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Carvedilol-liposome interaction: evidence for strong association with the hydrophobic region of the lipid bilayers

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Abstract

Carvedilol (Kredex, Coreg) is a multiple action antihypertensive drug that has been shown to protect cell membranes from lipid peroxidative damages. In this study the physical and structural effects of carvedilol on lipid bilayers are investigated by fluorescence techniques, differential scanning calorimetry and other physical methods. Carvedilol binds to liposomal membranes (9:1 DMPC:DMPG) strongly with an apparent binding constant on the order of 10^4 M^{-1} in PBS (pH 7.4). The characteristic changes in its intrinsic fluorescence properties when bound to liposomes suggest that this compound is situated in a non-polar environment. The Stern-Volmer and bimolecular quenching constants, determined using nitrate as the fluorescence quencher, for the free and bound carvedilol indicate that the carbazole moiety is at a depth of > 11 Å in the lipid bilayer. Fluorescence anisotropy measurements show that, unlike the membrane probes DPH and TMA-DPH, carvedilol is relatively mobile, and does not have a rigidly-defined molecular orientation in the bilayers. Differential scanning calorimetry results indicate that carvedilol is an effective membrane 'fluidizer' as it dose-dependently lowers the gel to liquid crystalline transition temperature and broadens the endothermic transition. Comparative studies of interactions of carbazole, 4-OH carbazole and carvedilol with the model liposomal membranes reveal a possible role of membrane-partitioning in their antioxidant efficacy. These findings are discussed in perspective with the membrane biophysical properties of different classes of therapeutic significant lipid antioxidants in mind.

Keywords: Carvedilol; Lipid peroxidation; Drug–membrane interaction; Liposome binding; Fluorescence; Scanning calorimetry

1. Introduction

Carvedilol (Kredex, Coreg) is a multiple action antihypertensive drug for treatment of mild to moderate hypertension [1], and currently being evaluated in clinical trials for utilities in chronic unstable angina and congestive heart failure. This compound has been shown to reduce infarct size in different models of acute myocardial infarction [2–4]. There is significant evidence in the literature suggesting that oxygen free radicals are involved in myocardial damage during ischemic insults [5,6] and that antioxidant compounds are effective in minimizing the damage [7–10]. We have investigated the antioxidant properties of carvedilol in various in vitro models. Carvedilol inhibits oxygen radical-mediated lipid peroxidation in swine ventricular membranes [11], human low-density lipoproteins [12] and bovine and human endothelial cells [13]. We concluded from EPR spin-trapping and structure-activity redox potential correlation studies that carvedilol is a free radical scavenger and its antioxidant activity resides mainly in its carbazole moiety [13–15].

The physical state of lipid bilayers has been shown to affect the rate of lipid peroxidation in liposomes [16]. A number of antioxidants of therapeutic significance, such as the 'lazaroids' [17,18], probucol [19,20] and α-tocopherol [21–23], are known to selectively interact with a certain domain or region within the lipid bilayers and induce modifications in the molecular packing and dynamics of liposomal membranes. It has been proposed that the location and nature of drug:bilayer interaction may play a role

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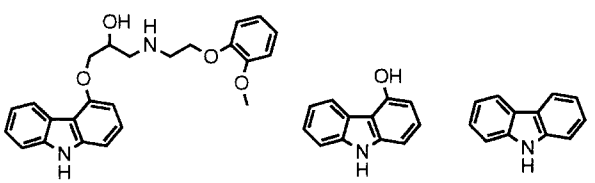
in the effectiveness of these compounds as lipid peroxidation antioxidants [17–23]. To further understand the mechanism involved in the protection of cell membranes from peroxidative damage by carvedilol, we investigate here the physical interaction of carvedilol with model lipid bilayers. Fluorescence techniques and differential scanning calorimetry are used to study the effect of low concentrations of carvedilol on the temperature-dependent dynamic and packing properties of synthetic liposomal membranes. The characteristic intrinsic fluorescence of carvedilol is exploited to provide quantitative information about the drug-liposome binding and the location of the drug within the bilayer. Comparisons are made for carvedilol and two structural analogs, and these findings are discussed in the context of biophysical implications relevant to their antioxidant properties.

2. Materials and methods

2.1. Chemicals

Carvedilol (I) and 4-OH carbazole (II) were obtained from SmithKline Beecham Pharmaceuticals. Carbazole (III) was obtained from Aldrich.



(I) (II) (III)

Dimyristoylphosphatidylcholine (DMPC) and the sodium salt of dimyristoylphosphatidylglycerol (DMPG) were obtained from Avanti (Pelham, AL). Lipids were stored at −20°C and used without further treatment. The fluorescence probes 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) were obtained from Molecular Probes (Eugene, OR). All other chemicals were reagent grade.

2.2. Preparation of liposomes

The procedures for preparing the synthetic phospholipid liposomes are similar to those described in the literature [24,25]. In short, DMPC and DMPG of a pre-determined ratio, with or without the drug and the fluorescence probe, were dissolved in chloroform. The solvent was removed with a rotary evaporator forming a thin film of lipids on the wall of the round bottom flask. The flask was stored in a vacuum desiccator overnight to remove traces of chloroform. An appropriate volume of phosphate-buffered saline (PBS) was then added into the flask and the solution was incubated at 35°C for 20 min to allow hydration of the lipids at above its phase transition temperature. The solution was vortexed for 3 min, and the incubation/vortex process was repeated. The resulting multilamellar vesicles (MLVs) were used for scanning calorimetry studies and for the preparation of small unilamellar vesicles (SUVs). The SUVs for fluorescence studies were prepared by sonication of the MLV solution using a high power bath sonicator (Model G112SP, General Laboratory Supplies Co., Hicksville, NY). The temperature during sonication was maintained above the phase transition temperature of the lipids. A model PCS-4700 photon correlation spectrometer (Malvern Instrument, Southborough, MA) equipped with a Spectra Physics argon laser was used to determine the average size of the liposomes and to ensure the batch-to-batch reproducibility.

2.3. Fluorescence spectroscopy

Fluorescence experiments were carried out using a Model LS-100 Luminescence Spectrophotometer (Photon Technology International, South Brunswick, NJ) equipped with a stroboscopic fluorescence lifetime measurement system with the Glan-Thompson polarizers anisotropy attachment. Software routines for fluorescence lifetime, polarization, anisotropy and G-factor calculations are incorporated in the data acquisition software provided by the manufacturer. All measurements were carried out in 1-cm thermostated cuvettes. For drug–liposomes interaction studies, carvedilol and fluorescence probes (DPH or TMA-DPH) were dissolved in methanol first, then diluted into the PBS or liposome solution. The final solutions contain 1% methanol. The procedure for the determinations of the bimolecular quenching constants for carvedilol, carbazole and 4-OH carbazole was the same as that described in the literature for carazolol [28]. Small aliquots of concentrated stock solution of sodium nitrate (2 M) in PBS were sequentially added into the PBS or liposome solution containing 10 μM of the test compound. The emission spectra were recorded from 335 to 380 nm with the excitation wavelength set at 325 nm. The emission intensity was corrected for 'inner optical filter effect' based on the following equation:

$$I_{corr} = I \times 10^{(A_{ex}+A_{em})/2}$$

where A_{ex} and A_{em} are the absorbance value at the excitation (325 nm) and emission (355 nm) wavelength, respectively. The fluorescence quenching was analyzed using the Stern-Volmer equation:

$$F_o/F = 1 + K_d [Q]$$

where F_o/F is the intensity ratio in the absence and presence of quencher concentration $[Q]$. The Stern-Volmer quenching constant K_d was determined from the slope.

The bimolecular quenching constant K_q was determined using the following equation, $K_q = K_d/\tau$, where τ is the fluorescence lifetime of the particular carbazole compound determined in the absence of the quencher.

2.4. Determination of drug-MLV binding

Drug:MLV binding constants were determined by the centrifugation method as previously described [25]. DMPC:DMPG (9:1) MLV solutions, at a fixed total drug concentration of 10 μM and systematically varied lipid concentration in the range of 0.25 mg/ml to 2 mg/ml, were prepared as described in the previous section. The resulting solutions were incubated for 2 h at 37°C and subsequently transferred into the micro-ultracentrifuge tubes. Separation of the liposomes from the aqueous phase was achieved using a Model TL-100 Ultracentrifuge (Beckmann Instruments, Fullerton, CA) at 50 000 rpm with rotor model TLA 100.2 for 2 h at room temperature. The drug concentration in the supernatant was determined by measuring the intrinsic fluorescence of the carbazole, and the amount of drug bound to liposomes was determined from the difference. The drug-liposome apparent binding constant K was analyzed using the double reciprocal plot. A plot of 1/(fraction bound) vs. 1/[lipid concentration] would yield a straight line of slope $1/K$.

2.5. Differential scanning calorimetry

DSC studies were carried out with a Microcal MC-2 Microcalorimeter (Amherst, MA). Samples were scanned from 5 to 40°C at a rate of 20°C/h or 60°C/h. Scans were repeated on the same sample to check reversibility. In all cases PBS was used in the reference cell. Data were analyzed using the ORGIN software provided by Microcal. The drug and liposome concentrations were as stated in the text and figure captions.

3. Results

In our previous study of the chemical integrity of C-14-labelled carvedilol in cell-based lipid peroxidation assays, we have found that a large fraction of the radiolabel was concentrated in the cell membranes after only a short period of incubation (Cheng, H.Y. and Yue, T-L., unpublished data). Carvedilol has an apparent partition coefficient (Log D octanol/pH 7.4 buffer) of 3.22. We therefore anticipated that this lipophilic compound would have high affinity for phospholipid vesicles. Carvedilol (10 μM) was incubated for 2 h at 37°C with a series of liposome solutions, which contained MLVs ranging from 0.25 mg/ml to 2 mg/ml (0.37 mM to 2.95 mM based on the average molecular weight of 679 for the 9:1 DMPC:DMPG liposomes). The MLVs were separated from the PBS by ultracentrifugation, and the amount of bound carvedilol was determined as described in Section 2. The fraction of drug bound to the liposomes can be calculated from the known total concentration. As shown in Fig. 1, more than 90% of the total drug was bound to the liposomes even at the relatively low lipid concentration of 0.75 mM. A plot of the inverse of the fraction of drug bound vs. the inverse of the liposome concentration was linear. An apparent binding constant of 9200 M^{-1} was obtained from the reciprocal of the slope.

Having determined that carvedilol binds to the liposomes with a very high equilibrium affinity, we began to address the issues concerning the nature of the drug-bilayer interactions and the effect of the drug on the dynamics and packing order of the bilayers. Conveniently, carvedilol has strong intrinsic fluorescence and its emission characteristics are sensitive to the change in its physical environment. Carvedilol has a relatively long fluorescence decay lifetime and its emission is effectively quenched by such collision quenchers as nitrate and nitrite. These properties allow carvedilol to serve as its own membrane probe. The following fluorescence studies were carried out with small unilamellar vesicles (SUVs) of 9:1 DMPC:DMPG composition. The SUVs are optically clear up to 100 mg/ml and have been extensively used as a bilayer model in drug-membrane studies using fluorescence techniques [26].

In the aqueous buffer, carvedilol displays dual emission maxima at 345 (λ_1) and 355 (λ_2) nm with the excitation wavelength at 325 nm. This is shown in Fig. 2b for a 10 μM solution of carvedilol in PBS. When SUVs are added into the solution, both the overall emission intensity and the relative intensity of λ_1/λ_2 are increased (i.e., blue shifted, Fig. 2c), indicating that carvedilol had been incorporated into a less polar environment. The liposome solution by itself has negligible fluorescence emission in the

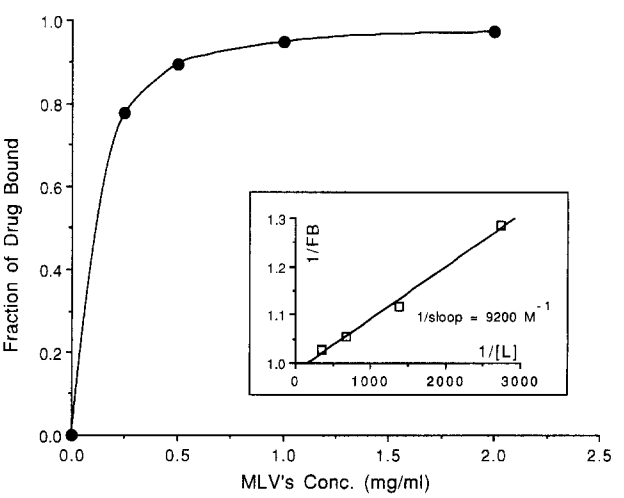


Fig. 1. Binding of carvedilol to DMPC:DMPG (9:1) multilamellar vesicles in PBS (pH 7.4). The fraction bound is determined by the centrifugation method, as described in Section 2. The double reciprocal plot is shown in the inset.

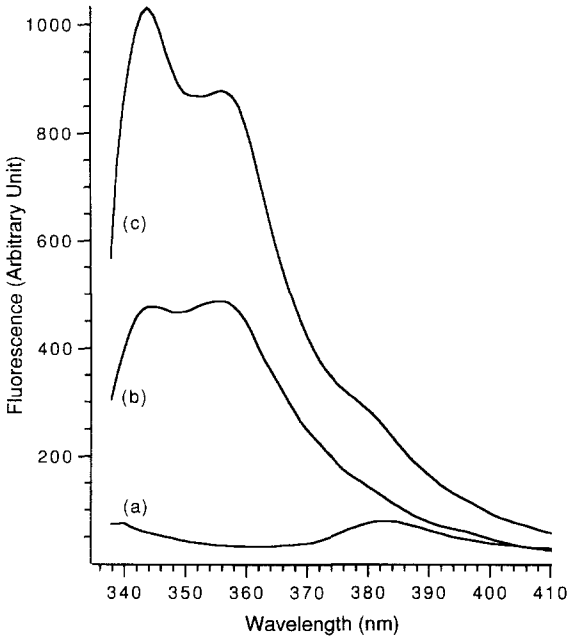


Fig. 2. Fluorescence emission spectra of (a) 20 mg/ml (29.5 mM) DMPC:DMPG SUVs in PBS, (b) 10 μM of carvedilol in PBS, (c) 10 μM carvedilol + 20 mg/ml SUVs. Excitation wavelength is at 322 nm.

wavelength range of interest (Fig. 2a). A series of solvents were selected and the fluorescence emission spectra of carvedilol in these solvents were acquired at the excitation wavelength of 325 nm. Summarized in Table 1 are the emission wavelength maxima and the intensity ratio of λ_1/λ_2. The systematic blue shift of λ_1/λ_2 emission is clearly established following the decreasing polarity order of PBS, methanol, acetonitrile and (water-saturated) octanol. A comparison with the values obtained for the liposome solution would suggest that the carvedilol molecule is likely to be located in an environment with a solvent polarity between that of octanol and acetonitrile.

Fluorescence polarization measurements show that carvedilol in the presence of 20 mg/ml SUVs has an anisotropy value of about 0.1 at room temperature. An aqueous solution of carvedilol has a negligible anisotropy value. Fig. 3 shows the titration of a fixed-concentration (10 μM) of carvedilol solution with liposomes in the 0 to 25 mg/ml range. The binding behavior is similar to that observed for the MLVs using centrifugation to separate

Table 1
Solvent effect on carvedilol fluorescence properties

Solvent	λ_1, λ_2 (nm)	Intensity (λ_1/λ_2)	Rel. Fl. Yield [a]
PBS	345,355	0.96	1
Methanol	340,353	1.11	1.23
Acetonitrile	340,354	1.12	1.21
Octanol	341,354	1.17	1.34
Liposomes [b]	344,355	1.15	2.19

[a] Not corrected for solvent absorption or sample scattering.
[b] 20 mg/ml SUVs in PBS (pH 7.4).

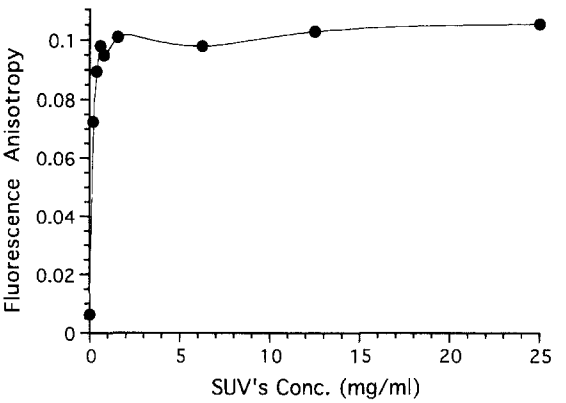


Fig. 3. Fluorescence anisotropy for a 10 μM carvedilol solution in the presence of various amounts of DMPC/DMPG liposomes.

free from liposome-bound drug. More than 95% of the total carvedilol is bound to liposomes at the relatively low lipid concentration of 2 mg/ml (2.95 mM total lipid concentration). For the purpose of interpreting the fluorescence and calorimetry results, we consider that the carvedilol is totally bound to the liposomes at > 2 mg/ml.

The anisotropy value for carvedilol in the lipid bilayers is significantly lower than that for the rigidly-oriented membrane probes DPH and TMA-DPH. As shown in Fig. 4, in the 5°C to 40°C temperature range the carvedilol anisotropy remains constant while the DPH and TMA-DPH fluorescence anisotropy follow the characteristic gel to liquid crystalline phase transition with the steepest part around 25°C. The experiments were carried out with each solution containing 2 mg/ml SUVs and 5 μM of the designated fluorescence probe (about 0.16% probe:lipid mole ratio). Co-incubation of 5 μM of DPH or TMA-DPH and up to 50 μM of carvedilol with the SUV solution

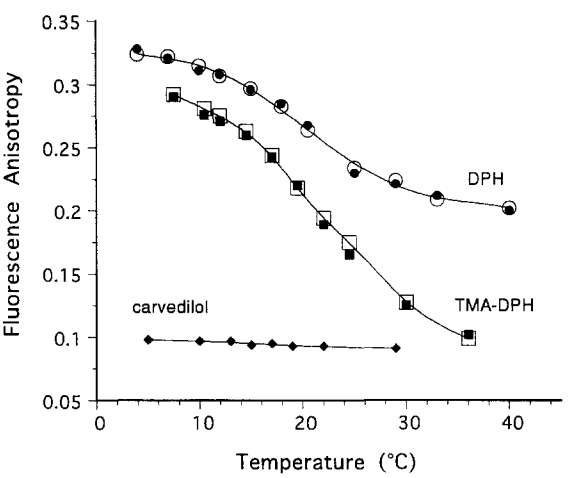


Fig. 4. Fluorescence anisotropy measurements in the phospholipid bilayer phase transition temperature range. Carvedilol alone (5 μM, filled diamond), DPH alone (5 μM, open circle), DPH + carvedilol (+50 μM, filled circle), TMA-DPH alone (5 μM, open square), TMA-DPH + carvedilol (+50 μM, filled square). The DMPC/DMPG SUV concentration was at 2 mg/ml.

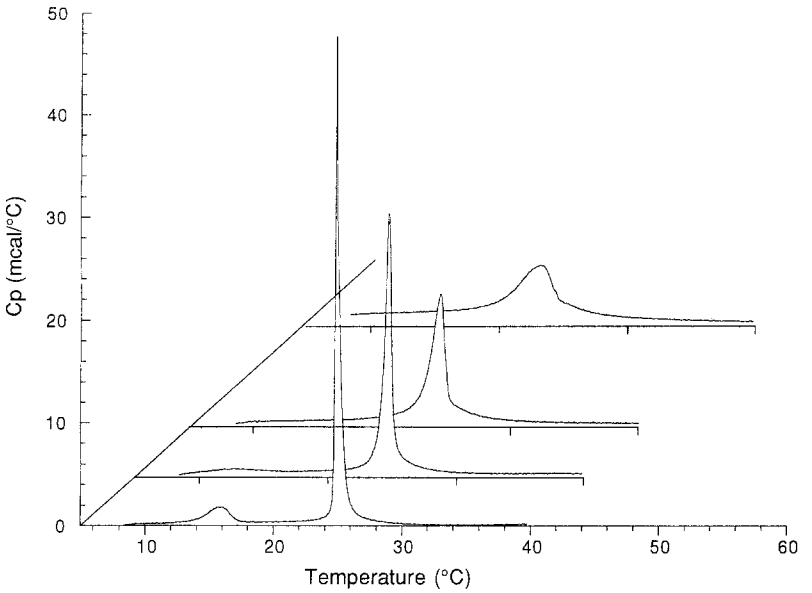


Fig. 5. Differential scanning calorimetry thermograms of DMPC:DMPG (9:1) MLVs. Front to back, incorporation of carvedilol with mole ratio = (1) drug-free, (2) 1.7%, (3) 3.4%, (4) 6.8%. The liposome concentration was 2 mg/ml, scan rate was 60°C per h; no significant differences in trends were observed at 20°C/h.

(1.6% drug:lipid mole ratio) does not change the temperature-dependent phase transition behavior of the liposomes, as shown in Fig. 4. The limited aqueous solubility of carvedilol prevented the use of higher drug concentrations in the fluorescence membrane probe experiments.

Differential scanning calorimetry proved to be a much more sensitive technique for studying the effect of the drug on the packing order of the model bilayers. Carvedilol was incorporated in the lipid/chloroform mixture and subjected to the same evaporation/hydration/dispersion procedure for the preparation of MLVs. The differential thermogram in Fig. 5a shows a sharp peak at 24.8°C for the main gel to liquid crystalline transition for the drug-free 9:1 DMPC:DMPG liposome solution. Also observed is the smaller and broader 'pre-transition' peak at 16°C. This calorimetric behavior is consistent with that observed in previous studies using similar synthetic liposomes [27]. By incorporation of 1.7%, 3.3% and 6.7% mol ratio of the drug in the bilayers, the thermotropic phase transitions are shifted toward lower temperatures accompanied by a systematic broadening of the peaks. The total peak areas, corresponding to the enthalpy involved, remained approximately constant (5.5 ± 1.2 kcal/mol). The pre-transition peaks followed the same trend, although their positions are more difficult to determine due to the broader peak shape and the much lower enthalpy. The thermotropic behavior of the carvedilol-doped liposomes is similar to that observed with other lipophilic drugs and suggests that the drug behaves like a membrane 'fluidizer' [25,27].

We have thus far established that carvedilol binds strongly to the lipid membrane and induces significant changes in the bilayer structure. However, the data do not reveal information about the location of the drug within the lipid bilayers. It has been demonstrated that nitrate quenches the fluorescence of carazolol, a carbazole-derived β-adrenoceptor antagonist, and such fluorescence property has been used for the estimation of the depth of a buried binding site of the receptor [28]. In aqueous solution, carvedilol fluorescence is also effectively quenched by nitrate, as shown in Fig. 6 with the rapidly diminishing fluorescence intensity. By contrast, in the presence of 20 mg/ml SUVs the fluorescence intensity of carvedilol is minimally affected by the sequential additions of nitrate. Clearly, the liposome-bound drug is not accessible to the collisional quencher which exists only in the aqueous phase. The Stern-Volmer quenching constant, K_d, can be determined from the slope of the plot shown in the inset.

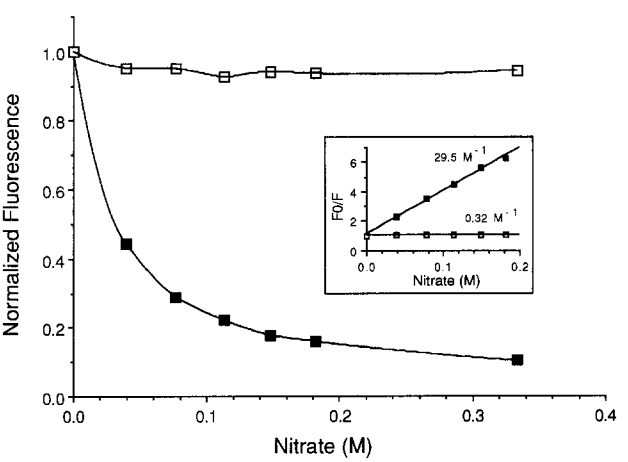


Fig. 6. Quenching of carvedilol fluorescence by sodium nitrate in PBS (filled squares) and in the presence of 20 mg/ml SUVs (open squares). The Stern-Volmer plots are shown in the inset.

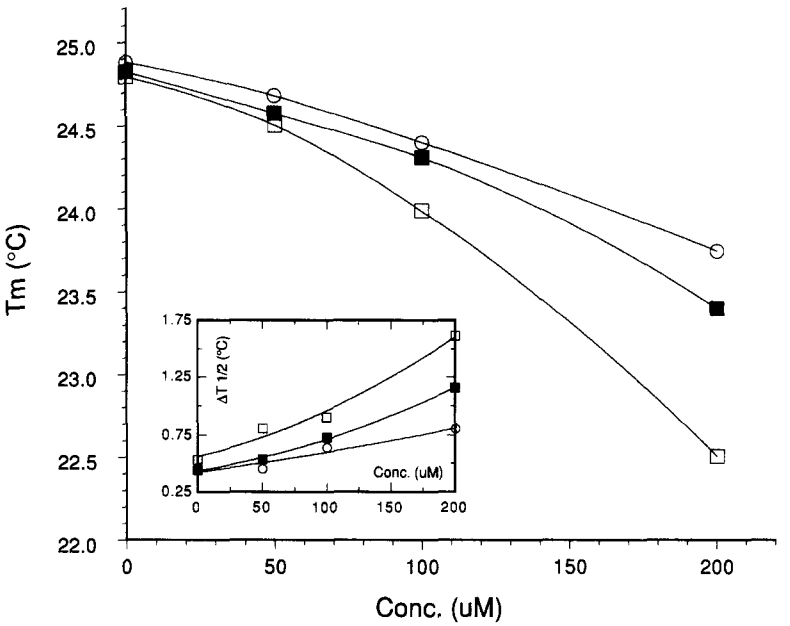


Fig. 7. The lowering of main transition temperature (Tm) and broadening of half-height peak width ($\Delta T_{1/2}$ shown in the inset) of MLVs with the incorporation of 4-OH carbazole (open circles), carbazole (filled squares) and carvedilol (open squares). The liposome concentration was 2 mg/ml (2.95 mM).

The K_d for free carvedilol is determined to be 29.5 M^{-1}, practically the same as the value reported (30.7 M^{-1}, Ref. [28]) for carazolol. The K_d for the liposome-bound carvedilol is determined to be 0.32 M^{-1}. The fluorescence lifetime for the free and the bound carvedilol is determined to be 8.2 and 9.0 ns, respectively. These values yield the bimolecular quenching constant 3.6×10^9 and 3.6×10^7 $M^{-1}s^{-1}$ for the free and the bound carvedilol, respectively.

Table 2
Lipophilicity, fluorescence and antioxidant properties of carbazole compounds

	Carvedilol	Carbazole	4-OH Carbazole
Log P	3.22	3.53	2.86
K (MLV)	9200	11800	2200 (M^{-1})
Anisotropy (SUV)	0.098	0.055	0.078
K_d (aqueous)	29.5	25.7	24.2 (M^{-1})
τ (aqueous)	8.2	10.3	7.3 (ns)
K_q (aqueous)	3.58	2.50	3.33 (1/M·ns)
K_d (SUV)	0.32	0.16	0.72 (M^{-1})
τ (SUV)	9.0	13.7	8.3 (ns)
K_q (SUV)	N.S.	N.S.	N.S.
E (vs. S.C.E.)	1.10	1.25	1.12 (volts)
TBARS IC_{50}	8.1	15	1.9 (μM)

Log P = octanol/PBS pH 7.4 partition coefficient.
K = apparent liposome binding constant.
K_d = Stern-Volmer quenching constant.
τ = fluorescence lifetime.
kq = K_d / τ; bimolecular quenching constant.
E = oxidation potential in acetonitrile.
TBARS = thiobarbituric acid reactive species (i.e., ipid peroxidation assay).
N.S. = no significant quenching. The calculated K_q's are 0.036, 0.011 and 0.087 (1/M·ns). According to Ref. [28], these values fall below the detection limit for quenching constant error measurement.

In the above quenching experiments, only the location of the carbazole fluorophore is being probed. Therefore, the effect of the side chain at the 4-position on the carbazole ring is not addressed by the fluorescence technique. The differential scanning calorimetry data, on the other hand, should reflect physical interactions of the entire chemical structure of carvedilol with the lipid bilayers. Two additional compounds, 4-OH carbazole (II) and carbazole (III), were selected and subjected to various physico-chemical measurements in order to investigate this aspect. Fig. 7 compares the thermotropic main transitions (T_m) and peak width at half height ($\Delta T_{1/2}$) for different preparations of MLVs incorporating compound I, II or III at several mol ratios. The ranking of carvedilol > carbazole > 4-OH carbazole is clearly established in terms of the drug's effect on the membrane melting characteristics. Other relevant physical parameters, each reflecting some aspect of the drug-membrane interactions, were determined for the three compounds and listed in Table 2. They include octanol/water partition coefficient (by shake flask method), drug-MLVs binding constant (by ultracentrifugation), fluorescence anisotropy (polarization measurement) and bimolecular quenching constant. Also included for comparison are our previously reported brain homogenate lipid peroxidation inhibition IC_{50}'s and the oxidation peak potentials [14].

4. Discussion

The results from the centrifugation of MLVs and the fluorescence anisotropy titration of SUVs clearly indicate that carvedilol binds to the negatively charged phospholipid vesicles with a very high equilibrium affinity. With the apparent binding constant at approximately 10^4 M^{-1}, a rough calculation would show that, at 10 nM drug concentration and 10 mg/ml lipid, 90% of the total drug would be bound to the lipid phase. This is a reasonable scenario of plasma concentrations for a relatively bioavailable drug dosed at the multi mg/kg levels. Carvedilol also binds to human low density lipoprotein with a binding constant about 1/4 of that determined in this work using the DMPC/DMPG liposomes ([13], Cheng and Yue unpublished data). Taken together, the strong membrane association of the cardioprotective drug should enhance the compound's potency as a lipid peroxidation inhibitor in that the drug is concentrated in cell membranes where the lipid peroxidation chain reactions propagate. The results from this study also set carvedilol apart from those water-soluble reducing agents, such as ascorbate and thiol-containing peptides, in terms of their antioxidant mechanisms of action.

Carvedilol has an acid dissociation constant (pK_a) of 7.9 for the secondary amino function on the side chain. A large proportion (76%) of the compound would carry a positive charge in pH 7.4 buffer. The DMPC/DMPG liposomes are negatively charged, thus an immediate question would be whether the binding of carvedilol to liposomes is simply electrostatic interaction on the surface of the vesicles. Using carvedilol itself as a fluorescence membrane probe, we demonstrated that the carbazole moiety is not bound to the outer leaflet of the lipid bilayer. The blue shift of its fluorescence emission and the inability of nitrate to quench the liposome-bound drug indicates that the carbazole backbone is located in the hydrophobic interior of the bilayer. Unlike the membrane probes DPH and TMA-DPH, which are rod-shaped and thought to be rigidly oriented with the lipid acyl chains, carvedilol does not seem to occupy a particular molecular orientation in the bilayer structure. This is evidenced by the contrast in the fluorescence anisotropy responses over the bilayer phase transition temperature range (Fig. 4). Both DPH and TMA-DPH probe the gel to liquid-crystalline fluidity change, while the anisotropy values for carvedilol remain unchanged. The smaller anisotropy values also suggest that carvedilol is more mobile than the rigidly aligned DPH and TMA-DPH.

We did not perform the rigorous experiments necessary for a theoretical calculation of the Foster radius R_0 for the carvedilol-nitrate fluorescence energy transfer complex. However, since the bimolecular quenching constants (K_q's) for carvedilol are almost identical to those obtained for carazolol, we use the published values for carazolol as an approximation for our discussion here [28]. The calculated K_q value for liposome-bound carvedilol is below the detection limit of error measurement for bimolecular quenching constant. Tora and Strader [28] concluded that the failure to detect any quenching of bound carazolol indicates that the molecule is buried in the receptor protein at a depth of > 11 Å. The thickness of a lipid bilayer in binary DMPC/DMPG mixtures has been estimated at 40–50 Å with a 5 Å interfacial 'hydrogen-bonding' region, on either side of the bilayers, consisting mainly of the glycerol bridge. If we hypothesize that the carvedilol molecules are buried in the lipid bilayer at a depth of > 11 Å, this would place the drug deep in the hydrophobic domain. The same conclusion can be reached for carbazole and 4-OH carbazole.

Incorporation of a few mol% of carvedilol into liposomes results in a disruption of the packing order of the lipid bilayers. This is reflected in the lowering of the gel to liquid crystalline transition temperature and the broadening of the endothermic transition. It should be noted that the thermotropic behavior of the carvedilol-doped liposomes is comparable to that observed for the anthracycline antibiotics [27]. Insertion of the molecule in the hydrophobic region of the bilayer affect bilayer dynamics such that the motion of the acyl chains become less restrictive. One possible role of this membrane fluidizer effect in the antioxidant action is that it could change the mobility of the reactive oxygen species in the membrane and affect the rate of lipid peroxidation [16].

The comparative studies on carbazole, 4-OH carbazole and carvedilol reveal subtle differences in their interactions with lipid bilayers. The rank order of the MLV binding constants and SUV fluorescence quenching constants follow the lipophilicity scale (as octanol/water partition coefficient) of carbazole > carvedilol > 4-OH carbazole. The DSC results, however, show that carvedilol affects the gel to liquid crystalline phase transition of the liposomes to a much greater extent than either carbazole or 4-OH carbazole. The difference may be explained if we consider that the intrinsic fluorescence reflects only the lipid environment around the carbazole backbone while the DSC result represents the effect of the whole molecule on the bilayers. The carbazole moiety interacts strongly with the lipophilic region of the bilayer and largely dominates the overall binding affinity of the molecule to liposomes. The greater effect of carvedilol than carbazole on the liposome phase transition temperature, despite the fact that carvedilol has a somewhat weaker binding constant, suggests that the long methoxyphenoxyl amino side chain indeed contributes to the disordering of the bilayer structure. The relatively small fluorescence anisotropy values for the liposome-bound compounds, on the other hand, suggest that these molecules probably do not have a rigidly-defined molecular orientation in the bilayers.

The amino side chain of carvedilol has a pK_a value of 7.9, indicating that the compound would be present mostly in cationic form at physiological pH. This implies that the

contribution of electrostatic interaction, which we have not established in this study, may play a role in determining the binding behavior of carvedilol to liposomal membranes. Investigations using cationic and neutral liposomes at different pH's, as well as membrane structural studies incorporating carvedilol or analogs, would provide further insights into the mechanism of action of carvedilol in the inhibition of lipid peroxidation.

This study has demonstrated that (1) carvedilol has a high affinity for model lipid bilayer membranes, (2) the carbazole moiety is located deep in the hydrophobic region, (3) the drug molecule decreases the cooperativity of the phospholipid chain melt and (4) carvedilol is in a relatively fluid environment with no preferential molecular orientations. The results from the comparative study on carbazole, 4-OH carbazole and carvedilol are consistent with a physico-chemical model based on hydrophobic interactions between the drug and the fatty acyl chain domain of the bilayers. To put things in perspective, we compare here the conclusions derived from comparable studies of drug-membrane interactions on several therapeutic significant lipid peroxidation antioxidants. In phosphatidylcholine vesicles, α-tocopherol is hydrogen-bonded and situated in a polar region of the membrane [29]. It causes a decrease in membrane fluidity [22] and stabilizes the liquid-crystalline phase [23]. The 21-aminosteroids (U-74500 and U-74006) have been shown to increase the molecular packing order of cell membranes [18]. By contrast, the 21-aminosteroids are associated with the hydrophobic domain of the bilayers. The effect of Probucol on the temperature-induced phase transition of liposomes is qualitatively similar to that for carvedilol, i.e., causing an increase in membrane fluidity and decrease in molecular packing order [30,31]. Probucol is probably located in the hydrophobic region as it does not exhibit synergy with ascorbate in the protection of membrane peroxidation. Regeneration of ascorbate has been demonstrated for phenolic antioxidants which are associated with the polar region [31]. All these lipophilic antioxidants have comparably low μM IC_{50} values in the inhibition of cell membrane peroxidation.

It is interesting to note that the efficacy of the inhibition of brain lipid peroxidation, listed in Table 2, follows the inverse lipophilicity order for the three compounds. While tempting, it would be highly speculative to suggest that a structure-activity correlation existed. As discussed above, no general rules governing the biomembrane interactions and activities can be derived from the different classes of lipid antioxidants. Each class of compounds should be considered individually based on their molecular structures and functionalities. From our previous EPR spin trapping and electrochemistry data [14], we believe that the redox activity of the carbazole moiety plays a dominant role in determining the lipid peroxidation inhibition activity. Synthesis of a series of ring-substituted carbazole compounds with systematically-varied structures, such as hydrocarbon chain length and head group charges, could yield molecular entities which would place the carbazole ring in a designated region of the bilayers with a defined structural orientation. Further investigations may reveal optimal locations and molecular conformations for the design of better antioxidants for the inhibition of peroxidative cell damage.

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