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Biodistribution and safety studies of hDel-1 plasmid-based gene therapy in mouse and rabbit models

Abraham Quezada, Jeff Larson, Martha French, Rafael Ponce, Jerry Perrard, Ross Durland and Michael Coleman

Valentis, Inc., Burlingame, CA, USA

A. Quezada, M. French, J. Perrard, R. Durland, M. Coleman

Sierra Biomedical, Inc., San Diego, CA, USA

J. Larson

SNBL USA, Ltd, Seattle, WA, USA

R. Ponce

Correspondence: A. Quezada, STILLMEADOW, Inc, 12852 Park One Drive, Sugar Land, TX 77478, USA. E-mail: aquezada@stillmedow.com

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Abstract

A plasmid encoding the human developmentally regulated endothelial locus-1 (hDel-1) protein formulated with poloxamer 188 is a potential gene therapy for peripheral arterial disease in man. As a prelude to clinical trials, the biodistribution and safety of this therapy were evaluated after intramuscular and intravenous administration in mice and rabbits. In mice, plasmid DNA persisted at the intramuscular injection site for at least 28 days, but was barely detectable in distal tissues by 24 h and essentially cleared by 28 days. By 24 h after intravenous administration, plasmid DNA was readily detected in blood, muscle, and lungs but sporadically and at low levels in other tissues. At 28 days, plasmid DNA was readily detectable only at the intravenous injection site (tail) after low- and high-dose administration, and sporadically in blood and muscle after high-dose administration. In rabbits, the highest intramuscular (4.2 mg kg^{-1}) or intravenous (3.7 mg kg^{-1}) dose caused no deaths; no treatment-related clinical signs; no changes in body weight, clinical pathology parameters, ophthalmology, ECG, or histopathology; and no detectable increase in antinuclear antibodies by 28 days. The results supported testing of hDel-1 plasmid–based gene therapy in phase I clinical trials.

Introduction

Peripheral arterial disease affects 1 in 20 Americans over age 50 (roughly eight million people) and is more common in men than women (Spittell & Spittell 1993; Creager 2001). It accounts for a sizable portion of annual health-care expenditures and is a major cause of disability, loss of work or wages, and lifestyle limitations (Rosenfield & Isner 1998). The disease is caused by stenosis and/or occlusion of the iliac-femoral-popliteal arterial axis that in turn reduces perfusion of the muscles and skin of the lower limbs and causes progressive tissue ischaemia (Novo 1996). Unfortunately, there is currently no effective pharmacological treatment for vascular defects in the lower limbs, and many patients who present with persistent ischaemic ulcers are not appropriate candidates for surgical or endovascular therapies (Rissanen et al 2001). One potential therapeutic alternative is angiogenic arterial gene transfer (Isner 1998; Kanno et al 1999; Liau et al 2001). The ability to maintain a suitably high local concentration of a gene medicine over days or weeks makes gene transfer very attractive (Isner & Asahara 1999), and various angiogenic approaches are already being investigated in clinical trials (Carmeliet & Jain 2000).

Developmentally regulated endothelial locus-1 (Del-1) is a cell-specific matrix protein expressed primarily in the endothelium of the vasculature and immediately adjacent cell types during embryological development of the vascular tree (Hidai et al 1998; Penta et al 1999). A gene therapeutic agent comprised of a plasmid encoding the human Del-1 protein (hDel-1) formulated with a non-ionic polymer (VLTS-589; Valentis Inc., Burlingame, CA) has been synthesized for localized treatment of peripheral limb arterial disease. The polymeric delivery system of the hDel-1 plasmid, poloxamer 188, is used widely (O'Keefe et al 1996; Moghimi & Hunter 2000) and is non-viral, which makes it easier to use, easier to produce on a large scale, and less likely to produce a host immune response when compared with viral vectors (Nishikawa & Huang 2001).

The rationale for developing an hDel-1-based gene medicine for treatment of peripheral arterial disease is based on research into local administration of angiogenic growth factors in ischaemic tissue and angiogenic gene transfer. On the one hand, animal studies have suggested that local administration of angiogenic growth factors such as fibroblast growth factor (FGF) or vascular endothelial growth factors (VEGF) can enhance angiogenesis and tissue perfusion after surgical induction of ischaemia. In preclinical studies, recombinant murine Del-1 protein and formulated Del-1 plasmid produced effects comparable with those produced by FGF-2 and VEGF-165 (Howell et al 2000), and formulated Del-1 plasmids caused no overt toxicities or gross pathologies, even at the elevated pharmacological levels of Del-1 expression achieved by electroporation (Wilson et al 2000). On the other hand, recent clinical results suggested that the delivery of angiogenic factors by gene transfer instead of by recombinant protein therapy might enhance efficacy, allow less frequent dosing, and reduce systemic toxicity. Recent phase II trials of recombinant FGF-2 (Chiron Corporation 2000) and VEGF-165 (Henry et al 1998) protein therapy in patients with myocardial ischaemia have been disappointing, suggesting that recombinant proteins do not reside in the tissue long enough to obtain the desired therapeutic effect.

In this light, we hypothesized that gene transfer of our hDel-1-based plasmid would result in sustained, local expression of Del-1 protein in a manner that would mimic the autocrine/paracrine modes of action associated with endogenous Del-1, and that local drug delivery would reduce the systemic exposure and potential toxicity associated with systemic administration of a gene medicine. This study in mice and rabbits was performed to assess the potential toxicity of the hDel-1 plasmid formulated with poloxamer 188 when administered intramuscularly and intravenously, as a prelude to phase I clinical trials.

Materials and Methods

Plasmid composition

The gene medicine used in this study (VLTS-589) was comprised of a plasmid expression system and a polymeric gene delivery system. The plasmid expression system was comprised of an eukaryotic expression cassette encoding the full-length hDel-1 protein. The polymeric delivery system was comprised of the non-viral, non-ionic poloxamer 188. Tris(hydroxymethyl)aminomethane USP (Tris) and Tris-(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) were used as excipients. All components were aseptically mixed using an in-line process and sterilized by filtration. The resulting mixture was poured into glass vials, lyophilized under sterile conditions, and finally stored at 2–8 °C until used.

Biodistribution studies in mice

Intramuscular administration

Twelve male and 12 female CD-1 mice (2–3 weeks-old, 20–25 g), were obtained from Charles River (Raleigh, NC) and allowed to acclimatize for nine days before dosing at the facilities of Valentis, Inc. (The Woodlands, TX). These mice were housed in micro-isolators in a highly ventilated, HEPA-filtered unit, three mice per micro-isolator segregated by sex and treatment group. The housing unit was maintained on a 12-h light–dark cycle at 18–26 °C and 30–70% relative humidity, with 30 air exchanges h^{-1}. All mice were provided with rodent chow (Purina, St Louis, MO) and clean water was freely available. All animals were housed and handled in accordance with the regulations of the Institutional Animal Care and Use Committee of Valentis, Inc., and with generally accepted animal care guidelines. On day zero, mice were divided into two groups, six per sex per group. Each mouse was given a single injection of either 25 μL 0.9% saline solution as vehicle (negative control) (group 1) or 25 μL of the drug formulation (group 2). Injections were given in the right gastrocnemius muscle, with the volume divided between the two bellies of the muscle. The total plasmid dose was 25 μg, corresponding to approximately 1.25 mg kg^{-1} body weight. Three mice per sex per group were killed at 24 h and the rest at 28 days after injection. At necropsy, tissues were collected from each mouse in the following order: blood, gonads, spleen, liver, kidneys, heart, lungs, brain, non-injected contralateral gastrocnemius muscle, and injected gastrocnemius muscle. Extensive precautions were taken to minimize cross-contamination of individual tissue samples with plasmid DNA. These precautions included spraying work surfaces, gloves, and the skin and fur of anaesthetized mice with a 10% bleach solution before sample collection to inactivate any plasmid DNA. Gloves were changed between mouse handlings and at prescribed time points, and all instruments were soaked in a 10% bleach solution for at least 30 s between each tissue harvest. Each solid tissue sample was transferred to a labelled tube, snap-frozen in liquid nitrogen, and stored at approximately −80 °C. Blood samples were collected in an EDTA-containing tube on wet ice and subsequently stored at 2–8 °C.

Total DNA was isolated from each of the harvested tissues by phenol–chloroform extraction. DNA from blood samples was isolated using the Invitrogen Easy-DNA kit (San Diego, CA). The DNA concentration in each sample was determined by UV absorption at 260 nm, and the samples were stored at approximately 4 °C. Each DNA sample was tested for the presence of hDel-1 DNA using a quantitative polymerase chain reaction (qPCR) assay specific for hDel-1 plasmid DNA. PCR reagents and oligonucleotide probes were obtained from Perkin Elmer (Boston, MA) and PCR primers were obtained from Sigma-Genosys (The Woodlands, TX). For each tissue specimen, 200 ng sample DNA was added to each PCR reaction and tested in duplicate wells. Each run included a standard curve consisting of duplicate samples of seven concentrations of hDel-1 DNA diluted in a matrix containing 200 ng mouse liver genomic DNA per reaction. Plasmid concentrations for the standard curve ranged from 0.001 to 1000 pg per reaction, in 1-log increments. For all PCR reactions in this study, the baseline concentration was determined during amplification cycles

3–10, and the threshold was set at 0.02 pg per reaction. A standard curve was calculated for each run by linear regression analysis using the threshold cycle (Ct) values and the log values of the known amounts of hDel-1 DNA present in each standard reaction. Quantitative values for hDel-1 DNA were calculated from the Ct value of each reaction that was within the range of the standard curve (0.001–1000 pg of hDel-1 DNA per reaction). PCR reactions that were below the range of the standard curve but still resulted in a Ct value less than the total number of amplification cycles (45 cycles) were considered positive for hDel-1 but below the limit of quantification (LOQ). Reactions in which the fluorescence did not cross the threshold by the 45th cycle were considered negative. The LOQ was 0.001 pg of hDel-1 DNA per reaction, corresponding to approximately 200 copies of plasmid per reaction or 1000 copies μg^{-1} genomic DNA. As a control, samples mixed with 0.003 pg purified hDel-1 plasmid DNA were subjected to PCR analysis to confirm that there was no inhibition of the qPCR reaction.

Intravenous administration

The intravenous biodistribution study was conducted at the facilities of SNBL USA, Ltd (Seattle, WA) in compliance with Good Laboratory Practices (GLP). Twenty-seven male and 27 female CD-1 mice (3–4 weeks-old, 18.5–25.8 g) were purchased from Charles River (Hollister, CA) and allowed to acclimatize for seven days before dosing. These mice were housed in solid-bottom, polycarbonate cages with contact bedding. The housing unit was maintained on a 12-h light–dark cycle at 21–24 °C and 30–70% relative humidity, with at least 10 air exchanges h^{-1}. The mice were fed rodent chow (PicoLab 20-5K75; Purina, St Louis, MO) which was freely available. The mice were housed and handled in accordance with the Guide for the Care and Use of Laboratory Animals. On day zero, the mice were divided into three groups, nine per sex per group. Each mouse received a single injection of 20 μL 0.9% sodium chloride USP (Baxter Healthcare, Deerfield, IL) as vehicle (negative control) (group 1); 5 μL of the drug formulation at a dose of 0.2 mg kg^{-1} (group 2, low dose); or 20 μL of the drug formulation at a dose of 0.8 mg kg^{-1} (group 3, high dose). Table 1 shows the dosing and kill schedule for this intravenous biodistribution study. Animal necropsies, tissue collection, DNA isolation, and qPCR analysis of tissue samples were performed as described above.

Safety studies in rabbits

Safety studies in rabbits were conducted to determine the potential toxicity of a single administration of the drug formulation given by multiple intramuscular injections or a single intravenous injection and the potential reversibility of any drug effects after a four-week observation period. These safety studies were performed at Sierra Biomedical, Inc. (San Diego, CA) in compliance with GLP and the Standard Operating Procedures established at Sierra Biomedical, Inc., in accordance with the USDA Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals. The Animal Care and Use Committee of Sierra Biomedical, Inc., approved the safety study protocols. The New Zealand White rabbit model was chosen to assess the potential toxic effects of VLTS-589 because it is a species commonly used in angiogenesis research, and preclinical studies have shown that hDel-1 is efficacious and easily expressed in this animal model (Howell et al 2000).

Rabbits, 6–7 months-old, were obtained from Myrtle's Rabbitry (Thompson Station, TN). Rabbits used for the intramuscular study weighed 4.1–4.6 kg each; those used for the intravenous study weighed 3.6–4.5 kg each. All rabbits were housed in stainless steel cages in an isolated room maintained on a 12-h light–dark cycle at 16–22 °C, with more than 12 air exchanges h^{-1}. All rabbits were provided a standard diet (Certified High Fiber Rabbit Diet 5325; PMI Feeds, Inc., St. Louis, MO) and filtered, UV light-treated water was freely available.

Intramuscular administration

A total of 80 rabbits (five groups of 16 rabbits, eight per sex per group) were assigned to the intramuscular safety study. Target organ toxicity was evaluated by killing half of the animals at day 8 and the rest on day 29. Table 2 shows the dosing and kill schedule for the intramuscular safety study.

Before dosing, all rabbits were anaesthetized with ketamine HCl and shaved at the intended injection sites. Rabbits in groups 1, 2, and 5 received eight intramuscular injections on the inside of each thigh along the femoral artery line and one injection in each leg in the dorsal half of the tibialis cranialis muscle. The injection volume at each site was 1 mL (a total of 18 mL). Rabbits in group 3 received four intramuscular injections on the inside of the right thigh. The injection volume at each site was 0.3 mL

Table 1 Intravenous biodistribution study in mice: dosing and kill schedule.

Group	No. M/F	Agent	Dose ($mg\,kg^{-1}$)[a]	Dose volume (μL)	Kill schedule		
					Day 2	Day 8	Day 29
1	9M/9F	Saline	0	20	3M/3F	3M/3F	3M/3F
2	9M/9F	VLTS-589	0.2	5	3M/3F	3M/3F	3M/3F
3	9M/9F	VLTS-589	0.8	20	3M/3F	3M/3F	3M/3F

M, male; F, female. [a]Dose levels were based on day 1 body weights and a fixed administration volume.

Table 2 Intramuscular safety study in rabbits: dosing and kill schedule.

Group	No. M/F	Agent	Injection sites/leg	Total injection volume (mL/rabbit)	Total DNA dose (mg/rabbit)	DNA dose ($mg\,kg^{-1}$)[b]	Kill schedule	
							Day 8	Day 29
1	8M/8F	Vehicle (control)[a]	9	18.0	18.0	4.2	4M/4F	4M/4F
2	8M/8F	Saline	9	18.0	0	0	4M/4F	4M/4F
3	8M/8F	VLTS-589	4	1.2	1.2	0.3	4M/4F	4M/4F
4	8M/8F	VLTS-589	8	9.0	9.0	2.1	4M/4F	4M/4F
5	8M/8F	VLTS-589	9	18.0	18.0	4.2	4M/4F	4M/4F

M, male; F, female. [a]Empty plasmid in 5% poloxamer 188. [b]Assuming a mean weight of 4.3 kg/rabbit.

(a total of 1.2 mL). Rabbits in group 4 received seven intramuscular injections on the inside of each leg and one injection in the dorsal half of the tibialis cranialis muscle. The injection volume at each site was 0.5 mL for the thigh and 1 mL for the tibialis (a total of 9 mL). All rabbits were evaluated at least once daily for clinical signs of toxicity, beginning the day before dosing and continuing until the day of their scheduled killing. Body weights were measured weekly. All rabbits underwent ophthalmic examinations, electrocardiography, blood chemistry, haematology, and antinuclear antibody testing before dosing and again on the day of scheduled kill. Immediately after killing, a full necropsy was performed, during which a complete panel of tissues was collected, weighed, preserved, processed to slides, and examined by light microscopy. Urinalysis and bone marrow examinations were performed.

Intravenous administration

A total of 50 rabbits (five groups of 10 rabbits, five per sex per group) were assigned to treatment groups as shown in Table 3. Each rabbit was temporarily restrained for the dosing procedure. Rabbits in groups 1, 2, and 5 had a catheter placed in the marginal ear vein, through which the drug formulation or control solution was delivered by an infusion pump at a rate of approximately $1\,mL\,min^{-1}$. Rabbits in groups 3 and 4 also had a catheter placed in the marginal ear vein, but received the drug formulation or control solution by hand-delivered slow bolus infusion as the dose volume was small. Rabbits in all groups were examined at least once daily for clinical signs of toxicity and killed at day 29. Body weights were recorded weekly. Blood specimens were collected for evaluation of serum chemistry, haematology, and coagulation before dosing and on days 3, 15, and 29. Necropsy, tissue harvesting, and histopathology were conducted as described above. Urine and blood for evaluation of antinuclear antibodies were collected at necropsy only. A bone marrow smear was made and evaluated microscopically.

Statistical analysis

Group means and standard deviations were calculated for all numerical data. Statistical evaluations were performed on body and organ weights, haematology, and serum chemistry. For body weight, the change per day from the pretreatment day to the last unfasted weight before termination was analysed. Clinical pathology time points included baseline, day 8 and day 29 for the intramuscular administration study (Table 4), and baseline, day 3, 15, and 29 for the intravenous administration study (Table 5). Body and organ weights were analysed using a two-way analysis of variance with dose and sex as factors, while the clinical pathology was analysed using a three-way analysis of variance with dose, time, and sex as factors. The baseline values were included as a covariate for clinical pathology.

Table 3 Intravenous safety study in rabbits: dosing schedule.

Group	No. M/F	Agent	Total injection volume (mL/rabbit)	Total DNA dose (mg/rabbit)	DNA dose ($mg\,kg^{-1}$)[b]	Kill schedule
1	5M/5F	Vehicle (control)[a]	14.0	14.0	14.0	Day 29
2	5M/5F	Saline	14.0	0.0	0.0	Day 29
3	5M/5F	VLTS-589	0.35	0.35	0.089	Day 29
4	5M/5F	VLTS-589	1.4	1.4	0.36	Day 29
5	5M/5F	VLTS-589	14.0	14.0	3.70	Day 29

M, male; F, female. [a]Empty plasmid in 5% poloxamer 188. [b]Based on group mean body weight before dosing.

Table 4 Selected clinical chemistry and haematology parameters in rabbits following a single intramuscular administration of hDel-1 plasmid-based gene medicine.

Groups time points (day)	Group 1			Group 2			Group 3			Group 4			Group 5		
	0	8	29	0	8	29	0	8	29	0	8	29	0	8	29
Na ($mmol\,L^{-1}$)	144 ± 2	142 ± 2	140 ± 1	144 ± 1	141 ± 1	141 ± 1	143 ± 2	141 ± 1	142 ± 1	143 ± 1	142 ± 1	143 ± 2	144 ± 1	142 ± 1	142 ± 1
K ($mmol\,L^{-1}$)	5.7 ± 0.6	5.1 ± 0.9	4.5 ± 0.3	5.7 ± 0.6	4.6 ± 0.5	4.4 ± 0.3	5.8 ± 0.6	4.3 ± 0.2	4.2 ± 0.2	5.7 ± 0.5	4.3 ± 0.2	4.3 ± 0.2	5.7 ± 0.4	4.2 ± 0.2	4.3 ± 0.3
Cl ($mmol\,L^{-1}$)	106 ± 2	105 ± 2	105 ± 1	106 ± 2	103 ± 1	105 ± 1	106 ± 2	105 ± 1	104 ± 3	105 ± 2	104 ± 1	104 ± 1	106 ± 1	106 ± 2	106 ± 2
Ca ($mg\,dL^{-1}$)	14 ± 1.9	13 ± 0.2	12 ± 0.3	13 ± 0.5	13 ± 0.2	12 ± 0.3	14 ± 0.8	13 ± 0.2	13 ± 0.2	13 ± 0.2	13 ± 0.2	13 ± 0.2	13 ± 0.6	13 ± 0.2	12 ± 0.2
P ($mg\,dL^{-1}$)	3.5 ± 0.4	4.5 ± 0.4	4.1 ± 0.4	3.6 ± 0.3	4.3 ± 0.6	4.5 ± 0.5	3.7 ± 0.4	4.2 ± 0.4	4.2 ± 0.5	3.4 ± 0.3	4.4 ± 0.5	4.3 ± 0.2	3.3 ± 0.4	4.6 ± 0.7	4.1 ± 0.5
BUN ($mg\,dL^{-1}$)	17 ± 2.8	14 ± 2.9	13 ± 2.4	16 ± 2.9	14 ± 2.7	13 ± 3.1	16 ± 2.3	13 ± 1.8	14 ± 3.2	15 ± 3.2	14 ± 2.0	12 ± 3.0	16 ± 2.9	14 ± 2.2	12 ± 1.9
Creatinine ($mg\,dL^{-1}$)	1.7 ± 1.2	1.4 ± 0.1	1.4 ± 0.1	1.0 ± 0.1	1.4 ± 0.2	1.5 ± 0.1	1.1 ± 0.2	1.4 ± 0.2	1.4 ± 0.1	0.9 ± 0.1	1.2 ± 0.1	1.2 ± 0.3	0.9 ± 0.1	1.3 ± 0.1	1.3 ± 0.3
T. Bilirubin ($mg\,dL^{-1}$)	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.6	0.4 ± 0.1	0.4 ± 0.1	0.9 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
Glucose ($mg\,dL^{-1}$)	110 ± 15	120 ± 9	122 ± 8	103 ± 10	123 ± 19	130 ± 7	101 ± 8	118 ± 8	121 ± 6	113 ± 11	121 ± 11	121 ± 6	110 ± 10	117 ± 10	116 ± 8
Cholesterol ($mg\,dL^{-1}$)	15 ± 26	25 ± 35	15 ± 27	11 ± 23	15 ± 28	14 ± 25	15 ± 23	31 ± 46	7.6 ± 22	3.2 ± 9.2	0.0 ± 0	14 ± 39	11 ± 24	8 ± 22	15 ± 28
T. Protein ($g\,dL^{-1}$)	5.7 ± 0.4	5.8 ± 0.4	5.6 ± 0.2	5.8 ± 0.2	5.9 ± 0.1	5.7 ± 0.3	5.7 ± 0.2	5.6 ± 0.2	5.7 ± 0.3	5.8 ± 0.2	5.9 ± 0.2	5.8 ± 0.2	5.8 ± 0.3	5.7 ± 0.3	5.7 ± 0.3
Albumin ($g\,dL^{-1}$)	3.6 ± 0.4	3.7 ± 0.3	3.4 ± 0.2	3.6 ± 0.1	3.7 ± 0.1	3.4 ± 0.2	3.9 ± 0.2	3.5 ± 0.2	3.4 ± 0.2	3.7 ± 0.2	3.8 ± 0.2	3.5 ± 0.2	3.7 ± 0.2	3.6 ± 0.2	3.4 ± 0.3
Globulin ($g\,dL^{-1}$)	2.9 ± 1.3	2.1 ± 0.2	2.2 ± 0.1	2.2 ± 0.1	2.2 ± 0.0	2.3 ± 0.1	2.1 ± 0.1	2.1 ± 0.2	2.3 ± 0.1	2.1 ± 0.8	2.1 ± 0.1	2.3 ± 0.1	2.2 ± 0.1	2.1 ± 0.1	2.3 ± 0.1
A/G Ratio	1.7 ± 0.2	1.7 ± 0.3	1.5 ± 0.1	1.7 ± 0.9	1.7 ± 0.1	1.5 ± 0.1	1.7 ± 0.1	1.7 ± 0.2	1.5 ± 0.1	1.8 ± 0.1	1.8 ± 0.2	1.5 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	1.5 ± 0.2
Creatine kinase ($IU\,L^{-1}$)	424 ± 114	525 ± 204	453 ± 203	620 ± 590	723 ± 743	360 ± 108	468 ± 134	505 ± 140	490 ± 132	717 ± 806	670 ± 79	1434 ± 2612	467 ± 173	533 ± 389	622 ± 451
AST ($IU\,L^{-1}$)	16 ± 3.2	21 ± 5.7	14 ± 4.1	14 ± 2.7	15 ± 1.9	13 ± 2.6	15 ± 3.1	16 ± 3.8	14 ± 2.4	13 ± 1.8	16 ± 2.0	14 ± 3.0	13 ± 2.5	15 ± 4.9	14 ± 2.5
ALT ($IU\,L^{-1}$)	49 ± 17	42 ± 22	57 ± 17	43 ± 6.8	35 ± 8.9	59 ± 15	46 ± 9.4	45 ± 8.6	45 ± 7.4	40 ± 11	42 ± 14	38 ± 6.2	41 ± 7.6	41 ± 10	43 ± 6.9
WBC ($10^3\,mm^3$)	8.7 ± 1.4	8.2 ± 2.0	7.7 ± 1.2	8.1 ± 1.6	8.0 ± 1.6	7.4 ± 1.0	8.8 ± 2.0	7.5 ± 1.3	7.7 ± 2.4	8.1 ± 1.9	7.0 ± 1.3	7.3 ± 1.9	8.0 ± 1.3	7.4 ± 1.7	7.3 ± 1.0
Neutrophils (%)	37 ± 9.5	37 ± 9.0	39 ± 8.2	32 ± 6.9	33 ± 12	39 ± 3.5	30 ± 8.4	33 ± 18	40 ± 13	35 ± 10	45 ± 14	34 ± 14	30 ± 8.1	32 ± 8.2	41 ± 12
Lymphocytes (%)	56 ± 9.9	59 ± 7.9	51 ± 11	60 ± 9.0	61 ± 11	53 ± 4.7	62 ± 9.9	61 ± 18	48 ± 13	57 ± 10	48 ± 14	56 ± 16	63 ± 8.8	59 ± 8.7	51 ± 11
Monocytes (%)	1.1 ± 1.1	3.1 ± 1.7	4.3 ± 2.7	1.7 ± 1.6	3.0 ± 1.5	3.6 ± 2.3	1.8 ± 1.8	2.5 ± 1.3	6.1 ± 2.5	1.4 ± 0.1	4.5 ± 2.6	5.3 ± 2.3	2.0 ± 0.9	4.5 ± 2.4	4.0 ± 2.1
Eosinophils (%)	1.2 ± 1.4	0.0 ± 0.0	0.0 ± 0.0	1.5 ± 1.0	0.0 ± 0.0	0.0 ± 0.0	1.3 ± 0.8	0.0 ± 0.0	0.0 ± 0.0	1.1 ± 0.9	0.0 ± 0.0	0.0 ± 0.0	1.3 ± 1.0	0.0 ± 0.0	0.0 ± 0.0
Basophils (%)	4.6 ± 2.8	0.4 ± 0.5	5.4 ± 3.4	5.3 ± 3.9	2.9 ± 1.6	4.1 ± 2.0	4.7 ± 3.1	3.6 ± 1.6	6.0 ± 3.1	5.1 ± 2.2	2.6 ± 1.8	4.8 ± 1.5	3.4 ± 2.2	4.1 ± 1.9	4.3 ± 2.1
Reticulocytes (%)	1.4 ± 0.6	1.9 ± 0.3	1.5 ± 0.2	1.3 ± 0.4	1.9 ± 0.3	1.6 ± 0.3	1.4 ± 0.4	1.9 ± 0.5	1.5 ± 0.4	1.2 ± 0.5	1.8 ± 0.3	1.7 ± 0.6	1.4 ± 0.4	2.3 ± 0.9	1.3 ± 0.3
RBC ($10^6\,mm^3$)	6.5 ± 0.3	6.3 ± 0.4	6.2 ± 0.3	6.5 ± 0.4	6.2 ± 0.3	6.3 ± 0.5	6.4 ± 0.4	6.0 ± 0.5	6.1 ± 0.5	6.5 ± 0.4	6.3 ± 0.5	6.6 ± 0.4	6.2 ± 0.4	5.9 ± 0.5	6.0 ± 0.4
Haematocrit (%)	41 ± 2.4	39 ± 1.6	38 ± 2.0	40 ± 3.0	38 ± 2.9	37 ± 2.7	39 ± 2.2	37 ± 2.5	37 ± 2.6	40 ± 2.2	38 ± 2.0	40 ± 2.6	39 ± 2.5	37 ± 2.5	37 ± 2.8
Platelets ($10^3\,mm^3$)	273 ± 63	298 ± 70	226 ± 38	242 ± 97	259 ± 72	244 ± 67	242 ± 53	248 ± 83	223 ± 42	224 ± 71	223 ± 44	216 ± 44	241 ± 98	241 ± 63	221 ± 76
Prothrombin time (s)	6.8 ± 0.2	6.9 ± 0.4	6.9 ± 0.2	6.9 ± 0.2	6.9 ± 0.2	6.8 ± 0.2	6.7 ± 0.2	6.9 ± 0.2	6.9 ± 0.2	6.7 ± 0.2	17 ± 25	6.7 ± 0.1	6.9 ± 0.6	6.9 ± 0.1	6.9 ± 0.1
APTT (s)	67 ± 16	66 ± 18	65 ± 16	75 ± 21	67 ± 10	82 ± 17	78 ± 21	68 ± 15	92 ± 14	88 ± 15	80 ± 49	74 ± 6.7	86 ± 33	65 ± 12	89 ± 19

Values are mean ± s.d.; BUN, blood urea nitrogen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; WBC, white blood cells; RBC, red blood cells; APTT, activated partial thromboplastin time.

Table 5 Selected clinical chemistry and haematology parameters in rabbits following a single intravenous administration of hDel-1 plasmid-based gene medicine. Data from day 15 are not shown.

Groups time points (day)	Group 1			Group 2			Group 3			Group 4			Group 5		
	0	3	29	0	3	29	0	3	29	0	3	29	0	3	29
Na (mmol L^{-1})	144 ± 1	143 ± 1	139 ± 2	144 ± 2	142 ± 1	140 ± 1	143 ± 1	144 ± 1	139 ± 1	144 ± 2	143 ± 2	139 ± 2	144 ± 2	144 ± 2	139 ± 2
K (mmol L^{-1})	4.9 ± 0.2	4.6 ± 0.3	4.1 ± 0.2	4.7 ± 0.3	4.5 ± 0.2	4.1 ± 0.2	4.7 ± 0.4	4.7 ± 0.3	3.9 ± 0.3	5.1 ± 0.4	4.3 ± 0.5	4.2 ± 0.6	4.8 ± 0.3	4.0 ± 0.2	4.9 ± 0.2
Cl (mmol L^{-1})	106 ± 2	105 ± 1	104 ± 3	105 ± 2	105 ± 2	105 ± 2	105 ± 2	106 ± 2	105 ± 2	105 ± 2	105 ± 2	105 ± 1	104 ± 2	107 ± 2	105 ± 1
Ca (mg dL^{-1})	14 ± 0.5	14 ± 0.8	13 ± 0.2	14 ± 0.9	14 ± 0.9	13 ± 0.2	14 ± 0.6	14 ± 0.6	13 ± 0.2	14 ± 0.7	13 ± 1.2	13 ± 0.3	14 ± 0.6	13 ± 0.2	13 ± 0.3
P (mg dL^{-1})	3.8 ± 0.6	3.5 ± 0.5	4.7 ± 0.4	4.2 ± 0.3	3.6 ± 0.3	4.8 ± 0.5	3.7 ± 0.3	3.7 ± 0.3	4.4 ± 0.5	3.8 ± 0.3	4.6 ± 1.1	4.2 ± 0.5	3.8 ± 0.7	5.0 ± 0.4	4.6 ± 0.5
BUN (mg dL^{-1})	17 ± 1.9	17 ± 2.6	14 ± 2.9	16 ± 2.9	16 ± 3.0	13 ± 3.2	17 ± 2.7	20 ± 4.1	15 ± 1.7	16 ± 3.2	15 ± 6.4	14 ± 2.1	17 ± 2.5	14 ± 1.5	15 ± 2.7
Creatinine (mg dL^{-1})	1.0 ± 0.1	1.0 ± 0.1	1.4 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.3 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.3 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	1.3 ± 0.1	1.5 ± 0.2
T. Bilirubin (mg dL^{-1})	0.5 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	0.1 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.5 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
Glucose (mg dL^{-1})	113 ± 10	121 ± 11	124 ± 5	110 ± 7	120 ± 7	120 ± 6	108 ± 4	108 ± 9	122 ± 5	111 ± 7	120 ± 8	119 ± 6	112 ± 9	125 ± 8	125 ± 15
Cholesterol (mg dL^{-1})	27 ± 38	20 ± 34	32 ± 35	13 ± 29	13 ± 29	45 ± 33	19 ± 30	18 ± 30	31 ± 33	12 ± 25	12 ± 24	19 ± 30	19 ± 31	12 ± 26	32 ± 35
T. Protein (g dL^{-1})	5.7 ± 0.3	5.6 ± 0.3	5.8 ± 0.2	5.7 ± 0.3	5.5 ± 0.2	5.8 ± 0.1	5.6 ± 0.2	5.7 ± 0.3	5.7 ± 0.2	5.6 ± 0.2	5.5 ± 0.3	5.6 ± 0.2	5.7 ± 0.2	5.4 ± 0.2	5.6 ± 0.3
Albumin (g dL^{-1})	3.6 ± 0.1	3.4 ± 0.2	3.4 ± 0.2	3.5 ± 0.3	3.3 ± 0.2	3.4 ± 0.1	3.4 ± 0.1	3.5 ± 0.2	3.3 ± 0.2	3.6 ± 0.2	3.5 ± 0.3	3.3 ± 0.1	3.5 ± 0.2	3.4 ± 0.2	3.3 ± 0.2
Globulin (g dL^{-1})	2.1 ± 0.1	2.1 ± 0.1	2.4 ± 0.1	2.1 ± 0.2	2.1 ± 0.2	2.4 ± 0.1	2.2 ± 0.2	2.2 ± 0.2	2.4 ± 0.1	2.0 ± 0.1	2.0 ± 0.1	2.3 ± 0.1	2.1 ± 0.2	2.0 ± 0.2	2.3 ± 0.1
A/G Ratio	1.7 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	1.7 ± 0.2	1.5 ± 0.1	1.4 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	1.4 ± 0.1	1.8 ± 0.2	1.8 ± 0.2	1.5 ± 0.1	1.6 ± 0.2	1.7 ± 0.2	1.4 ± 0.1
Creatine kinase (IU L^{-1})	295 ± 83	462 ± 231	254 ± 85	395 ± 140	495 ± 301	254 ± 129	333 ± 79	436 ± 91	241 ± 34	350 ± 117	901 ± 1400	322 ± 174	487 ± 202	505 ± 213	250 ± 154
AST (IU L^{-1})	13 ± 2.4	13 ± 2.5	15 ± 2.6	13 ± 3.6	13 ± 5.2	14 ± 3.8	13 ± 3.7	14 ± 3.5	17 ± 5.7	12 ± 3.1	13 ± 2.5	15 ± 2.9	14 ± 3.6	14 ± 2.8	14 ± 4.6
ALT (IU L^{-1})	46 ± 9.5	49 ± 10	45 ± 11	43 ± 13	45 ± 17	44 ± 9.7	44 ± 13	50 ± 16	50 ± 12	43 ± 7.7	52 ± 12	44 ± 9.0	40 ± 7.6	46 ± 8	40 ± 15
WBC (10^3 mm^3)	9.1 ± 2.2	8.9 ± 1.4	8.8 ± 1.5	9.3 ± 2.2	9.5 ± 2.8	9.6 ± 2.1	8.1 ± 1.4	8.0 ± 1.3	7.5 ± 1.1	10 ± 1.7	8.4 ± 1.6	9.9 ± 1.9	10 ± 2.3	8.2 ± 1.5	8.1 ± 1.7
Neutrophils (%)	33 ± 8.3	33 ± 8.3	39 ± 9.0	34 ± 13	34 ± 13	38 ± 13	29 ± 7.8	29 ± 7.8	36 ± 5.1	27 ± 8.6	27 ± 8.6	30 ± 11	27 ± 5.8	35 ± 10	23 ± 5.1
Lymphocytes (%)	57 ± 9.1	60 ± 5.6	57 ± 8.6	58 ± 12	56 ± 8.9	56 ± 13	60 ± 11	43 ± 17	57 ± 5.1	64 ± 9.7	51 ± 17	64 ± 11	64 ± 7.6	58 ± 10	71 ± 5.9
Monocytes (%)	3.0 ± 1.6	1.2 ± 1.0	1.2 ± 0.8	2.4 ± 1.6	1.2 ± 1.4	3.1 ± 1.8	4.5 ± 3.1	1.3 ± 1.2	2.8 ± 1.7	1.9 ± 1.7	2.2 ± 1.6	2.0 ± 1.3	2.3 ± 1.3	1.2 ± 1.1	2.2 ± 0.8
Eosinophils (%)	1.1 ± 1.7	1.2 ± 1.7	0.3 ± 0.5	0.9 ± 1.2	0.9 ± 1.2	0.4 ± 0.5	0.5 ± 1.1	0.0 ± 0.0	0.6 ± 1.1	1.4 ± 1.6	0.7 ± 1.3	1.3 ± 0.9	1.4 ± 2.5	1.3 ± 1.3	1.6 ± 1.5
Basophils (%)	5.9 ± 3.0	5.2 ± 2.5	2.3 ± 2.2	4.1 ± 2.5	4.9 ± 2.4	2.1 ± 1.8	5.8 ± 3.5	1.6 ± 1.2	3.9 ± 1.7	5.4 ± 3.1	2.5 ± 2.6	3.2 ± 2.3	4.7 ± 3.9	5.0 ± 3.4	2.7 ± 2.1
Reticulocytes (%)	1.2 ± 0.4	1.8 ± 0.6	1.7 ± 0.3	1.2 ± 0.4	1.6 ± 1.0	1.7 ± 0.2	1.3 ± 0.3	2.0 ± 0.5	1.5 ± 0.3	1.3 ± 0.4	1.7 ± 0.4	1.7 ± 0.3	1.0 ± 0.4	1.8 ± 0.5	1.7 ± 0.3
RBC (10^6 mm^3)	6.6 ± 0.5	6.2 ± 0.5	6.3 ± 0.4	6.8 ± 0.3	6.2 ± 0.2	6.5 ± 0.3	6.6 ± 0.3	6.5 ± 0.5	6.5 ± 0.2	6.7 ± 0.5	6.4 ± 0.3	6.3 ± 0.3	6.8 ± 0.3	6.2 ± 0.4	6.3 ± 0.3
Haematocrit (%)	39 ± 2.0	37 ± 2.3	38 ± 1.9	40 ± 1.3	37 ± 0.9	38 ± 2.4	39 ± 3.0	38 ± 3.2	38 ± 2.1	41 ± 2.4	39 ± 1.2	39 ± 1.9	41 ± 2.0	38 ± 2.0	38 ± 2.0
Platelets (10^3 mm^3)	256 ± 100	250 ± 79	306 ± 77	197 ± 66	284 ± 75	281 ± 100	245 ± 106	301 ± 91	245 ± 86	220 ± 65	258 ± 81	297 ± 70	266 ± 96	291 ± 87	301 ± 75
Prothrombin time (s)	6.7 ± 0.2	6.7 ± 0.2	6.3 ± 0.2	6.7 ± 0.2	6.6 ± 0.2	6.3 ± 0.3	6.9 ± 0.4	6.8 ± 0.2	6.4 ± 0.2	6.8 ± 0.2	6.5 ± 0.2	6.3 ± 0.1	6.7 ± 0.2	6.4 ± 0.4	6.3 ± 0.2
APTT (s)	67 ± 15	67 ± 15	79 ± 16	67 ± 16	71 ± 13	81 ± 12	60 ± 15	73 ± 9.3	81 ± 19	67 ± 15	77 ± 17	60 ± 23	67 ± 12	73 ± 11	73 ± 7

Values are mean ± s.d.; BUN, blood urea nitrogen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; WBC, white blood cells; RBC, red blood cells; APTT, activated partial thromboplastin time.

The randomization block was included as a random effect for all analysis. If a significant term was identified, unadjusted contrasts were performed to locate those groups that differed statistically from the vehicle control (group 1), within a gender and/or time point for significant interaction terms. If, for any parameter, group 3, 4 or 5 were significantly different from group 1, a comparison of these groups to group 2 (negative control group) was performed to determine the potential role of adjuvant to any effects. All statistical comparisons were performed at the 0.05 significance level. SAS version 6.12 (SAS Institute, Cary, NC) was used to perform all analysis.

Results

Biodistribution studies in mice

Intramuscular administration

At 24 h after drug administration, the injected muscle was the only tissue containing quantifiable levels of plasmid DNA (mean, 1.1 pg plasmid DNA/μg total DNA). This applied to all six mice that received the drug formulation. Plasmid DNA was detected in the non-injected muscle, blood, lung, and brain of a few animals, though the levels were below the LOQ (< 1000 copies/μg) and some discordant replicates were observed. No plasmid DNA was detected in the gonads, liver, spleen, heart, or kidney. At four weeks after drug administration, the injected muscle was again the only tissue that contained quantifiable levels of plasmid DNA (mean, 0.02 pg plasmid/μg total DNA), an approximately 50-times decrease from day 1. Again, this applied to all six mice that received the drug formulation. No plasmid was detected in the gonads, brain, kidney, heart, lung, blood, or non-injected muscle. One female liver and one male spleen contained detectable levels of plasmid DNA, but discordant replicates were observed in both cases. Of 120 tissue samples from the control group, 117 were negative for hDel-1 plasmid DNA. The remaining tissue samples were positive only at the level of detection and exhibited discordant replicates.

Intravenous administration

The results of the intravenous biodistribution study were similar to those of the intramuscular biodistribution study. Twenty-four hours after drug administration, in the mice that received the low dose (0.2 mg kg^{-1}) plasmid DNA was detected at the injection site (tail) in all cases; at levels below the LOQ in the blood (5/6), muscle (3/6), lung (1/6), spleen (1/6), heart (1/6), and liver (1/6); and not at all in the gonads, kidneys, or brain. In the mice that received the high dose (0.8 mg kg^{-1}), plasmid DNA was detected at quantifiable levels at the injection site in all cases and at levels below the LOQ in the blood (6/6), lungs (5/6), liver (2/6), spleen (2/6), heart (1/6), gonads (1/6), and brain (1/6).

One week after drug administration, in the low-dose group, plasmid DNA was detected at quantifiable levels at the injection site in all cases and at levels below the LOQ in five of six mice and in the blood of only one mouse. In the high-dose group, plasmid DNA was detected at quantifiable levels at the injection site in all cases; at levels below the LOQ in muscle (2/6) and blood (3/6); and not at all in the lung, liver, spleen, heart, kidney, brain, and gonads.

Four weeks after drug administration, those tissues that had contained detectable levels of hDel-1 plasmid DNA at day 8 (i.e. the tail (injection site), blood, and muscle) were analysed again by PCR. In the low-dose group, plasmid DNA was detected at levels below the LOQ in tissue from the injection site (2/6, both males). In the high-dose group, plasmid DNA was detected at levels below the LOQ in tissue from the injection site (5/6), blood (3/6), and muscle (1/6).

Of the 138 tissue samples tested, 125 were negative for hDel-1 plasmid DNA. The remaining samples contained plasmid DNA at levels below the LOQ and exhibited discordant replicates.

Safety studies in rabbits

Intramuscular administration

Intramuscular drug administration to rabbits at doses up to 18 mL (18 mg hDel-1 plasmid DNA) caused no deaths, caused no clinical signs of toxicity, or changes in body weight. No appreciable adverse effects were seen on electrocardiographic and ophthalmic examination. Clinical pathologic evaluations showed that there were no demonstrable differences between rabbits killed on day 8 compared with day 29. Antinuclear antibody analysis was negative for all rabbits, and no treatment-related histopathological changes in tissues were noted at necropsy.

Intravenous administration

Intravenous drug administration to rabbits at doses up to 14 mL (14 mg hDel-1 plasmid DNA) caused no deaths, caused no clinical signs of toxicity, or appreciable changes in body weight. Differences in mean values were seen in the results of clinical chemistry, haematology, coagulation, or urinalysis during the study. However, since these differences were not observed consistently at any dose or at any time point, they were considered incidental and not directly related to the administration of drug or vehicle (control). Antinuclear antibody analysis was negative. No gross lesions related to drug or vehicle (control) administration, no differences in organ weights, and no histopathological changes were observed at necropsy in rabbits killed at day 29.

Discussion

Administration of the hDel-1 plasmid-based gene medicine VLTS-589 by either the intramuscular or intravenous route was well tolerated by mice and rabbits. No clinical or pathological signs of adverse effects and no immunological responses were observed in the studies.

In mice, a single intramuscular administration of VLTS-589 at a dose of up to approximately 1.25 mg kg^{-1} resulted in persistent expression of the plasmid DNA in the injected muscle for at least 28 days. However, the mean level of plasmid/μg total DNA at 28 days was approximately 50-times less than the mean at 24 h after administration. Some distal tissues contained trace amounts of plasmid DNA, but most of these exhibited discordant replicates on PCR analysis. Intravenous administration of the drug in mice yielded similar results: plasmid DNA levels at the injection site were quantifiable at one week but were unquantifiable at four weeks after administration. Together, these results suggested a localized effect of hDel-1, where the protein could be detected at a higher level at the site of administration but not in distal tissues at levels that would suggest an unexpected effect. These findings were consistent with those of Howell et al (2000), who found that Del-1 transgene expression was limited to tissues near the site of intramuscular injection in rabbits.

In the clinic, drugs that are supposed to be delivered intramuscularly are sometimes inadvertently delivered intravenously. Therefore, in this experiment, the intravenous route was used to study the possible adverse effects of just such a scenario involving the hDel-1 plasmid drug formulation. Rabbits appeared to tolerate well doses of 4.2 mg kg^{-1} intramuscularly and 3.56 mg kg^{-1} intravenously. There were several statistically significant differences between groups in terms of mean serum chemistry and haematology values, but these differences were considered to be incidental and unrelated to drug administration. All other measured parameters were considered normal, including those measured by ophthalmic examination, electrocardiography, urinalysis, and antinuclear antibody testing. Together, the findings suggested that any potential side effects of transgene-expressed hDel-1 protein were likely to be minimized by the local expression of the transgene at the site of administration. In addition, since the doses administered to rabbits were significantly higher than the highest dose expected to be tested in human clinical trials (e.g. the intramuscular administration of 4.2 mg kg^{-1} was approximately 100-times higher than the starting dose and 3.5-times higher than the highest dose proposed for phase I clinical trials), the findings suggested that phase I clinical trials may be safely undertaken.

Conclusion

Biodistribution and safety studies in mice and rabbits indicated that VLTS-589 administered by the intramuscular or intravenous route at evaluated doses was not associated with adverse effects. The observed changes in clinical chemistry and haematology were considered to be incidental and not directly related to the administration of VLTS-589. Thus, no significant deleterious consequences are expected to occur in man. This paves the way for FDA-approved phase I clinical trials of VLTS-589 in patients with peripheral arterial disease.

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