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Research paper

Characterization of the physicochemical properties of the micelles by the novel platelet activating factor receptor antagonist E5880

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Abstract

E5880, a novel platelet activating factor receptor antagonist, was dispersed in the buffer solution (4.8 mM citric acid, 10% lactose, pH 2.8) for the preparation of an injectable formulation and the physicochemical properties of the micelles were characterized. The critical micelle concentration of E5880 was 0.12 mM. Using the area per molecule results, the critical packing parameter was calculated and showed that the structure was spherical and the number of molecules in the aggregates was 46. The diameter of the micelle was 5.6 nm. The micropolarity around the hydrocarbon region of the micelle was similar to that of isobutanol.

Keywords: Micelle; Critical micelle concentration; Size; Structure; Micropolarity

1. Introduction

Platelet activating factor (PAF), 1-alkyl-2-acetylglyceryl-phosphocholine, is a group of biologically potent active phopsphoglyceride with actions more diverse than those of eicosanoids [1]. PAF exhibits a variety of biological activities including activation of platelets [2], neutrophils [3], bronchoconstriction [4], hypermeability in peripheral veins [5], hypotension [6], and cardiac dysfunction [7]. Because these biological activities of PAF are extremely potent, it is generally accepted that PAF is a mediator of inflammation [8] and plays important roles in the pathology of thrombosis, asthma or hypotension in shock [9–11]. Consequently, it is expected that specific PAF receptor antagonists may be beneficial for the treatment of these diseases, and many efforts to develop potent and specific PAF antagonists have been made.

Several PAF antagonists, such as CV-3988[12], CV-6209 [13], SRI63-072 [14], U66985 [15] were synthesized and the biological activities were evaluated. These compounds have hydrocarbon chains ($C_{18:0}$) and are amphiphilic, indicating that some aggregates will be formed in aqueous media. They were dissolved in an aqueous medium and injected into animals for evaluation of their biological activities, however, their physicochemical properties were not reported.

E5880, a newly synthesized PAF antagonist (Fig. 1), is more potent in PAF receptor binding than PAF [16]. This compound is amphiphilic and it is expected to form the micelles in aqueous media. For the treatment of the above diseases, an injectable formulation would be extremely useful. In order to develop the injectable formulation, the clarification of the characteristics of the physicochemical properties for E5880 micelles is important.

In this study, in order to clarify the behavior of E5880 aggregates in the formulation, the critical concentration for the formation of aggregates was determined by measurement of fluorescence intensity. The 'critical packing parameter' was determined and the size, structure and the number of the molecules per aggregate were calculated and compared with the results from dynamic and static light scattering. In addition, the micropolarity around the hydrophobic region of the micelles was determined by fluorescence techniques.

2. Materials and methods

2.1. Materials

E5880 was obtained from Eisai Chemical Co. Ltd. (Ibaraki, Japan). Lactose was purchased from Mallinkrodt (Paris, KT), and citric acid was purchased from Kozakai-

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Seiyaku (Tokyo, Japan). *N*-phenyl-1-naphtylamine (NPN) was purchased from Tokyo-kasei Co. Ltd. (Tokyo, Japan). Nile red (NR) was purchased from Lambda (Graz, Austria).

2.2. Sample preparation

Sixty milligrams of E5880 was dissolved in 100 ml of the buffer solution (4.8 mM citric acid, 10% lactose, pH 2.8) by stirring at room temperature. Based on the stability of the compound, the pH (2.8) was determined for the formulation.

2.3. Determination of the critical micelle concentration using fluorescence spectroscopy

As the NPN molecule partitions into the hydrophobic compartment of the E5880 micelle its emission peak shifts from about 475 nm to 425 nm and the quantum yield increases [17,18]. The increase in fluorescence was measured from 5 μM NPN (E5880 varying from 0 to 500 μM.). The samples were incubated for 30 min at 25°C, prior to fluorescence measurements, with excitation wavelength at 350 nm, emission at 425 nm with slit set at 5.0 nm resolution in a F-4500 fluorescence spectrophotometer (Hitachi, Tokyo).

2.4. Determination of the surface pressure of the E5880 micelles

Monolayer measurements at the air/water interface were performed with a firm balance. E5880 was spread on the aqueous subphase (4.8 mM citric acid, 10% lactose, pH 2.8) with the spreading solutions made of reverse phase lipid and benzene using a surface tension meter (Model CBVP-A3, Kyowa Kaimenkagaku Co. Ltd., Tokyo, Japan) at 25°C. The details of the monolayer techniques have been described elsewhere [19].

2.5. Determination of the molecular weight of the E5880 micelles

The molecular weight of the E5880 micelles in buffer solution (4.8 mM citric acid, 10% lactose, pH 2.8) was determined using static light scattering techniques. Measurements were carried out with the DLS-7000DL analyzer (Ohtsuka Electronics Co. Ltd., Osaka, Japan) at 25°C. The results of the static light scattering measurements were analyzed according to the well-known equation [20]:

$$\left(Kc/R_q\right) = \left[k'(dn/dc)2 \times c\right]/R_q = \left(1/M_w\right)$$

$$\times\left\{1 + \left[16\pi 2n_0^2/3l_0^2\right]\left(R_g^2 \sin^2(q/2)\right) + 2A_2c\right\} \quad (1)$$

where K is the scattering coefficient, R_q is the reduced scattering intensity of the solution in excess over the solvent at the scattering angle q, k' is the optical constant, c is the weight concentration of the solution (in this case, 2 mg/ml), n_0 is the solvent refractive index, l_0 is the wavelength in vacuum, R_g^2 is the mean square radius of gyration, A_2 is the second virial coefficient (in this case, A_2 is almost equal to zero), and M_w is the weight-averaged molecular weight of the system. All light scattering measurements were performed for scattering angles in the range of 30–150°. The refractive index increments (dn/dc) of the aggregates were determined individually with DRM-1020 (Ohtsuka Electronics Co. Ltd., Osaka, Japan) at 25°C.

2.6. Determination of the size of the E5880 micelles

The size distribution of E5880 micelles was determined by the dynamic light scattering (DLS) technique using a laser particle analyzer equipped with an Ar laser (Model DLS-7000DL, Ohtsuka Electronics Co. Ltd., Osaka, Japan) at 25°C. The data were analyzed by the histogram method [21] and the weight-averaged size of the aggregates was evaluated.

2.7. Measurement of zeta potentials

The zeta potentials of the E5880 micelles in the buffer solution (pH 2.8) was measured at 25°C using an Ohtsuka Electronics (Osaka, Japan) model ELS-800 zeta-potential analyzer. The data are presented as the mean values of triplicate measurements.

2.8. Determination of the micropolarity around NR in the hydrocarbon region of the E5880 micelles

The micropolarity of hydrocarbon regions in E5880 micelles was determined using a fluorescence technique (probe: NR). NR exhibits a strong environment-dependent blue shift, a high quantum yield and low fluorescence in water [22,23]. The fluorescence spectra were measured using a fluorescence spectrophotometer (model F-4500, Hitachi Co. Ltd., Tokyo, Japan) upon excitation at 549 nm at 25°C. The micropolarity of NR incorporated into the lipid aggregates was evaluated using the wavelength of maximum intensity of emission. A 3.8 mg of NR were dissolved in 10 ml of acetone (100 μM). Five microliters of each solution were then diluted with 5 ml of 10 mM of E5880 aqueous solutions, methanol, ethanol, propanol, butanol, isobutanol, acetone, tetrahydrofuran and acetonitorile, respectively. The wavelengths at the maximum fluorescence intensity of each solution was plotted against the polarity of each solvent [24]. The micropolarity around the probe was determined using this standard curve.

3. Results and discussion

3.1. Critical micelle concentration of the E5880 micelles

Steady-state fluorescence spectroscopy measurements of NPN intensity with increasing E5880 concentration indicate that at a specific E5880 concentration in the solution the NPN intensity increases dramatically. The change of fluor-

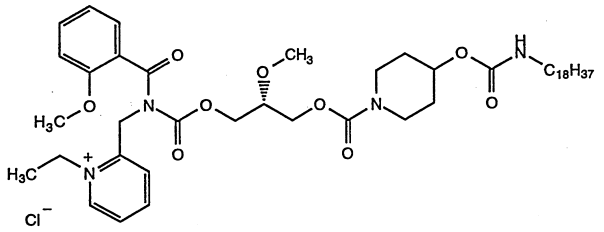

Fig. 1. Chemical structure of the platelet activating factor (PAF) antagonist, E5880.

escence intensity depends on the preferential partitioning of NPN molecules into the hydrophobic core of E5880 micelles. The critical micelle concentration (cmc) for E5880 was determined by the relative NPN fluorescence (Fig. 2), 0.12 mM (0.10 mg/ml). The cmc is found at the point where the two slopes of the curve intersect. The IC_{50} value in the septic shock model was 0.66 nM [16], so that the IC_{50} value was much lower than cmc and the biological activity of E5880 will be observed in its monomer state.

3.2. Monolayer properties

In order to determine the molecular projection area/molecule for E5880 on the air-water interface, the monolayer behavior was evaluated. The minimum area per molecule at the air/water interface can in turn be used for an estimation of the 'critical packing parameter' [25]. Fig. 3 shows the surface pressure/area diagram measured at 25°C with subphases (4.8 mM citric buffer, 10% lactose, pH 2.8). The form of the diagram is characteristic for a liquid-expanded monolayer. The collapse of E5880 occurs at a surface pressure of 30 mN/m and the corresponding area is 0.90 nm^2.

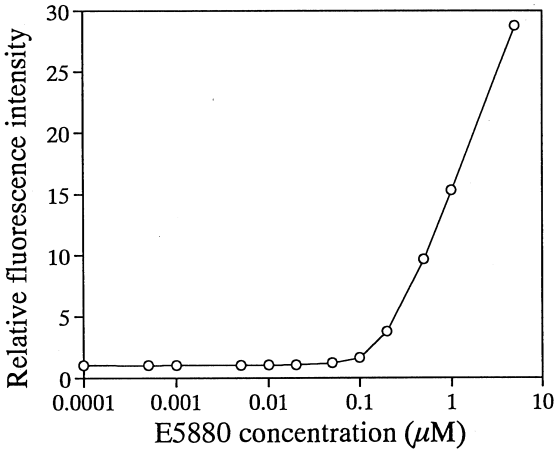

Fig. 2. Cmc determination for E5880. Relative fluorescence intensity of 5 μM NPN at 425 nm as a function of concentration of E5880 in the buffer (4.8 mM citric acid, 10% lactose, pH 2.8). The NPN was excited at 350 nm and the fluorescence peak was normalized to 1.0. The intensity of NPN increases sharply in the range of Cmc.

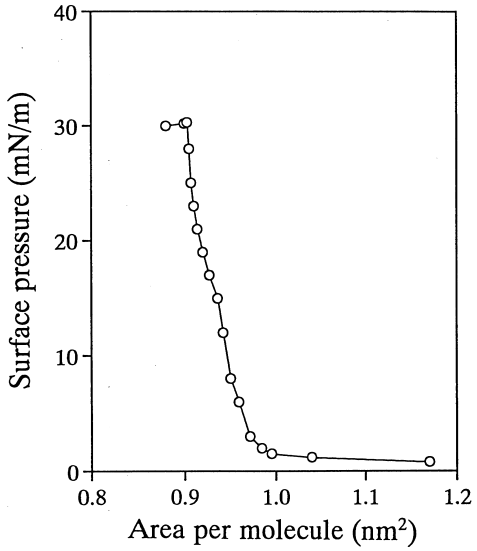

Fig. 3. Surface pressure/area diagram for E5880 aggregates in the buffer (4.8 mM citric acid, 10% lactose, pH 2.8).

3.3. Molecular weight of the E5880 micelles

The refractive index increments (dn/dc) of the aggregates were determined for calculation of the molecular weight. The values for E5880 were 0.15 ml/g. Table 1 shows the molecular weight of E5531 aggregates, 3.96×10^4. The molecular weight of E5880 is 861.6 and using these values, the number of molecules per aggregate can be calculated and shown in Table 1.

3.4. Critical packing parameters for the E5880 micelles

The 'critical packing parameters' [26] for E5880 were calculated based on the area per molecule results (Fig. 3), the volume of the hydrophobic part and the length of the acyl chain. For the formation of closed lamellar bilayer structures the effective cross-section of the hydrocarbon moiety must be lower than that of the hydrophilic head-group region. This assumption is confirmed by an estimation of the 'critical packing parameter' according to the formula [26] $x = v/a \times l$ (v is the volume of the hydrophobic part, a the area of the hydrophilic head group and l the length of the acyl chain). When $v/a \times l < 1/3$ spherical micelles form, $1/3 < v/a \times l < 1/2$ tubular micelles form, $1/2 < v/a \times l < 1$ vesicles form and $1 < v/a \times l$ hexagonal H_{II} structures form. The volume of the hydrocarbon domain (v) and the length of hydrocarbon (l) were calculated using the following equations:

$$v = (27.4 + 26.9n) \times 10^{-3} \ \left(\mathrm{nm}^3\right) \quad (2)$$

$$l = (0.15 + 0.1256n) \ \text{(nm)} \quad (3)$$

where n represents the number of the carbons in the hydrocarbon chains. For E5880, $n = 18$, $v = 0.51$ (nm^3), $l = 2.4$ (nm), $a = 0.90$ nm^2 and $x = 0.24$. Therefore, it can be

Table 1
Characterization of E5880 micelles in the buffer (4.8 mM citric acid, 10% lactose, pH 2.8)

Size (nm)	5.6 ± 1.4
Zeta potential (mV)	+6.3 ± 1.2
Critical micelle concentration (mM) determined by the fluorescence intensity (probe: NPN)	0.12
Critical packing parameter	0.24 (spherical micelle)
Molecular weight	3.96×10^4
The number of molecules/micelle	46
Emission maximum of DSHA (nm)	621
Microporality around the hydrocarbon region in the micelle	Similar to that of isobutanol (emission maximum of DSHA in isobutanol: 622 nm)

expected that these three lipids form spherical micelle structures. The number of molecules in the spherical micelles (N) can be calculated by the following equations [25]:

$$N = 36\pi\nu^2/a^3 \quad (4)$$

For E5880 micelles, $N = 40$ and this result was similar to that obtained from the static light scattering ($N = 46$).

3.5. Determination of the size and zeta potential of the E5880 micelles

Table 1 shows the size and the zeta potential of E5880 micelles (pH 2.8). The weight-averaged diameters for E5880 was 5.6 nm. This value was in good agreement with the ones calculated using the 'critical packing parameters' (4.8 nm). The zeta potential of the E5880 micelles (pH 2.8) was +6.3 mV. The positive charge of the head group of E5880 is thought to be responsible for the positive value of the zeta potential of E5880 micelles.

3.6. Micropolarity around NR in the E5880 micelles

The micropolarity around NR in the E5880 micelles was determined by measurement of the emission maxima of NR embedded in the micelles. It has been reported that the fluorescence characteristics of NR depend on the micropolarity around the probe and it is located in a hydrophobic region in the lipid aggregates [22,23]. Therefore it is expected that the emission maxima of NR in the lipid aggregates will provide information on the micropolarity around the hydrocarbon chains. Fig. 4 shows the relationship between solvent polarity and emission maximum of NR at 25°C. The emission maxima of E5880 micelles was 622 nm, indicating that the micropolarity around the probe in the lipid aggregates is comparable to that of isobutanol (621 nm).

4. Conclusions

The physicochemical characteristics of E5880 aggregates in the formulation (4.8 mM citric acid, 10% lactose, pH 2.8) were determined and are summarized in Table 1. The critical concentration for formation of the aggregates at 25°C was determined to be 0.12 mM by fluorescence intensity measurements. Based on the results from light scattering, the diameter of the micelle was 5.6 nm and the number of the molecules per micelle was 46. Using the area/molecule results, the structure of the micelle was spherical. The diameter of the micelles was 4.8 nm and the number of molecules per aggregate was calculated to be 40. These results were similar to those obtained from the light scattering. The microporality of the hydrocarbon regions in the micelle was similar to isobutanol. Further studies are planned to clarify the effects of environmental changes such as pH and ionic strength on the micelle properties and pharmacokinetics of E5880 in vivo and its biological activity as a PAF receptor antagonist.

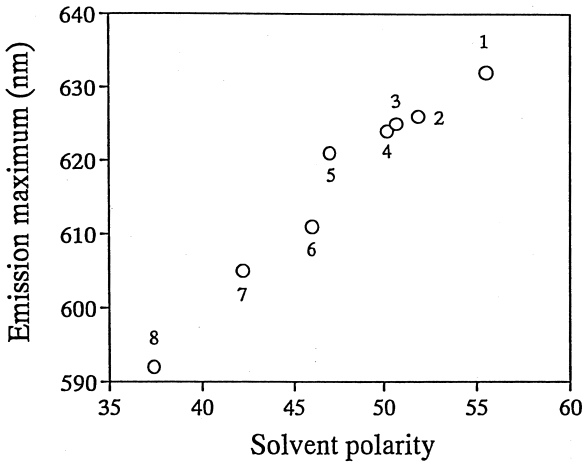

Fig. 4. Relationship between solvent polarity and emission maximum of NR (100 nM) at 25°C. 1, methanol; 2, ethanol; 3, propanol; 4, butanol; 5, isobutanol; 6, acetone; 7, tetrahydrofuran; and 8, acetonitorile.

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