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Multidrug resistance modulator interactions with neutral and anionic liposomes: membrane binding affinity and membrane perturbing activity

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Abstract

A variety of cationic lipophilic compounds (modulators) have been found to reverse the multidrug resistance of cancer cells. In order to determine the membrane perturbing efficacy and the binding affinity of such drugs in neutral and anionic liposomes, the leakage of Sulfan blue induced by five modulators bearing different electric charges was quantified using liposomes with and without phosphatidic acid ($x_{EPA} = 0$ and 0.1), at four lipid concentrations. The binding isotherms were drawn up using the indirect method based on the dependency of the leakage rate on the modulator and the lipid concentrations. Upon inclusion of negatively charged lipids in the liposomes: (i) the binding of cationic drugs was favoured, except in a case where modulator aggregation occurred in the lipid phase; (ii) the drugs with a net electric charge greater than 1.1 displayed a greater enhancement in their potency to produce membrane perturbation; and (iii) the EPA effect on membrane permeation was due mainly to that on membrane perturbation ($\geq 50\%$) and, to a lesser extent, to that on the binding affinity ($\leq 50\%$). The present study provides evidence that drug–membrane interactions are the result of a complex interplay between the structural and electrical characteristics of the drugs and those of the membranes.

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Introduction

Studies on peptides bearing a fixed positive electric charge have shown that the presence of electric dipoles and net electric charges at the membrane surface can enhance or weaken their binding affinity and affect the hydrophobic partitioning processes (Breschiaschvili & Seelig 1990b). The binding of these peptides to neutral phosphatidylcholine membranes generates a positive electric charge at the membrane surface and a re-orientation of the phosphocholine dipole. Hence, a surface potential (Ψ) is created that repels ions of like charge, making the binding of these peptides increasingly difficult (Breschiaschvili & Seelig 1990a, b and references cited therein). These effects can be induced by all positively charged agents that bind to the membrane surface (Altenbach & Seelig 1985; Breschiaschvili & Seelig 1990a and references cited therein). When peptides of this kind interact with negatively charged membranes, the binding process partially neutralizes the membrane charge. If complete neutralization occurs, the repulsion of like charge again becomes the predominant mechanism (Matzusaki et al 1989a, 1991; Breschiaschvili & Seelig 1990a, b).

A variety of substances (modulators), especially the Ca^{2+}-blocker verapamil, have been found to reverse the multidrug resistance (MDR) of cancer cells (Sharom 1997). Since most MDR modulators exist in various states of ionization, their interactions with membranes may differ from those of peptides bearing a fixed positive charge. This study was designed to assess the effects of the membrane surface potential on MDR modulator efficiency in terms of drug–membrane interactions. The ability of five modulators to induce dye leakage from neutral and anionic liposomes was determined at various drug and liposome concentrations. By analysing the dependence of the leakage rates on these two parameters, the respective contributions of the membrane binding affinity of the modulators and the membrane perturbing activity per membrane-bound molecule to the permeability changes were quantified. The effects of the net electric charge of the modulators were also studied by

comparing the dye leakage induced by mono-basic (diltiazem and verapamil: Klohs et al 1986) and di-basic (thioacridine: Hevér et al 1998; mepacrine: Inaba & Maruyama 1988) modulators with that of the non-ionic detergent Triton X-100 (Sharom 1997). Since two of the compounds under investigation exhibit fluorescence, the method based on carboxyfluorescein leakage was not applicable here. Sulfan blue was therefore used as a membrane permeation indicator (Castaing et al 2000).

Materials and Methods

Chemicals

L-α-Phosphatidylcholine prepared from fresh egg yolk (EPC), L-α-phosphatidic acid prepared from egg yolk lecithin (EPA), diltiazem hydrochloride (DZ), verapamil hydrochloride (VR) and Triton X-100 (TR; *t*-octylphenoxypolyethoxyethanol) were purchased from Sigma (St Louis, MO, USA). Mepacrine dihydrochloride hydrate (MP) and the dye anhydro-4-4′-bis(diethylamino)triphenyl-methanol-2″,4″-disulfonic acid monosodium salt (Sulfan blue) were purchased from Aldrich (Steinheim, Germany). Cholesterol was obtained from Fluka (Buchs, Switzerland). Diethyl ether, $Na_2HPO_4{\cdot}2H_2O$ and $NaH_2PO_4{\cdot}H_2O$ were purchased from Merck (Darmstadt, Germany), Sephadex PD-10 columns (G-25M) from Pharmacia (Uppsala, Sweden) and polycarbonate porous membranes from Nuclepore Corporation (Pleasanton, CA, USA). Thioacridine ether (TH) was prepared from the corresponding thioacridinone (compound 1q in Hevér et al 1998).

VR, DZ, TH and TR were dissolved in water. MP was dissolved in 100 mM phosphate buffer (pH 7.4).

Liposomes loaded with Sulfan blue

Large unilamellar vesicles (LUV) composed of EPC/EPA/cholesterol (9:0:1 and 8:1:1, i.e. $x_{EPA} = 0$ and 0.1) were prepared as previously described (Castaing et al 2000). The removal of the external dye was carried out by passage through two Sephadex G-25 columns eluted with 100 mM phosphate buffer (pH 7.4).

Permeability measurements

The Sulfan blue absorbance at 640 nm was determined by recording the visible absorption spectra of the samples with a Uvikon 933 spectrophotometer (Milan, Italy). Permeability measurements were performed on a 1-mL LUV suspension as previously described (Castaing et al 2000). The leakage was induced by 0–24 mM compound (0–18 mol compound (mol lipid)$^{-1}$) from neutral and anionic liposomes. It was quantified following a 3-min incubation (initial leakage rate) at 37°C (pH 7.4). As the translocation rate of MDR modulators through membranes is high, their binding equilibrium at membrane–solution interfaces is essentially complete within 1 min (Eytan et al 1996). Therefore, the changes in PD_{50} determined here should reflect post-binding processes.

Dose–response curves

The variations in dye leakage (%) with different drug concentrations, $[D]_0$, were fitted to the following equation by non-linear least squares regression:

$$\text{leakage}\ (\%) = 100 \times [D]_0^{\,h}/(PD_{50}{}^{h} + [D]_0^{\,h}) \quad (1)$$

where PD_{50} is the drug dose inducing 50% dye leakage from the liposomes and h is the Hill coefficient (i.e. the parameter characterizing the cooperativity of the permeation process).

Drug concentration inducing a given leakage extent

Equation 1 can be rearranged in order to calculate the drug concentration inducing a given dye leakage extent from the liposomes as follows:

$$[D]_0 = PD_{50} \times [\text{leakage}\ (\%)]^{1/h}/[100 - \text{leakage}\ (\%)]^{1/h} \quad (2)$$

Net electric charge of the drugs

With the exception of TR, the drugs studied were weak bases existing in various states of ionization at pH 7.4. Their net electric charge, z, (i.e. the mean net electric charge per drug molecule) was therefore calculated according to the following equation:

$$z = (1/[D]_0) \times \Sigma[D]_i \times z_i. \quad (3)$$

where $[D]_0$ is the total concentration of the drug in the aqueous phase and $[D]_i$ is the concentration of species i, with valence z_i.

Non-linear binding isotherms

When non-linear, the variations of the membrane-bound drug per phospholipid ($r = [D]_b/[L]$) with those of the free drug concentration dissolved in the aqueous phase ($[D]_f$) were fitted to the equation derived by Brunauer, Emmett and Teller (BET equation) (Adamson 1967):

$$r = r_{max} \times C \times [D]_f{}^{max} \times [D]_f/\{([D]_f{}^{max} - [D]_f) \times [[D]_f{}^{max} + (C-1) \times [D]_f]\} \quad (4)$$

where r_{max} and $[D]_f{}^{max}$ are the maximal membrane-bound fraction of the drug per phospholipid and free drug concentration in the aqueous phase, respectively, and $C = K_1/K_2$, the ratio between the binding constants of the drug to the binding sites at the surface of the membrane (K_1) and to the lipophilic side chains of the phospholipids in the core of the membrane (K_2). For large C values (i.e. $K_1 >> K_2$) the BET equation reduces to the Langmuir equation (type I isotherms that are bent downwards), and for small C values (i.e. $K_1 \leq K_2$) type III isotherms that are bent upwards result.

In the present study, the binding isotherms of MP were bent downwards at $x_{EPA} = 0$ and 0.1 (type I isotherms),

whereas the isotherm of TH was bent upwards at $x_{EPA} = 0$ (type III isotherm). The r vs $[D]_f$ relationships of these drugs were fitted to Equation 4, with $C \geq 100$ for MP data and $C \leq 1$ for TH data, until the value of the determination coefficient (R^2) reached a maximum. The best fits were obtained with $C = 10\,000$ for MP and $C = 0.1$ for TH.

The value of the apparent binding constant (K_{app}) of the drug to the membrane is equal to that of the slope of the r vs $[D]_f$ curve for a given value of $[D]_f$ (i.e. to the value of the derivative of the function for a given $[D]_f$):

$$K_{app} = \frac{r_{max} \times C \times [D]_f{}^{max} \times [[D]_f{}^{max\,2} + (C-1) \times [D]_f^2]}{\{([D]_f{}^{max} - [D_f]) \times [[D]_f{}^{max} + (C-1) \times [D]_f]\}^2} \quad (5)$$

At 50% leakage extent, $[D]_f$ can be approximated by $[D]_f^{50} = r_{50}/(K^{50}_{app} \times K^{0}_{app})^{0.5} = r_{50}/(k \times K^{50}_{app})$, where $k = (K^{0}_{app}/K^{50}_{app})^{0.5}$ is the constant characterizing the bending of the isotherm.

Statistical analysis

Linear and non-linear regressions were calculated using the least-square method (F test). Regressions lines were compared by performing slope comparison and covariance analysis, and the non-linear fitted parameters were compared by the t-test. Values of $P < 0.05$ were considered significant.

Results

The choice of an adequate incubation time to quantify the initial leakage rate is usually a compromise to minimize errors while taking into account the constraints of the system. When the incubation time is decreased, the systematic error associated with the quantification decreases, whereas the random errors increase to a large extent. As the present study focused on post-binding processes of modulators to liposomes, the incubation had to be long enough to ensure their binding was complete at membrane–solution interfaces (~1 min: Eytan et al 1996). A 3-min period of incubation seemed therefore to be the best compromise. After this time period, the permeation dose (PD_{50}) was lower, the Hill coefficient (h) unchanged, the membrane perturbing activity (r_{50}) lower, and the membrane binding affinity ($K^{50}{}_{app}$) higher than after a 1-min period of incubation. Note in particular that the shape of the binding isotherm was the same. The results presented here being consistent, the kinetic conclusions drawn remain entirely valid.

Drug permeation properties (PD_{50} and h) depending on [L] and x_{EPA}

The PD_{50} and h values are given in Table 1. Whatever x_{EPA}, the PD_{50} increased linearly and significantly with the lipid concentration, [L] ($R^2 = 0.855$–0.999; $P < 0.01$; $n = 8$). Covariance analysis showed that, except for TR, the PD_{50} decreased significantly with increasing x_{EPA} ($P < 0.001$). This result indicates that the efficiency of cationic modulators to induce dye leakage increased at a negative Ψ. The permeation was a cooperative process and under all of the experimental conditions used here, h did not vary with [L]. This result indicates that the number of drug molecules interacting at the same membrane site to induce dye leakage was similar at both high and low [L]. Covariance analysis showed that, except for TR, h increased significantly with x_{EPA} ($P < 0.001$). Therefore, except for TR, the number of drug molecules interacting at the same membrane site to induce dye leakage increased at a negative Ψ.

Membrane binding affinities ($K^0{}_{app}$ and $K^{50}{}_{app}$) and membrane perturbing activity (r_{50}) depending on x_{EPA}

The dye leakage occurring through membranes in response to a drug involves interactions with both the phospholipid head groups and with the phospholipids in the core of the membrane. Accordingly, the two determinants of leakage are the membrane binding affinity of the drug (binding isotherm) and its membrane perturbing activity. These two factors can be determined by analysing the $[D]_0$ and [L] dependence of the leakage rate. The leakage rate was enhanced by increasing $[D]_0$ or by decreasing [L], suggesting that the amount of membrane-bound drug per phospholipid ($r = [D]_b/[L]$) determines the leakage rate. This rate was assumed here to depend only on r. When the binding equilibrium is reached, r is determined by the free drug concentration, $[D]_f$. The concentration of the drug added, $[D]_0$, being the sum of the free and membrane-bound fractions, the material balance equation is given by:

$$[D]_0 = [D]_f + [D]_b = [D]_f + r \times [L] \quad (6)$$

where $[D]_0$ and [L] are known variables. For a given leakage extent, the constants $[D]_f$ and r can thus be expressed as the intercept and the slope, respectively, of the linear $[D]_0$ vs [L] regression (Thorn 1964; Matzusaki et al 1989a). Therefore, the $[D]_0$ values corresponding to different leakage extents were calculated at each [L] (Equation 2). Then, the $[D]_0$ vs [L] plots obtained for each drug were used to calculate a series of r values and corresponding $[D]_f$ values for different leakage extents. The $[D]_0$ vs [L] plots obtained at various leakage extents did indeed give linear relations ($R^2 \geq 0.913$; $P < 0.05$).

The relationship thus obtained between r and $[D]_f$ for each drug (i.e. the binding isotherm) is shown in Figure 1A and B. The linear binding isotherm of TR, DZ and VR at $x_{EPA} = 0$ and 0.1, and that of TH at $x_{EPA} = 0.1$ was explained in terms of partition. The $K^{50}{}_{app}$ ($r/[D]_f = K^{0}_{app}$) of these drugs was calculated from the slope of the linear r vs $[D]_f$ regressions (Table 1). By contrast, the binding isotherm of TH was bent upwards at $x_{EPA} = 0$, suggesting the occurrence of TH aggregation in the membrane, and the isotherms of MP at $x_{EPA} = 0$ and 0.1 were bent downwards, suggesting the occurrence

Table 1 Effects of x_{EPA} and lipid concentration ([L]) on the permeation dose (PD_{50}), the Hill coefficient (h), the overall membrane binding affinity (K^{50}_{app}), and the membrane perturbing activity (r_{50}) of multidrug resistance modulators bearing different net electric charges (z)

Compound	x_{EPA}	[L] (mM)	PD_{50} (mM)	h	$K^{50}app$ (mM^{-1})	r_{50}	z
Triton X-100	0.0	2.67	0.86 ± 0.01	5.61 ± 0.41	1.90 ± 0.127	0.26 ± 0.001	0.00
		4.00	1.21 ± 0.01	5.62 ± 0.29			
		5.33	1.54 ± 0.02	5.61 ± 1.54			
		6.67	1.91 ± 0.01	5.53 ± 0.23			
	0.1	2.67	0.75 ± 0.03	4.94 ± 1.28	1.59 ± 0.089	0.23 ± 0.001	0.00
		4.00	1.05 ± 0.03	5.58 ± 0.98			
		5.33	1.35 ± 0.05	5.57 ± 1.15			
		6.67	1.63 ± 0.05	5.17 ± 1.20			
Diltiazem	0.0	2.67	3.05 ± 0.04	5.08 ± 0.31	0.11 ± 0.001	0.27 ± 0.001	0.67
		4.00	3.35 ± 0.02	4.94 ± 0.19			
		5.33	3.74 ± 0.03	5.07 ± 0.26			
		6.67	4.11 ± 0.04	5.06 ± 0.25			
	0.1	2.67	2.05 ± 0.05	5.74 ± 0.84	0.16 ± 0.002	0.23 ± 0.001	0.67
		4.00	2.32 ± 0.06	6.23 ± 0.91			
		5.33	2.66 ± 0.06	5.73 ± 0.80			
		6.67	2.95 ± 0.09	5.92 ± 1.01			
Verapamil	0.0	2.67	1.62 ± 0.03	4.00 ± 0.35	0.22 ± 0.002	0.22 ± 0.001	0.97
		4.00	1.91 ± 0.02	3.92 ± 0.23			
		5.33	2.21 ± 0.04	4.06 ± 0.34			
		6.67	2.51 ± 0.03	3.97 ± 0.26			
	0.1	2.67	0.84 ± 0.01	5.18 ± 0.35	0.32 ± 0.008	0.14 ± 0.002	0.97
		4.00	1.02 ± 0.01	5.14 ± 0.20			
		5.33	1.21 ± 0.01	5.05 ± 0.22			
		6.67	1.41 ± 0.01	5.16 ± 0.22			
Thioacridine	0.0	1.33	7.20 ± 0.12	1.93 ± 0.08	0.66 ± 0.109	2.30 ± 0.001	1.33
		2.67	10.22 ± 0.21	1.99 ± 0.10			
		4.00	13.19 ± 0.32	1.98 ± 0.14			
		5.33	16.45 ± 0.40	1.86 ± 0.10			
	0.1	1.33	2.77 ± 0.02	3.61 ± 0.13	0.38 ± 0.001	0.67 ± 0.001	1.33
		2.67	3.57 ± 0.07	3.74 ± 0.35			
		4.00	4.63 ± 0.05	3.56 ± 0.17			
		5.33	5.41 ± 0.07	3.61 ± 0.17			
Mepacrine	0.0	1.33	28.84 ± 0.33	1.10 ± 0.02	0.03 ± 0.005	2.16 ± 0.028	1.68
		2.67	31.10 ± 0.63	1.15 ± 0.03			
		3.07	33.34 ± 2.34	1.15 ± 0.08			
		4.00	34.56 ± 0.97	1.23 ± 0.04			
	0.1	2.67	2.94 ± 0.04	2.39 ± 0.07	0.14 ± 0.017	0.41 ± 0.001	1.68
		4.00	3.52 ± 0.03	2.44 ± 0.06			
		5.33	4.04 ± 0.03	2.46 ± 0.06			
		6.67	4.57 ± 0.04	2.58 ± 0.06			

Data are given ± s.e., n = 10–12 for PD_{50} and h; n = 12–14 for K^{50}_{app} and r_{50}.

of MP aggregation in the aqueous phase (Albert 1966). All these non-linear r vs $[D]_f$ relationships were fitted to Equation 4 and the apparent binding constants corresponding to the infinite dilution (K^0_{app} = intrinsic binding affinity) and to 50% leakage (K^{50}_{app} = overall binding affinity) were calculated from Equation 5 (Table 1). Except for TR, x_{EPA} significantly modified the K^{50}_{app} of the drugs, increasing that for DZ, VR and MP ($P < 0.01$), and decreasing that for TH ($P < 0.02$). Consequently, the binding of the cationic drugs was modified by a negative Ψ. Since TH aggregated in membranes at $x_{EPA} = 0$, its K^{50}_{app} was ~1.5-times greater than its K^0_{app} ($0.47 \pm 0.037\,mM^{-1}$), whereas the MP aggregation occurring in the aqueous phase resulted in the K^{50}_{app} of this modulator being ~10-times lower than its K^0_{app} ($0.28 \pm 0.024\,mM^{-1}$) at $x_{EPA} = 0$, and ~3-times lower at $x_{EPA} = 0.1$ ($K^0_{app} = 0.36 \pm 0.049\,mM^{-1}$).

The r vs leakage extent relationships observed with the various drugs were fitted to Equation 1, with $[D]_0$ replaced by r. They are shown in Figure 1C and D, illustrating the membrane perturbing activity of the membrane-bound drug. This activity is independent from [L], and thus from the K_{app} of each drug. The amount of membrane-bound drug per lipid inducing 50% leakage is taken here to be r_{50}, where the lower r_{50} values correspond to the higher membrane perturbing efficiencies (Table 1).

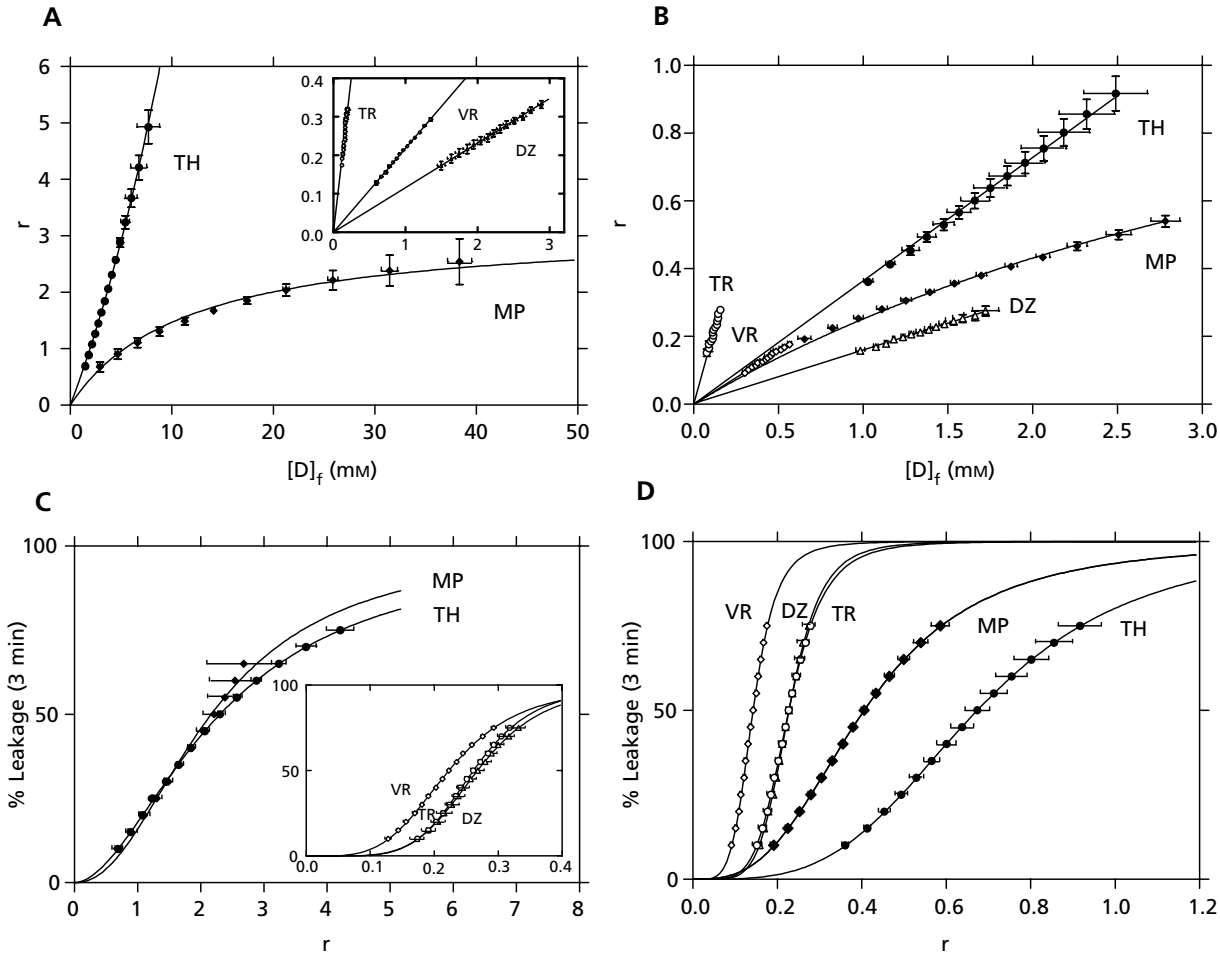

Figure 1 Quantitative analysis of Sulfan blue leakage induced by multidrug resistance modulators (VR, verapamil; DZ, diltiazem; TH, thioacridine; TR, Triton X-100; MP, mepacrine) at $x_{EPA} = 0$ and 0.1. The relationships between the amount of membrane-bound drug per lipid, r, and the free drug concentration, $[D]_f$, (i.e. the binding isotherm) at $x_{EPA} = 0$ (A) and $x_{EPA} = 0.1$ (B), and between the leakage extent and r at $x_{EPA} = 0$ (C) and $x_{EPA} = 0.1$ (D) are shown. Data are given ± s.e.

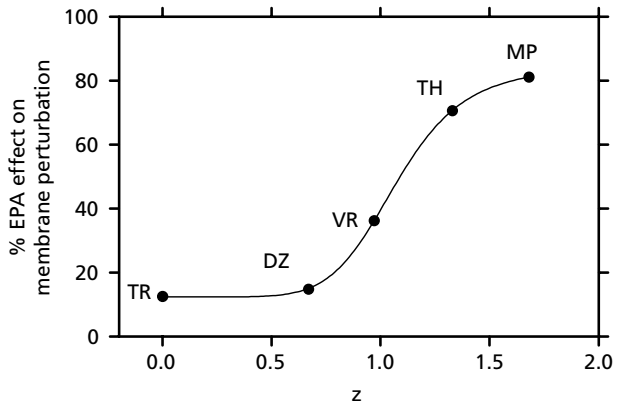

Figure 2 Effect of the net electric charge, z, of multidrug resistance modulators (VR, verapamil; DZ, diltiazem; TH, thioacridine; TR, Triton X-100; MP, mepacrine) on the EPA effect on their membrane perturbation efficiency. For each modulator, the $1 - r_{50}$ ($x_{EPA} = 0.1$)/r_{50} ($x_{EPA} = 0$) value (± s.e.) was calculated using the data given in Table 1.

Whatever z, x_{EPA} significantly decreased the r_{50} of the drugs ($P < 0.01$). Figure 2 shows the $1 - r_{50}$ ($x_{EPA} = 0.1$)/r_{50} ($x_{EPA} = 0$) values (EPA effect on membrane perturbation) of the drugs with z. This relationship was highly significant ($R^2 = 1.000$; $P < 0.001$). Therefore, 100% of the EPA effect on membrane perturbation could be explained here in terms of z. This result indicates that a negative Ψ increased the membrane perturbation potency of drugs bearing a net electric charge above $z = 1.08 \pm 0.005$ more than those of drugs with charges below this value.

Respective effects of K^{50}_{app} at a given [L] and of r_{50} on the EPA-induced changes in the PD_{50} of the modulators

At 50% dye leakage, Equation 6 can be written as follows:

$$PD_{50} = \frac{r_{50}}{k \times K^{50}_{app}} + r_{50} \times [L] \qquad (7)$$

where $r_{50}/(k \times K^{50}_{app}) = [D]_f^{50}$ is the free drug concentration at 50% leakage and $k = (K^{0}_{app}/K^{50}_{app})^{0.5}$, the constant characterizing the bending of the isotherm ($k = 1$ for ideally partitioning modulators, $k < 1$ for TH at $x_{EPA} = 0$, and $k > 1$ for MP at $x_{EPA} = 0$ and 0.1; Equation 5). Therefore, the EPA-induced changes in the PD_{50} and the r_{50} of a modulator can be expressed by:

$$\frac{PD_{50}(x_{EPA} = 0.1)}{PD_{50}(x_{EPA} = 0)} = \frac{[L] + 1/[k\ (x_{EPA} = 0.1) \times K^{50}_{app}(x_{EPA} = 0.1)]}{[L] + 1/[k\ (x_{EPA} = 0) \times K^{50}_{app}(x_{EPA} = 0)]} \times \frac{r_{50}(x_{EPA} = 0.1)}{r_{50}(x_{EPA} = 0)} \quad (8)$$

In this equation, $PD_{50}\ (x_{EPA} = 0.1)/PD_{50}\ (x_{EPA} = 0) = 1$ when EPA has no effect on r_{50} and $k \times K^{50}_{app}$ (binding affinity), and tends towards zero when EPA induces a large decrease in r_{50}. In order to picture the EPA effects more clearly, the changes in $1 - [PD_{50}\ (x_{EPA} = 0.1)/PD_{50}\ (x_{EPA} = 0)]$ (EPA effect on permeation) with those in $1 - [r_{50}\ (x_{EPA} = 0.1)/r_{50}\ (x_{EPA} = 0)]$ (EPA effect on membrane perturbation) were therefore plotted in Figure 3. In Figure 3, the dashed line (identity line) describes the EPA effect on permeation due solely to that on membrane perturbation. The vertical distance between the dashed line and the experimental points reflects the EPA effect on permeation resulting from that on the binding affinity at a given [L]. Since EPA did not affect the binding affinity of TR, the corresponding experimental points are on the identity line. The data corresponding to DZ, VR and MP are above the identity line. This indicates that EPA improved the permeation efficiency of these drugs by increasing their membrane perturbation efficiency as well as their binding affinity. By contrast, the data obtained for TH are under the identity line. In this case, the EPA effect on permeation resulting from a favourable effect on membrane perturbation was slightly counterbalanced by the concomitant unfavourable effect of EPA on the binding affinity. Based on the data plotted in Figure 3, it was calculated that over the 2.67–6.67 mM [L] range, the EPA effect on permeation resulting from that on membrane perturbation amounted to: 96% with TR, 50% with DZ, 80% with VR, 110% with TH, and 90% with MP. Therefore, the concomitant effect of EPA on the binding affinity of the modulators amounted to: 4% with TR, 50% with DZ, 20% with VR, −10% with TH and 10% with MP.

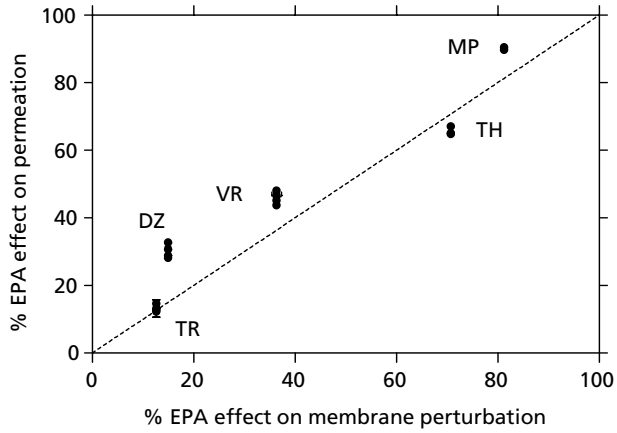

Figure 3 Respective effects of the membrane perturbation and of the overall membrane binding affinity (K^{50}_{app}) at a given lipid concentration, [L], on the EPA effect on permeation. VR, verapamil; DZ, diltiazem; TH, thioacridine; TR, Triton X-100; MP, mepacrine. Using the data given in Table 1, the $1 - PD_{50}\ (x_{EPA} = 0.1)/PD_{50}\ (x_{EPA} = 0)$ and $1 - r_{50}\ (x_{EPA} = 0.1)/r_{50}\ (x_{EPA} = 0)$ values (± s.e.) were calculated for each modulator. [L] (mM) = 2.67, 4.00, 5.33 and 6.67, except for TH (PD_{50} not determined at 6.67) and MP (PD_{50} not determined at 5.33 and 6.67). The error bars are drawn at [L] = 4.00 mM.

Discussion

In this study, the binding isotherms were drawn up using the indirect method based on the dependence of the leakage rate on $[D]_0$ and [L] (Thorn 1964; Paternostre et al 1988; Matzuzaki et al 1989a). This method has the advantage of allowing the concomitant determination of the membrane binding affinity and the membrane perturbing activity since the binding isotherm can be directly correlated with the leakage rate (Matzuzaki et al 1989a). With this approach, a fast binding equilibrium is assumed to occur at membrane–solution interfaces, which was confirmed by the linearity of the $[D]_0$ vs [L] plots obtained (Matzuzaki et al 1989b).

TR was used here as the control substance since its net electric charge is zero. Whatever x_{EPA}, the binding results from hydrophobic interactions with the bilayer. An ideal partitioning process (linear isotherm) was thus observed, as already found to occur (Paternostre et al 1988).

At $x_{EPA} = 0$ (Figure 1A), the binding isotherms of DZ and VR were linear. The positive Ψ generated by the binding of these mono-basic drugs to neutral membranes was therefore low enough for their partitioning to be ideal. The isotherm of the di-basic TH was bent upwards. Type III isotherms of this kind have been obtained when neutral peptides or steroids bind to neutral membranes (Matzuzaki et al 1989b; Onishi & Tachibana 1997). The Ψ generated upon TH binding may therefore not have played a key role. Due to its small size and very particular shape, that is a tricyclic planar aromatic ring with an aliphatic side chain, TH may have reduced the hydrophobic interactions in the very beginning of the aliphatic chains, which enhanced the partitioning. Since the isotherm of TH rose to infinity, an ideal partitioning of this modulator was maintained in the presence of aggregation (Rizzo et al 1987). The isotherm of MP was of the Langmuir type, suggesting the existence of repulsive interactions with the associated MP molecules. Also, the binding of MP molecules rotates the phosphocholine dipole out of the membrane plane (Altenbach & Seelig 1985; Breschiaschvili & Seelig 1990a and references cited

therein). The specific spatial positioning of the rigid tricyclic aromatic ring of MP within the phospholipid head groups may have further enhanced the extent of the dipole reorientation (Breschiaschvili & Seelig 1990a). Thus, the binding became increasingly more difficult, which in turn favoured the aggregation of MP molecules in the aqueous phase (Albert 1966) and reduced their thermodynamic activity.

At $x_{EPA} = 0.1$ (Figure 1B), electrostatic interactions took place between the cationic drugs and the anionic phosphate. The membrane charges were partially neutralized upon drug binding. Since the isotherms of DZ, VR and TH were linear, their hydrophobic partitioning may have been carried out mainly by the neutral species. At the VR concentrations used here, the partitioning of this modulator is known to be an ideal process in platelets (Retzinger & Cohen 1992). The isotherm of MP was still bent downwards. The binding of MP was mainly due to electrostatic effects rather than chemical effects. However, a positive Ψ was probably generated by the binding after the membrane charges had been completely neutralized. Subsequently, MP aggregation occurred in the aqueous phase.

For practical purposes, the overall binding constant K^{50}_{app} is often preferred to the intrinsic K^{0}_{app}. Except for TR, the K^{50}_{app} of the cationic drugs was found to be modified by EPA. At a negative Ψ, the K^{50}_{app} of DZ, VR and MP was enhanced, whereas that of TH was decreased. The K^{50}_{app} values recorded here with TR are within the range of those obtained in EPC multilamellar vesicles ($0.11\,mM^{-1}$: Ruiz et al 1988) and EPC/EPA LUV ($3.5\,mM^{-1}$: Paternostre et al 1988). When estimated by a direct method, the binding affinity of VR for human platelets ($0.95\,mM^{-1}$: Retzinger & Cohen 1992) is greater than that observed here at $x_{EPA} = 0.1$ ($0.32\,mM^{-1}$), probably due to the large amount of anionic lipids in the platelets (Kawasaki et al 1984).

At $x_{EPA} = 0$, the r_{50} of TH and MP was 8- to 10-times higher than that of TR, DZ and VR. The size and shape of TH and MP prevented them from penetrating deeply into the membrane. In addition, the neutral form of these modulators may be positioned with the rigid acridine nucleus either parallel or perpendicular to the membrane surface. At $x_{EPA} = 0.1$, the occurrence of electrostatic interactions oriented the acridine nucleus perpendicularly to the membrane surface and more deeply inside the membrane. This resulted in bilayer destabilization, which favoured the partitioning and, in turn, the large increase in the membrane perturbation potency (Figure 2). The r_{50} values determined here with TR are similar to those obtained in EPC/EPA LUV (0.05: Paternostre et al 1988) and EPC SUV (0.39: Ruiz et al 1988).

Conclusions

The partitioning of MDR modulators in membranes can be either ideal or modulated by the electrostatic effects occurring upon binding and by the specific spatial position of the drug with respect to the membrane surface. The electrostatic interactions induced by the presence of EPA enhanced the binding affinity of the cationic modulators, except when modulator aggregation occurred in the lipid phase, and increased their potency to produce membrane perturbation in a z-dependent manner. These interactions promoting an efficient orientation of the modulators with respect to the membrane surface led to a deeper insertion into the core of the membrane. The greater potency of the modulators to induce permeation in the presence of EPA resulted mainly from the EPA effect on membrane perturbation (≥50%) and, to a lesser extent, from that on the binding affinity (≤50%).

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