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Effects of HPE-101, a skin penetration enhancer, on human erythrocyte membranes

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Abstract

The primary aim of this study was to investigate the skin permeation-enhancing mechanism of HPE-101 using erythrocyte ghost cells prepared from human whole blood as a biomembrane model. The extent of hemolysis of erythrocytes induced by HPE-101 was measured using a spectrophotometer at 540 nm. The effect of HPE-101 on lipid fluidity was examined by observing the change of intramolecular excimer formation and fluorescence polarization using an intramolecular probe (1,3-*bis*(pyrene) propane) and a lipid probe (1,6-diphenyl 1,3,5-hexatriene), respectively. Hemolysis of erythrocytes was observed at 0.01 mM and completed at 1.0 mM of HPE-101. The fluorescence polarization of the ghost membrane decreased with the addition of HPE-101, whereas the intramolecular excimer formation increased. HPE-101 thus enhanced the rotational mobility and the lateral diffusion, thereby decreasing the microviscosity of ghost membranes, implying that HPE-101 increases the lipid fluidity of ghost membranes. Therefore, HPE-101 seems to cause an increase in fluidity of the lipid bilayers in the stratum corneum of the skin, resulting in the reduction of diffusion resistance.

Keywords: Penetration; Enhancer; HPE-101; Ghost membrane; Fluorescence; Polarization; Microviscosity

1. Introduction

The human skin has attracted considerable interest as an alternative administering route of systemically active drugs in recent years. A major problem, however, is in the control of drug flux which arises from

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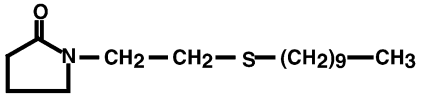
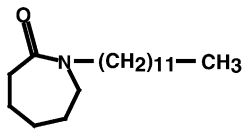
the impermeability of the skin as well as in its biological variability. The importance of the barrier function of the skin's stratum corneum is well established, with the entire horny layer providing the major rate-limiting lipophilic barrier. To increase the skin permeation rate of various drugs, several approaches have been attempted including the use of penetration enhancers, synthesis of prodrugs, iontophoresis, and phonophoresis (Sugibayashi et al., 1992). Among these, addition of penetration enhancers is one of the most convenient and relatively effective methods, which explains the tremendous amount of work found on the development of safe and effective enhancers in the last few decades. The nature of research with enhancers include that in which the effect of known enhancers such as alcohols, polyols, surfactants or fatty acids/amines are investigated or in which novel compounds like Azone are developed (Walker and Smith, 1996).

HPE-101 [1-(2-(decylthio)ethyl)azocyclopentan-2-one], which is similar in structure to Azone, was synthesized by a collaborative effort of Kumamoto University and Hisamitsu Pharmaceutical Co., Inc. in Japan (Yano et al., 1992) (Fig. 1). HPE-101 is known to enhance the percutaneous absorption of various drugs, such as indomethacin (Yano et al., 1992), alprostadiol (Adachi et al., 1992, 1993), cyclosporine (Nakashima et al., 1996), valproate (Matsuyama et al., 1994a), methotrexate (Matsuyama et al., 1994b), nicotinic acid and triamcinolone acetonide (Yano et al., 1993). However, little is known on how HPE-101 interacts with the skin barrier and how it enhances the percutaneous absorption of drugs. In a report by Katsu et al. (1989), the skin permeation-enhancing mechanism of Azone was investigated by looking into the interaction of the compound with erythrocyte ghost membrane. Hemoglobin-depleted human erythrocyte ghost cells were first developed by Dodge et al. (1963), and have been used as a model lipid bilayer, like liposomes, due to their ready accessibility, ease of preparation and wealth of available information (Katsu et al., 1989; Lieber and Steck, 1982; Kahana et al., 1991; Suwalsky et al., 2002). Considering the chemical similarity of HPE-101 and Azone, using erythrocyte ghost membrane would be a good starting point for its mechanism study. Herein, we report the results of the interaction of HPE-101 with erythrocyte ghost membrane using fluorescence polarization and intramolecular excimer formation techniques, which could provide valuable information on understanding the interaction of this enhancer with the lipid region of the stratum corneum of the skin.



1-[2-(decylthio)ethyl]azacyclopentan-2-one
(HPE-101)



1-dodecylazocycloheptan-2-one (Azone)

Fig. 1. The chemical structures of HPE-101 and Azone.

2. Materials and methods

2.1. Materials

HPE-101 was supplied from Hisamitsu Pharmaceutical Co. (Saga, Japan). 1,6-Diphenyl-1,3,5-hexatriene (DPH), and mineral oil were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 1,3-Di(1-pyrenyl)propane (py-3-py) was purchased from Molecular Probes, Inc. (Eugene, OR, USA). All other chemicals were reagent grade and used as purchased.

2.2. Hemolysis of erythrocyte induced by HPE-101

Erythrocytes were collected from human whole blood by centrifugation at 1630 × *g* for 30 min. The buffy coat was discarded and the cells were washed three times with phosphate buffer saline (PBS). Erythrocytes were suspended in 4 ml of PBS at the final concentration of 0.5% (v/v). Then, HPE-101 dissolved in 2% ethanol solution were added to the erythrocyte suspensions in the concentration range of 0.001–100 mM and the suspensions were incubated at 37 °C for 30 min. After centrifuging the suspensions, the degree of hemolysis was determined by measuring the absorbance of the supernatant at 540 nm, as previously reported (Katsu et al., 1989). The percentage of hemolysis was calculated by comparing with a sample treated in hypotonic solution without HPE-101.

2.3. Preparation of erythrocyte ghost cells

Erythrocyte ghost cells were prepared by a slightly modified method described in the literature (Kahana et al., 1991). Briefly, heparinized human whole blood was obtained from healthy volunteers, and was centrifuged at 4 °C for 30 min at 1630 × *g* to collect erythrocytes. After removing the plasma and buffy layer containing leukocytes and platelet, the packed erythrocytes were washed three times with isotonic PBS. Then, the erythrocytes were hemolyzed with hypotonic phosphate buffer and the mixture was centrifuged at 12,000 rpm for 15 min at 4 °C to remove water-soluble fragments. The supernatant was discarded and this process was repeated three to four times. The resulting open erythrocyte ghost membranes were resuspended in 9 volume of PBS containing 0.1 mM $MgCl_2$, and incubated for 30 min at 37 °C with gentle shaking. The resealed erythrocyte ghost cells were harvested by centrifugation at 4500 rpm for 30 min. The amount of the ghost membrane protein was analyzed by the Lowry et al. (1951) using bovine serum albumin as a standard. The ghost cells were suspended in PBS to make 100 μg protein of ghost membrane/ml of PBS and were store at 4 °C until used for experiments within 2 days.

2.4. Effect of HPE-101 on intramolecular excimer formation

Intramolecular excimer formation of py-3-py was employed to measure the fluidity change of membrane caused by the lateral diffusion of lipids (Zachariasse et al., 1982). An aliquot of py-3-py ethanol stock solution (5×10^{-5} M) was added to the ghost cell suspension so that the final probe concentration was less than 5×10^{-7} M. The suspension was then incubated at 4 °C for 18 h with gentle magnetic stirring. Previous reports from this laboratory showed that this method resulted in reproducible incorporation of the probes into the membranes (Lee and Kim, 1994; Park et al., 1995). Then, HPE-101 stock solution in 2% ethanol was spiked into the ghost cell-probe suspension to make 0.01–1.0 mM of HPE-101, and the excimer to monomer fluorescence intensities were measured within 1 min at 25 °C. A blank ghost cell suspension was prepared under identical conditions without py-3-py to serve as the control of the fluorometric measurements. The measurements were carried out using an AB2 spectrofluorometer (SLM-Aminco Instruments Inc., Urbana, IL, USA) with the excitation wavelength at 330 nm. The excimer to monomer fluorescence intensity ratio (I'/I) was calculated from the 480 to 379 nm signal ratio (Zachariasse et al., 1982).

2.5. Determination of fluorescence polarization

In order to confirm the results of the intramolecular excimer fluorescence technique, the estimation of fluorescence polarization was also carried out using 1,6-diphenyl 1,3,5-hexatriene (DPH) as a lipid probe. A half μl of DPH stock solution in THF (2 mg/ml) was added to ghost cell suspension (100 μg protein of ghost membrane/ml of PBS) to make the final probe concentration as 1/100 μg of membrane protein. The ghost cell-probe suspension was shaken in the dark at 37 °C for 30 min. A blank ghost cell suspension was prepared under identical conditions without probe. HPE-101 was added to the ghost cell-probe suspensions in the concentration range of 0.01–1.0 mM, and the fluorescence intensity was measured at 37 °C using a Kontron Spectrofluorometer (Model SPF-25, Kontron, Swiss) equipped with polarizers in both the excitation and emission beams. The excitation and the emission wavelengths for DPH were set at 362 and 430 nm, respectively, with a slit width of 5 nm. The fluorescence polarization (P) was obtained from the intensity measurements using the following equation (Kohmoto et al., 1994):

$$P = \frac{I_{vv} - I_{hv}G}{I_{vv} + I_{hv}G}$$

where G is the emission grating factor which corrects for the monochromator's transmission efficiency for vertically and horizontally polarized light, and is given by the ratio of the fluorescence intensities of the vertical to horizontal direction. I_{vv} and I_{hv} are the fluorescence intensities measured when the polarizer and analyzer prisms are in the vertical or horizontal position, respectively.

2.6. Evaluation of erythrocyte ghost membrane microviscosity

The viscosity of white mineral oil, a viscous mixture of high molecular weight aliphatic hydrocarbons, was determined at the temperature range of 0–40 °C

using the Physica-Rheometer (Model Rheolab MC10, Germany) with a thermostatic circulator (Model Viscotherm VT100). Py-3-py was incorporated into white mineral oil in the same manner as performed in the ghost cell suspensions, and excimer to monomer fluorescence intensity ratio (I'/I) was measured at the temperature range of 0–40 °C. The calibration curve of log η (viscosity) versus I'/I was obtained from both plots of temperature versus microviscosity of mineral oil and temperature versus I'/I of mineral oil. The microviscosity of ghost membranes was evaluated from the calibration curve of log η versus I'/I.

3. Results and discussion

3.1. Effect of HPE-101 on hemolysis of erythrocytes

In a preliminary study, 2% ethanol in PBS resulted in a negligible hemolysis of erythrocytes while completely dissolving HPE-101 up to 100 mM (data not shown). Thus, it was selected as a solvent for HPE-101. Fig. 2 shows the effect of HPE-101 concentration on hemolysis of human erythrocytes. Hemolysis of erythrocytes began to occur at as low as 0.01 mM of HPE-101 in 2% ethanol in PBS. Since complete hemolysis was observed at concentrations higher than 1.0 mM, the concentration range of 0.01–1.0 mM of HPE-101 was chosen to study its effect on the ghost membrane fluidity.

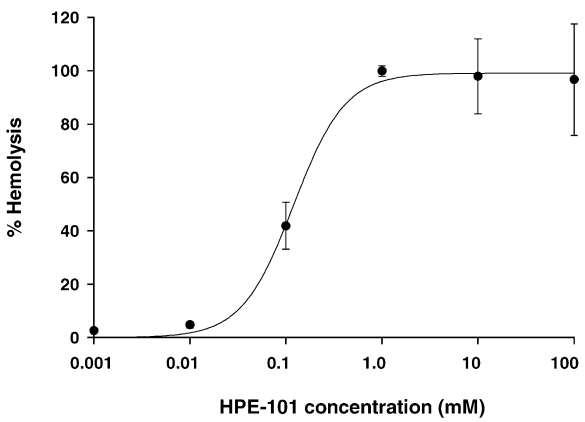


Fig. 2. The effect of HPE-101 on the hemolysis of erythrocytes suspended in isotonic phosphate buffer saline (0.5% (v/v)) containing various concentrations of HPE-101. Hemolysis was determined after incubating for 30 min at 37 °C.

3.2. Effect of HPE-101 on the lateral mobility of lipid bilayer in ghost membrane

Intramolecular excimer formation of py-3-py is known to be sensitive to the lateral mobility changes, and thus has been used to study the fluidity change of biomembranes (Kang et al., 1996; Zachariasse et al., 1982). An increase in the excimer (I') to monomer (I) intensity ratio (I'/I) is an indication of increased lateral mobility of the probe (i.e., py-3-py) within the membrane. In a preliminary study to determine the probe incorporation time, the value of $I'_{480}/I_{379\,nm}$ rapidly decreased for the initial 10 h of incubation at 4 °C, then reached a constant value thereafter for at least 25 h (data not shown). Thus, the effect of HPE-101 concentration on the lateral mobility was investigated after incubating the probe for 18 h with ghost cells at 4 °C. As shown in Fig. 3, HPE-101 increased the lateral mobility of ghost membrane in a dose-dependent manner.

3.3. Effect of HPE-101 on the rotational mobility of lipid bilayer in ghost membrane

Fluorescence polarization technique was employed using DPH as a probe dye to measure the rotational mo-

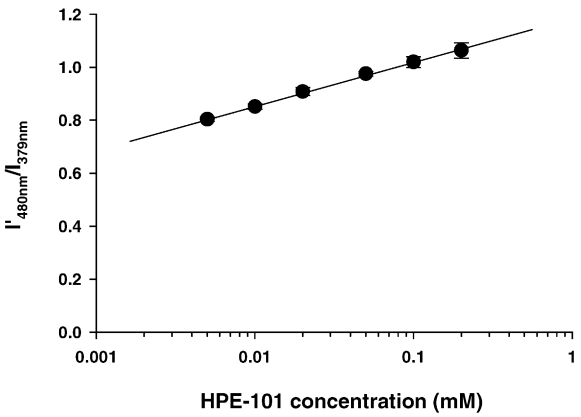


Fig. 3. The effect of HPE-101 on the lateral diffusion. HPE-101 increased the excimer to monomer fluorescence intensity ratio (I'/I) of py-3-py in erythrocyte ghost cells. The excitation wavelength was 330 nm and I'/I was calculated from the 480 to 379 nm signal ratio. Fluorescence measurements were performed at 25 °C. Each point represents the mean ± S.D. of three to nine determinations.

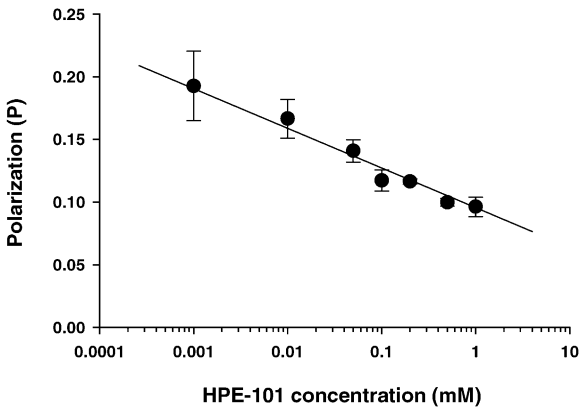


Fig. 4. The effect of HPE-101 on the rotational mobility. HPE-101 decreased the polarization of DPH incorporated in ghost membranes. The excitation and emission wavelengths were 362 and 430 nm, respectively. Fluorescence measurements were performed at 37 °C. Each point represents the mean ± S.D. of three measurements.

bility of lipid bilayer in ghost membrane. In a highly viscous membrane, the DPH molecule is held essentially motionless, and a fluorescence polarization value of 1 is observed. The polarization value near 1 thus is observed in highly rigid membranes, whereas the value near 0, in extremely fluid membranes. Therefore, there is an inverse relationship between membrane fluidity and fluorescence polarization (Kohmoto et al., 1994). As represented in Fig. 4, the values of fluorescence polarization (P) in ghost membranes decreased with an increase of HPE-101 concentration from 0.001 to 1 mM. This result implies that the rotational mobility of lipid in ghost membranes increased with an increase of HPE-101 concentration.

In a recent study using stratum corneum lipids in langmuir monolayers (Lopez-Castellano et al., 2000), Azone is known to increase the fluidity within monolayer. A liposome composed of lipid components of stratum corneum was also used as a system to evaluate the enhancing ability of Azone. Changes in the lipid fluidity of the model lipid liposome membrane, consisting of ceramide (40%), cholesterol (25%), palmitic acid (25%) and cholesterol 3-sulfate (10%), by the addition of Azone were also measured by a fluorescence polarization method (Kai et al., 1993). The basis for the skin permeation-enhancing mechanisms of Azone was proposed to be the change in diffusion constant of a drug in the rat skin caused by the increment of the fluidity of the model lipid liposome. Thus, considering the similarity in structure of HPE-101 with that of Azone, this may also be the possible mechanism for HPE-101.

3.4. Effect of HPE-101 on microviscosity of ghost membrane

The excimer to monomer fluorescence intensity ratio (I'/I) of py-3-py in the erythrocyte ghost membranes can be translated into microviscosity values by comparing this intensity ratio with those measured in an appropriate solvent or solvent mixture of known viscosity (Cogan et al., 1973). The fluorescence spectra of py-3-py are identical in membranes and white mineral oil, thus it was used as a reference system for this purpose. Both the excimer to monomer fluorescence intensity

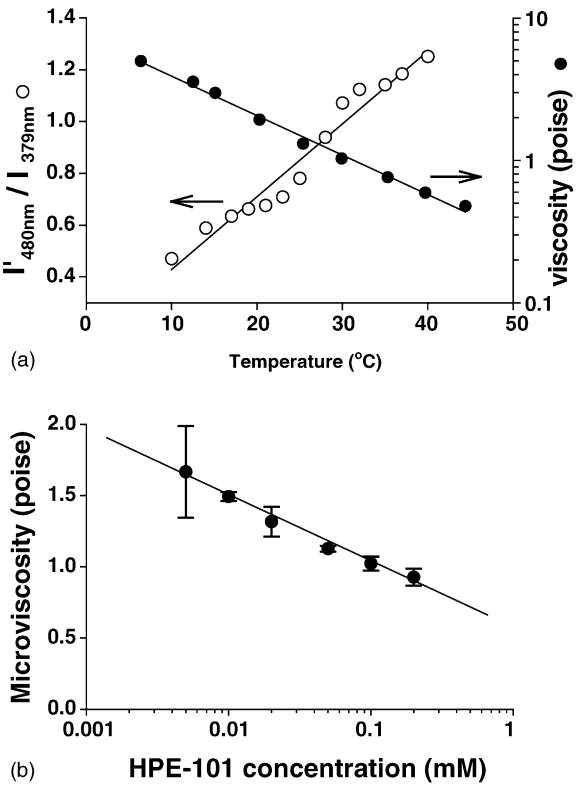


Fig. 5. The Effect of HPE-101 on the microviscosity of ghost membranes. (a) The viscosity (○) and excimer to monomer fluorescence intensity ratio (I'/I) of py-3-py (●) in white mineral oil were determined as a function of temperature. (b) The I'/I values in Fig. 3 were replotted using the linear equation obtained from the relationship between the I'/I ratio and $\log \eta$ in Fig. 5(a).

ratio of py-3-py (I'/I) and the viscosity (poise) of white mineral oil were obtained at various temperatures, and were plotted as a function of temperature (Fig. 5(a)). While the viscosity of white mineral oil decreased as the temperature increased, the fluidity of white mineral oil (expressed as I'/I) increased with the increase of temperature. Since a linear relationship existed between the I'/I values and log η, this could be served as a calibration curve in determining the microviscosity of the ghost membranes from the I'/I value. When the result of Fig. 3 was replotted using this calibration curve, the effect of HPE-101 on the microviscosity of the direct environment of py-3-py in the membranes could be expressed as shown in Fig. 5(b). HPE-101 significantly decreased the microviscosity of ghost membrane in a concentration-dependent manner.

Thus, ghost erythrocytes seem to be useful as a model for studying enhancer-membrane interaction and can be extended to studies of numerous biological systems. However, the limitation that should be pointed out when they are used as a model skin is that the human stratum corneum contains a complex and heterogenous mixture of lipids and lacks phospholipids, compared to the ghost cells which consist of more homogenous lipids. Further studies need to be conducted to evaluate the correlation between results from the ghost erythrocytes with actual human skin.

4. Conclusions

In order to study the skin permeation-enhancing mechanism of HPE-101, the erythrocyte ghost membrane was employed as a model biomembrane. The effect of HPE-101 on the fluidity of human erythrocyte ghost membranes was investigated by employing the intramolecular excimer formation and the fluorescence polarization techniques. The results showed that HPE-101 induced the hemolysis of erythrocytes at the concentration range of 0.01–1.0 mM. HPE-101 also enhanced the lateral and rotational diffusion of phospholipids in a concentration-dependent manner at this range, which was determined by using the intramolecular excimer formation of py-3-py and the fluorescence polarization of DPH, respectively. Microviscosity of the ghost membrane also decreased with the addition of HPE-101. Thus, it can be concluded that HPE-101 may enhance the skin permeation of drugs by increasing the fluidity of the lipid bilayers in the stratum corneum of the skin, which resulted in the reduction of diffusion resistance. Further studies are underway to understand other mechanism(s) of HPE-101, such as its effects on the lipid depletion and/or partitioning into the skin.

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