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Potential application of plant lipid transfer proteins for drug delivery

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Abstract

Ligand-binding proteins show an increasing interest as drug carriers and delivery systems [Wolf FA, Brett GM. Pharmacol Rev, 2000;52:207–36]. The wide binding properties of plant non-specific lipid transfer proteins such as LTP1 also offer many unexplored possibilities for such a task. In the present paper, by using intrinsic tyrosine LTP1 fluorescence, we survey, for the first time, the binding of wheat LTP1 with various ligands having cosmetic or pharmaceutical applications. LTP1 was found to bind skin lipids such as sphingosine, sphingomyelin, and cerebroside with an affinity of about one micromolar, low enough to allow a slow release of these molecules. Ether phospholipids and an azole derivative BD56 having antitumoral and/or antileishmania properties were also shown to bind LTP1 with similar affinity. Finally, amphotericin B, which is widely used as an antifungal drug, was shown to form a complex with LTP1, although no affinity could be determined. This binding study is a prerequisite for further work aimed at developing applications in LTP-mediated transport and controlled release of low molecular weight drugs.

Keywords: Ligand-binding proteins; Drug delivery; Carriers; Ether phospholipids; Cosmetics; Pharmaceutics; Amphotericin B; Sphingolipids; Conazole

1. Introduction

Many active agents used in pharmaceutics and cosmetics need a protective shielding from sensitive environment. This can be achieved by using carrier systems which include ligand-binding proteins [1], liposome or emulsion technology [2–5], nano-particles [6,7], and polymers [8–11]. The growing interest in the field of ligand-binding proteins originates from their accessibility to engineering via modern genetic techniques. By this way, these carriers can be modified to become drug-specific binding systems. Moreover, it is possible to circumvent their potential immunogenicity by incorporating these proteins into other carrier systems such as liposomes. In the present paper, we survey the potential of plant lipid transfer proteins, LTP1, in view of their application in drug delivery. These small (9 Kd) proteins, which are present in high amounts in plants, are known for their ability to transfer *in vitro* phospholipids through membranes [12]. Moreover, they are already accessible to genetic modifications [13]. The three-dimensional structure of wheat LTP1 has shown the presence of a hydrophobic cavity surrounded by four α-helices [14,15]. LTP1s possess 8 cysteins, all implicated in disulfide bridges affording the stabilisation of the structure. The binding with fatty acids and phospholipids has now been widely investigated [12, 15–17] and it was confirmed that LTP1s are non-specific lipid-binding proteins. The first work dealing with potential application of LTP1 in drug binding was carried out through the structure determination of the complex with prostaglandin B_2 [17]. It revealed that the ligand embedded in the protein cavity is well shielded from the environment, suggesting promising applications of LTP1 as drug carriers. The aim of the present paper is to screen the binding of LTP1 with various hydrophobic molecules having cosmetic or pharmaceutical interest. Among these ligands, sphingolipids are potential molecules entering this category because they are found in the external epidermal layer of human skin, the *stratum corneum* [18]. Moreover, they are sphingosine derivatives, in other words, they exhibit a rod-like amphiphilic structure and should then interact with LTP1s. In the same way, ether phospholipid derivatives are potential candidates for interacting with those proteins. As an

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example, edelfosine is an ether lipid active against human protozoal pathogens such as *Leishmania* [19]. Leishmaniasis, diagnosed for more than 12 million people worldwide, represents a serious public health problem [20,21]. Edelfosine and ether phospholipids analogues have now entered a phase II clinical trial [22], whose success could represent a dramatic progress in the treatment of leishmaniasis, taking into account the lack of availability of a wholly satisfactory chemotherapeutic agent. Among antifungal conazole derivatives, ketoconazole, itraconazole, and more recently 1-indolyl-1-phenylmethylazoles [23] have confirmed their interest as antileishmanial drugs. Studying the binding of such molecules to LTP1 should also help for better understanding the lipid protein interactions. Indeed, these compounds depart from lipids studied so far [24] since they do not possess long acyl chains. Finally, we investigated the complex formation with amphotericin B, a very efficient antifungal drug also clinically used for treatment of antimonial resistant cases of leishmaniasis [25].

This work relating to the complex formation between LTP1 and various drugs represents a prerequisite study liable to allow further applications of LTP1 as drug carriers.

2. Materials and methods

2.1. Purification of wheat LTP1 and ligand preparation

LTP1 was purified according to previously published work [15] and prepared in 20 mM Tris pH 7.4 buffer at a concentration of 0.5 mg/mL (50 μM). Sphingolipids, purchased from Sigma, were solubilised at 3 mg/mL in methanol or in 20 mM Tris pH 7.4 buffer. The alkyl phosphorylethanol (see Fig. 2B) was a gift from Prof. Y. Letourneux (Université de La Rochelle, France) and was prepared at a concentration of 3 mg/mL in ethanol. Edelfosine and Amphotericin B, purchased from Sigma, were prepared at a concentration of 3 mg/mL in ethanol and in methanol, respectively. Conazole BD56, synthesized as in [23], was prepared at a concentration of 3 mg/mL in DMSO.

2.2. Binding assay

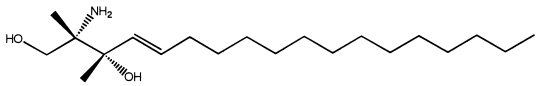
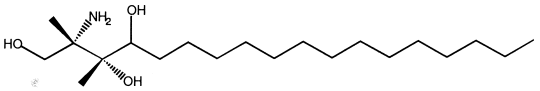
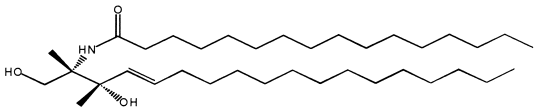
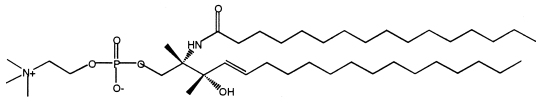
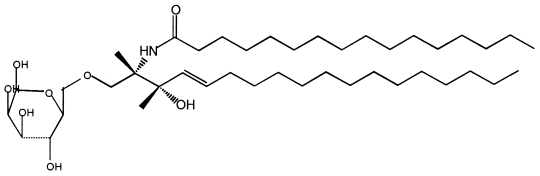
Fluorescence spectra were recorded with a Spex Fluoromax spectrophotometer. One millilitre of LTP1 solution was placed in a fluorescence cuvette at 25°; studied ligands were added in a step-wise manner. The evolution of LTP1 emission spectrum was followed from 280 nm to 500 nm after excitation at 275 nm. The fluorescence intensity was recorded at 304 nm and represented as a function of the lipid–protein ratio, Ri. Dissociation constant, *Kd*, and n, characterising the lipid–LTP1 interaction, were calculated by fitting experimental data with a mathematical model assuming independent and identical sites [24].

3. Results and discussion

3.1. Binding assay with sphingosine derivatives

The binding study between LTP1 and sphingosine, phytosphingosine, *N*-palmitoyl sphingosine, *N*-palmitoyl sphingomyelin, and *N*-palmitoyl cerebroside was investigated by fluorescence spectroscopy. This technique was successfully used for probing the interaction between LTP1 and phospholipids [24]. It is based on the variation of the fluorescence of the LTP1 tyrosine residues upon binding of ligands. Titration curves afford the determination of the dissociation constant, *Kd*, and the number of binding site, n. The structure of the sphingosine derivatives is depicted in Table 1 together with *Kd* and n as obtained by fitting the fluorescence data. In case of sphingosine at the endpoint titration, we observed a 1.5-fold increase in the fluorescence intensity, while the saturation occurred at about Ri = 4. This variation in fluorescence was analogous to that reported for the binding of fatty acids [24]. Regarding the number of binding sites in the case of sphingosine derivatives, our present data seem rather surprising. Until now, LTP1 was known to bind two lipid monomers [24] but no more acyl chains within the cavity. However, it must be remembered that lipids form aggregates or micelles in solution. As a consequence, there is a competition between the binding to LTP1 and formation of micelles that cannot be accounted for in the fitting procedure. It is then probable that LTP1 binds two monomers of sphingosine but also interacts with lipid aggregates in solution, yielding an overestimation of the number of binding sites. For phytosphingosine, whose structure is analogous to that of sphingosine except that it possesses a hydroxyl group instead of a double bond (Table 1), we observed a 2-fold increase in the fluorescence intensity of LTP1 tyrosine residues. Fitting the experimental data yielded a *Kd* of 5 μM and n of 2.7. As shown for other proteins [26], such a difference in the increase in fluorescence at saturation comes from the fact that the additional hydroxyl group of phytosphingosine probably makes a hydrogen bond with the Tyr79 residue. Moreover, this is due to the fact that lipids are differently positioned within the protein cavity and then the tyrosine residue. However, it must be remembered that it does not interfere with the fitting procedure for obtaining *Kd* and n [24]. In the same way, we investigated the complex formation of LTP1 with sphingolipids having two aliphatic chains (Table 1). Only *N*-palmitoyl sphingomyelin was prepared in buffer and because *N*-palmitoyl sphingosine and *N*-palmitoyl cerebroside were insoluble, there were both solubilised in methanol. It was previously verified that methanol did not interfere with the intrinsic fluorescence of LTP1 at a concentration lower than 10% (not shown). For these sphingolipids, we observed a lesser increase in fluorescence intensity than with sphingolipids possessing one acyl chain (Fig. 1). The affinity for *N*-palmitoyl sphingosine is comparable to that of mono-acyl sphingosine derivatives while for both

Table 1
Chemical structure of the sphingosine derivatives used for the binding study with LTP1. *Kd* (± 1 μM) and n (± 0.1) are also given as obtained by fitting the fluorescence data.

		Structure	*Kd* (μM)	n
1 chain	sphingosine		2	3.9
	phytosphingosine		5	2.7
2 chains	N-palmitoyl sphingosine*		1	2.6
	N-palmitoyl sphingomyelin		61	1.7
	N-palmitoyl cerebroside*		68	2.1

* Ligand solubilised in methanol.

other lipids, the affinity markedly decreases. The fact that LTP1 are capable of binding cerebroside is interesting because this lipid possesses a bulky polar head. Such a polar head certainly cannot cross into the LTP1 cavity, suggesting that lipids interact with the protein via their acyl chains. Taken together, these experiments show that LTP1 binds to skin lipids with a low affinity, which is a prerequisite for lipid transfer. As a consequence, this makes them attractive for controlled delivery of such molecules in membranes. Moreover, LTP1s are also known to bind oleic acid and the C18 fatty acid family [24] which are present in the skin sebum [27]; such data support the interest of further work aimed at testing their cosmetic or drug transport capacities.

3.2. Binding assay with ether phospholipid derivatives

The binding of LTP1 with two ether phospholipids including edelfosine (Fig. 2A) and an ilmofosine derivative (Fig. 2B) was performed as above. The interaction with wheat LTP1 induced a 2-fold increase in tyrosine fluorescence intensity at saturation (Fig. 3A). Emission spectra in the presence of edelfosine showed an additional emission intensity at about 400 nm due to impurities in the commer-

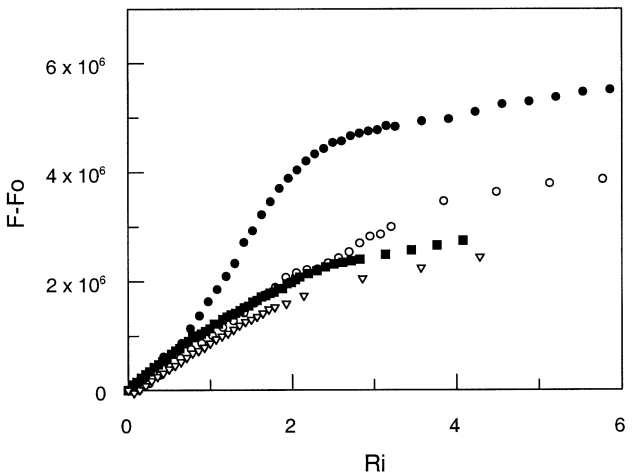


Fig. 1. Variation of fluorescence as a function of the molar ratio lipid/LTP, Ri, for the various sphingolipids: sphingosine (○), phytosphingosine (●), *N*-palmitoyl sphingosine (▽), and *N*-palmitoyl sphingomyelin (■). Standard deviation is within the symbol size.

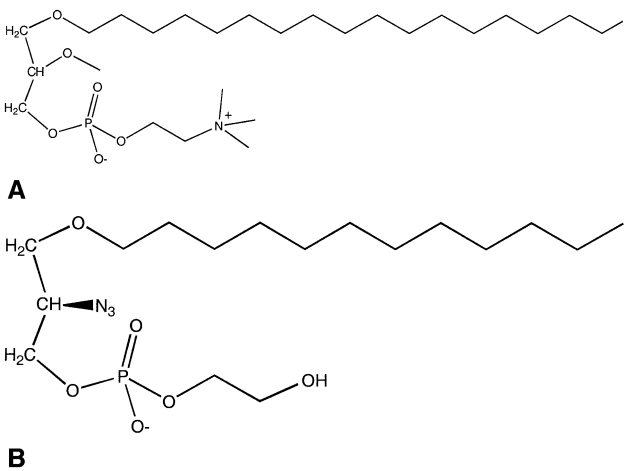


Fig. 2. (A) Chemical structure of edelfosine. (B) Chemical structure of the alkylphosphotrylethanol, an ilmofosine derivative.

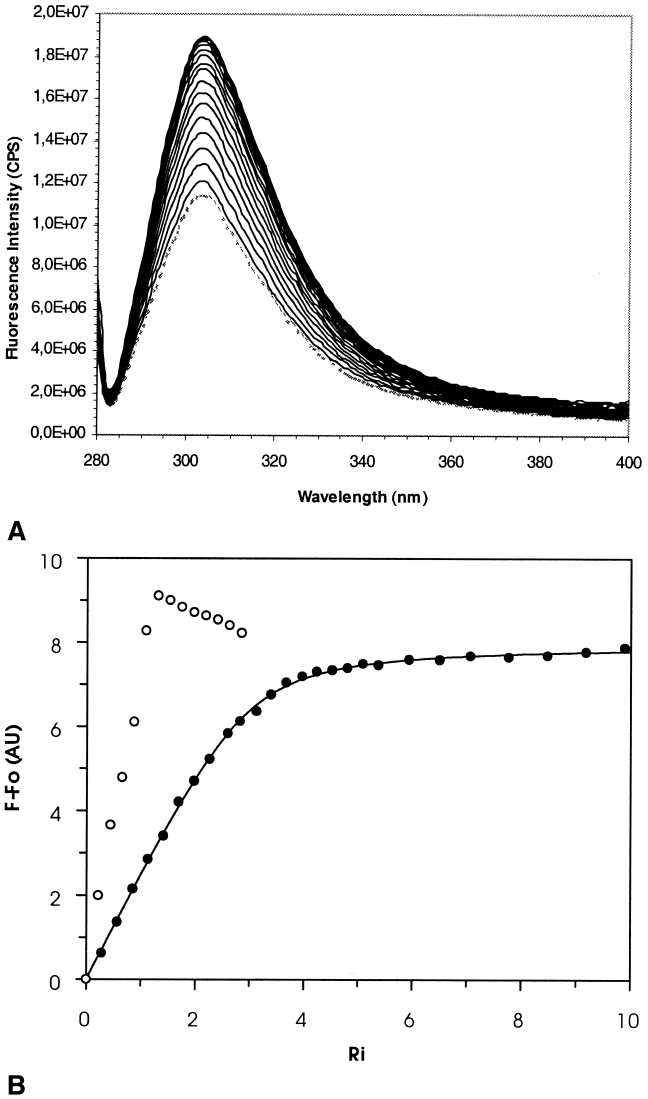


Fig. 3. (A) Intrinsic tyrosine fluorescence spectra of wheat LTP1, free (—) and complexed with the ilmofosine derivative (—) by the procedure described in the text. (B) Variation of fluorescence as a function of the molar ratio lipid/LTP, Ri, for etherphospholipids: edelfosine (○) and $C_{12}N_3GPOH$ (●). The curve represents the best fit obtained with the non-cooperative model.

cial product (not shown). The variation in fluorescence intensity recorded at 304 nm is presented in Fig. 3B as a function of Ri. LTP1 indeed interacted with edelfosine, although data could not be fitted for the reasons mentioned above. A *Kd* of 3.4 ± 0.1 μM and n = 3.0 ± 0.1 were obtained by fitting experimental data obtained with the alkylphosphorylethanol.

Such ether phospholipid analogues are known to be of strong interest since they are proposed as antileishmanial drugs [22]. However, they generate serious side-effects including hemolysis [28], pulmonary oedema, impairment of hepatic function [29], and cytotoxicity [30], which hampers their optimal use. Once again, the binding of such lipids by LTP1 suggests a potential application for these proteins as antileishmanial drug carrier systems.

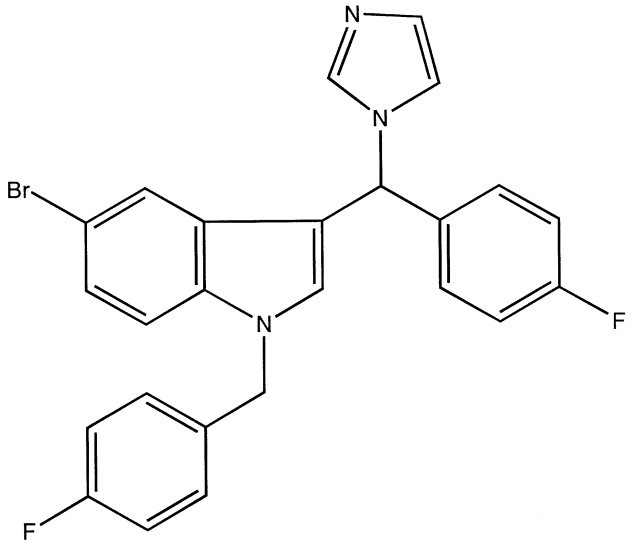


Fig. 4. Chemical structure of BD56 according to the French Patent FR 62937L.

3.3. Binding assay with conazole BD56

As above, the intrinsic fluorescence of LTP1 and BD56 [23] was used for probing the complex formation. However, BD56 possesses an imidazole group (Fig. 4) so that it has analogous fluorescence characteristic as tryptophane residue. Then, upon excitation at 280 nm, an emission spectrum can be observed from 310 to 350 nm depending on the hydrophobicity of the medium. This is illustrated in Fig. 5, where BD56, solubilised in methanol, was added in the cuvette containing only the buffer. In such a polar medium, the maximum of pure BD56 fluorescence intensity was observed at 325 nm. Upon addition of BD56 in buffer containing LTP1, the signal corresponding to LTP1 emission decreased because the absorbence coefficient of BD56 was greater than that of the tyrosine residues of LTP1. In the same way, the emission spectrum of BD56 increased (because it was added to the cuvette) together with a shift in the maximum fluorescence signal (Fig. 5). This red-shift showed that the environment of BD56 changed, suggesting that it was bound within the hydrophobic cavity of the protein. However, the dissociation constant of the complex could not be determined in such a case. Competition fluorescence studies were also performed (not shown) according to Zachowski *et al*. (1998), confirming that the binding did occur. The binding of such a bulky molecule is also of strong interest and should contribute to our understanding of the lipid/LTP1 interactions. Indeed, for steric hindrance reasons, it appears improbable that the molecule can be fully loaded within the protein cavity. We suggest that only a part of the BD56 molecule fits within the protein core so that the other part would be exposed to the solvent.

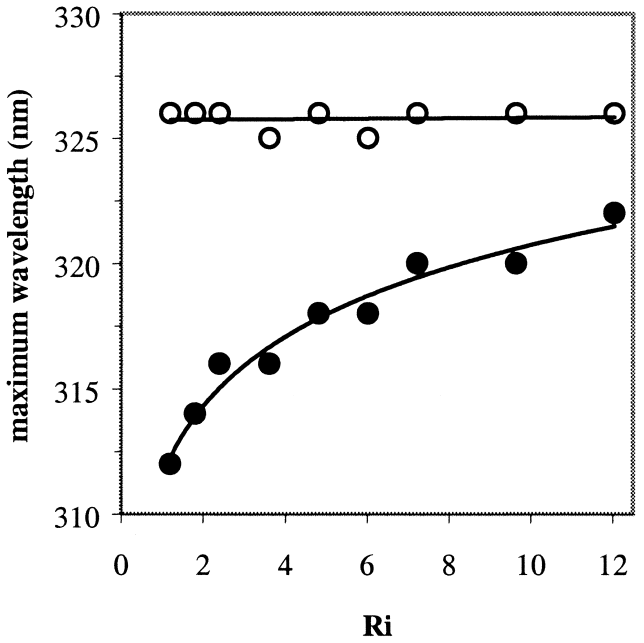


Fig. 5. Maximum wavelength of BD56 as a function of the molar ratio BD56/LTP1, in absence (○) and in presence (●) of LTP1.

3.4. Binding assay with amphotericin B

The interaction between LTP1 and amphotericin B was studied as previously for other lipids. It must be reminded here that amphotericin B is a polyene macrolide antibiotic [31] used in the treatment of systemic fungal infections and in drug-resistant visceral leishmaniasis [32]. When it was added to the LTP1-containing cuvette, fluorescence spectrum showed a marked decrease in tyrosine fluorescence (Fig. 6). This was accompanied by the appearance of a large peak at 450 nm. However, pure amphotericin B was not shown to fluoresce upon excitation at 275 nm. Rather, a broad peak with a maximum intensity at 450 nm appeared upon excitation at 305 nm (not shown). This revealed that, upon addition of amphotericin B to LTP1 solution (Fig. 6), a fluorescence transfer occurs. Such a feature only happens if tyrosine residues and the ligand are located at a distance

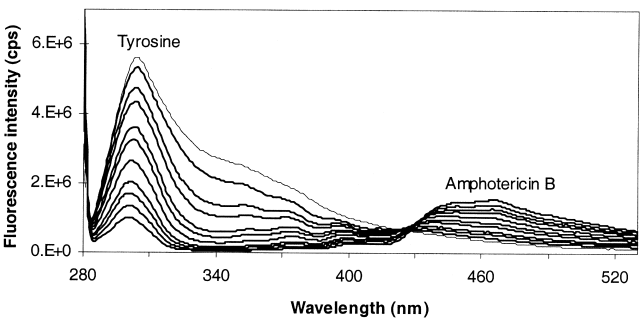


Fig. 6. Intrinsic tyrosine fluorescence spectra of wheat LTP1, free state (—) and complexed with amphotericin B (—), as obtained by the procedure described in the text.

called the Forster distance which is of few Å. This clearly shows that amphotericin B is bound by LTP1, although the dissociation constant could not be determined. Compared to BD56, amphotericin B is also a bulky and rigid molecule, and it appears improbable that it is fully loaded within the protein cavity.

The encapsulation of amphotericin B continues to attract considerable interest. Indeed, at therapeutical doses, amphotericin B exhibits severe side-effects such as renal dysfunction with both tubular and peritubular damage within the kidney. Traditionally, commercial amphotericin B is complexed with sodium desoxycholate (Fungizone®), while other formulations (Ambisome®, Amphocil®, and Intralipid®) have also shown a great potential [33]. However, systemic use of amphotericin B is often limited to treatment of invasive fungal infections as pulmonary aspergillosis [34] in AIDS and cancer disease [35]. Our present work on the binding of amphotericin B by plant LTP1 points out that such proteins could also be used as carrier systems for this drug.

In the present note, the survey of the binding between plant LTP1 and various molecules having cosmetic or pharmacological interest brings to the fore that these proteins have potential applications in the field of drug delivery. This work represents a prerequisite for further studies including *in vitro* and *in vivo* tests of the complex between various drugs and LTP1. Moreover, these proteins exhibit a transfer activity that should make their use attractive when drugs have to penetrate the lipid membrane.

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