J. Pharm. Pharmacol. 1999, 51: 577–584
Received February 23, 1998
Accepted April 15, 1998

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Control of the Dispersing Process and Pharmacokinetics in Rats for Lipid A Analogue, E5531

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Abstract

E5531 is a synthetic disaccharide analogue of lipid A which has a low toxicity but retains the ability to reduce production of tumour necrosis factor. This analogue has potential for use in the treatment of septic shock. An injectable formulation of E5531 would be useful, but dispersion in aqueous solution is a problem. In the present study the dispersing process for E5531 was evaluated using the pH-jump method (pH 11·0→7·3). The size of the aggregates was decreased (reaching 20 nm) with increasing dispersing time in 0·003 M NaOH (pH 11·0). The membrane fluidity of the aggregates increased with increasing dispersing time. When prepared by the normal dilution method (pH 7·3→7·3), the size of the aggregates remained constant at 140 nm and the membrane fluidity was smaller than that of samples prepared by the pH-jump method. This indicates that during dispersing at basic pH, the hydration proceeded in a normal manner, but then stopped, just after adjustment of the pH to 7·3. This suggests that the degree of hydration of the membrane is dependent on the dispersing time at pH 11·0. Using samples with different degrees of hydration and different membrane fluidity prepared by the pH-jump method, the pharmacokinetics and stability of the aggregates were evaluated after intravenous injection into rats. The data thus obtained confirmed that the membrane fluidity was correlated with the pharmacokinetics and stability in rat plasma.

It was concluded that the pharmacokinetics of E5531 in rats can be controlled by changing the degree of hydration and membrane fluidity by means of using different dispersing times in alkaline solution (pH 11·0).

Lipid A is a lipid anchor in lipopolysaccharide (LPS), which exists on the outer membrane of Gram-negative bacteria. The elucidation of the chemical constitution and conformation of this highly complex lipid is of great scientific interest. This is not only because of its crucial role in the barrier function of the outer membrane (e.g. to certain antibiotics) (Labischinski et al 1985; Nikaido & Vaara 1985), and its various biological activities such as the induction of fever and Schwartzmann bleeding reaction (Vogel et al 1984), but also because of the expression of remarkable polymorphism, including lamellar and non-lamellar phases, which are dependent on the experimental conditions such as hydration, temperature, pH, etc. (Naumann et al 1987).

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Numerous attempts have been made to synthesize analogues of lipid A with low toxicity. Christ et al (1995) reported that E5531 (Figure 1), a synthetic disaccharide analogue of lipid A, has low toxicity but retains some useful biological activities such as reduction of tumour necrosis factor (TNF) production. This compound has been found to be a specific LPS antagonist in an LPS-binding assay, and it inhibits LPS-induced TNF production in monocytes and macrophages. Thus, the compound has great potential for use in the treatment of septic shock.

An injectable E5531 formulation would be extremely useful but the dispersion of E5531 in aqueous solution represents a major problem. E5531, like many lipid A analogues, cannot be dispersed at neutral pH and cannot be obtained as a transparent solution. Sonication has been used to disperse lipid A analogues (Labischinski et al 1990; Hofer et al 1991) and LPS (van Alphen et al 1980)

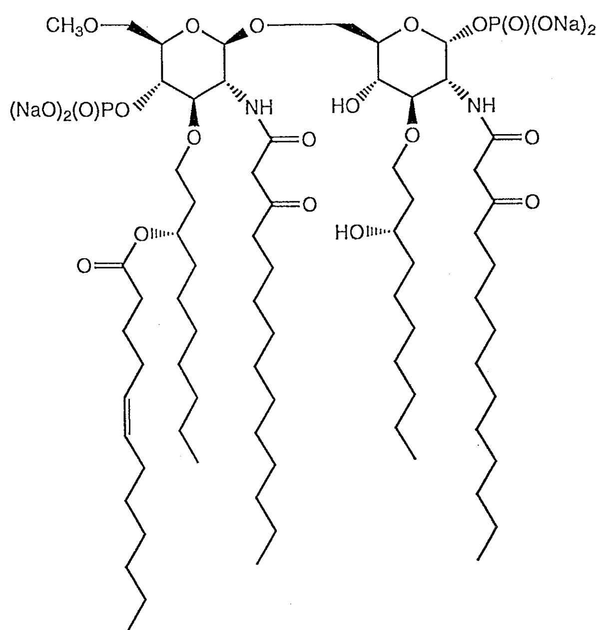

Figure 1. Chemical structure of the synthetic lipid A analogue, E5531.

for investigational use. However, sonication has two major drawbacks — it is not suitable for large-scale production and sonication power and dispersing time is difficult to control (Huang 1969; Johnson et al 1971). The present study describes the development of a new pH-jump method for dispersing E5531 (Asai et al 1998). The advantages of this method are that it is suitable for large-scale production (without mechanical input such as sonication), it can be used at neutral pH and gives rise to aggregates which are small in size. The physicochemical properties of E5531 have been determined during the development of the pH-jump method. The pK_a values of E5531 were determined to be 6·0 (pK_1) and 9·3 (pK_2). The phase transition temperatures at pH 11·0 and 7·3 were determined to be 31·3°C and 32·3°C, respectively. Based on these results, the developed method involves the dispersion of E5531 in 0·003 N NaOH solution (pH 11·0; above pK_2) at 50°C (above the phase transition temperature) and subsequent mixing with phosphate–NaOH buffer to adjust the pH to 7·3.

In this study, the dispersing process for E5531 using the pH-jump method is investigated in more detail. E5531 was dispersed using the pH-jump method (pH 11·0→pH 7·3) and the normal dilution method (pH 7·3→pH 7·3). In addition, in order to investigate the effect of the charge of the head phosphate group on the dispersion of E5531 at pH 11·0 in the pH-jump method, a divalent cation (Ca^{2+}) was added and the dispersing process was monitored.

We also investigated the physicochemical properties of E5531 aggregates, which were prepared under several dispersion conditions. Finally, the pharmacokinetics in rats after an intravenous injection of E5531 aggregates prepared by the various methods were evaluated. The relationship between the physicochemical properties of E5531 aggregates, such as the aggregate size and membrane fluidity, and the pharmacokinetic profiles in rats are discussed.

Materials and Methods

Materials

E5531 was obtained from Eisai Chemical Co. Ltd (Ibaraki, Japan). 1,6-Diphenyl-1,3,5-hexatriene (DPH), calcium chloride ($CaCl_2$) and ethylenediamine-*N*,*N*,*N′*,*N′*-tetraacetatic acid, disodium salt (EDTA) were purchased from Wako-Chemical Co. Ltd (Osaka, Japan). Calcein (3,3′-bis [*N*,*N*-bis (carboxymethyl)aminomethyl]-fluorescein) was purchased from Dojin Co. Ltd (Kumamoto, Japan). Lactose hydrate, monobasic and dibasic sodium phosphate and sodium hydroxide were purchased from Mallinckrodt Co. Ltd (Paris, KY).

Preparation of E5531 samples

The size of the E5531 aggregates and the fluidity and micropolarity of E5531 membranes prepared using the pH-jump or normal dilution methods (described below) were determined. In addition, in order to investigate the effect of the charge of the phosphate headgroup of the E5531 molecule on the dispersing process at pH 11·0, a divalent cation (Ca^{2+}) and EDTA were added to the alkaline solution.

pH-jump method (pH 11·0→pH 7·3). Two hundred milligrams of E5531 were dispersed in 100 mL of 0·003 N NaOH solution (pH 11·0) with stirring at 50°C.

Normal dilution method (pH 7·3→pH 7·3). Two hundred milligrams of E5531 were dispersed in 100 mL of buffer solution (4·25 mM phosphate–NaOH buffer containing 10% lactose, pH 7·3) with stirring at 50°C.

pH-jump method with Ca^{2+} (pH 11·0→pH 7·3). Two hundred milligrams of E5531 were added to 100 mL of 3·9 mM $CaCl_2$ solution (pH 11·0). The molar ratio of E5531 and $CaCl_2$ was 1 : 3. The solution was then dispersed by stirring at 50°C.

pH-jump method with Ca^{2+} and EDTA (pH 11·0→pH 7·3). Two hundred milligrams of E5531 were added to 100 mL of a solution containing 3·9 mM $CaCl_2$ and 3·9 mM EDTA (pH 11·0). The molar ratio of E5531, $CaCl_2$ and EDTA was 1 : 3 : 3. The solution was then dispersed with stirring at 50°C.

Determination of the size of the E5531 aggregates
After stirring for 3, 8, 15, 30 and 90 min, 10-mL samples of the solution were taken from the above E5531 solutions and the size-distribution of E5531 aggregates was determined at 25°C by the dynamic light-scattering technique using a laser particle analyser (Model DLS-7000DL, Ohtsuka Electronics Co. Ltd, Osaka, Japan). The sampled solutions were then mixed with phosphate–NaOH buffer containing lactose. The volume was adjusted to 200 mL by adding water to the formulated solution (E5531: 100 μg mL^{-1}, 4·25 mM phosphate–NaOH buffer containing 10% lactose solution, pH 7·3). Then, 5 mL of the solution was placed in glass vials and lyophilized. The lyophilized vials were reconstituted with 5 mL of water (E5531: 100 μg mL, pH 7·3) and the size-distribution of E5531 aggregates measured at 25°C. The data were analysed by the histogram method (Gulari et al 1979) and the weight-averaged sizes evaluated.

Determination of trapped volume of E5531 aggregates
In order to obtain information on the structure of the E5531 aggregates prepared by the pH-jump method, the normal dilution method, pH-jump method with divalent cations (Ca^{2+}) and the method with Ca^{2+} and EDTA at various dispersing times, the trapped volumes inside the aggregates were determined. E5531 was dispersed with a 70 mM calcein solution in the above four preparation methods. The untrapped calcein was removed by gel filtration (Sephadex G-50) at 25°C. The volume of the calcein solution trapped in the dispersed aggregates was then determined fluorimetrically (Allen & Cleland 1980) after solubilization of the E5531 aggregates by the addition of 10% Triton X-100, and the aqueous volume trapped per mole of E5531 was determined. The amount of E5531 in the dispersion was determined by high-performance liquid chromatography (HPLC) (with fluorescence detection at 254 nm wavelength).

Measurement of the membrane fluidity of E5531 aggregates
The hydration process and the relationship between dispersing method and dispersing time for E5531 were also evaluated by measurement of the membrane fluidity of E5531 aggregates. Fluorescence anisotropy of DPH for E5531 alkaline solutions (2 μg mL^{-1}, pH 11·0) and reconstituted E5531 solutions (100 μg mL^{-1}, pH 7·3) were measured as described previously (Shinitzky et al 1984) using a H-4500 spectrophotometer (Hitachi Co. Ltd, Tokyo, Japan) at 25°C. DPH was added at 1 mol% to the total lipids. The excitation and emission wavelengths used were 360 nm and 428 nm, respectively. Fluorescence polarization for reconstituted E5531 solutions prepared by the pH-jump method was determined at the various temperatures in particular detail. The steady-state anisotropy (r_s) can be defined by the following equation:

$$r_s = (I_{vv} - C_f \times I_{VH})/(I_{vv} + 2C_f \times I_{VH}) \quad (1)$$

where I is the fluorescence intensity and subscripts V and H indicate the vertical and horizontal orientations of excitation (first) and analysis (second) polarizers, respectively. C_f ($= I_{VH}/I_{HH}$) represents the grating correction factor. The order parameter, S, was calculated using the following equation (Heyn 1979):

$$S = (r_s/r_o)^{1/2} \quad (2)$$

where r_o represents the maximal and limiting fluorescence anisotropy. For DPH, r_o has been estimated to be 0·398 by Heyn (1979) using nanosecond time-resolved fluorescence techniques. In this work, equation 2 was used to estimate the order parameter, S.

Pharmacokinetics in rats for E5531 aggregates prepared by the pH-jump method with different membrane fluidity
In order to investigate the relationship between the membrane fluidity and pharmacokinetics in rats, reconstituted E5531 solutions (100 μg mL^{-1}, pH 7·3) prepared by the pH-jump method, dispersed in 0·003 N NaOH solution (pH 11·0) for 3, 8, 15, 30 and 90 min were administered as a bolus injection (0·3 mg kg^{-1}) via a femoral vein. Blood samples were collected before dosing and at 2, 10 and 30 min and 1 and 2 h after dosing. The concentrations of E5531 in plasma (100 μL) were measured by HPLC analysis with fluorescence detection. The AUC (0–2 h) was calculated from the plasma concentration at 2 h after administration by the trapezoidal method. The plasma concentration at time zero for AUC calculations was estimated to be equal to the concentration at the first time-point measured (2 min).

Determination of permeability in rat plasma of E5531 aggregates prepared by the pH-jump method using different dispersing times
In order to investigate the stability of E5531 aggregates after intravenous injection into rats, the leakage of calcein from the aggregates in plasma was evaluated according to the method of Kirby et al (1980) and Kiwada et al (1988). Two hundred milligrams of E5531 were dispersed in 100 mL of

70 mM calcein solution (pH 11·0) with stirring at 50°C. After stirring for 3, 8, 15, 30 and 90 min, 10-mL samples of the solution were removed and after cooling to 25°C, the pH of the solution was adjusted to 7·3 with 1 N HCl.

The untrapped calcein was eluted with the void fraction from Sephadex G-50 gel with 4·25 mM phosphate–NaOH and 10% lactose buffer solution (pH 7·3) and 0·5 mL of the fraction was added to 2·5 mL of rat plasma. The permeability of the E5531 aggregates was evaluated fluorimetrically by monitoring the leakage of calcein during incubation with rat plasma at 37°C. The percent leakage of calcein was calculated according to the following equation.

$$\text{Leakage (\%)} = [(F - F_0)/(F_\infty - F_0)] \times 100 \quad (3)$$

where F_0 represents the initial fluorescence intensity at time zero, F the fluorescence intensity monitored during the incubation at 37°C and F_∞ denotes the maximum fluorescence intensity after lysis of the aggregates by the addition of 0·1 mL of 10% Triton X-100.

Results and Discussion

Comparison of the size of E5531 aggregates

Figure 2 shows the relationship between dispersing time and the size of E5531 aggregates prepared by the pH-jump method (pH 11·0→7·3), the normal dilution method (pH 7·3→7·3), the pH-jump method with Ca^{2+} (pH 11·0→7·3) and the pH-jump method with Ca^{2+} and EDTA (pH 11·0→7·3). E5531 has two pKs, $pK_1 = 6{\cdot}0$ and $pK_2 = 9{\cdot}3$ (Asai et al 1998). At pH 11·0 in aqueous solution, E5531 was fully ionized and present in the dissociated form. At basic pH above pK_2 (9·3), E5531 was fully ionized and hydration could be accelerated via the loss of intermolecular hydrogen bonds in the head phosphate group. Figure 2a shows that in the pH-jump method, the size of the aggregates in 0·003 N NaOH decreased with increasing dispersing time and reached a size of approximately 20 nm. Just after adjustment of the pH to 7·3, the salt form of E5531 changed to the semi-ionized form, but the size of the aggregates were similar to those obtained in the alkaline solutions. However, the size of the aggregates prepared by the normal dilution method (pH 7·3→7·3) was 130 nm. Because of the intermolecular hydrogen bonds in the head phosphate group, hydration does not proceed directly when E5531 is dispersed at pH 7·3 straightaway. This indicates that when E5531 is dispersed in alkaline solution, hydration proceeds, but further hydration stops just after neutralization to pH 7·3.

Figure 2b shows that, even at pH 11·0, when Ca^{2+} was added, the aggregates size was approximately 240 nm after a 3-min dispersal period and remained unchanged for 90 min. This suggests that due to neutralization of the charge of the head phosphate group of the E5531 molecule by Ca^{2+}, the hydration cannot proceed and, as a result, the size of the aggregates remain unchanged at 240 nm. However, by adding EDTA and fully masking the Ca^{2+} at pH 11·0, the hydration can be accelerated and the size of the aggregates decreased to approximately 20 nm, a size similar to that obtained

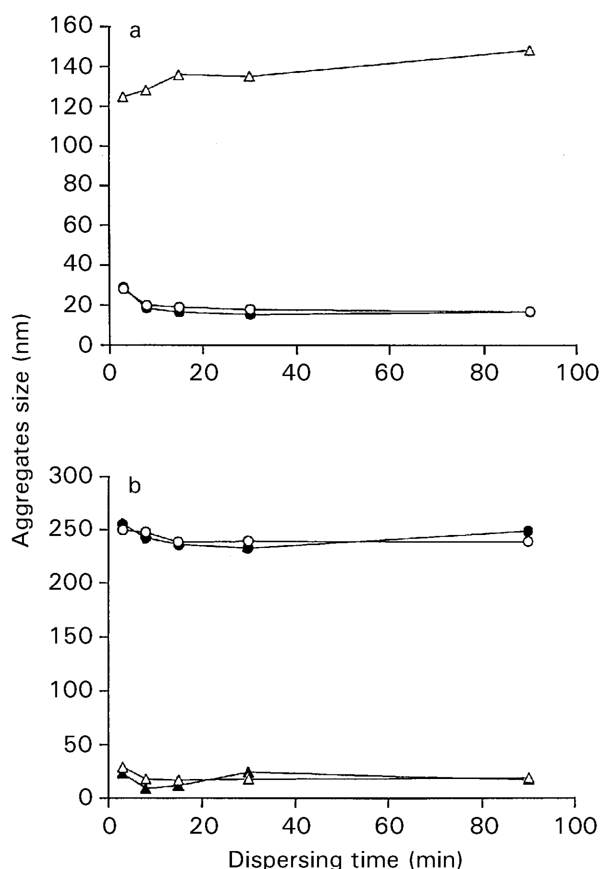

Figure 2. Relationship between dispersing time and size of E5531 aggregates. a. ○ E5531 was dispersed at pH 11·0 using the pH-jump method at 50°C (E5531: 2 mg mL^{-1}), ● after adjustment of the pH from 11·0 to 7·3 by the addition of a phosphate–NaOH buffer containing lactose (E5531: 100 μg mL^{-1}, 4·25 mM phosphate NaOH, 10% lactose, pH 7·3) △ prepared by the normal dilution method—E5531 was dispersed in phosphate–NaOH buffer containing lactose (E5531: 2 mg mL^{-1}, 4·25 mM phosphate–NaOH, 10% lactose, pH 7·3) and diluted with the same buffer (E5531: 100 μg mL^{-1}, pH 7·3). b. ○ E5531 was dispersed at pH 11·0 in the pH-jump method with Ca^{2+} (E5531: 2 mg mL^{-1}; [E5531] : [Ca^{2+}] = 1 : 3), ● after adjustment of the pH from 11·0 to 7·3 by the addition of phosphate–NaOH buffer (E5531: 100 μg mL^{-1}; [E5531] : [Ca^{2+}] = 1 : 3), ▲ dispersed at pH 11·0 in the pH-jump method with Ca^{2+} and EDTA (E5531: 2 mg mL^{-1}; [E5531] : [Ca^{2+}] : [EDTA] = 1 : 3 : 3), △ adjustment of the pH from 11·0 to 7·3 by the addition of phosphate–NaOH buffer (E5531: 100 μg mL^{-1}; [E5531] : [Ca^{2+}] : [EDTA] = 1 : 3 : 3).

by the pH-jump method. These finding indicate that the charge on, and dissociation of, the head phosphate group of the E5531 molecule are important in the dispersion process.

Structure of E5531 aggregates

Figure 3 shows the volumes of trapped inner space in the aggregates per mole of E5531 prepared by the pH-jump method (pH 11·0→7·3), the normal dilution method (pH 7·3→7·3), the pH-jump method with Ca^{2+} (pH 11·0→7·3) and the pH-jump method with Ca^{2+} and EDTA (pH 11·0→7·3) as a function of dispersing time. The trapped volumes of small unilamellar vesicles (diameter: 20–50 nm), large unilamellar vesicles (diameter: 200–1000 nm) and multilamellar vesicles (diameter: 400–3500 nm) of phosphatidylcholine have been estimated to be 0·2–0·5, 7–10 and 3–4 L mol^{-1}, respectively (Szoka & Papahadjopoulos, 1978). E5531 aggregates prepared by the pH-jump method (diameter: 20 nm) after a 3-min dispersal period contained a trapped volume of approximately 0·4 L mol^{-1}. The volume decreased to approximately 0·3 L mol^{-1} after 8 min of dispersing and then remained unchanged for dispersing times of up to 90 min. These data indicate that E5531 molecules prepared by the pH-jump method have liposome-like structures (small unilamellar vesicles). E5531 aggregates prepared by the normal dilution methods (diameter: 130 nm) and the pH-jump method with Ca^{2+} (diameter: 240 nm) after a 3-min dispersal period contained trapped volumes of approximately 1·0 and 1·9 L mol^{-1}, respectively. These volumes decreased to 0·9 and 1·8 L mol^{-1}, respectively and then remained unchanged for dispersing times of up to 90 min. These data indicate that the structures of these aggregates are oligolamellar vesicles. However, E5531 aggregates prepared by the pH-jump method with Ca^{2+} and EDTA (diameter: 20 nm) contained a trapped volume of approximately 0·4 L mol^{-1}. These data indicate that E5531 molecules prepared by this method have liposome-like structures (small unilamellar vesicles).

The progress of hydration and membrane fluidity of the E5531 aggregates

The hydration process and the relationship between the dispersing method and dispersing time were determined by measurements of the membrane fluidity of E5531 aggregates. Figure 4 shows the relationship between dispersing time and the order parameters of E5531 aggregates at 25°C prepared by the pH-jump method (pH 11·0→7·3), the normal dilution method (pH 7·3→7·3), the pH-jump

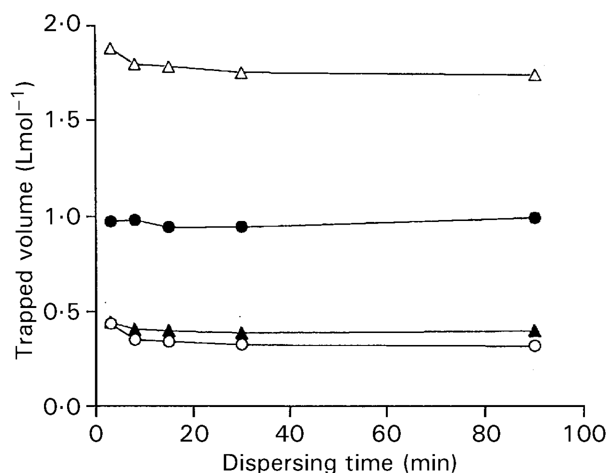

Figure 3. Relationship between dispersing time in 0·003 N NaOH solution and the trapped volume of the E5531 aggregates. ○ Prepared by the pH-jump method (pH 11·0→7·3), ● prepared by the normal dilution method (pH 7·3→7·3), △ prepared by the pH-jump method (pH 11·0→7·3) with Ca^{2+} ([E5531] : [Ca^{2+}] = 1 : 3), ▲ prepared by the pH-jump method (pH 11·0→7·3) with Ca^{2+} and EDTA ([E5531] : [Ca^{2+}] : [EDTA] = 1 : 3 : 3).

method with Ca^{2+} (pH 11·0→7·3) and the pH-jump method with Ca^{2+} and EDTA (pH 11·0→7·3). The order parameter of E5531 aggregates during dispersing at pH 11·0 in the pH-jump method decreased with increasing dispersing time. Just after the pH was adjusted to 7·3, the order parameter increased due to the conversion of the ionized form from semi-ionized to fully ionized. However, the order parameter of aggregates prepared by the normal dilution method remained unchanged with progress of dispersing time. This indicates that the hydration of the E5531 membrane is increased at the basic pH as a result of dissociation of the head phosphate group and, just after neutralization to pH 7·3, hydration is stopped by the formation of intermolecular hydrogen bonds in the head phosphate group.

Even at pH 11·0, when the charge of the head phosphate group is neutralized by the addition of Ca^{2+}, hydration did not progress and the order parameter was larger than that for the normal dilution method. However, after fully masking the Ca^{2+} by the addition of EDTA at pH 11·0, hydration progressed with dispersing time and the order parameter was similar to that for the pH-jump method. These findings indicate that the charge and dissociation of the head phosphate group of the E5531 molecule are important for dispersing.

Figure 5 shows the temperature dependence of the order parameter of the E5531 aggregates (100 μg mL^{-1}, pH 7·3) prepared by the pH-jump method as a function of the dispersing time in 0·003 N NaOH (pH 11·0). The order parameter of DPH decreased markedly at 30°C, indicating the

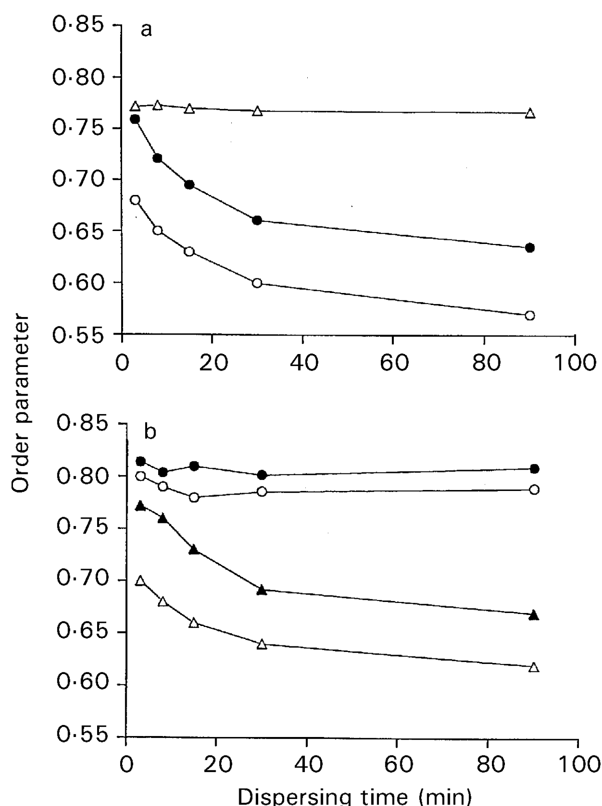

Figure 4. Relationship between dispersing time in 0·003 N NaOH solution and the order parameter of DPH for the E5531 aggregates at 25°C. a. ○ Dispersed at pH 11·0 in the pH-jump method (E5531: 2 mg mL^{-1}), ● adjustment of the pH from 11·0 to 7·3 by the addition of phosphate–NaOH buffer containing lactose (E5531: 100 μg mL^{-1}, 4·25 mM phosphate–NaOH buffer, 10% lactose, pH 7·3), △ prepared by the normal dilution method (pH 7·3→7·3). b. ○ Dispersed at pH 11·0 in the pH-jump method with Ca^{2+} (E5531: 2 mg mL^{-1}; [E5531] : [Ca^{2+}] = 1 : 3), ● after adjustment of the pH from 11·0 to 7·3 by the addition of phosphate-NaOH buffer containing lactose (E5531: 100 μg mL^{-1}, 4·25 mM phosphate–NaOH buffer, 10% lactose, pH 7·3; [E5531] : [Ca^{2+}] = 1 : 3), △ dispersed at pH 11·0 in the pH-jump method with Ca^{2+} and EDTA (E5531: 2 mg mL^{-1}; [E5531] : [Ca^{2+}] : [EDTA] = 1 : 3 : 3), ▲ after adjustment of the pH from 11·0 to 7·3 by the addition of phosphate–NaOH buffer containing lactose (E5531: 100 μg mL^{-1}, 4·25 mM phosphate–NaOH buffer, 10% lactose, pH 7·3; [E5531] : [Ca^{2+}] : [EDTA] = 1 : 3 : 3).

phase transition of the E5531 membrane from a gel to liquid-crystal state at this temperature. As dispersing time increased in the 0·003 N NaOH solution (pH 11·0), the order parameter after adjustment of the pH to 7·3 also decreased and membrane fluidity increased, indicating that, as dispersion progressed, more fluid E5531 membranes were formed.

Pharmacokinetics in rats for E5531 aggregates with different membrane fluidity

In order to investigate the relationship between the membrane fluidity of E5531 aggregates and the pharmacokinetic profile, reconstituted E5531 solutions (100 μg mL^{-1}, pH 7·3) with differing membrane fluidity prepared by changing the dispersing time in the pH-jump method was intravenously injected into rats. As shown in Figure 6 and Table 1, the plasma concentrations at 2 h after dosing and AUC (0–2 h) increased with an increase in the dispersion time. Figure 7 illustrates the relationship between the order parameter of an E5531 reconstituted solution at 37°C and AUC (0–2 h). The order parameter enables changes in AUC to be clearly observed.

We obtained clearly different pharmacokinetic profiles in rats using samples with differing membrane fluidity. This suggests that the fluidity of the E5531 membranes increased as dispersion progressed and that fluidity affects the pharmacokinetics in rats. In other words, pharmacokinetics in rats can be controlled by changing the dispersing time in the alkaline solution (pH 11·0) and the degree of hydration and membrane fluidity of the aggregates.

Hampton & Raets (1991) reported on the interactions between the metabolism of lipid A-like molecules by macrophages and their response to these molecules. They concluded that the uptake of the molecules is mediated by scavenger receptors. To date, the metabolic pathway of E5531 is not known with certainty. It is assumed that when E5531 molecules are bound to the receptors and incorporated into the macrophages, the more rigid membrane is more easily bound to the receptor and uptake of the molecules therefore occurs more rapidly.

Stability of E5531 aggregates in rat plasma

To investigate the stability of E5531 aggregates after intravenous injection into rats, the permeability of E5531 aggregates prepared by the

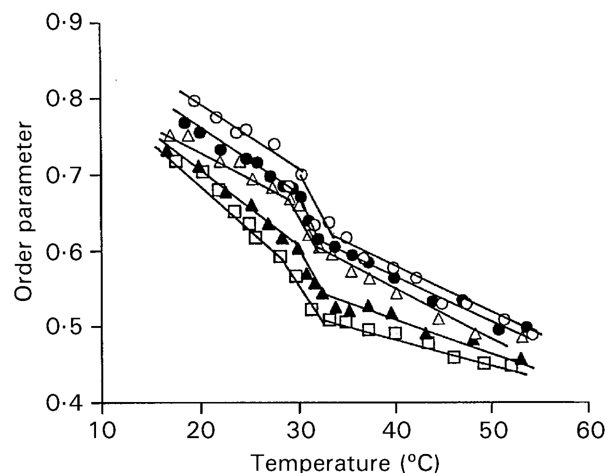

Figure 5. Relationship between temperature and order parameter of DPH for the E5531 aggregates prepared by the pH-jump method using various dispersing times. ○ Dispersing for 3 min, ● dispersing for 8 min, △ dispersing for 15 min, ▲ dispersing for 30 min, □ dispersing for 90 min.

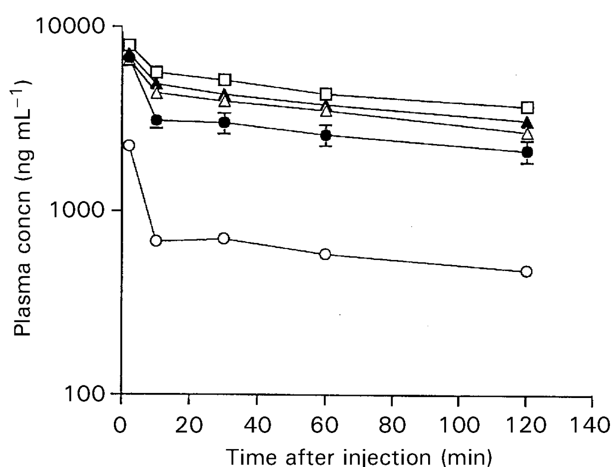

Figure 6. Plasma concentration of E5531 after intravenous administration of E5531 to male rats (as a bolus injection via the femoral vein; 0·3 mg kg^{-1}). Each point represents the mean ± s.e.m. of three animals. ○ Dispersing for 3 min, ● dispersing for 8 min, △ dispersing for 15 min, ▲ dispersing for 30 min, □ dispersing for 90 min.

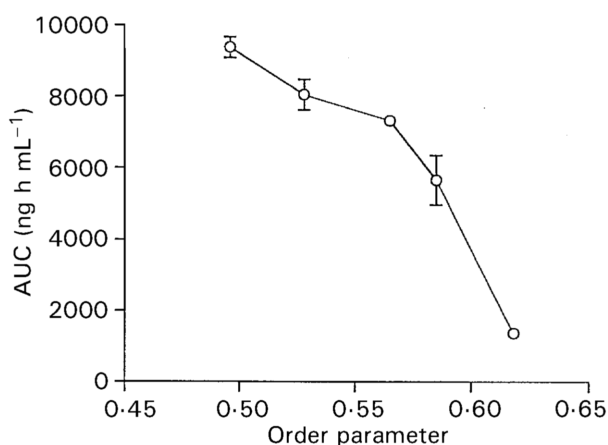

Figure 7. Relationship between the order parameter of DPH for E5531 aggregates at 37°C and AUC (0–2 h) in rats. The aggregates were prepared by the pH-jump method with different dispersing times. E5531 was administered as a bolus injection (0·3 mg kg^{-1}) via a femoral vein.

pH-jump method in rat plasma was evaluated by means of the leakage profile of calcein at 37°C. Figure 8 shows the time-course for the leakage of calcein from E5531 aggregates, prepared using different dispersing times by the pH-jump method. These data indicate that the permeability of E5531 aggregates for calcein increase with increasing dispersing time. The leakage of calcein from E5531 aggregates at 37°C after a 30-min incubation is shown in Table 1.

Based on membrane fluidity data (Figure 5), the order parameters of E5531 aggregates at 37°C decreased with increasing dispersing time, indicating that the membrane fluidity of E5531 aggregates increased. Kakee et al (1994) studied the relationship between permeability and membrane fluidity and reported that the permeability of egg phosphatidylcholine liposomes containing the lipid A analogue, GLA-60, in rat plasma increased with an increase membrane fluidity. In a similar manner, we can assume that the permeability of E5531 aggregates in rat plasma increased with an increase in membrane fluidity. The difference in hydration will affect membrane properties such as fluidity and permeability and the stability of the aggregates after intravenous injection in rats would be expected to be dependent on dispersing time during formulation. In other words, the stability of E5531 aggregates after intravenous injection into rats can be controlled by changing the dispersing time in the alkaline solution (pH 11·0).

Conclusions

The dispersing process for E5531 using the pH-jump method (pH 11·0→7·3) was evaluated. The size of the aggregates decreased to approximately 20 nm and the membrane fluidity increased with increasing dispersing time in 0·003 N NaOH (pH 11·0). However, when prepared by the normal dilution method (pH 7·3→7·3), the size of the aggregates was 140 nm and the membrane had less fluidity than that prepared by the pH-jump method and was independent of the dispersing time. This indicates that during dispersing at a basic pH, the hydration progressed and, just after adjustment of the pH to 7·3, the hydration stopped and the

Table 1. Process parameters for E5531 dispersion with different dispersing times in the pH-jump method.

Parameters (after neutralization to pH 7·3)	Dispersing time in 0·003 M NaOH (min)				
	3	8	15	30	90
Aggregate size (nm)	29·0 ± 14·0	18·4 ± 6·8	16·5 ± 6·0	15·3 ± 5·0	16·7 ± 5·3
Trapped volume (L mol^{-1})	0·44	0·35	0·34	0·33	0·32
Order parameter for DPH at 37°C	0·62	0·59	0·57	0·53	0·50
Plasma concn at 2 h in rats (ng mL^{-1})	481·8 ± 42·6	2141·4 ± 291·2	2707·3 ± 19·9	3119·7 ± 120·1	3741·1 ± 16·6
AUC (0–2 h) (ng h mL–1)	1362 ± 63	5667 ± 692	7333 ± 84	8051 ± 426	9380 ± 290
Leakage of calcein in rat plasma at 37°C after 30 min (%)	11·5	14·6	16·9	20·3	22·3

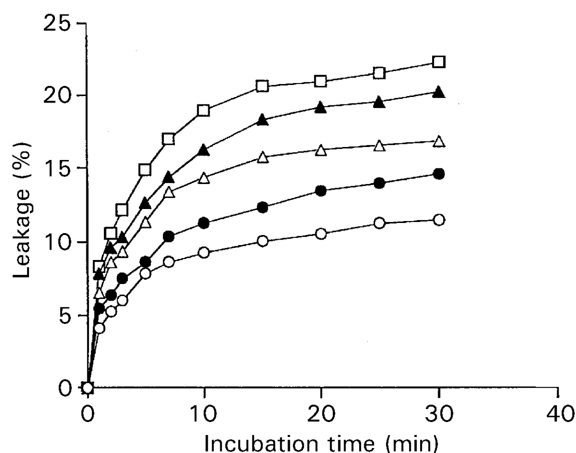

Figure 8. Leakage profile of calcein from E5531 aggregates in rat plasma at 37°C. Aggregates were prepared by the pH-jump method using different dispersing times. ○ Dispersing for 3 min, ● dispersing for 8 min, △ dispersing for 15 min, ▲ dispersing for 30 min, □ dispersing for 90 min.

aggregates' size and membrane fluidity remained constant thereafter. In other words, the degree of hydration of the membrane is dependent on the dispersing time at pH 11·0. Even at pH 11·0, the hydration did not proceed after the addition of Ca^{2+} but after completely masking the Ca^{2+} with EDTA, the hydration proceeded in a manner similar to that observed in the pH-jump method. These findings indicate that the charge and dissociation of the head phosphate group of the E5531 molecule are important for dispersing. Using samples with differing degrees of hydration and differing membrane fluidity prepared by changing the dispersing times in the pH-jump method, the pharmacokinetics in rats was evaluated and it was confirmed that the membrane fluidity is correlated with pharmacokinetics and stability in the plasma. Thus, the pharmacokinetics of E5531 in rats appears to be controllable by changing the degree of hydration and membrane fluidity by means of altering the dispersing time in alkaline solution (pH 11·0).

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