The anti-von Willebrand factor aptamer ARC1779 increases von Willebrand factor levels and platelet counts in patients with type 2B von Willebrand disease

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Summary
Blockade of hyperactive von Willebrand factor (VWF) by ARC1779 blunted the platelet drop induced by desmopressin in patients with type 2B von Willebrand disease (VWD). Thus, we hypothesised that ARC1779 may increase VWF levels and correct thrombocytopenia. Three thrombocytopenic patients suffering from type 2B VWD received a loading dose of 0.23 mg/kg ARC1779 followed by 4 μg/kg/min intravenously for 72 hours in a prospective clinical trial. ARC1779 was well tolerated and safe. Plasma concentrations of ARC1779 increased to 76 μg/ml (59–130) leading to an immediate decrease of free VWF A1 domains. VWF/FVIII levels increased as early as 12 h after start of infusion, peaked near the end of infusion, and returned to baseline at follow-up.

VWF ristocetin cofactor activity (VWF:RCo) showed a median 10-fold increase 8 hours after end of infusion, while the median VWF-antigen and FVIII increase was less (5-fold and 4-fold, respectively). Most importantly inhibition of hyperactive VWF rapidly increased platelet counts from 40x10⁹/l (38–58 x10⁹/l) to a maximum of 146 x10⁹/l (107–248 x10⁹/l). In conclusion, ARC1779 markedly increases VWF/FVIII levels and most importantly improves or even corrects thrombocytopenia in VWD type 2B patients. This underscores the in vivo potency of ARC1779.

Keywords
von Willebrand disease, thrombocytopenia, aptamer

Introduction
Von Willebrand disease type 2B (VWD type 2B) is a rare disorder which is caused by gain of function mutations of VWF(1). The molecular defects in VWD type 2B enhance the affinity of VWF for glycoprotein Ib which results in increased platelet binding (2). The mutant VWF molecules spontaneously bind to normal platelets, and the high-molecular-weight multimers are digested by the metalloprotease ADAMTS-13 (3) to form inactive proteolytic fragments (4). As a consequence, VWD type 2B patients become deficient in both large VWF multimers and platelets, which deficiencies are established risk factors for bleeding in VWD type 2B (5). As with other types of VWD, spontaneous mucosal bleedings occur (e.g. epistaxis, menorrhagia, or gastrointestinal bleeding), and severe bleeding can result from trauma, surgery, or childbirth.

Aptamers are a new class of oligonucleotide drugs (6) that, similar to antibodies, are able to block protease activity and/or protein-protein interactions, including those with pro- or anticoagulant functions. ARC1779 is a synthetically manufactured aptamer which binds to the A1 domain of human VWF with high affinity, preventing interaction with platelet GpIb; the crystal structure of its parent compound has recently been characterised (7). The core aptamer portion of ARC1779 (molecular weight, ≈13.7 kDa without pegylation), is a 40-mer modified DNA/RNA oligonucleotide, which is conjugated with a 20-kDa polyethylene glycol moiety to form the active pharmaceutical ingredient, ARC1779 (molecular weight, ≈33 kDa) (8). The potent anti-thrombotic activity of ARC1779 has been demonstrated in vivo in a cynomolgus monkey carotid electrical injury thrombosis model (9). ARC1779 dose-dependently prolongs the bleeding time, although to a lesser degree than abciximab (9). Thus, similar to other VWF antagonists it has little effect on the bleeding time and may potentially confer a lower risk of bleeding than other classes of anti-thrombotic agents (10).

Based on the demonstration that ARC1779 prevented the desmopressin induced drop in platelet counts and enhanced VWF levels after desmopressin infusion in VWD type 2B patients (11), we hypothesized that ARC1779 would correct the thrombocytopenia and increase VWF levels by inhibiting excessive VWF activity (12) in patients with VWD type 2B.
Methods

Study design

The Ethics Committee of the Medical University of Vienna and the National Competent Authority approved the trial protocol, and the trial was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients before study entry. Thrombocytopenic patients with type 2B VWD were eligible: in 2010 we enrolled three patients with VWD type 2B into this clinical trial, one male (aged 48 years) and two females (aged 53 and 58 years). Patients were identified based on their medical history of typical VWF multimer patterns in combination with exaggerated platelet-induced aggregation in response to low concentrations of ristocetin, a combination of findings which has been used to diagnose this disease (13). Inclusion criteria were chronic thrombocytopenia <100 x 10^9/l and adequate contraception, and exclusion criteria were pregnancy and co-existing conditions which are associated with an increased risk of intraparenchymal bleeding (e.g. recent major surgery). The infusion regimen was based on previous studies (14), including one that demonstrated an increase in platelet counts in patients with congenital TTP (15). We estimated that the free A1 domains could be blocked with a continuous infusion of 4 μg/kg/minute (min). Patients received a 30-min infusion (0.23 mg/kg) plus a 72-hour (h) continuous infusion of 4 μg/kg/min ARC1779 injection (total of 0.47 mg/kg). Blood samples were taken before ARC1779 infusion (time point: 0 h), then every 12 h until the end of infusion, and then 96 h and 192 h after the start of infusion. For platelet counts and VWF indices additional blood samples were collected at 2 h intervals after end of infusion until 84 h.

Laboratory methods

Samples for analysis were obtained into evacuated tubes containing 129 mM (3.8%) citrate (Vacuette tubes; Greiner Bio-One, Kremsmunster, Austria). Platelet counts were analysed in the hospital’s clinical laboratory with a cell counter (Sysmex XE 2100, Kyoto, Japan). Blockade of the A1 domains of VWF was measured with a quantitative direct ELISA kit (REAADS VWF Activity ELISA Test Kit, Corgenix, Inc, Westminster, CO, USA). (8) This ELISA utilises a purified murine anti-VWF monoclonal antibody which recognises a functional epitope on the VWF molecule to assess VWF activity levels. Results are reported in percent (%) of normal, relative to a calibrator that has been standardised against the third International Standard for Factor VIII and von Willebrand Factor in Plasma (91/666). Intra-assay and inter-assay variability are <6% and <8%, respectively. The detection limit was <3%, when this was reached the level of inhibition is set to 97% to provide a conservative estimate.

ARC1779 plasma concentrations were determined with a validated high-performance liquid chromatography assay with ultraviolet detection (linear range – 0.25 to 200 μg/ml) (8, 16). This assay measures all ARC1779 in plasma. A collagen binding assay was used (enzyme immuno assay with collagen type I from Hemochrom Diagnostica GmbH, Essen, Germany) (11). VWF antigen concentration was measured with a fully automated simultaneous thermal analyser using the STA Liatest VWF (Diagnostica Stago, Paris, France). Plasma activity of VWF ristocetin cofactor activity (VWF:RCo; primary endpoint) was assayed by turbidimetry using a commercial kit (BC von Willebrand reagent; Dade Behring, Marburg, Germany) (17). In contrast to the REAADS assay, VWF:RCo cannot be used to quantify the levels or activity of the aptamer, because the aptamer directly binds to ristocetin. The platelet function analyzer PFA-100, as described in more detail elsewhere (18, 19), was used to assess primary haemostasis because closure times are highly sensitive to the deficiency of high-molecular-weight VWF in VWD (18, 20), and are inversely related to inhibition of VWF in the REAADS assay (8). Platelet aggregation was measured with multiple electrode aggregometry (Roche Diagnostics, Munich, Germany) (21) using the following concentrations of agonists: ADP (6.4 μM), collagen (3.2 μg/ml), arachidonic acid (0.5 mM), and TRAP (30 μM).

Factor VIII (FVIII) activity was measured on a Sysmex CA 7000 analyser using FVIII-deficient plasma (Hyland Baxter Immuno, Vienna, Austria) and Dade® Actin®-FS (Dade Behring) in a one stage clotting assay. Quantification of VWF multimers was performed by sodium dodecyl sulphate-agarose discontinuous gel (1.2%) electrophoresis (22) followed by Western-Blotting and consequent quantification with a luminescence image analyser (LAS-3000, Raytest GmbH, Berlin, Germany). Final analysis was performed using AIDA Image Analyzer software version 4.11. Rotational thrombelastometry was carried out as previously described (23). Blood samples were spiked in vitro with ARC1779 concentrations of 30–300 μg/ml or unfractionated heparin (0.25, 0.5, and 1 U/ml) as a positive control to rule out anticoagulant effects of ARC1779 in the presence of an unspecific aptamer induced prolongation of aPTT. Activated partial thromboplastin time

Table 1: Demographics and laboratory parameters at baseline.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age (years)</th>
<th>PLT x 10^9/l</th>
<th>VWF:Ag (%)</th>
<th>VWF:RCo (%)</th>
<th>VWF: CBA (%)</th>
<th>FVIII:C (%)</th>
<th>CACP-CT (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>53</td>
<td>38</td>
<td>10</td>
<td>32</td>
<td>3</td>
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<td>&gt;300</td>
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<tr>
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<td>40</td>
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<td>45</td>
<td>19</td>
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<td>&gt;300</td>
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<tr>
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<td>58</td>
<td>66</td>
<td>26</td>
<td>38</td>
<td>43</td>
<td>&gt;300</td>
</tr>
</tbody>
</table>

Ag, antigen; CACP-CT, collagen adenosine diphosphate closure time; CBA, collagen binding activity; FVIII:C, factor VIII activity; PLT, platelets; RCo, ristocetin cofactor activity; VWF, von Willebrand factor.
(aPTT) was determined with reagents of Diagnostica Stago, Asnieres, France (STA APTT and STA Fibrinogen, respectively) (24, 25) on a STA-R Evolution analyzer (Diagnostica Stago).

Sample size and statistical analysis

Simulations of trial results indicated that a minimum of three patients would be needed for the primary endpoint (change in platelet count). While descriptive statistics were used primarily, data were also log transformed for further statistical analysis by repeated measures of variance analysis and the LSD test was used for post-hoc comparisons.

Results

Baseline characteristics

All patients had moderate thrombocytopenia (Table 1; Fig. 1), low levels of VWF:RCo, VWF:CBA, and FVIII:C (Fig. 2) and prolonged collagen adenosine diphosphate closure times with the PFA-100 (>300 seconds [s]). Patients were seropositive for hepatitis C, and 2/3 patients had evidence of chronic hepatitis C. All patients had an extensive history of severe bleedings.

ARC1779 was well tolerated without relevant adverse events in any of the patients. One of the patients suffered from active chronic gastrointestinal bleeding from angiodysplasias before start of ARC1779. While bleeding continued during infusion of ARC1779, ARC1779 did not visibly enhance bleeding. All patients completed the trial.

Pharmacokinetics of ARC1779 and blockade of the A1 domains of VWF (REAADS)

Patients received a bolus-primed (0.23 mg/kg bolus) continuous infusion of ARC1779 which increased ARC1779 plasma concentrations to 12 µg/ml after 1 h ( Fig. 1), 50 µg/ml after 24 h, and peaked at 76 µg/ml (59–130) at the end of infusion. This equals 3,500 nM or a 100-fold molar excess of ARC1779 as compared to VWF when a molecular weight of 225 kD for mature VWD (26) is assumed.

ARC1779 decreased free VWF A1 domains to below limits of detection (<3%) in one patient who had baseline levels of 13%, in the other patients whose baseline levels were 62–70%, 7–28% remained during continuous infusion (Fig. 1).

Consistent with a normalisation in the half-life of VWF, VWF/FVIII levels increased as early as 12 h after start of infusion, peaked near the end of infusion, remained at a plateau as long as free A1 domains were blocked, and had returned to baseline at subsequent follow-up (Fig. 2). That was best exemplified by VWF:RCo levels which increased from 32% (26–45%) 10-fold to 367% (215–418%) 8 h after end of infusion, and decreased again to 33% (20–40%) during follow-up. Similarly, VWF:Ag levels increased 5.2 fold (3- to 27-fold), and FVIII levels 4.3 fold (3- to 6-fold) and ARC1779 also normalised large VWF multimers (Fig. 3). Most importantly inhibition of hyperactive VWF rapidly increased platelet counts from 40 x10^9/l (38–58 x10^9/l) by 70% within 12 h and to a maximum of 146 x10^9/l (107–248 x10^9/l). This was observed although patients were suffering from chronic liver disease due to hepatitis C infection (2/3) or associated immune thrombocytopenia (1/3). Hence, these co-existing diseases did not appear to be the major cause of thrombocytopenia in these patients. Repeated measures ANOVA showed that all changes were statistically significant (p<0.007).
aPTT, Platelet function analyzer PFA-100 and whole blood aggregometry

aPTT values increased from 39.6 s (35.6–40.9 s) to 44.3 s within 1 h, and to 76 s (69–89 s; p<0.01) at 12 h, remained at that level until the end of infusion, and returned to 34.8 s (34.0–35.6 s) after stop of infusion. As expected (27), basal collagen adenosine diphosphate-induced closure times were >300 s in all patients, and consistently remained in that range, because ARC1779 prolongs closure times to >300 s (14, 28). ARC1779 did not decrease platelet response to various agonists as assessed by whole blood aggregometry (data not shown).

Discussion

This is the first clinical trial with a pharmacologic approach to increase platelet counts in thrombocytopenic patients suffering from type 2B VWD.

A dose-ranging trial was previously conducted in patients with congenital TTP, where the high-dose intravenous (i.v.) infusion (0.004–0.006 mg/kg/min) was most effective (15); on the basis of those results the loading dose of 0.23 μg/kg, the rate of continuous infusion (4 μg/kg/min) and duration of ARC1779 infusion for 72 h were chosen for use in this trial. Plasma concentrations of ARC1779 were comparable between the previous trial in TTP patients (15) and the current one. In previous studies when ARC1779 was infused in healthy volunteers (8), in patients with type 2B VWD (11), or in patients with acute TTP (29) the half-life of ARC1779 was approximately 2 h. In the current study, ARC1779 levels continually increased in samples taken from the subjects from 12 h to 72 h during continuous infusion (Fig. 1). Thus, the time needed to reach steady state ARC1779 concentrations suggests an increase in half-life to 4–8 h when higher infusion rates (4 μg/kg/min) are used. This is consistent with the pharmacokinetics observed in a recent trial in patients with congenital TTP who received ARC1779 at identical infusion rates (15).

Free A1 domains were reduced by ARC1779 within 1 h, blocking the clearance of VWF that is characteristically accelerated in VWD type 2B (30). ARC1779 steadily increased plasma levels of VWF:Ag, RCo, FVIII:C until 84 h (Fig. 2). A steady state can be expected for these proteins after approximately 4–5 half-lives when these endogenous proteins are continuously released into the circulation. Thus, the increase in VWF over 72 h is consistent with five half-lives of normal VWF (16 h according to Casonato et al. [31]), and thus a normalisation in the half-life of VWF in the circulation. Plasma concentrations of VWF, FVIII:C and platelet counts still increased for several hours after cessation of the infusion. This likely indicates that effective drug concentrations were maintained for several hours despite discontinuation of study drug.

One may ask for the reason of the strong increase in VWF. Patients suffering from VWD type 2B produce normal amounts of VWF as measured by VWF propeptide levels but show a markedly shortened half-life of VWF after desmopressin infusion (31), which, however, is normalised by concomitant infusion of ARC1779 (11).

Initial experiments suggested that VWF sorting to WPB in endothelial cells is a very inefficient process with only 5% to 10% of newly synthesised VWF being sorted, the rest (90%-95%) being secreted through the constitutive secretory pathway (32). In contrast, a study based on the analysis of VWF multimers concluded that constitutive secretion of VWF was insignificant (33), which is consistent with the high multimers seen in healthy individuals.

Along these lines, a significant proportion of newly synthesised VWF is retained within HUVECs for at least 24 h, and most mature VWF spontaneously released into the medium by non-stimulated HUVECs is not secreted by the constitutive secretory pathway.
This basal secretion is a non-stimulated secretion of regulated secretory cargo which has been described for other molecules before (35, 36). Alternatively, and not mutually exclusively, ARC1779 could work as a carrier molecule for VWF.

Termination of ARC1779 infusion swiftly increased free A1 domains (Fig. 1) and a reciprocal decrease of VWF:RCo levels (Fig. 2) occurred after 24 h. In contrast, VWF:Ag was still elevated and FVIII was in the normal range 24 h after cessation of infusion.

The different behaviour of the VWF:RCo and REAADS assays measuring free A1 domains (Fig. 1) and VWF:RCo levels (Fig. 2) occurred after 24 h. In contrast, VWF:Ag was still elevated and FVIII was in the normal range 24 h after cessation of infusion.

The different behaviour of the VWF:RCo and REAADS assays measuring free A1 domains needs some explanation. Free A1 domains and VWF:RCo levels correlate reasonably well in healthy subjects and patients with acute myocardial infarction (14). However, there is one important difference between assays. Whereas the REAADS assay is sensitive to inhibition by ARC1779 (Fig. 1), the VWF:RCo assay is not, because ARC1779 directly binds to ristocetin. Hence ARC1779 blocks free A1 domains in the REAADS assay, but not VWF:RCo. Quite on the contrary VWF:RCo increases in vitro, because consumption of hyperactive large VWF multimers is prevented by ARC1779.

As observed after infusion of other therapeutic oligonucleotides (37), ARC1779 increased the aPTT in a non-specific manner, but not clotting times measured by thrombelastometry.

Considering all necessary caveats regarding the increasing platelet counts, whole blood aggregometry suggested that blockade of VWF by ARC1779 does not negatively affect TRAP, ADP, or arachidonic acid-induced aggregation, which is in line with previous findings (14). This supports the concept that platelet counts are haemostatically competent. Results from the current study may have two potential clinical implications for patients with VWD type 2B. First, autologous platelets could be collected before elective surgery after treatment with ARC1779. Such platelets could be used peri-operatively after washing off ARC1779. However, such platelets would be expected to be cleared as rapidly as those contained in allogeneic platelet concentrates, and would therefore mainly reduce the risk for allo-immunisation or infections. Second, correction of thrombocytopenia is an unmet medical need of VWD type 2B patients: an anti-VWF strategy could be used as adjunctive treatment for hepatitis C. Antiviral therapy with interferon increases VWF levels (38, 39), as expected for an acute-phase reaction (40, 41), but induces thrombocytopenia. In fact, one of our type 2B VWD patients previously had to discontinue interferon treatment because of worsening thrombocytopenia according to the recommendations in the summary of product characteristics for peg-interferon. As ARC1779 needs to be infused, and is thus not suitable for long-term treatment, blockade of VWF by a longer acting aptamer with subcutaneous bioavailability such as ARC15105 would be needed (42, 43). This could allow the start or continuation of peg-interferon treatment in case of aggravated thrombocytopenia. However, potential usefulness of anti-VWF aptamers for VWD type 2B remains to be determined in future.

Figure 3: ARC1779 normalises VWF multimer patterns in patients with VWD type 2B. Lanes were loaded with pooled plasma (first and last lane) and patient’s plasma obtained 0, 1, 12, 24, 36, 48, 60 and 72 h after start of ARC1779 infusion (lanes 2–9, respectively). ARC1779 corrected multimer patterns within 12 h. Changes in multimer patterns were similar in the other patients with VWD type 2B (data not shown).

What is known about this topic?
- Thrombocytopenia is a contraindication for interferon therapy of hepatitis C.
- Thrombocytopenia in type 2B von Willebrand disease (VWD type 2B) is considered to be due to release of large and hyper-adhesive von Willebrand factor (VWF) multimers.
- ARC1779 is a novel potent VWF inhibitor which inhibited desmopressin-induced thrombocytopenia in VWD type 2B.

What does this paper add?
- Blockade of free A1 domains by ARC1779 increased VWF/FVIII indices four- to 10-fold in VWD type 2B.
- Blockade of VWF by ARC1779 improved or even corrected thrombocytopenia in VWD type 2B suffering from hepatitis C.
- Therapeutically, an anti-VWF aptamer could be useful as an adjuvant treatment for type 2B VWD patients suffering from hepatitis C and requiring interferon therapy. This is an unmet medical need which merits further trials.
clinical trials. Also, anti-VWF aptamers cannot replace standard therapy such as VWF concentrates or heterologous platelet concentrates for emergency treatment of spontaneous bleeding.

One possible limitation is the lack of genotyping in our patients, which has been proposed as a new standard for the diagnosis of VWD type 2B (44). Although the demographic characteristics of our patients are typical of VWD type 2B suggesting our results should be consistent in a larger patient population, it is likely that some variability in response to ARC1779 treatment may be seen between different patients.

In conclusion, ARC1779 markedly increases VWF/FVIII levels and most importantly improves or even corrects thrombocytopenia in VWD type 2B patients. This underscores the in vivo potency of ARC1779.

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Conflicts of interest
Dr. J. Gilbert was employed by Archemix Corp. None of the other authors declare any conflicts of interest.

References


