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Pharmacodynamics and pharmacokinetics studies of phenoxazinium derivatives for antimalarial agent

Mei Yang [a], Jian-Feng Ge [a], Chika Arai [a], Isamu Itoh [a,b], Qiang Fu [c], Masataka Ihara [a,b,*]

[a] Research Centre of Medicinal Sciences, Hoshi University, 2-4-41 Ebara, Shinagawa, Tokyo 142-8501, Japan
[b] Synstar Japan Co., Ltd, 2-18-3 Nishigotanda, Shinagawa, Tokyo 141-0031, Japan
[c] College of Pharmacy, Chosun University, Gwangju 501-759, Republic of Korea

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ABSTRACT

In vivo antimalarial drug candidates screening test was carried out on a series of water-soluble 3,7-bis(dialkylamino)phenoxazin-5-ium derivatives. Among them, 3-(diethylamino)-7-(piperidin-1-yl)phenoxazin-5-ium chloride (SSJ-206) showing highest efficacy was chosen for further pharmcodynamics and pharmacokinetics study. It was supported from these data that the phenoxazinium salts, SSJ-206, would be one of hopeful candidates as an oral antimalarial drug.

1. Introduction

Malaria is a major public health concern in tropical and subtropical regions. This disease causes up to 2 million deaths each year and affects the lives of most people across the continent, especially children under the age of five and pregnant women.[1] Recently, even inhabitants of temperate zones may be exposed to the danger of malaria infection owing to global warming. Today, there is still no effective vaccine for malaria.[2] Furthermore, the spread of drug-resistant parasites and insecticide resistant mosquitoes causes more serious problem.[3–6] Therefore, antimalarial compounds with novel mechanisms of action, which might circumvent the issue of structurally related drug-resistant, are urgently needed.

Many new drug substances with high antimalarial efficacy in vitro have been discovered.[7–11] However, most of them can only maintain the high bioactivities by intraperitoneal administration when evaluated in vivo.[12–19] Since malaria often takes place in the poorest region of the world, where is often lacked of medical care and equipments, drugs with high efficacies that can be dosed orally are very strongly desired. In our early study, we found some phenoxazinium salts, which were designed by using DLC (π-delocalized lipophilic cation) hypothesis,[20] exhibited potent in vitro antimalarial activity against *Plasmaodium falciparum* K_1 strain (chloroquine-resistant).[21] Recently, we reported a series of low cost and water-soluble 3,7-bis(dialkylamino)phenoxazin-5-ium derivatives with high in vitro antiprotozoal activities.[22] These outstanding pharmacological profiles encouraged us for further development. In this study, we carried out the evaluation with in vivo antimalarial activity and selected the most hopeful candidate of phenoxazinium SSJ-206, which showed high activity and selectivity (Table 1) in order to examine further pharmacodynamics and pharmacokinetics study.

2. Results

2.1. In vivo antimalarial activities

The phenoxazinium derivatives were tested for the in vivo antimalarial activity in mice infected with *Plasmodium berghei NK-65*. Tables 2a and b show the suppression ratio at day-4 after inoculated intravenously with 1×10^6 parasitized erythrocytes and treated with positive control drug (chloroquinine) and phenoxazinium salts by intraperitoneal (ip) and oral administration (po). The results showed that all phenoxazinium derivatives strongly suppressed the growth of malaria parasites. By the ip administration (20 mg/kg/day × 3 days), all phenoxaziniums except SSJ-208–SSJ-210 showed high parasitemia suppression (>90%) on day-4, while chloroquine showed 90.6% suppression with a dose of 10 mg/kg. By the ip administration of SSJ-208–SSJ-210, the mice died before day-4. By the po administration (100 mg/kg for a single dose), SSJ-199, 202, 203 and 204 showed moderate parasitemia suppression 90.3%, 84.9%, 66.3% and 68.9%, respectively and other phenoxazinium derivatives showed high parasitemia suppression (>95%),

* Corresponding author.
E-mail address: m-ihara@hoshi.ac.jp (M. Ihara).

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Table 1
In vitro antimalarial activity and selectivity of SSJ-206[a]

	P. falciparum K_1	
	IC_{50} (nM)	SI[b]
Chloroquine	147.72	NT[c]
SSJ-206	2.69	2390

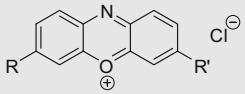
[a] The in vitro data was obtained by the earlier described methods.[22]
[b] Selectivity Index = (IC_{50} value for the cytotoxicity against L6)/(IC_{50} value for the efficacy against *P. falciparum* K_1). L6 is a rat skeletal myoblast cell line, which represents a model of host.
[c] NT means not tested.

which were similar to that of the positive drug chloroquine (98.4% suppression by the po administration with 30 mg/kg). Among them, SSJ-206 showed the best mean survival day (MSD) both by the ip and po administration, and no acute toxicity was found through the experiment.

2.2. Pharmacodynamics study of SSJ-206

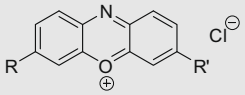
Due to the high in vivo parasitemia suppression and mean survival day, SSJ-206 was considered as one of the most hopeful candidates. Further pharmacodynamics study was carried out by checking the infection ratio daily after inoculation for 24 h until the mice died or the parasitemia level became more than 50%. As shown in Figure 1, the suppression was still high at day-4 (96.2%) even the dose was decreased to 30 mg/kg/day for 3 days by the po administration. Treated with 4 mg/kg/day for 3 days of intravenous injection or oral administration, the parasitemia suppression were 85.9% and 76.0% on day-4, respectively.

Table 2a
In vivo antimalarial activity against *P. berghei* by intraperitoneal administration[a]

ID no.			Medication program	In vivo	
	R	*R′*		% supp.	MSD[c] (days)
CQ[b]			10 mg/kg/day × 1 day	90.6 ± 0.7	22.7 ± 2.6
SSJ-133	$CH_2(CH_2CH_2)_2N$	$N(CH_2CH_2)_2CH_2$	20 mg/kg/day × 3 days	97.2 ± 0.8	16.8 ± 0.6
SSJ-131	$(CH_2CH_2)_2N$	$N(CH_2CH_2)_2$	20 mg/kg/day × 3 days	95.4 ± 0.9	8.3 ± 0.6
SSJ-199	$(CH_2CH_2)_2N$	$N(CH_3)_2$	20 mg/kg/day × 3 days	95.7 ± 0.5	7.8 ± 2.4
SSJ-201	$(CH_2CH_2)_2N$	$N(C_3H_7\text{-}n)_2$	20 mg/kg/day × 3 days	95.2 ± 0.3	14.8 ± 5.3
SSJ-132	$O(CH_2CH_2)_2N$	$N(C_2H_5\text{-}n)_2$	20 mg/kg/day × 3 days	90.0 ± 3.7	12.3 ± 2.0
SSJ-198	$O(CH_2CH_2)_2N$	$N(C_3H_7\text{-}n)_2$	20 mg/kg/day × 3 days	95.7 ± 0.3	18.0 ± 0.9
SSJ-202	$S(CH_2CH_2)_2N$	$N(CH_3)_2$	20 mg/kg/day × 3 days	94.5 ± 0.4	9.8 ± 1.7
SSJ-203	$S(CH_2CH_2)_2N$	$N(C_2H_5\text{-}n)_2$	20 mg/kg/day × 3 days	99.2 ± 0.2	11.5 ± 3.1
SSJ-204	$S(CH_2CH_2)_2N$	$N(C_3H_7\text{-}n)_2$	20 mg/kg/day × 3 days	96.5 ± 0.3	12.5 ± 1.5
SSJ-205	$CH_2(CH_2CH_2)_2N$	$N(CH_3)_2$	20 mg/kg/day × 3 days	96.4 ± 0.5	15.5 ± 2.4
SSJ-206	$CH_2(CH_2CH_2)_2N$	$N(C_2H_5\text{-}n)_2$	20 mg/kg/day × 3 days	95.9 ± 0.5	23.3 ± 6.4
SSJ-207	$CH_2(CH_2CH_2)_2N$	$N(C_3H_7\text{-}n)_2$	20 mg/kg/day × 3 days	93.2 ± 0.6	12.3 ± 2.9
SSJ-208	$CH_2(CH_2CH_2)_2N$	$N(C_4H_9\text{-}n)_2$	20 mg/kg/day × 3 days	Toxicity[d]	
SSJ-209	$CH_2(CH_2CH_2)_2N$	$N(C_5H_{11}\text{-}n)_2$	20 mg/kg/day × 3 days	Toxicity[d]	
SSJ-210	$CH_2(CH_2CH_2)_2N$	$N(C_6H_{13}\text{-}n)_2$	20 mg/kg/day × 3 days	Toxicity[d]	

[a] In the in vivo screening, for each group, $n = 3$.
[b] CQ = chloroquine.
[c] MSD: mean survival days. MSD for untreated mice (negative control) was 5.6 ± 0.6 days.
[d] Toxicity means a death of mice before day-4.

Table 2b
In vivo antimalarial activity against *P. berghei* by oral administration[a]

ID no.			Medication program	In vivo	
	R	*R'*		% supp.	MSD[c] (days)
CQ[b]			30 mg/kg/day × 1 day	98.4 ± 0.4	9.6 ± 3.0
SSJ-133	$CH_2(CH_2CH_2)_2N$	$N(CH_2CH_2)_2CH_2$	100 mg/kg/day × 1 day	99.8 ± 0.1	11.3 ± 0.3
SSJ-131	$(CH_2CH_2)_2N$	$N(CH_2CH_2)_2$	100 mg/kg/day × 1 day	95.3 ± 0.2	8.3 ± 0.9
SSJ-199	$(CH_2CH_2)_2N$	$N(CH_3)_2$	100 mg/kg/day × 1 day	90.3 ± 3.4	10.0 ± 2.5
SSJ-201	$(CH_2CH_2)_2N$	$N(C_3H_7\text{-}n)_2$	100 mg/kg/day × 1 day	99.6 ± 0.1	10.0 ± 2.6
SSJ-132	$O(CH_2CH_2)_2N$	$N(C_2H_5\text{-}n)_2$	100 mg/kg/day × 1 day	96.3 ± 0.8	13.0 ± 3.0
SSJ-198	$O(CH_2CH_2)_2N$	$N(C_3H_7\text{-}n)_2$	100 mg/kg/day × 1 day	99.5 ± 0.1	10.0 ± 0.7
SSJ-202	$S(CH_2CH_2)_2N$	$N(CH_3)_2$	100 mg/kg/day × 1 day	84.9 ± 0.7	11.3 ± 2.0
SSJ-203	$S(CH_2CH_2)_2N$	$N(C_2H_5\text{-}n)_2$	100 mg/kg/day × 1 day	66.3 ± 1.7	6.3 ± 0.3
SSJ-204	$S(CH_2CH_2)_2N$	$N(C_3H_7\text{-}n)_2$	100 mg/kg/day × 1 day	68.9 ± 5.8	7.0 ± 0.6
SSJ-205	$CH_2(CH_2CH_2)_2N$	$N(CH_3)_2$	100 mg/kg/day × 1 day	97.8 ± 0.4	7.6 ± 1.6
SSJ-206	$CH_2(CH_2CH_2)_2N$	$N(C_2H_5\text{-}n)_2$	100 mg/kg/day × 1 day	97.8 ± 1.1	12.7 ± 0.7
SSJ-207	$CH_2(CH_2CH_2)_2N$	$N(C_3H_7\text{-}n)_2$	100 mg/kg/day × 1 day	97.6 ± 1.3	10.3 ± 1.5

[a] In the in vivo screening, for each group, $n = 3$.
[b] CQ = chloroquine.
[c] MSD: mean survival days. MSD for untreated mice (negative control) was 5.6 ± 0.6 days.

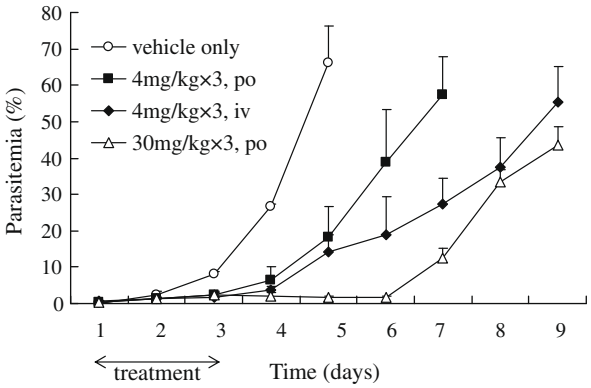


Figure 1. Parasitemia-time profile in ICR mice treated with SSJ-206. For each group, $n = 3$. The mice were inoculated with 1×10^6 *P. berghei*-parasitized erythrocytes. Administered by iv or po after 24 h of inoculation with once daily for 3 consecutive days.

The parasitemia-time profile (Fig. 1) showed that both iv or po administration with SSJ-206 can inhibit the parasite growth during 3 days therapy (parasitemia increased from 0.71% to 1.56% and 0.43% to 2.41%, respectively) at the dosage of 4 mg/kg × 3 days. After stopping treatment on day-4, the levels of parasitemia increased sharply. However, the levels of parasitemia remained significantly descent when compared with the mice which treated with vehicle only. For the group of the mice treated with 30 mg/kg × 3 of SSJ-206 by the po administration, the level of parasitemia was very low (0.35–1.56%) and the inhibition of parasitemia was sustained until day-6. A recrudescence of parasites erupted on day-7 and led to the mice death.

2.3. Pharmacokinetics study of SSJ-206

The drug concentration-time profiles of SSJ-206 in rats for single dosage are illustrated with the mean ± standard deviation (SD) in Figure 2. By the iv administration of SSJ-206 with a single dose of 4 mg/kg, the peak plasma concentration of 467.6 ± 42.9 ng/mL was observed after 10 min and the plasma concentration quickly declined to 96.6 ± 45.8 ng/mL after 4 h. By the po administration with 4 mg/kg or 30 mg/kg, the drug concentration reached the peak after about 2 h (157.9 ± 108.0 ng/mL) or 1 h (658.5 ± 215.3 ng/mL). It is noteworthy that the second peak appeared after 25 h of the po administration with 30 mg/kg.

The pharmacokinetic parameters were calculated by noncompartmental analysis and shown in Table 3. The oral bioavailability of SSJ-206 is very high (89.6%) by the dosage of 4 mg/kg, while elevated dose to 30 mg/kg resulted in a decreased bioavailability of 60.7%. SSJ-206 has a clearance rate of 2.67 L/h/kg and a high distribution volume of 35.3 L/kg following the iv injection, and similar values were observed for the po administration.

Table 3
Pharmacokinetic parameters of SSJ-206 in single-dose administration ($n = 3$)

Entry	$T_{1/2}$ (h)	T_{max} (h)	CL (L/h/kg)	V (L/kg)	AUC_{0-t} (μg h/mL)	A.B. (%)
4 mg/kg, iv	8.82 ± 3.00	—	2.67 ± 0.51	35.3 ± 16.0	1.54 ± 0.31	—
4 mg/kg, po	9.84 ± 3.15	0.99 ± 0.36	2.60 ± 0.00	36.9 ± 11.8	1.38 ± 0.04	89.6 ± 2.9
30 mg/kg, po	10.3 ± 6.65	0.98 ± 0.00	2.60 ± 0.00	38.5 ± 14.9	7.00 ± 0.10	60.7 ± 0.9

$T_{1/2}$ = terminal elimination half-life.
T_{max} = Time to attain maximum plasma concentration.
CL = apparent clearance.
V = apparent volume of distribution.
AUC = Area under plasma concentration–time curve. AUC_{0-t} means AUC from time 0 to the last sampling time.
A.B. = absolute bioavailability.

A multiple-dose pharmacokinetic study was carried out with the same dosage scheme as pharmacodynamic study (30 mg/kg/day for three consecutive days by po administration). Similar to single dose (Fig. 3), the drug concentration reached the peak after 1 h of each administration (298.9 ± 91.3 ng/mL, 419.9 ± 121.5 ng/mL and 583.3 ± 159.0 ng/mL at 1 h, 25 h and 49 h respectively), and decreased to the trough after 9 h of each administration. The appearances of the second peaks were also observed since the drug concentrations at 24 h, 48 h and 72 h were higher than those at 9 h, 33 h and 57 h, respectively.

3. Discussion

From the result of in vivo antimalarial screening test, it seemed that whether the structure was symmetric or not didn't affect the efficacy. However, the bulkiness of the wing substituents affected the toxicity dramatically. The latter was evidenced by the toxicity of SSJ-208–SSJ-210 (introduction of bulky group). This phenomenon was correlated to the in vitro test.[22] As previously reported, we found compounds with short alkyl chain (such as methyl, ethyl) showed high selectivity due to low cytotoxicity and high activity for in vitro tests; However, when the length of alkyl carbon chain exceeds three (such as propyl or more, SSJ-208–SSJ-210), the selectivity decreased and toxicity dramatically increased. The present in vivo screening data showed that if the alkyl chain was less than three carbons, the efficacy would increase along with the length of the chain (SSJ-199 and SSJ-201, SSJ-132 and SSJ-198). In the case of

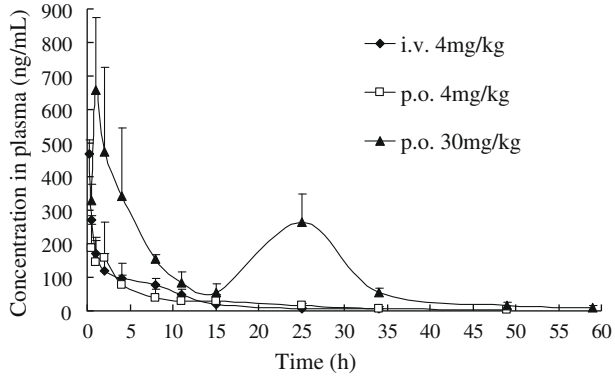


Figure 2. Concentration-time curve of SSJ-206 in rat after single-dose administration. Data are given as mean ± SD plasma SSJ-206 concentrations ($n = 3$).

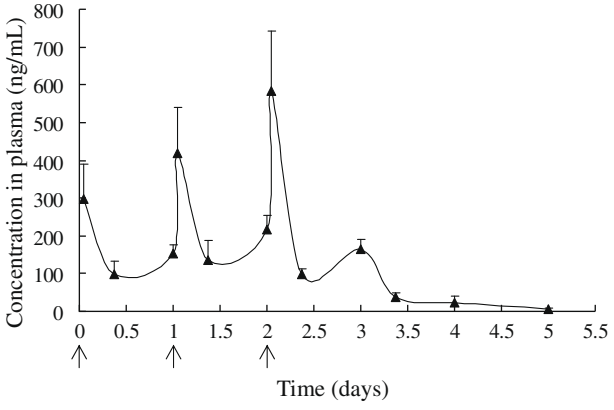


Figure 3. Drug concentration-time curve of SSJ-206 in rat after multiple-dose administration. Data are given as mean ± SD plasma SSJ-206 concentrations ($n = 3$). Arrows indicate the administration of SSJ-206.

ip administration, the activities are not so much different in each side chain. But in the case of po administration, when the alkyl chain is increased within three carbons, the efficacy would increase. Furthermore, the introduction of thiomorpholino group might increase the polarity and hydrophilicity of those compounds to decrease efficacy by po (SSJ-202–SSJ-204). It would be considered that the introduction of longer chain would lead to higher lipophilicity and flexibility, which would make better absorption and bioactivity. Once the side chain would be became (over three carbons), however, it would break the flat structure of the central tricyclic moiety leading to increase toxicity and decrease efficacy.

In the pharmacodynamic study of SSJ-206, both dose of 4 and 30 mg/kg following iv and po administration showed significant inhibition of parasitemia until day-4. The parasitemia repopulated from day-5 at a dose of 4 mg/kg, suggesting that a dose of 4 mg/kg may be too low to exert prolonged effect. Oral administration at a dose of 30 mg/kg could maintain suppression over 90% for 6 days before recrudescence occurred progressively, indicating that long-period administration is required. The result of the multiple-dose pharmacokinetic study also suggested that high drug concentration in plasma could only maintain for 5 days even after 3 consecutive days' administration. To eradicate all of the parasites in the body, an effective antimalarial drug concentration [at least the minimum inhibitory concentration (MIC) but preferably the minimum parasiticidal concentration (MPC)], which can be achieved by long-period administration, needs to be present in the blood until either the last parasite has been removed or immune defenses are able to deal with the residuum.[23] Therefore, a long-period administration is required.

Comparing the pharmacodynamic and pharmacokinetic profiles of SSJ-206 with single dose of 4 mg/kg, the bioavailability is as high as 89.6% by oral administration. However, the bioactivity of the po administration was poorer than that of the iv administration. Antimalarial efficacy depends on the lasting of the effective drug plasma concentration, which are sufficient to exceed parasiticidal concentration to eradicate parasitemia in the blood.[23] It was supposed that the higher peak concentration following iv injection as compared to the po administration (467.6 ng/mL vs 157.9 ng/mL, respectively) contributed to the strong inhibition of parasitemia.

In the pharmacokinetic study, the concentration–time profile of SSJ-206 exhibited double peaks following oral administration with 30 mg/kg (the second peak appeared at 25 h). SSJ-206 is water-soluble cationic drug and may be absorbed in the large intestine. Therefore, the double peak phenomenon may arise from absorption in the large intestine since blue feces were found after about 12 h of the oral administration. Furthermore, blue feces were also observed on the next day for iv group, this phenomenon suggested SSJ-206 might have enterohepatic recycling property. However, further studies should be carried out to confirm this hypothesis.

4. Conclusions

In conclusion, many 3,7-bis(dialkylamino)phenoxazin-5-ium derivatives show potent antimalarial activity both in vitro and in vivo. Among them, SSJ-206 presented high bioactivity and oral bioavailability. These results indicated that SSJ-206 was a potential new drug substance for malaria treatment and it warranted further investigation.

5. Experimental

5.1. Drugs

The phenoxazinium derivatives were synthesized as previously described.[22] For all the following in vivo pharmacodynamic and pharmacokinetic study, the sample were dissolved in the solution of ethanol–5% aqueous glucose solution = 1:9 (v/v).

5.2. Animals

The animal study was approved by the Hoshi University Animal Experimentation Ethics Committee. All the animals were obtained from Japan SLC Inc (Hamamatsu, Japan). Female ICR mice (19 – 25 g) were used for pharmacodynamics study and male Wistar rats (220–290 g) were used for pharmacokinetics study. They were kept under conditions of constant temperature (23 °C) and relative humidity of approximately 55% with a standard 12 h light-dark cycle. Animals were fed with a standard rodent diet (Labo. MR Stock, Nosan Corp., Yokohama, Japan) and allowed free access to tap water.

5.3. Parasite enumeration in infected mice

Peripheral blood smears were prepared by using blood obtained from the tail veins of infected experimental mice. The thin films were stained with Diff-Quick (Sysmex Corp., Koube, Japan). Blood smears were examined at a magnification of ×100 by oil immersion light microscopy with a Leica DM6000B microscope (Leica Microsystems K.K., Tokyo, Japan). Parasitemia was determined by counting over 10 fields of view.

5.4. In vivo antimalarial activities

The in vivo evaluation of antimalaria efficacies of phenoxazinium salts were carried out using rodent malaria *P. berghei NK-65* in female ICR mice (for each group, $n = 3$).[24] The mice were inoculated intravenously with 1×10^6 parasitized erythrocytes (resuspended in 200 μL of normal saline solution) on day-0. Phenoxazinium derivatives were administered by ip (20 mg/kg/day, 10 mL/kg for 3 consecutive days) or oral gavage (100 mg/kg/day, 10 mL/kg for single dosage) after 24 h of inoculation. Control mice were treated with an equal volume of vehicle. The infection ratio and the suppression ratio were checked at day-4 by comparing to the parasite growth in the control group. The suppression ratio = (parasitemia % control − parasitemia % sample)/parasitemia % control. All results are expressed as means ± standard deviation (SD).

5.5. Pharmacodynamic study of SSJ-206

The mice were inoculated intravenously with 1×10^6 parasitized erythrocytes (resuspended in 200 μL of normal saline solution) on day-0. SSJ-206 was administered by iv (4 mg/kg/day, 10 mL/kg) or oral gavage (4 mg/kg/day, 10 mL/kg or 30 mg/kg/day, 10 mL/kg) after 24 h of inoculation with once daily for 3 consecutive days. Control mice were treated with an equal volume of vehicle. The infection ratio were checked daily after 24 h of inoculation until the mice died or the parasitemia >50%. The suppression ratio was calculated as compared to the parasite growth in the control group. All results are expressed as means ± SD ($n = 3$).

5.6. Pharmacokinetic study

5.6.1. Dosing and blood sampling

To determine the pharmacokinetic behavior of SSJ-206, twelve male rats (Wistar, 220–290 g) were used for intravenous injection or oral administration. They were averagely separated to 4 groups randomly. One group of rats received the SSJ-206 (4 mg/kg, 5 mL/kg) by intravenous injection through tail vein. Blood samples were collected terminally by orbital sinus venipuncture at 10 and 30 min, 1, 2, 4, 8, 11, 15, 25 and 34 h followed by anesthetization with diethyl ether (Et_2O). For oral administration, the rats were fasted

for 12 h before administration, and after administration, they could take food and water freely. The second group of rats received SSJ-206 (4 mg/kg, 10 mL/kg) by oral gavage. Blood samples were collected terminally by orbital sinus venipuncture at 30 min, 1, 2, 4, 8, 11, 15, 25, 34 and 49 h followed by anesthetization with Et_2O. The third group of rats received SSJ-206 (30 mg/kg, 10 mL/kg) by oral gavage. Blood samples were collected terminally by orbital sinus venipuncture at 30 min, 1, 2, 4, 8, 11, 15, 25, 34, 49 and 59 h followed by anesthetization with Et_2O. For the multiple-dose administration, the fourth group of rats received SSJ-206 (30 mg/kg, 10 mL/kg) by oral gavage for 3 consecutive days at 0, 24 and 48 h respectively. Blood samples were collected terminally by orbital sinus venipuncture at 1, 9, 24, 25, 33, 48, 49, 57, 72, 81, 96 and 120 h followed by anesthetization with Et_2O. All the blood samples were collected into tubes pretreated by heparin and the plasma was separated by centrifugation at 4000 rpm for 10 min. After centrifugation, 100 μL of plasma was transferred to polypropylene screw-cap tubes, and then stored in the dark at −20 °C until analysis.

5.6.2. Plasma pretreatment and assay

Quantity of SSJ-206 was measured by LC–MS. Cresyl violet perchlorate (Sigma–Aldrich Japan K.K., Tokyo, Japan) was used as an internal standard. Before quantification, the plasma samples were thawed to room temperature, then 10 μL of internal standard (0.5 μg/mL) was added to each sample, and 400 μL of acetonitrile was added in order to extract the drug and precipitate the proteins. After centrifugation at 10,000*g* for 10 min, 400 μL of supernatant was transferred to a clean polypropylene tube and dried under N_2 flow. The residues were reconstituted in 100 μL of LC mobile phase (acetonitrile–0.1% aqueous trifluoroacetic acid = 20:80, v/v). After vortexed for 30 s and centrifuged at 10,000*g* for 10 min, the supernatant was taken for analysis. Drug concentrations were calculated from calibration curves at SSJ-206 concentrations that ranged from 1 to 1000 ng/mL. The calibration curve was prepared using blank plasma and processed in the same way as the samples.

A Shimadzu LCMS-2010 EV (Shimadzu Corp., Kyoto, Japan) was used, and data were collected using a LCMS solution version 3. After auto-injection of a 20 μL sample, the compounds were separated using a 3 μM, 2.0 mm I.D. × 50 mm CAPCELLPAK C_{18} column (Shiseido Co., Ltd, Tokyo, Japan) maintained at room temperature. The mobile phase consisted of 0.1% aqueous trifluoroacetic acid and acetonitrile, and all compounds were eluted under gradient conditions. Mass spectrometry was conducted under ESI conditions with detection by selected ion monitoring. HPLC detection was at 600 nm, and the flow rate was 0.2 mL/min. The retention times for SSJ-206 and internal standard were 10.2 and 6.7 min, respectively.

5.6.3. Pharmacokinetic analysis

Non-compartmental pharmacokinetic analysis was performed by using WINNONLIN version 5.2 (InnaPhase Corp., Philadelphia, PA, USA). The maximum plasma concentration (C_{max}) and the time to reach C_{max} (T_{max}) of drug were determined directly from the observed data. The elimination rate constant (k_e) was obtained by linear regression analysis by use of at least three sampling points of the terminal log-linear declining phase to the last measurable concentration. The elimination half-life ($T_{1/2}$) was calculated as 0.693 divided by k_e. The area under the plasma concentration–time curve from time 0 to the last sampling time (AUC_{0-t}) was calculated by the trapezoidal rule. The apparent clearance (CL) was obtained from the equation CL = Dose/AUC, and the apparent volume of distribution (V) was calculated from the equation $V = CL/k_e$. The absolute bioavailability (A.B.) of drug was calculated by $AUC_{po}/AUC_{iv} \times Dose_{iv}/Dose_{po} \times 100\%$. Pharmacokinetic parameters were expressed as means ± SD, of which each value was calculated separately for each mouse.

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