LP1-mPAH) has been described previously [9]. 1×10^{11} vector genomes of the recombinant AAV were dissolved in 0.5 ml of saline and injected into the peritoneal cavity of a PKU mouse at 8 weeks of age.

Brain sampling and biochemical analysis

Mice were killed by cervical dislocation, and the removed brain was immediately frozen in liquid nitrogen and stored at −80°C until used. The brain was homogenized in 10 volumes of 0.2 M of perchloric acid containing 0.1 mM of EDTA for deproteination. Protein concentrations were determined using a DC protein assay kit (Bio-Rad, Hercules, California, USA). Catecholamine and 5-HT levels were measured by high-performance liquid chromatography using an electrochemical detector ECD-100 (EICOM, Kyoto, Japan) as described elsewhere [12]. Amino acid levels were analyzed using an L-8500 amino acid analyzer (Hitachi, Tokyo, Japan). Data are presented as means ± SDs in the text and figures. An unpaired *t*-test was performed using the StatView 5.0 software for Macintosh (SAS Institute, Cary, North Carolina, USA) for comparison between two groups, and a P value of less than 0.05 was considered to be significant.

Results

Phenotypic correction after gene transfer

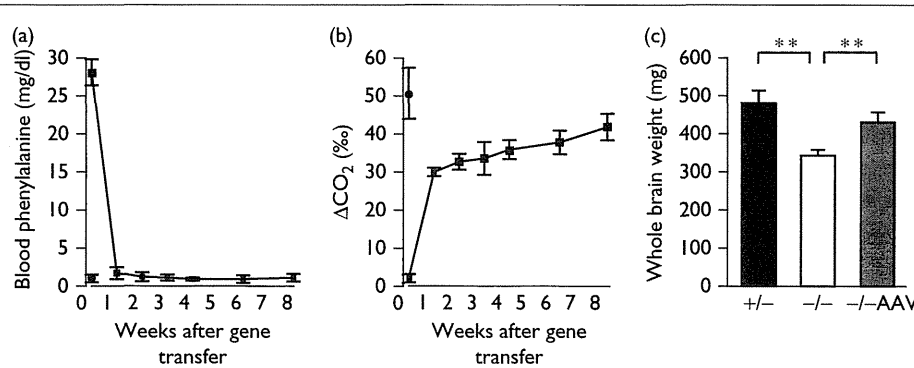
As shown in the original BTBR-*Pah*^{enu2} mice [9], the scAAV8/LP1-mPAH vector exhibited remarkable efficacy in restoring phenylalanine catabolism in C57BL/6-*Pah*^{enu2} mice. Before the gene transfer, PKU mice had elevated blood phenylalanine levels (28.1 ± 1.7 mg/dl; $n = 3$) compared with their heterozygous (+/−) littermates (0.3 ± 0.2 mg/dl; $n = 6$), whereas blood phenylalanine levels in heterozygous mice were indistinguishable from those in wild-type homozygous (WT, +/+) mice (0.3 ± 0.1 mg/dl; $n = 6$). After a single injection of the AAV vector to PKU mice, the blood phenylalanine concentration rapidly decreased to a near-normal level in 1 week (1.7 ± 0.8 mg/dl) and remained within the normal range from weeks 2 to 8 (Fig. 1a). In parallel, we evaluated phenylalanine-oxidizing capacity by conducting a ^{13}C-phenylalanine-loading breath test (Fig. 1b). In this assay, the production of $^{13}CO_2$ (ΔCO_2) is associated with PAH activity, although we were not able to distinguish heterozygous mice (50.6 ± 6.7‰; $n = 6$) from WT mice (52.6 ± 10.5‰; $n = 6$), presumably due to other limiting factors such as phenylalanine transport and cofactor availability *in vivo*. Before the gene transfer, PKU mice produced very little, if any, ΔCO_2 (2.0 ± 1.1‰; $n = 3$). One week post-AAV injection, ΔCO_2 was increased to 2/3 of the control level (29.9 ± 1.1‰) and the value gradually increased to a near-normal level (41.8 ± 3.5‰ at week 8).

The AAV-treated PKU mice ($n = 3$) were euthanized at week 8 after injection along with heterozygous littermates ($n = 6$) and age-matched, untreated PKU mice ($n = 4$) for further analysis. First, we measured the whole brain weight of these animals (Fig. 1c). As reported [13], the weight of the brain in untreated PKU mice was significantly decreased compared with the control level (343 ± 15 vs. 481 ± 33 mg; $P = 0.00005$). In contrast, the brains of AAV-treated PKU animals regained weight significantly (431 ± 26 mg; $P = 0.0023$ vs. untreated PKU), reaching a level comparable level to that in heterozygous mice ($P = 0.55$).

Amino acid analysis

In the amino acid analysis, we confirmed that the untreated PKU mice had a marked imbalance of phenylalanine and tyrosine in the brain [13] (Fig. 2a). The phenylalanine content was nearly 10 times that of heterozygous mice (6.24 ± 0.81 vs. 0.66 ± 0.08 nmol/mg protein; $P = 0.0008$), whereas the tyrosine content was lower (0.31 ± 0.08 vs. 0.77 ± 0.09 nmol/mg protein; $P = 0.0005$). In the AAV-treated PKU mice, the amount of phenylalanine in the

Fig. 1



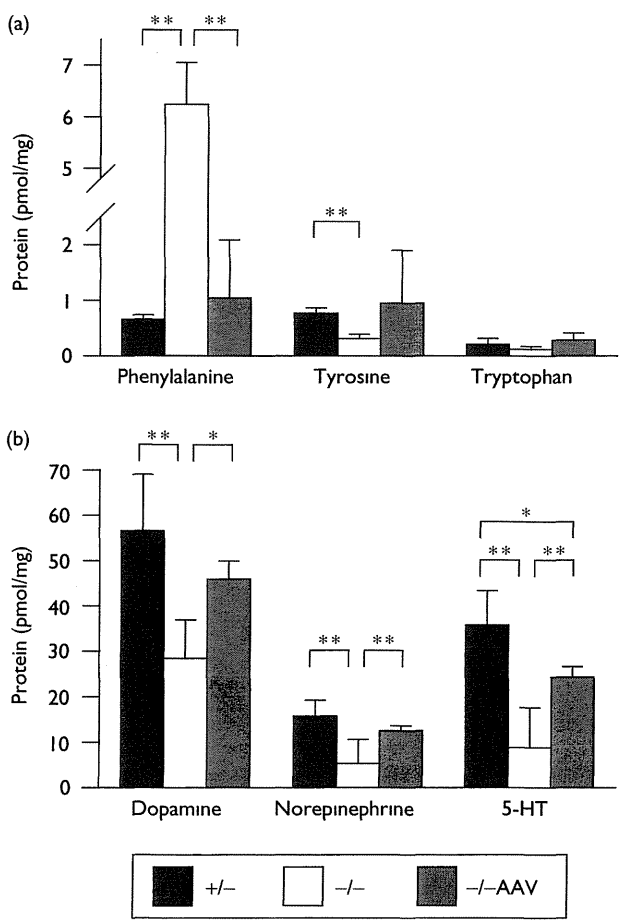
Phenotypic correction in phenylketonuria (PKU) mice after gene transfer. (a) Blood phenylalanine levels in adeno-associated virus (AAV)-treated PKU mice (squares) and heterozygous controls (circle). (b) $^{13}CO_2$ production (ΔCO_2) by AAV-treated PKU mice (squares) and heterozygous controls (circle) in a [1-^{13}C]L-phenylalanine-loading test. (c) Whole brain weights of heterozygous control (+/−), untreated PKU (−/−), and AAV-treated PKU (−/−AAV) mice. Data are shown as the mean ± SD. $^{**}P < 0.01$.

brain was decreased (1.04 ± 0.50 nmol/mg of protein; $P = 0.001$ vs. untreated PKU) in accordance with that in blood. The treated mice also had increased levels of tyrosine, but the elevation was not significant because one animal had a supranormal tyrosine content (1.55 nmol/mg of protein) that resulted in a relatively large SD for this group. As for tryptophan, the untreated PKU mice had a lower average level (0.12 ± 0.05 nmol/mg protein) than the heterozygous and AAV-treated mice (0.21 ± 0.11 and 0.28 ± 0.13 nmol/mg of protein, respectively), but the difference was not significant as reported previously [7].

Monoamine neurotransmitters and metabolites

The levels of catecholamines, serotonin, and metabolites are summarized in Table 1. We confirmed that the amounts of dopamine, norepinephrine, and 5-HT in the untreated PKU mice were significantly decreased compared with those in the heterozygous controls ($P = 0.004$, 0.0005, and 0.00008, respectively) [6,7]. Eight weeks after gene transfer, such aminergic deficits were markedly ameliorated ($P = 0.022$, 0.0007, and 0.0004 vs. untreated PKU, respectively; Fig. 2b). Accordingly, the levels of some catecholamine metabolites increased in the AAV-treated mice. In PKU mice, 3-4-dihydroxyphenylacetic acid decreased significantly compared with the heterozygous controls, and it recovered partly after gene transfer. 3-methoxytyramine (3-MT) content in PKU mice was not significantly lower than that in the heterozygous controls, but it may have actually been lower compared with WT homozygotes [6]. Otherwise, a compensatory dopamine release to the synapse may take place in PKU mice, resulting in a relatively small 3-MT decrease. As shown in Table 1, we found a significant increase in 3-MT after gene therapy, presumably due to an improved dopamine synthesis. In contrast, homovanillic acid did not increase after gene transfer ($P = 0.749$ vs. PKU; $P = 0.023$ vs. heterozygous). Overall, we assumed that catecholamine synthesis in the AAV-treated mice was restored to approximately 80–90% of the level in heterozygous mice. Similarly, the levels of serotonin and its metabolite 5-hydroxyindoleacetic acid recovered to 60–70% of those in heterozygous mice ($P = 0.039$ for 5-HT and $P = 0.092$ for 5-hydroxyindoleacetic acid).

Fig. 2



Aromatic amino acids and neurogenic amines in the phenylketonuria (PKU) mouse brain. (a) Phenylalanine, tyrosine, and tryptophan levels in the brain of heterozygous control (+/−), untreated PKU (−/−), and adeno-associated virus (AAV)-treated PKU (−/−AAV) mice. (b) Dopamine, norepinephrine, and serotonin (5-HT) levels in the brain of heterozygous control (+/−), untreated PKU (−/−), and AAV-treated PKU (−/−AAV) mice. Data are shown as the mean ± SD. *$P<0.05$, **$P<0.01$.

Discussion

The present study showed an overt reversal of the aminergic deficit in PKU mouse brain after liver-targeted gene therapy. In untreated PKU mice, HPA may disturb monoamine synthesis through at least two mechanisms. One is that excess phenylalanine may hamper the neuronal uptake of tyrosine (dopamine and norepinephrine precursor) and tryptophan (5-HT precursor) through competition for transport across the blood–brain barrier by the L-type amino acid carrier [14,15]. The other is that a high concentration of phenylalanine interferes with tyrosine hydroxylase and tryptophan hydroxylase [16,17]. For the catecholamine pathway, we observed a significant decrease in the amount of tyrosine in PKU mice, which may play some role in the dopamine and norepinephrine deficit. However, Joseph and Dyer [18] reported an increase in dopamine despite low tyrosine levels in PKU mice on a low-phenylalanine diet, which may suggest that HPA causes a lack of catecholamine primarily by inhibiting the hydroxylation of tyrosine. As for the serotonin pathway, we found a limited decrease in tryptophan in the PKU mouse brain. Pascucci *et al.* [7] found a similar decrease in tryptophan and observed a significant decrease in 5-hydroxytryptophan in the brain of PKU mice. Therefore, they speculated that HPA impedes 5-HT synthesis mainly by inhibiting tryptophan hydroxylation, which is the rate-limiting step in this pathway. We previously showed that phenylalanine acted as an inhibitor more strongly against tryptophan hydroxylase than against tyrosine hydroxylase [17], further

Table 1 Dopamine, norepinephrine, 5-hydroxytryptamine, and metabolites (pmol/mg protein) in adeno-associated virus-treated *Pah*$^{enu2/enu2}$ and control mouse brain

	Pah$^{enu2/+}$ ($n=6$)	*Pah*$^{enu2/enu2}$ ($n=4$)	*Pah*$^{enu2/enu2}$ + adeno-associated virus ($n=3$)
Dopamine	56.6 ± 12.4[a]	28.4 ± 8.5	45.9 ± 4.0[a]
3-Methoxytyramine	11.9 ± 4.6	9.9 ± 2.1	13.7 ± 1.2[b]
3-4-Dihydroxyphenylacetic acid	10.4 ± 2.2[a]	4.7 ± 1.2	7.9 ± 1.6[b]
Homovanillic acid	41.9 ± 12.1[b]	25.1 ± 5.1	26.2 ± 2.7[c]
Norepinephrine	15.7 ± 3.5[a]	5.3 ± 1.4	12.5 ± 1.1[a]
5-Hydroxytryptamine	35.8 ± 7.5[a]	8.8 ± 2.5	24.3 ± 2.3[a,c]
5-Hydroxyindoleacetic acid	24.3 ± 7.9[a]	5.3 ± 1.5	15.0 ± 2.0[a]

Values are represented as mean ± SD.
[a] $P<0.01$ vs. *Pah*$^{enu2/enu2}$
[b] $P<0.05$ vs. *Pah*$^{enu2/enu2}$
[c] $P<0.05$ vs. *Pah*$^{enu2/+}$

supporting their speculation. By either mechanism, correction of HPA would reset amine metabolism and thereby improve the relevant brain function, as we demonstrated here and previously [8].

Untreated PKU patients have smaller brains, and the primary pathologic finding is hypomyelination and gliosis of central nervous system white matter. A similar pathologic change is observed in *Pah*^{enu2} mice, which may result from aberrant glial cell differentiation induced by HPA [19]. It has also been documented that cerebral protein synthesis is decreased in PKU mice, which presumably contributes to the underdevelopment and degeneration of the PKU brain [13]. We observed a marked recovery in brain weight in the PKU mice only 8 weeks after gene transfer. Correction of HPA may facilitate protein synthesis and reset glial cell plasticity to reconstitute myelin. In addition, it may reduce oxidative stress and induce neuronal regeneration as shown by Embury *et al.* [20].

The results demonstrate that liver-targeted gene therapy for PKU would restore the structural and biochemical fitness of the brain. Current gene transfer technology has achieved a partial reconstitution of coagulation factor IX in the human liver to ameliorate hemophilia B [21]. Further development should lead to broader applications of this modality including PKU. Preventing HPA without a restrictive diet would make it easier to meet nutritional requirements for the physical and neuronal development of patients as well as to maintain sociopsychological well-being.

Conclusion

Liver-targeted gene therapy for PKU reverses the aminergic deficit in the brain and improves the neuropsychological function.

Acknowledgements

The authors thank Mr. Sho Shimaguchi (Tokyo Institute of Technology) for assisting with brain amine analysis and Prof. Kazunao Kondo (Fujita Health University) for assisting with amino acid analysis. They also thank Prof. Hiroyuki Yoshikawa and Dr. Hiromi Hamada (University of Tsukuba) for enthusiastic support and thoughtful discussion. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (20591230).

Conflicts of interest

There are no conflicts of interest.

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Haemophilia

Haemophilia (2012), 1–8 DOI: 10.1111/hae.12024

ORIGINAL ARTICLE

Results of clot waveform analysis and thrombin generation test for a plasma-derived factor VIIa and X mixture (MC710) in haemophilia patients with inhibitors—phase I trial: 2nd report

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Summary. We reported the results of a clinical pharmacological study of MC710 (a mixture of plasma-derived FVIIa and FX) in haemophilia patients with inhibitors during a non-haemorrhagic state. This report provides the results of a clot waveform analysis (CWA) and thrombin generation test (TGT) using blood samples obtained in this study. CWA and TGT were conducted using blood samples obtained from a pharmacokinetic and pharmacodynamic study in which MC710 (five dose rates: 20, 40, 80, 100 and 120 μg kg^{-1}) was compared with NovoSeven (120 μg kg^{-1}) and FEIBA (two dose rates: 50 and 75 U kg^{-1}) as control drugs in 11 haemophilia patients with inhibitors without haemorrhagic symptoms. CWA showed that MC710 provided significantly greater improvement than the control drugs in activated partial thromboplastin time (APTT) at 80 μg kg^{-1}; maximum clot velocity and maximum clot acceleration were more enhanced by MC710 than by control drugs. TGT revealed that MC710 significantly shortened the initiation time of thrombin generation in comparison to FEIBA and induced greater thrombin generation potency than NovoSeven. It was not clear whether or not MC710 caused significant dose-dependent changes in the two measurements; however, differences between MC710 and the control drugs were clarified. MC710 was confirmed to have superior coagulation activity and thrombin productivity and is expected to have superior bypassing activity.

Keywords: bypassing agent, clot waveform analysis, factor VIIa, factor X, haemophiliacs with inhibitors, thrombin generation test

Introduction

Bleeding events in haemophilia patients with inhibitors (particularly patients with high titres and high-responder types) are controlled predominantly with haemostatic bypassing agents, recombinant activated factor VII (rFVIIa; NovoSeven, Novo Nordisk A/S,

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Accepted after revision 15 August 2012

Bagsværd, Denmark) and activated prothrombin complex concentrate (APCC; FEIBA, Baxter International Inc., Deerfield, IL, USA), and sequential therapy with both agents [1]. However, these agents do not always achieve haemostasis; therefore, the development of a new agent or method of administration for more effective haemostasis is required. MC710 is a novel bypassing agent containing activated factor VII (FVIIa) and factor X (FX) highly purified from human plasma by affinity chromatography using anti-FVII and anti-FX monoclonal antibodies and mixed at a protein weight ratio of 1:10 [2]. MC710 is designed to administer FVIIa and its substrate FX concomitantly. Thus, MC710 is expected to be more potent than agents containing FVIIa alone, and longer acting due to the long half-life of FX [3].

We conducted a clinical pharmacological study (Phase I trial) of MC710 in haemophilia patients with inhibitors to evaluate its effects on pharmacokinetic (PK) and pharmacodynamic (PD) parameters and safety [4]. A clot waveform analysis (CWA) and thrombin generation test (TGT) were conducted in patients treated with MC710, NovoSeven and FEIBA in addition to measurements of conventional activated partial thromboplastin time (APTT) and prothrombin time (PT). CWA provides a chronological coagulation profile through real-time monitoring of changes in turbidity during coagulation and, in addition to measuring clotting time, calculates maximum clot velocity and maximum clot acceleration after the initiation of coagulation by first and second differentiation [5]. TGT monitors real-time changes in fluorescence intensity that chronologically increases during the thrombin generation process and quantitatively analyses thrombin production using parameters obtained in a thrombogram [6].

This report provides an analysis of the dose dependency of CWA and TGT in plasma samples obtained from a clinical pharmacological study of MC710 given as single doses at five dose rates in haemophilia patients with inhibitors. The changes in parameters were compared with those generated by control drugs (NovoSeven and FEIBA).

Materials and methods

Study design and drugs

This study was designed as a multi-centre, open-labelled, non-randomized, active controlled crossover clinical pharmacological study for Japanese male haemophilia patients with inhibitors. MC710 was administered intravenously as a single dose (20, 40, 80, 100, or 120 $\mu g\ kg^{-1}$) to male congenital haemophilia patients with inhibitors, but without haemorrhage with re-administration at different dose rates allowed. All subjects provided written informed consent as approved by the institutional review board of each participating institute. Prior to the administration of MC710, a single clinical dose of NovoSeven and/or FEIBA was administered intravenously as a control. The dose of NovoSeven was set at 120 $\mu g\ kg^{-1}$, whereas the FEIBA dose was set at the usual clinical dose for each patient, 50 or 75 $U\ kg^{-1}$ [4].

Blood samples were drawn using evacuated blood collection tubes containing sodium citrate preadministration of the agents and 10 min, 2, 6, 12 and 24 h postadministration of MC710, 10 min, 2 and 6 h postadministration of NovoSeven and 10 min, 2, 6 and 24 h postadministration of FEIBA.

MC710, NovoSeven and FEIBA were provided by KAKETSUKEN (Kumamoto, Japan), Novo Nordisk A/S and Baxter International Inc. respectively. Normal human plasma (NHP; FACR, Georgeking, KS, USA) was used as a control.

Clot waveform analysis

Clot waveform analysis was performed by the standard method for determining APPT using a MDA® II automated coagulation analyser (bioMérieux, Marcy l'Etoile, France) [5]. After 50 μL of APTT reagent (ellagic acid and phospholipid (PL) solution, Baxter International Inc.) and 50 μL of $CaCl_2$ solution (final concentration: 5 mM) were added to 50 μL of patient plasma, the APTT clot waveform was monitored in real-time using an MDA® II. In this study, the maximum clot velocity (|Min1|) and the maximum clot acceleration (|Min2|) defined the minimum value of the first derivative of transmittance change (dT/dt) and the minimum value of the second derivative of transmittance change (d^2T/dt^2) respectively. The assay results were assured by confirming the parameter values of normal, FVIII-depleted, or FIX-depleted plasma in every measurement laid within 20% of the average values prior to the assay in six measurements ×3 days.

Thrombin generation test

Thrombin generation test was conducted as described previously [6]. Twenty microlitres of PPP-Reagent LOW (final concentration: 1 pM tissue factor (TF) and 4 μM PL, Thrombinoscope, Maastricht, Netherlands) and 20 μL of the fluorescent substrate Z-G-G-R-MCA (Calbiochem-Merck KGaA, Darmstadt, Germany) were added to 80 μL of human plasma. The change in fluorescence intensity caused by the release of aminomethylcoumarin hydrolysed by the generated thrombin was monitored in real-time using a Fluoroskan fluorescence plate reader (Thermo Electron Corp., Waltham, MA, USA) and that was calibrated using a thrombin standard reagent (Thrombinoscope BV, Maastricht, Netherlands). The following parameters

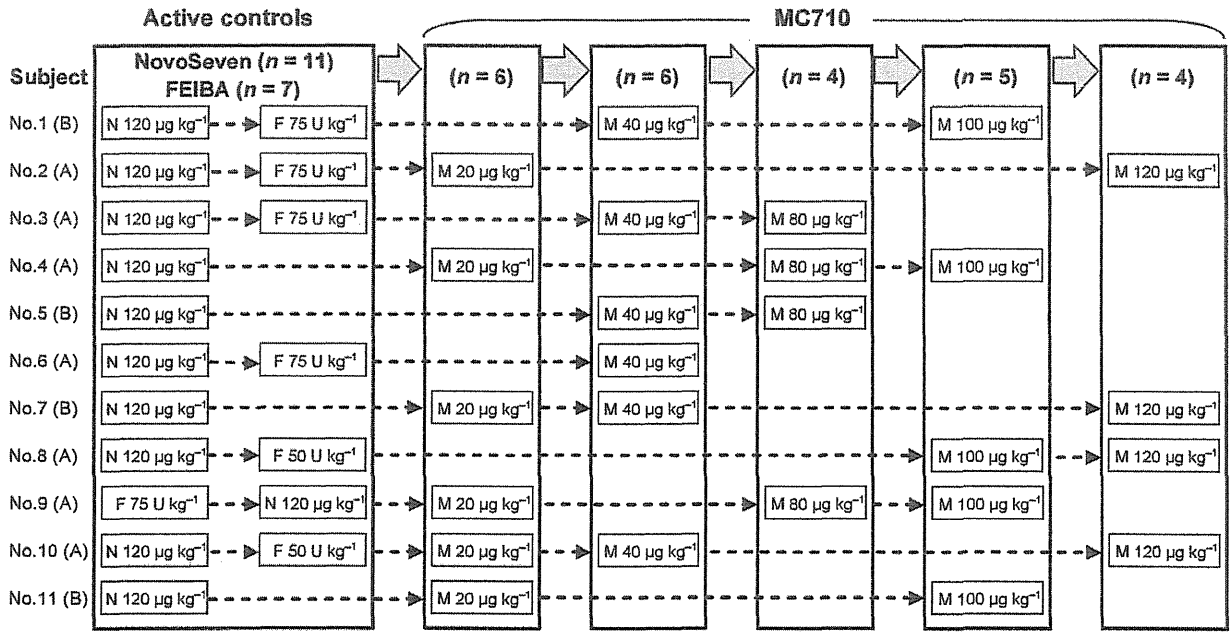

Fig. 1. Study flow chart. Study flow chart of the Phase I trial is shown in the figure. The letter within the parentheses after the subject No. indicates the type of haemophilia: (A) indicates a haemophilia A patient with inhibitors, and (B) indicates a haemophilia B patient with inhibitors. M, N and F mean MC710, NovoSeven and FEIBA respectively. Refer to the text for details.

were calculated from the profile obtained using TGT software (Thrombinoscope BV): (i) time from the beginning of measurement to the initiation of thrombin generation (Lag time); (ii) time until a peak was reached (time to peak: ttPeak); (iii) peak thrombin generation (Peak Th); and (iv) the integrated value of the area under the thrombin generation wave, reflecting the total amount of thrombin generated (endogenous thrombin potential: ETP). The assay results were assured by the same method described for CWA.

Statistical analysis

The MC710 dose-dependency of each parameter was analysed using a mixed-effects model with subject as a random effect and dose (including observation time in the parameter analysis) as a fixed effect. Differences between MC710 and the active control were analysed using a mixed-effects model with subject as a random effect and treatment and observation time as fixed effects. SAS Release 9.1 (SAS Institute Inc., Cary, NC,

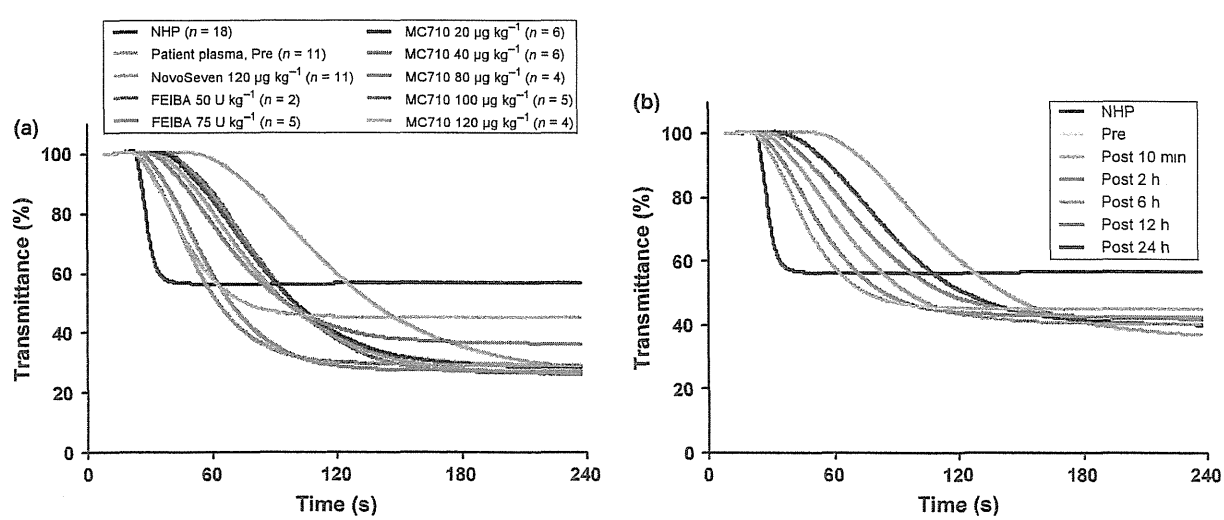

Fig. 2. APTT clot waveform profiles. Changes in mean profiles of APTT clot waveform at 10 min postadministration of control drugs and MC710 at five dose rates (a), and pre and postadministration (10 min, 2, 6, 12 and 24 h) of MC710 at 120 μg kg^{-1} (n = 4) (b) are shown. The black line indicates the mean profile for NHP (18 times repetitive measurement).

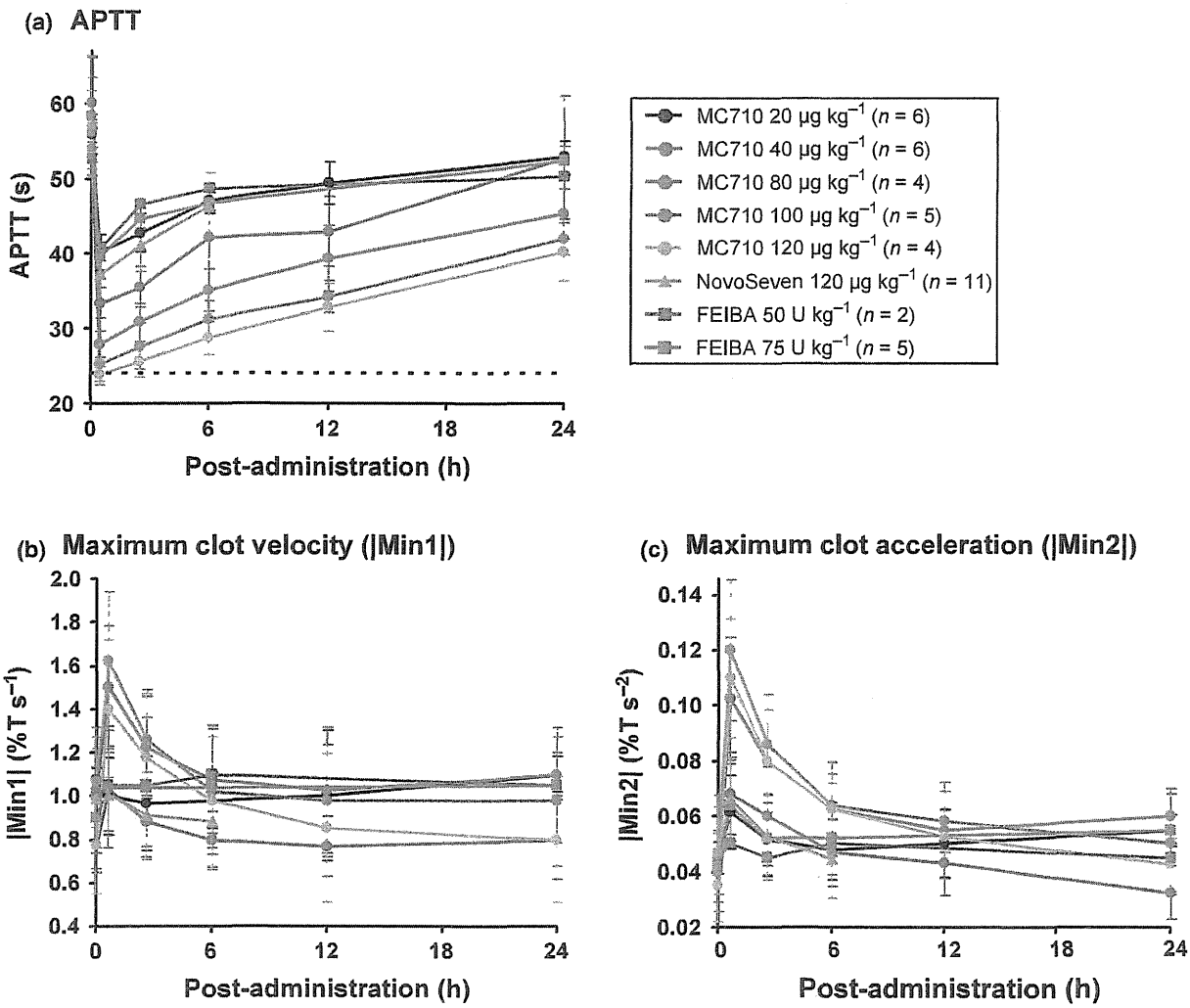

Fig. 3. Changes in CWA parameters. Time-dependent changes in CWA parameters obtained from APTT clot waveform profiles are shown. The assay was conducted using the plasma at pre and postadministration; 10 min, 2, 6, 12 and 24 h for five dose rates of MC710, 10 min, 2 and 6 h for NovoSeven, 10 min, 2, 6 and 24 h for FEIBA. (a) APTT; (b) |Min1|; (c) |Min2| (mean ± SD). The same symbols and lines are used in (a)–(c). Dashed line in (a) indicates the mean value of APTT for NHP (18 times repetitive measurement). Parameter values for NHP (mean ± SD) are APTT; 24.09 ± 0.28 s, |Min1|; 5.15 ± 0.13%T s^{-1} and |Min2|; 0.51 ± 0.01%T s^{-2}. Mean values of |Min1| and |Min2| for NHP are not denoted in (b) and (c).

USA) was used for statistical analyses. All reported *P*-values are two-tailed and not adjusted for multiple testing. $P < 0.05$ were statistically significant.

Results

Outline of the trial

The agents and doses were reported previously [4]. In brief, a total of 25 administrations doses of MC710 were given to 11 subjects (seven haemophilia A patients with inhibitors and four haemophilia B patients with inhibitors) at five dose rates after administration of active controls (Fig. 1). Subjects' mean age was 27.2 years (17–41 years) and the mean body weight was 61.3 kg (46.5–86.2 kg). The FVIII and FIX inhibitor titres immediately before administration of the investigational product ranged from 2.9 to 633 BU mL^{-1} and from 1.9 to 89.3 BU mL^{-1} respectively.

APTT clot waveform analysis

The mean clot waveform profiles for NHP and plasma obtained from patients at 10 min postadministration of each agent are shown in Fig. 2a. Transmittance of the analysis rapidly decreased with the initiation of coagulation and reached a plateau after clot formation. The mean profiles at 10 min postadministration, when the change in the waveform was greatest, showed similar transmittance reductions for 120 μg kg^{-1} NovoSeven, 50 and 75 U kg^{-1} FEIBA

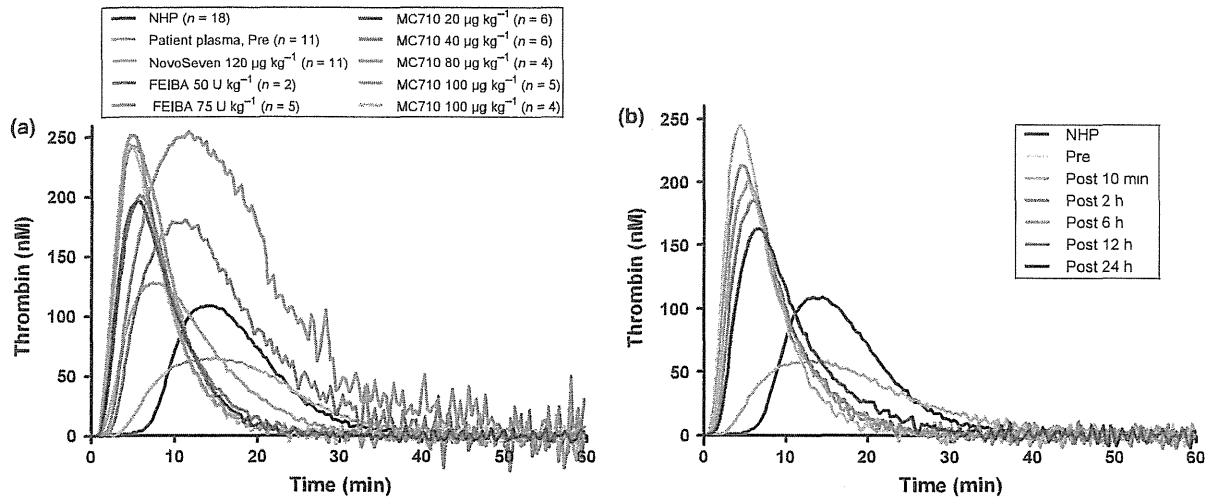

Fig. 4. Thrombin generation profiles. Changes in mean profiles of thrombin generation at 10 min postadministration of control drugs and MC710 at five dose rates (a), and mean profiles of thrombin generation pre and postadministration (10 min, 2, 6, 12 and 24 h) of MC710 at 120 μg kg^{-1} (n = 4) (b) are shown. The black line indicates the mean profile for NHP (18 times repetitive measurement).

and 20 and 40 μg kg^{-1} MC710, whereas the reductions for 80, 100 and 120 μg kg^{-1} MC710 were greater, but of a similar pattern (Fig. 2a). The profile for plasma obtained postadministration of MC710 at 120 μg kg^{-1} was closest to the NHP profile. The mean APTT clot waveform profiles for plasma obtained pre and postadministration of MC710 at 120 μg kg^{-1} showed a time-dependent change, i.e. with the most rapid decrease in transmittance being recorded 10 min postadministration followed by a gradual recovery to the preadministration profile (Fig. 2b).

In the CWA, the effect of improving prolonged APTT was significantly dependent on MC710 dose ($P < 0.001$) (Fig. 3a), consistent with previous results [4]. The effect of MC710 on APTT at doses of 80, 100 and 120 μg kg^{-1} was significantly greater than that of NovoSeven at 120 μg kg^{-1} and FEIBA at 75 U kg^{-1} (MC710 against NovoSeven: $P = 0.010$ at 80 μg kg^{-1}, $P < 0.001$ at 100 μg kg^{-1}, $P < 0.001$ at 120 μg kg^{-1}; MC710 against FEIBA: $P = 0.049$ at 80 μg kg^{-1}, $P = 0.022$ at 100 μg kg^{-1}, $P = 0.008$ at 120 μg kg^{-1}).

Both |Min1| and |Min2| were greatest 10 min postadministration of MC710 and had almost returned to preadministration levels after 12 h. The increases were not dose-dependent, but greater at ≥ 80 μg kg^{-1} MC710 than at 120 μg kg^{-1} NovoSeven or 50 or 75 U kg^{-1} FEIBA (Fig. 3b and c).

Thrombin generation test

The mean profiles of thrombin generation for NHP and for plasma obtained from patients administered each agent are shown in Fig. 4a. At 10 min postadministration, when the change in thrombin generation was greatest, there was one pattern for plasma collected postadministration of MC710 at 20 and 40 μg kg^{-1}, and another pattern for plasma collected postadministration of MC710 at 80, 100 and 120 μg kg^{-1}. In comparison with the controls, Peak Th levels were higher at all doses of MC710 than for NovoSeven at 120 μg kg^{-1} and ttPeaks were shorter at all doses of MC710 than with FEIBA at 50 or 75 U kg^{-1} (Fig. 4a). Compared to the mean thrombin generation profile preadministration, MC710 at 120 μg kg^{-1} produced the shortest time to peak generation and the highest peak. The mean profiles showed a time-dependent change, i.e. a gradual return to the preadministration profile; however, a higher peak than that obtained for NHP was maintained for up to 24 h postadministration (Fig. 4b).

In the TGT, reductions in Lag time and ttPeak were greatest 10 min postadministration and both parameters slowly returned to preadministration values (Fig. 5a and b). Similarly, enhancement of Peak Th and EPT was greatest at 10 min; the values gradually returned to preadministration levels (Fig. 5c and d). The changes in these parameters did not significantly depend on MC710 dose.

The effect of MC710 on Lag time and ttPeak was significantly greater than that of FEIBA at 75 U kg^{-1} (Lag time: MC710 against FEIBA: $P = 0.004$ at 20 μg kg^{-1}, $P = 0.017$ at 40 μg kg^{-1}, $P = 0.008$ at 80 μg kg^{-1}, $P = 0.014$ at 100 μg kg^{-1}, $P = 0.001$ at 120 μg kg^{-1}; ttPeak: MC710 against FEIBA: $P = 0.001$ at 20 μg kg^{-1}, $P = 0.001$ at 40 μg kg^{-1}, $P = 0.052$ at 80 μg kg^{-1}, $P = 0.013$ at 100 μg kg^{-1}, $P = 0.055$ at 120 μg kg^{-1}). The increase in Peak Th was significantly greater post MC710 administration than after NovoSeven at 120 μg kg^{-1} (MC710 against NovoSeven:

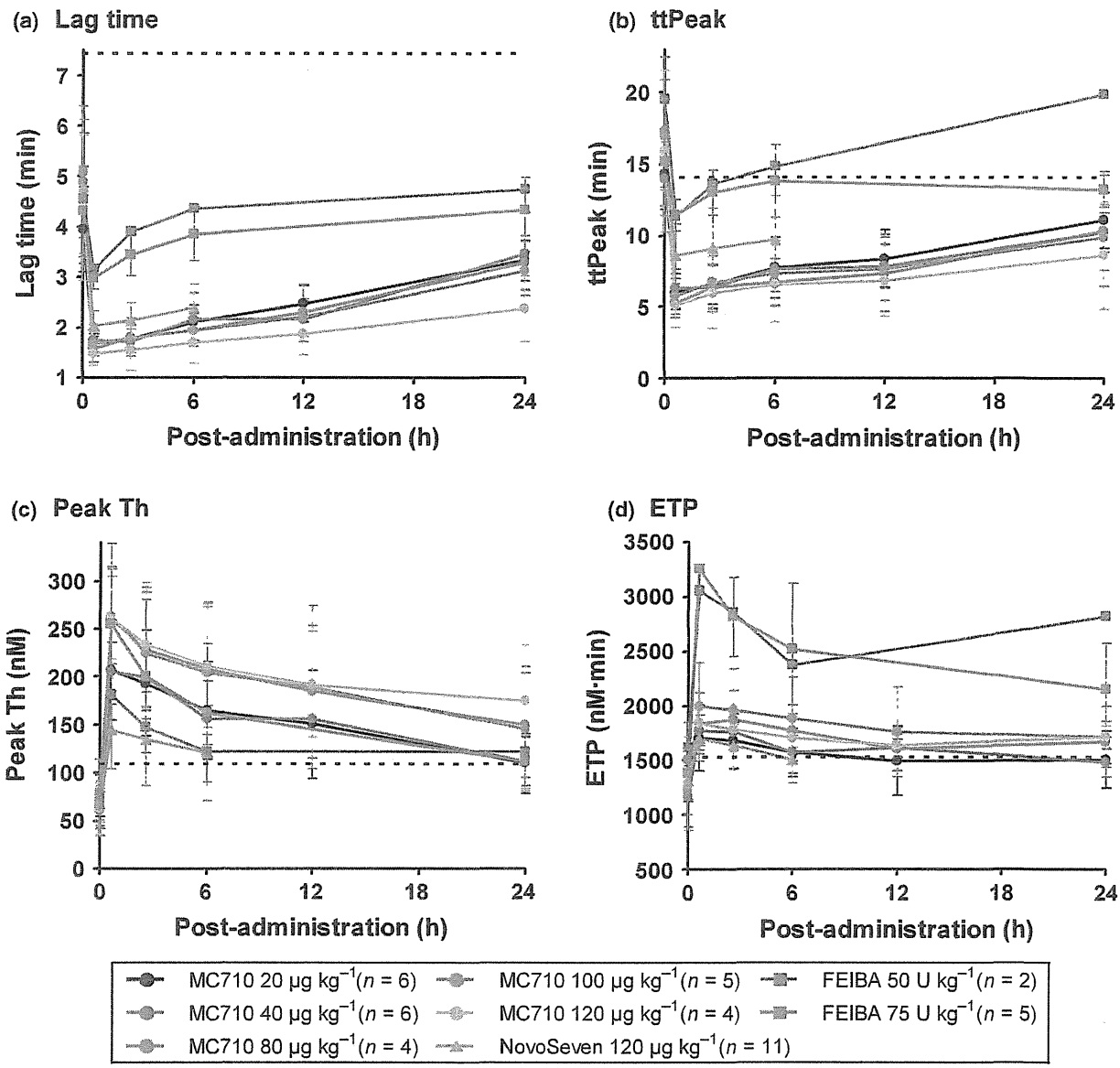

Fig. 5. Changes in TGT parameters. Time-dependent changes in TGT parameters obtained from thrombin generation profiles are shown. The assay was conducted using the plasma at pre and postadministration; 10 min, 2, 6, 12 and 24 h for five dose rates of MC710, 10 min, 2 and 6 h for NovoSeven, 10 min, 2, 6 and 24 h for FEIBA. (a) Lag time; (b) ttPeak; (c) Peak Th; (d) ETP (mean ± SD). The same symbols and lines are used in (a)–(d). Dashed lines in (a)–(d) indicate the mean values for NHP (18 times repetitive measurement). Parameter values for NHP (mean ± SD) are Lag time; 7.42 ± 0.27 min, ttPeak; 14.07 ± 0.43 min, Peak Th; 108.99 ± 8.92 nM and ETP; 1531.28 ± 73.27 nM min.

$P = 0.039$ at 20 μg kg^{-1}, $P = 0.041$ at 40 μg kg^{-1}, $P = 0.049$ at 80 μg kg^{-1}, $P = 0.031$ at 100 μg kg^{-1} and $P = 0.029$ at 120 μg kg^{-1}). Conversely, postadministration of FEIBA produced a marked increase in ETP; the effect was approximately double that of MC710 and NovoSeven. Enhanced ETP was significantly greater post FEIBA administration than after all doses of MC710 (75 U kg^{-1} FEIBA against MC710: $P = 0.002$ at 20 μg kg^{-1}, $P = 0.004$ at 40 μg kg^{-1}, $P = 0.005$ at 80 μg kg^{-1}, $P = 0.004$ at 100 μg kg^{-1}, $P = 0.003$ at 120 μg kg^{-1}).

Discussion

CWA with an MDA® II has been used to examine the pathophysiology of disseminated intravascular coagulation (DIC) [7,8], quantify trace concentrations of FVIII (<1.0 IU dL^{-1}) in the plasma of severe haemophilia A patients, and analyse the coagulation process induced by rFVIIa in clotting factor-deficient plasma [5,9]. In this study, we evaluated the effectiveness of CWA using plasma from haemophilia patients with inhibitors who were given bypassing products includ-

ing MC710. We confirmed the change in APTT post MC710 administration was dose dependent (Fig. 3a) and that the clot waveform profile at 10 min postadministration of MC710 at 120 μg kg^{-1} was close to that of NHP (Fig. 2a).

The values of |Min1| and |Min2| immediately postadministration of MC710 at doses of ≥ 80 μg kg^{-1} were greater than those for NovoSeven at 120 μg kg^{-1} g or FEIBA at 75 U kg^{-1} (Fig. 3b and c), suggesting that MC710 has superior bypassing activity. However, mean |Min1| and |Min2| were not MC710 dose-dependent and were higher at 100 μg kg^{-1} (given to five subjects) than at 120 μg kg^{-1} (given 4 subjects). The three of four subjects administered 120 μg kg^{-1} MC710 were not administered 100 μg kg^{-1}; therefore, the 100 μg kg^{-1} and 120 μg kg^{-1} subject groups were not considered the same, as this might influence the statistical analysis.

TGT evaluates thrombin production triggered by the TF/FVIIa complex. The coagulation reaction consists of (i) an initiation phase from the beginning of the coagulation reaction to the beginning of thrombin generation; (ii) a propagation phase with increasing thrombin production; and (iii) a termination phase, in which thrombin generation is suppressed by the protein C pathway or inhibitors. Lag time is an indicator of the initiation phase, and Peak thrombin and ttPeak reflect the propagation phase [10,11]. As shown in Figs 4a and 5a and b, Lag time and ttPeak were shortened in the NovoSeven and MC710 groups compared to NHP and FEIBA groups. TGT reflects extrinsic coagulation; thus, NovoSeven and MC710, which contain a large amount of FVIIa, were expected to have a shorter initiation phase. On the other hand, Peak Th was higher at all doses of MC710 than with NovoSeven at 120 μg kg^{-1} (Fig. 5c). These results suggest that explosive thrombin generation, which promotes the shift to the propagation phase, is more quickly established in plasma after administration of MC710 than with FVIIa alone. There was a more marked increase in ETP post FEIBA administration than after MC710 or NovoSeven, suggesting more sustained thrombin production is induced by FEIBA (Fig. 5d).

Clot waveform analysis evaluates the whole process of fibrin clot formation by thrombin generated in the 'intrinsic coagulation pathway' triggered by ellagic acid. TGT evaluates thrombin generation in the 'extrinsic coagulation pathway' using the TF/PL complex as a trigger of coagulation. Both methods directly or indirectly evaluate the amount of thrombin although the thrombin generation pathways differ. High doses of rFVIIa activate FX independently of TF in the absence of FVIII, generating a FXa-induced thrombin burst and thus providing bypassing activity [12–14]. FVIII or FIX in the intrinsic coagulation pathway does not function in APTT of the plasma of inhibitor patients; consequently, we conclude the FVIIa-activated reaction of FX depends on PL added to the measurement system. The specific characteristics of MC710 enable FVIIa to function more TF-independently by increasing the FX concentration in blood, resulting in changes in APTT. The increased FX concentration in the extrinsic coagulation pathway after MC710 administration also eased access of FX to the TF/FVIIa complex [15,16] and, as shown in the TGT results, the bypassing activity via TF by MC710 is expected to be greater than that achieved by monotherapy with FVIIa.

In this report, we described CWA and TGT in haemophilia patients with inhibitors after administration of bypassing agents NovoSeven, FEIBA and MC710. We concluded these measurement methods are appropriate for monitoring the procoagulant potential specific to bypassing agents and are available analytical tools for assessing bypassing activity in haemophilia patients with inhibitors. Measurements using both methods revealed MC710 at doses of ≥ 80 μg kg^{-1} induced higher |Min1| and |Min2| than did the control drugs; more rapid thrombin generation than FEIBA and greater thrombin production than NovoSeven. These results suggest that MC710 has superior bypassing activity in comparison to control drugs.

Acknowledgements

This study was supported by KAKETSUKEN (The Chemo-Sero-Therapeutic Research Institute). Other participants in the MC710 clinical trial were described in the first report [4].

Disclosures

Akira Shirahata and Yasuo Ohashi received a fee from KAKETSUKEN for the implementation of the trial. The other authors have no conflicts of interest to declare.

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Journal of Thrombosis and Haemostasis, 10: 81–89 DOI: 10.1111/j.1538-7836.2011.04549.x

ORIGINAL ARTICLE

Recombinant factor VIIa analog (vatreptacog alfa [activated]) for treatment of joint bleeds in hemophilia patients with inhibitors: a randomized controlled trial

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To cite this article: De Paula EV, Kavakli K, Mahlangu J, Ayob Y, Lentz SR, Morfini M, Nemes L, Šalek SZ, Shima M, Windyga J, Ehrenforth S, Chuansumrit A, for the 1804 (adept™1) investigators. Recombinant factor VIIa analog (vatreptacog alfa [activated]) for treatment of joint bleeds in hemophilia patients with inhibitors: a randomized controlled trial. *J Thromb Haemost* 2012; **10**: 81–9.

Summary. *Background:* A recombinant factor VIIa analog (NN1731; vatreptacog alfa [activated]) was developed to provide safe, rapid and sustained resolution of bleeds in patients with hemophilia and inhibitors. *Patients/Methods:* This global, prospective, randomized, double-blinded, active-controlled, dose-escalation trial evaluated and compared one to three doses of vatreptacog alfa at 5, 10, 20, 40, and 80 μg kg^{-1} with one to three doses of recombinant FVIIa (rFVIIa) at 90 μg kg^{-1} in the treatment of acute joint bleeds in hemophilia patients with inhibitors. The primary endpoint comprised adverse events; secondary endpoints were evaluations of immunogenicity, pharmacokinetics, and efficacy. *Results and Conclusions:* Overall, 96 joint bleeds in 51 patients (> 12 years of age) were dosed. Vatreptacog alfa was well tolerated, with a low frequency of adverse events. No immunogenic or thrombotic events related to vatreptacog alfa were reported. A high efficacy rate of vatreptacog alfa in controlling acute joint bleeds was observed; 98% of bleeds were controlled within 9 h of the initial dose in a combined evaluation of 20–80 μg kg^{-1} vatreptacog alfa. The efficacy rate observed for rFVIIa (90%) is consistent with data from published clinical trials. The trial was not powered to compare efficacy, and further trials are needed to investigate the efficacy of vatreptacog alfa as compared with that of rFVIIa. The trial was registered at ClinicalTrials.gov (Registration Number: NCT00486278).

Keywords: hemophilia, inhibitors, rFVIIa analog, safety, vatreptacog alfa (activated).

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Presented in abstract form at the 52nd annual meeting of the American Society of Hematology, Orlando, FL, 6 December 2010. E. de Paula, K. Kavakli, J. Mahlangu, *et al.*; on behalf of the 1804 (ADEPT-1) Investigators. Safety and preliminary efficacy of recombinant activated FVII analog (NN1731) in the treatment of joint bleeds in congenital hemophilia patients with inhibitors. *Blood* 2010; 116(21): 719.

[1]A complete list of members of the 1804 investigators appears in Appendix S1.

Received 5 August 2011, accepted 21 October 2011

Introduction

The development of neutralizing antibodies (inhibitors) against factor (F) VIII or FIX is a serious complication of replacement therapy in patients with congenital hemophilia. The use of FVIII/FIX-bypassing agents is the preferred treatment option for acute bleeds in patients with high-responding inhibitors. Two bypassing agents are currently available to treat patients with inhibitors, recombinant FVIIa (rFVIIa) (NovoSeven®; Novo Nordisk A/S, Bagsværd, Denmark) and plasma-derived activated prothrombin complex concentrate (pd-aPCC) (FEIBA VH®; Baxter AG, Vienna, Austria). Both agents have

well-established efficacy and safety profiles. However, the hemostatic efficacy of these agents in patients with inhibitors does not reach the rates obtained with FVIII or FIX replacement therapy in patients without inhibitors [1–3], and may display considerable intraindividual and interindividual variability [4–6].

An rFVIIa analog was developed by Novo Nordisk with the aim of providing an improved bypassing agent offering more rapid, reliable and sustained resolution of acute bleeds in patients with hemophilia and inhibitors. This would be of clinical benefit, given the reduced need for retreatment of insufficiently treated bleeds, fewer venipunctures, reduced pain and consumption of hemostatic medication and analgesics, and less interruption to daily activities. The INN name of the active pharmaceutical ingredient of rFVIIa analog (formerly designated as NN1731) is 'vatreptacog alfa (activated)', and the term 'vatreptacog alfa' is used as the name of the drug.

Vatreptacog alfa is an activated recombinant human FVIIa analog produced biosynthetically with a CHO cell line cultured in serum-free medium. No raw materials or excipients of human or animal origin are used in the production of vatreptacog alfa. It is structurally similar to rFVIIa, with the exception of three amino acid substitutions (V158D, E296V, and M298Q) affecting the protease domain, resulting in increased tissue factor (TF)-independent activity as compared with wild-type FVIIa [7]. The mutations introduced into the vatreptacog alfa molecule mimic the effects of binding to TF, and allow the molecule to express greater proteolytic activity in the absence of TF than wild-type FVIIa. This translates into greater activity than rFVIIa on the surface of activated platelets. The enhanced platelet-dependent (TF-independent) activity of vatreptacog alfa was confirmed in several non-clinical studies [7–9].

Non-clinical studies indicate that vatreptacog alfa may provide effective, rapid and lasting cessation of bleeds [8–13]. On activated platelets, vatreptacog alfa shows increased enzymatic activity, resulting in faster and more pronounced thrombin generation, and subsequently faster and stronger clot formation with increased stability against fibrinolytic degradation [7–13]. Unlike rFVIIa, vatreptacog alfa normalizes the thrombin generation rate and clot formation in several models of hemophilia [8–13]. In a severe bleeding model in hemophilia A mice, vatreptacog alfa demonstrated significantly greater efficacy and faster bleeding resolution than rFVIIa, pd-aPCC, or rFVIII [13].

In the first human dose trial in healthy subjects, rapid thrombin generation was observed immediately after vatreptacog alfa administration [14]. Approximately 73% of vatreptacog alfa was eliminated in the initial phase, with a half-life of ~ 20 min, and the remaining 27% was eliminated in the terminal phase, with a half-life of ~ 3.1 h. A single dose of vatreptacog alfa appeared to be safe and well tolerated in doses up to 30 $\mu g\ kg^{-1}$. No serious adverse events, including immunogenic or thromboembolic events, occurred [14].

The aim of this phase 2 trial was to evaluate the safety and preliminary efficacy of vatreptacog alfa for treatment of joint bleeds in hemophilia patients with inhibitors.

Patients, materials and methods

Trial design and objectives

The trial was a prospective, global, multicenter, randomized, double-blinded, active-controlled, dose-escalation trial, conducted from June 2007 to June 2010. The objective of the trial was to evaluate the safety and preliminary efficacy of five escalating dose levels of vatreptacog alfa (one to three doses at 5, 10, 20, 40 and 80 $\mu g\ kg^{-1}$) vs. one to three doses of rFVIIa at 90 $\mu g\ kg^{-1}$ in the treatment of joint bleeds in hemophilia patients with inhibitors. The randomization ratio was 4 : 1 (vatreptacog alfa/rFVIIa) in all dose tiers. Vatreptacog alfa (activated), rFVIIa and rFVIIa placebo were manufactured by Novo Nordisk (Hillerød, Denmark), and were provided as a sterile freeze-dried powder in single-use vials of 1.2 mg to be reconstituted with 2.2 mL of sterile water for injection.

Sequential dose escalation followed safety evaluations by an independent external data monitoring committee (DMC). Patients who experienced several joint bleeds during the trial period were randomized and treated in subsequent dose tiers for a maximum of five qualifying joint bleeds.

The primary endpoint was frequency of adverse events; secondary endpoints included evaluations of immunogenicity, pharmacokinetics and efficacy of vatreptacog alfa.

The trial was performed in accordance with the Declaration of Helsinki and its amendments in force at trial initiation [15], and the International Conference on Harmonization [16] and Japanese [17] guidelines on Good Clinical Practice. The trial was registered at ClinicalTrials.gov (Number: NCT00486278).

Eligibility criteria

The trial population included adolescent and adult males above 12 years of age with congenital hemophilia A or B complicated by high-responding inhibitors to FVIII or FIX (current or historical titer above above 5 Bethesda units mL^{-1}). Furthermore, for all patients, a documented bleeding frequency of at least two joint bleeds over 6 months or four joint bleeds over 12 months was required for inclusion in the trial.

Patients were ineligible if they had a low platelet count (< 50 000 μL^{-1}), active pseudotumors, advanced atherosclerotic disease, severe liver disease, coagulation disorders other than congenital hemophilia, or a history of thromboembolic events.

In order for a joint bleed to qualify for trial product administration, the following had to be fulfilled: (i) the patient should not have received any intravenous hemostatic treatment for a minimum of 5 days prior to trial product administration; and (ii) the patient should not have had any other bleeds within 7 days of onset of the qualifying joint bleed. Qualifying bleeds included hemorrhages into elbows, knees, and ankles.

When experiencing a qualifying joint bleed, the patient had to attend the clinic and receive the initial dose of trial product within 3 h (+ 30 min) of onset of bleed. If the patient could

not meet this requirement, the bleed was not eligible for treatment with trial product.

Trial procedures

All patients experiencing a qualifying joint bleed were randomly allocated to treatment (vatreptacog alfa or rFVIIa), and always to the lowest dose tier available.

The initial dose of trial product was to be administered in a hospital setting, and this was to be followed by continuous assessment of the bleed. If the bleeding was not controlled 3 h after initial trial product administration, up to two additional doses of trial product could be given (3-h dosing interval). Other hemostatic agents could be given according to the local standard of care if, according to the investigator, the bleed was not controlled with trial product. Patients remained at the clinic for at least 12 h after the initial dose for monitoring and evaluation of clinical response. Seven days after treatment, patients attended a follow-up visit for evaluation of general safety parameters and screening for formation of antibodies towards trial product. Screening for antibodies was repeated 28 days after each trial product administration.

Outcomes

Safety parameters were adverse events including thromboembolic events, laboratory safety data (hematology, coagulation-related parameters, clinical chemistry, and urinalysis), presence of antibodies against vatreptacog alfa, physical examination, and vital signs. Coagulation-related parameters (prothrombin fragment [F_{1+2}], prothrombin time [PT], activated partial thromboplastin time [aPTT], D-dimers, and fibrinogen) were measured predose, and 10 min, 30 min, 1 h, 3 h, 8 h and 12 h postdose.

PT analysis was performed with STA Stago®, with STA-neoplastin and calcium as reagents. aPTT analysis was performed with Pathrombin SL reagents (Dade Behring, Deerfield, IL, USA). D-dimer levels were measured by ELISA: VIDAS® D-Dimer ExclusionTM (bioMérieux, Marcy l'Etoile, France). F_{1+2} was measured by ELISA: Enzygnost® F_{1+2} (monoclonal; Dade Behring). Fibrinogen levels were measured with the STA Stago®–Clauss method.

Samples for anti-vatreptacog alfa and anti-rFVIIa antibody screening were measured with a screening assay for detection of binding antibodies and a functional assay for detection of neutralizing antibodies. A radioimmunoassay with an ^{125}I-labeled vatreptacog alfa/rFVIIa tracer was used to measure binding antibodies. The presence of antibodies was confirmed by inhibition with excess unlabeled vatreptacog alfa or rFVIIa. Antibody-positive samples were characterized with in vitro clotting assays for vatreptacog alfa and FVII neutralizing antibodies.

Blood sampling for assessment of the pharmacokinetic profile was obtained in a subset of patients enrolled in dose tiers 3, 4 and 5 predose and at intervals up to 12 h postdose (24 h in the United States). The FVIIa activity was determined with a one-stage clotting assay, using the Staclot® VIIa-rTF assay, and an ACL Advance® Analyzer (Instrumentation Laboratory, Milan, Italy), as described previously [14,18]. Standard pharmacokinetic endpoints were determined from the FVIIa activity profiles after a single intravenous injection, and included area under the curve $(AUC)_{0-t}$, AUC, mean residence time (MRT), $t_{½}$, clearance (CL) and V_{ss}, with non-compartmental methods. In order to convert the dose in μg kg^{-1} to IU kg^{-1}, the specific activities (FVIIa activity per amount of drug) of vatreptacog alfa and rFVIIa were determined in reconstituted vials.

The main preliminary efficacy endpoint was the number of bleeds successfully controlled with a single dose of trial product. Overall treatment efficacy was assessed by the need for additional hemostatic medication concomitantly with the per-protocol treatment regimen. Treatment failure was defined as bleeds where additional hemostatic medication was administered to control bleeding within 12 h of the initial dose. Assessment of efficacy also included the number of doses of trial product administered to achieve and maintain hemostatic control within the scheduled 9-h dose period, and change in pain over time after the first trial product administration.

Statistics

Data from patients exposed to at least one dose of trial product were included in the safety evaluation, and data from patients with at least one efficacy evaluation postdose were included in the efficacy evaluation.

The safety of vatreptacog alfa was based on descriptive statistics. In order to ensure an adequate number of patients for addressing the efficacy endpoints, the sample size was set to 20–25 patients per dose tier.

Statistical analyses were conducted on the efficacy endpoints. Control of bleeding with a single dose of vatreptacog alfa was analyzed by logistic regression, with treatment as a factor and target joint status and time from the start of the trial as covariates. Overall treatment efficacy within 9 h was analyzed with Fisher's exact test, as data were categorical and the success rate was high. However, it should be noted that the trial was not powered for efficacy.

Results

Characteristics of the cohort

Fifty-one male patients were randomized (Fig. 1) from 28 centers in 13 countries (see Appendix S1).

At enrollment, ages ranged from 12 to 69 years, with a mean of 28 years, and the trial population included 11 patients aged 12–17 years. The majority of eligible patients had hemophilia A (n = 48). Patient demographics and baseline assessments (including hemophilia type, inhibitor level, and bleeding episode characteristics) were well balanced in the vatreptacog alfa and rFVIIa treatment groups, and were comparable between the vatreptacog alfa dose level groups.

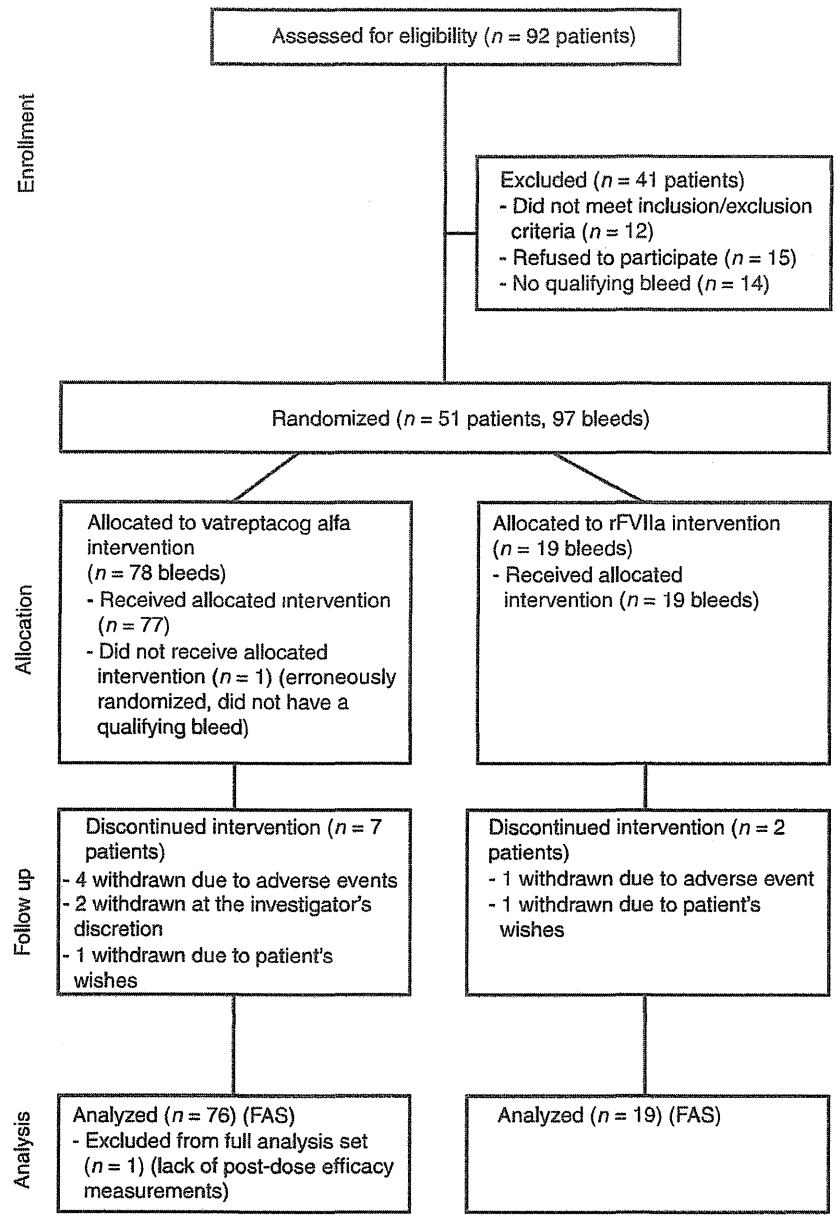

Fig. 1. CONSORT diagram showing the flow of patients and number of bleeds. FAS, full analysis set.

Differences were seen, however, in baseline joint status, which was evaluated before trial product administration for each qualifying bleed. The baseline symptoms, including swelling, pain, and loss of range of motion, were more severe for bleeds treated with rFVIIa. The proportion of target joint bleeds (defined as joints with three or more bleeds in the past 6 months) was higher among vatreptacog alfa-treated bleeds (49.4%) than among rFVIIa-treated bleeds (31.6%).

Exposure to trial products

Each dose tier was planned to enroll 25 bleeds. After DMC evaluation of the first 20 bleeds in dose tier 1, the sponsor decided to discontinue this tier, as the data indicated that this dose was subtherapeutic. Dose tier 2 included the planned 25 bleeds. Because of slow recruitment of patients into the trial, dose tiers 3 and 4 were reduced from 25 to 20 bleeds each, and dose tier 5 to 12 bleeds.

Overall, 96 bleeds were treated within the trial, including 77 bleeds with vatreptacog alfa, and 19 bleeds with rFVIIa (Fig. 1).

Fifty-one patients received at least one dose of trial product(s), and 46 of these were given vatreptacog alfa (Table 1). Two patients included in dose tier 1 received higher doses than expected, owing to errors in the reconstitution of the trial product; one patient received three doses of 19.4 μg kg^{-1} vatreptacog alfa, and one received a single dose of 26.7 μg kg^{-1} vatreptacog alfa.

Table 1 Number of exposures to trial products

	Vatreptacog alfa	rFVIIa	Total*
Number of patients	46	17	51
Exposure days† for trial product, *N* (%)			
1	25 (54.3)	15 (88.2)	24 (47.1)
2	13 (28.3)	2 (11.8)	15 (29.4)
3	7 (15.2)	–	7 (13.7)
4	–	–	4 (7.8)
5	1 (2.2)	–	1 (2.0)
Total dose (μg kg^{-1})			
Mean	86	214.8	
Minimum; maximum	5; 459	85; 528	
Total number of doses			
Mean	3.57	2.41	4.02
Minimum; maximum	1; 15	1; 6	1; 15

rFVIIa, recombinant FVIIa. *Total exposure days is not a sum of the numbers for vatreptacog alfa and rFVIIa directly, because, for example, if a patient had one exposure to vatreptacog alfa and one to rFVIIa, he had two exposures in total, and is included under the two row for total exposure days. †Exposure days = total number of days in which patients were treated with trial product.

Evaluation of safety: adverse events, including immunogenicity

Overall, vatreptacog alfa was well tolerated, with a low frequency of adverse events in all dose groups (Table 2). Most adverse events were related to concomitant illnesses and the underlying disease (hemophilia) and consequences thereof. The type and frequency of adverse events reported following exposure to vatreptacog alfa were comparable to the well-established adverse event profile of rFVIIa.

The majority of adverse events were rated as mild, and were judged by the investigator as unlikely to be related to the trial products. Three episodes of errors during reconstitution and administration of trial products (5 μg kg^{-1} vatreptacog alfa), and one episode of discomfort (after 90 μg kg^{-1} rFVIIa) were evaluated as being related to the trial products.

Fifteen serious adverse events were reported. All events started more than 2 weeks (range, 16–677 days) after treatment with trial product, and were evaluated as not being related to the trial product as judged by the investigator, DMC, and sponsor.

One thrombotic event was reported among patients exposed to trial product during the trial. In a 23-year-old patient exposed to vatreptacog alfa, a deep vein thrombosis of the right superficial femoral vein was reported as a serious adverse event with an onset 199 days after administration of 10 μg kg^{-1} vatreptacog alfa. The event, which occurred shortly after the patient suffered from cholecystitis requiring intensive care and after he had received alternative hemostatic medication according to local standard care, was judged as being unrelated to vatreptacog alfa exposure by the investigator, DMC, and sponsor.

A total of five patients were withdrawn from the trial because of adverse events, including three misdosing events (tier 1), one episode of elevated alanine aminotransferase (ALAT) level (tier 3), and the above-described venous thrombosis event (tier 2). No clinical symptoms were reported in relation to the dosing errors or the elevated ALAT value, which was present predose and was assessed as resulting from chronic hepatitis C. No formation of antibodies against vatreptacog alfa or rFVIIa was observed in any patients exposed to trial product.

Evaluation of safety: laboratory assessments

No safety concerns were revealed by any of the laboratory parameters investigated in the trial (troponin, hematology, including platelet count, biochemistry, including ALAT, and coagulation-related parameters).

No clinically relevant differences between predose and postdose values or differences between treatment groups were observed for platelet count (predose range, 126–474 × 10^9; 12-h postdose range, 127–502 × 10^9) or fibrinogen (predose range, 0.8–5.7 g L^{-1}; postdose range, 1.0–7.4 g L^{-1}; reference range,

Table 2 Overview of adverse events

	Vatreptacog alfa, *N* (%), *E*						rFVIIa, *N* (%), *E*
	5 μg kg^{-1}	10 μg kg^{-1}	20 μg kg^{-1}	40 μg kg^{-1}	80 μg kg^{-1}	Total	
Total bleeds	16	19	16	16	10	77	19
Adverse events	8 (50.0), 10	5 (26.3), 8	5 (25.0), 5	3 (18.8), 5	0 (0), 0	20 (26), 28	10 (52.6), 11
Serious adverse events*	2 (12.5), 3	3 (15.8), 5	2 (12.5), 2	2 (12.5), 2	0 (0), 0	9 (11.7), 12	2 (10.5), 3
Adverse events with onset within 7 days postdose	6 (37.5), 7	3 (15.8), 3	3 (18.8), 3	1 (6.3), 3	0 (0), 0	13 (16.9), 16	8 (42.1), 8
Possibly/probably related adverse events	3 (18.8), 3	0 (0.0), 0	0 (0), 0	0 (0), 0	0 (0), 0	3 (3.9), 3	1 (5.3), 1
Adverse events leading to withdrawal	3 (18.8), 3	1 (5.3), 1	0 (0), 0	0 (0), 0	0 (0), 0	4 (5.2), 4	1 (5.3), 1

E, number of adverse events; *N*, number of bleeds with an adverse event; %, proportion of bleeds with adverse event; rFVIIa, recombinant FVIIa. Non-serious adverse events include events occurring from the initial dose of trial product (for treatment of bleed) until 7 days after the initial dose. Serious adverse events include all events collected from the first administration of trial product to the end of patients' participation in the trial. *All serious adverse events had an onset more than 2 weeks (range, 16 days to almost 2 years) after treatment with trial product, and were evaluated as not related to the trial product as judged by the investigator, DMC, and sponsor.

2–4 g L^{-1}). No evidence of consumption of platelets or fibrinogen was apparent in any patients.

Postdose changes of D-dimers (Fig. 2A), F_{1+2} (Fig. 2B), PT and aPTT were considered to be consistent with the expected pharmacologic effect of both vatreptacog alfa and rFVIIa on the coagulation system.

Pharmacokinetic profile of vatreptacog alfa

Thirty patients had assessments of pharmacokinetic data, including a total of 42 bleeds treated in dose tier 3, 4, or 5. The FVIIa activity in plasma during the first 3 h after the initial intravenous dose of trial product is shown in Fig. 3. After the end of vatreptacog alfa infusion, FVIIa activity declined in an exponential way. A three-fold to four-fold higher peak activity was seen for 80 μg kg^{-1} vatreptacog alfa as compared with 90 μg kg^{-1} rFVIIa. At 1 h after the initial dose, mean plasma activities of vatreptacog alfa (20, 40 and 80 μg kg^{-1}) were below the levels obtained after a single dose of 90 μg kg^{-1} rFVIIa.

The total exposure during the initial 30 min ($AUC_{0-30\ min}$) and 3 h (AUC_{0-t}) postdose increased with increasing dose level of vatreptacog alfa, indicating dose linearity. The estimates of AUC_{0-t} and corresponding 95% confidence intervals did not indicate any statistically significant deviations from dose linearity for vatreptacog alfa.

The clearance of vatreptacog alfa was approximately three times faster than the clearance of rFVIIa.

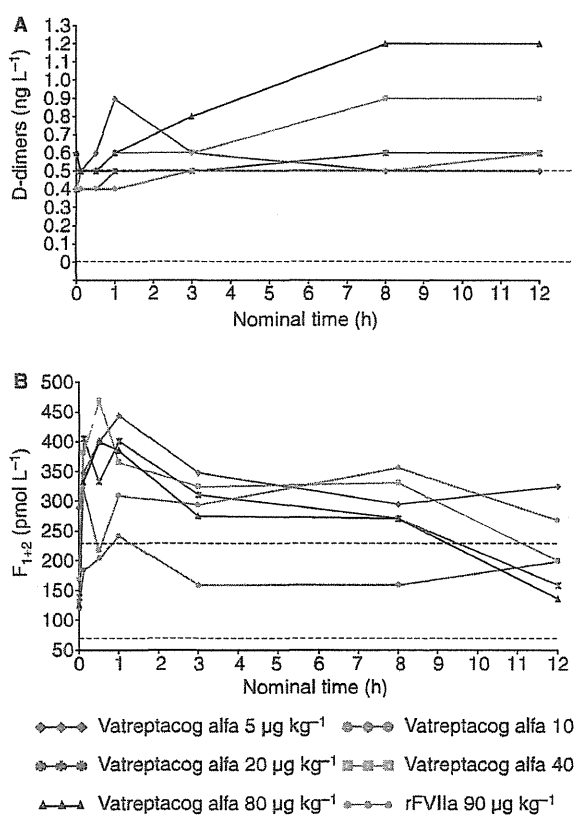

Fig. 2. Coagulation-related parameters of thrombin formation and fibrinolysis (A) Mean levels of D-dimers (ng L^{-1}) by dose. (B) Mean levels of F_{1+2} (pmol L^{-1}) by dose. Horizontal lines indicate the normal reference range. rFVIIa, recombinant FVIIa.

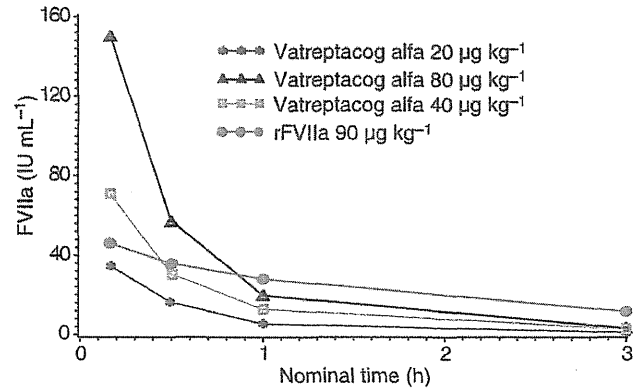

Fig. 3. Mean single-dose pharmacokinetic profiles of FVIIa activity vs. time for vatreptacog alfa (20, 40 or 80 μg kg^{-1}) and recombinant FVIIa (rFVIIa) (90 μg kg^{-1}).

Evaluation of efficacy

Ninety-five bleeds were included in the evaluation of efficacy (Fig. 1).

For the majority of joint bleeds (86/95 bleeds), effective and sustained control was obtained with the per-protocol one to three doses of trial product(s) (Table 3).

Overall, 98% of the joint bleeds were controlled successfully with vatreptacog alfa in a combined evaluation of 20–80 μg kg^{-1} dose groups, as compared with 90% of bleeds treated with rFVIIa (90 μg kg^{-1}).

The number of doses needed to obtain hemostatic control decreased with increasing dose of vatreptacog alfa, 40% of bleeds being effectively treated with a single dose of 80 μg kg^{-1} vatreptacog alfa (Table 3).

Discussion

Vatreptacog alfa was well tolerated in actively bleeding hemophilia patients with inhibitors enrolled in the current trial, with a low frequency of adverse events and no safety concerns being observed at any dose level. In particular, no immunogenic or thrombotic events related to the trial product were reported. In addition, no dose relationship was evident with respect to the incidence or nature of the adverse events reported following exposure to vatreptacog alfa. Notably, the type and frequency of adverse events reported were similar to the well-established safety profile of rFVIIa in patients with hemophilia. Hence, no adverse events specific to vatreptacog alfa were identified in the trial setting.

In vatreptacog alfa, three amino acids have been substituted vs. rFVIIa. Therefore, the formation of antibody against vatreptacog alfa was closely monitored. In the current trial, 45 patients received vatreptacog alfa and were followed with

Table 3 Treatment efficacy as evaluated by number of doses of trial product administered to control bleeds within 9 h from initial dose

	Vatreptacog alfa, *N* (%)					rFVIIa, *N* (%)
	5 μg kg^{-1}	10 μg kg^{-1}	20 μg kg^{-1}	40 μg kg^{-1}	80 μg kg^{-1}	90 μg kg^{-1}
Total bleeds	15	19	16	16	10	19
Treatment successes*	12 (80.0)	16 (84.2)	16 (100)	15 (93.8)	10 (100)	17 (89.5)
Bleeds controlled with a single dose	3 (20.0)	3 (15.8)	2 (12.5)	5 (31.3)	4 (40.0)	6 (31.6)
Bleeds controlled with two doses	5 (33.3)	9 (47.4)	7 (43.8)	6 (37.5)	4 (40.0)	4 (21.1)
Bleeds controlled with three doses	4 (26.7)	4 (21.1)	7 (43.8)	4 (25.0)	2 (20.0)	7 (36.8)

rFVIIa, recombinant FVIIa. *Bleeds successfully controlled with one to three doses of trial product.

antibody measurements for up to 28 days after the last trial product administration. The mean total number of vatreptacog alfa doses received during the trial was 3.6, ranging from 1 to 15. No antibody development was detected in any patients exposed to vatreptacog alfa in this trial. It should be borne in mind, however, that patients could receive vatreptacog alfa for a maximum of five bleeds with up to three doses per bleed, which may not be sufficient for the evaluation of immunogenic risk. To further evaluate the potential immunogenic risk of vatreptacog alfa, repeated assessment of potential antibody formation will be performed in future clinical trials.

One patient experienced a thrombosis of the right superficial femoral vein 199 days after administration of 10 μg kg^{-1} vatreptacog alfa, which was judged by the investigator, DMC and sponsor as being unrelated to the trial product. It should also be noted that his event occurred shortly after cholecystitis requiring intensive care, and after the patients had received hemostatic medication according to local standard care for treatment of a large iliopsoas bleed. Vatreptacog alfa exerts its pharmacologic action on activated platelets at the site of injury [10], and is not capable of directly activating platelets [8], so its action is limited to the site of injury. In addition, vatreptacog alfa is susceptible to the same plasma inhibitors (TF pathway inhibitor [TFPI] and antithrombin [AT]) as FVIIa, and, when bound to TF, is inhibited similarly to rFVIIa. In the absence of TF, vatreptacog alfa is inhibited more readily by TFPI and AT than rFVIIa. Approximately 70% of vatreptacog alfa is eliminated in the initial phase, with a half-life of ~ 20 min. This rapid elimination may contribute to a low risk of thromboembolic events in patients with hemophilia following exposure to or treatment with vatreptacog alfa. As the trial included first exposure of vatreptacog alfa in bleeding patients with hemophilia, a cautious dose-escalation trial was chosen, and the trial population comprised relatively young patients (age range, 12–69 years; mean, 28 years) without any known risk factors for thrombosis/arterial thrombosis. Therefore, assessment of the thrombogenic risk of vatreptacog alfa will be further evaluated in future trials.

The pharmacokinetic data obtained in bleeding patients with hemophilia and inhibitors are consistent with the pharmacokinetic profile of vatreptacog alfa previously reported in healthy subjects [14]. The FVIIa activity declined with a biexponential decay pattern, with initial rapid elimination being followed by a less rapid elimination phase. Dose proportionality could be concluded within the dose range studied (20–80 μg kg^{-1}). The pharmacokinetic profile obtained after intravenous administration of vatreptacog alfa was distinctly different from that obtained after the same dose of rFVIIa. As expected, the initial postdose concentration was significantly increased after administration of vatreptacog alfa. An approximately three-fold to four-fold higher initial activity for the 80 μg kg^{-1} dose than for 90 μg kg^{-1} rFVIIa was shown at 10 min postdose. At 1 h after injection of trial product, the mean plasma activities of vatreptacog alfa were below the levels obtained after rFVIIa administration. The clearance of vatreptacog alfa was approximately three times faster than the clearance of rFVIIa, as reflected in the initial half-life of vatreptacog alfa. The shorter half-life and faster clearance of vatreptacog alfa may be explained by the greater inhibition of vatreptacog alfa by AT, as suggested by recent studies [19,20].

Non-clinical data have shown that vatreptacog alfa displays increased procoagulant activity on activated platelets, resulting in a normalization of thrombin generation and formation of a stable clot [10,11]. Furthermore, animal data obtained in a mouse hemophilia model [12,13] showed that vatreptacog alfa efficiently shortened the bleeding time and reduced blood loss. This current trial expands upon these findings, showing a high efficacy rate of vatreptacog alfa in controlling acute joint bleeds at all dose levels studied, with an apparent dose relationship being observed for vatreptacog alfa efficacy endpoints.

The observed 90% efficacy rate in the rFVIIa comparator group is consistent with data from published clinical trials and experience, supporting the appropriateness of the trial design and the trial population.

In summary, a favorable safety profile of vatreptacog alfa with a low frequency of adverse events was observed in the patient population studied in this trial. In addition, preliminary efficacy evaluation showed a high efficacy rate of vatreptacog alfa, with 98% of joint bleeds being successfully controlled in a combined evaluation of the 20–80 μg kg^{-1} dose groups. However, as the trial was not powered for efficacy, assessment of the relative efficacy of vatreptacog alfa will need to be verified in a larger confirmatory trial.

On the basis of the clinical data obtained in the current trial and the pharmacologic characteristics, vatreptacog alfa may represent an improved option for the treatment of acute bleeding episodes in hemophilia patients with inhibitors.

Addendum

S. Ehrenforth: made substantial contributions to the conception and design of the trial; E. V. De Paula, K. Kavakli, J. Mahlangu, Y. Ayob, S. R. Lentz, M. Morfini, L. Nemes, S. Z. Šalek, M. Shima, J. Windyga, S. Ehrenforth, and A. Chuansumrit: performed research/contributed to the acquisition of data; E. V. De Paula, K. Kavakli, J. Mahlangu, Y. Ayob, S. R. Lentz, M. Morfini, L. Nemes, S. Z. Šalek, M. Shima, J. Windyga, S. Ehrenforth, and A. Chuansumrit: collected data; E. V. De Paula, K. Kavakli, J. Mahlangu, Y. Ayob, S. R. Lentz, M. Morfini, L. Nemes, S. Z. Šalek, M. Shima, J. Windyga, S. Ehrenforth, and A. Chuansumrit: analyzed and interpreted the data; E. V. De Paula and S. Ehrenforth: co-wrote the manuscript; E. V. De Paula, K. Kavakli, J. Mahlangu, Y. Ayob, S. R. Lentz, M. Morfini, L. Nemes, S. Z. Šalek, M. Shima, J. Windyga, S. Ehrenforth, and A. Chuansumrit: critically reviewed the manuscript for important intellectual content; E. V. De Paula, K. Kavakli, J. Mahlangu, Y. Ayob, S. R. Lentz, M. Morfini, L. Nemes, S. Z. Šalek, M. Shima, J. Windyga, S. Ehrenforth, and A. Chuansumrit: approved the final version of the manuscript. E. V. De Paula, the lead author, assumes full responsibility for the integrity and interpretation of the data.

Acknowledgements

We thank the 1804 investigators for their participation in this trial; a complete membership list is given in Appendix S1. The authors would like to thank H. F. Andersen (employed by Novo Nordisk A/S) for performing the statistical analyses, and L. K. Amby (employed by Novo Nordisk A/S) for providing medical writing and editorial assistance to the authors during the preparation of this manuscript.

Disclosure of Conflict of Interests

This trial was supported by research funding from Novo Nordisk to E. V. De Paula, K. Kavakli, J. Mahlangu, Y. Ayob, S. R. Lentz, M. Morfini, L. Nemes, S. Z. Šalek, M. Shima, J. Windyga, and A. Chuansumrit. E. V. De Paula: Novo Nordisk – consultancy, research funding, Speakers' Bureau; Bayer – consultancy, Speakers' Bureau. K. Kavakli: Novo Nordisk – membership of an entity's Board of Directors or advisory committees, research funding, honorarium, costs of travel to scientific events; Baxter, Bayer – honorarium, costs of travel to scientific events; Pfizer – costs of travel to scientific events. J. Mahlangu: Novo Nordisk – honoraria, research funding, Speakers' Bureau, costs of travel cost to scientific meeting; Bayer – honoraria, research funding, consultancy, Speakers' Bureau, costs of travel to scientific events. Y. Ayob: Novo Nordisk – research funding. S. R. Lentz: Novo Nordisk – consultancy, research funding; Celgene– equity ownership. M. Morfini: Novo Nordisk – honoraria, research funding, Speakers' Bureau; Bayer – honoraria, Speakers' Bureau; Baxter – honoraria, Speakers' Bureau; CSL Behring – honoraria, membership of an entity's Board of Directors or advisory committees, Speakers' Bureau; Wyeth/Pfizer – membership of an entity's Board of Directors or advisory committees. L. Nemes: Novo Nordisk – research funding. S. Z. Šalek: Novo Nordisk – consultancy, research funding, Speakers' Bureau; Baxter – consultancy, Speakers' Bureau. M. Shima: Novo Nordisk – research funding; Baxter – research funding; Bayer – research funding. J. Windyga: Novo Nordisk – honoraria, research funding, Speakers' Bureau, costs of travel to attend scientific meetings. S. Ehrenforth: Novo Nordisk – employment. A. Chuansumrit: Novo Nordisk – honoraria, research funding.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Participating principal investigators and sites.

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