P-Selectin Inhibition Therapeutically Promotes Thrombus Resolution and Prevents Vein Wall Fibrosis Better Than Enoxaparin and an Inhibitor to von Willebrand Factor


Objective—Aptamers are oligonucleotides targeting protein–protein interactions with pharmacokinetic profiles and activity reversal options. Although P-selectin and von Willebrand factor (vWF) have been implicated in the development of venous thrombosis (VT), no studies have directly compared aptamer efficacy with standard of care in VT. In this study, ARC5692, an anti-P-selectin aptamer, and ARC15105, an anti-vWF aptamer, were compared with low–molecular-weight heparin, enoxaparin, to test the efficacy of P-selectin or vWF inhibition in promoting thrombus resolution and preventing vein wall fibrosis, in a baboon model of VT.

Approach and Results—Groups were as follows: treatment arm: animals received P-selectin or vWF aptamer inhibitors or enoxaparin (n=3 per group). Controls received no treatment (n=3). Prophylactic arm: animals received P-selectin inhibitor (n=4) or vWF inhibitor (n=3). Treatment arm: P-selectin-inhibitor demonstrated a significant improvement in vein recanalization by magnetic resonance venography (73% at day 21), and significantly decreased vein wall collagen, compared with all groups. Anti–P-selectin equaled enoxaparin in maintaining valve competency by ultrasound. All control animals had compromised valve competency post thrombosis. Prophylactic arm: animals receiving P-selectin and vWF inhibitors demonstrated improved vein recanalization by magnetic resonance venography versus controls (80% and 85%, respectively, at day 21). Anti–P-selectin protected iliac valve function better than anti-vWF, and both improved valve function versus controls. No adverse bleeding events were observed.

Conclusions—The P-selectin inhibitor aptamer promoted iliac vein recanalization, preserved valve competency, and decreased vein wall fibrosis. The results of this work suggest that P-selectin inhibition maybe an ideal target in the treatment and prophylaxis of deep VT, warranting clinical trials. (Arterioscler Thromb Vasc Biol. 2015;35:829-837. DOI: 10.1161/ATVBAHA.114.304457.)

Key Words: animal models ▪ inflammation ▪ P-selectin ▪ venous thromboembolism ▪ venous thrombosis

Deep vein thrombosis (VT) and pulmonary embolism, known collectively as venous thromboembolism, affect an estimated 900,000 people in the United States each year, resulting in ≈300,000 deaths.1 Most of those deaths occurred within 1 month of the initial diagnosis.2 Anticoagulation is the current standard treatment for the prevention of VT, VT recurrence, pulmonary embolism, and the progression of the thrombus. However, it does not lyse the thrombus or prevent the development of post-thrombotic syndrome. In addition, it carries with it significant bleeding risks, such as intracranial hemorrhage, which occurs in 1.15% patients per year with a case fatality rate (major bleeding) of 13%.3 Thus, new treatment options that limit bleeding risk, while decreasing thrombotic risk, should be investigated. Recently, it has been demonstrated that P-selectin and von Willebrand factor (vWF) can mediate events associated with VT.4,5 Both proteins are stored by platelets and endothelial cells (P-selectin is a component of the membrane and also the matrix of the α granule and the Weibel–Palade bodies), can be released from platelets and endothelial cells by prothrombotic and inflammatory factors generated on cell activation, and can be localized to the surface of the cells. The surface that expresses P-selectin and vWF can promote accumulation of plasma-derived, cell-associated procoagulant factors and cells (platelets and leukocytes) that will promote vein wall injury and thrombus formation. Previous studies have demonstrated that P-selectin and vWF...
Inhibition of P-selectin and vWF are attractive therapeutic approaches. In addition, increased levels of these molecules are being used clinically to direct treatment.8–10 In this study, we wanted to compare P-selectin and vWF inhibition by aptamers to standard-of-care low–molecular-weight heparin (LMWH) for VT to determine whether one or both would inhibit VT and promote thrombus resolution better than LMWH in a baboon model of VT (Figure 1).

Materials and Methods
Material and Methods are available in the online-only Data Supplement

Results
Anti–P-Selectin Aptamer Improved Vein Recanalization Over Anti–vWF Aptamer and Enoxaparin

Treatment Arm
Animals that were administered therapeutic anti–P-selectin aptamer (ARC5692) had a significant recanalization of 73% by day 21, versus controls. The animals that received therapeutic enoxaparin showed 0% on day 2 and a 42% vein recanalization by day 21. Of interest, the iliac veins of animals treated with the therapeutic anti-vWF aptamer (ARC15105) failed to recanalize at all by day 21. In comparison, the average percentage vein recanalization of control animal iliac veins was 0% on day 2 and only 13% by day 21 post thrombosis (Figure 2A).

Prophylactic Arm
Animals receiving both aptamers in a prophylactic protocol showed the best vein lumen recanalization of 50% at day 2, for both groups, and 80%, 85%, respectively, by 21 days post thrombosis (Figure 2A). Percentage of vein reopening was calculated using magnetic resonance venography as shown in Figure 2B.

Anti–P-Selectin Aptamer Decreases Fibrin Thrombus Content and Vein Wall Collagen
Animals receiving anti–P-selectin aptamer, both in a prophylactic and in a treatment protocol, showed a statistically significant decrease in thrombus fibrin ($P<0.05$). The anti-vWF aptamer in a prophylactic protocol showed significantly lower thrombus fibrin content (Figure 2C). Animals receiving anti–P-selectin aptamer, both in a prophylactic and in a treatment protocol, showed statistically significant decreases in vein wall collagen, versus control animals ($P<0.05$). Also, anti–P-selectin aptamer treatment primates showed significantly lower vein wall collagen when compared with treatment animals given LMWH, and the anti-vWF aptamer ($P<0.05$; Figure 2D).

Anti–P-Selectin Aptamer Did Not Decrease Circulating or Leukocyte Recruitment to the Vein Wall
Animals receiving anti-P-selectin aptamer, both in a prophylactic and in a treatment protocol, showed increases in vein wall

Nonstandard Abbreviations and Acronyms

- aPTT: activated partial thromboplastin time
- BT: bleeding time
- LMWH: low–molecular-weight heparin
- vWF: von Willebrand factor

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Figure 1. Nonhuman primate venous thrombosis model and experimental design. Schematic representation of the baloon vein occlusion technique. A, A balloon is placed and inflated in the iliac vein, just below the iliac bifurcation; another balloon is placed and inflated near the pelvic crest (yellow arrows); both balloons remain in initial position for 6 hours (T6) promoting thrombus formation. Baseline venogram; balloons inflated; hourly ultrasound follow-up; balloons removed; final ultrasound to confirm the presence of the thrombus at T6 and that a thrombus remains in a vein segment of ≈3.5 cm in length. B, Percentage vein reopening magnetic resonance venography (MRV), vein valve function ultrasound, vein lumen patency, ascending phlebography, and bleeding risk assessment coagulation tests were evaluated at each time point: days 2, 6, 14, and 21 after thrombosis. C, At day 21, after the follow-up, a harvest of the tissue for gross anatomy and histology (for leukocyte counts, quantification of percentage of thrombus fibrin, and vein wall collagen deposition) were performed.
leukocyte counts, versus control animals (polymorphonuclear leukocytes $P<0.05$ in the group receiving prophylaxis). Animals receiving anti-vWF aptamer in a prophylactic protocol and LMWH showed decreases in vein wall monocyte counts, versus control animals ($P<0.05$) and increases in vein wall lymphocyte counts, versus control animals ($P<0.05$; Figure 3A). No differences were observed in circulating leukocyte counts (Figure 3B).

**Iliac Valve Function by Ultrasound**

**Treatment Arm**

Both anti–P-selectin aptamer and LMWH given in a treatment protocol (n=3 each group) demonstrated a 33% iliac venous valve competency. All control and anti-vWF aptamer treatment animals had 0% iliac valve competency.

**Prophylactic Arm**

Ultrasound analysis demonstrated that animals given the anti P-selectin aptamer in the prophylactic protocol (n=4) had 100% iliac valve competency, at day 21, and those given anti-vWF aptamer (ARC15105) in a prophylactic manner had 67% valve competency (Figure 4).

**Contrast Venography**

Contrast venography showed complete occlusion of the iliac vein at day 2 in all animals with abundant collateral circulation. Contrast venography confirmed the magnetic resonance venography results and demonstrated that the collateral circulation disappeared by day 21 in those animals where recanalization occurred (Figure 5).

**Histology, Gross Anatomy, and Venography**

Representative corresponding vein wall histology and gross anatomy sections showed a strong correlation with the contrast venography at day 21 (Figure 6). The presence of thrombus was observed in all specimens except for the anti–P-selectin prophylaxis group, where only a small remnant of the thrombus was observed in the distal vein segment (Figure 6).

**Aptamer Effects on Coagulation**

Fibrinogen and activated partial thromboplastin time followed the same nonsignificant trends for all groups, with levels of fibrinogen and activated partial thromboplastin time peaking at 2 days post thrombosis. No adverse bleeding events occurred because of the anti–P-selectin or anti-vWF aptamers.

**Treatment Arm**

Animals receiving the anti–P-selectin aptamer and controls were within normal reference ranges. $^4$ $^5$ Thrombin clotting times in animals treated with enoxaparin showed transient increases of 77% (day 2), 53% (day 6), and a 69% (day 14) above baseline levels. Coagulation values at peak activity
time, along with anti-Xa levels between 0.5 and 1.01 IU/mL, confirm that these animals were anticoagulated sufficiently during the study. Animals that received anti-vWF in a treatment protocol had also increased bleeding time (BT) (Table).

**Prophylactic Arm**

Animals receiving the anti–P-selectin aptamer, in the prophylactic protocol, were within normal reference ranges for this thrombosis model.4,5 Animals receiving anti-vWF aptamer in a prophylactic manner had significantly increased BT values over baseline (Table).

**Anti-vWF Aptamer Modulates Platelet Aggregation**

Anti-vWF aptamer significantly decreased platelet aggregation when compared with baseline (nontreated), 60 minutes post administration (212.0±10.63 versus 92.0±12.7; closure time col/ADP seconds, \( P < 0.05 \)). Platelet aggregation was significantly inhibited at days 2, 6 (in both treatment and prophylactic groups), 10, and 14 (only in the treatment group) compared with baseline (\( P < 0.05 \)), thus confirming the inhibitory effects of ARC15105 on platelet aggregation.

**Discussion**

Deep VT (DVT) remains a significant healthcare problem today. Even with the most effective current therapies, there remains a significant risk of recurrence, extension of a primary DVT, and the development of pain and swelling leading to post thrombotic syndrome. A recurrence rate of 29% to 47% is observed with iliofemoral DVT without anticoagulation, 5% to 7% with full heparin anticoagulation, 4% to 5% with LMWH anticoagulation, and 3% to 9% with direct thrombin inhibitors.11–13 The incidence of the post-thrombotic syndrome is \( \approx 29\% \) after 8 years in treated patients, with the development of ipsilateral recurrent DVT strongly associated with an increased risk of this syndrome.14 In this study, we hypothesized that the inhibition of P-selectin or of vWF would be at least equal to, if not an improvement, over the most commonly used standard-of-care agent clinically used for VT prophylaxis and treatment, enoxaparin sodium, a LMWH. Nonhuman primate thrombosis models have flow hemodynamics and hematologic parameters that closely resemble human vasculature making them valuable models to determine the clinical usefulness of therapies for humans.15–19

The anti–P-selectin aptamer ARCS692 was effective in accelerating vein recanalization and decreasing vein wall collagen deposition, without adverse bleeding events post thrombosis, compared with animals receiving treatment with anti-vWF or LMWH, the standard-of-care therapy. Animals receiving anti–P-selectin aptamer therapy had vast improvements in vein recanalization that was significantly better than enoxaparin and controls. In addition, we observed a decreased effect after 6 days in animals receiving prophylactic P-selectin
aptamer. Day 6 was the final day of P-selectin inhibitor administration in the prophylactic protocol. We think that during the first 6 days, the continuous administration of the P-selectin inhibitor favors vein reopening. After its administration to the animals was stopped, a small rebound in thrombus deposition was observed.

Figure 4. Duplex ultrasound analysis: iliac vein valve function. The evaluation of the iliac vein valve was assessed in all groups at each time point. Results at day 21 post thrombosis. In the iliac veins that were occluded, the assessment was not possible (NA). Valve competency was defined as a valve closure time ≤1 s. Anti–Psel-Aptamer-Px indicates P-selectin inhibitor prophylaxis group; anti–Psel-Aptamer-Tx, P-selectin inhibitor treatment group; anti-vWF-Aptamer-Px, von Willebrand factor inhibitor prophylaxis group; anti-vWF-Aptamer-Tx, von Willebrand factor inhibitor treatment group; CTR, control group; LMWH, low–molecular-weight heparin treatment group; NA, not available; and VCT, valve closure time.

Figure 5. Contrast venography. Selected pictures from contrast venography, performed at days 2, 6, 14, and 21 after thrombosis, that show the recanalization process. Occlusion of the iliac vein and collateral circulation was observed in all groups at day 2. Of note, recanalization was evident directly by the channel in the iliac vein and indirectly by the reduction of collateral circulation. Anti–Psel-Aptamer-Px indicates P-selectin inhibitor prophylaxis group; anti–Psel-Aptamer-Tx, P-selectin inhibitor treatment group; anti-vWF-Aptamer-Px, von Willebrand factor inhibitor prophylaxis group; anti-vWF-Aptamer-Tx, von Willebrand factor inhibitor treatment group; CTR, control group; and LMWH, low–molecular-weight heparin treatment group.
There is supporting evidence for the probable mechanism that anti–P-selectin therapy decreases vein wall and thrombus fibrin deposition. Platelet–leukocyte interactions, mediated by P-selectin, have been implicated in thrombus amplification and stabilization. We observed a significantly decreased fibrin content in the thrombi in animals receiving anti–P-selectin in both prophylactic and treatment protocols. Our results are supported by Palabrica et al.\textsuperscript{20} that demonstrated that leukocyte accumulation and fibrin deposition in thrombi are P-selectin dependent. Also, the adhesion of monocytes and platelets through P-selectin lead to tissue factor release from monocytes, which initiates further coagulation and conversion of fibrinogen to fibrin.\textsuperscript{21} In addition, the localization of fibrin, platelets, and leukocytes in a developing thrombus supports this mechanism of fibrin deposition during VT.\textsuperscript{22} Our laboratory and others have previously shown, in rodent models of VT, that inhibition of P-selectin or its receptor P-selectin glycoprotein ligand-1 significantly decreases profibrotic mediators, such as interleukin-13, which selectively stimulates transforming growth factor-β and subsequent collagen deposition, and monocyte chemotactic protein-1. Monocyte chemotactic protein-1 directs monocytes to areas of inflammation and initiates a profibrotic environment.\textsuperscript{23,24} Anti–P-selectin therapy decreased vein wall collagen, fibrosis, and intrathrombus fibrin deposition, whereas conferring either decreased thrombosis or enhanced thrombus resolution\textsuperscript{5,25,26} In the present study, fibrin deposition and vein wall collagen were significantly decreased in animals treated with the anti–P-selectin aptamer, and these animals had significant vein reopening and retention of venous valve competency. In addition, the anti–P-selectin aptamer does not impair inflammatory recruitment to the vein wall. Paradoxically, we observed increased numbers of polymorphonuclear leukocytes, monocytes, and lymphocytes in the thrombotic area, in animals receiving anti–P-selectin aptamer. These results are consistent with previous work on VT using the same species.\textsuperscript{4,27} Interestingly, we did not find significant differences between groups on circulating leukocyte counts. Six hours after the thrombotic process was initiated, we observed an increase of circulating leukocytes that returned to baseline levels by day 21 in all groups.

Post-thrombotic syndrome has 2 underlying mechanisms that promote venous pathology, persistent venous obstruction, and valvular reflux.\textsuperscript{28} Ultrasound analysis demonstrated nonfunctioning valves in controls and animals treated with anti-vWF aptamer, whereas animals treated with anti–P-selectin aptamer had an equal rate of valve competency to enoxaparin. When agents were on board at the time of thrombus formation, both anti–P-selectin and anti-vWF aptamers demonstrated improved iliac vein valve competency, with the best results in the anti–P-selectin treated animals. In addition, we think that it is more likely that the efficiency of the agent to rapidly remove (or promote quick reduction in) the thrombus from the valve area was critical in improving valve function. vWF mediates the initial contact of platelets with the injured vessel wall and is necessary for normal hemostasis and
thrombosis. Its role in arterial disease has been well characterized. However, the role of vWF during VT and resolution is still under investigation.

In this study, anti-vWF aptamer treatment failed to promote vein reopening or limit the proliferation of vein wall collagen, 21 days post VT. The therapeutic levels of anti-vWF significantly inhibited platelet aggregation, as determined by the PFA-100 analysis, whereas it increased template BTs by 43% to 80% over control animals, yet did not promote vein recanalization. In arterial thrombosis, one of the most important differences is that platelet thrombus formation in vivo is initiated by endothelial injury, such as that occurring after atheroma rupture.29 Brill et al7 recently evaluated the role of vWF in 2 mouse models of VT. The authors found that vWF inhibition protected mice from VT more effectively in the presence of disturbed blood flow in one of the IVC stenosis model.30,31 In our nonhuman primate 6-hour balloon occlusion model of VT, all animals had confirmed occlusive thrombosis 2 days post induction.

This investigation indicated that the therapeutic dosing regimen for anti–P-selectin aptamer in our thrombosis model did not elevate any coagulation test versus the nontreated controls. Also, enoxaparin-treated animals, while having anti-Xa activity within the reported target range of 0.5 to 1 U/mL,32 had increases in thrombin clotting time, indicating the bleeding potential of this compound. Animals receiving the anti-vWF aptamer showed increased BTs for both prophylaxis and treatment protocols.4,5,26,33

Limitations

The number of animals per group in this work is small (n=4); however, we have found in our previous studies a valid sample size to discriminate statistical significances between those animals not given selectin inhibitors and those given the inhibitors using the same animal species.26

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<td>T6 15.7</td>
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<td>D6 20.6</td>
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<td>T6 133.5</td>
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<td>D2 321.7</td>
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<td>D6 298.3</td>
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<td>D14 185.0</td>
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<td>D21 163.3</td>
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TCT, fibrinogen, aPTT, and bleeding time were assessed in all groups at baseline, T6, and days 2, 6, 14, and 21. Anti–P-sel-Aptamer-Px indicates P-selectin inhibitor prophylaxis group; anti–P-sel-Aptamer-Tx, P-selectin inhibitor treatment group; anti–vWF-Aptamer-Px, von Willebrand factor inhibitor prophylaxis group; anti–vWF-Aptamer-Tx, von Willebrand factor inhibitor treatment group; aPTT, activated partial thromboplastin time; CTR, control group; D2, 2 days after thrombosis; D6, 6 days after thrombosis; D14, 14 days after thrombosis; D21, 21 days after thrombosis; and LMWH-Tx, low–molecular-weight heparin treatment group; and T6, 6 hours after thrombosis.

*Values out of range.
Our Data Bring Insights on Venous Thrombus Physiopathology

P-selectin inhibition was effective in both prophylactic and treatment applications. This suggests that the inflammatory and procoagulant factors involved with thrombus initiation and resolution are associated with P-selectin localization on platelets and endothelial cells. vWF inhibition was effective only in prophylactic application. This suggests that vWF has a greater participation in the early stages of thrombogenesis and plays a less important role in the later pathophysiology events of VT. In addition, the prolongation of BTs with vWF inhibition may make bleeding a higher potential side effect for the use in VT than in P-selectin inhibition.

Conclusions

The P-selectin inhibitor treatment promoted iliac vein recanalization better than enoxaparin and the vWF inhibitor treatment. The P-selectin inhibitor preserved valve competency equal to enoxaparin and better than the vWF inhibitor. Only the P-selectin inhibitor decreased vein wall fibrosis and exclusively did not cause any increase in bleeding parameters. The results of this work suggest that P-selectin inhibition may be an ideal target in the treatment and prophylaxis of DVT, warranting a clinical trial.

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Disclosures

R.G. Schaub, PhD, is currently Senior Vice President and Chief Scientific Officer at NKT Therapeutics, Waltham, MA. He worked for Archemix Corporation, Cambridge, MA, who supplied the aptamers used in this work, but no financial support was provided. The other authors report no conflicts.

References


**Significance**

New treatment options for venous thrombosis are needed because the current standard-of-care only prevents recurrence, pulmonary embolism, and the progression of the primary thrombus. Anticoagulation options do not prevent the development of post-thrombotic syndrome, and unfortunately carry with them significant bleeding risks.³ It is clear that an improved clinical approach is necessary. In an effort to gain new treatment options, our group has been studying P-selectin biology for the past 20 years. The results of this work, in the most translational animal model of venous thrombosis, support the benefits of P-selectin inhibition and the necessity for clinical trials.
P-Selectin Inhibition Therapeutically Promotes Thrombus Resolution and Prevents Vein Wall Fibrosis Better Than Enoxaparin and an Inhibitor to von Willebrand Factor


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