—Reviews—

Development of an Injectable Formulation for the Novel Lipid A Analog E5531 Using a 'pH-jump Method'

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(Received June 28, 2004; Accepted August 19, 2004)

In order to design an injectable formulation of E5531, a novel synthetic disaccharide analog of novel lipid A, for the treatment of septic shock, a 'pH-jump method' was developed. In this method, E5531 was dispersed in 0.003 mol/l NaOH (pH 11.0, above pK_{a2}) at 50°C (above phase transition temperature) and then mixed with a buffer to neutralize the pH to 7.3. E5531 was dispersed as particles, and the size was approximately 20 nm. The structure of the particles was vesicular. After dispersal, the solution was sterilized using a filter, filled aseptically into vials, and lyophilized. The size of the particles did not change before and after lyophilization. The relationship between the physicochemical properties of the particles and the pharmacokinetics in rats after intravenous administration was investigated. The membrane fluidity of the particles was affected by the dispersal methods, the dispersal time in 0.003 mol/l NaOH in the pH-jump method, and the addition of Ca^{2+} to the solution. The membrane fluidity was correlated with the pharmacokinetics in rats.

Key words——lipid A analog; injectable formulation; pH-jump method; membrane fluidity; pharmacokinetics

INTRODUCTION

Lipid A is a component of bacterial lipopolysaccharide (LPS), which is present in the major amphiphilic constituents of the leaflet of Gram-negative bacteria. Lipid A is a potent biologically active site[1,2] and induces prostaglandins, and cytokines such as interferon,[3] interleukin 1[4] and tumor necrosis factor (TNF)[5] in mammalian cells such as macrophages and lymphocytes. This compound also induces undesirable toxic effects, such as fever and the Schwartzmann bleeding reaction.[6,7]

Many attempts have been made to synthesize low toxic lipid A analogs. Christ and others have indicated that E5531 (Fig. 1), a novel synthetic disaccharide analog of lipid A, has low toxicity and retains some of the biological activities of lipid A such as the reduction of TNF production.[8—11] This compound has been found to be a specific LPS antagonist in an LPS-binding assay, and is an inhibitor of LPS-induced TNF production in monocytes/macrophages, so it is expected that E5531 will be useful for the treatment of septic shock.

An injectable E5531 formulation would be extremely useful; however, the dispersion of E5531 in aqueous solution was a major problem. E5531, like any lipid A analog, does not disperse well by only stirring in solutions with a neutral pH. Sonication has been used to disperse lipid A analogs[12,13] and LPS[14] for investigational use. However, sonication has two major drawbacks-its is not suitable for large scale production, and it is difficult to control the sonication

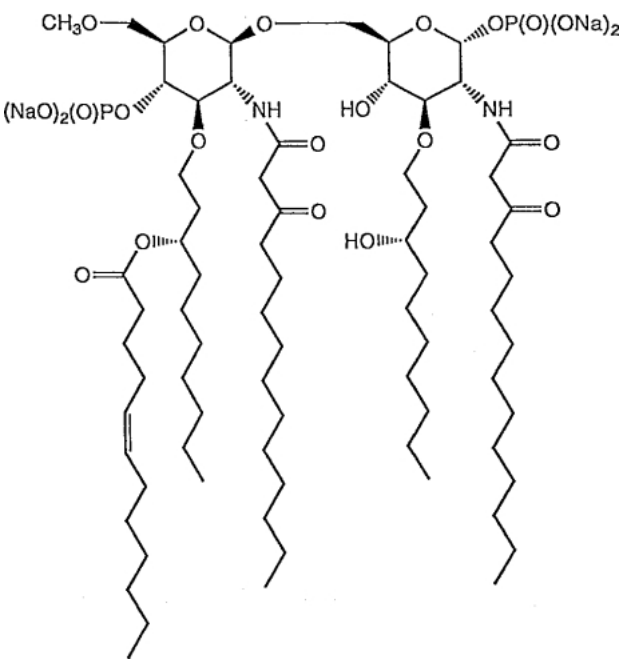

Fig. 1. Chemical Structure of the Synthetic Lipid A Analog E5531

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energy and dispersal time.[15,16] Ideally, the pH of E5531 pharmaceutical injection should be neutral since the injection of alkaline or acidic pH solutions is undesirable. It is also important to control the size of the particles because the size of colloidal particles is correlated with their hepatic uptake, and a smaller particle size (less than 100 nm) may result in reduced hepatic uptake.[17] When the compound is not stable for steam sterilization, the particle size should be at least less than 220 nm for sterilization by filtration in the production of an injectable dosage form.

In order to overcome these problems, a novel 'pH-jump method' for dispersing E5531 has been developed. The advantages of this method include the suitability for large scale production (without any mechanical input such as sonication) and the preparation of smaller sized particles (approximately 20 nm) at neutral pH. The physicochemical properties of E5531 particles, such as the size, structure and membrane fluidity, were determined. The phramacokinetics in rats after intravenous administration were evaluated, and the relationship between them are discussed.

DEVELOPMENT OF A DISPERSAL PROCEDURE FOR THE LIPID A ANALOG E5531[18]

The dispersal procedures for E5531 were investigated from the measurements of pK_a and the gel-liquid crystal phase transition temperature.[18,19] Figure 2 represents the titration curve for the E5531 dispersion (0.2 mM) with 0.2 mM HCl, and shows two pK_a values, at approximately 6.0 (pK_{a1}) and 9.3 (pK_{a2}), respectively. At neutral pH, E5531 exists almost entirely in the semi-ionized form (2Na-form). At a pH of 10 or above in aqueous solution, E5531 is almost completely present in the fully ionized form. This result indicates that it will be difficult to disperse E5531 at neutral pH but that it will be easily dispersed in alkaline solution, especially above pH 10.0.

DSC measurements revealed that the phase transition temperature of E5531 in 4.25 mM phosphate-NaOH buffer containing 10% lactose (pH 7.3) and 0.003 N NaOH solution (pH 11.0) were 31.7℃ and 31.6℃, respectively.[18] Lactose was popularly used in the lyophilization of the lipid particles and selected in this study. Based upon the results, it is expected that the hydration will be accelerated above the phase transition temperature. So, the dispersal temperature was set for 50℃ and the effect of pH on the dispersal of E5531 was evaluated.

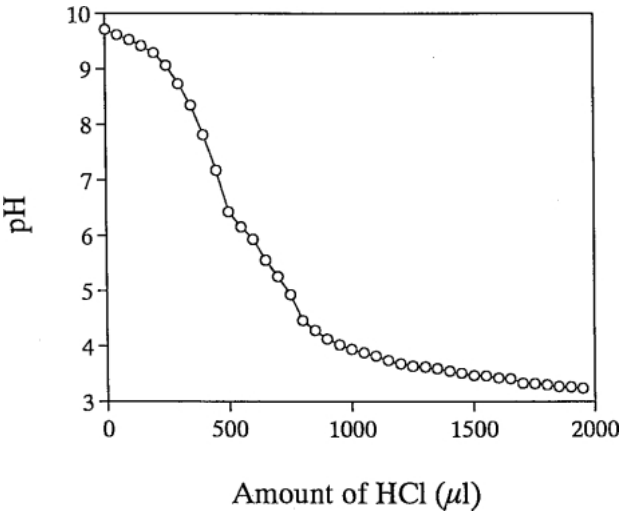

Fig. 2. pH Titration Curve for E5531
A 0.2 mM solution of E5531 (10 ml) dispersed by sonication was titrated with 0.2 mM HCl. The pH was recorded as a function of the added volume of HCl. pK_{a1}=6.0 and pK_{a2}=9.3.

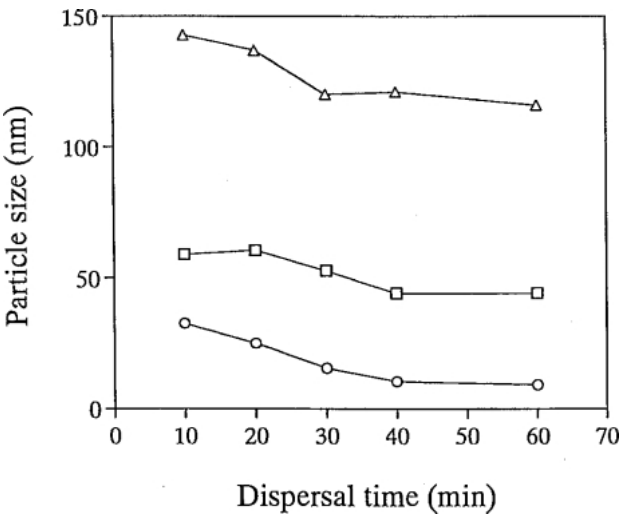

Fig. 3. Relationship between Dispersal Time and the E5531 Particle Size Evaluated by Dynamic Light Scattering (DLS) Methods (in weight) at 25℃
○: pH-jump method, △: stirring method, □: sonication method.

Figure 3 represents the relationship between dispersal time and the size of the E5531 particles determined by dynamic light scattering (DLS), prepared, respectively, by the pH-jump method, stirring method and sonication method. The size of the E5531 particles prepared by the stirring method, sonication method and pH-jump method after a 10-minute dispersal period, was approximately 140, 60 and 35 nm, respectively. The size decreased with increasing dispersal

Table 1. Diameter Evaluated by Dynamic Light Scattering (DLS), Trapped Volume and the Estimated Structure of E5531 Particles Prepared by Stirring, Sonication and pH-jump Methods

	Particle diameter (nm) (mean±S.E.)	Trapped volume (liter/mol)	Estimated structure
E5531 particles prepared by stirring method	128.9±75.3	0.91	Oligolamellar vesicle
E5531 particles prepared by sonication method	47.1±19.9	0.66	Oligolamellar vesicle
E5531 particles prepared by pH-jump method	13.1±4.4	0.27	Small unilamellar vesicle

time and reached constant values of approximately 120, 45 and 15 nm, respectively, after 40 minutes. The size of the E5531 particles prepared by the pH-jump method after a 60-minute dispersal period was also evaluated by electron microscopy. This method indicates the size of 17 nm, similar to the result obtained from the DLS method.

Table 1 shows the volumes of trapped inner space in the particles per mole of E5531 prepared by the pH-jump, stirring and sonication methods. The trapped volumes of small unilamellar vesicles (diameter: 20—50 nm), large unilamellar vesicles (200—1000 nm) and multilamellar vesicles (400—3500 nm) of phosphatidylcholine have been estimated to be 0.2—0.5, 3—4 and 7—10 liter per mole, respectively.[20] E5531 particles prepared by the pH-jump method (diameter: 13.1 nm) had a trapped volume of 0.27 liter per mole. This data indicates the E5531 molecules prepared by the method have a liposome-like structure (small unilamellar vesicles). E5531 particles prepared by the stirring method (diameter: 128.9 nm) and sonication method (diameter: 47.1 nm) had a trapped volume of 0.91 and 0.66 liters per mole, respectively. These data indicate that the structure of the particles is an origolamellar vesicle.

The hydration process of the three dispersal methods and time were compared by measurement of the membrane fluidity of the particles.[21] Figure 4 shows the relationship between dispersal time and the order parameter of E5531 particles determined by a fluorescence polarization technique[22] at 25°C prepared by the pH-jump and stirring methods. The order parameter of E5531 particles during dispersal at pH 11.0 in the pH-jump method decreased with increasing time. Just after the pH was adjusted to pH 7.3, the order parameter increased due to the conversion of the ionized form from fully ionized to semi-ionized. However, the order parameter of the particles prepared by the stirring method remained unchanged with the progression of dispersal time. This

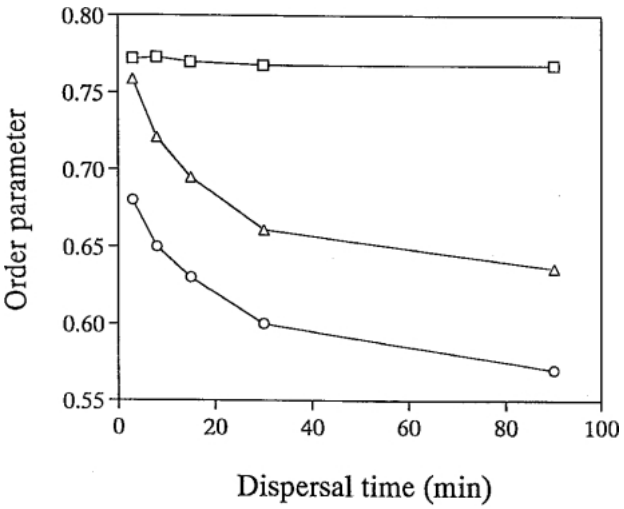

Fig. 4. Relationship between Dispersal Time in 0.003 N NaOH Solution and the Order Parameter of DPH for the E5531 Particles at 25°C

(○): dispersed at pH 11.0 in the pH-jump method (E5531: 2 mg/ml), (△): adjustment of the pH from 11.0 to 7.3 by the addition of phosphate-NaOH buffer containing lactose (E5531: 0.1 mg/ml, 4.25 mM phosphate-NaOH buffer, 10% lactose, pH 7.3), (□): prepared by the stirring method (4.25 mM phosphate-NaOH buffer, 10% lactose, pH 7.3).

result indicates that the hydration of the E5531 membrane is increased at basic pH as a result of dissociation of the head phosphate group, and just after neutralization to pH 7.3, hydration will be stopped by the formation of intermolecular hydrogen bonds in the head phosphate group.

Based on the above results, the hydration of E5531 is increased in the alkaline solution (0.003 N NaOH, pH 11.0), the size of the particles is decreased to approximately 20 nm, and a more fluid membrane is formed with a subsequent increase in the dispersal time in the alkaline solution.

CHARACTERIZATION OF E5531 PARTICLES PREPARED BY THE pH-JUMP METHOD

In order to clarify in detail the behavior of E5531 particles during dispersion in alkaline solution (0.003 N NaOH, pH 11.0) and after neutralization to pH

7.3, various physicochemical properties of E5531 particles were measured and compared to that of L-α-dipalmitoylphosphatidylcholine (DPPC) liposomes.[23] DPPC liposomes were prepared by the sonication method for 60 min at 50°C (64.7 μM DPPC, 4.25 mM phosphate-NaOH buffer containing 10% lactose, pH 7.3).

The physicochemical characteristics of E5531 particles and DPPC liposomes are summarized in Table 2. The critical packing parameters[24] for E5531 were calculated based on the area per molecule results, the volume of the hydrophobic part and the length of the acyl chain in the molecular structure. The critical packing parameter (x) **is determined by the following formula:**

$$x = v/a \cdot l$$

where, v is the volume of the hydrophobic part, a is the area of the hydrophilic head group and l is the length of the acyl chain, when $x < 1/3$ discoidal micelles form, $1/3 < x < 1/2$ tubular micelles form, $1/2 < x < 1$ vesicles form and $1 < x$, hexagonal H_{II} structures form.

The x values for E5531 particles and DPPC liposomes in pH 7.3 were 0.87 and 0.70, respectively. Therefore, it can be expected that the structure of E5531 particles and DPPC liposomes are of the vesicle structure, respectively. These data agree with the results of the trapped inner space of E5531 particles.

Fluorescence quenching techniques[25] were used to obtain information on the structure of E5531 particles and DPPC liposomes. Acrylamide was used as a nonpenetrating quencher for DSHA (*N*-dansylhexadecylamine) fluorescence embedded in the E5531 particles. DPPC liposomes which served as a control for the vesicles had a value of 0.58, which is in agreement with the molar ratio of phosphatidylcholine molecules at the outer and inner surface of small unilamellar vesicles.[26] The value for E5531 (=0.65) is similar to that of DPPC, indicating that the structure of the E5531 particle is also small unilamellar vesicles. Based on the results, the number of molecules which exist in the internal and external membrane can be calculated, and this is shown in Table 2.

THE QUALITY OF THE E5531 LYOPHILIZED VIALS PREPARED BY THE pH-JUMP METHOD[27]

The pH-jump method does not need a mechanical input such as sonication, and is suitable for large-scale production of an injectable dosage form. No degradated products of E5531 were observed at 50°C for 3 hr during dispersal in the alkaline solution. As shown in Table 3, the size of the particles, order parameters and chemical purity did not change before and after lyophilization, or after 24 months of storage in the lyophilized cake at room temperature.

Table 2. Characterization of E5531 Particles and DPPC Liposomes

	E5531 particles	DPPC liposomes
Weight averaged particle size (nm)	22.9±9.1	27.1±10.3
Zeta potential (mV)	−45.1	−1.3
Trapped volume (liter/mol)	0.27	0.28
Ratio of external membrane (%)	65.0	58.1
Molecular weight	4.72×10^6	3.13×10^6
The number of molecules per particle	3062	4209

Table 3. Stability of E5531 Lyophilized Vials

Parameters	Storage at room temperature after lyophilization (months)				
	Before lyophilization	0 (After lyophilization)	6	12	24
Particle size (nm) (mean±S.E.)	19.6±5.3	19.1±6.3	20.6±5.5	21.4±5.6	20.3±5.3
Order parameter determined by DPH polarization at 37°C	0.60	0.62	0.63	0.62	0.62
Chemical purity determined by HPLC (%)	99.3	99.5	99.4	99.1	99.3

THE EFFECTS OF DIVALENT CATIONS ON THE PHYSICOCHEMICAL PROPERTIES OF E5531 PARTICLES

In the case of anionic phospholipids, membrane fluidity is affected by divalent cations, resulting in an increase in the phase transition temperature.[28] E5531 is also classified as an anionic lipid and, hence, it would be expected that the membrane fluidity of E5531 particles would be altered by the addition of cations. In order to investigate the effect of divalent cations on E5531 particles, Ca^{2+} was added to the E5531 reconstituted solution and the physicochemical properties were evaluated.[29]

Figure 5 shows the weight averaged size of E5531 particles in the presence of different molar ratios of $[Ca^{2+}]/[E5531]$, as evaluated by DLS measurements. At molar ratios $[Ca^{2+}]/[E5531]=0$, 1 and 3, the mean diameters were all nearly 20 nm. At a ratio of $[Ca^{2+}]/[E5531]=5$ or greater, the particle size increased with increasing $[Ca^{2+}]$. In order to evaluate the effect of Ca^{2+} on physicochemical properties under conditions in which the size of the particles is similar (approximately 20 nm), the zeta potentials and membrane fluidity were determined using the samples having molar ratios $[Ca^{2+}]/[E5531]=0$, 1 and 3. Table 4 shows the zeta potentials as a function of the $[Ca^{2+}]/[E5531]$. Since E5531 is negatively charged in neutral aqueous solution, the phosphate group at the head sugar moiety had a net negative charge and minus values for the zeta potentials. The addition of Ca^{2+}, in part, neutralizes the negative charge of the head phosphate group, thus causing the zeta potential to be increased. An increase of the zeta potential, in some cases, induces instability of the colloidal particles30) and their fusion.[31] However, at the molar ratio of $[Ca^{2+}]/[E5531]=0$, 1 and 3, these phenomena were not observed.

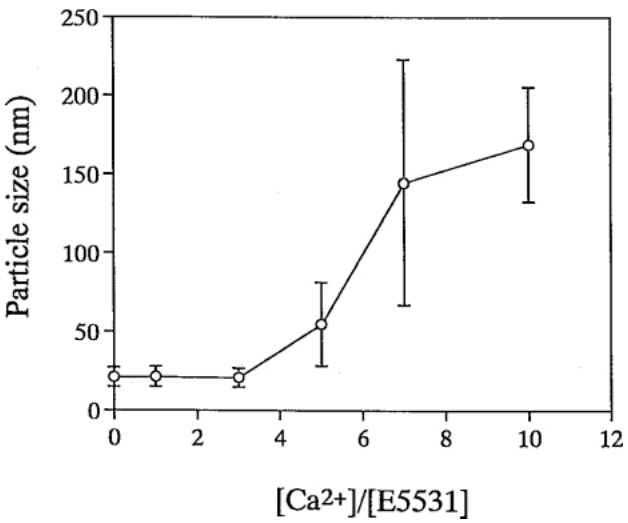

Fig. 5. Relationship between the Molar Ratio of $[Ca^{2+}]/[E5531]$ and the Size of E5531 Particles
Each point represents the mean diameter±S.E. of the measurements.

RELATIONSHIP BETWEEN THE PHYSICOCHEMICAL PROPERTIES OF E5531 PARTICLES AND THE PHARMACOKINETICS AFTER INTRAVENOUS ADMINISTRATION IN RATS

In order to investigate the relationship between the membrane fluidity of E5531 particles and the pharmacokinetic profile, a reconstituted E5531 solution (100 μg/ml, pH 7.3) with a different membrane fluidity prepared by changing the dispersal time in the pH-jump method was intravenously administered to rats.[21] As shown in Table 5, the plasma concentration at 2 hr after dosing and the AUC (0—2 hr) increased with an increase in the dispersal time. Figure 6 illustrates the relationship between the order parameter of E5531 reconstituted solution at 37°C and AUC (0—2 hr), respectively. The order

Table 4. Effect of Ca^{2+} on the Physicochemiucal Properties of E5531 Particles and the Pharmacokinetics in Rats

$[Ca^{2+}]/[E5531]$	Aggregates size (nm) (mean±S.E.)	Zeta potential (mV) (mean±S.E.)	Order parameter at 37°C determined by DPH polarization	AUC (0-2 hr) (ng·hr/ml) (mean±S.E.)	Plasma half life ($t_{1/2}$, 0.5 hr～) (mean±S.E.)
0	20.9±6.2	−48.7±0.6	0.434	11384±376	4.0±1.0
1	21.2±6.4	−39.3±0.7	0.558	8582±665	4.3±1.3
3	20.6±6.2	−35.1±0.5	0.702	5321±419	4.0±1.2
5	54.8±26.6	NP	NP	NP	NP
7	144.9±78.0	NP	NP	NP	NP
10	169.0±36.2	NP	NP	NP	NP

NP: Not performed.

Table 5. Process Parameters for E5531 Samples with Different Dispersing Times in 0.003N NaOH (pH 11.0)

Parameters (after neutralization to pH 7.3)	Dispersing time in 0.003N NaOH (min)				
	3	8	15	30	90
Particle size in reconstituted solution (nm) (mean±S.E.)	29.0±14.0	18.4±6.8	16.5±6.0	15.3±5.0	16.7±5.3
Order parameter determined by DPH polarization at 37℃	0.62	0.59	0.57	0.53	0.50
Plasma concentration at 2 hr in rats (ng/ml) (mean±S.E.)	481.8±42.6	2141.4±291.2	2707.3±19.9	3119.7±120.1	3741.1±16.6
AUC in rats (0-2hr) (ng·hr/ml) (mean±S.E.)	1362±63	5667±692	7333±84	8051±426	9380±290

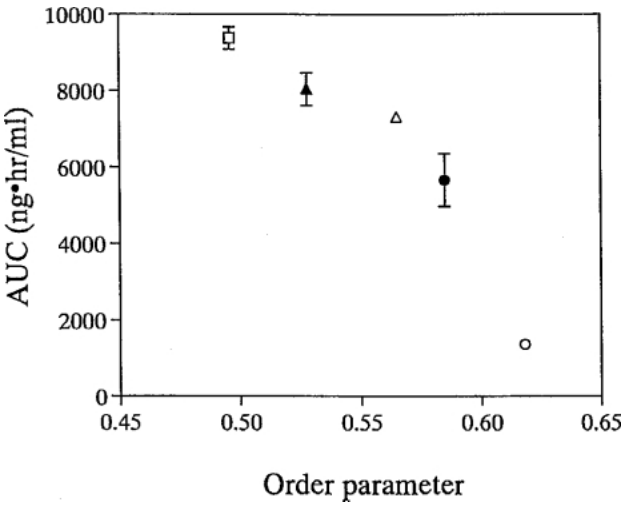

Fig. 6. Relationship between the Order Parameter of DPH for the E5531 Membranes at 37℃ (The Particles were Prepared by the pH-jump Method with Different Dispersing Times) and AUC (0—2 h) in rats

○: dispersed for 3 min, ●: dispersed for 8 min, △: dispersed for 15 min, ▲: dispersed for 30 min, □: dispersed for 90 min. Each point represents the mean±S.E. of three animals.

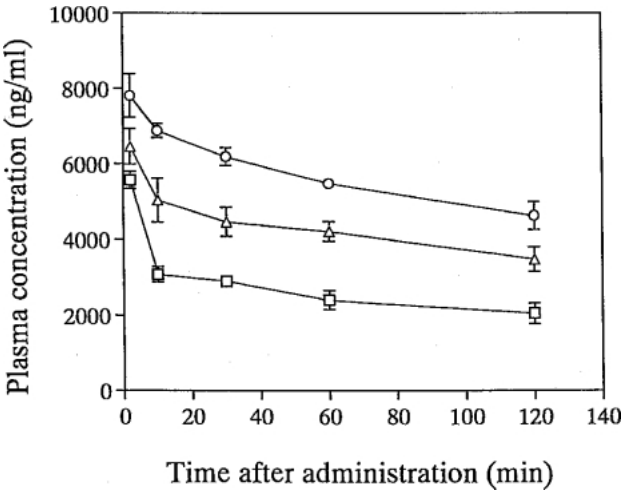

Fig. 7. Plasma Concentrations of E5531 after Intravenous Administration of E5531 to Male Rats as a Function of the Molar Ratio of $[Ca^{2+}]/[E5531]$

$[Ca^{2+}]/[E5531]=0$ (○), 1 (△), 3 (□). Each point represents the mean ±S.E. of three animals.

parameter enables changes in the AUC to be clearly observed. This result suggests that the fluidity of the E5531 membrane increased as dispersion progressed, and that the fluidity affected the pharmacokinetics in rats. In other words, pharmacokinetics in rats can be controlled by changing the dispersal time in the alkaline solution (pH 11.0), the degree of hydration and the membrane fluidity of the particles.

In addition, in order to investigate the relationship between the membrane fluidity of E5531 particles with the same particle size and the pharmacokinetic profile, a reconstituted E5531 solution (100 μg/ml, pH 7.3) with different membrane fluidities prepared by the addition of Ca^{2+} was intravenously injected into rats.[29] As shown in Fig. 7 and Table 4, the plasma concentrations at 2 hr after doing, and the AUC (0—2 h) decreased with increasing in $[Ca^{2+}]$. Figure 8 illustrates the relationship between the order parameter of a reconstituted E5531 solution at 37℃ and AUC (0–2 h), respectively. Distinctly different pharmacokinetic profiles were obtained in rats using the samples obtained via changing both $[Ca^{2+}]$ and the membrane fluidity. As shown in Table 4, the $t_{1/2}$ of the three samples were similar, and the fluidity affected on the distribution phase.

The reported metabolic pathway for natural lipid A suggests that lipid A after administration into a vein will bind with LBP (lipopolysaccharide binding protein) and then be metabolized in the liver.[32,33] From ^{14}C–E5531 biodistribution study, most of the E5531 was metabolized in the liver (data not shown). On the other hand, the complex will bind with the macrophrages, HDL and CD14 (glycoprotein, molecular

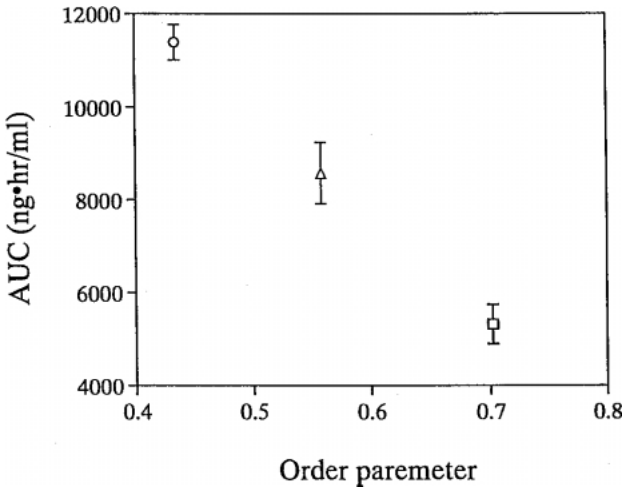

Fig. 8. Relationship between the Order Parameter of DPH for the E5531 Membranes with a Molar Ratio of $[Ca^{2+}]/[E5531]$ at 37°C and AUC (0—2 h) in Rats

$[Ca^{2+}]/[E5531]=0$ (○), 1 (△), 3 (□). Each point represents the mean ±S.E. of three animals.

weight: 60 kDa) in the vein and will prompt the biological effects.[32,33] At this time, the effects of the membrane fluidity of E5531 particles on binding with LBP and the characterization of the particles in the vein are not known with certainty. Of course, the particles will interact with divalent cations such as Ca^{2+} and Mg^{2+} in the vein, and the E5531 particle size and the membrane fluidity will be changed in vivo. However, the effects of divalent cations in the vein will be smaller than the metabolizing speed in the liver.

CONCLUSION

E5531, a novel synthetic lipid A analog, was dispersed using a pH-jump method, which involves dispersing E5531 in an alkaline solution (0.003 N NaOH, pH 11.0, above $pK_{a2}=9.3$) at 50°C (higher than the phase transition temperature of approximately 30°C) and then mixing the solution with a buffer to neutralize the pH to 7.3. This method does not require the mechanical power and is suitable for large-scale production of an injectable formulation. E5531 particles form a vesicle-like structure. The size was approximately 20 nm and did not change before or after lyophilization, or after 24 months of storage in the lyophilized cake at room temperature. The degree of hydration of the membrane was dependent on the dispersing time at pH 11.0. With an increase in the dispersal time in the alkaline solution, the membrane fluidity of the particles after neutralization of the pH to 7.3 was increased. The membrane fluidity was correlated with pharmacokinetics in rats.

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