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Interaction of arginine oligomer with model membrane

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Abstract

Short oligomers of arginine (R8) have been shown to cross readily a variety of biological barriers. A hypothesis was put forward that inverted micelles form in biological membranes in the presence of arginine oligomer peptides, facilitating their transfer through the membranes. In order to define the role of peptide–lipid interaction in this mechanism, we prepared liposomes as the model membrane to study the ability of R8 inducing calcein release from liposomes, the fusion of liposomes, R8 binding to liposomes and membrane disturbing activity of the bound R8. The results show that R8 binding to liposome membrane depends on lipid compositions, negative surface charge density and interior water phase pH values of liposomes. R8 has no activity to induce the leakage of calcein from liposomes or improve liposome fusion. R8 does not permeabilize through the membrane spontaneously. These peptides delivering drugs through membranes may depend on receptors and energy.

Keywords: Interaction; Arginine oligomer; Peptide-membrane; Partition coefficient; Liposomes

Nuclear transcription activator protein (Tat) is encoded by HIV type1 (HIV-1), a 101-aa protein that is required for viral replication [1,2]. Of particular interest for drug delivery is that exogenously added HIV-1 Tat efficiently crosses the plasma membranes of cells localizes to the nucleus, and is functional, stimulating HIV-long terminal repeat-driven RNA synthesis [1–6]. The sequence responsible for the cellular uptake of HIV-1 Tat consists of highly basic region amino acid residues 49–57 (RKKRRQRRR) [7–10]. Through covalent attachment to $Tat_{49–57}$, several proteins have been delivered into cells [8–13].

Tat is an arginine-rich peptide. Arginine oligomers, potentially structural analogies to $Tat_{49–57}$, were synthesized and their cellular uptake were compared [14–16]. Interestingly, short oligomers of arginine were much more efficient at entering cell than the corresponding short oligomers of histidine, lysine and ornithine [14], furthermore, a 9mer of L-arginine (R9) was 20-fold more efficient than $Tat_{49–57}$ at cellular uptake as determined by Michaelis–Menten kinetic analysis. The D-arginine (r9) exhibited an even greater uptake rate enhancement (>100-fold) [16]. One arginine oligomer has been used to deliver cycloporin into skin. A conjugate of heptamer of arginine to cyclosporin (R7-CsA) was efficiently transported into cells in mouse and human skin, reaching dermal T lymphocytes and inhibiting cutaneous inflammation [17].

We are interested in the arginine oligomers acting as a potential transporter to deliver drug across biological membrane. Previous studies showed oligomers of L- or D-arginine containing six or more amino acids entered cells far more effectively than oligomers of equal length composed of lysine, ornithine and histidine. Peptides of fewer than six amino acids were ineffective [14,16]. These studies suggested that the guanidinium groups in these peptides play a greater role in facilitating cellular uptake than either charge or backbone structure. Guanhidinium group in arginine has higher pK (12.48) than amino group in lysine (pK = 10.79). A hypothesis was put forward that inverted micelles form in biological membrane in the presence of arginine oligomers, facilitating their transfer through the

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membrane. Because of the formation of reversed micelles in the membrane, the membrane is disrupted transiently. This process is spontaneous and energy-independent [18]. In order to examine the hypothesis, large unilamellar vesicles(LUVs) were prepared and used as a model of biological membrane to study the interaction of membrane with octamer of arginine (R8). We used fluorospectrophotometry method to study the ability of R8 inducing calcein release from liposomes, the fusion of liposomes, R8 binding to liposomes and membrane disturbing activity of the bound R8.

Materials and methods

Materials. Octamer of arginine (R8) and R8 labeled with fluorescent isothiocyanate (FITC-R8) were provided kindly by CellGate Inc. Egg phosphotidylcholine (PC), distearoyl phosphatidylglycerol (DSPG) and cholesterol (Ch) were purchased from Avanti polar lipids (Alabaster, AL, USA). Water was deionized and then distilled. All other chemicals were reagent grade.

Liposomes preparation. Large unilamellar liposomes were prepared with egg PC, or along with DSPG or Ch. The molar ratio was 7:3, 7:0.5 and 7:3 for PC/DSPG(7:3) liposomes, PC/DSPG (7:0.5) liposomes and PC/Ch (7:3) liposomes, respectively. Lipid was dried first under a stream of argon gas and then vacuum dried for a minimum of several hours. The thin lipid film was then dispersed in 1.0 ml of buffer (5 mM Hepes, 140 mM NaCl, 10 mM EDTA, pH 7.4) to a concentration of 35 mM. For liposome leakage studies, the lipid suspension buffer contained 70 mM calcein. The suspension was subjected to five freeze-thaw cycles and subsequently extruded through two stacked 0.1 μm polycarbonate filters 20 times at 45 °C. The unentrapped Calcein was removed by passing the liposome suspension through a sephadex G-120 gel filtration column. The liposome fractions were stored at 4 °C and used within 3 days after preparation. Phospholipid concentrations were determined by a phosphate assay that has been modified to use sulfuric acid in place of perchloric acid [19].

Binding isotherm. FITC-R8 was employed to study R8 binding to liposomes. Fluorescence-derived binding isotherms were determined from the fluorescence intensity change of peptide solution after adding liposomes. For measuring the binding of FITC-R8 to liposomes, excitation and emission wavelength were set at 490 and 520 nm, respectively. Aliquots of a concentrated stock solution of liposomes (7.0 mM) were added to the peptide solution (0.5 μM), each measurement was performed after equilibrium was reached. Binding dynamics was also measured at certain interval time. For determination of binding isotherms, the following formulas were used [20]: (1) $r = \mathrm{Kp} \times C_f$, Where r is defined as the molar ratio of bound peptide per total lipid, Kp corresponds to the partition coefficient and C_f represents the equilibrium concentration of peptide in the solution. (2) $F = F_\infty(C_B/C_P) = F_\infty(C_L/C_P)r$, where F is the fluorescence value, F_∞ is F of completely bound peptide, C_L the lipid concentration, C_P the total peptide concentration, C_B the lipid-bound peptide concentration and $r = C_B/C_L$.

Calcein release assay. Liposome leakage promotion induced by R8 was monitored by an increase in calcein fluorescence intensity [21]. Liposomes were diluted in buffer to a final lipid concentration of 30–45 μM. Exact phospholipid concentrations were measured after leakage experiments. R8 was added to the liposome suspension. After 60-min incubation at 25 °C, samples were analyzed for fluorescence with a F-2000 fluorometer (HITACHI, Japan), set at an excitation wavelength of 490 nm and an emission wavelength of 520 nm. Percentage leakage was calculated as $[(F - F_0)/F_{100} - F_0] \times 100$, where F_{100} *is* the F due to total release of calcein induced by addition of 0.5% (v/v) Triton X-100, and F_0 is the initial F before addition of calcein. For calcein leakage of time-dependent fashion, R8 was added to the liposome suspension in a 1:200 molar ratio, and at indicating time, samples were measured for fluorescence intensity.

Lipid-mixing experiments. Mixing of liposome membrane was followed by measuring the fluorescence increase of FITC-DHPE (Avanti polar lipids), occurring after the fusion of labeled and unlabeled liposome. Labeled liposomes were obtained by incorporating FITC-DHPE in the dry lipid film at a concentration of 6.3% of the total lipid weight. R8 was added to a mixture of labeled and unlabeled liposomes (1:4 w/w). Final lipid concentration was 105 μM in Hepes buffer (pH 7.4). In a control experiment, the same volume of buffer is added to the liposome mixture. Fluorescence was recorded at room temperature on a fluorometer (λex 490 nm, λem 520 nm).

Results and discussion

An important element of current interest is the identification of short specific domains in messenger proteins that are used to internalize cargoes into live cells and across the biomembrane. These transporter peptides deliver cargoes across biomembrane barrier to the intracellular specific sites, such as cytoplasma and nucleus. The cargo size can be variable from small molecules to 200 nm particles [17,23,24]. Recent studies exhibited that short oligomers of arginine were more efficient than $Tat_{49–57}$ and oligomers of equal length composed of lysine, ronithine and histidine at cellular uptake. This study reported herein attempts to address a mechanism for the translocation through biological membrane of arginine oligomer peptides. Liposomes are lipid bilayer models suitable for determining by fluorescence the influence of lipid on bound peptides affinity [25].

Binding isotherms

The FITC fluorescence is sensitive to the polarity of its environment. The alteration of fluorescence intensity was observed when FITC-R8 was in a different environment at 25 °C. Fluorescence spectra analysis shows that shift in maximum fluorescence in different composition liposomes (maximum fluorescence at 520 nm) did not occur except for PC/DSPG (7:3, molar ratio) liposomes, which quenched the fluorescence of FITC-R8 at 520 nm.

The FITC-labeled R8, at a concentration of 0.5 μM, was titrated with the liposomes of different membrane compositions. The binding curves were obtained by plotting the resulted increase in fluorescence intensity of FITC-R8 against lipid to R8 molar ratio (Fig. 1A). An increase in the florescence intensity was observed when FITC-R8 was titrated with PC, PC/Ch (7:3) and PC/DSPG (7:0.5) liposomes (except for PC/DSPG (7:3) liposomes). It should be noted that the partition coefficient depends on the concentration of lipid accessible to peptide. The fluorescence intensity of FITC-R8 titrated by PC/DSPG (7:3) liposomes decreased with the increasing lipid/peptide molar ratio (Fig. 1A). At the ratio of 200:1, the fluorescence intensity of FITC-R8 is entirely extinguished, indicating that the most of R8 bind at the surface of liposomes. In contrast, using PC/DSPG (7:0.5) liposomes titrating FITC-R8, the FITC fluorescence intensity was enhanced with the increasing lipid/peptide molar ratio. This complete opposite result suggests that when electrostatic force between the peptide

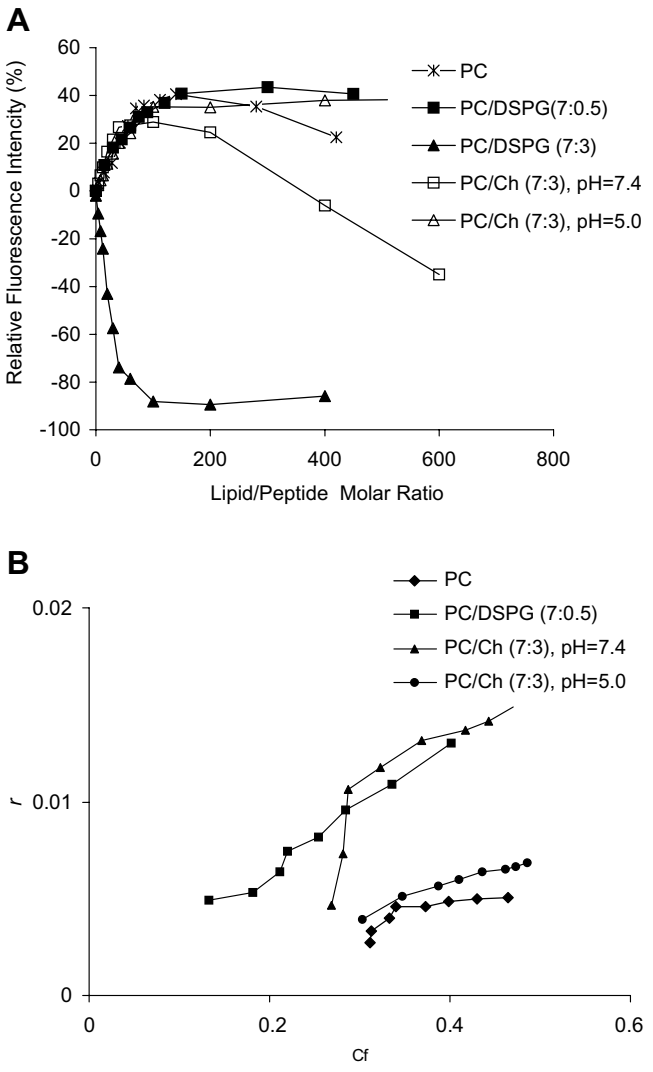

Fig. 1. (A) Fluorescence intensity of lipid-binding affinity of FITC-R8 to PC (∗), PC/DSPG (7:0.5) (■), PC/DSPG (7:3) (▲), PC/Ch (7:3, interior pH = 7.4) (□), and PC/Ch (7:3, interior pH = 5.0) liposomes (Δ) in buffer (10 mM Hepes, 140 mM NaCl, 1 mM EDTA, pH = 7.4) at 25 °C. The data represent mean values of three independent experiments. (B) Binding isotherm of FITC-R8 with PC (♦), PC/DSPG (7:0.5) (■), PC/Ch (7:3, interior pH = 7.4) (▲), and PC/Ch (7:3, interior pH = 5.0) (●) liposomes. The data represent mean values of three independent experiments.

and the liposomes become strong enough, a majority of FITC-R8 in solution accumulated at the surface of liposome where FITC-R8 concentration could be too high. The fluorescence intensity of FITC is dependent on the distance between fluorophore species, when FITC is accumulated at the surface of liposomes, the distance between the FITC molecules is comparatively small. This proximity of FITC species leads to self-quenching behavior and then a low fluorescence signal, even entirely quenching.

Cholesterol is one of the most important components of biomembrane. The results show that the effect of cholesterol on fluorescence intensity of FITC-R8 binding to liposomes was contradictory. For PC/Ch (7:3) liposomes, at lower lipid/R8 molar ratio (⩽100:1), the increase in FITC fluorescence intensity was observed with the increasing lipid/R8 molar ratio, but when the lipid/R8 molar ratio was higher than 100:1, FITC fluorescence was quenched significantly. Mixture of PC and Ch is known to form two liquid-crystalline phases, liquid ordered phase and disordered phase. The phase diagrams for binary mixture of Ch with PC show that liquid ordered phase and disordered phase exist in the Ch concentration range 0 to 7–23 mol % and 25–33 to 50 mol %, respectively [26]. We used Ch concentration of 30 mol % in liposomes, the liposomes could form liquid ordered phase and have low membrane fluidity. Corresponding fluidity of FITC-R8 molecules binding to PC/Ch (7:3) liposomes could be decreased, leading to low fluorescence intensity. Interestingly, reducing the pH value of interior water phase of the liposomes from 7.4 to 5.0, fluorescence quenching induced by cholesterol was inhibited in part, indicating that the addition of Ch to the PC liposomes alternates the behavior of association of R8-liposomes.

The curves obtained by plotting r (the molar ratio of bound R8 per total lipid) versus C_f (the equilibrium concentration of free peptide in the solution) are referred to conventional binding isotherms (Fig. 1B, except for PC/DSPG (7:3) liposomes). The shape of the binding isotherm of FITC-R8 to PC/DSPG (7:0.5) liposomes and PC/Ch (7:3) liposomes with interior water phase pH 5.0 is linear, indicating that R8 accumulation at the surface is a simple phenomenon without cooperation association [22]. The shape of the binding isotherm of FITC-R8 to PC liposomes and PC/Ch (7:3) liposomes with interior water phase pH 7.4 is nonlinear, revealing that cooperative association may be involved.

The surface partition coefficient Kp was estimated by extrapolating the initial slopes of the curves to C_f values of zero for PC/DSPG (7:0.5) liposomes and PC/Ch (7:3) liposomes with interior water phase pH 5.0. For PC liposomes and PC/Ch liposomes with interior water phase pH 7.4, the Kp was estimated by extrapolating the slopes of higher C_f concentrations, because when the C_f values were lower than 0.26 and 0.31 μM, respectively, the r values sharply decreased, we cannot estimate the Kp by extrapolating the initial slopes of the curves to C_f values of zero. The Kp of FITC-R8 binding to PC liposomes is about 4900 M^{-1}; addition of DSPG to PC liposomes (PC/DSPG, 7:0.5) increases the binding affinity of the FITC-R8, and the corresponding Kp is 6-fold higher. This suggests that electrostatic interaction between R8 and the phospholipid is important for R8 anchoring into the membrane. The binding affinity of R8 increases with addition of cholesterol to the PC liposomes. The Kp of R8 to PC/Ch (7:3) liposomes with interior water phase pH 7.4 is 4-fold higher than that to PC liposomes. However, adjusting the interior water phase pH value of PC/Ch (7:3) liposomes by encapsulating 250 mM ammonium sulfate, pH 5.0 solution, decreases the binding affinity of the R8, and the corresponding Kp is 2.5-fold higher than that determined with PC liposomes. Activation energies associated with diffusion

across membranes have been rationalized on the basis of the need to break extramolecular hydrogen bonds to enter the hydrocarbon matrix and the ability to reform intramolecular hydrogen bonds in the hydrocarbon [27,28]. Activation energies can then be estimated according to the number of hydrogen bonds that must be broken, which is 5 kcal/mol. On this basis activation energies can be calculated between R8 and cholesterol (data no shown). Hydrogen bonds between R8 and hydrocarbon can not form due to R8 located at surface of membrane. PC/Ch liposomes with acidic interior have a transmembrane pH gradient ($\Delta pH = 2.4$, 1 $\Delta pH = 5.74$ kJ/mol) that gives the energy to break hydrogen bonds between R8 and cholesterol. Obviously, in addition to electrostatic forces, our experiments provide proof principle for the effect of cholesterol on the interaction of R8 with phospholipid bilayer.

Calcein leakage

Liposomes made from different components were created with calcein, a fluorophore, trapped in the interior water phase. Calcein fluorescence intensity increases when it releases from the liposomes and is thereby an indicator of leakage. Fluorescence change was plotted against either time (Fig. 2A) or the R8 concentration (Fig. 2B). It can be seen that R8 does not have the activity to induce the leakage of calcein from all the liposomes (Fig. 2A). No activity of R8 inducing the leakage of calcein from the liposomes was observed with the molar ratio of R8/lipid from 0.001 to 0.01 (Fig. 2B). For PC/DSPG (7:3) liposomes, the R8 quickly precipitates out of solution when the molar ratio of R8 to lipid is $\geqslant 0.01$. For PC, PC/DSPG (7:0.5) and PC/Ch (7:3) liposomes, increasing the molar ratio of R8/lipid to 0.1, R8 inducing an increase of the calcein fluorescence was not evident (data not shown). These data suggest that R8 has no activity to induce leakage of calcein from phospholipid bilayers, not only independent of membrane compositions also independent of surface charge density. This observation gives rise to a challenge for the hypothesis that inverted micelles form in biological membrane in the presence of translocation peptides, facilitating their transfer through the membrane. The membrane is disrupted transiently. This process is spontaneous and energy-independent. If the hypothesis is correct, extracellular solutes can enter cell in the presence of arginine oligomers, corresponding reversible process is possible that intracellular solutes can efflux out of cell into extracellular medium. This would lead to the release of the calcein loaded in liposomes [29,30]. In addition, we loaded FITC-R8 in the liposomes with various membrane compositions, in order to avoid the electrostatic interaction between the liposome and the R8, DSPG was not used. An increase in the Fluorescence intensity of FITC-R8 loaded in the liposomes in the release experiment during 24 h at 25 or 37 °C was not observed (data not shown), which suggests that FITC-R8 does not have membrane-permeabilizing activity. With these results, we could conclude that R8 does not penetrate through

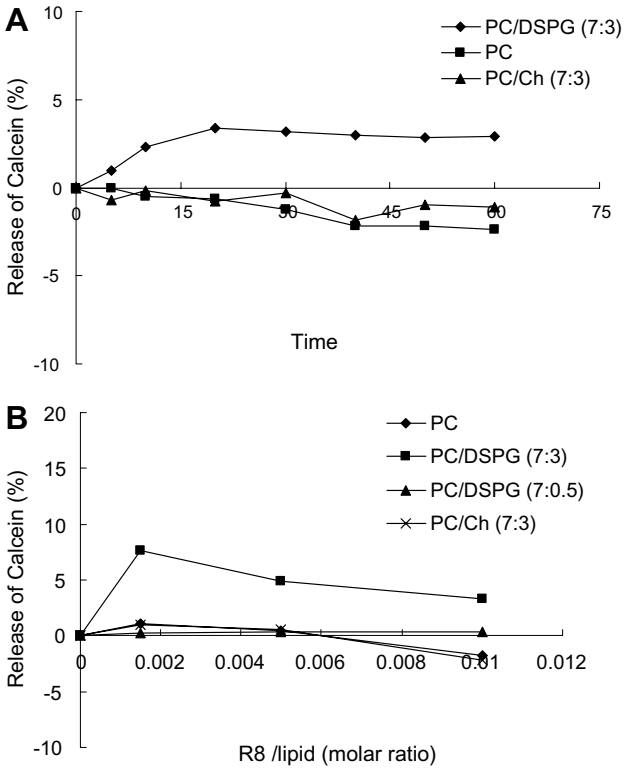

Fig. 2. (A) Time course of percentage leakage of calcein from PC (■), PC/DSPG (7:3) (♦) and PC/Ch (7:3) (▲) liposomes induced by R8 for different time at 25 °C. R8 is added to the liposome suspension in a 1:200 molar ratio. The data represent mean values of three independent experiments. (B) Influence of the R8 concentration on the leakage of calcein from PC (♦), PC/DSPG (7:0.5) (▲), PC/DSPG (7:3) (■) and PC/Ch (7:3) (×) liposomes.

phospholipid bilayer, only interacts with lipid polar head groups and locates at the hydrophilic surface of phospholipid membrane.

Lipid mixing assay

The capacity of R8 inducing membrane fusion between liposomes was determined, by mixing FITC-labeled and FITC-free liposomes and the increase in fluorescence intensity due to the dequenching of the probe is indicator of lipid fusion. Incubation of labeled and unlabeled liposomes in buffer alone did not modify the fluorescence intensity (Fig. 3A). At a 1:20 molar ratio of R8 to lipid, the increase in fluorescence was at the same level as in the buffer alone, which supports the fact that the R8 has no fusogenic activity. Increasing amounts of R8 were added to liposomes, and the FITC fluorescence intensity change recorded 30 min after mixing was plotted against the R8 concentration (Fig. 3B). The increase of the fluorescence intensity induced by R8 for PC, PC/Ch (7:3) and PC/DSPG (7:0.5) liposomes was not observed either.

In conclusion, the results presented here suggest that the arginine oligomer peptides do not permeabilize

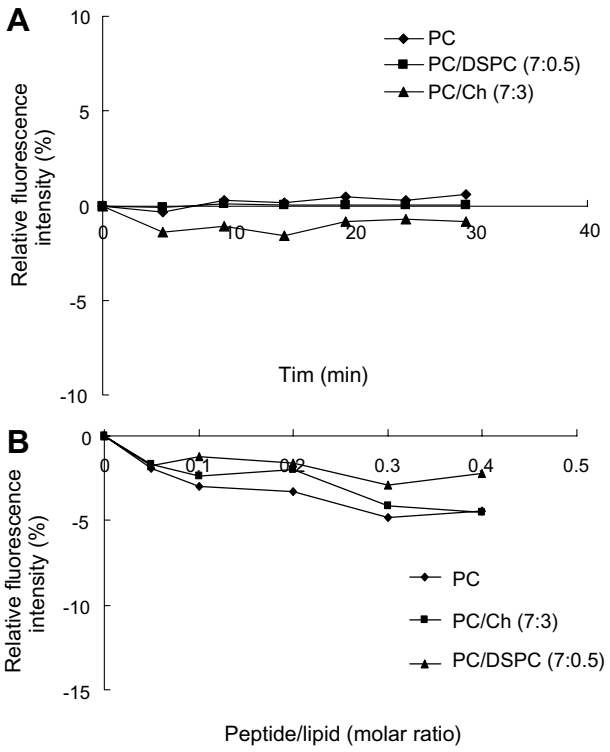

Fig. 3. (A) Time course of lipid mixing induced by R8 in a 1:20 (R8/lipid) mol/mol ratio. The fluorescence intensity was measured at 25 °C. PC (♦), PC/DSPG (7:0.5) (■) and PC/Ch (7:3) (▲) liposomes. The data represent mean values of three independent experiments. (B) Influence of the R8 concentration on lipid mixing. The relative fluorescence intensity of FITC probe is measured after 30 min incubation. PC (♦), PC/DSPG (7:0.5) (▲) and PC/Ch (7:3) (■) liposomes. The data represent mean values of three independent experiments.

through the phospholipid bilayer spontaneously. These transporter peptides delivering drugs through biological barriers may depend on both of receptors and energies. Recent studies provide genetic and biochemical evidences that cell-associate heparan sulfate proteoglycans function as cell surface receptors for extracellular Tat internalization [31]. Arginine oligomer peptides may have the same mechanism as Tat that translocation through cell membrane is mediated by heparan sulfate proteoglycans. It will be important to develop peptides transporter for drug delivery basing on specific domains in messenger proteins that enhance and enable the delivery of drugs through biological barrier. Thus, understanding the molecular basis of the preference arginine oligomer peptides has for biomembrane can lead to new insights for drug design.

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