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A calorimetric evaluation of the interaction of amphiphilic prodrugs of idebenone with a biomembrane model

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Abstract

Lipoamino acids (LAA) are useful promoieties to modify physicochemical properties of drugs, namely lipophilicity and amphiphilicity. The resulting membrane-like character of drug–LAA conjugates can increase the absorption profile of drugs through cell membranes and biological barriers. To show the role of amphiphilicity with respect to lipophilicity in the interaction of drugs with biomembranes, in the present study we evaluated the mode of such an interaction of lipophilic conjugates of LAA with the antioxidant drug idebenone (IDE). DSC analysis and transfer kinetic studies were carried out using dimyristoylphosphatidylcholine (DMPC) multilamellar liposomes (MLVs) as a model. For comparison, two esters of IDE with alkanoic acids were synthesized and included in the analysis. The experimental results indicate that based on their different structure, IDE–LAA conjugates interacted at different levels with respect to pure IDE with DMPC bilayers. In particular, a progressive penetration inside the vesicles was observed upon incubation of IDE–LAA compounds with empty liposomes. The enhanced amphiphilicity of the drug due to the LAA moieties caused more complex interactions with DMPC bilayers, compared to those registered with the native drug or IDE alkanoate esters.

Keywords: Idebenone; Lipoamino acids; Lipophilicity; Amphiphilicity; DSC; Biomembrane model

1. Introduction

In previous studies, some ester prodrugs of idebenone (IDE) with short-chain 2-alkylamino acids were described. These prodrugs were characterized by their biological activity and stability profile [1,2].

IDE [2-(10-hydroxydecyl)-5,6-dimethoxy-3-methylbenzo-1,4-quinone] is a synthetic analogue of coenzyme Q_{10} (Fig. 1). IDE is a potent antioxidant agent and, thanks to its ability to inhibit lipid peroxidation, it protects cell and mitochondrial membranes from oxidative damage [3]. This drug has clinical applications in many central nervous system degenerative diseases associated with oxidative stress, such as Parkinson's and Alzheimer's diseases, as well as cerebral ischemia and brain aging [4,5], and especially in the therapy of Friedreich's ataxia [6].

The above-mentioned studies belong to a wider project aimed at evaluating the advantages of conjugating drugs to lipoamino acids (LAA). Conjugation of LAA to drug molecules can modify many physicochemical properties of the latter, namely lipophilicity and amphiphilicity. The resulting *membrane-like character* of these conjugates can ultimately affect the absorption of drugs through cell membranes and biological barriers, as well as increase their stability in the bloodstream [7,8].

LAA have been conjugated to many drugs, including antitumor and anti-inflammatory agents [9–12]. Shorter-chain LAA

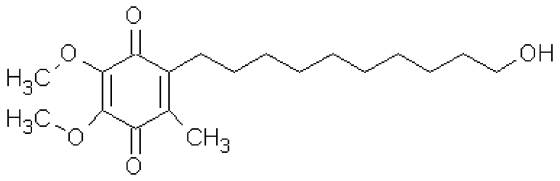

Fig. 1. Chemical structure of idebenone.

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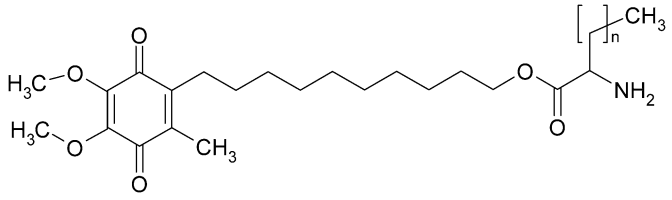
Table 1
Chemical structure and properties of IDE–LAA esters



Compound	n	MW	$c\log P$[a]	$c\log P$[b]	$c\log D_{7.4}$[c]	Water solubility[b] ($\log S$)[d]
IDE–LAA4	1	423.55	4.52	4.08	4.13	−3.79
IDE–LAA5	2	437.58	5.06	4.54	4.53	−4.06
IDE–LAA6	3	451.61	5.59	5.00	4.95	−4.33
IDE–LAA7	4	465.64	6.12	5.47	5.17	−4.60
IDE	–	*338.45*	*3.49*	*4.21*	–	*−3.16*

[a] ACD log *P* 5.15 software.
[b] Osiris Property Explorer.
[c] Pallas 3.1.1.2, CompuDrug Chemistry Ltd.
[d] Unit stripped logarithm (base 10) of the solubility measured in mol/l.

have more recently been used by us instead of the originally proposed long-chain ones [7]. Short- and medium-chain 2-alkylamino acids (C_4 to C_8) can, in fact, still exert the lipophilicity modifier properties of LAA without dramatically reducing the solubility of conjugates in biological and experimental media.

Studies on IDE were aimed at exploring the effects caused by LAA conjugation upon an already lipophilic drug (IDE has a log *P* around 3.5–4, Table 1). Most studies on LAA have, in fact, been focused on peptides and hydrophilic drugs [13–15]. However, by their nature LAA are able to enhance not only the lipophilic character of drugs, but also their amphiphilicity, a critical property associated with crossing through and interaction with biological membranes [16,17]. For instance, in a recent study we demonstrated that conjugation of a model drug with LAA allows the drug to interact in a complex mode and at different levels with a biomembrane model, consisting of pure phospholipid liposomes [18]. Compared to LAA conjugates, the corresponding drug derivatives with simple fatty (alkanoic) acids gave poorer interactions with such a model.

DSC analysis of the degree of interaction of xenobiotics, such as drugs, with such an anisotropic biphasic system has already been shown to correlate with their biological behavior [19–21]. The partitioning into and binding of a drug to cell membranes/barriers, as well as to models such as liposomes, follow complex mechanisms and are related to so-called "anisotropic lipophilicity" [17]. The latter results from the hydrophobicity of the drug, but also from its ability to make polar and ionic bonds with the membranes. In IDE–LAA conjugates the presence of a free ionizable amine group allows polar and ionic interactions to enhance the partition of these compounds within DMPC bilayers, causing deep changes in the thermotropic parameters of the pure phospholipid.

Therefore, in the present study we evaluated the mode of interaction of IDE lipophilic prodrugs, described in Table 1, with a biomembrane model. DSC analysis and transfer kinetic studies were performed using pure dimyristoylphosphatidylcholine (DMPC) multilamellar liposomes (MLVs) as a model. Two esters of IDE with alkanoic acids were synthesized and included in the study for comparison.

2. Materials and methods

2.1. Materials

IDE was kindly provided by Wyeth Lederle SpA (Catania, Italy). 1,2-Dimyristoyl-*sn*-glicero-phosphocholine (DMPC) (purity ⩾99.0% by TLC) is a Fluka product (No. 41803; Sigma-Aldrich Chimica Srl, Milan, Italy). IDE–LAA esters (Table 1) were synthesized as previously described [1]. Purity was checked by TLC on silica gel aluminum sheets ($F_{254+356}$, Merck); spots were detected either by UV light, ninhydrin, or acid–base reactant treatment. Reactants and solvents were all commercial products of at least analytical grade. HPLC-grade water was used throughout the study.

IR spectra were registered in nujol with an FT-IR Perkin–Elmer 1600 spectrophotometer. ^{1}H NMR spectra were obtained in DMSO-d_6 with a 200-MHz Brüker instrument; chemical shifts are reported in ppm with TMS as the internal standard. Mass spectra were recorded on a Perkin–Elmer Sciex API 3000 triple quadrupole mass spectrometer, checked using a Sample Control 1.4 software package in selected ion monitoring mode (SIM). Data were analyzed using Multiview and MacQuan 1.6 software packages (Perkin–Elmer Sciex, Toronto, Canada).

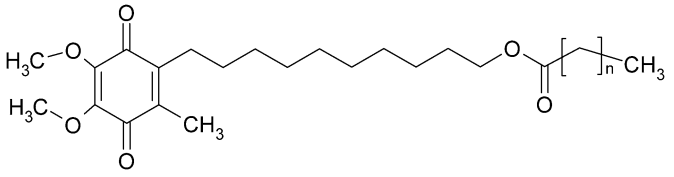
2.2. Synthesis of IDE esters with alkanoic acids (IDE–FA)

Butyric (C_4) or caproic acids (C_6) (0.15 mmol) were dissolved in 5 ml of dry dichloromethane and 1-hydroxybenzotriazole (0.3 mmol, 46 mg), triethylamine (0.375 mmol, 52 µl), and 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide (EDAC) hydrochloride (0.45 mmol, 64 mg) were added to the solution. The mixture was stirred for 2 h in an iced-water bath and then a solution of IDE (0.15 mmol, 25.5 mg) in 5 ml of dry dichloromethane was added. The reaction mixture was magnetically stirred at room temperature for an additional 24 h. At the end of this period, the solvent was removed under vacuum and the residue dissolved in 30 ml of dichloromethane and extracted with a 5% aqueous sodium bicarbonate solution (30 ml) and then brine (2 × 30 ml). The organic phase was desiccated with anhydrous sodium sulphate and then filtered and evaporated to dryness in vacuo. The structure and properties of the obtained esters are reported in Table 2.

IDE–FA4: 10-(4,5-dimethoxy-2-methyl-3,6-dioxocyclohexa-1,4-dien-1-yl)decyl butyrate. IR (nujol; cm^{-1}): 1736 (ester C=O). MS (m/z): 409 [M + 1]. ^{1}H-NMR (ppm, δ): 4.09 (m, 2H, O–CH_2), 3.79 (s, 6H, CH_3), 2.50 (t, 2H, ∅–CH_2), 1.99 (t, 3H, CH_3), 1.66 (m, 2H, CH_3–*CH_2*), 1.33–1.25 (broad m, 16H, CH_2).

IDE–FA6: 10-(4,5-dimethoxy-2-methyl-3,6-dioxocyclohexa-1,4-diene-1-yl)decyl hexanoate. IR (nujol; cm^{-1}): 1709 (ester C=O). MS (m/z): 437 [M + 1]. ^{1}H NMR (ppm, δ): 4.12 (m, 2H, O–CH_2), 3.65 (s, 6H, CH_3), 2.51 (t, 2H, ∅–CH_2), 1.88 (t,

Table 2
Chemical structure and properties of IDE–FA esters



Compound	n	MW	$c\log P$[a]	$c\log P$[b]	Water solubility[b] ($\log S$)[c]
IDE–FA4	2	408.54	5.45	6.02	−4.24
IDE–FA6	4	436.59	6.51	6.95	−4.78

[a] ACD log P 5.15 software.
[b] Osiris Property Explorer.
[c] Unit stripped logarithm (base 10) of the solubility measured in mol/l.

3H, CH_3), 1.64 (m, 2H, CH_3–CH_2), 1.38–1.15 (broad m, 22H, CH_2).

2.3. Calculation of lipophilicity and solubility

Different software packages were used to make separate calculations of the log P and log $D_{7.4}$ values for IDE and the ester conjugates: the ACD log P package (ACD log P, ver. 5.15; Advanced Chemistry Development Inc., Toronto, Canada), the Pallas 3.1.1.2 (CompuDrug Chemistry Ltd.), and the Osiris Property Explorer (http://www.organic-chemistry.org/prog/peo/index.html). The latter was also used to estimate the aqueous solubility of the compounds ($\log S$). The calculated values are reported in Tables 1 and 2.

2.4. Calorimetric experiments

2.4.1. Preparation of liposomes

MLV suspensions were obtained by rehydrating pure DMPC or drug–phospholipid mixtures, according to the classical method of Bangham's thin layer evaporation. Five milligrams of DMPC and the amount of IDE or esters required to achieve different molar fractions ($X_0 = 0.01$; 0.015; 0.03; 0.045; 0.06; 0.09) were dissolved in 1 ml of chloroform and dried on tube walls by gentle rotation under a nitrogen stream. Tubes were then kept overnight at 30 °C under vacuum (Buchi T-50 oven) for complete solvent removal. Rehydration of the films was obtained by adding 300 µl of isotonic phosphate buffer (PBS, pH 7.4; Sigma) in a water bath at 40 ± 2 °C for 2 min, followed by vortex agitation for a further 2 min. The entire cycle was repeated three times, and the suspensions were then left for 2 h at room temperature to reach a drug partition equilibrium between the aqueous and lipid phases.

2.4.2. DSC analysis

Calorimetric experiments were performed with a Mettler DSC 12E calorimeter, connected to a Lauda Ecoline RE207 thermocryostat. The detector consisted of a chromel–constantan sensor with a thermometric sensitivity of 56 µV/°C, a calorimetric sensitivity of about 3 µV/mW, and a noise less than 60 nV (<20 µW) at 100 °C. DSC scans showed an accuracy of ±0.4 °C, with a reproducibility and resolution of 0.1 °C.

Table 3
Program of kinetic transfer experiment of IDE esters into empty DMPC liposomes

Step	Thermal change	Scan rate	Time length	DSC scan	Total time[a]
1	Isothermal, 5 °C	–	5 min		
2	Heating 5 → 35 °C	1 °C/min	30 min	√	5 min
3	Isothermal, 35 °C	–	30 min		
4	Cooling 35 → 5 °C	2 °C/min	15 min		
5	Heating 5 → 35 °C	1 °C/min	30 min	√	1 h 20 min
6	Isothermal, 35 °C	–	30 min		
7	Cooling 35 → 5 °C	2 °C/min	15 min		
8	Heating 5 → 35 °C	1 °C/min	30 min	√	2 h 35 min
9	Isothermal, 35 °C	–	1 h		
10	Cooling 35 → 5 °C	2 °C/min	15 min		
11	Heating 5 → 35 °C	1 °C/min	30 min	√	4 h 20 min
12	Isothermal, 35 °C	–	1 h		
13	Cooling 35 → 5 °C	2 °C/min	15 min		
14	Heating 5 → 35 °C	1 °C/min	30 min	√	6 h 05 min
15	Isothermal, 35 °C	–	17 h		
16	Cooling 35 → 5 °C	2 °C/min	15 min		
17	Heating 5 → 35 °C	1 °C/min	30 min	√	23 h 50 min
18	Cooling 35 → 5 °C	5 °C/min	6 min		

[a] Total time of incubation at the beginning of each scan step.

For the DSC runs, each sample was sealed in a 40 µl aluminum pan, while a pan containing 40 µl of PBS, pH 7.4 was used as the reference. Each sample was submitted to three cycles of analysis (in heating and cooling mode) at a running rate of 2 °C/min in the range 5–37 °C. A Mettler TA89A system software package (version 4.0) was used to evaluate the data from each DSC run, i.e., transition peak temperature (T_m), enthalpy changes as a function of heating (ΔH), and full width at half height of endothermal peaks ($\Delta T_{1/2}$). Further mathematical analysis was carried out on a PC using the Origin 7 SR2 software package (OriginLab Corporation, Northampton, MA, USA), by which multiple peak curve fitting, subtraction of the y-offset, and correction for time-based drift were performed. The experimental DSC data are available as Supplementary material.

2.4.3. Permeation experiments

The time-course ability of TCP derivatives to dissolve in the aqueous phase, to absorb onto liposome surface, and then to penetrate within the inner DMPC bilayers was also evaluated on IDE esters. A weighed amount of each compound, finely powdered, was placed at the bottom of a 100-µl aluminum DSC crucible and covered with 80 µl of a 5 mg/ml pure DMPC liposome suspension to obtain a 0.06 drug molar fraction vs DMPC. This drug concentration was chosen after some preliminary tests, in order to obtain reproducible results. Once sealed, the pan was gently vortex-mixed for 5 min, kept for 5 min at 5 °C in the calorimeter oven, and then submitted to repeated heating and cooling cycles (between 5 and 35 °C), separated by isothermal steps at 35 °C, to allow the migration of the drug into the phospholipid bilayers.

DSC scans were performed on each sample at predetermined times (5 min and about 1, 2, 4, 6, and 24 h), according to the

schedule shown in Table 3, to obtain the rate of diffusion of the drugs within the liposome bilayers.

3. Results and discussion

3.1. DSC experiments

The experimental values registered in the DSC analysis of IDE and its esters with LAA or alkanoic acids are shown in Figs. 2–7. The experimental calorimetric data are available as Supplementary material. All compounds, including pure IDE, first caused the sudden disappearance of the pretransition endothermic signal (at about 15 °C for DMPC). This peak is attributed to the rotation of phospholipid polar heads or to the transformation in bilayer structures and changes in the packing of hydrocarbon chains. The pretransition peak is quite sensitive to the presence of foreign molecules in the bilayers [22]. Thus, the depression of this peak, already observed at the lowest molar fraction of compounds, indicates that IDE and its esters interact with the polar region of DMPC and alter the inclination of the phospholipid acyl chains [23,24].

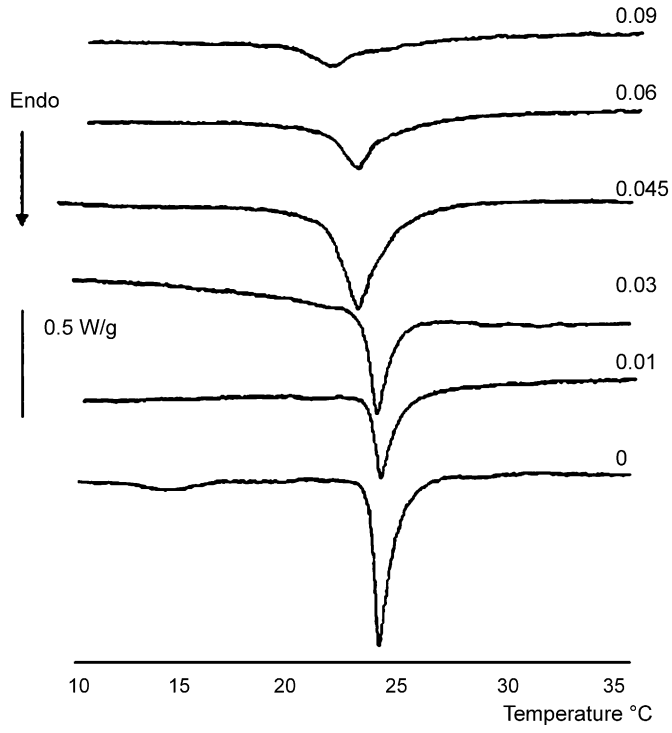


Fig. 2. DSC curves of DMPC MLVs loaded with different IDE molar fractions.

Pure IDE caused a significant change of the shape of the main endothermic phase transition peak only at relatively high concentrations (above an $X_0 = 0.045$). At the highest tested molar fraction ($X_0 = 0.09$) such a peak was profoundly altered, indicating a strong penetration of the drug within the bilayers. The transition temperature (T_m) was not affected by pure IDE at most of the tested molar fractions; this behavior indicates that the interaction between IDE and the bilayers mainly occurs at the level of lipid (acyl chains) domains. IDE–LAA conjugates instead caused a strong deformation of the transition peak, associated with a relevant reduction of the T_m.

These findings will be discussed below. However, they help to confirm our initial hypothesis: the presence of the lipoamino acid moiety, apart from increasing the lipophilicity of the drug (which, in this case, already has a high log P value; see Table 1), is also able to generate polar interactions with the choline head groups of DMPC bilayers, thanks to the presence of its amino acid head. In other words, the "anisothropic lipophilicity" described by Plemper van Balen et al. [17] took place; it could account for a better interaction with cell membranes and/or passage across biological barriers.

In Figs. 8–12 the changes of the same thermotropic markers are plotted as a function of the molar fraction of each drug or the length of the LAA alkyl side chain.

In particular, Fig. 8 shows that the T_m depression is proportional to the molar fraction of both IDE and IDE–LAA conjugates in the vesicles. The effect on T_m tended to increase without reaching a *plateau*, even at the highest tested molar fraction (0.09). Only at this concentration was an initial sign of

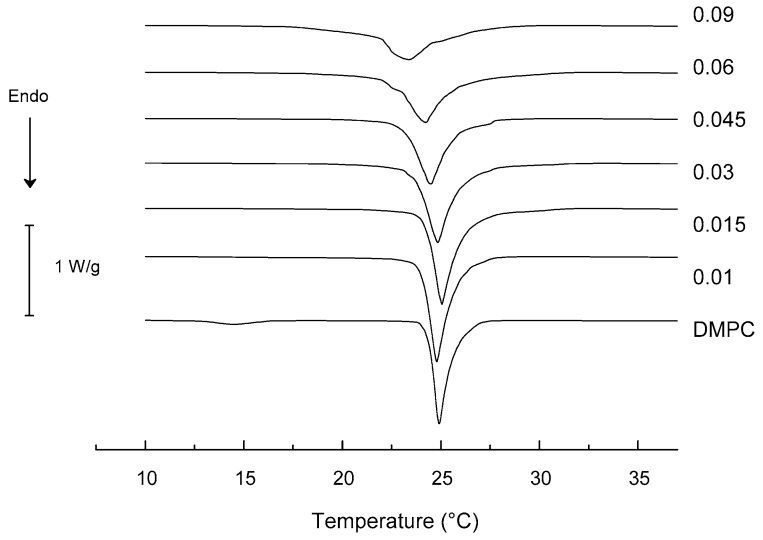


Fig. 3. DSC curves of DMPC MLVs loaded with different IDE–LAA4 molar fractions.

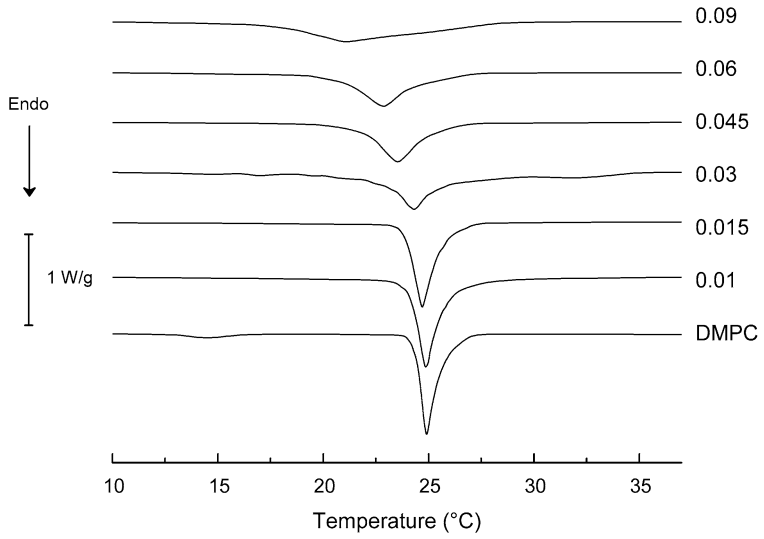


Fig. 4. DSC curves of DMPC MLVs loaded with different IDE–LAA5 molar fractions.

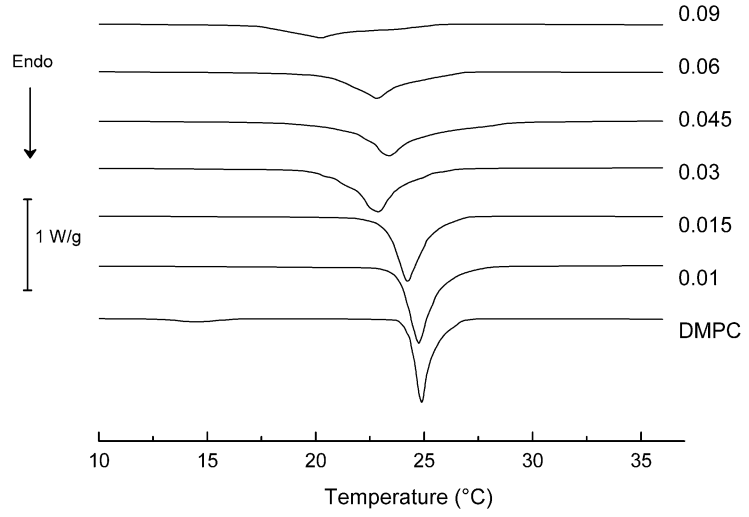


Fig. 5. DSC curves of DMPC MLVs loaded with different IDE–LAA6 molar fractions.

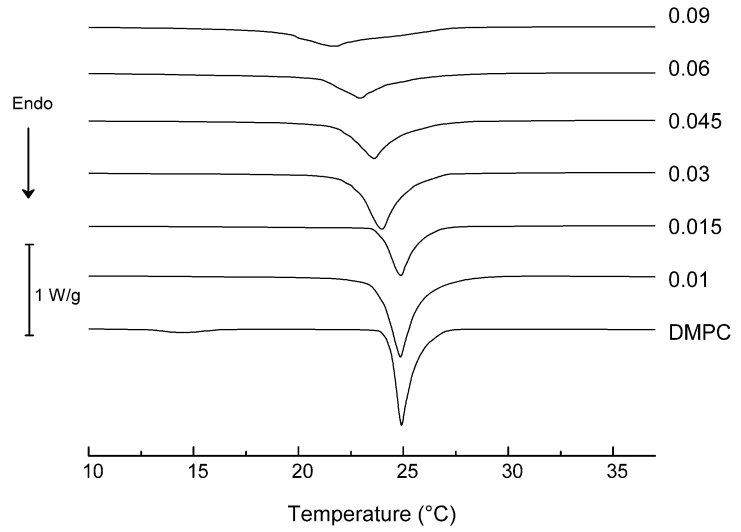


Fig. 6. DSC curves of DMPC MLVs loaded with different IDE–LAA7 molar fractions.

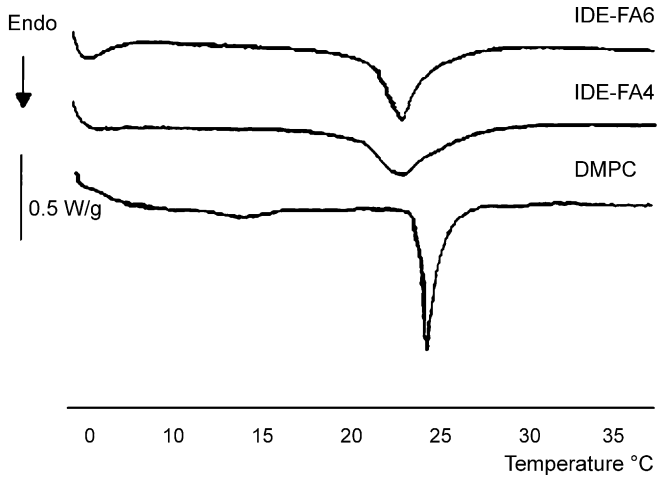


Fig. 7. DSC curves of DMPC MLVs loaded with different IDE–FA4 and IDE–FA6 molar fractions.

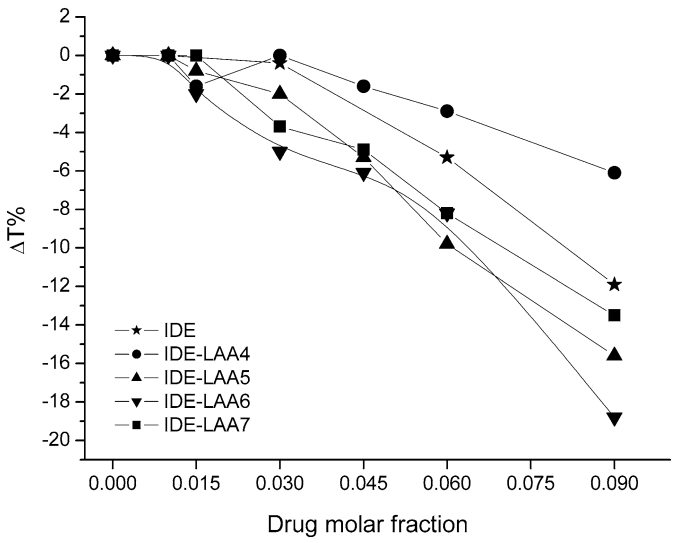


Fig. 8. Correlation between T_m changes and drug molar fraction.

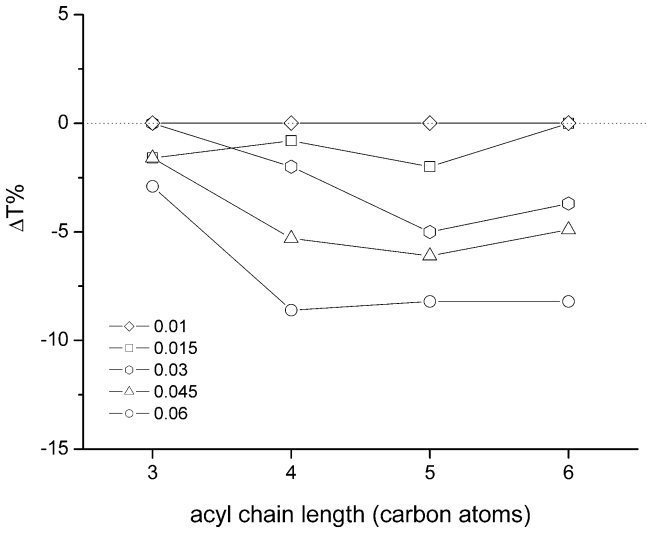


Fig. 9. Correlation between T_m changes and alkyl side-chain length of the lipo-amino acid residue.

phase segregation observed. Such behavior suggests that these compounds are still soluble in the DMPC bilayers at these concentrations.

It is noteworthy that the lower term of the series, IDE–LAA4, lowered the DMPC T_m value less than pure IDE (Fig. 8). It is conceivable that the introduction of the 2-aminobutyric moiety in the IDE molecule, although increasing the lipophilicity (see Table 1), increased its amphiphilic character. The resulting IDE–LAA4 conjugate was then able to accommodate more homogeneously within the phospholipid bilayers, without significantly altering their packing and the consequent thermotropic parameters.

The effect of the alkyl side chain length in IDE–LAA conjugates on T_m was limited and, except for the lower IDE–LAA4 term, considerable differences were not seen (Fig. 9).

Such a behavior indicates that the hydrophilic interaction with the polar head groups of DMPC, upon which T_m changes are mainly based, is related to the presence of the amino acid portion in IDE–LAA conjugates and is unaffected by the lengthening of the alkyl chain of the LAA used.

As regards the effects caused by IDE or its esters on the enthalpy changes associated with the main phase transition (ΔH), a less linear and more complex trend was observed. IDE depressed the phospholipid enthalpy changes, indicating its ability to penetrate within the bilayers and alter their cooperativity. When an LAA moiety was conjugated to IDE, such a reduction effect was always lower, as confirmed by the positive $\Delta(\Delta H)$ values shown by IDE–LAA esters. The increment of lipophilicity and/or steric hindrance given by the LAA probably reduced the capacity of conjugates to penetrate into the phospholipid bilayers or disturb their ordered structure.

In particular, the lower terms (IDE–LAA4 and IDE–LAA5) induced limited enthalpy changes, regardless of their molar fraction, whereas the more lipophilic terms IDE–LAA6 and IDE–LAA7 showed a biphasic profile: at low concentrations they gave small ΔH changes or even lower than those measured for pure DMPC, while at molar fractions above 0.03 the enthalpy changes were more marked.

These findings suggest that the latter conjugates can disturb the order of phospholipid bilayers only at high concentrations. In these conditions, conjugate molecules tended to aggregate in separate domains, which exerted a stronger disordering effect on the bilayers (Figs. 5 and 6). As a result, the measured effect on ΔH was much more evident than that registered for the lower homologues. In contrast, at lower molar fractions IDE–LAA6 and IDE–LAA7, because of their particular structure, could be well accommodated in the bilayers, without then altering their thermotropic profile and, instead, stabilizing the structure of DMPC vesicles (as confirmed by ΔH value increase).

A further comparison was made by plotting the molar fraction of conjugates in the liposomes vs the width at half height of the main phase transition peak ($t_{1/2}$). Such a value is known to numerically describe the shape of the peak, and thus represents a useful marker to understand the effects of a guest molecule within the phospholipid bilayers. Lipid-phase transitions are co-

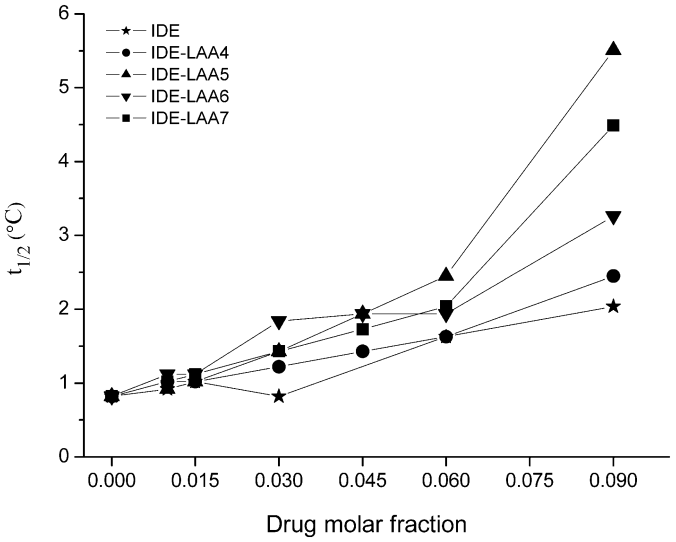


Fig. 10. Correlation between $t_{1/2}$ (main transition peak half-height width) and the drug molar fraction.

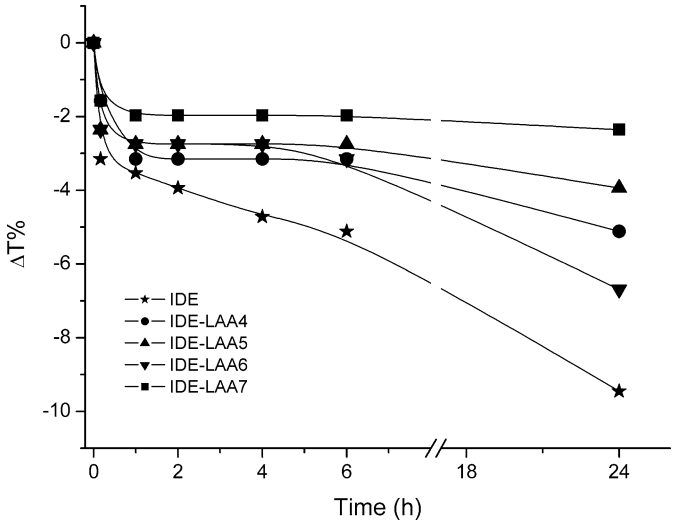


Fig. 11. T_m changes registered during the kinetic interaction experiments, performed by incubating IDE or IDE–LAA conjugates with empty DMPC MLVs at 35 °C.

operative phenomena, meaning that the behavior of a particular compound in one phase depends on the state of the surrounding molecules and on the number that are forced to cooperate in the transition itself. As a consequence, the broadening of the transition peak in the presence of foreign compounds indicates that the latter can intercalate within the phospholipid bilayers, reducing the homogeneity of the system and, at the same time, modifying the number of lipid molecules that a single phospholipid unit is able to influence.

In our experiments the $t_{1/2}$ value grew linearly while increasing the molar fraction of each IDE–LAA conjugate (Fig. 10). This behavior accounted for a progressive loss of cooperativity of the DMPC bilayers, as a consequence of the gradual penetration of conjugates into them. However, only slight differences were registered for the various terms of the series, as if the length of the alkyl side chain would have a limited significance for this calorimetric parameter. Only at the highest molar fraction (0.09) were marked differences among the conjugates observed, probably related to the above discussed phenomena of phase segregation (Figs. 3–6).

3.2. Calorimetric evaluation of the kinetics of interaction

Solid IDE or IDE–LAA conjugates were incubated with preformed empty DMPC MLVs. The amount of each compound and DMPC were chosen to achieve a 0.06 molar fraction of the former in the case of a complete mixing with the phospholipid. Periodic DSC scans were then performed in heating mode (after 5 min and then about 1, 2, 4, 6, and 24 h), joined by isothermal phases at 35 °C, as shown in Table 3.

In this kind of experiment, the solid drug can gradually enter into contact with the phospholipid bilayers. These studies have widely been used to monitor the gradual absorption of a compound from an aqueous phase to the biomembrane model [25–29]. The amount of drug that penetrates into the bilayers during the incubation can induce proportional and measurable changes in DMPC thermotropic parameters [17]. If a complete transfer of the drug from the aqueous medium to the phospholipid vesicles occurs, a DSC profile will be obtained that is similar to that registered when the liposomes are prepared in the presence of the same molar fraction of the drug (preparation of vesicles in organic phase).

Upon incubation of IDE–LAA conjugates with DMPC at 35 °C, the T_m of pure DMPC showed an initial shift to slight lower values (with a ΔT around 2–3%), thereafter remaining constant for the following 6 h. After 24 h, for most conjugates a further reduction of T_m was observed, approaching the value measured when the liposomes were prepared in organic phase with the same molar fraction of each IDE–LAA. Only the highest homologue IDE–LAA7 was unable to further reduce the T_m value of DMPC, probably because of its low solubility in the external aqueous medium and consequent difficulty in being adsorbed onto the bilayers. In any case, the progressive effect exerted by these conjugates was always less than that given by pure IDE, which instead showed a linear depression of T_m during the incubation (Fig. 11). This behavior indicates that the higher lipophilicity of IDE–LAA conjugates with respect to the parent drug, mainly because of their reduced solubility (cf. Table 1), hindered an equilibrium being reached between the aqueous phase and DMPC bilayers.

The $t_{1/2}$ variations measured during the kinetic transfer experiments are shown in Fig. 12. All the conjugates, as well as pure IDE, caused a sudden broadening of the main transition peak (i.e., an increase of $t_{1/2}$ value), particularly evident for the IDE–LAA4 conjugate. The two intermediate terms of the series, IDE–LAA5 and IDE–LAA6, caused a further broadening of the peak during incubation, reaching a similar $t_{1/2}$ value after 24 h. The last results indicate that all the IDE–LAA conjugates are able to alter the geometry of DMPC bilayers in a similar way, until an equilibrium between aqueous phase/vesicles is reached. It is noteworthy, however, that the lowest homologue, IDE–LAA4, is able to achieve such an equilibrium suddenly af-

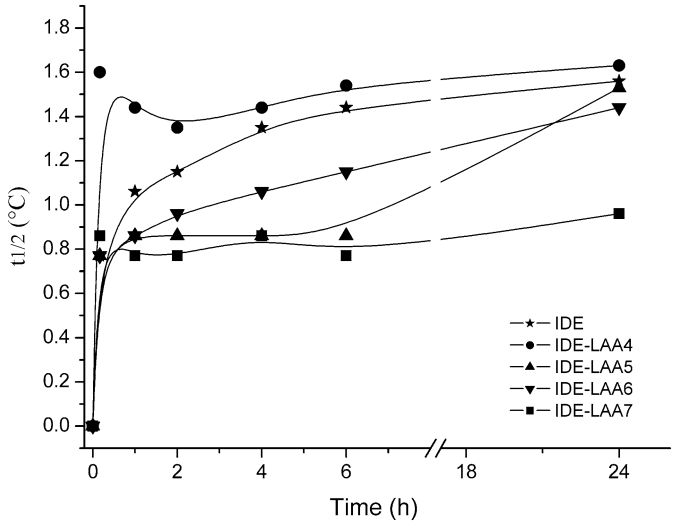


Fig. 12. Change of the $t_{1/2}$ value registered during the kinetic interaction experiments, performed by incubating IDE or IDE–LAA conjugates with empty DMPC MLVs at 35 °C.

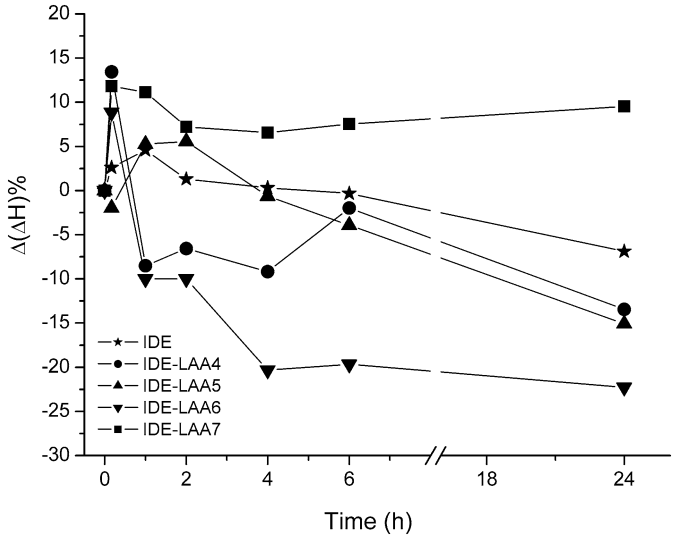


Fig. 13. ΔH changes registered during the interaction kinetic experiments, performed by incubating at 35 °C IDE or IDE–LAA conjugates with empty DMPC MLVs.

ter the first hour of incubation, while the other more lipophilic terms required a longer time of contact with the liposomes.

Finally, Fig. 13 shows the effects on enthalpy changes measured during the kinetic transfer experiments. Pure IDE affected such a parameter only marginally, in the same way as the highest homologue IDE–LAA7. Conversely, the other IDE–LAA conjugates induced a gradual reduction of DMPC ΔH values, from about 33 to 22–25 J/g. The depressant effect was particularly evident for the IDE–LAA6 conjugate (Fig. 13).

By considering the meaning of ΔH changes consequent to the interaction of phospholipids with external molecules, and the fact that multilamellar liposomes were used in these experiments, the progressive depression of ΔH exerted by IDE–LAA4, IDE–LAA5, and, mainly, IDE–LAA6 provided for their ability to penetrate gradually inside the DMPC bilayers.

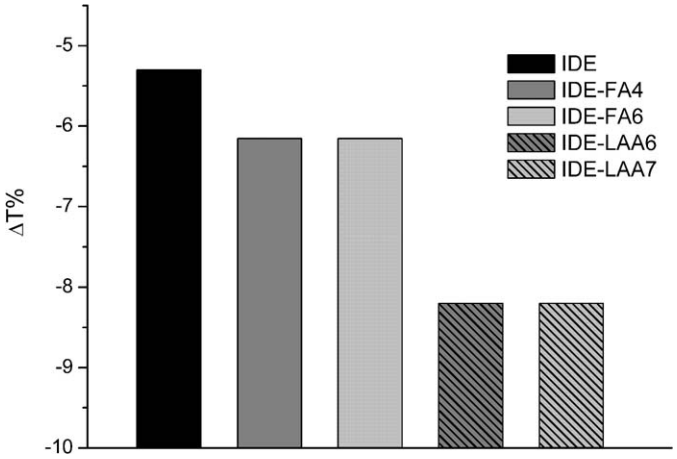


Fig. 14. Comparison between T_m changes induced on DMPC liposomes by a 0.06 IDE–LAA6 and IDE–LAA7 molar fraction, compared with those caused by the same molar fraction of pure IDE or IDE–alkanoate esters.

All these data led to a conclusion on how IDE or its lipophilic esters spontaneously interacted with preformed DMPC vesicles. The pure drug seemed to develop only a superficial interaction with the liposomes, but it was not able to diffuse spontaneously inside the inner bilayers, despite its lipophilic character. The IDE–LAA conjugates, as a result of their enhanced amphiphilicity due to the LAA moiety, although they were less able to interact with the phospholipid polar head groups (as shown by the lower effects on T_m), could slowly penetrate inside the vesicles, progressively altering the ordered geometry of DMPC acyl chains.

3.3. Comparison between DSC analysis of IDE–LAA and IDE–FA esters

A last comparison was made between the thermotropic behavior of DMPC MLVs loaded with the same 0.06 molar fraction of either the two IDE alkanoates IDE–FA4 and IDE–FA6, or the corresponding IDE–LAA6 and IDE–LAA7 conjugates, which display close $c\log P$ values (in the range 5–6; see Tables 1 and 2).

These compounds share a structural homology, at least with respect to the alkyl side chain length. However, the absence of a free amine group in the α position in the former pair of esters dramatically changes their physicochemical properties, as also shown by the calculated $\log P$ and solubility values (Tables 1 and 2). As a consequence, a different mode of interaction with the biomembrane model could be anticipated.

The DSC pattern of IDE–FA4 and IDE–FA6, at a 0.06 molar fraction in DMPC liposomes is shown in Fig. 7. Both compounds caused a strong deformation of the main transition peak, with a similar shift of DMPC T_m toward lower values. Such a trend can easily be correlated with the strong enhancement of lipophilicity induced by the presence of the FA moieties (see Table 1).

The two pairs of esters—IDE–LAA and IDE–FA—caused similar variations in the DMPC T_m value (Fig. 14). The effect was greater than that exerted by pure IDE and was more

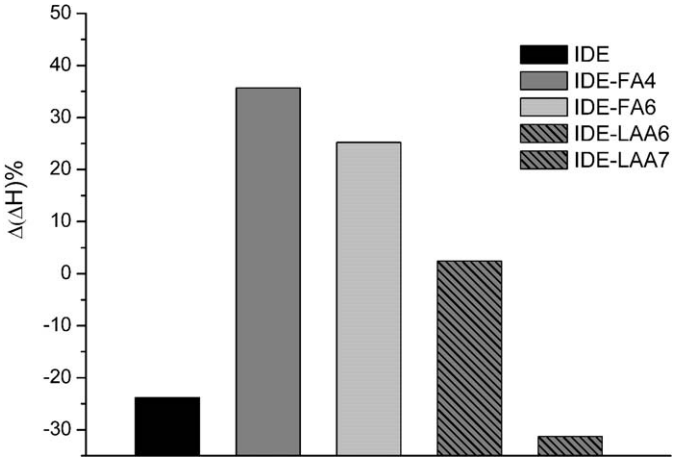


Fig. 15. Comparison between ΔH changes induced on DMPC liposomes by a 0.06 IDE–LAA6 and IDE–LAA7 molar fraction, compared with those caused by the same molar fraction of pure IDE or IDE–alkanoate esters.

marked for the IDE–LAA esters. The variations of this parameter are mainly associated with the interaction of drugs with the phospholipid polar heads. Thus, we can conclude that for compounds with close lipophilicity values, the free amine group of the LAA moiety was also strongly involved in this mode of interaction with DMPC bilayers.

The effects of enthalpy changes were much more complex (Fig. 15). The two alkanoic esters did not induce significant changes in the ΔH associated with the main DMPC phase transition, whereas pure IDE deeply altered the bilayer structure. IDE–LAA conjugates also depressed DMPC ΔH, although less than pure IDE. The comparison between the two pairs of esters with respect to this calorimetric parameter suggested that the mere increase of lipophilicity obtained by the esterification of IDE with alkanoic acids resulted in a substantial incapacity to interact in an ordered manner with the DMPC bilayers, either at the level of polar head groups, or by being evenly placed within DMPC acyl chains. The only registered effect was a strong alteration of the shape of the endothermic peak (see Fig. 7), which, however, indicated a loss of cooperativity of the liposomal system. These results further delineate the more complete mode of interaction with DMPC liposomes allowed by LAA moieties, with respect to the alkanoic acid residues.

4. Conclusions

LAA have been demonstrated to impart some amphiphilic properties to drug molecules, the so-called "membrane-like character," which can greatly affect their mode of interaction with biological membranes. They allow a more complete range of phenomena to occur, i.e., electrostatic and polar interactions, which can facilitate cellular uptake and passage across absorption barriers. These aspects become particularly interesting when applied to a drug that already possesses a high lipophilic character, such as IDE. For this kind of drug, in fact, lipophilicity can strongly hinder solubility in biological fluids and overall bioavailability. In contrast, conjugation to LAA, by converting lipophilicity into amphiphilicity, can enhance the affinity of the drug for cells and tissues.

The biomembrane model used in the present study, consisting of multilamellar liposomes made up of a pure phospholipid, is once more confirmed to be a reliable tool for studying the interactions with a foreign compound. With respect to the parent drug IDE and its lipophilic alkanoic esters, IDE–LAA conjugates developed a complex interaction with DMPC bilayers at different levels. In particular, with respect to pure IDE a greater ability to change the phospholipid main phase transition temperature was observed, with lower effects on the enthalpy changes associated to it. These different behaviors suggested a different site of interaction within DMPC bilayers as a results of the structure changes caused by LAA conjugation.

When IDE–LAA conjugates were incubated with empty liposomes in the liquid crystalline state, they showed an ability to penetrate slowly inside the bilayers, whereas pure IDE interacted only at the vesicle surface.

In conclusion, in agreement with similar results obtained for methotrexate [19] and tranylcypromine conjugates with LAA [18], the DSC analysis helped outline the usefulness of LAA moieties in increasing the affinity of drug molecules for cell membranes.

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Supplementary material

The online version of this article contains additional supplementary material.

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