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Thermal Dependence of Multidrug-resistant-modulator Efficiency: a Study in Anionic Liposomes

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Abstract

This study was designed to test the hypothesis that there exists a correlation between the ability of lipophilic drugs to mediate the reversal of multidrug-resistance (MDR) by interacting with the membrane phospholipids and the metabolic level in tissues. The permeation properties of five MDR-modulators were studied by quantifying their ability to induce the leakage of Sulphan blue through unilamellar liposomes, over the temperature range 27–42°C.

The dye leakage induced by a non-ionic detergent (Triton X-100), two calcium blockers (diltiazem and verapamil) and two antiparasitic agents (thioacridine derivative and mepacrine) was temperature-dependent. The permeation process was a co-operative one ($1{\cdot}1 <$ Hill coefficient $< 7{\cdot}5$) and the permeation doses inducing 50% dye leakage (PD50) were 1·5–14·9 mM. The permeation ability of the MDR-modulators (log(1/PD50)) decreased significantly as the net electric charge (z) increased. The passive dye leakage ($\Delta G < 0$) was found to be an endothermic process ($\Delta H > 0$), favoured by an increase in the membrane disorder ($\Delta S > 0$). The apparent enthalpy factor (ΔH_{50}) associated with 50% dye leakage increased with the net electric charge of the compound, and this energetically non-favoured event was entirely offset by the concomitant increase in the entropy factor (ΔS_{50}). The apparent permeation enthalpy (ΔH_{50}) and entropy (ΔS_{50}) showed the lowest values for Triton X-100 ($\Delta H_{50} = 7{\cdot}1 \pm 0{\cdot}53$ kJ mol^{-1}, $\Delta S_{50} = 76{\cdot}9 \pm 1{\cdot}86$ J mol^{-1} K^{-1}), and the highest values for mepacrine ($\Delta H_{50} = 79{\cdot}5 \pm 3{\cdot}80$ kJ mol^{-1}, $\Delta S_{50} = 306{\cdot}7 \pm 5{\cdot}97$ J mol^{-1} K^{-1}). When the temperature was increased from 27 to 42°C, the apparent Gibbs free energy (ΔG_{50}) of the dye leakage induced by Triton X-100 decreased by less than 10% of the initial value, and that induced by mepacrine decreased by nearly 40%.

The results provide evidence that in tissues with high metabolic levels and therefore high temperatures, MDR-reversal is likely to be enhanced via favourable drug-membrane interactions controlled by the electric charge of the modulators.

The multidrug-resistance (MDR) of cancer cells has attracted increasing attention in recent years. MDR is characterized by cross-resistance of the cell towards a broad range of structurally and functionally unrelated drugs after cell exposure to a single drug (Ford & Hait 1990; Sharom 1997). The MDR process is generally associated with over-expression of a cell-surface P-glycoprotein (P-gp). This protein acts as an energy-dependent efflux pump, extruding cytotoxic agents (anthracyclines, Vinca alkaloids and other compounds) from the tumour cell, thus abolishing their cytotoxic effects (Beck 1987; Ford & Hait 1990).

A variety of substances have been found to inhibit P-gp-mediated drug efflux and thus induce the reversal of MDR. These modulators include calcium channel blockers, calmodulin antagonists, antimalarials, cyclic peptides, steroids and hormonal analogues and amphiphiles such as Triton X-100 (Ford & Hait 1990; Sharom 1997).

Given the considerable structural heterogeneity of the MDR-modulators, several biochemical mechanisms have been thought to explain their anti-MDR action. The most commonly suggested

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explanation is that competition may occur between MDR-modulators and cytotoxic agents for binding to P-gp (Ford & Hait 1990; Sharom 1997). Another hypothesis is that P-gp may function like a flippase, extruding a wide range of chemically unrelated compounds from the cell (Higgins & Gottesman 1992). According to Eytan et al (1996), anticancer drugs and MDR-modulators enter cells by a process of passive diffusion through the plasma membrane, at different rates. The active efflux rate by P-gp may successfully overcome the slow passive influx rate of anticancer drugs, whereas it would fail to overcome the fast re-entry of MDR-modulators into the cell. The P-gp ATPase activity may be stimulated and the ATP consumption increased by the futile recycling of the modulators, yet there is no net transport. It is therefore possible that MDR-modulators may function like uncouplers. In addition, most of the MDR-modulators show similar high levels of lipophilicity and have a basic nitrogen atom (Zamora et al 1988). It has therefore been speculated that interactions between these compounds and the membrane phospholipids might contribute to the mechanism underlying MDR modulation (Seydel et al 1994). According to Pajeva et al (1996), drug-membrane interactions might lead to the reversal of MDR either directly by changing the membrane permeability and fluidity, or indirectly by inhibiting protein kinase C and therefore, P-gp phosphorylation, or by changing the structural organization of the lipids surrounding membrane-integrated proteins, thus modifying their conformation and functional modes. Several studies have proved that drug–membrane interactions play a key role in the reversal of MDR (Callaghan & Riordan 1995; Drori et al 1995; Pajeva et al 1996). Given the existence of physiological thermal gradients of up to 20°C in man (Bazett et al 1948), studies on the thermal dependence of MDR-modulator efficiency via drug–membrane interactions are now needed.

In this study, to assess the changes in the efficiency of MDR-modulators, depending on the metabolic level of tissues, the strength of drug-membrane interactions was quantified at various temperatures. The ability of five modulators to induce dye leakage from anionic liposomes was determined over the temperature range 27–42°C. The effect of the net electric charge of the drugs was also studied by comparing the dye leakage induced by mono- and di-basic amines with that induced by the amphiphile Triton X-100, a non-ionic polyoxyethylene detergent. Triton X-100 has been found to reverse MDR-resistance (Cano-Gauchi & Riordan 1987; Drori et al 1995), and to behave like a P-gp substrate (Doige et al 1993; Loe & Sharom 1993; Sharom 1997). Since two of the modulators in this study exhibit fluorescence, the method based on carboxyfluorescein leakage could not be used. Sulphan blue was therefore used as the membrane permeation indicator (Castaing et al 2000). This method appears to be suitable for studying MDR-reversal by modulators in tumour cells, via their ability to interact with the membrane phospholipids. MDR-modulators, but not non-modulators, were found to induce the leakage of Sulphan blue through the membrane of anionic liposomes (Castaing et al 2000).

Materials and Methods

Chemicals

Triton X-100 was purchased from Sigma (St Louis, MO). Thioacridine ether (Figure 1) was prepared from the corresponding thioacridinone (Hevér et al 1998). L-α-phosphatidyl choline prepared from fresh egg yolk (EPC), L-α-phosphatic acid prepared from egg yolk lecithin (EPA), diltiazem hydrochloride were purchased from Sigma (St Louis, MO). Mepacrin dihydrochloride hydrate and the dye anhydro-4-4′-bis(ditheylamino)triphenyl-methanol-2″,4″-disulphonic acid monosodium salt (Sulphan blue or Patent blue VF) were purchased from Aldrich (Steinheim, Germany). Verapamil hydrochloride was a gift from Knoll France Laboratory (Levallois-Perret, France) and cholesterol was obtained from Fluka (Buchs, Switzerland). Diethyl ether, $Na_2HPO_4.2H_2O$ and $NaH_2PO_4.H_2O$ were purchased from Merck (Darmstadt, Germany), Sephadex PD-10 columns (G-25M), from Pharmacia (Uppsala, Sweden) and polycarbonate porous membranes, from Nucleopore Corporation (Pleasanton, CA).

Verapamil hydrochloride, diltiazem hydrochloride, thioacridine dihydrochloride and Triton X-100 were dissolved in water. Mepacrine dihydrochloride was dissolved in 100 mM phosphate buffer (pH 7·4).

Preparation of liposomes

Large unilamellar vesicles (LUV) containing L-(-phosphatidyl choline), L-(phosphatidic acid) and cholesterol in an 8 : 1 : 1 molar ratio were prepared by reverse-phase evaporation, as previously described (Castaing et al 2000).

Permeability measurements

The absorbance of Sulphan blue at 640 nm was determined by recording the visible absorption spectra of the samples with a Uvikon 933 spectrophotometer (Milan, Italy). Permeability mea-

surements were performed on a 1-mL LUV suspension as previously described (Castaing et al 2000). The leakage of Sulphan blue entrapped was induced by 0–27 mM modulator (or 0–4·0 mol modulator/mol lipid, i.e., approx. 0–27·2 μmol modulator/m^2 surface membrane) through negatively-charged LUV membranes. The leakage was quantified after a 3-min incubation at 27, 32, 37 and 42°C (pH 7·4). Results were plotted as the percentage of total dye leaked as a function of drug concentration.

Data analysis

The variations in the percentage of total dye leaked with different drug concentrations were fitted to the dose-response curves described by:

$$\%\text{Total dye leaked} = 100 \times [\text{Drug}]^h/(\text{PD50}^{\,h} + [\text{Drug}]^h) \quad (1)$$

where PD50 is the dose inducing 50% dye leakage from the liposomes and h is the Hill coefficient, characterizing the co-operativity of the permeation process.

Hydrophobicity of the modulators

The Pallas 2.0 software program (Compudrug Chemistry Ltd, Budapest, Hungary) was used to calculate the octanol–buffer partition coefficient (P) and the ionization constant (pK_a) of Triton X-100 and of the thioacridine derivative at 25°C (pH 7·4 and $\mu = 0{\cdot}22$). In the case of diltiazem, verapamil and mepacrine, the experimental P and pK_a values given by Craig (1990) at the same temperature were taken into account. Since drug ionization is a temperature-dependent process influencing drug distribution, the pK_a values of the compounds were calculated at each temperature according to the temperature coefficients ($-dpK_a/dT$) estimated by Perrin (1964) for the ionization of bases. Using these temperature-corrected pK_a values, the temperature-dependent octanol-buffer distribution coefficients (D) of the compounds were then calculated according to the equations given by Bowden (1990) for mono- and di-basic drugs (Eqn 2 and 3, respectively):

$$\log D = \log P - \log (1 + 10^{pKa-pH}) \quad (2)$$

$$\log D = \log P - \log (1 + 10^{pKa1-pH} + 10^{pKa1+pKa2-2\,pH}) \quad (3)$$

With pKa1 > pKa2.

Since no reliable data exist, the octanol–buffer distribution coefficients calculated according to equations 2 and 3 did not take into account the temperature-dependence of the octanol–buffer partition coefficient of the drugs.

Net electric charge of the modulators

With the exception of Triton X-100, the modulators studied were weak bases existing in various states of ionization at the pH used. The net electric charge (z) of the various drugs (i.e. the mean net electric charge per drug molecule) was therefore calculated according to the following equation (pH 7·4):

$$z = (1/C_T) \times \Sigma(C_i \times z_i) \quad (4)$$

where C_T is the total concentration of the drug in the aqueous phase, and C_i is the concentration of species i, with valence z_i.

Statistical analysis

Data are expressed as mean ± s.e.m. Linear and non-linear regressions were calculated using the least-square method. Results were considered significant at $P < 0{\cdot}05$.

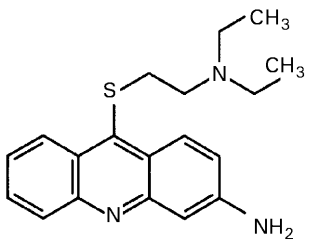

Figure 1. Chemical structure of the thioacridine ether derivative.

Results

Drug permeation properties (PD50 and h) are temperature-dependent. The leakage of Sulphan blue induced by the five modulators through the membrane of negatively-charged liposomes was quantified after a 3-min incubation at 27, 32, 37 and 42°C (pH 7·4). All the modulators induced a temperature-dependent leakage at concentrations ranging from 0 to 27 mM (Table 1). At the PD50 of the modulators, the lipid:modulator molar ratios ranged from 0·5 (mepacrine at 27°C) to 4·5 (Triton X-100 at 42°C).

The co-operativity (h) of the permeation process varied with the temperature depending on the modulator involved. Membrane permeation was a highly co-operative process ($4{\cdot}0 < h < 7{\cdot}5$) characterized by a low temperature-dependence when induced by Triton X-100, diltiazem and verapamil.

The permeation induced by mepacrine and the thioacridine derivative was almost non co-operative at 27°C (h = 1·1–1·2), although slight co-operativity existed at 42°C (h = 1·7–1·9) (Table 1).

It is generally agreed that several MDR-modulators are cationic compounds. However, it has been clearly established that amphiphiles such as Triton X-100 can behave as chemosensitizers (Sharom 1997). The net electric charge (z) of the modulators in this study was therefore calculated (pH 7·4) at each temperature using either the experimental pK_a (Craig 1990) or the calculated pK_a value at 25°C, and the temperature coefficients estimated by Perrin (1964) (Table 1). The $\log(1/PD_{50})$ vs z linear regression was highly significant (slope = $-0{\cdot}40 \pm 0{\cdot}07$, y-intercept = $2{\cdot}81 \pm 0{\cdot}07$, $F_{(1,38)} = 38{\cdot}9$, $S_{residual} = 0{\cdot}222$, r = $-0{\cdot}711$ and $P < 0{\cdot}0001$). Approximately 51% of the permeation properties of the drugs observed at various temperatures could be explained in terms of their temperature-dependent net electric charge ($r^2 = 0{\cdot}506$). Table 1 also shows that the higher the net electric charge (z) of the drug, the greater the temperature-induced change in its permeation ability.

As previously discussed (Castaing et al 2000), the dye leakage through membranes is prevented by the ionization of the drug, both directly by decreasing the non-ionized form of the drug, which is the only species able to partition into the organic phase (Taylor 1990), and indirectly because the properties of lipid–buffer interfaces can differ considerably from those of octanol–buffer interfaces, especially when the membranes bear electric charges (Tocanne & Teissié 1990; Romsicki & Sharom 1999). As a result, the permeation properties of cationic drugs varied significantly with their octanol–buffer distribution per unit net electric charge ((log D)/z) (Castaing et al 2000). The temperature-dependent octanol–buffer distributions (log D) of the modulators in this study were therefore calculated at each temperature (Table 1). The log(1/PD50) vs (log D)/z linear regression was highly significant (slope = $0{\cdot}13 \pm 0{\cdot}04$, y-intercept = $2{\cdot}04 \pm 0{\cdot}12$, $F_{(1,30)} = 8{\cdot}8$, $S_{residual} = 0{\cdot}264$, r = 0·476 and $P < 0{\cdot}006$). However, only 23% of the permeation properties of the modulators could be explained in terms of their temperature-dependent net electric charge because in this regression ($r^2 = 0{\cdot}227$), the data obtained with Triton X-100 could not be taken into account (z = 0) and the log D values described this parameter only approximately, since the temperature-dependence of the octanol–buffer partition of the modulator is unknown.

The induction of dye leakage through bilayer membranes by a drug is a passive process, and is therefore characterized by a negative apparent

Table 1. Permeation dose (PDSO), Hill coefficient (h), ionization constants (pK_a), net electric charge (z) and octanol–water distributions (log D) of the MDR-modulators.

Compounds	Temp°C	PD50 (mM)	h	pK_a[a]	z	log D[a]
Non-ionic detergent						
Triton X-100	27	1·7 ± 0·03	7·5 ± 0·74	non-ionizable	0·00	2·98[b]
	32	1·6 ± 0·02	7·0 ± 0·64	non-ionizable	0·00	2·98[b]
	37	1·6 ± 0·03	7·3 ± 1·64	non-ionizable	0·00	2·98[b]
	42	1·5 ± 0·04	6·7 ± 0·57	non-ionizable	0·00	2·98[b]
Calcium-channel blockers						
Diltiazem	27	3·8 ± 0·14	4·9 ± 0·30	7·65	0·64	2·26
	32	3·6 ± 0·19	5·6 ± 0·89	7·54	0·58	2·32
	37	3·3 ± 0·23	5·6 ± 0·45	7·43	0·52	2·38
	42	3·2 ± 0·11	5·6 ± 0·22	7·33	0·46	2·43
Verapamil	27	2·0 ± 0·19	4·6 ± 0·23	8·87	0·97	2·31
	32	1·9 ± 0·12	4·3 ± 0·31	8·73	0·96	2·44
	37	1·7 ± 0·18	4·2 ± 0·35	8·61	0·94	2·55
	42	1·6 ± 0·14	4·0 ± 0·39	8·48	0·92	2·68
Antiparasitic agents						
Thioacridine	27	12·9 ± 0·78	1·2 ± 0·15	7·07[b]; 9·23[b]	1·31	1·77[b]
	32	8·7 ± 0·36	1·4 ± 0·14	6·97[b]; 9·10[b]	1·25	1·93[b]
	37	6·3 ± 0·35	1·8 ± 0·13	6·87[b]; 8·96[b]	1·20	2·09[b]
	42	4·7 ± 0·51	1·9 ± 0·25	6·77[b]; 8·84[b]	1·16	2·23[b]
Mepacrine	27	14·9 ± 1·63	1·1 ± 0·01	7·68; 10·12	1·65	3·00
	32	7·2 ± 0·78	1·3 ± 0·01	7·57; 9·97	1·59	3·22
	37	4·5 ± 0·45	1·6 ± 0·05	7·46; 9·82	1·53	3·43
	42	3·2 ± 0·22	1·7 ± 0·02	7·36; 9·68	1·47	3·62

The parameters PD50 and h are expressed as mean ± s.e.m. of the permeation parameters obtained from the study of two large unilamellar vesicle preparations.[a]Calculated from the data given by Craig (1990), except when noted.[b]Calculated from the data predicted by the Pallas 2.0 software program.

Gibbs free energy ($\Delta G < 0$). The well-known Gibbs equation relates the Gibbs free energy (ΔG), the enthalpy (ΔH) and the entropy (ΔS) as follows: $\Delta G = \Delta H - T\Delta S$, where T is in K. As shown in Table 1, the dye leakage process was favoured (1/PD50 increase) by increasing the temperature. Membrane permeation is clearly an endothermic process ($\Delta H > 0$). Dye leakage can be expected to result from a decrease in molecular packing of the membrane phospholipids (increased disordering of lipids $\Delta S > 0$). It therefore seemed of interest to quantify the respective parts played by enthalpy and entropy in the permeation process induced by drugs bearing different net electric charges.

Over the 27–42°C temperature range, the reverse logarithmic value of the permeation dose (log(1/PD50)) characterizing 50% dye leakage varied linearly with the reciprocal absolute temperature (1/T). With all the modulators studied, the log(1/PD50) vs 1/T regression was statistically significant. The apparent enthalpy (ΔH_{50}) and entropy (ΔS_{50}) of the permeation by each drug were therefore calculated from the slope and y-intercept of the Van't Hoff plots, respectively (Table 2). Using these values, the apparent Gibbs free energy (ΔG_{50}) of the dye leakage was calculated at the four temperatures studied according to the Gibbs' equation (Table 2).

As shown in Table 2, the apparent permeation enthalpy (ΔH_{50}) and entropy (ΔS_{50}) increased with the net electric charge of the drug inducing dye leakage. The lowest values were obtained with the non-ionic detergent Triton X-100 ($\Delta H_{50} = 7{\cdot}1 \pm 0{\cdot}53$ kJ mol^{-1}, $\Delta S_{50} = 76{\cdot}9 \pm 1{\cdot}86$ J mol^{-1} K^{-1}), and the highest values with mepacrine ($\Delta H_{50} = 79{\cdot}5 \pm 3{\cdot}80$ kJmol^{-1}, $\Delta S_{50} = 306{\cdot}7 \pm 5{\cdot}97$ J mol^{-1} K^{-1}). The ΔS_{50} vs ΔH_{50} linear regression was highly significant (slope $= 3{\cdot}16 \pm 0{\cdot}55 \times 10^{-3}$ K^{-1}, y-intercept $= 5{\cdot}19 \pm 0{\cdot}24 \times 10^{-2}$ kJ mol^{-1}K^{-1}, $F_{(1,8)} = 3348$, $S_{residual} = 0{\cdot}005$, $r = 0{\cdot}999$ and $P < 0{\cdot}0001$) (Figure 2).

As a result of the low apparent entropy (ΔS_{50}) of the membrane permeation by Triton X-100, diltiazem and verapamil, the apparent Gibbs free energy (ΔG_{50}) decreased only slightly (approx. 10%) when the temperature was increased from 27 to 42°C (Table 2). The efficiency of the non-ionic detergent Triton X-100 and that of the two calcium-channel blockers thus increased only slightly when the temperature was raised. Due to the high apparent entropy (ΔS_{50}) of the membrane permeation by the thioacridine derivative and mepacrine, the apparent Gibbs free energy (ΔG_{50}) of these drugs was approximately 30–40% lower at 42°C than at 27°C. It can therefore be concluded that the higher the temperature, the greater the efficiency of the MDR-modulators to induce dye leakage through membranes.

Discussion

In this study, the temperature-induced changes in the interactions between five MDR-modulators (Klohs et al 1986; Inaba & Maruyama 1988; Sharom 1997; Hevér et al 1998) and the membrane of anionic liposomes were investigated. The compounds were chosen on the basis of their very different electric charges at physiological pH; the P-gp substrate Triton X-100 (Sharom 1997) is a non-ionic detergent, whereas the calcium-channel blockers (diltiazem and verapamil) and the anti-parasitic agents (mepacrine and thioacridine derivative) are mono- and di-basic amines, respectively. Over the temperature range 27–42°C, all the modulators affected the lipid bilayer of the liposomes sufficient for the passage of the entrapped polar dye, Sulphan blue, to occur.

Table 2. Apparent enthalpy (ΔH_{50}), entropy (ΔS_{50}) and Gibbs free energy (ΔG_{50}) of membrane permeation by the MDR-modulators.

Compounds	ΔH_{50}(kJ mol^{-1})	ΔS_{50} (J mol^{-1} K^{-1})	ΔG_{50} (kJ mol^{-1})			
			27°C	32°C	37°C	42°C
Non-ionic detergent						
Triton X-100	$7{\cdot}1 \pm 0{\cdot}53$	$76{\cdot}9 \pm 1{\cdot}86$	$-16{\cdot}0 \pm 0{\cdot}03$	$-16{\cdot}4 \pm 0{\cdot}05$	$-16{\cdot}8 \pm 0{\cdot}06$	$-17{\cdot}2 \pm 0{\cdot}07$
Calcium channel blockers						
Diltiazem	$10{\cdot}3 \pm 0{\cdot}25$	$80{\cdot}9 \pm 1{\cdot}03$	$-14{\cdot}0 \pm 0{\cdot}06$	$-14{\cdot}4 \pm 0{\cdot}07$	$-14{\cdot}8 \pm 0{\cdot}07$	$-15{\cdot}2 \pm 0{\cdot}06$
Verapamil	$10{\cdot}9 \pm 0{\cdot}26$	$88{\cdot}5 \pm 0{\cdot}10$	$-15{\cdot}6 \pm 0{\cdot}23$	$-16{\cdot}1 \pm 0{\cdot}23$	$-16{\cdot}5 \pm 0{\cdot}23$	$-17{\cdot}0 \pm 0{\cdot}23$
Antiparasitic agents						
Thioacridine	$53{\cdot}6 \pm 2{\cdot}71$	$215{\cdot}8 \pm 8{\cdot}86$	$-11{\cdot}1 \pm 0{\cdot}06$	$-12{\cdot}2 \pm 0{\cdot}01$	$-13{\cdot}3 \pm 0{\cdot}04$	$-14{\cdot}4 \pm 0{\cdot}08$
Mepacrine	$79{\cdot}5 \pm 3{\cdot}80$	$306{\cdot}7 \pm 5{\cdot}97$	$-12{\cdot}5 \pm 2{\cdot}01$	$-14{\cdot}0 \pm 1{\cdot}98$	$-15{\cdot}6 \pm 1{\cdot}95$	$-17{\cdot}1 \pm 1{\cdot}91$

Results are expressed as mean ± s.e.m. of the permeation parameters obtained from the study of two large unilamellar vesicle preparations.

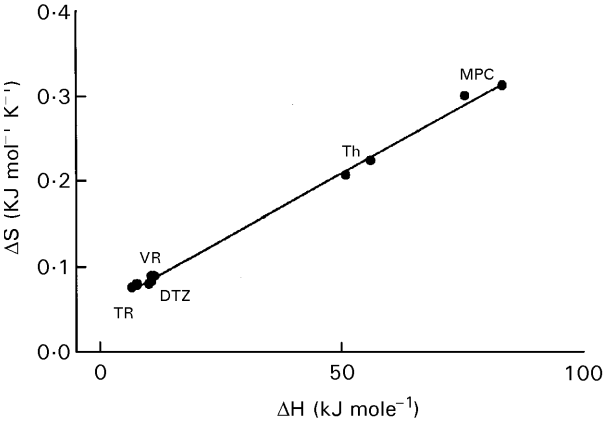

Figure 2. Dependence of the apparent entropy (ΔS_{50}) on the apparent enthalpy (ΔH_{50}) of membrane permeation by the MDR-modulators. Each point is the result obtained from the study of one large unilamellar vesicle preparation. TR, Triton X-100 ; DTZ, diltiazem; VR, verapamil; Th, thioacridine derivative; MPC, mepacrine.

When induced by Triton X-100, dye release from liposomes has been found to involve either leakage or membrane disruption, depending on the concentration of the detergent used and on the membrane packing (Liu & Regen 1993). Below its critical micelle concentration (200 μM), Triton X-100 induced dye release via a leakage mechanism from tightly packed gel-phase liposomes, whereas release via a rupture mechanism was promoted above this critical micelle concentration. Whatever the detergent concentration, dye-release by Triton X-100 always involved a leakage mechanism from liposomes in their fluid phase, except at cholesterol mole fractions as high as 45 mol % (Liu & Regen 1993). In this study, the membranes of large unilamellar vesicles composed of egg phosphatidyl choline, egg phosphatidic acid and cholesterol in an 8 : 1 : 1 molar ratio were equilibrated at temperatures well above their transition point from the solid to liquid-crystalline phase (Lee 1990). It therefore seems likely that Triton X-100 induced the release of Sulphan blue through these membranes via a leakage mechanism. The PD50 values determined with Triton X-100 (1·5–1·7 mM) were in reasonably good agreement with previous data on sonicated egg phosphatidyl choline vesicles (2·6 mM), after correcting the latter to allow for the lipid concentration used in this study (Ruiz et al 1988). The similarity between these results confirmed that cholesterol seems to only slightly affect the bilayer permeability when present in low concentrations (Ruiz et al 1988). We also observed that the PD50 values of Triton X-100 and verapamil were of the same order of magnitude at all the temperatures investigated. Similar results were found by Drori et al (1995) at 37°C (verapamil induced 30% dye leakage from large anionic unilamellar vesicles at concentrations 1·4-times higher that those of Triton X-100).

Over the temperature range 27–42°C, the log(1/PD50) vs 1/T regressions were found to be linear for each of the modulators. It was therefore concluded that a single apparent enthalpy (ΔH_{50}) characterized the overall process of dye leakage by these modulators. This finding implies that no phase transition occurred throughout the entire temperature range investigated. According to Ginsburg & Noble (1974), it also means that between 27 and 42°C, the rate-limiting processes for dye leakage by each drug were always the same.

The apparent enthalpy (ΔH_{50}) characterizing dye leakage by the five MDR-modulators studied was found to have positive values (Table 2), that is increasing the temperature favoured the binding of the drug to the membrane (PD50 decrease) (Table 1). When the temperature was raised, the dissociation of the drug from its receptors (phospholipid head groups) at the membrane interface was enhanced. This direct effect of temperature on the drug affinity caused an increase in the PD50 value. Concomitantly, the ionization of the amine groups was reduced. In turn, the concentration of unprotonated drug molecules increased, thus favouring the partition of the drug into the lipid core of the membrane by both increasing the concentration of the only species able to partition into the organic phase (Taylor 1990), and decreasing the electrostatic interactions occurring at the membrane interface (Castaing et al 2000). This indirect effect of temperature on the drug affinity caused a decrease in the PD50 value. It should noted that the membrane fluidity increased with increasing temperature, which may have greatly favoured the entry into the lipid core of the drugs with the largest steric hindrance and/or the lowest affinity for the acyl chains of the phospholipids. This direct effect of temperature on the physical state of the membrane induced a decrease in the PD50 values. All these molecular events probably explain why the apparent enthalpy (ΔH_{50}) of the dye leakage induced by Triton X-100 (in the absence of electrostatic interactions) was the lowest (7·1 kJ mol^{-1}) of the modulators studied. Due to the strong electrostatic interactions between their two amines in the region of the phospholipid head groups, the thioacridine derivative and mepacrine might have been prevented from penetrating into the lipid bilayer, and thus from inducing dye leakage, especially at low temperatures. Dye leakage by these compounds was thus mainly under the control of the indirect effects of temperature on drug affinity (pK_a decrement). Since the enthalpy for amine group ionization ranges from 36 to 53 kJ mol^{-1} (Perrin 1964), the apparent enthalpy (ΔH_{50}) of the

dye leakage induced by the thioacridine derivative and mepacrine had therefore very high values (50–80 kJ mol^{-1}).

The results of this study showed that the membrane phospholipid disorder associated with 50% dye leakage (ΔS_{50}) increased with the net electric charge borne by the MDR-modulators (Table 2). The passage of a dye across a continuous bilayer of lipid depends on the probability of the lipid molecules having an appropriate configuration for the dye molecules to be able to enter and leave the lipid. Hill (1974) has established that when a drug is present in the membrane, the entropy term is changed by a factor ($R.n.C_m$) which is proportional to the gas constant (R), to the number of lipid molecules involved in the passage of one dye molecule (n), and to the drug membrane concentration in moles of drug per mole of lipid (C_m). Since the drug partition into the lipid core was reduced when high electrostatic interactions occurred between the cationic drugs and the anionic phospholipid head groups, the membrane drug concentration (C_m) necessary to induce 50% dye leakage increased. As a result, the entropy term (ΔS_{50}) of the dye leakage also increased with the net electric charge of the drugs.

It can be concluded that dye leakage through anionic membranes results from an energetically-favourable balance between enthalpic and entropic variations controlled mainly by the net electric charge of MDR-modulators. Hence, high temperatures increase the membrane permeation efficiency of these compounds and probably also their ability to reverse MDR in tumour cells with a high metabolic level.

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References

Bazett, H. C., Love, L., Newton, M., Eisenberg, L., Day, R., Froster, R. (1948) Temperature changes in blood flowing in arteries and veins in man. J. Appl. Physiol. 1: 3–19

Beck, W. T. (1987) The cell biology of multiple drug resistance. Biochem. Pharmacol. 36: 2879–2887

Bowden, K. (1990) Electronic effects. In: Ramsden, C. A. (ed.) Comprehensive Medicinal Chemistry. Vol 4. Pergamon Press, Oxford, pp 205–239

Callaghan, R., Riordan, J. R. (1995) Collateral sensitivity of multidrug resistant cells to narcotic analgesics is due to effects on the plasma membrane. Biochim. Biophys. Acta 1236: 155–162

Cano-Gauci, D. F., Riordan, J. R. (1987) Action of calcium antagonists on multidrug resistant cells. Specific cytotoxicity independent of increased cancer drug accumulation. Biochem. Pharmacol. 36: 2115–2123

Castaing, M., Brouant, P., Loiseau, A., Santelli-Rouvier, C., Santelli, M., Alibert-Franco, S., Abdallah, M., Barbe, J. (2000) Membrane permeation by MDR-modulators and non-modulators: effects of hydrophobicity and electric charge. J. Pharm. Pharmacol. 52: 289–296

Craig, P. N. (1990) Drug compendium. In: Drayton, C. J. (ed.) Comprehensive Medicinal Chemistry. Vol 6. Pergamon Press, Oxford, pp. 237–991

Doige, C. A., Yu, X., Sharom, F. J. (1993) The effects of lipids and detergents on ATPase-active P-glycoprotein. Biochim. Biophys. Acta 1146: 65–72

Drori, S., Eytan, G. D., Assaraf, Y. G. (1995) Potentiation of anticancer-drug cytotoxicity by multidrug-resistance chemosensitizers involves alterations in membrane fluidity leading to increased membrane permeability. Eur. J. Biochem. 228: 1020–1029

Eytan, G. D., Regev, R., Oren, G., Assaraf, Y. G. (1996) The role of passive transbilayer drug movement in multidrug resistance and its modulation. J. Biol. Chem. 271: 12897–12 902

Ford, J. M., Hait, W. N. (1990) Pharmacology of drugs that alter multidrug resistance in cancer. Pharmacol. Rev. 42: 155–199

Ginsburg, S., Noble, D. (1974) The activation enthalpies for ion conductance systems in lipid bilayer membranes. J. Membr. Biol. 18: 163–176

Hevér, A., Santelli-Rouvier, C., Brouant, P., El Khyari, S., Molnar, J., Barra, Y., Barbe, J. (1998) Effect of new thioacridine derivatives on P-gp function and on mdr1 gene expression. Anticancer Res. 18: 3053–3058

Higgins, C. F., Gottesman, M. M. (1992) Is the multidrug transporter a flippase? Trends Biochem. Sci. 17: 18–21

Hill, M. W. (1974) The Gibbs free energy hypothesis of general anaesthesia. In: Halsey, M. J., Millar, R. A., Sutton, J. A. (eds) Molecular Mechanism in General Anaesthesia. Churchill Livingstone, London, pp 132–144

Inaba, M., Maruyama, E. (1988) Reversal of resistance to vincristine in P388 leukemia by various polycyclic clinical drugs, with a special emphasis on quinacrine. Cancer Res. 48: 2064–2067

Klohs, W. D., Steinkampf, R. W., Havlick, M. J., Jackson, R. C. (1986) Resistance to anthrapyrazoles and anthracyclines in multi-resistant P388 murine leukemia cells: reversal by calcium blockers and calmodulin antagonists. Cancer Res. 46: 4352–4356

Lee, A. G. (1990) Structure and function of cell membranes. In: Emmet, J. C. (ed.) Comprehensive Medicinal Chemistry. Vol 3. Pergamon Press, Oxford, pp 1–43

Liu, Y., Regen, S. L. (1993) Control over vesicle rupture and leakage by membrane packing and by the aggregation state of an attacking surfactant. J. Am. Chem. Soc. 115: 708–713

Loe, D. W., Sharom, F. J. (1993) Interaction of multidrug-resistant Chinese hamster ovary cells with amphiphiles. Br. J. Cancer 68: 342–351

Pajeva, I. K., Wiese, M., Cordes, H. P., Seydel, J. K. (1996) Membrane interactions of some catamphiphilic drugs and relation to their multidrug-resistance-reversing ability. J. Cancer Res. Clin. Oncol. 122: 27–40

Perrin, D. D. (1964) The effect of temperature on pK values of organic bases. Aust. J. Chem. 17: 484–488

Romsicki, Y., Sharon, F. J. (1999) The membrane lipid environment modulates drug interactions with the P-glycoprotein multidrug transporter. Biochemistry 38: 6887–6896

Ruiz, J., Goni, F. M., Alonso, A. (1988) Surfactant-induced release of liposomal contents. A survey of methods and results. Biochim. Biophys. Acta 937: 127–134

Seydel, J. K., Coats, E. A., Cordes, H. P., Wiese, M. (1994) Drug membrane interaction and the importance for drug transport, distribution, accumulation, efficacy and resistance. Arch. Pharm. (Weinheim) 327: 601–610

Sharom, F. J. (1997) The P-glycoprotein efflux pump: how does it transport drugs? J. Membr. Biol. 160: 161–175

Taylor, P. J. (1990) Hydrophophobic properties of drugs. In: Ramsden, C. A. (ed.) Comprehensive Medicinal Chemistry. Vol 4. Pergamon Press, Oxford, pp 241–294

Tocanne, J. F., Teissié, J. (1990) Ionization of phospholipids and phospholipid-supported interfacial lateral diffusion of protons in membrane model systems. Biochem. Biophys. Acta 1031: 111–142

Zamora, J. M., Pearce, H. L., Beck, W. T. (1988) Physical-chemical properties shared by compounds that modulate multidrug resistance in human leukemic cells. Mol. Pharmacol. 33: 454–462