Biochemical Pharmacology, Vol. 33, No. 15, pp. 2499–2503, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

ELLIPTICINE DERIVATIVES INTERACTING WITH MODEL MEMBRANES. INFLUENCE OF QUATERNARIZATION OF NITROGEN-2

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(*Received* 22 *December* 1983; *accepted* 22 *February* 1984)

Abstract—Four compounds of the ellipticine family were examined in their interaction with liposomes and with an isolated bacterial membrane. The physicochemical methods used detected only minor differences between the properties of the amphiphilic drugs (ellipticine and 2-methyl-ellipticinium) and the two dipolar drugs (9-hydroxy-ellipticine and 2-methyl 9-hydroxy-ellipticinium). The amphiphilic drugs were able to become associated to anionic liposomes in a 20–30% excess of charge neutralization, and seem to penetrate deeper into the lipid layer than the two dipolar drugs. It was also shown that ellipticine penetrates deeper into liposomes membrane than into natural membrane used.

In contrast with what can be postulated from the literature dealing with the behaviour of quaternarized drugs, it seems that ellipticine and its quaternarized analogues present fast diffusion through multilayered vesicles.

On the whole, the membrane effects of the ellipticines studied here are not different for quaternarized drugs and for drugs not permanently charged, but are influenced by the existence on the molecules of a second polar function.

Ellipticine [5, 11 dimethyl-6H-pyrido (4, 3-b) carbazole] and some of its derivatives (Fig. 1) are DNA-intercalating substances, endowed with anticancer properties [1]. It was shown by our group that these drugs are also able to strongly interact with negatively charged membranes, and that ellipticine can completely neutralize phosphatidylglycerol, an anionic phospholipid [2, 3]. We have also shown that, depending on the substituents on the carbon-9, ellipticine derivatives penetrated at different depths into the lipidic phase of liposome or of a natural membrane [4]. Such interactions have to be considered to understand the origins of the pharmacological properties of these drugs, since drastic effects can be observed *in vitro* on model and natural membranes [5].

Recent clinical trials have been performed mainly with 2-methyl 9-hydroxyellipticinium, because, among the active ellipticine derivatives, this compound is one of the most active and one of the least toxic [6]. It was thus interesting to investigate a possible influence of quaternarization of nitrogen-2 on the drug–membrane interactions. Some effects of ellipticine and of 9-hydroxyellipticine were compared to the effects of their quaternary analogues, 2-methyl ellipticinium and 2-methyl 9-hydroxyellipticinium.

As it is generally accepted that substances with a permanent positive charge cannot easily permeate membranes, the penetration of ellipticine and of ellipticinium into liposomes was followed.

MATERIALS AND METHODS

Ellipticine and 9-hydroxyellipticine were synthesized and purified by P. Lecointe (Centre de Pharmacologie et Toxicologie Fondamentales du CNRS, Toulouse). Ellipticinium and 9-hydroxy-ellipticinium were synthesized and purified by J. Chenu (SANOFI and Centre de Pharmacologie et Toxicologie Fondamentales du CNRS, Toulouse). Egg yolk phosphatidylcholine and phosphatidylglycerol from *Micrococcus luteus* were prepared according to our usual procedures [3–5]. Dimyristoylglycerophosphoglycerol was purchased from Medmark (D.B.R.). 2-(9-Anthroyloxy)palmitate was synthesized and purified according to [7]. Liposomes and the bacterial membrane isolated from *Micrococcus luteus* (ATCC 4698) were prepared as

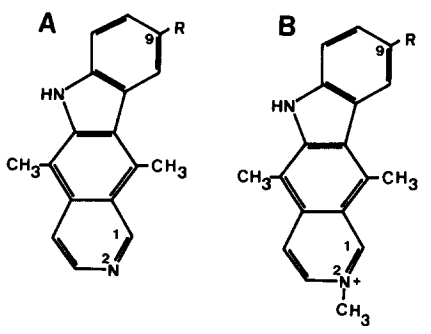

Fig. 1. Ellipticine (A) and 2-methylellipticinium (B). In the present work–R was either –H or –OH.

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previously described [3, 8]. They were used in a 45 mM NaCl, 5 mM phosphate, pH 7.2.

Fluorescence spectra and fluorescence intensity measurements were performed with a non-corrected JY3D apparatus (Jobin et Yvon, France). The quenching ratios were calculated from F and Fo, the fluorescence intensities which are respectively measured in the presence or in the absence of the quencher at concentration Q. The calculated $(Fo/F) - 1$ values were plotted against Q (Stern–Vollmer plot) [9].

Surface pressure ($\Delta\pi$) and surface potential (ΔV) of phosphatidylglycerol (extracted and purified from *Micrococcus luteus*) were measured as described in [2, 10]. Ellipticine or its derivatives were added stepwise to the water subphase (50 mM NaCl), under a film at an initial surface pressure of 10 mN/m.

RESULTS AND DISCUSSION

Interaction with monolayers of phosphatidylglycerol. The surface pressure allows to detect a penetration of molecules into the phospholipid layer. The surface potential responds to changes in the orientation of the lipid polar head-groups at the air/water interface, as well as to changes in the surface charge density.

As it can be seen in Fig. 2, 9-hydroxyellipticine (Fig. 2a) and 2-methyl, 9-hydroxyellipticinium (Fig. 2b) did not change significantly the surface pressure, a result which allows us to exclude a penetration of these molecules within the film. In contrast, the corresponding non-hydroxylated compounds did penetrate between the lipid molecules, since a film expansion was observed with these two substances. However, the four compounds studied interacted with the phospholipidic surface, since they increased the surface potential. The hydroxy derivatives gave a 30–60% increase of the potential, while the non-hydroxylated compounds increased ΔV by 140%.

Stoichiometry of the interaction. These experiments were carried out by taking advantage that in the presence of anionic phospholipids, ellipticine derivatives obey the Beer-Lamber law with an absorbance coefficient lower than that in water solution. Moreover ellipticine derivatives in water give stacking, which leads to a deviation from the law [3]. Since the same behaviour was observed with the quaternarized drugs (Fig. 3), this is at least partly due to the lipid phase itself, and not only to a greater proportion of protonated drug molecules in the presence of the negatively charged surface.

The absorbance coefficients can be calculated from the slopes of curves in Fig. 3: at the wavelength used (300 nm), the free 2-methylellipticinium and its 9-hydroxy derivative had exactly the same coefficient (3.5×10^4/M/cm), while the coefficient measured for 2-methylellipticinium in the presence of anionic liposomes was 2.3×10^4/M/cm and the coefficient of the hydroxy derivative was 1.4×10^4/M/cm. This indicates that the two compounds are not in the same environment when interacting with anionic liposomes. This conclusion is in agreement with the results of the monolayer study showing a penetration into the lipid layer by the two amphiphilic drugs, but not by the hydroxy derivatives.

The effect of anionic liposomes on the absorbance coefficients allowed the determination of the stoi-

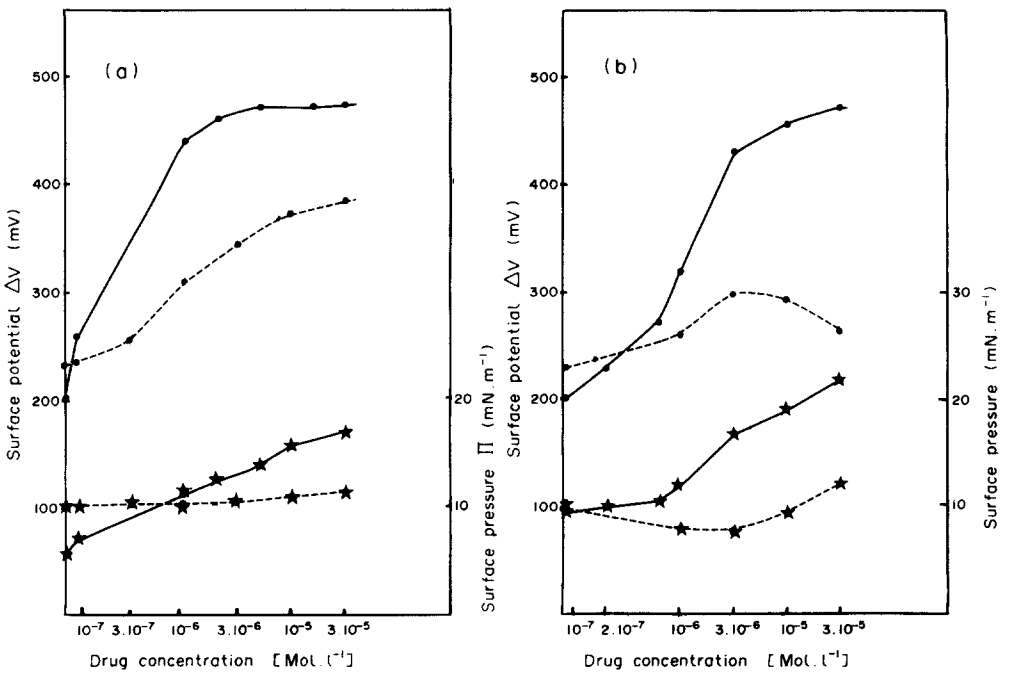

Fig. 2. Interaction of ellipticine derivatives with a monolayer of phosphatidylglycerol (from *Micrococcus luteus*). Full lines: non-hydroxylated compounds; dotted lines: hydroxylated compounds. Stars: effect on surface pressure ($\Delta\pi$, in mN/m), circles: effect on surface potential (ΔV, in mV). Water subphase 50 mM NaCl. (a) Ellipticine and 9-hydroxyellipticine; (b) 2-methylellipticinium and 2-methyl 9-hydroxyellipticinium.

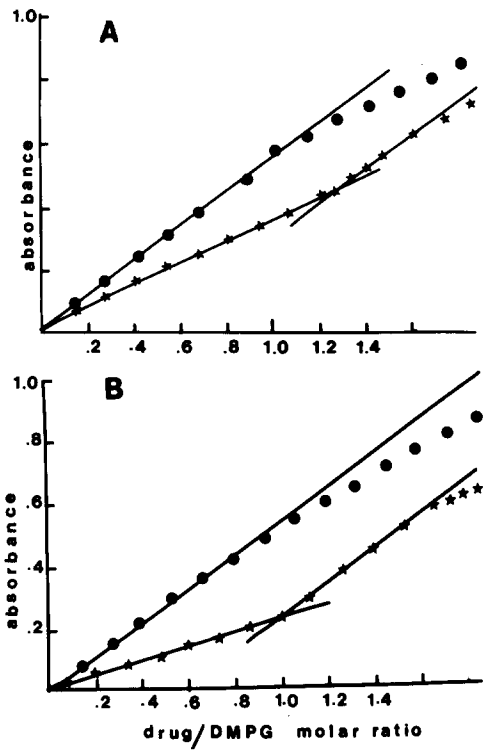

Fig. 3. Stoichiometry of the drug dimyristoylglycerophosphoglycerol (DMPG) interaction. DMPG 1.5×10^{-5} M, Temperature 33°. Absorbance measured at 300 nm. Circles: free drugs. Stars: drugs in the presence of DMPG-liposomes. (A) 2-methylellipticinium; (B) 2-methyl 9-hydroxy-ellipticinium.

chiometry of the interaction between the drug and dimyristoylglycerophosphoglycerol [3]. As seen in Fig. 3(A) this interaction achieve a stoichiometry of about 1.2–1.3 molecule of 2-methylellipticinium per phospholipid, but only of 1.0 molecule of 2-methyl 9-hydroxy-ellipticinium per phospholipid molecule (Fig. 3B). Exactly the same results were obtained (figure not shown) with ellipticine (1.2) and 9-hydroxy-ellipticine (1.0). This means that the two amphiphilic drugs, ellipticine and ellipticinium, are able to be incorporated into anionic liposomes up to a 20–30% excess of charge neutralization, while the two dipolar drugs, 9-hydroxy-ellipticine and 2-methyl 9-hydroxy-ellipticinium, cannot be associated with anionic lipids above the exact neutralization. It is thus likely that, after completion of charge neutralization, a few molecules of the two amphiphilic drugs can bind to the lipid layer, owing to drug lipid hydrophobic interactions, while the two dipolar drugs cannot.

Drug localization in model and natural membranes. Ellipticine derivatives are able to quench fluorescent probes [4]. Dimyristoylglycerolphosphoglycerol liposomes and membrane isolated from *Micrococcus luteus* were labeled by 2-(9-anthroyloxy)palmitate. This fluorescent probe is anchored at the lipid–water interface by its carboxyl group, and has its anthracene moiety in the region of the carbonyl functions and of the first methylene groups of the acyl chains [11]. As seen in Fig. 4, the four drugs are very efficient quenchers, and, for low drug concentrations, the quenching curves are deflected upwards. This corresponds to the so-called "static quenching", that can occur when the quencher and the fluorophor are permanently close enough to give immediate quenching of the excited fluorophores [9].

The four drugs gave static quenching, but the two amphiphilic ones, ellipticine and 2-methyl-ellipticinium, were more efficient than their two dipolar hydroxylated derivatives. As the quenching efficiencies of these drugs in a homogeneous solution, i.e. in alcohol, are not significantly different, these results suggest that the amphiphilic drugs penetrate in the lipid layer better than the two dipolar analogues.

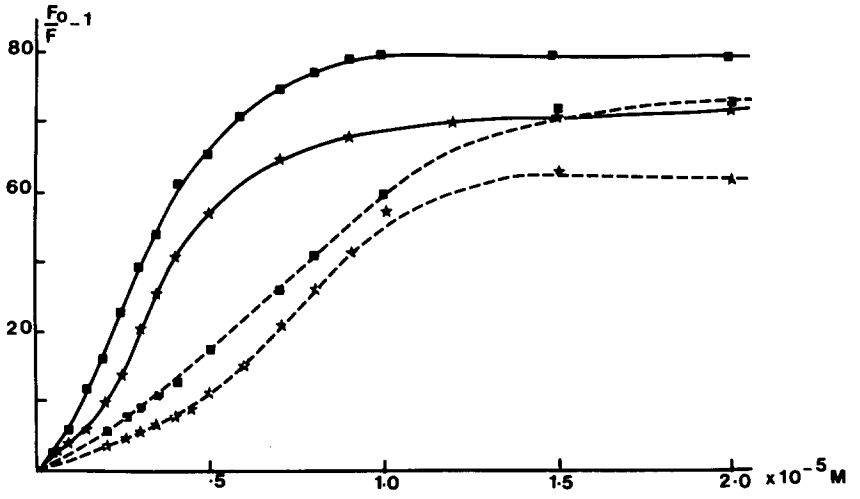

Fig. 4. Quenching of the fluorescence of 2-(9-anthroyloxy)palmitate by drugs. *F*, fluorescence intensity in the presence of a drug; *Fo*, fluorescence intensity in the absence of a drug. Excitation wavelength 367 nm, emission wavelength 450 nm; 10^{-6} M 2-(9-anthroyloxy)palmitate; 7.10^{-6} M dimyristoylglycerophosphoglycerol; Temperature 35°. ———, quenching by ellipticine (squares); quenching by 2-methylellipticinium (stars). – – –, quenching by 9-hydroxyellipticine (squares) quenching by 2-methyl 9-hydroxyellipticinium (stars).

Ellipticine is a fluorescent compound (excitation maximum 298 nm, emission maximum 517 nm), the fluorescence of which can be quenched by the water soluble (but not membrane-permeant) cupric ion. In the absence of membrane, quenching of ellipticine by cupric ion was barely detectable. In the presence of the natural membrane (Fig. 5, upper curve) an efficient static quenching of ellipticine fluorescence by cupric ion was observed. In the presence of liposomes (Fig. 5, lower curve), the quenching was weaker, and looked like a "solvation-type" quenching. This latter type is observed when the quencher molecules surround the fluorophor without permanent contact between the two molecules. In this case, it is assumed that only the first layer of the quencher is active in a dynamic quenching process [12]. From data of Fig. 5. it can be concluded that ellipticine is in permanent contact with cupric ions in the presence of the natural membrane, at the membrane interface. In the presence of liposomes, ellipticine is somehow remote from the layer of cupric ions that faces the anionic surface of liposomes. This indicates that ellipticine does not penetrate the natural membrane as deep as the liposomal membrane. The same conclusions have been reached in a previous paper, for an amphiphilic derivative of ellipticine, the 9-methoxyellipticine, by using it as a quencher of various membrane fluorescent probes [4].

Diffusion of ellipticine derivatives through liposomal membranes. Diffusion of these compounds through liposomal membranes was followed by taking advantage of the spectral properties of these molecules: (i) There is a decrease of the absorbance coefficient of these compounds in the presence of acidic liposomes; (ii) These compounds quench the fluorescence of the membrane probe 2-(9-anthroyloxy)palmitate; (iii) The fluorescence of ellipticine measured at 517 nm increased in the presence of negatively charged membrane surface.

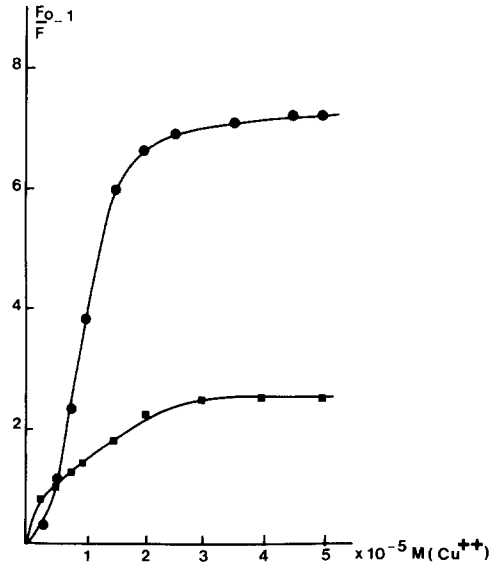

Fig. 5. Quenching of the fluorescence of ellipticine by cupric ions. *F*, fluorescence intensity in the presence of cupric ions; *Fo*, fluorescence intensity in the absence of cupric ions. Excitation wavelength 300 nm; emission wavelength 520 nm. 10^{-5} M ellipticin—temperature 32°. Upper curve (circles), membrane isolated from *M. luteus*. Lower curve (squares), liposomes (DMPG).

The absorbance or the fluorescence intensities were recorded continuously with drugs alone, then after addition of multilayered liposomes of dimyristoylglycerophosphoglycerol (DMPG) at a temperature above the phase transition temperature of the lipid (24° for DMPG). The amount of drug was calculated to neutralize about 75% of the negative charges of phospholipids. For quenching assays, the drug was added to multilayered vesicles labeled by 2-(9-anthroyloxy)palmitate.

A stable absorbance or fluorescence intensity was established within 30–60 sec for ellipticine and 2-methylellipticinium, within 2 or 3 min for 9-hydroxyellipticine, and it took approximately 1 hr for 2-methyl 9-hydroxyellipticinium.

Multilayered liposomes were used in these experiments. As the drug achieved a 1 to 1 stoichiometry interaction with anionic lipids, it is clear that a fraction of the drug molecules had to cross several lipid bilayers before interacting with all the phospholipid molecules. Thus, the diffusion of these drugs through liposomes was fast, compared to the several hours needed to reach an inside/outside equilibrium for entrapped neutral compounds like glucose or sucrose, and compared to the two or three days needed when an anionic compound like 6-carboxyfluorescein is encapsulated in phospholipids. As it is generally accepted that natural membranes have a higher passive permeability than liposomal membrane, it is likely that ellipticine, 2-methylellipticinium and their hydroxy derivatives diffuse easily through membranes.

The above result is interesting because it is accepted that cationic substances permeate under their non-ionized form, and, as a consequence of this, it is generally said that permanent cations penetrate cells or cell organelles much more slowly than non-permanent cations (see for instance ref. 13). Differences noted in the properties of chlorpromazin and of its quaternary analogue methochlorpromazine have been explained using the above reasoning [14]. It seems that this cannot be applied to ellipticine derivatives.

On the whole, it appears that amphiphilic as well as dipolar ellipticine derivatives interact with anionic phospholipids in membranes, the amphiphilic derivatives being able to penetrate deeper in the lipid matrix than the dipolar derivatives.

The membrane effects of ellipticine derivatives are much more influenced by the existence of a second polar function on molecules than by quaternarization. As a permanent charge on nitrogen-2 does not seem to prevent the crossing of the lipid layer, the cytoplasmic membrane as well as the inner membranes of cells have to be considered as normal targets of this family of anti-cancer drugs.

Acknowledgements—This work was supported by a P.I.R.M.E.D. grant (A.S.P. antitumoraux).

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