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Partitioning of (±)-5,6-dihydro-6-phenyl-2-*n*-alkyl-imidazo-[2,1-*b*]thiazoles into large unilamellar liposomes: a steady-state fluorescence quenching study

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The interaction of the tetramisole derivative (±)-5,6-dihydro-6-phenyl-imidazo[2,1-*b*]thiazole and a number of its 2-*n*-alkyl homologues (-ethyl through -*n*-pentyl and -*n*-heptyl) with large unilamellar phosphatidylcholine/phosphatidylethanolamine/dipalmitoylphosphatidic acid (2:1:0.06, w/w) vesicles was studied by means of steady-state fluorescence quenching using 8-(2-anthryl)octanoic acid as membrane probe. Linear Stern-Volmer plots were obtained for each derivative, indicating dynamic quenching. The slopes of the plots decreased with increasing liposomal concentration. For four short-chain homologues (-H, -ethyl, -*n*-propyl and -*n*-butyl), the respective membrane partition coefficients K_p and bimolecular quenching rate constants k_q were determined from the plots of the reciprocal of the apparent quenching rate constant $(k_0^{app})^{-1}$ against the lipid volume fraction c_L of the liposomes. The partition coefficients increased with increasing chain-length of the tetramisoles. A linear relationship was found between the free energy of partitioning and the number of methylene units of the homologues ($-\Delta G^\circ$ per methylene group = 1.6 ± 0.1 kJ mol^{-1}). For the *n*-pentyl and *n*-heptyl derivatives, the fluorescence quenching technique did not allow one to determine their membrane partition coefficients. Analysis of the fluorescence intensity measurements with Scatchard plots gave further evidence for the partitioning nature of the tetramisole derivatives' association with the liposomal membranes.

Abbreviations: H-TETR, (±)-5,6-Dihydro-6-phenyl-imidazo[2,1-*b*]thiazole hydrochloride; C2-TETR, (±)-5,6-dihydro-6-phenyl-2-ethyl-imidazo[2,1-*b*]thiazole oxalate; C3-TETR, (±)-5,6-dihydro-6-phenyl-2-*n*-propyl-imidazo[2,1-*b*]thiazole cyclohexyl-aminosulphonic acid; C4-TETR, (±)-5,6-dihydro-6-phenyl-2-*n*-butyl-imidazo[2,1-*b*]thiazole hydrochloride; C5-TETR, (±)-5,6-dihydro-6-phenyl-2-*n*-pentyl-imidazo[2,1-*b*]thiazole oxalate; C7-TETR, (±)-5,6-dihydro-6-phenyl-2-*n*-heptyl-imidazo[2,1-*b*]thiazole oxalate; C10-TETR, (±)-5,6-dihydro-6-phenyl-2-*n*-decyl-imidazo[2,1-*b*]thiazole hydrochloride; C13-TETR, (±)-5,6-dihydro-6-phenyl-2-*n*-tridecyl-imidazo[2,1-*b*]thiazole hydrochloride; PC, egg L-α-phosphatidylcholine; PE, egg phosphatidylethanolamine; DPPA, dipalmitoylphosphatidic acid; DCM, 4-dicyanomethylene-2-methyl-6-*p*-dimethylaminostyril-4H-pyran; dimethylPOPOP, 2,2′-*p*-phenylenebis(4-methyl-5-phenyloxazole).

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Introduction

The distribution of amphipathic or lipophilic molecules between an aqueous phase and the lipid region of biological membranes or artificial bilayer systems has been the subject of many reports in the literature. Membrane associations of lipid-soluble compounds have been considered as a partition process [1–8] or as a binding equilibrium [9–12]. The interaction of homologous series of compounds with phospholipid bilayers is of special interest. In several studies, the lipophilic character of the homologues, defined by their organic solvent/water or membrane partition coefficient, has been correlated to the biological activity of the homologues [8,13–15].

Fluorescence quenching is a powerful technique to examine quantitatively the distribution of molecules

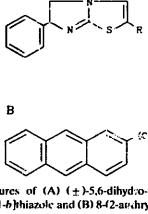

Fig. 1. Structures of (A) (±)-5,6-dihydro-6-phenyl-2-*n*-alkyl-imidazo[2,1-*b*]thiazole and (B) 8-(2-anthryl)octanoic acid.

between an aqueous phase and a lipid bilayer. The method has been used to study the membrane interaction of several compounds including organochlorine insecticides [16–19], local anaesthetics [20] dimethylaniline [21] and copper(II) [22].

The current paper reports on the membrane association of an homologous series of tetramisole derivatives, namely (±)-5,6-dihydro-6-phenyl-2-*n*-alkyl-imidazo[2,1-*b*]thiazoles (Fig. 1), studied with steady-state fluorescence quenching of 8-(2-anthryl)octanoic acid (Fig. 1) in large unilamellar liposomes. Tetramisole ((±)2,3,5,6-tetrahydro-6-phenyl-imidazo[2,1-*b*]-thiazole), and in particular its laevorotatory enantiomer, levamisole, has potent broad-spectrum anthelmintic properties [23,24]. Besides, levamisole has been found to be an immunopotentiating agent [25] and an inhibitor of alkaline phosphatase [26,27].

The quenching data were analysed in two different ways. In the first method, apparent quenching rate constants k_q^{app} were determined from the Stern-Volmer plots. By plotting $(k_q^{app})^{-1}$ versus the lipid volume fraction α_L, the membrane partition coefficients K_p and bimolecular quenching rate constants k_q, for several of the homologues, were calculated. In the second method, the quenching data were analysed with Scatchard plots. The results indicated that the interaction of the tetramisole derivatives with liposomal membranes can be considered as a pure partitioning process.

Materials and Methods

Reagents

(±)-5,6-Dihydro-6-phenyl-imidazo[2,1-*b*]thiazole hydrochloride, (±)-5,6-dihydro-6-phenyl-2-ethyl-imidazo[2,1-*b*]thiazole oxalate, (±)-5,6-dihydro-6-phenyl-2-*n*-propyl-imidazo[2,1-*b*]thiazole cyclohexylaminosulphonic acid, (±)-5,6-dihydro-6-phenyl-2-*n*-butyl-imidazo[2,1-*b*]thiazole hydrochloride, (±)-5,6-dihydro-6-phenyl-2-*n*-pentyl-imidazo[2,1-*b*]thiazole oxalate, (±)-5,6-dihydro-6-phenyl-2-*n*-heptyi-imidazo[2,1-*b*]thiazole oxalate, (±)-5,6-dihydro-6-phenyl-2-*n*-decyl-imidazo[2,1-*b*]thiazole hydrochloride and (±)-5,6-dihydro-6-phenyl-2-*n*-tridecyl-imidazo[2,1-*b*]thiazole hydrochloride were kindly provided by Janssen Pharmaceutica (Beerse, Belgium). Egg L-α-phosphatidylcholine and egg phosphatidylethanolamine were purchased from Avanti Polar Lipids (Birmingham, AL, USA). Dipalmitoylphosphatidic acid was from Sigma Chemical Co. (St. Louis, MO, USA). The phospholipids gave a single spot on thin-layer chromatography (solvent, $CHCl_3/CH_3OH/H_2O$ (65:25:4, v/v); I_2 staining) and were used without further purification. *n*-Octyl-β-D-glucopyranoside was purchased from Boehringer GmbH (Mannheim, FRG). 8-(2-Anthryl)octanoic acid was synthetised according to the method of Kaplun et al. [28]. Glycine, gold label (Aldrich Chemie, Brussels, Belgium), and chloroform and methanol, both of fluorimetric grade (Merck, Darmstadt, FRG) were used as received.

Preparation of liposomes

Liposomes were prepared according to a detergent dialysis method [29,30]. A chloroform solution of 38.25 mg of phospholipid (PC/PE/DPPA (2:1:0.06, w/w)) was evaporated as a film in a small conical vessel under argon and stored *in vacuo* for 1 h to eliminate residual solvent. 75 mg of *n*-octyl-β-D-glucopyranoside in 6 ml of 0.1 M glycine buffer (pH 9.5), containing 0.2 mM EDTA (referred to as 'standard buffer'), was added and the mixture was gently stirred until the lipid was completely dissolved. Mixed micelle solutions were dialysed for 16 h at room temperature against 3 litres of the standard buffer using a Lipoprep-apparatus (Diachema, Langnau a.A./Zürich, Switzerland). After dilution of the liposomal suspension to the desired lipid concentrations, the fluorescent probe was added in a small volume (< 10 μl) of methanol to each diluted vesicle suspension so that the probe to lipid molar ratio varied between 1:100 and 1:800. Uptake of the probe was effected by vortex mixing for 15 s.

Stock solutions of the tetramisole derivatives (500 mM for H-TETR, C2-TETR and C3-TETR; 250 mM for C4-TETR and C5-TETR and 100 mM for C7-TETR) were made in 50% aqueous methanol (by volume). Aliquots (total volume < 20 μl) of the quenchers were added to the liposomes using Agla micrometer syringes (Wellcome Research Laboratories, Beckenham, UK). The total concentration of methanol in the vesicle dilutions, after addition of probe and increasing amounts of quencher, did not exceed 1% (by volume). At this small concentration, methanol did not effect the fluorescence spectrum of 8-(2-anthryl)octanoic acid.

Phospholipid concentrations were determined by quantitation of inorganic phosphate [31]. For the density of the vesicles, a value of 1 g/ml was accepted [32]. The average relative molecular mass of the phospho-

lipids was taken as 770. Assuming that the vesicles prepared by *n*-octyl-β-D-glucopyranoside dialysis had a mean diameter of 176 nm [30], a bilayer thickness of 4 nm [33], and that the total bilayer volume of the liposomes was available for partition, it was calculated that there are $2.9 \cdot 10^5$ phospholipid molecules per vesicle and that a vesicle suspension of 1 mg/ml correspond to a $4.5 \cdot 10^{-6}$ millimolar concentration of vesicles.

The pK_a values of the tetramisole derivatives in 50% metnanol were 8.2, 8.5, 8.4, 8.3, 8.3 and 8.3 for H-TETR, C2-TETR, C3-TETR, C4-TETR, C5-TETR, C7-TETR, respectively, and in water, the pK_a values of H-TETR and C2-TETR amounted to 8.7 and 9.0, respectively (communicated by Janssen Pharmaceutica). Based on a difference of 0.5 between the pK_a value in water and the pK_a value in 50% aqueous methanol for H-TETR and C2-TETR, the pK_a values in water of the 2-*n*-propyl, 2-*n*-butyl, 2-*n*-pentyl and 2-*n*-heptyl homologues were assumed to be 8.9, 8.8, 8.8 and 8.8, respectiveiy.

Fluorescence experiments

Fluorescence spectra of 8-(2-anthryl)octanoic acid labelled vesicles were recorded with a Spex Fluorolog 212/Datamate at an excitation wavelength of 360 nm. All fluorescence measurements were carried out with 2 ml of liposomal solution in standard buffer, contained in 1 cm × 1 cm quartz cuvettes, at 30°C. The probe's fluorescence lifetime was determined by time-correlated single photon counting [34,35] using a Spectra-Physics mode-locked, cavity-dumped, synchronously pumped, frequency-doubled DCM dye laser. Excitation was at 330 nm. A detailed description of the instrumentation has been given elsewhere [35,36]. Correction for the wavelength dependence of the instrument response function was performed using the delta function convolution method [37,38]. DimethylPOPOP was used as reference compound. The graphical methods for residual analysis were plots of weighted residuals, the autocorrelation function and the normal probability plot. Numerical criteria for the goodness-of-fit included the reduced chi-square χ^2 and its standard normal deviate Z_{χ^2}, the ordinary runs test statistic Z and the Durbin-Watson test statistic d [36,39].

Theory

Dynamic fluorescence quenching in an homogeneous solution can be described by the Stern-Volmer equation:

$$I_0/I = 1 + k_q \tau_0 [Q]_T \quad (1)$$

where I_0 and I are the fluorescence intensities in the absence and presence of quencher, respectively. $[Q]_T$ is the total quencher concentration. τ_0 represents the fluorescence lifetime of the probe in the absence of quencher and k_q is the bimolecular rate constant of quenching.

If the fluorophore is solubilized in a lipid bilayer, the fluorescence quenching is dependent on the concentration of molecules present in the lipid phase, $[Q]_L$. Thus the Stern-Volmer equation becomes:

$$I_0/I = 1 + k_q \tau_0 [Q]_L \quad (2)$$

$[Q]_L$ is given by:

$$[Q]_L = K_p [Q]_A \quad (3)$$

where $[Q]_A$ refers to the concentration of quencher molecules in the aqueous phase and K_p is the membrane partition coefficient.

For an ionizable quencher Q undergoing an acid-base equilibrium in the aqueous phase, $QH_A^+ \rightleftharpoons Q_A + H_A^+$, the aqueous concentration of neutral quencher, $[Q]_A$, is a function of its dissociation constant K_a and of the pH of the aqueous phase:

$$[Q]_A = [QH^+]_A 10^{(pH - pK_a)} \quad (4)$$

where $[QH^+]_A$ is the concentration of ionized quencher in the aqueous phase. The total mass of quencher can be expressed as:

$$[Q]_T V_T = ([QH^+]_A + [Q]_A) V_A + [Q]_L V_L \quad (5)$$

where V_A and V_L denote the volume of the aqueous and the lipid phase, respectively. If only the neutral form of the quencher is capable of partitioning into the lipid phase, substitution of Eqns. 3–5 into Eqn. 2 gives the Stern-Volmer relationship, written as a function of the total quencher concentration, $[Q]_T$

$$I_0/I = 1 + k_q^{app} \tau_0 [Q]_T \quad (6)$$

with k_q^{app} given by

$$1/k_q^{app} = \left[1/k_q - \frac{10^{(pK_a - pH)} + 1}{k_q K_p}\right] \alpha_L + \frac{10^{(pK_a - pH)} + 1}{k_q K_p} \quad (7)$$

where α_L ($= V_L/V_T$) is the lipid volume fraction. Thus, the apparent quenching constant k_q^{app} is dependent on the lipid volume fraction α_L of the liposomes. k_q^{app} values are obtained as the slopes of the Stern-Volmer plots, divided by τ_0. By plotting $1/k_q^{app}$ versus α_L, the partition coefficient K_p and the bimolecular quenching rate constant k_q can be calculated from Eqn. 7.

Partition and binding

An alternative model can be applied to distinguish between binding and/or partition of a quencher [40,41].

$$Q_a + v \underset{}{\overset{K_{eq}}{\rightleftharpoons}} Q_l$$

$$K_a \Big\| H^+$$

$$QH_a^+$$

Scheme I.

The method assumes that I_0/I at a particular quencher concentration is only dependent on the average number of quenchers per vesicle, $\langle Q \rangle$, regardless of the quenching mechanism. Scheme I describes the uptake of a quencher into a lipid bilayer: Q_a and QH_a^+ refer to the neutral and charged form of the quencher in the aqueous phase, respectively, Q_l represents neutral quencher in the lipid phase, v denotes vesicle and K_{eq} is an equilibrium distribution constant defined as

$$K_{eq} = \frac{[Q_l]}{[Q_a][v]} \tag{8}$$

$[Q_l]$, $[Q_a]$ and $[v]$ are concentrations related to the total volume V_T. The dissociation constant K_a is defined with respect to the aqueous volume V_A. Assuming that $V_A \approx V_T$, one obtains

$$[QH_a^+] = [Q_a]10^{(pK_a - pH)} \tag{9}$$

The average number of quencher molecules per vesicle, $\langle Q \rangle$, is given by

$$\langle Q \rangle = [Q_l]/[v] \tag{10}$$

The total concentration of quencher $[Q]_T$ is expressed as

$$[Q]_T = [Q_a] + [QH_a^+] + [Q_l] \tag{11}$$

Combining Eqns. 8–11 gives

$$[Q]_T = \langle Q \rangle (1 + 10^{(pK_a - pH)})/K_{eq} + \langle Q \rangle [v] \tag{12}$$

When both binding and partition of neutral quencher occur, the equilibrium distribution constant is given by [41]

$$K'_{eq} = \frac{\langle Q \rangle}{[Q_a]} = \bar{V}_L K_p + \frac{nK_b}{1 + K_b[Q_a]} \tag{13}$$

where K_b is the binding constant, $\bar{V}_L$ is the molar volume of the liposomes and n is the number of equivalent binding sites. For an ionizable quencher, the binding of both the charged and the neutral form should be considered.

Application of the analysis involves obtaining Stern-Volmer plots at several lipid concentrations. At each particular level of I_0/I, a plot of $[Q]_T$ versus $[v]$ yields one pair of values for K_{eq} and $\langle Q \rangle$. Secondary plots of K_{eq} against $\langle Q \rangle$ which are, in fact, Scatchard plots, allows one to distinguish between binding and/or partition. Independence of K_{eq} on $\langle Q \rangle$ indicates that the quencher partitions into the vesicles while a linear relationship with a negative slope indicates binding. When binding and partition occur simultaneously, the Scatchard plots show a decreasing dependence of K_{eq} with increasing $\langle Q \rangle$ asymptotically approaching to a constant value of $\bar{V}_L K_p$.

Results

Characterisation of the 8-(2-anthryl)octanoic acid labelled vesicles

Large unilamellar liposomes were obtained, using the *n*-octyl-β-D-glucopyranoside dialysis method. Their diameter, estimated from electron micrographs of negatively stained liposomes, varied between 150 and 250 nm (results not shown), which is in accordance with values published elsewhere [30,42].

The fluorescence decay of 8-(2-anthryl)octanoic acid in the PC/PE/DPPA (2:1:0.06, w/w) liposomes was single-exponential with a lifetime $\tau_0 = 3.50 \pm 0.03$ ns (Fig. 2). τ_0 was independent of the probe/lipid molar ratio, indicating that no excimers were formed. Measurements of the fluorescence decay in the presence of C3-TETR showed that the quencher did not effect the single exponential decay mode of the fluorophore although its lifetime decreased with increasing quencher concentrations (Vermeir, M. and Boens, N., unpublished data). Since only one lifetime contributed to the fluorophore's decay, this indicated that the probe was entirely taken up into the liposomal membrane.

Partition coefficients

Fluorescence intensities of 8-(2-anthryl)octanoic acid incorporated into PC/PE/DPPA (2:1:0.06, w/w) liposomes in the presence of H-TETR or one of its 2-*n*-alkyl homologues (C2-TETR, C3-TETR, C4-TETR, C5-TETR or C7-TETR) were measured at several liposomal lipid volume fractions. The fluorescence quenching titrations were carried out at pH 9.5 to ensure that most of the quencher molecules were in the neutral form which enables sufficient penetration of the quenchers in the membrane. Fig. 3 depicts the quenching of the fluorescence intensity of 8-(2-anthryl)octanoic acid in liposomes by C4-TETR. None of the quenchers induced the formation of an exciplex band in the fluorescence spectrum. Linear Stern-Volmer plots were obtained for each homologue which indicated that the quenching resulted from diffusion collisions and not from complex formation. Figs. 4A, B and C show the Stern-Volmer plots for the quenching experiments with C2-TETR, C4-TETR and C7-TETR, respectively. The apparent quenching rate constants

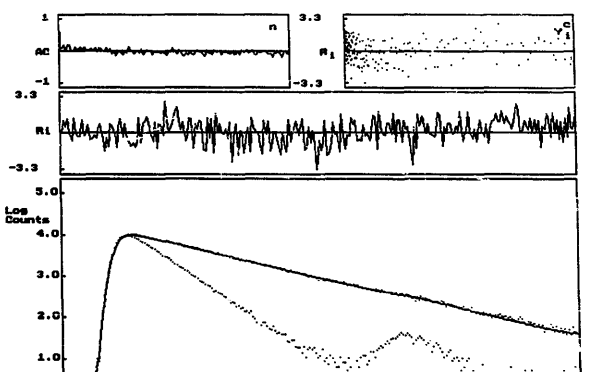

Fig. 2. Fluorescence decay of 8-(2-anthryl)octanoic acid in liposomes of PC/PE/DPPA (2:1:0.06, w/w) (λ_{exc} = 330 nm; λ_{em} = 405 nm; channel width 0.098 ns). The experimental decay (point plot) was fitted to a single-exponential decay function (solid line). Estimated decay parameters were: α = 0.77, τ_1 = 3.5 ns and τ_2 = 1.25 ns. The fluorescence decay of the reference compound (dimethylPOPOP in isooctane) is shown as a point plot. Plots of the autocorrelation function, AC, and of the weighted residuals, R_i, versus channel number i and versus calculated values y_i^c are depicted at the top. Test statistics: χ^2 = 1.19, Z_{χ^2} = 2.03, ordinary runs test statistic Z = 0.31 and Durbin-Watson test statistic α = 1.90.

k_q^{app}, obtained from the slopes of the Stern-Volmer plots, decreased with increasing lipid volume fraction of the liposomes (Table I). The quenching efficiency increased with increasing hydrophobicity of the homologues. For each tetramisole derivative, a linear relationship was found when the reciprocal of the apparent

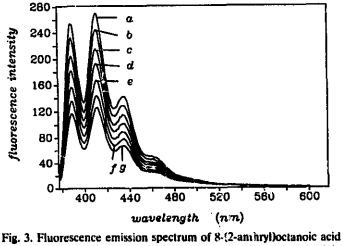

Fig. 3. Fluorescence emission spectrum of 8-(2-anthryl)octanoic acid incorporated into liposomes of PC/PE/DPPA (2:1:0.06, w/w) in the presence of (a) 0 mM, (b) 0.075 mM, (c) 0.150 mM, (d) 0.225 mM, (e) 0.300mM, (f) 0.375 mM and (g) 0.450 mM (±)-5,6-dihydro-6-phenyl-2-*n*-butyl-imidazo[2,1-*b*]thiazole. The vesicles had a phospholipid concentration of 2.32 mg/ml suspension and a lipid to probe molar ratio of 400. Excitation was at 360 nm. The measurements were performed in 0.1 M glycine buffer pH 9.5, containing 0.2 mM EDTA.

TABLE I

Values of the apparent quenching rate constants k_q^{app} ($\times 10^{-10}$ $M^{-1}s^{-1}$) at various lipid volume fractions α_L ($\times 10^{-2}$), obtained from the steady-state quenching of liposomal incorporated 8-(2-anthryl)octanoic acid by (±)-5,6-dihydro-6-phenyl-2-n-alkyl-imidazo[2,1-b]thiazoles

H-TETR		C2-TETR		C3-TETR	
α_L	k_q^{app}	α_L	k_q^{app}	α_L	k_q^{app}
		0.51	7.91	0.57	18.22
1.15	1.34	1.02	7.31	1.13	16.90
1.73	1.30	1.54	6.71	1.70	15.77
2.30	1.27	2.05	6.28	2.26	14.04
2.88	1.23	3.07	5.89	3.40	11.64
		4.10	5.23	4.53	9.48

C4-TETR		C5-TETR		C7-TETR	
α_L	k_q^{app}	α_L	k_q^{app}	α_L	k_q^{app}
0.58	113.21	0.49	93.93	0.44	166.06
1.16	84.22	0.97	51.91	0.88	82.75
1.74	73.28	1.46	45.04	1.32	56.36
2.32	64.57	1.95	30.22	1.76	41.07
3.48	47.54	2.92	17.47	2.64	24.56
4.64	37.80	3.89	12.99	3.52	16.96

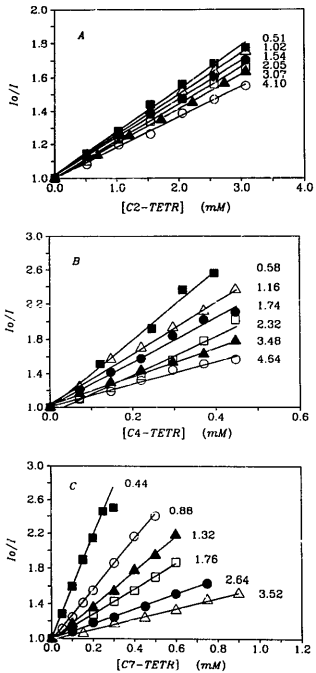

Fig. 4. Stern-Volmer plots of fluorescence intensity quenching of 8-(2-anthryl)octanoic acid in liposomes of PC/PE/DPPA (2:1:0.06, w/w) by (A) C2-TETR, (B) C4-TETR and (C) C7-TETR at different lipid volume fractions. The numbers denote the phospholipid concentration of the vesicles in mg/ml.

quenching rate constant $(k_q^{app})^{-1}$ was plotted against the lipid volume fraction α_L of the liposomes (Fig. 5), indicating that for each compound, a true partition process occurred. On the assumption that only neutral quencher molecules partition into the lipid membrane, the membrane partition coefficients K_p of H-TETR, C2-TETR, C3-TETR and C4-TETR, and the respective bimolecular quenching rate constants k_q for the 8-(2-anthryl)octanoic acid quenching by H-TETR, C2-TETR, C3-TETR, C4-TETR, C5-TETR and C7-TETR

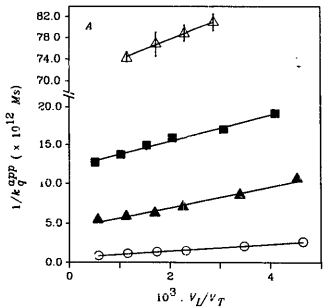

Fig. 5. Dependence of the reciprocal of the apparent bimolecular quenching rate constant $(k_q^{app})^{-1}$ on the lipid volume fraction α_L of the vesicles for the 8-(2-anthryl)octanoic acid quenching by the tetramisole derivatives. The symbols refer to: H-TETR (△); C2-TETR (■); C3-TETR (▲) and C4-TETR (○). The error bars represent ±one standard deviation.

TABLE II

Membrane-partition coefficients K_p ± standard deviation and bimolecular quenching rate constants k_q ± standard deviation of 2-n-alkyl substituted tetramisole derivatives in liposomes of PC/PE/DPPA (2:1:0.06, w/w) determined by fluorescence quenching of 8-(2-anthryl)octanoic acid

	K_p	k_q ($\times 10^{-8}$ $M^{-1}s^{-1}$)
H-TETR	63 ± 2	1.71 ± 0.09
C2-TETR	191 ± 15	5.7 ± 0.3
C3-TETR	365 ± 38	7.7 ± 0.5
C4-TETR	807 ± 78	23.4 ± 0.8
C5-TETR		5.0 ± 0.3
C7-TETR		5.8 ± 0.2

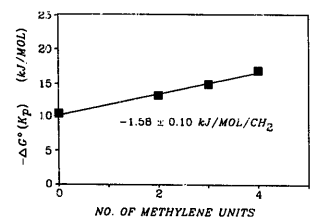

Fig. 6. Size dependence of the free energy of transfer $-\Delta G°$ between 0.1 M glycine buffer (pH 9.5), containing 0.2 mM EDTA and PC/PE/DPPA (2:1:0.06, w/w) liposomes for several 2-n-alkyl substituted (±)-5,6-dihydro-6-phenyl-imidazo[2,1-b]thiazoles.

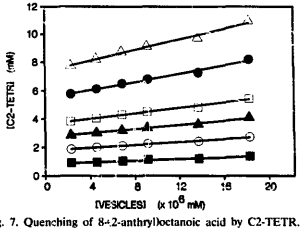

Fig. 7. Quenching of 8-(2-anthryl)octanoic acid by C2-TETR. The data were plotted according to Eqn. 12 at I_0/I values of 1.25 (■), 1.50 (○), 1.75 (▲), 2.00 (□), 2.50 (●) and 3.00 (△).

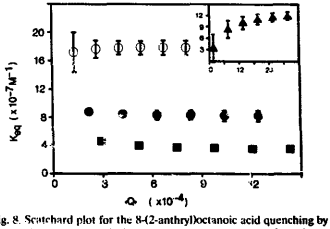

Fig. 8. Scatchard plot for the 8-(2-anthryl)octanoic acid quenching by 2-*n*-alkyl substituted (±)-5,6-dihydro-6-phenyl-imidazo[2,1-*b*]thiazoles in vesicles of PC/PE/DPPA (2:1:0.06, w/w). The symbols refer to: H-TETR (▲); C2-TETR (■); C3-TETR (●) and C4-TETR (○). The error bars represent ± one standard deviation.

were determined according to Eqn. 7 (Table II). Quenching measurements with C3-TETR at several pH values demonstrated that, indeed, only the neutral species of the quencher is taken up into the membrane (Vermeir, M. and Boens, N., unpublished data). The k_q values for H-TETR through C4-TETR increased with elongation of the alkyl side chain of the homologues. For C5-TETR and C7-TETR, smaller quenching rate constants were determined than for C4-TETR. The intercepts of the $(k_q^{app})^{-1}$ versus α_L plots on the $1/k_q^{app}$-axis decreased with increasing lipophilicity of the tetramisole derivatives (Fig. 5). Negative intercepts were obtained for C5-TETR (-0.2 ± 0.3 (S.D.)) and C7-TETR (-0.4 ± 0.2 (S.D.)). As the membrane partition coefficient of a quencher is obtained from the ordinate intercept (Eqn. 7), it was not possible to calculate the K_p value of the latter two compounds. The partition coefficients of the four shortest-chain homologues increased with increasing hydrophobicity of the quenchers (Table II). For these compounds, the free energy of partition between the aqueous phase and the liposomal membrane was determined according to $\Delta G^\circ = -RT \ln K_p$. The free energy of partition was linearly dependent on the number of methylene units in the alkyl chain of the tetramisole derivatives (Fig. 6). From the slope of the $-\Delta G^\circ(K_p)$ versus $(CH_2)_n$ plot, a value of -1.6 ± 0.1 kJ/mol/methylene group was calculated for the size dependence of the free energy of partition.

Quenching experiments were also performed with C10-TETR and C13-TETR in standard buffer. Upon addition of either one of both derivatives to a liposomal suspension, precipitation of quencher in the cuvette was observed. C10-TETR showed only a minor quenching efficiency, and for C13-TETR, quenching was completely absent. As a consequence, neither K_p values nor k_q values could be determined for the latter compounds.

Analysis of the quenching data with Scatchard plots

For each tetramisole derivative, the quencher concentrations $[Q]_T$ as a function of the vesicle concentration [v] at various levels of I_0/I, were determined from the Stern-Volmer plots. Fig. 7 show the plots of $[Q]_T$ *versus* [v] for C2-TETR. Negative ordinate intercepts were obtained for the plots of C5-TETR and C7-TETR. Hence, values of the average number of quenchers per

TABLE III

Average number of quencher molecules ⟨Q⟩ per vesicle ± standard deviation at different values of I_0/I for the quenching of 8-(2-anthryl)octanoic acid

I_0/I	⟨Q⟩ (×10⁻⁵)			
	H-TETR	C2-TETR	C3-TETR	C4-TETR
1.25	0.2 ± 0.2	0.29 ± 0.01	0.22 ± 0.01	0.06 ± 0.01
1.50	0.8 ± 0.2	0.52 ± 0.02	0.43 ± 0.02	0.13 ± 0.01
1.75	1.4 ± 0.2	0.75 ± 0.04	0.63 ± 0.04	0.20 ± 0.01
2.00	2.0 ± 0.3	0.98 ± 0.05	0.84 ± 0.05	0.27 ± 0.01
2.25	2.6 ± 0.3	–	–	–
2.50	3.2 ± 0.3	1.43 ± 0.08	1.25 ± 0.08	–
3.00	–	1.9 ± 0.1	1.7 ± 0.1	0.54 ± 0.01
4.00	–	–	–	0.81 ± 0.02

vesicle $\langle Q \rangle$ (Table III) and of the equilibrium distribution constant K_{eq} could only be determined for H-TETR, C2-TETR, C3-TETR and C4-TETR. For each of the four short-chain homologues, the Scatchard plots (K_{eq} versus $\langle Q \rangle$) were horizontal (Fig. 8), characteristic of a partition process. The equilibrium distribution constants K_{eq} increased with increasing hydrophobicity of the tetramisole compounds. The values of K_{eq} at a I_0/I level of 2.5 amounted to $1.20 \cdot 10^7$ M^{-1}, $3.45 \cdot 10^7$ M^{-1}, $8.24 \cdot 10^7$ M^{-1} and $17.78 \cdot 10^7$ M^{-1} for H-TETR, C2-TETR, C3-TETR and C4-TETR, respectively. A plot of the free energy of the quenchers' association with the vesicles, $\Delta G^\circ = -RT \ln K_{eq}$, versus the number of carbon atoms of the alkyl side chain of the quenchers resulted in a linear relationship with $r = 0.99$. From the slope of the $-\Delta G^\circ(K_{eq})$ vs. $(CH_2)_n$ plot, a value of -1.7 ± 0.1 kJ/mol per CH_2 was determined for the change of free energy per methylene unit for the quencher-vesicle association, which is similar to the value calculated for the size dependence of free energy of partition.

Discussion

Various techniques are being used in the study of solute–membrane interactions. To examine the membrane association of a series of (±)-5,6-dihydro-6-phenyl-2-*n*-alkyl-imidazo[2,1-*b*]thiazoles, we used fluorescence quenching because this technique offers several advantages over more commonly used methods like centrifugation or filtration. The technique is very sensitive and allows one to investigate if a quencher is actually taken up into the interior lipid region of a membrane. Moreover, fluorescence quenching is an equilibrium method that does not require the separation of membrane-associated and free solute.

8-(2-Anthryl)octanoic acid forms a valuable alternative membrane probe for the anthroyloxy fatty acids which have frequently been used in fluorescence quenching studies [20–22,43,44], and will align with the fatty acyl chains of the phospholipids allowing a regular packing in the bilayer. The fluorophore is highly lipophilic. From its single-exponential fluorescence decay, it appeared that the probe entirely incorporates into the lipid moiety of the membrane. Eximer formation was not observed at the probe to lipid molar ratios used. The fluorophore proved very useful in examining the interaction of the homologous series of tetramisole derivatives with the PC/PE/DPPA liposomes. Addition of quenchers induced a decrease of the probe's fluorescence intensity, indicating that the compounds locate in the hydrophobic core of the liposomal membranes. No formation of an exciplex-band could be observed in the spectra. Furthermore, linear Stern-Volmer plots were obtained, ruling out the partition of fluorophores between different membrane phases or the non-accessibility of the fluorophore molecules to the quenchers or a static quenching mechanism. Our data were consistent with a model that ionized quencher molecules are unable to partition into the lipid membrane and that the quenching of 8-(2-anthryl)octanoic acid by the tetramisole derivatives is a dynamic process which takes place via an intermediate excited-state non-emitting complex (Vermeir, M. and Boens, N., unpublished results).

The interaction of small amphipathic or lipophilic molecules with lipid membranes has been considered as a binding or a partition or a combination of both types of association. Treating the solute–membrane interaction as a partition process implies a constant ratio, at equilibrium, between the solute's concentration in the lipid and the aqueous phase. Such approach does not take into account the anisotropic structure of the lipid bilayer. Due to this non-uniform membrane architecture, solute molecules may locate at different depths within the bilayer [21,45,46]. Experimental membrane partition coefficients relate to the total volume of the membrane and thus may differ from local partition coefficients applicable to various subvolumes of the membrane.

At pH 9.5, DPPA bears two negative charges per molecule [47], so that the liposomes were negatively charged under the experimental conditions. Hence, there was a possibility of electrostatic interaction between cationic quenchers and the negatively charged vesicles. Nevertheless, for each quencher, linear Stern-Volmer plots were obtained and the reciprocal of the apparent quenching rate constants was linearly dependent on the lipid volume fraction of the liposomes, indicating that a pure partition occurred for each tetramisole derivative. The analysis of the fluorescence quenching data with Scatchard plots supported this conclusion: for each derivative, the equilibrium distribution constants were independent of the average number of quencher molecules per vesicle. Probably, no binding was observed because of the low charge density of the membranes and the relatively high ionic strength of the glycine buffer, reducing the negative surface potential of the liposomes.

The membrane partition coefficients of H-TETR, C2-TETR, C3-TETR and C4-TETR were determined with a good level of accuracy: their standard deviations were about 10% or less. $\text{Log}(K_p)$ increased linearly with the number of methylene units in the alkyl side chain of the tetramisole derivatives. A similar relationship has been observed for the membrane interaction of homologous series of *n*-alcohols and aromatic hydrocarbons [2,8,48,49]. The value of -1.6 ± 0.1 kJ/mol per methylene unit, determined for the size dependence of the free energy of partition is realistic. For comparison, values of -1.72 kJ/mol (-0.41 kcal/mol) and -1.13 kJ/mol (-0.27 kcal/mol) have been re-

ported for the change of the free energy per methylene unit for the membrane partitioning of *n*-alcohols in lecithin and of aromatic hydrocarbons in rat liver microsomes, respectively [49,50]. In accordance with the increase of the partition coefficients of the tetramisole derivatives, an increase of the equilibrium distribution constants K_{eq}, upon elongation of the alkyl side chain of the homologues, was observed. The influence of the alkyl chain-length of the tetramisoles on their K_p and K_{eq} values was identical: comparable values were found for the size dependence of partitioning and of the quenchers' association with the vesicles.

Although fluorescence quenching proved very convenient for the determination of membrane partition coefficients, application of the technique was restricted to the short-chain members of the homologous series of tetramisole derivatives. A serious drawback of the technique, especially for highly lipophilic molecules, is that the partition coefficient of a lipid-soluble compound is obtained from the ordinate intercept of the k_q^{app} versus α_L plot. Since K_p is inversely correlated to the ordinate intercept, high partition coefficients correspond to small intercepts. Experimental errors may cause the ordinate intercept to become negative, making the calculation of the partition coefficient impossible, as was the case for C5-TETR and C7-TETR. It is easy to realize that for highly lipophilic compounds, a minor change of the ordinate intercept may cause a substantial change of its K_p value. As a consequence, high standard deviations can be expected for such compounds. For example, standard errors up to 300% were reported for the membrane partition coefficient of lindane obtained from fluorescence quenching measurements using carbazole derivatives as membrane probes [18,19]. For each tetramisole derivative, the standard deviation of its quenching rate constant was less than 10%. The values of the quenching rate constants for the four shortest-chain homologues increased with the number of methylene units in the alkyl side chain (Table II). The highest k_q value was measured for C4-TETR, implying that the formation of the non-fluorescent quencher-probe complex is fastest for this compound. In analogy, an increase of quenching rate constants with increasing alkyl chain length has been reported for the quenching of 16-(9-anthryloxy)palmitic acid by a series of *n*-alkyl-*p*-aminobenzoate derivatives [20].

The decrease of the quenching capacity of the long-chain tetramisole derivatives C10-TETR and C13-TETR may be rationalized by their limited aqueous solubility. Indeed, upon addition of either one of both homologues to liposomal suspensions, quencher precipitation was clearly visible, reducing the concentration of quencher monomers in the aqueous phase and, as a consequence, also the number of quencher molecules that partition into the membrane. This could account for the decreased quenching of C10-TETR and the absence of quenching for C13-TETR.

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