

Pitavastatin-Incorporated Nanoparticles for Chronic Limb Threatening Ischemia: A Phase I/IIa Clinical Trial

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Aim: To assess the results of a phase I/IIa open-label dose-escalation clinical trial of 5-day repeated intramuscular administration of pitavastatin-incorporated poly (lactic-co-glycolic acid) nanoparticles (NK-104-NP) in patients with chronic limb threatening ischemia (CLTI).

Methods: NK-104-NP was formulated using an emulsion solvent diffusion method. NK-104-NP at four doses (nanoparticles containing 0.5, 1, 2, and 4 mg of pitavastatin calcium, $n=4$ patients per dose) was investigated in a dose-escalation manner and administered intramuscularly into the ischemic limbs of 16 patients with CLTI. The safety and therapeutic efficacy of treatment were investigated over a 26-week follow-up period.

Results: No cardiovascular or other serious adverse events caused by NK-104-NP were detected during the follow-up period. Improvements in Fontaine and Rutherford classifications were noted in five patients (one, three, and one in the 1-, 2-, and 4-mg dose groups, respectively). Pharmacokinetic parameters including the maximum serum concentration and the area under the blood concentration–time curve increased with pitavastatin treatment in a dose-dependent manner. The area under the curve was slightly increased at day 5 compared with that at day 1 of treatment, although the difference was not statistically significant.

Conclusions: This is the first clinical trial of pitavastatin-incorporated nanoparticles in patients with CLTI. Intramuscular administration of NK-104-NP to the ischemic limbs of patients with CLTI was safe and well tolerated and resulted in improvements in limb function.

Clinical Trial Registration Number: UMIN000008011

Key words: Drug delivery system, Angiogenesis, Arteriogenesis, Peripheral arterial disease, Statin

Introduction

More than 20 years have passed since therapeutic angiogenesis was proposed to promote reparative collateral growth as an alternative therapy for ischemic diseases in patients for whom neither surgical revascularization nor endovascular therapy were considered a suitable option. Initial efforts in

therapeutic angiogenesis involved the local administration of recombinant growth factor proteins^{1, 2)} or gene-encoding growth factors³⁻⁶⁾ to endothelial cells. Since then, autologous stem cells⁷⁾, endothelial progenitor cell transplantation therapy⁸⁾, and novel nucleic acid analogs⁹⁾ have been developed.

Peripheral arterial disease is a typical phenotype of progressive and systemic atherosclerosis, leading to

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disability of limb function (intermittent claudication) and serious pain, toe ulcers, and gangrene due to chronic ischemic changes (chronic limb threatening ischemia; CLTI). The primary goals of CLTI treatment are relief of ischemic pain, resolution of ischemic ulcers, prevention of limb amputation, and prolonged survival. There is a global focus on therapeutic angiogenesis as treatment for CLTI, with the aim of recovering blood flow in ischemic tissue by inducing angiogenesis. Following pre-clinical success, a number of clinical trials of therapeutic angiogenesis strategies have been conducted; however, to date, no clinically relevant drug therapy has been established.

Because the vascular endothelium plays a central role in therapeutic angiogenesis/arteriogenesis, we hypothesized that nanoparticle (NP)-mediated drug delivery systems targeting endothelial cells may represent an innovative treatment strategy. We also hypothesized that HMG-CoA reductase inhibitors, or statins, are appropriate candidate drugs for a nanotechnology approach, given their variety of pleiotropic vasculoprotective effects that are independent of their lipid-lowering activity^{10, 11}. Statins increase the angiogenic activity of mature endothelial cells¹², as well as that of endothelial progenitor cells^{13, 14}, and augment angiogenesis/arteriogenesis in ischemic heart¹⁵ and limb in experimental animals¹⁶. Statins are widely used as a cholesterol-lowering drug, and their safety profile is well established.

Most of the beneficial effects of statins on therapeutic angiogenesis/arteriogenesis have been observed after the daily administration of high doses in experimental animals, a regimen that could lead to serious adverse side effects in a clinical setting¹²⁻¹⁴. To optimize the therapeutic effects of statins in the induction of therapeutic neovascularization, we therefore formulated pitavastatin-incorporated poly (lactic-co-glycolic acid) (PLGA) NPs (NK-104-NP)¹⁷. We have reported that NP-mediated pitavastatin delivery into vascular endothelial cells effectively increased therapeutic neovascularization with no serious side effects in a murine model of acute hind limb ischemia after a single intramuscular injection¹⁶. The beneficial effects induced by pitavastatin-NP were mediated by increased activity of endothelial nitric oxide synthase and multiple endogenous angiogenic growth factors, suggesting that NP-mediated cell-selective delivery produces a well-harmonized integrative system for therapeutic neovascularization. Importantly, this NP-mediated delivery system was effective at a dose approximately 100–300 times lower than the cumulative systemic dose¹⁸. Tissue concentrations of pitavastatin were increased by 7 to 8

times in skeletal muscles injected with pitavastatin-NP compared with those injected with pitavastatin after intramuscular administration, whereas serum levels of pitavastatin were comparable between animals injected with pitavastatin-NP and those injected with pitavastatin.

Efficacy was also demonstrated in a rabbit model¹⁸ and a cynomolgus monkey model of chronic lower limb ischemia¹⁹. Repeated intramuscular administration began 4 weeks after the induction of lower limb ischemia in the monkey model, and 6-day repeated intramuscular administration demonstrated a higher development of collateral circulation than the 1- or 3-day repeated administration. To translate our experimental findings to clinical medicine, we have established the production of pitavastatin-NP in compliance with GMP regulations, performed required toxicity and pharmacokinetics studies in compliance with GLP regulations, and confirmed the safety of pitavastatin-NP¹⁷. After an Investigational New Drug application to the Japanese regulatory agency (Pharmaceutical and Medical Device Agency; PMDA), we have completed a phase I/II open-label, dose-escalation, investigator-initiated trial of 5-day repeated intramuscular administration of NK-104-NP to treat CLTI.

Here, we report the safety and efficacy of intramuscular administration of NK-104-NP in 16 patients with CLTI.

Aim

The aim of this study was to assess the safety, tolerability, and therapeutic efficacy of the intramuscular administration of NK-104-NPs for five consecutive days in patients with CLTI.

Methods

Study Population

Patients were eligible to participate in the study if they had CLTI (atherosclerosis obliterans), including pain at rest and non-healing ischemic ulcers. Specifically, patients were enrolled if they (1) were classified as having Fontaine grade III or IV disease; 2) were unsuitable for revascularization of the femoral artery or a lower artery and did not show an improvement in Fontaine classification after 2 weeks of drug therapy (with vasodilators, anti-platelet agents, or prostaglandins); and (3) were aged ≥ 20 years at the time of consent. Candidates for catheter intervention or surgical revascularization were excluded from the study. A vascular surgical specialist determined prior to enrollment if patients were

candidates based on vein length and diameter, or the presence of appropriate peripheral arteries to complete the reconstruction. This study intentionally excluded Rutherford 6 (major tissue loss) patients and only included Fontaine IV patients classified as Rutherford 5.

This decision was made because the inclusion of Rutherford six patients would affect the efficacy of this treatment.

All eligible participants provided written consent prior to enrollment.

Drug Formulation (NK-104-NP)

The lactide/glycolide copolymer PLGA with an average molecular weight of 20,000 and a lactide-to-glycolide copolymer ratio of 75:25 (PLGA7520; Wako Pure Chemical Industries, Osaka, Japan) was used as the substrate material for NPs because of its bioabsorption half-life of 2 weeks in rat tissue. Pitavastatin (Kowa Company Ltd., Nagoya, Japan) was incorporated into PLGA NPs using a GMP-compliant emulsion solvent diffusion method in purified water, as reported previously^{20, 21}. Pitavastatin-loaded PLGA NPs contained 12% pitavastatin and were preserved as freeze-dried material. The mean particle size was determined using a light scattering method (Microtrack UPA150; Nikkiso, Tokyo, Japan). The mean diameter of NPs was 196 nm.

Study Design

This study was conducted as a phase I/IIa, multicenter, open-label, dose-escalation study. NK-104-NPs containing 0.5, 1, 2, or 4 mg of pitavastatin calcium were intramuscularly administered for 5 days repeatedly to patients with CLTI. The pharmacokinetics of the parent compound and lactone bodies of pitavastatin was measured in plasma and urine. The studies were conducted at two participating centers after approval by the PMDA and the ethics committee for each center. The trial was registered under UMIN CTR number UMIN000008011.

Primary Endpoints

The primary endpoints were the safety of treatment, determined as adverse events (AEs) coded according to the Medical Dictionary for Regulatory Activities/Japanese version (MedDRA/JTM version 17.0), with summation performed for each system organ class and each preferred term; pharmacokinetics of pitavastatin and pitavastatin lactone in plasma and urine; and efficacy of treatment as change in Fontaine and Rutherford classifications.

Secondary Endpoints

The secondary endpoints were safety as measured using physiological tests (body weight, body temperature, blood pressure, and pulse), clinical laboratory tests, and cardiac function tests and efficacy measured using ankle-brachial index (ABI), toe-brachial index (TBI), ankle pressure, pulse volume recording, laser Doppler blood flow (LDPI), angiography (IA-DSA), ulcer size, degree of pain (VAS), transcutaneous oxygen pressure (TcPO₂), and number of cases of minor and major amputation.

Dose Escalation Criteria

Dose escalation was according to the judgment of the principal investigator on the basis of safety and pharmacokinetic results up to 2 weeks after final administration to all subjects in each treatment group.

Observation Period

Follow-up was continued for 26 weeks after the final administration of NK-104-NP.

Administration of NK-104-NP

NK-104-NP was supplied by Kowa Company Ltd. (Nagoya, Japan) as a sterile powder within a vial. The total dose of NK-104-NP was suspended in 20 mL of physiologic saline (Otsuka Pharmaceutical Factory, Inc., Tokyo, Japan) and administered over a 1-h period. A specified amount of injectable saline was added to the vial, suspended, and administered intramuscularly. Patients were treated for five consecutive weekdays. Treatment was administered to 20 sites on the lower limb, which were determined in accordance with the administration manual for the test product (**Supplemental Fig. 1**). The dose per site was approximately 1 mL, and markings were made on the limb to ensure that injection sites did not overlap. The lower limb in worse condition was selected for treatment.

Pharmacokinetic Sampling and Analysis

Blood samples for determining pitavastatin and pitavastatin lactone concentrations were collected prior to treatment administration (blank sample) and at 0.5, 1, 2, 4, 8, 12, and 24 h after the initial administration on day 1. Blood samples were also collected on day 5 prior to the final administration (blank sample) and at 0.5, 1, 2, 4, 8, 12, 24, and 36 h after treatment. Urine samples for determining pitavastatin, pitavastatin lactone, and pitavastatin conjugate concentrations were collected prior to the initial administration of the test product (blank sample) and at 12 h after treatment initiation (12-h urine collection). Samples were also collected from 12

Table 1A. Individual baseline characteristics ($n=16$)

Cohort	Patients code	Age	Gender	Target limb	Fontaine stage	Rutherford stage	Ulcer area (mm ²)	Smoking history	Alb	BMI	DM	HD	Non-ambulatory status
NP0.5	NP-001	41	M	L	IV	III-5	0.57 (1 st toe)	-	3.0	19.6	-	+	-
	NP-004	77	M	L	III	II-4	-	past	4.0	23.6	-	-	-
	NP-006	81	M	L	III	II-4	-	past	3.5	23.0	-	-	-
	NP-008	66	F	L	III	II-4	-	-	3.7	28.3	+	+	-
NP1	NP-009	79	M	L	IV	III-5	0.68 (1 st toe lateral), 0.48 (1 st toe), 0.51 (2 nd toe)	-	4.5	21.9	+	-	-
	NP-010	59	M	R	IV	III-5	2.29 (2 nd toe), 0.52 (4 th toe)	past	3.8	21.1	-	+	-
	NP-011	63	M	R	III	II-4	-	current	3.6	28.6	+	-	-
	NP-013	77	M	R	IV	III-5	0.34 (1 st toe), 0.34 (between 4 th and 5 th toe)	past	3.2	21.5	-	-	-
NP2	NP-014	78	F	R	III	II-4	-	-	3.5	23.8	-	-	-
	NP-015	63	M	R	III	II-4	-	past	4.1	19.1	+	-	-
	NP-017	76	F	R	IV	III-5	4.53 (1 st toe), 1.81 (2 nd toe)	-	3.8	29.2	-	-	-
	NP-018	79	M	L	IV	III-5	0.53 (heel)	past	4.0	29.5	-	+	-
NP4	NP-019	73	F	R	III	II-4	-	-	3.9	22.4	-	-	-
	NP-020	83	F	R	IV	III-5	3.86 (heel lateral)	-	2.8	21.4	+	-	+
	NP-021	80	F	R	IV	III-5	1.45 (1 st toe), 0.29 (4 th toe)	-	3.9	24.0	-	-	-
	NP-024	71	M	R	III	II-4	-	past	3.8	16.3	-	+	-

NP0.5: NK-104-NP containing 0.5 mg of pitavastatin calcium was intramuscularly administered in each limb once a day for 5 days.

NP1: 1 mg of pitavastatin calcium; NP2: 2 mg; NP4: 4 mg Alb: Albumin, BMI: Body mass index, DM: Diabetes mellitus, HD: Hemodialysis

to 24 h (24-h urine collection) and from 24 to 48 h (48-h urine collection) after treatment initiation. Blood samples were collected and centrifuged to obtain plasma within 30 min of sampling. The plasma samples were stored at $-80 \pm 20^\circ\text{C}$ until analysis. Plasma concentrations of pitavastatin and pitavastatin lactone and urine concentrations of pitavastatin, pitavastatin lactone, and pitavastatin conjugate were determined using a validated liquid chromatography tandem mass spectrometry method after liquid-liquid extraction. The range of quantification, accuracy, and inter-run precision was 0.5–500 ng/mL. The lower limit of quantification of pitavastatin and pitavastatin lactone was 0.5 ng/mL.

Data Collection and Statistical Analysis

All data were collected and analyzed at the Center for Clinical and Translational Research at Kyushu University Hospital. Pharmacokinetic parameters were established at the Department of Clinical Pharmacokinetics. Subjects were included in the safety evaluation if they received the test product and had plasma pharmacokinetic data available. For patient characteristics, the basic statistics of each group, as well as of the entire study cohort, were calculated. All data were expressed as means \pm SD. For

comparisons with baseline values, the data were examined using paired *t*-tests where appropriate. Values of $P < 0.05$ were considered significant.

Results

Baseline Patient Characteristics

A total of 24 eligible patients with chronic CLTI were screened; 17 patients met the inclusion criteria and were enrolled, and the remaining seven patients were excluded. A total of 17 limbs, 1 limb per patient, were treated five times with NK-104-NP in a dose-escalation fashion. For 16 subjects (Table 1A), NK-104-NP (0.5, 1, 2, and 4 mg) was administered according to the protocol schedule; one patient who received NK-104-NP in an unaffected nonischemic limb was excluded from the analysis. As shown in Table 1, the cohort consisted of 11 men and six women (mean age, 72.4 ± 11.1 years; range, 41–85 years). Nine patients (52.9%) were classified as Fontaine III/Rutherford II-4 and 8 patients (47.1%) were classified as Fontaine IV/Rutherford III-5. There were no patients with major tissue loss (Rutherford III-6). Risk factors were not different between the groups. The characteristics of the patients are shown in Table 1B.

Table 1B. Patient Demographics and Characteristics

Variables	(n = 16) (%)	Variables	(n = 16) (%)
Age (y, range)	72.4 ± 11.1	Cerebrovascular disease	4 (25.0)
Sex	Male 10 (62.5) Female 6 (37.5)	Hemodialysis	5 (31.3)
BMI (kg/m ²)	22.8 ± 0.89	Hyperlipidemia	7 (43.8)
Alb(g/dl)	3.7 ± 0.4	Medications	
Smoking Status		Aspirin	8 (50.0)
Never	8 (50.0)	Thienopyridines/clopidogrel	6 (37.5)
Former	7 (43.8)	Cilostazol	3 (18.8)
Current	1 (6.2)	Sarpogrelate	5 (31.3)
Hypertension	14 (87.5)	Beraprost	3 (18.8)
Coronary Artery Disease	3 (18.8)	Warfarin	8 (50.0)
Diabetes Mellitus	5 (31.3)	Statins	6 (37.5)

Continuous data are presented as mean ± standard deviation.

Table 2. Details of the 4 patients who died during follow-up

patients code	Age	Sex	Fontaine stage	Rutherford stage	Dose of NK-104-NP (mg/kg)	survival time after treatment	cause of death
NP-001	41	M	IV	III -5	NP0.5	114	sepsis
NP-013	77	M	IV	III -5	NP1	96	acute myocardial infarction
NP-017	76	F	IV	III -5	NP2	215	chronic heart failure and pneumonia
NP-024	71	M	III	II -4	NP4	65	perforated intestine and skin ulcer

The 16 patients were divided into four groups: NP0.5, NP1, NP2, and NP4 groups that received NK-104-NP containing 0.5, 1, 2, and 4 mg of pitavastatin calcium, respectively.

Primary Endpoint: Safety and Efficacy of Treatment Survival

A total of four patients (NP-001, NP-013, NP-017, and NP-024 in the NP0.5, NP1, NP2, and NP4 groups, respectively) died during the observation period, although none of the deaths were deemed to be treatment related. The causes of death were septic shock 114 days after treatment, acute myocardial infarction 96 days after treatment, chronic heart failure and pneumonia 215 days after treatment, and a perforated intestine and skin ulcer 65 days after treatment (Table 2).

AEs

Seven patients had nineteen serious AEs (SAEs) during the observation period (Table 3). No cardiovascular events or other SAEs that could be definitively attributed to NK-104-NP were observed.

A total of 111 AEs (41, 25, 32, and 13 in the NP0.5, NP1, NP2, and NP4 groups, respectively) were reported, but no dose–response relationship was observed. Each reported AE occurred in two patients

or more. The AEs were skin ulcers (seven subjects; two, two, two, and one in the NP0.5, NP1, NP4, and NP2 groups, respectively), limb pain (five subjects; four and one in the NP0.5 and NP1 groups, respectively), gangrene (three subjects; two and one in the NP0.5 and NP1 groups, respectively), ingrown toenail (two subjects in the NP0.5 group), and hematuria (two subjects in the NP2 group).

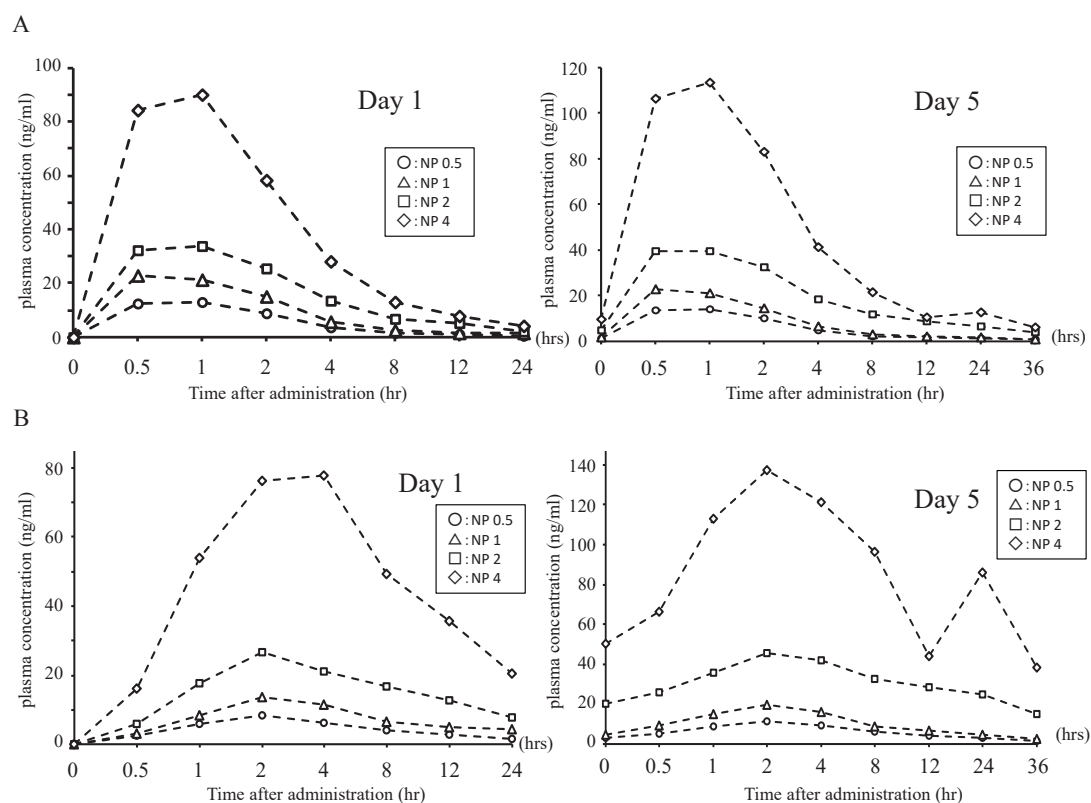
Pharmacokinetics of Pitavastatin and its Metabolite (Pitavastatin Lactone) in Plasma/Urine

Pitavastatin and its metabolite pitavastatin lactone were confirmed in blood 30 min after administration in all four groups on day 1 of administration, and the average plasma concentrations peaked (maximal concentration) 1 and 2 h after administration, respectively, and gradually decreased over time. The average plasma concentrations of pitavastatin and pitavastatin lactone on day 5 of administration followed the same trend as for day 1 (Fig. 1A and 1B).

The maximum serum concentration (C_{max}) of pitavastatin (average ± SD) in the NP0.5, NP1, NP2, and NP4 groups was 13.76 ± 3.41, 23.00 ± 8.52, 34.58 ± 3.81, and 93.71 ± 7.99 ng/mL, respectively, on day 1 and 14.54 ± 5.86, 23.19 ± 8.58, 40.95 ± 7.86, and 114.48 ± 12.69 ng/mL, respectively, on day 5 day

Table 3. Serious adverse events (SAEs) reviewed by the principal investigator or sub investigators

patient code	SAEs	onset after treatment (days)	severity	prognosis	review result
NP-001	Left limb gangrene	113	severe	not recovered	not related
	Septic shock	114	severe	dead	not related
NP-006	Left 2 nd toe ulcer	83	severe	recovered	not related
	Left 5 th toe gangrene	107	severe	recovered	not related
	Bypass graft occlusion	166	severe	recovered	not related
NP-013	Right limb lymphatic fistula	19	moderate	not recovered	not related
	anorexia	82	mild	uncertained	not related
	Acute myocardial infarction	96	severe	dead	not related
NP-015	Listlessness	12	mild	recovered	not related
NP-017	Left limb stasis skin ulcer	58	moderate	not recovered	not related
	right 2 nd toe ulcer	75	moderate	not recovered	not related
	Stevens-johnson syndrome	157	severe	not recovered	not related
	gastrointestinal bleeding	178	severe	not recovered	not related
	chronic heart failure exacerbation	215	severe	dead	not related
	pneumonia	215	severe	dead	not related
NP-018	left facial nerve palsy	-18	moderate	recovered	not related
NP-024	right 3 rd toe and heel ulcer	43	moderate	dead	not related
	pneumonia	61	moderate	not recovered	not related
	perforated intestine	65	severe	dead	not related

**Fig. 1.** (A) Plasma concentration of pitavastatin on days 1 and 5 of administration. (B) Plasma concentration of pitavastatin lactone on days 1 and 5 of administration

(Supplemental Table 1A).

The area under the blood concentration–time curve (AUC)_{0-τ} of pitavastatin (average ± SD) in the NP0.5, NP1, NP2, and NP4 groups was 56.24 ± 21.12, 90.67 ± 47.72, 195.94 ± 53.90, and 410.00 ± 89.23 h·ng/mL, respectively, on day 1 and 71.56 ± 36.52, 93.41 ± 49.45, 306.13 ± 145.73, and 637.02 ± 247.75 h·ng/mL, respectively, on day 5 (Supplemental Table 1A).

C_{max} of pitavastatin lactone (average ± SD) in the NP0.5, NP1, NP2, and NP4 groups was 8.35 ± 3.16, 13.77 ± 7.39, 26.63 ± 8.79, and 86.15 ± 45.27 ng/mL, respectively, on day 1 and 11.32 ± 4.14, 19.63 ± 11.65, 46.60 ± 18.77, and 137.11 ± 71.65 ng/mL, respectively, on day 5 (Supplemental Table 1B).

AUC_{0-τ} of pitavastatin (average ± SD) in the NP0.5, NP1, NP2, and NP4 groups was 85.27 ± 20.74, 146.53 ± 103.22, 331.89 ± 118.33, and 993.13 ± 670.43 h·ng/mL, respectively, on day 1 and 134.13 ± 53.27, 193.16 ± 141.19, 746.46 ± 383.83, and 2054.96 ± 1699.62 h·ng/mL on day 5 (Supplemental Table 1B).

From 0 to 1 h after dosing, C_{max} increased for pitavastatin and from 2 to 4 h after dosing, C_{max} increased for pitavastatin lactone. After 5 days of administration, the AUC was slightly increased compared with that on day 1, although the difference was not statistically significant.

The amount of pitavastatin in the urine was measurable in one subject each in the NP2 and NP4 groups but was not detectable in the other participants. The percentage of drug excreted unchanged into the urine, Ae(%), up to 24 h after administration was 0.3%–1.4% on day 1 and 0.2%–2.3% on day 5. The amount of pitavastatin lactone in the urine was measurable in two subjects in the NP0.5 group and one subject each in the NP2 and NP4 groups. Ae(%) up to 24 h after administration was 0.5%–2.3% on day 1 and 0.7%–3.6% on day 5.

Efficacy of Treatment

All patients enrolled in this trial were categorized as Fontaine grades III or IV at baseline. One subject in the NP0.5 group worsened in Fontaine grade from III (baseline) to IV (evaluated 88 days after administration). One subject in the NP2 group improved in Fontaine grade from III (baseline) to IIa (60 days) and IIa (88 days). Another subject in the NP2 group improved in Fontaine grade from III (baseline) to IIb (evaluated as IIb at 18, 60, and 88 days). One other subject in the NP2 group improved in Fontaine grade from IV (baseline) to IIa (88 days). The remaining subject in the NP2 group showed no change in Fontaine grade. One subject in the NP4

group improved in Fontaine grade from III (baseline) to I (evaluated as I at 32, 60, and 88 days). One subject in the NP4 group worsened in Fontaine grade from III (baseline) to IV. Rutherford grade changed in the same manner as that of the Fontaine grade for all applicable subjects. The improvement ratio was 0%, 25%, 75%, and 25% in the NP0.5, NP1, NP2, and NP4 groups, respectively (Table 4A, B).

Secondary Endpoints: Safety and Efficacy of Treatment

No changes in physiological tests (body weight, body temperature, blood pressure, and pulse) and cardiac function tests were observed at each time point compared with baseline data. Clinical laboratory data did not significantly change (Fig. 2) and remained within normal ranges before and after administration of NK-104-NP.

Significant leakage of angiogenic factors into the blood was not detected during the trial period (Supplemental Fig. 2). Among all groups, the value of ABI was lower in the affected limb when compared with that in the unaffected limb.

Four small, ischemic ulcers, each less than <0.5 cm² in size in three patients (three in the NP1 group) resolved during the study period (Supplemental Fig. 3). No significant change in VAS, ABI, TBI, LDPI, or TcPO₂ was observed at any time point up to 26 weeks after administration of NK-104-NP (Supplemental Fig. 4). Minor amputation was performed twice in one subject in the NP0.5 group and a major amputation was performed in one other subject. No other minor or major amputation was performed.

Discussion

We report here the first-in-human phase I/IIa open-label dose-escalation investigator-initiated trial of 5-day repeated intramuscular administration of NK-104-NP to treat CLTI.

The primary endpoints were treatment safety and the pharmacokinetics of pitavastatin and its metabolites. The key safety findings were (1) that intramuscular administration of NK-104-NP was feasible, with no drug-related SAE observed within the study period and (2) that no significant inflammatory reaction, leakage of angiogenic factors into the blood, or increase in CK were detected. Rhabdomyolysis is a known SAE associated with statin therapy, so we measured serial changes in its biomarkers (including CK) and found no significant increases in any patients included in this study. These findings indicate that intramuscular administration of

Table 4A. Efficacy of treatment; changes of Fontaine stage

Patient No.	Dose (mg)	Improvement ratio(%)	Fontaine					
			baseline	32D	60D	88D	186D	efficacy
NP-001	0.5	0	IV	IV	IV	IV	#N/A	no change
NP-004			III	III	III	III	III	no change
NP-006			III	III	III	IV	IV	worsen
NP-008			III	III	III	III	III	no change
NP-009	1	25	IV	IV	IV	IV	IV	no change
NP-010			IV	IV	IV	IV	IIb	improved
NP-011			III	III	III	III	IV	worsen
NP-013			IV	IV	IV	#N/A	#N/A	no change
NP-014	2	75	III	III	IIa	IIa	IIa	improved
NP-015			III	IIb	IIb	IIb	IIb	improved
NP-017			IV	IV	IV	IV	#N/A	no change
NP-018			IV	IV	IV	IIa	IIa	improved
NP-019	4	25	III	I	I	I	I	improved
NP-020			IV	IV	IV	IV	IV	no change
NP-021			IV	IV	IV	IV	IV	no change
NP-024			III	IV	#N/A	#N/A	#N/A	worsen

Table 4B. Summary of the response to treatment about each dose groups

	NP 0.5			NP 1			NP 2			NP 4		
	improved	no change	worsen	improved	no change	worsen	improved	no change	worsen	improved	no change	worsen
Fontaine/ Rutherford	0 (0%)	3 (75%)	1 (25%)	1 (25%)	2 (50%)	1 (25%)	3(75%)	1(25%)	0(0%)	1 (25%)	2 (50%)	1 (25%)

NK-104-NP for 5 days is safe and well tolerated in patients with CLTI. The pharmacokinetic parameters T_{max} , C_{max} , and $AUC_{0-\tau}$ increased in a dose-dependent manner but did not significantly differ between days 1 and 5 of administration, indicating an absence of pitavastatin accumulation in the systemic circulation after 5-day repeated intramuscular administration of NK-104-NP. No significant changes in the secondary endpoints of safety and efficacy were observed in any of the test groups after NK-104-NP administration.

Previously, we reported that NP is selectively delivered into vascular cells, especially the endothelium, and is retained in those cells/tissues for longer time after intramuscular administration of FITC-incorporated PLGA-NP compared with FITC alone in animal models of hind limb ischemia^{16, 18)}. Therefore, it is likely that the NP-mediated increased local concentration and retention time of pitavastatin in ischemic tissues are key determinants of the efficacy and safety of therapeutic neovascularization. Indeed, we reported that tissue concentrations of pitavastatin were 7 to 8 times greater in skeletal muscles injected

with pitavastatin-NP than in those injected with pitavastatin alone after intramuscular administration, whereas serum levels of pitavastatin were comparable between animals injected with pitavastatin-NP and those injected with pitavastatin¹⁶⁾.

The primary endpoints of efficacy were changes in Fontaine and Rutherford classifications. Improvements in Fontaine and Rutherford classifications were noted in five patients (one, three, and one in the NP1, NP2, and NP4 groups, respectively), indicating efficacy in limb function. However, caution is required when interpreting these results because worsening of Fontaine and Rutherford classification was observed in three patients (one each in the NP0.5, NP1, and NP4 groups, respectively). No significant changes in second endpoints of efficacy were noted in any of the test groups after administration of NK-104-NP. Therefore, additional placebo-controlled phase IIb and/or phase III studies are required in the future.

As described in the Introduction, daily administration of statins at high doses has been

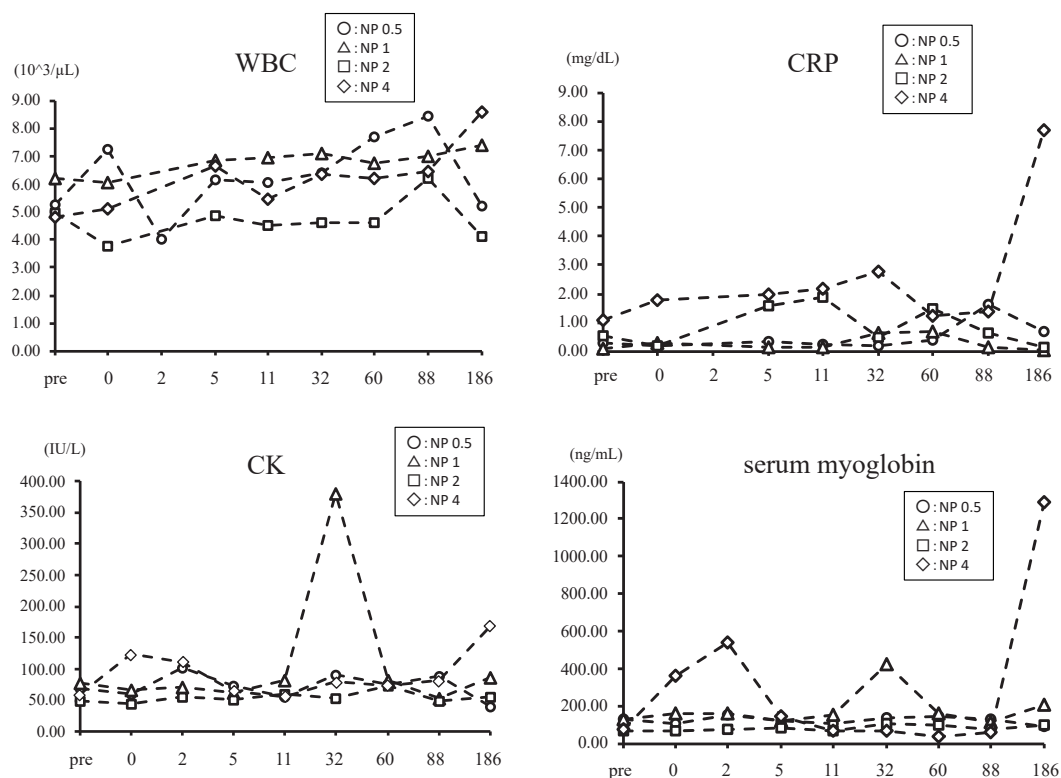


Fig. 2. Time course of inflammation response-related parameters and muscle damage markers, including serum CK and myoglobin
CK: Creatine Kinase

reported to augment arteriogenesis in mice^{12, 16} and rabbits¹⁸) with hind limb ischemia. These pleiotropic effects of statins are mediated through reduced levels of cholesterol biosynthesis pathway intermediates that serve as lipid attachments for post-translational modification (isoprenylation) of proteins, including Rho and Rac. Among clinically available statins, pitavastatin was selected as the NP compound because (1) it showed the highest HMG-CoA reductase activity in rat liver microsomes *in vitro* (unpublished observation in our laboratory), (2) it elicited the most potent effects on angiogenic activity of human endothelial cells *in vitro* compared with other statins, and (3) NP-mediated intracellular delivery of pitavastatin showed greater angiogenic activity in human endothelial cells compared with pitavastatin alone¹⁸).

Pitavastatin is minimally metabolized by CYP2C9, unlike other statins such as atorvastatin, fluvastatin, pravastatin, rosuvastatin, and simvastatin, which are mainly metabolized by CYP3A4²²).

We have also reported in an *in vivo* model that (1) a single intramuscular injection of pitavastatin-NP increased angiogenesis/arteriogenesis¹⁶) and (2) treatment with pitavastatin-NP selectively increased

expression of angiogenic growth factors such as vascular endothelial growth factor in vascular endothelial cells in the ischemic limb¹⁶). We found that FITC signals were localized mainly to the vascular endothelium for up to 2–4 weeks after the intramuscular injection of FITC-NP into ischemic skeletal muscles of mice¹⁶) and rabbits¹⁸) *in vivo*, indicating that this NP-mediated delivery system may be beneficial as an innovative therapeutic strategy targeting endothelial cells. Therefore, it is likely that after NP-mediated targeting of vascular (endothelial) cells, pitavastatin retained and reduced levels of cholesterol biosynthesis pathway intermediates in the cells, resulting in significant therapeutic effects.

We have recently reported that a single intravenous administration of NK-104-NP resulted in significant therapeutic effects on ischemia–reperfusion injury in the heart²³) and brain²⁴), remodeling after acute myocardial infarction²⁵), pulmonary arterial hypertension^{26, 27}), and atherosclerosis plaque destabilization/rupture^{28, 29}). We have also shown that NPs containing drugs that intervene in cytoprotective intracellular signaling (e.g., pioglitazone, irbesartan, imatinib, and toll-like receptor inhibitors) markedly attenuate ischemia–reperfusion injury and enhance

tissue healing³⁰). Because PLGA NPs are delivered selectively to inflammatory cells (inflammatory monocytes) and/or small arteries after intravenous administration, monocyte-mediated inflammation may play an important role in the mechanism by which NPs exerted beneficial therapeutic effects in these previous studies^{31, 32}).

We have completed phase I clinical studies of intravenous administration of NK-104-NP in healthy volunteers (UMIN 000014940, UMIN 000019189) and are now conducting a phase IIa clinical trial to investigate the safety and efficacy of intravenous administration of NK-104-NP in 12 patients with severe pulmonary arterial hypertension (UMIN000032531). NK-104-NP is a clinically feasible drug delivery system that may represent a significant advance in therapeutic modalities over current unsatisfactory approaches for the treatment of CLTI.

Conclusions

The intramuscular administration of NK-104-NP to ischemic limbs of patients with CLTI was safe and well tolerated and resulted in improvements in limb function. Further placebo-controlled phase II/III studies in larger patient cohorts are needed to verify the safety and efficacy of NK-104-NP in CLTI treatment.

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Conflicts of Interest

Kensuke Egashira holds a patent on the results reported in the present study (pharmaceutical composition containing statin-encapsulated nanoparticle, WO 2008/026702). The remaining authors report no conflicts of interest.

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Supplemental Fig. 1. Administration sites of injection in the lower limb

There were four injection sites on the inside of the thigh, 6 on the back of the thigh, 4 on the front of the calf, and 6 on the back of the calf. To ensure the same injection sites for all patients, a plastic mold that clearly defined the areas where the sites should be placed was made. The mold had six sites marked with “+” signs, and was applied in the same manner to every patient. On day 1, the middle of every “+” sign (0) was injected. On days 2, 3, 4, and 5, the top of the signs (1), right points (2), bottom points (3), and left points (4) were injected, respectively. This process was implemented to prevent overlap of injection sites.

Supplemental Table 1A. Pharmacokinetic parameters of pitavastatin after administration

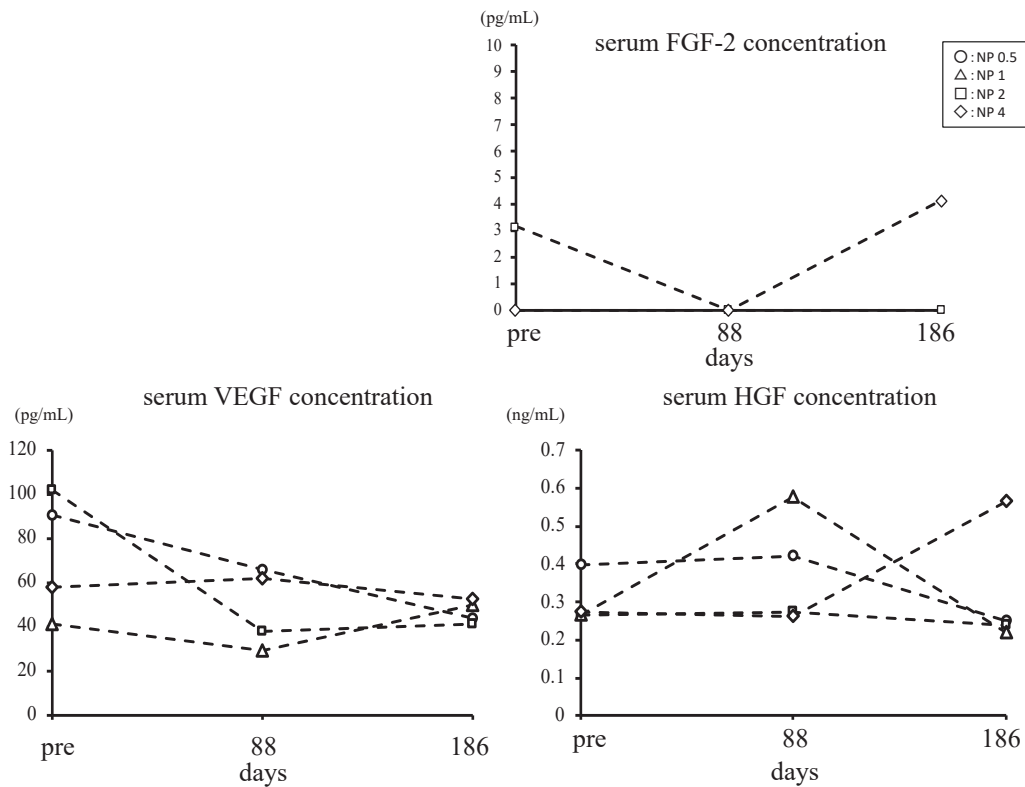
Dose (mg)	Patient No.	on the 1st day of administration				on the 5th day of administration			
		T _{max} (hr)	C _{max} (ng/mL)	AUC _{0-τ} (hr*ng/mL)	T _{1/2} (hr)	T _{max} (hr)	C _{max} (ng/mL)	AUC _{0-τ} (hr*ng/mL)	T _{1/2} (hr)
0.5	NP-001	0.50	17.76	81.66	9.75	0.50	20.84	112.71	14.01
	NP-004	0.50	9.80	46.75	14.30	0.50	9.79	52.98	12.84
	NP-006	1.00	15.01	63.86	9.37	1.00	18.21	89.51	14.31
	NP-008	0.50	12.47	32.67	2.59	0.50	9.33	31.06	2.90
	Ave.	0.63	13.76	56.24	9.00	0.63	14.54	71.56	11.01
1	NP-009	1.00	19.51	90.61	9.92	0.50	17.07	79.84	11.02
	NP-010	0.50	15.25	38.31	1.87	0.50	16.26	33.84	1.73
	NP-011	0.50	22.22	80.02	11.89	1.00	24.71	108.46	13.23
	NP-013	1.00	35.03	153.74	6.51	0.50	34.74	151.51	9.47
	Ave.	0.75	23.00	90.67	7.55	0.63	23.19	93.41	8.86
2	NP-014	1.00	38.22	261.68	12.96	1.00	41.52	403.13	22.54
	NP-015	1.00	29.24	142.86	7.55	0.50	31.30	143.78	12.19
	NP-017	0.65	34.99	216.96	9.35	1.00	50.52	452.40	17.22
	NP-018	1.05	35.86	162.26	6.52	0.50	40.48	225.23	13.71
	Ave.	0.93	34.58	195.94	9.09	0.75	40.95	306.13	16.42
4	NP-019	0.50	87.30	305.40	9.33	1.00	101.31	343.43	9.00
	NP-020	1.00	89.89	420.63	10.02	0.50	106.89	686.11	19.67
	NP-021	1.00	105.28	392.12	5.27	1.00	129.11	578.08	12.03
	NP-024	1.00	92.35	521.86	8.98	1.00	120.62	940.44	19.46
	Ave.	0.88	93.71	410.00	8.40	0.88	114.48	637.02	15.04

T_{max}: Time to maximum plasma concentration, C_{max}: Maximum observed concentration, AUC_{0-τ}: Area under the plasma concentration–time curve from time 0 to τ after administration, T_{1/2}: Biological half-life, Ave: Average

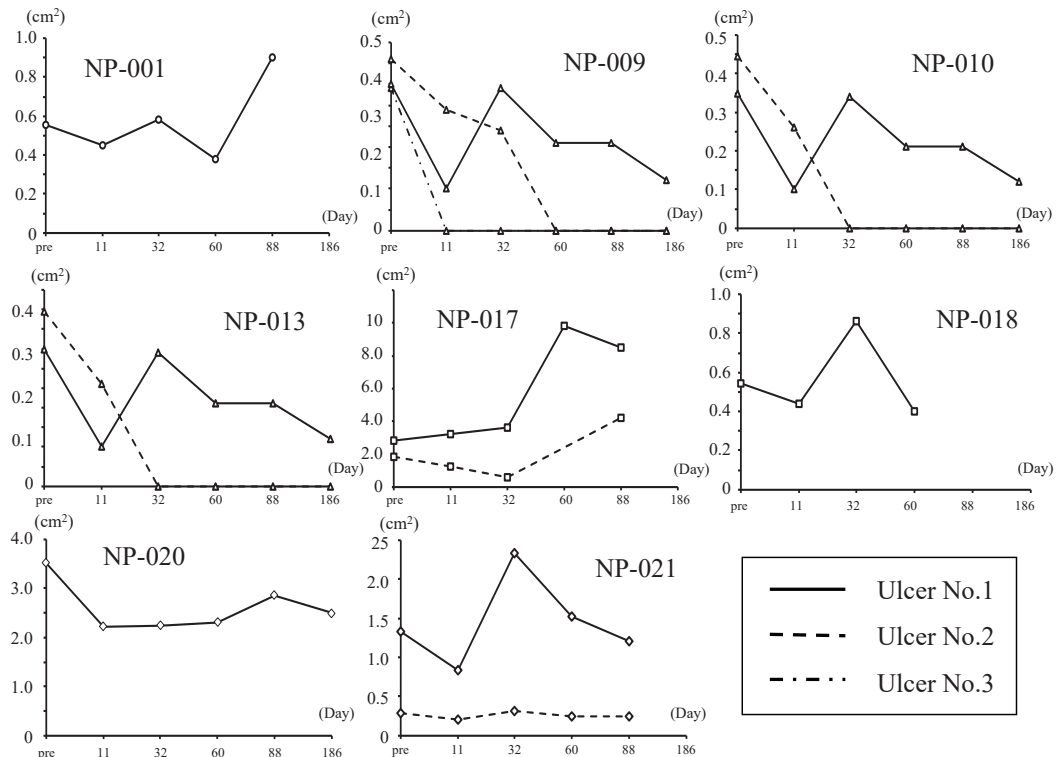
*: Data were excluded from the analysis.

Supplemental Table 1B. Pharmacokinetic parameters of pitavastatin lactone after administration

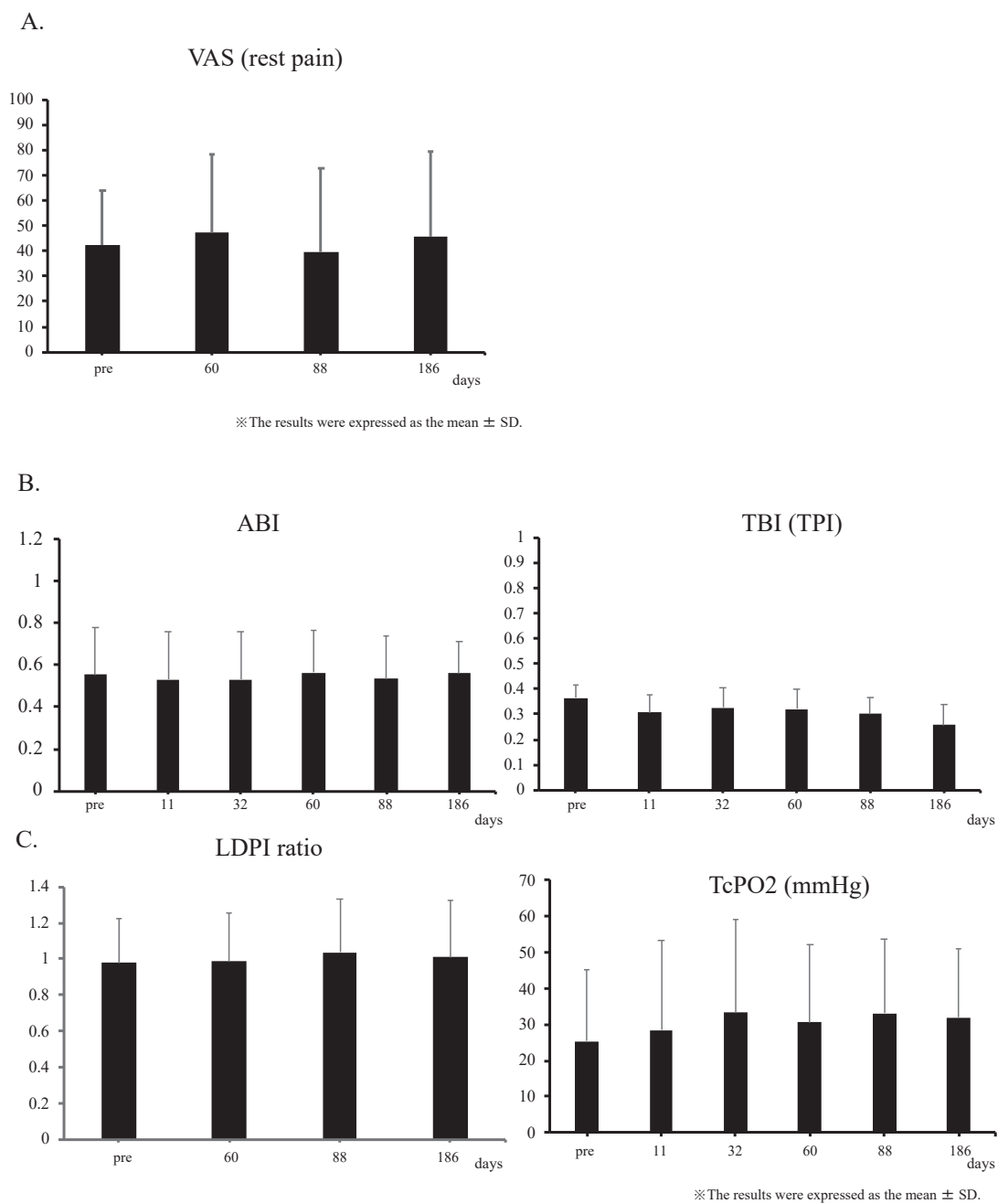
Dose (mg)	Patient No.	on the 1st day of administration				on the 5th day of administration			
		T _{max} (hr)	C _{max} (ng/mL)	AUC _{0-τ} (hr*ng/mL)	T _{1/2} (hr)	T _{max} (hr)	C _{max} (ng/mL)	AUC _{0-τ} (hr*ng/mL)	T _{1/2} (hr)
0.5	NP-001	2.00	12.64	115.73	10.28	2.00	17.42	208.64	16.10
	NP-004	2.00	5.73	79.07	20.14	2.00	8.25	110.64	13.59
	NP-006	2.00	6.21	76.97	15.53	2.00	10.05	132.15	15.45
	NP-008	2.00	8.80	69.30	7.84	2.00	9.57	85.08	8.08
	Ave.	2.00	8.35	85.27	13.45	2.00	11.32	134.13	13.31
1	NP-009	2.00	15.22	157.56	12.52	2.00	17.43	131.30	13.00
	NP-010	2.00	7.08	40.99	4.32	1.00	6.60	47.43	9.28
	NP-011	2.00	9.21	103.67	17.51	2.00	19.63	215.76	13.54
	NP-013	3.92	23.58	283.89	25.20	1.98	34.89	378.16	13.24
	Ave.	2.48	13.77	146.53	14.89	1.75	19.63	193.16	12.26
2	NP-014	2.27	36.39	508.04	15.22	1.90	69.01	1144.64	27.83
	NP-015	1.83	22.95	257.55	10.48	1.88	24.68	327.39	16.39
	NP-017	1.93	16.35	269.36	22.98	4.00	52.43	988.85	27.04
	NP-018	2.02	30.82	292.60	10.07	2.00	40.29	524.98	19.86
	Ave.	2.01	26.63	331.89	14.69	2.45	46.60	746.46	22.78
4	NP-019	1.85	31.49	279.85	14.12	1.88	45.76	307.24	9.04
	NP-020	2.17	91.81	1092.13	13.31	1.87	171.40	2563.99	20.94
	NP-021	1.00	79.60	734.07	8.02	1.87	119.55	1160.16	10.03
	NP-024	3.92	141.71	1866.48	12.75	2.05	211.74	4188.45	31.53
	Ave.	2.23	86.15	993.13	12.05	1.92	137.11	2054.96	17.89



Supplemental Fig. 2. Time courses of circulating angiogenic factors, including serum FGF-2, VEGF, and HGF
 FGF-2: Fibroblast growth factor-2, VEGF: Vascular endothelial growth factor, HGF: Hepatocyte growth factor



Supplemental Fig. 3. Changes in ischemic ulcer size pre- and post-administration of NK-104-NP



Supplemental Fig. 4. Degree of pain (VAS) (A), time courses of ankle-brachial pressure index (ABI), toe pressure index (TPI) (B), laser Doppler perfusion index (LDPI) ratio, and transcutaneous oxygen pressure (TcPO₂) (C) ($n = 12$, $p = n.s.$).