) and necessary (micromolar) concentration ranges observed for these molecules. The question of whether these compounds can alter membrane organization also merits consideration.

We thus performed a study of the interactions of these tamoxifen analogues with phospholipids in model membranes through surface pressure and surface potential measurements in monolayers, and by measuring the electrophoretic mobility of liposomes. Since the trypanosome membranes are mainly constituted of phosphatidylcholine and phosphatidylethanolamine with various amounts of phosphatidylserine and phosphatidylinositol [7], we

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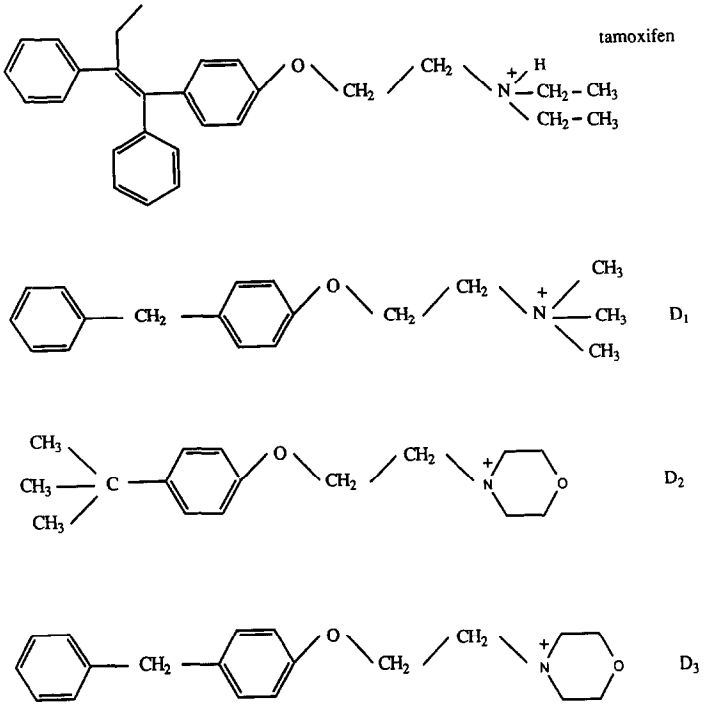

Fig. 1. Chemical structures of the drugs.

have used phosphatidylcholine and phosphatidylserine as representative of neutral and acidic phospholipids, respectively.

MATERIALS AND METHODS

Chemicals. Compounds D_1, D_2 and D_3* were synthesized according to Poling and Yokoyama [8] using slightly modified procedures (see Fig. 1). Samples were purified by preparative thin-layer chromatography on alumina gels (elution with dichloromethane/pentane, 1/1, v/v mixtures). The structure and purity of these compounds were assessed by chemical analysis, NMR and mass spectroscopy.

PS and PC, from the Sigma Chemical Co. (Poole, U.K.), were pure as checked by thin-layer chromatography. Salts were of analytical grade (Merck, Darmstadt, F.R.G.) and ultra-pure water was used (Milli-Q apparatus, Millipore).

Drug affinity and biological activity measurements. Affinity measurements were determining by displacement of a reference radioactive probe.†

Biological activities *in vitro* on *Trypanosoma equiperdum* populations were established using a standard medium described previously [9]. Increasing concentrations of drugs were solubilized in the medium and, after 24 hr of incubation (37°, 4% CO_2), viability was checked by direct counting of moving trypanosomes on a microscope grid.

Monolayer experiments. Compression isotherms, surface pressure π and surface potential ΔV were measured with an experimental device described previously [10], using experimental procedures detailed elsewhere [11]. Surface pressure was measured with a platinum plate connected to a torsion balance of our own manufacture, while surface potential was measured by means of two Americium electrodes connected to a Keithley electrometer. Reference surface potentials of aqueous solutions were around 10–20 mV. Lipids were spread in the form of chloroform:methanol (9/1, v/v) solutions as described previously [11]. Compounds D_1, D_2 and D_3 were added stepwise into the water subphase (10 mM NaCl solution buffered with 1 mM phosphate, pH 7.1). Experiments were carried out at 20°.

Electrophoretic mobility measurements. Multilamellar vesicles of PS were prepared by the Bangham method [12]. Electrophoretic measurements were carried out with a Mark II microelectrophoresis apparatus (Rank Brothers, Bottisham, U.K.). Data were obtained by applying the electric field at constant current intensity in a cylindrical cell equipped with a four electrode arrangement, two electrodes for applying the electric field and two

* Abbreviations: D_1, (4-benzyl)-phenoxy-2 trimethylammonium ethane iodide; D_2, (4-tertiobutyl)-phenoxy-2 morpholinium ethane chloride; D_3, (4-benzyl)-phenoxy-2 morpholinium ethane chloride; PC, phosphatidylcholine; PS, phosphatidylserine.

† Betbeder D, Périé J-J, Baltz T, Poirot M and Faye J-C, Characterization of a benzyl-phenoxy-ethanamine bound protein in Trypanosome: possible relation between binding affinity and trypanocidal activity. *Mol Biochem Pharmacol*, submitted.

electrodes for measuring the resulting voltage. Particles were alternatively timed in each electric field direction by inversing the field polarity at the upper stationary level, avoiding problems of liposome sedimentation.

Presented data are averaged on at least 20 determinations in each direction. The temperature of the cell was maintained at 20° in a thermoregulated bath. The results were interpreted in the light of the theory presented elsewhere [13, 14]. Briefly, the experimental ζ potentials were calculated from mobility determinations using Henry's equation [15]:

$$\zeta = (3/2)\eta\mu/\varepsilon_0\varepsilon_r \cdot f_1(\kappa a)$$

where η and μ are, respectively, the viscosity of the aqueous phase and the electrophoretic mobility of the particle; ε_0 and ε_r are permitivities of the free space and the aqueous phase, respectively; κ is the reciprocal of the Debye screening distance and a is the radius of the particle, f_1 being a polynomial function of both parameters.

The size of PS liposomes was heterogenous with predominantly small vesicles [16]. Taking a mean particle radius of 0.5 μm and a concentration of 10 mM for NaCl, one obtains a value of 1.484 for $f_1(\kappa a)$. The knowledge of the exact radius of the mobile particles is in fact not of critical importance. Thus, if the mean particle radius is taken as 0.1 μm, the value of $f_1(\kappa a)$ is 1.429 and the mobility values are 4% higher than with a radius of 0.5 μm. For a radius greater than 0.5 μm, the value of $f_1(\kappa a)$ is 1.5 and the mobility difference is only 1%.

The interpretation of the data in terms of the Gouy–Chapman–Stern theory [15, 17] of the double layer enables the ζ potential to be calculated. This potential is the value $\Psi(x)$ of the electrical potential at the plane of shear, at a given distance x from the interface:

$$\Psi(x) = (2kT/e)\ln\left[\frac{1 + \alpha \cdot \exp(-\kappa x)}{1 - \alpha \cdot \exp(-\kappa x)}\right]$$

$$\alpha = (\exp(e\Psi_0/2kT) - 1)/(\exp(e\Psi_0/2kT) + 1).$$

In these equations, k is the Boltzmann constant, T is the absolute temperature and e is the electron charge.

The distance of the plane of shear x depends on the size of the charged molecules which can adsorb to the surface of the liposomes. Taking into account the small size of the molecules used (discussed in Ref. 16), we adopted a value of 0.2 nm for x in the present calculation.

The electrostatic potential $\Psi(x)$ depends on the surface charge density σ which itself depends on the lipid molecular packing and is affected by cation and drug adsorption. Assuming a 1:1 stoichiometric interaction between these cationic species and the anionic phosphate group PO^-, the equilibria are thus:

$$PO^- + M^+ \leftrightarrows POM$$

with the association constant:

$$K_a(M) = [POM]/[PO^-](M^+)_0]$$

and

$$PO^- + D^+ \leftrightarrows POD$$

with:

$$K_a(D) = [POD]/PO^-](D^+)_0.$$

In these equations, square brackets refer to lipid surface concentrations while round brackets with subscripted zero indicate cation and drug volumic concentrations at $x = 0$.

These volumic concentrations are obtained from the Boltzmann equation:

$$(M^+)_0 = C_b \cdot \exp(-e\psi_0/kT)$$

$$(D^+)_0 = D_b \cdot \exp(-e\psi_0/kT)$$

where C_b and D_b are the bulk concentrations of Na^+ and drug molecules, respectively.

Finally, to account for possible changes in drug concentration in the bulk aqueous phase owing to binding to lipid molecules, the following relationship was established:

$$D_t = D_b + D_a$$

It assumes that the total drug concentration, D_t, is the sum of the bulk concentration in the aqueous phase (D_a) and that of molecules bound to lipids (D_b). D_a depends on both the drug-to-lipid adsorption constant $K_a(D)$ and the number of PS molecules which are involved in the drug binding equilibrium.

Calculations were carried out with a molecular area of 0.52 nm^2 for the lipids used which corresponds to a surface pressure of 30 nM/m for PS monolayer. A sodium-to-PS binding constant of 0.16 M^{-1} was taken, as previously determined [14]. The drug-to-PS binding constants were considered as variable parameters and were adjusted in order to obtain the best fit between calculated and measured ζ potentials.

RESULTS

D_1, D_2 and D_3 (Fig. 1) were selected for their different biological activities against MCF7 type cells and trypanosomes (Table 1).

D_1 was purposely chosen for its non-reactivity whereas D_2 is weakly active and D_3 has a significant activity. Experiments were performed with two different phospholipids, PS and PC, which are liable to interact differently with the three substrates owing to their charge; PS bears a net negative charge at pH 7.1 whereas PC is zwitterionic [18]. Note that PC is one of the main lipid components of the trypanosome membrane [7].

Monolayer experiments

First, in the absence of any phospholipid, the ability of D_1, D_2 and D_3 to form a film at the air–water interface was checked. This control experiment was required owing to the amphiphilic structure of these molecules. A too large tensio-activity might hamper the detection of interactions between these drug molecules and a phospholipid film. Curves (a) and (b) in Fig. 2 indicate that the formation of a film was observed for concentrations higher than 1 mM for D_1 and 0.1 mM for D_2 and D_3. This film formation cannot be accounted for by the appearance of drug micelles in the subphase since measuring the

Table 1. Biological activities of the drugs

Compound	MCF7 growth inhibition		Trypanocidal effect	
	K_i (nM)	% Inhibition of control	K_i (nM)	LD_{50} (μM)
D_1	—	0	Not measurable	—
D_2	200	10	1000	50
D_3	3	60	4	5

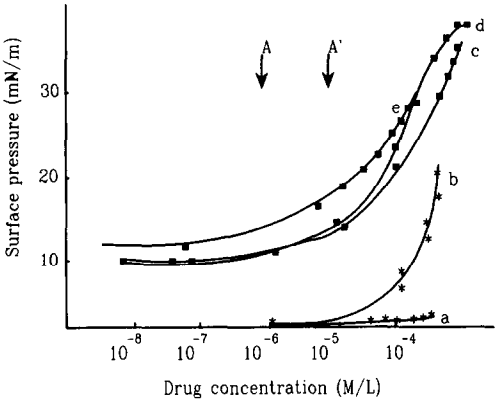

Fig. 2. Changes in surface pressure versus drug concentration in the absence (*) and in the presence (■) of PS. (a) D_1 alone; (b) D_2 and D_3 alone; (c) D_2 + PS; (d) D_3 + PS; (e) D_1 + PS. The arrows A and A' indicate drug concentrations at which biological activity and film formation are observed respectively.

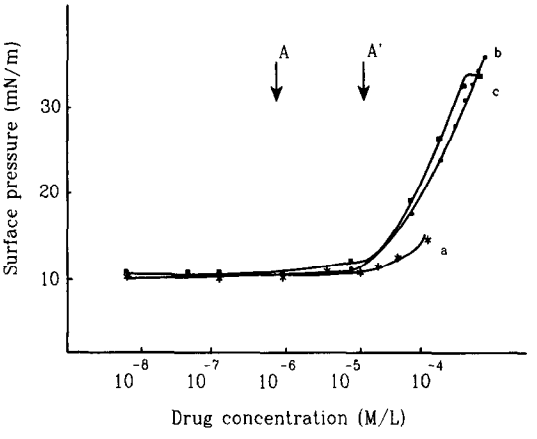

Fig. 3. Changes in surface pressure versus drug concentration for PC monolayers in the presence of: (a) D_1; (b) D_2; (c) D_3. Arrows A and A', meaning as in Fig. 2.

critical micellar concentration gave a value of 1.76×10^{-2} M for D_3. This determination was made by following the changes in water surface tension of solutions of increasing concentrations of drug (data not shown). The critical micellar concentration corresponds to the drug concentration above which no further decrease in surface tension was observed.

Monolayer experiments were next carried out in the presence of PS and PC films. Figure 2 shows the changes in surface pressure resulting from injection of the drugs in the subphase, underneath a PS film spread at an initial pressure of 10 mN/m.

For the three molecules, a significant increase in surface pressure was observed for subphase concentrations higher than 10^{-6} M. Changes in surface pressure $\Delta\pi$ reached values of 17, 26 and 25 nM/m for D_1, D_2 and D_3, respectively, for final drug concentrations of 2.5×10^{-5} M for D_1, and nearly 10^{-3} M for D_2 and D_3. These upper concentration values were imposed by the rather poor solubility of these molecules in water. For D_3, the existence of a saturation plateau allowed us to determine a drug-to-PS apparent binding constant of about 10^4 M^{-1}. It was taken as the concentration at which half-maximal film expansion occurred.

With PC, as with PS, for an initial surface pressure of 10 mN/m, a significant π increase was detected for drug concentrations higher than 10^{-5} M. (Fig. 3). $\Delta\pi$ remained very small in the presence of D_1 (5 mN/m) while it reached values of 25 and 24 mN/m in the presence of D_2 and D_3, respectively, similar to those observed with PS. A saturation plateau was also observed for D_3, leading to an apparent binding constant of this molecule to PC of about 10^4 M^{-1}.

To check whether the drug–lipid interactions depended on the lipid molecular packing, full compression isotherms of PS were recorded in the absence and in the presence of either D_2 or D_3 in the subphase. Similar results were obtained with the two drugs and, for the sake of clarity, only the results of isotherm compressions of PS in the presence of 10^{-6} M and 10^{-5} M of D_2 in the subphase are shown (Fig. 4). Film expansions observed were larger than those recorded after injecting the drug molecules underneath preformed monolayers of PS (Fig. 3). This strongly suggests that the capability of these molecules to interact with and penetrate the lipidic film depends largely on the initial lipid molecular packing. In addition, it should be observed that film expansion decreased with increasing film surface pressure. This indicates that part of the drug molecules embedded in the film were "squeezed out" from the lipid phase as the lipid molecular packing increased.

For both drug molecules D_2 and D_3 introduced

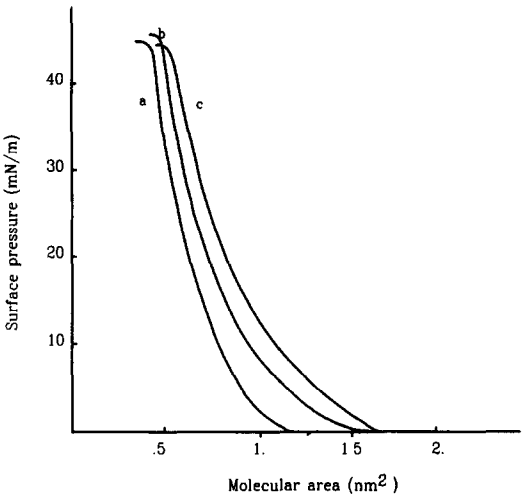

Fig. 4. Compression isotherms of PS monolayers on aqueous subphase containing different concentrations of D_2 drug. Curve (a) PS alone; (b) 10^{-6} M of D_2; (c) 10^{-5} M of D_2.

into the subphase, the value of the surface potential per molecule of PS monolayer decreased with increasing density of molecular packing (data not shown).

Electrophoretic mobility measurements

The results above indicate significant perturbations of the monolayer organization, the nature of which was monitored by electrophoretic mobility measurements using multilamellar vesicles of PS in the presence of either D_2 or D_3 drug molecules.

As can be seen in Table 2, observed electrophoretic mobilities are nearly constant in the presence of D_2. This absence of significant perturbation suggests a rather poor binding of the drug to the lipid and no attempt was made to calculate a binding constant. In contrast, results with D_3 indicate binding of the drug to PS. Within the limits of error in this kind of determination, the binding constant of D_3 to PS appeared to be independent of drug concentration and was evaluated to be about 2×10^4 M^{-1}.

DISCUSSION

The purpose of this work was to check whether the analogues of tamoxifen, D_1, D_2 and D_3, owing to their amphiphilic character, were able to interact with membrane lipids. Data in Figs 2 and 3 clearly indicate that the three drugs provoke film expansion with both PC and PS as host lipids.

Nevertheless, interpretation of monolayer results can be hampered by the highly amphiphilic character of the drug molecules under study. To avoid erroneous conclusions about the binding of these molecules with lipids, one has to discriminate carefully between the film expansion due to drug-to-lipid binding and that originating from adsorption of drug molecules at the air–water interface.

Thus, one can conclude from data in Figs 2 and 3 that D_1, which did not appear to be a film-forming molecule over the subphase concentration range tested (from 10^{-6} to 3×10^{-4} M), did not interact with PC but did interact with PS. The film expansion of $\Delta\pi = 15$ mN/m which was observed for a drug concentration of 10^{-4} M in the subphase, is most likely accounted for by the insertion of D_1 between PS and molecules in the monolayer.

With D_2, a more complex behaviour was observed. When recording the full compression isotherm of PS in the presence of D_2 in the subphase, film expansion was readily detected for drug concentrations of 10^{-6} M and 10^{-5} M. The same concentrations brought about only slight film expansion when the drug was added into the subphase, beneath a preformed lipid film. On the other hand, film expansions recorded in the presence of D_2 with preformed films of PC and PS paralleled the increase in surface pressure provoked by the drug alone, suggesting the occurrence of non-specific adsorption of drug molecules at the air–water interface. This strongly suggests that D_2 can penetrate a film of PS when the molecular area is large, as during the spreading step of the lipid molecules before the recording of a compression curve. Then, some intercalated drug molecules would be progressively squeezed out of the film as the film is compressed. For D_3, the increase in surface pressure resulting from an increase in the drug concentration in the subphase was more pronounced in the presence of a lipid film, especially of PS, than with the drug alone. This indicates that there was interaction between the drug and lipid molecules. In agreement with this conclusion, a binding constant for D_3 to PS of about 2×10^4 M^{-1} (Table 2) was evaluated from electrophoretic mobility measurements on PS liposomes. Such a value can be compared to that of

Table 2. ζ potentials, electrostatic potentials and affinity constants calculated from electrophoretic mobilities

	0	10^{-6}	10^{-5}	10^{-4}
		(mol/L)		
$\zeta(D_2)$ (mV)	−132 ± 11	−122 ± 11	−124 ± 11	−125 ± 17
$\zeta(D_3)$ (mV)	−142 ± 10	−100 ± 10	−94 ± 13	−55 ± 13
$\Psi(PS/D_2)$ (mV)	−164	−145	−149	−151
$\Psi(PS/D_3)$ (mV)	−182	−113	−105	−59
$K(D_3)$ (M^{-1})	0	4.6×10^4	7.7×10^3	1.5×10^4

about $10^4\ M^{-1}$ inferred from the S-shaped adsorption curve shown in Fig. 2. However, as for D_2, monolayer data obtained with preformed lipid films or with full compression curves indicate that interaction of D_3 with PS was more easily attained when the lipid was loosely packed.

In any case, it is worth stressing that with both D_2 and D_3, the number of drug molecules which are present within the lipid film depends on the lipid molecular packing. Such dependence has already been reported in the case of the anticancer drug Adriamycin® [19]. The differences which are observed in the way D_1, D_2 and D_3 interact with lipids can be connected to their chemical structure. First, due to their cationic nature and in view of simple electrostatic considerations, these drug molecules are expected to interact preferentially with an acidic lipid like PS, as compared to a zwitterionic one like PC [18]. This predicted difference was observed. Second, in the course of physical studies of lipids labelled with anthracene residues, it has been shown that aromatic rings with their axis lying along the main axis of the molecule, as is the case for D_1 and D_3, are no more bulky than their equivalent polymethylenic chain [20]. This would enable these molecules to penetrate the lipid phase, with their hydrophobic segment interacting with the hydrophobic part of the lipids and their positively charged heads located near the negatively charged groups of PS molecules. In such a model, drug–lipid interaction would be stabilized by both hydrophobic and electrostatic interactions.

In contrast, the absence of significant interaction of D_2 with preformed lipid phase can be explained by the presence on the molecule of the highly bulky tertiobutyl group which prevents the drug from being inserted to a significant extent between the lipid molecules.

With the three drugs, significant perturbation of PC and PS film monolayers was observed for a drug concentration in the subphase of at least 10^{-5} M, which is about one order of magnitude higher than the concentration required for antiproliferative [2] and trypanocidal effects.* Nevertheless, this is still three orders of magnitude higher than the concentration required for an effective binding of each of the three drugs to its purified protein receptor *in vitro*. It is worth emphasizing that owing to their positive charge, the molecules of these drugs can accumulate inside the cells and organelles in response to the transmembrane potential $\Delta\Psi$, as occurs in the case of the cationic anticancer drug Celiptium [21].

In the present case, drug accumulation at the surface of the different membranes in the cell may account for the discrepancy between the drug concentration required for biological activity at the membrane where the assumed target anti-oestrogen binding site is located [22], and the binding activity to the protein. Typically, a transmembrane potential of *ca.* 60 mV (negative inside) leads to a 10-fold accumulation of these drug molecules inside the cells. It is difficult to assess the actual concentration of these molecules *in vivo*, during a phase of administration. However, if they were allowed to reach an intracellular concentration of 10^{-5} M and above, the present study indicates that, at least for D_2 and D_3, they would be able to interact with membranes through their lipid components. As the relevant receptor is likewise associated with the membrane [5], this might facilitate the interaction of these drug molecules with their receptor sites, since the stronger interaction with phospholipids is seen with the more biologically active molecule D_3.

* Betbeder D, Périé J-J, Baltz T, Poirot M and Faye J-C, Characterization of a benzyl-phenoxy-ethanamine bound protein in Trypanosome: possible relation between binding affinity and trypanocidal activity. *Mol Biochem Pharmacol*, submitted.

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