Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochimica et Biophysica Acta 1611 (2003) 107–114
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
www.bba-direct.com

Interactions between verapamil and neutral and acidic liposomes: effects of the ionic strength

M. Castaing[a,*], A. Loiseau[b], G. Mulliert[c]

[a] GERCTOP-UMR6009, Faculté de Pharmacie, 27 Boulevard Jean Moulin, 13385 Marseille Cedex 05, France
[b] INSERM-IFR2, Faculté de Médecine Xavier-Bichat, Paris, France
[c] LCM³B, Université Henri Poincaré, Vandoeuvre-les-Nancy, France

Received 11 October 2002; received in revised form 9 January 2003; accepted 16 January 2003

Abstract

Patients with cancer often develop major electrolyte disorders, which are aggravated by radiation therapy and chemotherapy and by the concomitant impairment of the renal function and the development of drug resistance. In addition, tumour cells have membranes with more negative charges than normal eukaryotic cells. This study was designed to test the hypothesis that the ability of the Ca^{2+} blocker verapamil to mediate the reversal of multidrug resistance (MDR) by interacting with the membrane phospholipids may be correlated with the ionic strength and membrane surface potential in resistant tumours. The permeation properties of verapamil, which is the best-known MDR-modulator, were therefore studied by quantifying its ability to induce the leakage of carboxyfluorescein through unilamellar liposomes containing various mole fractions of phosphatidic acid (x_{EPA} = 0, 0.1 and 0.3), at four different ionic strengths (I = 0.052, 0.124, 0.204 and 0.318 M). The dye leakage induced by verapamil varied greatly with I, depending on x_{EPA}. The permeation process was a co-operative one (1.3 < Hill coefficient < 3.5) and the permeation doses inducing 50% dye leakage (PD_{50}) ranged between 0.2 and 1.8 mM. A highly significant multiple correlation was found to exist between the variations of $\log(1/PD_{50})$ with those of $1/\sqrt{I}$ and x_{EPA} ($d\log(1/PD_{50})/d(1/\sqrt{I}) = 0.15 \pm 0.01$, $d\log(1/PD_{50})/dx_{EPA} = 2.07 \pm 0.08$, y-intercept = 2.46 ± 0.03, $P < 0.000001$). Kinetic studies on the permeation process showed that it involved two steps. The apparent rate constants of the slow and fast kinetic steps, which were driven by electrostatic and hydrophobic interactions, respectively, increased with the verapamil concentrations, depending on x_{EPA}. The results provide evidence that in resistant tumours (high negative membrane surface potential), the MDR reversal by verapamil might be enhanced by favourable drug–membrane interactions in patients with severe hypo-electrolytic (Na^+ and K^+) disorders, whereas the MDR reversal might be reduced by unfavourable drug–membrane interactions in patients with severe hyper-electrolytic (Ca^{2+}, Na^+ and K^+) disorders.

Keywords: Drug resistance, multiple; Neoplasm; Water–electrolyte balance; Ionic strength; Dye leakage; Drug–membrane interaction

1. Introduction

Patients with cancer may develop major electrolyte disturbances due to local disorders caused by the tumour, substances produced by the tumour and surgical defects resulting from resection of the cancer. Radiation therapy and chemotherapy are additional stresses which may induce fluid and electrolyte problems that are seldom simple [1–5]. Benign and malignant neoplasms may also affect the renal function by: (i) anatomically involving the kidneys, ureters or bladder; (ii) secreting hormones that secondarily affect the renal function; (iii) destroying bone and endocrine organs, thus secondarily causing renal electrolyte abnormalities; (iv) responding to specific therapy that may produce renal electrolyte disorders [6]. In the advanced stages of the disease, the patient is debilitated, more susceptible to acid–base balance problems and fluid or electrolyte disorders and less capable of adjusting to or compensating for such disorders. Carcinoma is a complex problem requiring complex and often multiple curative or palliative efforts, espe-

Abbreviations: MDR, multidrug resistance; Triton X-100, *t*-octylphenoxypolyethoxyethanol; I, ionic strength of the external solution; ω_e and ω_i, osmolarity of the external and internal solution; x_{EPA}, mole fraction of phosphatidic acid; PD_{50}, dose that induces 50% permeation; h, Hill coefficient; C, verapamil concentration; CF, 4(5)-carboxyfluorescein; P-gp, P-glycoprotein; EPC, egg phosphatidylcholine; EPA, egg phosphatidic acid; PS, phosphatidylserine; PE, phosphatidylethanolamine

* Corresponding author. Tel.: +33-4-91-83-55-95; fax: +33-4-91-83-56-30.
E-mail address: gerctop@pharmacie.univ-mrs.fr (M. Castaing).

0005-2736/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0005-2736(03)00031-2

cially in those cases where clinical resistance to chemotherapeutic drugs develops during the therapy.

When treated with chemotherapeutic drugs many cancer cells take advantage of their ability to develop a resistance phenotype, as part of an adaptive mechanism. The multidrug resistance (MDR) is characterized by cross-resistance of the cells towards a broad range of structurally and functionally unrelated drugs after cell exposure to a single drug [7]. The MDR process is generally associated with the overexpression of a cell-surface P-glycoprotein (P-gp). This protein acts as an energy-dependent efflux pump, extruding cytotoxic agents from tumour cells, thus abolishing their cytotoxic effects [7]. Since the first report on the pharmacological reversal of MDR by the Ca^{2+} blocker verapamil [8], a variety of substance (modulators, chemosensitizers) have been found to inhibit P-gp-mediated drug efflux and thus to reverse MDR to chemotherapeutic agents. Among the mechanisms suggested to explain their anti-MDR action [7,9–12], it has been speculated that drug–membrane interactions between the MDR-modulators and the membrane phospholipids may play a role in the MDR reversal [11]. Because most of the MDR-modulators are highly lipophilic cationic compounds [13], they may affect the membrane permeability and fluidity, and modify the structural organization of the lipids surrounding embedded proteins, thus changing their functional modes [14]. Several studies have indeed proved that drug–membrane interactions play a key role in the reversal of MDR [15,16]. Since the development of a tumour and its therapy are often associated with large electrolyte disturbances, then the ionic environment within the cell membrane may be affected, which in turn may disturb these drug–membrane interactions and hence decrease the efficiency of the MDR-modulators.

In this study, it was proposed to assess the effects of the ionic environment on MDR modulator efficiency in terms of the drug–membrane interactions by testing the ability of the Ca^{2+} blocker verapamil, which was selected as the best known chemosensitizer, to induce dye leakage from unilamellar liposomes at ionic strengths ranging from 0.052 to 0.318 M. In addition, since tumour cells have membranes with more negative charges than normal eukaryotic cells [17–19], the effects of the negative electrostatic surface potential of the membrane were also studied by comparing the dye leakage induced by verapamil through membranes containing various amounts of EPA ($x_{EPA}=0$, 0.1 and 0.3) at each I level investigated. The method based on CF leakage was used here to quantify the interactions between verapamil and the liposome membrane.

2. Materials and methods

2.1. Materials

L-α-Phosphatidylcholine prepared from fresh egg yolk (EPC), L-α-phosphatidic acid prepared from egg yolk lecithin (EPA), verapamil hydrochloride and Triton X-100 were purchased from Sigma (St. Louis, MO, USA). The fluorescent dye 4(5)-carboxyfluorescein (dye content: 97.7%) was purchased from Aldrich (Steinheim, Germany), D-mannitol, from Research Organics Inc. (Cleveland, OH, USA) and cholesterol, from Fluka (Buchs, Switzerland). Diethyl ether, Na_2SO_4, $Na_2HPO_4{\cdot}2H_2O$ 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).

Four external solutions of various I were adjusted to the same osmolarity with D-mannitol ($\omega_e=360$ mosM): 20 mM phosphate, 290 mM D-mannitol ($I=0.052$ M), 20 mM phosphate, 227 mM D-mannitol, 30 mM Na_2SO_4 ($I=0.124$ M), 20 mM phosphate, 138 mM D-mannitol, 71 mM Na_2SO_4 ($I=0.204$ M), 20 mM phosphate, 136 mM Na_2SO_4 ($I=0.318$ M). All these solutions were adjusted to pH 7.4 at 37 °C. Internal vesicular solution was 100 mM phosphate, 50 mM CF, pH 7.4 at 37 °C ($\omega_i=360$ mosM). Verapamil hydrochloride and Triton X-100 were dissolved in water.

2.2. Liposomes loaded with CF

Large unilamellar vesicles (LUV) composed of EPC/EPA/cholesterol (9:0:1, 8:1:1, 6:3:1) were prepared by reverse-phase evaporation, as previously described [20]. The removal of the external CF was carried out by passage through four Sephadex G-25 columns eluted with the external solution (20 mM phosphate, 290 mM D-mannitol ($I=0.052$ M)). CF is a hydrophilic probe whose fluorescence is self-quenched at concentrations above 10 mM [21].

2.3. Estimation of the ionic strength (I) of the external solutions

The ionic strength of the external solutions was calculated according to the following equation:

$$I = 0.5 \times \sum (C_i \times z_i) \quad (1)$$

where C_i is the concentration of species i, with valence z_i.

Applying the Henderson–Hasselbalch equation to the sodium phosphate buffer dissociation allowed the calculation of the ionic concentrations (C_i) in Na^+, $H_2PO_4^-$ and HPO_4^{2-} at pH 7.4. The ionization constant ($pK_a=6.76$ at 37 °C) used in this calculation was estimated from the data given by Perrin and Dempsey [22], i.e., $pK_a=6.80$ at 25 °C and $dpK_a/dT=-0.0028$ u/°C.

As a weak electrolyte, Na_2SO_4 dissociates in aqueous solution depending on its concentration. At any given Na_2SO_4 concentration (C_T), the dissociation degree (α_T) is given by

$$\alpha_T = (\omega_T/2 \times C_T) - 0.5 \quad (2)$$

where ω_T, the osmolarity associated with C_T, was calculated from the data given by Wolf et al. [23]. Using these α_T values, then the ionic concentrations (C_i) in Na^+ ($C_{Na^+} = 2 \times C_T \times \alpha_T$) and SO_4^{2-} ($C_{SO_4^{2-}} = C_T \times \alpha_T$) could be estimated at each Na_2SO_4 concentration used.

2.4. Kinetic measurements

The kinetics of CF leakage from liposomes were performed using an SFM25 spectrofluorimeter connected to a 32-bit workstation and a software for Windows (Bio-Tek Kontron Instruments, Milan, Italy) was used. The excitation wavelength was 484 nm, and the fluorescence emission was measured at 512 nm (both slit widths at 10 nm).

Loaded liposomes (20 μl) were mixed with external solution (2 ml, final volume) and equilibrated at 37 °C using a Hetofrig CB 11E thermostat (Heto, Allerød, Denmark). Samples were stirred continuously and the reaction begun with the addition of 0–1.12 mM verapamil (or 0–8.4 mol verapamil/mol lipid). Total reaction time was 15 min at which point 50 μl of 10% (w/v) Triton X-100 was added to disperse the liposomes and allow determination of the total amount of fluorescence associated with the entrapped dye in each sample, i.e., 100% total dye leaked. Previous to the experiments, independent calibrations were performed to 100% relative fluorescence at each I investigated by adding 10% Triton X-100 in a liposome sample, and to 0% relative fluorescence on bi-distilled water (auto-blank). Results were plotted as the percentage of total fluorescence observed as a function of reaction time.

2.5. Data analysis

At each I, x_{EPA} and verapamil concentration, the variations with time in the fluorescence signal were fitted to the equation of a two-phase exponential association:

$$100 \times (F_t - F_0)/(F_\infty - F_0) = A_a \times (1 - e^{-k_a \times t}) + A_b \times (1 - e^{-k_b \times t}) \quad (3)$$

where F_t, F_∞ and F_0 are the relative fluorescence intensity at time t, time ∞ and zero time, respectively. A_a (%) and A_b (%) are the normalized amplitude, and k_a and k_b, the apparent rate constants (min^{-1}).

The variations in the percentage of total dye leaked with different drug concentrations were fitted to the following equation by nonlinear least squares regression:

$$\text{Total dye leaked (\%)} = 100 \times [\text{Drug}]^h/(PD_{50}^h + [\text{Drug}]^h) \quad (4)$$

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 co-operativity of the permeation process.

2.6. Statistical analysis

Data are expressed as mean ± S.E. Linear, bilinear and nonlinear regression lines were calculated using the least-square method. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Drug permeation properties (PD_{50} and h) depending on I and x_{EPA}

The kinetics of CF leakage induced by verapamil through the membrane of unilamellar liposomes (EPC/EPA/cholesterol) were quantified for 15 min (37 °C) at $I = 0.052$, 0.124, 0.204 and 0.318 M, and $x_{EPA} = 0$, 0.1 and 0.3. This leakage was induced from the majority of the vesicles since the observed values of $F_t - F_0$, as a function of time, increased gradually until $F_t - F_0 = F_\infty - F_0$, i.e., the kinetics of CF leakage always reached the same equilibrium level of 100% at t_∞, regardless of x_{EPA}, I and [verapamil] (Fig. 1). Fig. 2 illustrates the effects of I and x_{EPA} on the extent of the leakage induced by verapamil from the liposomes at the end of the incubation period (15 min). In all the sets of experiments, the mole fraction of cholesterol was the same ($x_{chol} = 0.1$), as was the sum of those of EPC (x_{EPC}) and EPA (x_{EPA}), i.e., $x_{EPC} + x_{EPA} = 0.9$. The PD_{50} and h values determined for the dye leakage induced by verapamil after a 3-min period of incubation (initial rate of CF leakage) with the liposomes are given in Table 1. At each x_{EPA} inves-

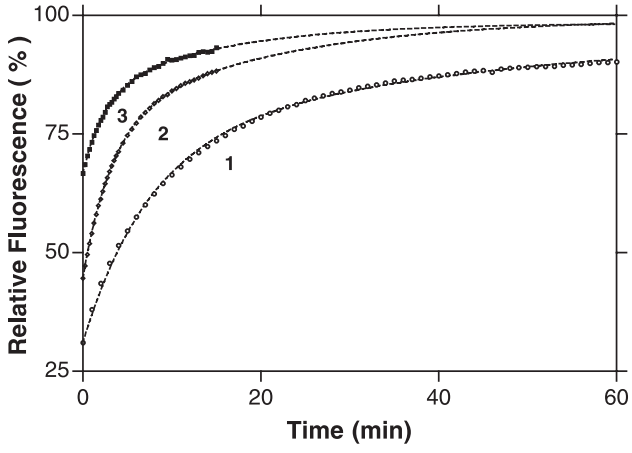

Fig. 1. Kinetics of the CF leakage induced by verapamil from liposomes: the leakage of CF entrapped was induced by (1) 1.12 mM verapamil at $x_{EPA} = 0$ and $I = 0.318$ M ($F_0 = 31.0\%$ and $F_\infty = 97.3\%$), (2) 0.37 mM verapamil at $x_{EPA} = 0.10$ and $I = 0.052$ M ($F_0 = 44.6\%$ and $F_\infty = 98.4\%$), and (3) 0.26 mM verapamil at $x_{EPA} = 0.30$ and $I = 0.124$ M ($F_0 = 66.7\%$ and $F_\infty = 99.4\%$). The units along the Y axis were determined previous to the experiments, by performing a calibration to 100% relative fluorescence at each I investigated by adding 10% Triton X-100 in a liposome sample, and an auto-blank to 0% relative fluorescence on bi-distilled water. The variations with time in the fluorescence signal were fitted to Eq. (3).

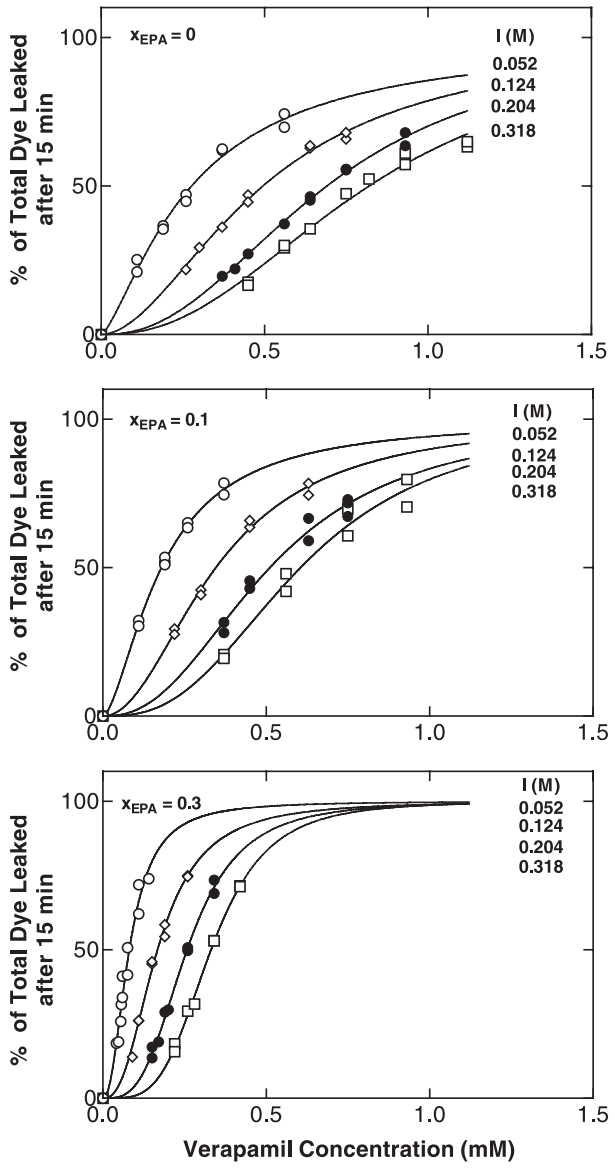

Fig. 2. Effects of I and x_{EPA} on the extent of CF leakage induced by verapamil from liposomes after a 15-min period of incubation: the leakage of CF entrapped was induced by 0 to 1.12 mM verpamil (or 0 to 0.15 mol verapamil in the membrane/mol lipid) through LUV membranes at I = 0.052, 0.124, 0.204 and 0.318 M, and x_{EPA} = 0, 0.1 and 0.3. The extent of CF leakage was quantified after a 15-min period of incubation at 37 °C (pH 7.4) and the total amount of fluorescence associated with the entrapped dye in each sample, i.e., 100% total dye leaked, was determined by dispersing the liposomes with 10% Triton X-100. Previous to the experiments, independent calibrations were performed to 100% relative fluorescence at each I investigated by adding 10% Triton X-100 in a liposome sample, and to 0% relative fluorescence on bi-distilled water (auto-blank). Each point is the result obtained from the study of one LUV preparation.

tigated, verapamil induced a significant ionic strength-dependent leakage when added at concentrations ranging from 0 to 1.12 mM. The overall $\log(1/PD_{50})$ vs. $1/\sqrt{I}$ linear regression was highly significant ($r^2 \geq 0.957$; $P < 0.0001$) at each x_{EPA} level and the value of the positive slope ($d\log(1/PD_{50})/d(1/\sqrt{I})$) of this regression increased significantly with x_{EPA} ($r^2 = 0.964$; $P < 0.001$). These results indicate that the efficiency of verapamil to induce dye leakage from unilamellar liposomes decreased with increasing I ($1/\sqrt{I}$ decrease) and that this effect was counteracted by increasing x_{EPA}, which in turn increased the negative surface potential (Ψ) of the membranes. With the ratio between the membrane volume and the external solution volume, which was estimated to be equal to 0.0001 in a sample, and the octanol-buffer distribution calculated by Castaing et al. [24] for verapamil ($\log D = 2.26$ at pH 7.4), the verapamil concentrations in the membranes per mol lipid were calculated. At the PD_{50} of verapamil, these lipid/drug molar ratios ranged from 1:0.02 (I = 0.052 M at x_{EPA} = 0.3) to 1:0.24 (I = 0.318 M at x_{EPA} = 0).

The membrane permeation induced by verapamil was a co-operative process ($1.3 < h < 3.5$), characterized by a Hill coefficient (h) increasing with I and x_{EPA} (Table 1). Whatever x_{EPA}, the overall $\log h$ vs. $1/\sqrt{I}$ linear regression was statistically significant ($r^2 \geq 0.708$; $P \leq 0.01$). However, there was no significant correlation between the negative slope value $d\log h/d(1/\sqrt{I})$ of these regressions and x_{EPA}. This result suggests that the binding of one verapamil molecule to the membrane somehow accelerated the binding of the subsequent molecules to a greater extent at low than at high $1/\sqrt{I}$. An alternative interpretation is that the number of verapamil molecules concomitantly interacting at the same site on the membrane and inducing a sufficiently strong destabilization of the bilayer for dye leakage to occur may be higher at low than at high $1/\sqrt{I}$. Since the x_{EPA} variations did not significantly affect the slope of the $\log h$ vs. $1/\sqrt{I}$ linear regressions, then it is possible that in the 0 to 0.3 x_{EPA} range investigated here, the changes in the negative surface potential (Ψ) of the membranes did not significantly

Table 1
Effects of x_{EPA} and I on the permeation dose (PD_{50}) and Hill coefficient (h) of verapamil

x_{EPA}	I (M)	PD_{50} (mM)	h
0.0	0.052	0.94 ± 0.08	1.27 ± 0.13
0.0	0.124	1.34 ± 0.05	1.48 ± 0.12
0.0	0.204	1.56 ± 0.08	1.88 ± 0.16
0.0	0.318	1.77 ± 0.02	2.15 ± 0.08
0.1	0.052	0.49 ± 0.03	1.81 ± 0.06
0.1	0.124	0.74 ± 0.03	1.99 ± 0.08
0.1	0.204	0.98 ± 0.03	2.28 ± 0.01
0.1	0.318	1.16 ± 0.09	2.56 ± 0.15
0.3	0.052	0.16 ± 0.01	2.41 ± 0.05
0.3	0.124	0.30 ± 0.01	2.47 ± 0.00
0.3	0.204	0.42 ± 0.01	2.98 ± 0.05
0.3	0.318	0.55 ± 0.01	3.54 ± 0.05

The leakage of CF entrapped was induced by 0 to 1.12 mM verapamil (or 0 to 0.15 mol verapamil in the membrane/mol lipid) through LUV membranes. The leakage was quantified following a 3-min incubation period (initial rate of CF leakage) at 37 °C (pH 7.4). The permeation parameters (PD_{50} and h) were determined according to Eq. (4). The parameters PD_{50} and h are expressed as mean ± S.E. of the permeation parameters obtained from the study of two LUV preparations.

modify the ionic strength-dependence of the co-operativity (h) of the dye leakage induced by verapamil.

3.2. Multiple correlation between the permeation dose (PD_{50}) of verapamil, $1/\sqrt{I}$ and x_{EPA}

The results presented above clearly indicate that CF leakage through membranes was prevented by a decrease of the absolute value of the negative surface potential realised either by increasing I or decreasing x_{EPA}.

Multiple correlation analysis was therefore performed between the efficiency ($\log(1/PD_{50})$ of verapamil, $1/\sqrt{I}$, and x_{EPA}. The results can be expressed by the following equation:

$$\log(1/PD_{50}) = 0.15(\pm 0.01) \times 1/\sqrt{I} + 2.07(\pm 0.08) \times x_{EPA} + 2.46(\pm 0.03) \quad (5)$$

This bilinear dependence of $\log(1/PD_{50})$ on $1/\sqrt{I}$ and x_{EPA} ($F_{(2,21)} = 420.0$, $s_{residual} = 0.050$, $r = 0.988$ and $P < 0.000001$) was highly significant. Nearly 98% of the efficiency of verapamil to induce dye leakage could be explained here in terms of $1/\sqrt{I}$ and x_{EPA} ($r^2 = 0.976$).

Fig. 3 shows the multiple correlation observed between $\log(1/PD_{50})$, $1/\sqrt{I}$ and x_{EPA}. It clearly illustrates the fact that the higher the $1/\sqrt{I}$ and x_{EPA}, the higher the ability of verapamil to induce dye leakage through membranes, whereas the lower the $1/\sqrt{I}$ and x_{EPA}, the lower the ability of verapamil to induce dye leakage through membranes.

3.3. Multiple correlation between the apparent rate constants k_a and k_b of the dye leakage, $1/\sqrt{I}$ and the verapamil concentration (C)

The induction of dye leakage through bilayer membranes by a drug can be expected to involve two stages: the interaction between the drug and its receptors at the membrane interface (first stage) and the interaction between the drug and the phospholipids in the core of the membrane (second stage) [20]. In the present study, the kinetics of CF leakage was fitted to the equation of a two-phase exponential association model. The apparent rate constants k_a and k_b of the leakage process were determined at each C, I and x_{EPA} investigated. The values of k_a (slow kinetic step) ranged between 0.011 and 0.063 min^{-1} (relaxation time $\tau_a = 16-91$ min), and those of k_b (fast kinetic step) between 0.10 and 0.50 min^{-1} (relaxation time $\tau_b = 2-10$ min).

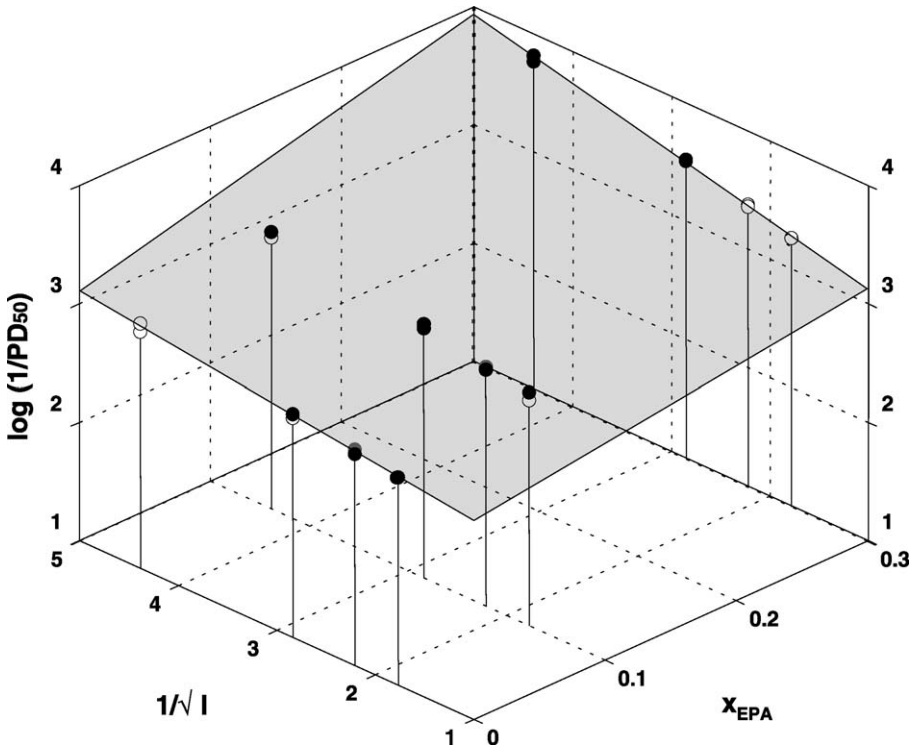

Fig. 3. Bilinear dependence of the permeation ability ($\log(1/PD_{50})$) of verapamil on $1/\sqrt{I}$ and x_{EPA}: the leakage of CF entrapped was induced by 0 to 1.12 mM verapamil (or 0 to 0.15 mol verapamil in the membrane/mol lipid) through LUV membranes. The leakage was quantified following a 3-min period of incubation (initial rate of CF leakage) at 37 °C (pH 7.4) at $I = 0.052$, 0.124, 0.204 and 0.318 M, and $x_{EPA} = 0$, 0.1 and 0.3. The permeation parameters (PD_{50} and h) were determined according to Eq. (4). Each point is the result obtained from the study of one LUV preparation. The experimental data above (filled circles) and below (open circles) the plane of the multiple regression are plotted on the figure.

The respective effects of C and I on these apparent rate constants were then quantified at each EPA level investigated by performing multiple correlation analysis between log$k$, C and $1/\sqrt{I}$. The values of the dlog$k$/dC and dlog$k$/d$(1/\sqrt{I})$ regression coefficients obtained are given in Table 2. Regardless of x_{EPA}, there was a significant bilinear dependence of logk_a on C and $1/\sqrt{I}$ ($r^2 \geq 0.680$; $P < 0.001$) (Table 2). Also, the dlogk_a/dC regression coefficient had a positive value which was 3.7–3.8 times higher at $x_{EPA} = 0.3$ than at $x_{EPA} = 0$–0.1, whereas the dlogk_a/d$(1/\sqrt{I})$ regression coefficients had positive values which increased with x_{EPA}. These results indicate that the slow kinetic step in the leakage process (logk_a) depended on C and $1/\sqrt{I}$, and that these relationships were strengthened at $x_{EPA} = 0.3$. It is therefore likely that this slow kinetic step was driven by the electrostatic interactions occurring between the cationic verapamil and the phospholipid head groups at the lipid/buffer interface. The data given in Table 2 also show that the bilinear dependence of logk_b on C and $1/\sqrt{I}$ was not significant at $x_{EPA} = 0$, whereas it was significant at $x_{EPA} = 0.1$ and 0.3 ($r^2 \geq 0.435$; $P \leq 0.01$). In addition, the value of the dlogk_b/dC regression coefficient was positive and increased with x_{EPA}, whereas that of the dlogk_b/d$(1/\sqrt{I})$ regression coefficient did not differ significantly from zero at any of the x_{EPA} levels investigated. These results indicate that the fast kinetic step in the dye leakage process (logk_b) depended on C, and that this effect was strengthened by increasing x_{EPA}, whereas this step did not depend on $1/\sqrt{I}$. It therefore seems quite likely that this fast kinetic step may have been driven by the hydrophobic interactions occurring between verapamil and the acyl chains of the phospholipids in the lipophilic core of the membrane.

Table 2
Bilinear dependence of the apparent rate constants k_a and k_b on $1/\sqrt{I}$ and C

k	x_{EPA}	dlog$k$/dC (mM^{-1})	dlog$k$/d $(1/\sqrt{I})$ ($M^{1/2}$)	y-intercept	r^2	P
k_a	0.0	1.08 ± 0.08	0.184 ± 0.021	−2.63 ± 0.10	0.825	< 0.001
	0.1	1.06 ± 0.13	0.197 ± 0.029	−2.51 ± 0.13	0.680	< 0.001
	0.3	3.97 ± 0.33	0.307 ± 0.034	−3.29 ± 0.15	0.820	< 0.001
k_b	0.0	0.09 ± 0.08	−0.014 ± 0.023	−0.46 ± 0.11	0.171	NS
	0.1	0.52 ± 0.12	0.001 ± 0.032	−0.81 ± 0.13	0.516	< 0.001
	0.3	0.80 ± 0.55	−0.081 ± 0.063	−0.51 ± 0.28	0.435	< 0.01

The leakage of CF entrapped was induced by 0 to 1.12 mM verapamil (or 0 to 0.15 mol verapamil in the membrane/mol lipid) through LUV membranes. The kinetics of dye leakage induced by verapamil at 37 °C (pH 7.4) were recorded during 15 min and the total amount of fluorescence associated with the entrapped dye in each sample, i.e., 100% total dye leaked, was determined by dispersing the liposomes with 10% Triton X-100. Prior to the experiments, independent calibrations were performed to 100% relative fluorescence at each ionic strength investigated by adding 10% Triton X-100 in a liposome sample, and to 0% relative fluorescence on bi-distilled water (auto-blank). The apparent rate constants (in min^{-1}) were determined according to Eq. (3). Means ± S.E. were determined by the simultaneous fitting of the data obtained from two LUV preparations. The determination coefficient (r^2) and p-level (P) of the regressions are also given.

4. Discussion

The Ca^{2+} blocker verapamil was the first compound found to reverse MDR in tumour cells [8]. The relevant aspects of its chemical structure are two planar aromatic rings and a basic nitrogen atom. In the present study, the ability of verapamil to induce the CF leakage from liposomes was quantified at various I and x_{EPA} levels. The kinetic approach used here to study the leakage process brought to light several aspects of the interactions between verapamil and the membranes.

At any given I and x_{EPA} investigated in the present study, verapamil induced 50% dye leakage within 15 min in the 80–820 μM range (Fig. 2). Verapamil has been previously found to be less efficient on PC/PS/PE (3:4:3) liposomes at $I = 0.170$ M [15] and on soybean lipids/PS/cholesterol (~ 5.7:1.4:2.9) liposomes at $I = 0.017$ M [16]. Bramhall et al. [25] have suggested, however, that the rate of CF leakage may depend on the nature of the base used to adjust the intravesicular pH. This may well explain why verapamil was found here to be more efficient than previous works reported it to be [15,16].

The results of the present study show the existence of a highly significant bilinear dependence of the efficiency (log(1/PD_{50})) of verapamil on the increase in x_{EPA} and $1/\sqrt{I}$. Ionization of the phospholipid head groups at the membrane interface generates an electrostatic surface potential Ψ, which in turn brings about a redistribution of the protons, cations and anions in the vicinity of the interface [26]. In the present study, increasing x_{EPA} favoured electrostatic interactions between the cationic verapamil and the anionic phosphate groups, both directly via the increase in the negative surface charge density (σ) and indirectly via the decrease in the interfacial pH and the increase in the interfacial pK of the drug. This indirect effect increased further the electric charge of verapamil. All in all, the dependence of the CF leakage observed here on x_{EPA} is in agreement with the data published by Majee and Chakabarti [27]. Our results are also in line with the findings obtained by Krämer et al. [28] showing that the liposome/buffer distribution of cationic drugs increases with the mole fraction of anionic lipids. As the ionic strength was increased, small ions in the external solution screened the electric charge of verapamil and that of the ionized groups of the membrane [29]. The electrostatic interactions therefore decreased due to the decrease in both the apparent electric charge of the drug and the surface potential (Ψ) of the membrane. According to the linearised Gouy–Chapman theory, the surface potential (Ψ) is proportional to the surface charge density (σ) and to $1/\sqrt{I}$ [26,29,30]. Consequently, the ability of verapamil (log(1/PD_{50})) to induce dye leakage through membranes was favoured by the enhanced electrostatic interactions occurring at high x_{EPA} and $1/\sqrt{I}$ levels, and reduced by the decrease in these interactions occurring at low x_{EPA} and $1/\sqrt{I}$ levels.

The kinetics of the CF leakage process induced here by verapamil can be fairly described by a two-step process. The rate constant of the slow kinetic step (k_a) was eight to nine times smaller than that of the fast kinetic step (k_b). Since no kinetic model describing this leakage seems to be available in the literature, the rate constants k_a and k_b determined here were apparent ones. Multiple correlation analysis showed that both $\log k_a$ and $\log k_b$ depended on C, whereas only $\log k_a$ depended on $1/\sqrt{I}$. Since k_a depended on $1/\sqrt{I}$ whereas k_b did not, it is possible that k_a might be ascribed to the first kinetic step in the leakage process (k_a apparent = k_1 apparent), whereas k_b might be ascribed to the second kinetic step (k_b apparent = k_2 apparent). However, further studies are required to confirm this statement. The slow kinetic step (k_a) driven by electrostatic interactions varied with C and the $\mathrm{d}\log k_a/\mathrm{d}C$ coefficient value was nearly four times higher at $x_{EPA}=0.3$ than at $x_{EPA}=0$ and 0.1 (Table 2). This effect might be due to the ability of verapamil to accumulate in the regions of the membrane where EPA molecules predominate. At $x_{EPA}=0.3$, it may then have reached the critical concentration on the membrane which is required to induce dye leakage in a co-operative manner at rather low concentrations. When x_{EPA} was low, verapamil might have dispersed more widely over the membrane and reached this critical concentration on the lipid at much higher concentrations [31]. The fast kinetic step (k_b) driven by hydrophobic interactions was also found to vary with C, depending on x_{EPA}. In fact, the $\mathrm{d}\log k_b/\mathrm{d}C$ coefficient value was nine times higher at $x_{EPA}=0.3$ than at $x_{EPA}=0$ (Table 2). This result suggests that at high EPA levels, the strong electrostatic repulsions occurring between the anionic phospholipid head groups at the membrane/buffer interface may have induced bilayer destabilization more deeply into the core of the membrane. Therefore, the efficiency of verapamil to increase further this destabilization favouring the occurrence of dye leakage was higher at $x_{EPA}=0.3$ than at $x_{EPA}=0$ and 0.1.

To sum up, the overall outcome of the molecular events described above seems to be that the ability of verapamil ($\log(1/PD_{50})$) to induce dye leakage was favoured by the enhanced electrostatic interactions occurring at high x_{EPA} and $1/\sqrt{I}$ levels, and reduced by the decrease in these interactions occurring at low x_{EPA} and $1/\sqrt{I}$ levels. Concomitantly, the efficiency of verapamil was increased by the enhanced hydrophobic interactions occurring at high x_{EPA} levels, and decreased by the weakening of these interactions occurring at low x_{EPA} levels. The results of the present study point to the importance of parameters such as the ionic strength and the membrane surface potential, which should be taken into account by authors using the method based on CF leakage to quantify the efficiency of drugs.

It was concluded from the results of this study that when the negative surface potential of the membranes is high, as occurs in tumour cells [17–19], the MDR reversal induced by verapamil might be enhanced by favourable drug–membrane interactions in patients with severe hypo-electrolytic (Na^+ and K^+) disorders, whereas the MDR reversal might be reduced by unfavourable drug–membrane interactions in patients with severe hyper-electrolytic (Ca^{2+}, Na^+ and K^+) disorders.

Acknowledgements

We are grateful to Professors M. Santelli and J. Barbe for encouraging the progress of this work. We also thank Dr. C. Santelli-Rouvier and Dr. P. Loiseau for their help during the course of this work. This work is dedicated to V. Meltzeim and G. Bosmans (UVK-LAB, previously Bio-Tek Kontron Instruments), and was supported by a grant from the Conseil Général des Bouches-du-Rhône, France.

References

[1] E. Scheiner, M. Isaacs, P. Vanamee, Med. Clin. North Am. 50 (1966) 711–732.
[2] A. Kessinger, H.M. Lemon, J.F. Foley, Geriatrics 27 (1972) 97–106.
[3] J. Takacs, Med. Clin. North Am. 59 (1974) 449–457.
[4] B.J. Trump, I.K. Berezesky, Carcinogenesis 8 (1987) 1027–1031.
[5] S.M. Mahon, D.S. Caperson, Oncol. Nurs. Forum 20 (1993) 937–946.
[6] M. Fichman, J. Bethune, Ann. N.Y. Acad. Sci. 230 (1974) 448–472.
[7] F.J. Sharom, J. Membr. Biol. 160 (1997) 161–175.
[8] T. Tsuruo, H. Iida, S. Tsukagoshi, Y. Sakurai, Cancer Res. 41 (1981) 1967–1972.
[9] Y. Raviv, H.B. Pollard, E.P. Bruggemann, I. Pastan, M.M. Gottesman, J. Biol. Chem. 265 (1990) 3975–3980.
[10] L. Homolya, Z. Holló, U.A. Germann, I. Pastan, M.M. Gottesman, B. Sarkadi, J. Biol. Chem. 268 (1993) 21493–21496.
[11] J.K. Seydel, E.A. Coats, H.P. Cordes, M. Wiese, Arch. Pharm. (Weinh.) 327 (1994) 601–610.
[12] G.D. Eytan, R. Regev, G. Oren, Y.G. Assaraf, J. Biol. Chem. 271 (1996) 12897–12902.
[13] J.M. Zamora, H.L. Pearce, W.T. Beck, Mol. Pharmacol. 33 (1988) 454–462.
[14] I.K. Pajeva, M. Wiese, H.P. Cordes, J.K.J. Seydel, Cancer Res. Clin. Oncol. 122 (1996) 27–40.
[15] R. Callaghan, J.R. Riordan, Biochim. Biophys. Acta 1236 (1995) 155–162.
[16] S. Drori, G.D. Eytan, Y.G. Assaraf, Eur. J. Biochem. 228 (1995) 1020–1029.
[17] W.J. Van-Blitterswijk, G. De-Veer, J.H. Krol, P. Emmelot, Biochim. Biophys. Acta 688 (1982) 495–504.
[18] A.J. Moore, W.D. Beazley, M.C. Bibby, D.A. Devine, J. Antimicrob. Chemother. 37 (1996) 1077–1089.
[19] H.M. Chen, W. Wang, D. Smith, S.C. Chan, Biochim. Biophys. Acta 1336 (1997) 171–179.
[20] M. Castaing, P. Brouant, A. Loiseau, C. Santelli-Rouvier, M. Santelli, S. Alibert-Franco, M. Abdallah, J. Barbe, J. Pharm. Pharmacol. 52 (2000) 289–296.
[21] J.N. Weinstein, S. Yoshikami, P. Henkart, R. Blumenthal, W.A. Hagins, Science 195 (1977) 489–491.
[22] D.D. Perrin, B. Dempsey, in: Chapman & Hall (Eds.), Buffers for pH and Metal Ion Control, Wiley, New York, 1974, pp. 128–163.

[23] A.V. Wolf, M.G. Brown, P.G. Prentiss, in: R.C. Weast (Ed.), Handbook of Chemistry and Physics, 55th ed., CRC Press, Cleveland, 1974–1975, pp. D194–D236.
[24] M. Castaing, A. Loiseau, M. Dani, J. Pharm. Pharmacol. 53 (2001) 1021–1028.
[25] J. Bramhall, J. Hofmann, R. DeGuzman, S. Montestruque, R. Schell, Biochemistry 26 (1987) 6330–6340.
[26] J.-F. Tocanne, J. Tessié, Biochim. Biophys. Acta 1031 (1990) 111–142.
[27] S. Majee, A. Chakrabarti, Biochem. Pharmacol. 57 (1999) 981–987.
[28] S.D. Krämer, A. Braun, C. Jakits-Deiser, H. Wunderli-Allenspach, Pharm. Res. 15 (1998) 739–744.
[29] C. Tanford, Physical Chemistry of Macromolecules, Wiley, New York, 1967, pp. 457–525.
[30] S. Richter, J. Hamann, D. Kummerow, I. Bernhardt, Biophys. J. 73 (1997) 733–745.
[31] W. Wang, D.K. Smith, K. Moulding, H.M. Chen, J. Biol. Chem. 273 (1998) 27438–27448.