Evaluation of efficacy and safety of the anti-vWF Nanobody ALX-0681 in a preclinical baboon model of acquired thrombotic thrombocytopenic purpura

Short title: ALX-0681 treatment of acquired TTP in baboons

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Abstract

ALX-0681 is a therapeutic Nanobody targeting the A1-domain of von Willebrand factor (vWF). It inhibits the interaction between ultra-large vWF and platelet GpIb-IX-V, which plays a crucial role in the pathogenesis of thrombotic thrombocytopenic purpura (TTP). This study reports the efficacy and safety profile of ALX-0681 in a baboon model of acquired TTP. In this model, acute episodes of TTP are induced by administration of an ADAMTS13-inhibiting monoclonal antibody. ALX-0681 completely prevented the rapid onset of severe thrombocytopenia and schistocytic hemolytic anemia. After induction of TTP, platelet counts also rapidly recovered upon administration of ALX-0681. This effect was corroborated by the full neutralisation of vWF activity. Also the schistocytic haemolytic anemia was halted and partially reversed by ALX-0681 treatment. Importantly, brain CT scans and post mortem analysis did not reveal any sign of bleeding, suggesting that complete neutralisation of vWF by ALX-0681 in conditions of thrombocytopenia was not linked with an excessive bleeding risk. Taken together, the results obtained in this study demonstrate that ALX-0681 can successfully treat and prevent the most important hallmarks of acquired TTP without evidence of a severe bleeding risk. Therefore, ALX-0681 offers an attractive new therapeutic option for acquired TTP in the clinic.
Introduction

von Willebrand Factor is a large adhesive glycoprotein circulating in plasma as multimers up to 20 000 kDa.\textsuperscript{1,2} It plays a key role in hemostasis, since vWF recruits circulating platelets to damaged vessels, through binding of the vWF A1-domain to the GpIb-IX-V platelet receptors. It also acts as a carrier for FVIII, thereby preventing its proteolytic degradation while inactive.\textsuperscript{3} vWF is synthesized predominantly by endothelial cells but also by megakaryocytes, and is secreted as ultra-large vWF multimers (ULvWF, > 20 000 kDa).\textsuperscript{4} These ULvWF multimers are abnormally adhesive and are able to spontaneously link platelets irrespective of vessel injury.\textsuperscript{5} In normal circulation, ULvWFs are cleaved by the protease ADAMTS13 into normal sized multimers (500 – 20 000 kDa).\textsuperscript{6,7} Thereby the agglutination properties of vWF are reduced, since the GpIb-IX-V platelet receptor binding site in the A1 domain of regular sized vWF is cryptic and only exposed under high shear conditions.\textsuperscript{8-12}

In the condition of thrombotic thrombocytopenic purpura (TTP), there is an inability to process ULvWF due to inhibition (acquired) or dysfunction (congenital) of the protease ADAMTS13.\textsuperscript{13,14} TTP is a rare but life-threatening disorder characterized by clinical features like thrombocytopenia, hemolytic anemia, fever and neurologic/organ dysfunction. All clinical symptoms are associated with excessive platelet aggregation and formation of platelet-rich thrombi occluding the microcirculation due to the lack of ADAMTS13 activity. Acquired TTP presents both as an acute idiopathic form with no clear underlying cause, and as a secondary form which develops in association with other clinical conditions (pregnancy, cancer, HIV, autoimmune disorders).\textsuperscript{14,15} The former is the predominant form, and is related to ADAMTS13 deficiency due to an autoimmune mechanism.

The current primary treatment for acquired TTP is plasma exchange (PE) therapy, which removes the adhesive ULvWF multimers and attached platelets as well as auto-antibodies to ADAMTS13.\textsuperscript{16,17} Corticosteroids and immunosuppressive therapy is also used in combination with plasma exchange to reduce the production of ADAMTS13 antibodies for refractory or relapsing disease.\textsuperscript{18} Although PE has significantly reduced mortality rates\textsuperscript{19}, acquired TTP still carries a considerable risk of mortality and morbidity. Therefore, new therapeutic approaches are urgently needed to better manage this disorder.\textsuperscript{19,20} ALX-0681 is a bivalent humanized Nanobody containing two identical monovalent building blocks targeting the A1 domain of vWF, as described for ALX-0081.\textsuperscript{21} Nanobodies are therapeutic proteins derived from the heavy-chain variable domains (VHH) that occur naturally in heavy-chain-only
immunoglobulins from Camelidae.\textsuperscript{22,23} ALX-0081 and ALX-0681 comprise of the same active drug targeting vWF, but are denominated according to delivery route (intravenous and subcutaneous, respectively). For clarity reasons, ALX-0681 is used in this paper to describe both forms. The anti-thrombotic Nanobody avidly binds to multimeric vWF, and blocks the interaction of any sizes and activation stages of multimeric vWF with the platelet GpIb-IX-V receptor. Given the potential role of vWF-dependent formation of platelet-rich thrombi in the pathogenesis of TTP, the anti-vWF Nanobody may provide an attractive new option for the treatment of acquired TTP. Data of the anti-vWF aptamer ARC1779 from a prematurely terminated phase II study in acute TTP patients have indeed demonstrated that blocking the A1 domain of vWF has the potential to increase platelet counts in conjunction with plasma exchange therapy.\textsuperscript{24,25} Given the well-established role of the vWF-platelet interaction during normal hemostasis, bleeding risk is a relevant safety concern for anti-vWF compounds. Yet, currently available \textit{in vivo} and \textit{in vitro} data on ALX-0681, ARC1779 or other anti-vWF antagonists have not demonstrated a significant impact of functional vWF neutralization on bleeding tendency.\textsuperscript{26,27} However, the effect of vWF inhibition in situations of low platelet counts has not yet been thoroughly assessed.

A baboon model mimicking the acute early episode of acquired TTP has been described in which the disease is induced by administration of an ADAMTS13-inhibiting monoclonal antibody 3H9.\textsuperscript{28} Functional inhibition of ADAMTS13 in this model was sufficient to induce severe thrombocytopenia, schistocytic hemolytic anemia and the appearance of platelet- and vWF-rich thrombi. This preclinical baboon model is therefore relevant to evaluate the \textit{in vivo} efficacy and safety of ALX-0681 treatment in the context of acquired TTP, and will further support its potential to treat patients suffering from this disorder.

This work describes the evaluation of ALX-0681 in the baboon model of acquired TTP. The efficacy of ALX-0681 to either prevent or treat acquired TTP was studied. In addition, safety (bleeding risk) related to ALX-0681 treatment in the context of thrombocytopenia with concomitant neutralization of vWF activity was monitored in this study by screening for intracranial bleeding and internal organ bleeding.
Material and methods

Materials

The humanized bivalent ALX-0681 Nanobody consists of two identical vWF-binding building blocks (PMP12A2h1), genetically linked to each other with a 3 alanine linker, and is produced in Escherichia coli, as described elsewhere. The ALX-0681 drug substance formulation buffer (D-PBS, 0.2M Glycine, 0.02% Tween 80, pH 7.1 ± 0.1) was used as vehicle in the study. The TTP-inducing monoclonal antibody 3H9 was produced and provided by the Katholieke Universiteit Leuven.

Animals

Housing, treatment, care and final protocol were approved by the Interfaculty Control Committee for Animal Experimentation of the University of the Free State (Bloemfontein, South Africa) in accordance with South African National Standard for the Care and Use of Animals for Scientific Purpose (SANS 10386). The animals (wild caught baboons supplied by Grootfontein Boerdery) were all male, weighed between 7.8-14.6 kg (average: 10.4 ± 2.1 kg), and were housed in the holding area on the west Campus of the University of the Free State in standard housing cages. An acclimatisation period of 14 days was applied before study start. Animals were fed dry formulated primate pellets supplied by AquaNutro (formulated by the University of Stellenbosch) for the duration of the experiment, and had free access to water. Animals were weighed daily and monitored twice daily for signs of TTP or discomfort (fever, lethargia, decreased feeding, bleeding or bruising, and blood in urine) and observations were noted on the Animal Welfare sheet. No symptoms were observed that warranted euthanasia or treatment interruption in any of the study animals. At the end of the study animals selected for postmortem analysis and histopathological evaluation (four animals of the therapeutic group and one control animal) were euthanized by pentobarbitone overdose.
Study design

This study combined a preventive and a therapeutic arm. The overall study outline is indicated in Table 1.

The study consisted of 3 groups (4 baboons per group, 12 baboons in total): (i) Control animals received daily vehicle and 3H9 at 48 hours intervals until day 9. These animals served as controls for both the preventive and therapeutic study arm. (ii) Preventive ALX-0681-treated animals received 3H9 on days 1 and 3 plus daily ALX-0681 from day 1 until day 5. Potential occurrence of TTP symptoms after withdrawal of ALX-0681 treatment was monitored from day 5 to day 11 every 48 hours. (iii) Therapeutic ALX-0681-treated animals received 3H9 on 48 hours intervals from day 1 to day 9. During the first 4 days, these animals were also daily injected with vehicle. Induction of TTP was confirmed by platelet count and haptoglobin testing. From day 5 onward, these animals received a daily dose of ALX-0681 for the remaining days.

In order to yield groups with comparable mean body weights, platelet counts and vWF levels, study animals were stratified into one of the 3 study groups based on prescreening results during the acclimatization period (Supplemental Table S1). The randomization of treatment status for each group was performed by sealed paper drawing. Animals were identified by cage number throughout the study.

Injections and blood sampling

Injections of the compounds (ALX-0681, vehicle and 3H9) and blood sampling were performed under anaesthesia (ketamine-hydrochloride intramuscular; 1 mg/kg every 30 minutes). Daily blood sampling was done by venipuncture from the femoral vein, using a 21 G Vacutainer blood collection set (Becton Dickinson). Injections were done subcutaneously (s.c) in the chest area (ALX-0681 or vehicle; approximately 2 minutes before blood sampling) or in the femoral vein (3H9; immediately after blood sampling). The 3H9 antibody was administered by an intravenous bolus injection of 600µg/kg in phosphate buffered saline every 48 hours. ALX-0681 was administered by a daily s.c. injection at a 2.5 mg/kg dose. Vehicle was administered s.c. using the same dosing volume compared to ALX-0681. Individual dosing volumes were based on the daily measurement of body weight. Animals were euthanized by pentobarbitone overdose (200 mg/kg).
**Blood analysis**

The following blood parameters were determined by automated and standardized methods in the National Health Laboratory Service Tertiary Laboratory (Universitas Hospital, Bloemfontein, South Africa): full blood count, LDH, haptoglobin, urea, creatinin, troponin-T, Factor FVIII clotting activity (FVIII:C) and DIC screen (Disseminated Intravascular Coagulation screen). Schistocytes were counted by an expert hematologist. Samples were blinded and counting was done manually.

von Willebrand Factor antigen (vWF:Ag) and total active ALX-0681 plasma concentrations were determined by validated ELISA assays. Ristocetin cofactor activity (RICO) was determined using a PAP-8E platelet aggregometer (Chrono-log 4 channel aggregometer, Kordia).

Other blood parameters were measured using ELISA kits for human markers: ADAMTS13:Ag (Technozym ADAMTS-13 Antigen ELISA Kit, Technoclone), ADAMTS:Act (Technozym ADAMTS-13 Activity ELISA Kit, Technoclone) and brain injury markers NSE (neuron specific enolase; ALPCO Diagnostics) and S100B (DRG International). These kits were validated before sample analysis, by analyzing parallelism and by monitoring precision and accuracy of calibrators, validation/QC samples and individual baboon samples (data not shown). Although the assays were found fit-for-purpose, data should be interpreted semi-quantitatively since the baboon plasma levels were determined against a calibrator curve derived from human plasma.

**Brain CT scans**

All animals of the therapeutic study arm were subjected to a brain CT scan on day 4, day 7 and day 11, under anaesthesia (ketamine-hydrochloride intramuscular; 1 mg/kg every 30 minutes or as needed). A CT brain scan was performed on a General Electric HD 750 CT Scanner. 5mm axial images were performed throughout the brain including the posterior fossa up to the cortex. These images were interpreted on an Advantage Windows Workstation (General Electric) by a staff radiologist skilled in interpreting brain injury and brain bleeding. If present, intracranial bleeding is represented by high density areas in the CT of the brain, and bleeding lesion classified as sub-dural, extra-dural, sub-arachnoid and intracerebral bleed.
Post-mortem analysis

Internal bleeding was evaluated through post mortem analysis by macroscopic examination and histopathology assessment of selected organs (major parenchymas). Only the animals of the therapeutic group and one control animal were evaluated. Histopathological evaluation of aggregates was included in these animals, as described previously. Briefly, wedges of lung, heart, brain, kidney, and spleen were dissected and fixed in 10% buffered formaldehyde for 24 hours. A Tissue Tek mictrotome/cryostat device (Bayer Healthcare) was used for processing and embedding. Hematoxylin and eosin staining was performed by standard techniques. Staining for vWF and platelets was done with a polyclonal anti-VWF (P0226) and the mAb antihuman glycoprotein IIIa clone Y2/51 (M0753), respectively, both peroxidase labelled (Dako Denmark). 3-3-Diaminobenzidine was used as a chromogenic substrate.

Statistical analysis

Separate models were constructed for the first period (day 1 up until day 5) and the second period (day 6 up until day 11). Based on the PK-profile of ALX-0681 in the animals, it was chosen to perform comparisons between groups when ALX-0681 was at steady state. Therefore, in the first period the comparison was done on days 3, 4 and 5, in the second period on days 9, 10 and 11. All analyses were performed in SAS version 9.2 (SAS Institute Inc., Cary, NC).

In the first period, the control animals (n=4) and the therapeutic treated animals (n=4) were pooled in one group for statistical analysis, since these animals were treated equally in that period. Consequently, the preventive treated group was compared to this pooled group during the first period. In the second period, comparisons were done compared to the control group.

The variable RICO was converted to categorical as the RICO was either considered as completely suppressed (RICO ≤ 20%) or not. The RICO results were analyzed with a χ² test, for the first and second period separately. Troponin-T was analysed by non-parametric statistics as the residuals were non-normally distributed. In the first period the Wilcoxon test was used and in the second period the Kruskal-Wallis test. All other variables were analysed with a mixed model having a random intercept per animal. For the fixed effects in the mixed model, the model selection started off from the model that includes day, treatment and
day\*treatment. Because in the mixed models a profile analysis without assumptions on the shape of the evolution over time was performed\textsuperscript{29}, day was entered in the model as a categorical variable. When not significant, the effect day\*treatment was removed. A post-hoc test with Tukey multiple comparison adjustment\textsuperscript{30} assessed which groups specifically differed and at which point in time. Log-transformation of the response was applied when needed to achieve normal distribution.
Results

3H9 completely neutralizes ADAMTS13:Act

Injection of 3H9 every 48 hours resulted in a rapid and sustained suppression (below LLOQ of 4.4%) of ADAMTS13:Act in all animals (Figure 1A). Remarkably, in the animals of the prevention study group who received 3H9 only twice (day 1 and day 3), ADAMTS13:Act remained inhibited for the entire study period, suggestive of a long half-life of the monoclonal antibody. Concomitantly, ADAMTS13:Ag levels moderately decreased in all study animals as well (Figure 1B), indicating partial clearance by injection of 3H9.

ALX-0681 prevents and treats the acute early episodes of acquired TTP in baboons

Inhibition of ADAMTS13:Act resulted in a rapid onset and pronounced development of thrombocytopenia and schistocytic hemolytic anemia in the control animals (Figure 2A-D). These major hallmarks of acquired TTP were confirmed by a fast and persistent suppression of platelet count and haptoglobin levels (marker of intravascular hemolysis), and increases in red blood cell fragmentation (schistocytes) and LDH levels (marker of tissue damage and hemolysis) (Figure 2A-D). Also red blood cell counts and hemoglobin concentrations were decreased as a result of TTP induction by 3H9 (Supplemental Figure S1). There were no signs of kidney failure (creatinin and urea) or brain injury (NSE or S100B) in any of the study groups (Supplemental Figure S2). There was one control animal with pronounced increases in troponin-T levels, suggesting myocardial infarction. This finding was confirmed by post-mortem analysis showing signs of myocardial damage (tissue necrosis) in this animal.

Prophylactic administration of ALX-0681 not only prevented the rapid onset of thrombocytopenia after 3H9 injection, but also schistocytosis, intravascular hemolysis and increases in LDH levels were completely and significantly prevented (Figure 2A-D). The decrease in red blood cell counts and hemoglobin concentrations was also avoided in the preventive study group (significant difference compared to control animals on day 9 and day 11 (Supplemental Figure S1). In parallel with the sustained suppression of ADAMTS13:Act in these animals, a decrease in platelet count could still be observed after treatment cessation (Figure 2A).

In the therapeutic arm of the study, induction of TTP was first confirmed on day 5 before initiating ALX-0681 treatment. A similar decrease in platelet count and haptoglobin levels,
and comparable increases in schistocyte count and LDH levels were noted in the animals of the therapeutic study group compared to control animals until day 5 (Figure 2A-D). Daily injections with ALX-0681 were started on day 5 and were followed by an immediate and steep increase of the platelet count. Predose platelet counts were reached on day 9 followed by an overshoot on day 11 (+80% mean increase on day 11 compared to day 1 counts). Also the induction of the schistocytic hemolytic anemia could be effectively treated with ALX-0681, as shown in Figure 2B-D. Further increases in schistocyte count after day 5 were entirely blocked. A trend towards normalization of red blood cell fragmentation could be noted, supported by significant differences between control and treated animals on day 9, 10 and 11 (Figure 2C). Along the same line, haptoglobin concentrations started to normalize in the therapeutic study group from day 10, together with significantly lower LDH concentrations from day 9 compared to control animals (Figure 2B,D). Hemoglobin and RBC count were similarly decreased in the therapeutic animals compared to the control animals (Supplemental Figure S1). Most probably the follow-up period was too short to observe a reversal of these parameters since haptoglobin concentrations started to recover only from day 10.

Immunohistochemical scoring of occluded vessels in the kidney, heart, spleen brain and lung of the animals of the therapeutic study group and one control animal confirmed the presence of vWF- and platelet-rich thrombi. A similar occlusion score was observed in treated animals compared to the control animal (Table 2). Occlusions were most prominently observed in the brain but also in the heart, kidney and spleen, but not in the lungs. These data suggest that ALX-0681 treatment was not able to dissolve already formed thrombi in the vessels, but rather prevents the progressing formation of aggregates as would be expected from the preventive mode of action. In fact, in vitro flow chamber data indeed showed that ALX-0681 completely blocked ULvWF-mediated platelet string formation when added to the platelet suspension prior to perfusion over stimulated endothelial cells (Supplemental Method and Supplemental Figure S3). However, ALX-0681 was not able to detach these platelets if they were allowed to form platelet strings prior to ALX-0681 addition (Supplemental Figure S3).
Pharmacodynamic markers and PK profile of ALX-0681

The prevention or reversal of the clinical signs of acquired TTP fully correlated with suppression of the pharmacodynamic marker RICO, as shown in Figure 3. RICO activity was suppressed (below the pharmacological threshold of 20%) in both treatment groups the day following the first administration of ALX-0681. In addition, the PD marker recovered above threshold level after treatment cessation from day 7 onward in the preventive study group. The latter is in line with the observed ALX-0681 plasma concentration-time profiles (Table 3).

There was a significant decrease in FVIII:C and vWF:Ag levels upon repeated administration of ALX-0681 in both the preventive and therapeutic study group (Figure 4A-B). The levels quickly returned to predose levels after stopping ALX-0681 treatment. The changes in FVIII:C and vWF:Ag concentration were manageable in terms of coagulation, since other markers for coagulation (PT, aPTT and TT) were all within normal ranges (Supplemental Table S2).

ALX-0681 treatment is not linked with bleeding events

Bleeding related to ALX-0681 treatment of acquired TTP in the context of thrombocytopenia and neutralization of vWF activity was monitored in the therapeutic study group. There was no indication of relevant bleeding events during the in-life phase, apart from the expected bruising at injection sites and mild transient gingival bleeding. Animals were also screened for intracranial bleeding and checked for internal organ bleeding through brain CT scans and post-mortem analysis of the organs, respectively. No signs of intracranial bleeding were seen in any of the scans of the four ALX-0681 treated animals. Moreover, the absence of any intracranial bleeding was further confirmed by the absence of any macroscopic and/or histological observation of bleeding in any of the organs of the animals examined.
Discussion

The formation of platelet-rich thrombi in the microvasculature is a key characteristic of TTP patients due to an acquired deficiency of ADAMTS13. In the absence of ADAMTS13, the processing of ULvWF multimers is impaired and leads to the spontaneous interaction of vWF and platelets. This enhanced platelet aggregation can cause severe organ damage and long term neurologic disorders and is linked with pronounced morbidity and mortality. Given the crucial role of this abnormal interaction of vWF with platelets in the pathophysiology of TTP, targeting the vWF A1 domain and preventing the formation of these thrombi offers an attractive new therapeutic concept for the treatment of acquired TTP.

A preclinical baboon model has been described that mimics the acute early episodes of acquired TTP. This model provided evidence for a direct relationship between the induction of TTP and ADAMTS13 deficiency, leading to the inability to process ULvWF and the formation of platelet-rich aggregates responsible for the clinical symptoms of acquired TTP. In line with this earlier report, our herein presented data show that administration of the ADAMTS13 inhibiting monoclonal antibody 3H9 resulted in a similar rapid onset of early acute episodes of acquired TTP. The pathophysiology of TTP in this preclinical model seems to differ to some extent from the human situation, as ADAMTS13 deficiency alone suffices to induce TTP in these animals. Additional exogenous triggers are hypothesized to accelerate and aggravate disease progression in humans, possibly leading to a more refractory disease, even though the exact triggers are not always known. Moreover, other typical clinical manifestations of TTP like neurologic symptoms and renal failure are not reported in this model, suggesting a mild expression of the disease. However, it has previously been suggested that the initial symptoms of TTP patients at presentation may be variable as well, and that not all patients present with neurological symptoms or renal failure. Therefore, this preclinical baboon model is a very suitable model to test the potential of new treatment opportunities for acquired TTP.

The antithrombotic Nanobody has previously been shown to inhibit the interaction between normal-sized vWF multimers and platelets in nonclinical and clinical studies. Based on its mechanism of action, it is hypothesized that ALX-0681 is also able to prevent the ULvWF-
mediated formation of platelet-rich microthrombi. The efficacy of ALX-0681 for targeting ULvWF and spontaneous platelet aggregation was therefore investigated in a baboon model of acquired TTP. The prophylactic effect of ALX-0681 seen in the current experiments on the induction of thrombocytopenia and schistocytic haemolytic anemia strongly supports the concept that ULvWF is a promising target to mediate the pathophysiology of TTP. The tendency towards thrombocytopenia and hemolytic anemia after cessation of ALX-0681 treatment further confirms the target-specific effect of ALX-0681 considering that the disease-inducing antibody 3H9 is expected to remain in circulation for a longer time. In the clinical situation treatment duration for ALX-0681 therapy is tailored with the disposition of anti-ADAMTS13 inhibitors via plasma exchange in mind.

The introduction of plasma exchange has dramatically reduced the mortality rates for TTP patients. However, the condition still carries a significant risk of mortality (10% to 30%) and morbidity, which is related to the high risk of complications linked to the procedure.\textsuperscript{16,17,19,41} Given this sustained level of mortality in the treatment of TTP, ALX-0681 offers a novel approach towards treating the disease and can reduce the need for plasma exchange. In fact, plasma exchange removes the auto-antibodies against ADAMTS13 as well as the ULvWF multimers, while simultaneously supplying ADAMTS13, but does not provide direct pharmacological targeting of the pathophysiology of TTP, being ULvWF-mediated platelet aggregation. A clinical study with the anti-vWF aptamer ARC1779, which neutralises the vWF A1 domain as well, has provided a preliminary proof-of-concept of the novel approach to target the A1 domain in the treatment of TTP.\textsuperscript{25} So far, only limited data are available showing clinically significant improvements of platelet counts and LDH levels in some but not all TTP patients treated with ARC1779.\textsuperscript{24} Given the higher potency and superior pharmacokinetic properties of ALX-0681 over ARC1779, it is expected that ALX-0681 shows a more favourable effect towards inhibition of ULvWF-mediated platelet aggregation. Data reported in this study indicate that treatment with the anti-vWF Nanobody rapidly reversed the pronounced drop in platelet count, reaching baseline levels after 4 days of treatment. Also the schistocytic haemolytic anemia was immediately stopped upon initiation of ALX-0681 treatment together with a significant normalization of LDH levels, which perfectly correlated with full neutralization of vWF activity. As a consequence, these data indicate that ALX-0681 has the potential to inhibit the progressing formation of platelet-rich
thrombi in the microvasculature. Also its marked effect on LDH and haptoglobin levels highlights the potential benefits on reducing ischemic and haemorrhagic complications.

In the clinical setting, early onset of treatment with ALX-0681 and sufficient treatment duration needs to be considered. The anti-vWF Nanobody does not eliminate the anti-ADAMTS13 antibodies as the root cause of idiopathic TTP, nor is it expected to dissolve platelet-rich ULvWF aggregates based on the vessel occlusion data and the in vitro perfusion data. It is anticipated that ALX-0681 may significantly reduce the time to normalization of platelet counts and number of plasma exchange sessions to achieve remission, and therefore may subsequently reduce the risk of complications linked to the plasma exchange procedure (systemic infections, catheter obstruction or insertion complications, hypotension, venous thrombosis).17 The inhibition of microvascular thrombosis is expected to reduce organ dysfunction, accelerate organ recovery and improve the long term neurological disorders.42 It may also provide an interesting alternative for immunsuppressive treatment, which is traditionally initiated for refractory or relapsing patients.18 Together, these therapeutic benefits of ALX-0681 in addition to plasma exchange are expected to largely impact on the quality of life of TTP patients.

Not only efficacy but also safety of ALX-0681 treatment for the acute onset of TTP was monitored in this study. The most relevant safety concern of the anti-vWF compound ALX-0681 in the treatment of TTP is an elevated bleeding risk in the context of low platelet counts and reduced vWF activity. Although this study was not adequately powered to fully address the safety profile of ALX-0681, the current data showed no bleeding events in any of the animals of the therapeutic group which would exclude a severe bleeding risk of this compound. These observations are further confirmed by a lower bleeding potential of ALX-0681 in a preclinical surgical bleeding model compared to other marketed antithrombotics21 and by the absence of spontaneous significant bleeding in phase I trials performed with ALX-0681.39,40 Repeated administration of ALX-0681 also resulted in significant decreases of vWF:Ag and FVIII:C concentrations, which were rapidly normalized upon treatment cessation. Mild and transient reductions in FVIII and vWF were also observed in single or multiple dosing phase I trials with the anti-thrombotic Nanobody.39,40 These changes can be attributed to the pharmacology of ALX-0681.21 PK simulation has pointed towards a change in the elimination rate of the ALX-0681-vWF-FVIII complex compared to the clearance of
vWF alone. Importantly, the observed reductions in vWF and FVIII correspond to the range of vWF levels in patients with type 1 or type 2 vWD. These patients typically have 15-50% of normal vWF plasma levels with proportional decreases in FVIII concentrations, and generally present mild bleeding symptoms. It has to be noted that TTP patients present with elevated vWF serum concentrations, a reduction to normal levels can support the beneficial effect of ALX-0681. Overall, these data suggest that ALX-0681 treatment has a manageable safety profile in terms of clinical bleeding risk, even in conditions of thrombocytopenia and full neutralization of vWF activity.

In conclusion, this study in a preclinical baboon model of acquired TTP convincingly demonstrated that ALX-0681 can prevent and treat the acute early episodes of acquired TTP in baboons, by targeting the vWF-platelet interaction. Full neutralization of vWF-activity by ALX-0681 resulted in efficacious treatment of the most important hallmarks of TTP being severe thrombocytopenia and schistocytic haemolytic anemia. Moreover, no signs of an excessive bleeding risk related to ALX-0681 treatment were noted. Together, these preclinical data further strengthen the promising novel concept of inhibiting ULvWF-mediated platelet aggregation as add-on treatment for TTP patients, and fully support the ongoing phase II clinical trial assessing the efficacy and safety of ALX-0681 as adjunctive treatment to patients with acquired TTP.
Acknowledgements

The authors thank Jasper Jacobs, Sarah De Pauw and Kris Cosyns for the technical support during sample analysis, and Antoine Thomas for supplying and characterizing ALX-0681 and formulation buffer. We are also grateful to Dr. Elson Mberi for counting schistocytes, Prof. Curt De Vries for performing and interpreting the brain CT scans, and Dr. Ton Lisman of the Department of Hematology of the UMC Utrecht for performing the flow chamber experiments.
Authorship Contributions

F.C. designed research, performed research, analyzed and interpreted data, and wrote the manuscript; J.R. designed research, performed research, analyzed and interpreted data and critically read the manuscript; WJvR and SL performed research; H.U., T.S., S.R., S.P. and J.-B.H. designed research and analyzed and interpreted data; W.W. analyzed data and performed statistical analysis.
Disclosure of Conflicts of Interest

The study was paid for by Ablynx N.V.
The remaining authors declare no competing financial interest.
Nanobody is a registered trademark of Ablynx N.V.
References


Tables

Table 1. Study design and dosing schedule. Overview of the timing of injections in the different study groups. 3H9: TTP-inducing monoclonal antibody; D: day; i.v.: intravenous; s.c.: subcutaneous.

<table>
<thead>
<tr>
<th>Group</th>
<th># animals</th>
<th>Administration - schedule</th>
<th>3H9</th>
<th>Vehicle</th>
<th>ALX-0681</th>
</tr>
</thead>
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<td>Control animals</td>
<td>4</td>
<td>0.6 mg/kg i.v. D1, D3, D5, D7, D9</td>
<td>Vehicle s.c. Daily (D1 to D11)</td>
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<tr>
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<td>4</td>
<td>0.6 mg/kg i.v. D1 and D3</td>
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<td>2.5 mg/kg s.c. Daily (D1 to D5)</td>
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<tr>
<td>Therapeutic ALX-0681 treated animals</td>
<td>4</td>
<td>0.6 mg/kg i.v. D1, D3, D5, D7, D9</td>
<td>Vehicle s.c. Daily (D1 to D4)</td>
<td>2.5 mg/kg s.c. Daily (D5 to D11)</td>
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Table 2. Occlusion scores for all organs. Organs were examined for occluded vessels rich in platelets (monoclonal mouse anti-human CD61) or vWF (polyclonal rabbit anti-human vWF). 10 random consecutive vessels were scored for occlusions in animals from the therapeutic study group (C4 to C7) and one control animal (D10); results are represented as percentage positive vessels. Mean (C4-C7) represents the average occlusion (%) per organ of the 4 animals in the therapeutic study group.

<table>
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<tr>
<th>organ</th>
<th>Animal C4</th>
<th>Animal C5</th>
<th>Animal C6</th>
<th>Animal C7</th>
<th>Mean (C4-C7)</th>
<th>Animal D10</th>
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<tr>
<td>Kidney</td>
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<td>0</td>
<td>0</td>
<td>10</td>
<td>2.5</td>
<td>0</td>
</tr>
<tr>
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<td>0</td>
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<tr>
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<td>10</td>
<td>10</td>
<td>0</td>
<td>7.5</td>
<td>10</td>
</tr>
<tr>
<td>Lung</td>
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<td>0</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>50</td>
<td>40</td>
<td>37.5</td>
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<tr>
<th>organ</th>
<th>Animal C4</th>
<th>Animal C5</th>
<th>Animal C6</th>
<th>Animal C7</th>
<th>Mean (C4-C7)</th>
<th>Animal D10</th>
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<tr>
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<tr>
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<td>20</td>
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<tr>
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Table 3. PK profile of ALX-0681 treated animals. Plasma concentrations of ALX-0681 (ng/mL) in animals of the preventive (animals D3 – D6) and therapeutic (animals C4 – C7) study group. BQL: below quantification limit of 20 ng/mL; D: day; ND: not determined

<table>
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<tr>
<th></th>
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<td>99.05</td>
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<td>155.87</td>
<td>204.31</td>
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<td>BQL</td>
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Figure legends

Figure 1. Inhibition of ADAMTS13 parameters by 3H9. (A) ADAMTS13:Activity (%) and (B) ADAMTS13:Antigen (µg/mL) were measured in plasma of the control (●, n=4), preventive (□, n=4) and therapeutic (▲, n=4) animals, in function of time (days). The lower limit of quantification (LLOQ) of the ADAMTS13:Activity assay was determined at 4.4%, and is indicated by the dashed line. Results were analysed with a mixed model and a post-hoc test with Tukey multiple comparison adjustment. Data are represented as mean±SD.

a significant difference between control and preventive animals (p < 0.05)

Figure 2. Preventive and therapeutic effect of ALX-0681 on the markers of thrombocytopenia and schistocytic hemolytic anemia. (A) Platelet counts (10^9/L), (B) haptoglobin levels (g/L), (C) schistocyte counts (%) and (D) LDH levels (U/L) were measured in control (●, n=4), preventive (□, n=4) and therapeutic (▲, n=4) animals in function of time (days). Results were analysed with a mixed model and a post-hoc test with Tukey multiple comparison adjustment. Data are represented as mean±SD.

a significant difference between control and preventive animals (p < 0.05)
b significant difference between control and preventive animals (p < 0.001)
c significant difference between control and therapeutic animals (p < 0.05)
d significant difference between control and therapeutic animals (p < 0.001)

Figure 3. ALX-0681 neutralizes vWF activity as shown by suppression of RICO. RICO activity was measured in plasma of the control (●, n=4), preventive (□, n=4) and therapeutic (▲, n=4) animals, in function of time (days). The lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) of the RICO assay were 10% and 145%, respectively, and are indicated by a dashed line. The pharmacological threshold is established at 20% activity, and is indicated by a dotted line. Results were analyzed with a \( \chi^2 \) test. Data are represented as mean±SD.

a significant difference between control and preventive animals (p < 0.01)
b significant difference between control and therapeutic animals (p < 0.01)
Figure 4. Repeated ALX-0681 administration reverses decreases FVIII:C and vWF:Ag concentrations. (A) FVIII clotting activity (FVIII:C, %) and (B) vWF antigen (vWF:Ag) were measured in baboon plasma of the control (●, n=4), preventive (□, n=4) and therapeutic (▲, n=4) animals, in function of time (days). Results were analysed with a mixed model and a post-hoc test with Tukey multiple comparison adjustment. Data are represented as mean±SD.

\(^a\) significant difference between control and preventive animals (\(p < 0.001\))

\(^b\) significant difference between control and therapeutic animals (\(p < 0.001\))
Figures

Figure 1

A. ADAMTS13: Activity

B. ADAMTS13: antigen
Figure 2

A. Platelet count

B. Haptoglobin

C. Schistocytes

D. LDH
Figure 3
Figure 4

A. FVIII:C

B. vWF:Ag

Controls
Preventive
Therapeutic
Evaluation of efficacy and safety of the anti-vWF Nanobody ALX-0681 in a preclinical baboon model of acquired thrombotic thrombocytopenic purpura

Filip Callewaert, Jan Roodt, Hans Ulrichs, Thomas Stohr, Walter Janse van Rensburg, Seb Lamprecht, Stefaan Rossenu, Sofie Priem, Wouter Willems and Josefin-Beate Holz