Original Article

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 proteins1,2 or gene-encoding growth factors3,4 to endothelial cells. Since then, autologous stem cells5, endothelial progenitor cell transplantation therapy6, and novel nucleic acid analogs7 have been developed. Peripheral arterial disease is a typical phenotype of progressive and systemic atherosclerosis, leading to...
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\(^\text{10, 11}\). Statins increase the angiogenic activity of mature endothelial cells\(^\text{12}\), as well as that of endothelial progenitor cells\(^\text{13, 14}\), and augment angiogenesis/arteriogenesis in ischemic heart\(^\text{15}\) and limb in experimental animals\(^\text{16}\). 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 setting\(^\text{12-14}\). 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\(^\text{17}\)). 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 injection\(^\text{16}\). 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 dose\(^\text{18}\). 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 model\(^\text{18}\) and a cynomolgus monkey model of chronic lower limb ischemia\(^\text{19}\). 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-NP\(^\text{17}\). 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 previously20, 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
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) (%) |
|--------------------------------|--------------|
| Age (y, range)                 | 72.4 ± 11.1  |
| Sex                            |              |
| Male                           | 10 (62.5)    |
| Female                         | 6 (37.5)     |
| BMI (kg/m²)                    | 22.8 ± 0.89  |
| Alb (g/dl)                     | 3.7 ± 0.4    |
| Smoking Status                 |              |
| Never                          | 8 (50.0)     |
| Former                         | 7 (43.8)     |
| Current                        | 1 (6.2)      |
| Hypertension                   | 14 (87.5)    |
| Coronary Artery Disease        | 3 (18.8)     |
| Diabetes Mellitus              | 5 (31.3)     |
| Cerebrovascular disease        | 4 (25.0)     |
| Hemodialysis                   | 5 (31.3)     |
| Hyperlipidemia                 | 7 (43.8)     |
| Aspirin                        | 8 (50.0)     |
| Thienopyridines/clopidogrel    | 6 (37.5)     |
| Cilostazol                     | 3 (18.8)     |
| Sarpogrelate                   | 5 (31.3)     |
| Beraprost                      | 3 (18.8)     |
| Warfarin                       | 8 (50.0)     |
| 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 (y) | 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 2nd toe ulcer                        | 83                           | severe   | recovered   | not related   |
|              | Left 5th 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     | uncertain   | 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 2nd 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 3rd 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
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 pitavastatin-NPs in PAD
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 NK-104-NP administration. 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 NK-104-NP for 5 days is safe and well tolerated in patients with CLTI. The pharmacokinetic parameters $T_{\text{max}}, C_{\text{max}},$ and $AUC_{0-\infty}$ 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 ischemia16, 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).

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      | 0.5       | 0                    | III      | III      | III | III | III | no change |
| NP-006      | 1         | 25                   | III      | III      | III | IV  | IV  | worsen |
| NP-008      | 1         | 25                   | III      | III      | III | IV  | IV  | no change |
| NP-009      | 2         | 75                   | III      | III      | IIa | IIa | IIa | improved |
| NP-010      | 2         | 75                   | III      | IIb      | IIb | IIb | IIb | improved |
| NP-011      | 2         | 75                   | III      | IIb      | IIb | IIb | IIb | no change |
| NP-012      | 2         | 75                   | IV       | IV       | IV  | IV  | #N/A| no change |
| NP-013      | 2         | 75                   | IV       | IV       | IV  | IV  | #N/A| no change |
| NP-014      | 2         | 75                   | IV       | IV       | IV  | IV  | #N/A| no change |
| NP-015      | 2         | 75                   | IV       | IV       | IV  | IV  | #N/A| no change |
| NP-016      | 2         | 75                   | IV       | IV       | IV  | IV  | #N/A| no change |
| NP-017      | 2         | 75                   | IV       | IV       | IV  | IV  | #N/A| no change |
| NP-018      | 2         | 75                   | IV       | IV       | IV  | IV  | #N/A| no change |
| NP-019      | 2         | 75                   | IV       | IV       | IV  | IV  | #N/A| no change |
| NP-020      | 2         | 75                   | IV       | IV       | IV  | IV  | #N/A| no change |
| NP-021      | 2         | 75                   | IV       | IV       | IV  | IV  | #N/A| no change |
| NP-022      | 2         | 75                   | IV       | IV       | IV  | IV  | #N/A| no change |
| NP-023      | 2         | 75                   | IV       | IV       | IV  | IV  | #N/A| no change |
| NP-024      | 2         | 75                   | IV       | IV       | IV  | IV  | #N/A| no change |

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

| NP | 0.5 | NP 1 | NP 2 | NP 4 | Fontaine/ Rutherford |
|----|-----|------|------|------|-----------------------|
| Improved | 0 (0%) | 1 (25%) | 1 (25%) | 1 (25%) | 1 (25%) |
| No change | 3 (75%) | 2 (50%) | 1 (25%) | 3 (75%) | 2 (50%) |
| Worsen | 1 (25%) | 1 (25%) | 0 (0%) | 1 (25%) | 1 (25%) |
expression of angiogenic growth factors such as vascular endothelial growth factor in vascular endothelial cells in the ischemic limb\(^\text{16}\). 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 mice\(^\text{16}\) and rabbits\(^\text{18}\) \(\text{in vivo}\), 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 heart\(^\text{23}\) and brain\(^\text{24}\), remodeling after acute myocardial infarction\(^\text{25}\), pulmonary arterial hypertension\(^\text{26, 27}\), and atherosclerosis plaque destabilization/rupture\(^\text{28, 29}\). 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 expression of angiogenic growth factors such as vascular endothelial growth factor in vascular endothelial cells in the ischemic limb\(^\text{16}\). 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 mice\(^\text{16}\) and rabbits\(^\text{18}\) \(\text{in vivo}\), 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.

Pitavastatin is minimally metabolized by CYP2C9, unlike other statins such as atorvastatin, fluvastatin, pravastatin, rosuvastatin, and simvastatin, which are mainly metabolized by CYP3A4\(^\text{22}\).

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\(^\text{12, 16}\) and rabbits\(^\text{18}\) 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-\(\text{CoA}\) reductase activity in rat liver microsomes \(\text{in vitro}\) (unpublished observation in our laboratory), (2) it elicited the most potent effects on angiogenic activity of human endothelial cells \(\text{in vitro}\) compared with other statins, and (3) NP-mediated intracellular delivery of pitavastatin showed greater angiogenic activity in human endothelial cells compared with pitavastatin alone\(^\text{18}\).

Pitavastatin is minimally metabolized by CYP2C9, unlike other statins such as atorvastatin, fluvastatin, pravastatin, rosuvastatin, and simvastatin, which are mainly metabolized by CYP3A4\(^\text{22}\).

We have also reported in an \(\text{in vivo}\) model that (1) a single intramuscular injection of pitavastatin-NP increased angiogenesis/arteriogenesis\(^\text{16}\) and (2) treatment with pitavastatin-NP selectively increased
tissue healing. 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.

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 |
|-----------|-------------|----------------------------------|----------------------------------|
|           |             | $\text{T}_{\text{max}}$ (hr)    | $\text{C}_{\text{max}}$ (ng/mL)   | $\text{AUC}_{0-\infty}$ (hr*ng/mL) | $\text{T}_{1/2}$ (hr) |
| 0.5       | NP-001      | 0.50                             | 17.76                           | 81.66                                | 9.75               |
|           | NP-004      | 0.50                             | 9.80                            | 46.75                                | 14.30              |
|           | NP-006      | 1.00                             | 15.01                           | 63.86                                | 9.37               |
|           | NP-008      | 0.50                             | 12.47                           | 32.67                                | 2.59               |
|           | Ave.        | 0.63                             | 13.76                           | 56.24                                | 9.00               |
| 1         | NP-009      | 1.00                             | 19.51                           | 90.61                                | 9.92               |
|           | NP-010      | 0.50                             | 15.25                           | 38.31                                | 1.87               |
|           | NP-011      | 0.50                             | 22.22                           | 80.02                                | 11.89              |
|           | NP-013      | 1.00                             | 35.03                           | 153.74                               | 6.51               |
|           | Ave.        | 0.75                             | 23.00                           | 90.67                                | 7.55               |
| 2         | NP-014      | 1.00                             | 38.22                           | 261.68                               | 12.96              |
|           | NP-015      | 1.00                             | 29.24                           | 142.86                               | 7.55               |
|           | NP-017      | 0.65                             | 34.99                           | 216.96                               | 9.35               |
|           | NP-018      | 1.05                             | 35.86                           | 162.26                               | 6.52               |
|           | Ave.        | 0.93                             | 34.58                           | 195.94                               | 9.09               |
| 4         | NP-019      | 0.50                             | 87.30                           | 305.40                               | 9.33               |
|           | NP-020      | 1.00                             | 89.89                           | 420.63                               | 10.02              |
|           | NP-021      | 1.00                             | 105.28                          | 392.12                               | 5.27               |
|           | NP-024      | 1.00                             | 92.35                           | 521.86                               | 8.98               |
|           | Ave.        | 0.88                             | 93.71                           | 410.00                               | 8.40               |

$\text{T}_{\text{max}}$: Time to maximum plasma concentration, $\text{C}_{\text{max}}$: Maximum observed concentration, $\text{AUC}_{0-\infty}$: Area under the plasma concentration–time curve from time 0 to $\tau$ after administration, $\text{T}_{1/2}$: 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 |
|-----------|-------------|----------------------------------|----------------------------------|
|           |             | $\text{T}_{\text{max}}$ (hr)    | $\text{C}_{\text{max}}$ (ng/mL)   | $\text{AUC}_{0-\infty}$ (hr*ng/mL) | $\text{T}_{1/2}$ (hr) |
| 0.5       | NP-001      | 2.00                             | 12.64                           | 115.73                               | 10.28              |
|           | NP-004      | 2.00                             | 5.73                            | 79.07                                | 20.14              |
|           | NP-006      | 2.00                             | 6.21                            | 76.97                                | 15.53              |
|           | NP-008      | 2.00                             | 8.80                            | 69.30                                | 7.84               |
|           | Ave.        | 2.00                             | 8.35                            | 85.27                                | 13.45              |
| 1         | NP-009      | 2.00                             | 15.22                           | 157.56                               | 12.52              |
|           | NP-010      | 2.00                             | 7.08                            | 40.99                                | 4.32               |
|           | NP-011      | 2.00                             | 9.21                            | 103.67                               | 17.51              |
|           | NP-013      | 3.92                             | 23.58                           | 283.89                               | 25.20              |
|           | Ave.        | 2.48                             | 13.77                           | 146.53                               | 14.89              |
| 2         | NP-014      | 2.27                             | 36.39                           | 508.04                               | 15.22              |
|           | NP-015      | 1.83                             | 22.95                           | 257.55                               | 10.48              |
|           | NP-017      | 1.93                             | 16.35                           | 269.36                               | 22.98              |
|           | NP-018      | 2.02                             | 30.82                           | 292.60                               | 10.07              |
|           | Ave.        | 2.01                             | 26.63                           | 331.89                               | 14.69              |
| 4         | NP-019      | 1.85                             | 31.49                           | 279.85                               | 14.12              |
|           | NP-020      | 2.17                             | 91.81                           | 1092.13                               | 13.31              |
|           | NP-021      | 1.00                             | 79.60                           | 734.07                               | 8.02               |
|           | NP-024      | 3.92                             | 141.71                          | 1866.48                               | 12.75              |
|           | Ave.        | 2.23                             | 86.15                           | 993.13                               | 12.05              |
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) ($n=12$, $p=\text{n.s.}$).