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BRIEF REPORT

Defibrotide Inhibits Antiphospholipid Antibody–Mediated Neutrophil Extracellular Trap Formation and Venous Thrombosis

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Objective. Defibrotide is a heterogenous mixture of polyanionic oligonucleotides currently approved for treatment of transplant-associated venoocclusive disease. While defibrotide has a known role in limiting endothelial cell activation, some studies have also demonstrated anti-leukocyte properties. In a recent study, we found that neutrophil extracellular traps (NETs) play a role in the thrombotic complications of antiphospholipid syndrome (APS). In the present study, we investigated the hypothesis that defibrotide might act to mitigate APS-relevant NET formation in vitro and in mouse models.

Methods. We used in vitro assays and a mouse model to determine the mechanisms by which defibrotide inhibits NET formation and venous thrombosis in APS.

Results. At doses ranging from 1 to 10 μg/ml, defibrotide significantly suppressed NET formation from control neutrophils stimulated with IgG isolated from patients with APS. Defibrotide increased levels of intracellular cyclic AMP in neutrophils, and its suppressive effects on NET formation were mitigated by blocking adenosine A_{2A} receptor or by inhibiting the cyclic AMP–dependent kinase protein kinase A. Defibrotide at doses ranging from 15 to 150 mg/kg/day inhibited NET formation and venous thrombosis in a model of antiphospholipid antibody–accelerated thrombosis—an effect that was reduced in adenosine A_{2A} receptor–knockout mice.

Conclusion. This study is the first to demonstrate mechanisms by which defibrotide counteracts neutrophil-mediated thrombotic inflammation inherent to APS.

INTRODUCTION

Antiphospholipid syndrome (APS) is a thromboinflammatory disease characterized by circulating antiphospholipid antibodies, classically anticardiolipin and anti–β_2-glycoprotein I (anti-β_2GPI). Meanwhile, additional relevant antibodies such as anti–phosphatidylserine/prothrombin can be detected by a functional screen called the lupus anticoagulant assay (1). APS is a leading acquired cause of both thrombotic events and pregnancy morbidity. Treatment of APS typically focuses on suppressing thrombosis with anticoagulation. However, anticoagulation does not fully protect against thrombotic events, conveys an increased risk of bleeding, and in many cases fails to restrain microvascular complications of APS such as diffuse alveolar hemorrhage, nephropathy, and livedoid vasculopathy.

Neutrophil extracellular traps (NETs) are web-like tangles of DNA, chromatin, and granule proteins released into the extracellular space by neutrophils in response to both infectious and sterile stimuli (2,3). NETs have been revealed as pathogenic actors in numerous autoimmune and thromboinflammatory diseases ranging from lupus to sepsis to COVID-19. To this end, recent work has pointed to a multifaceted (and generally deleterious) intersection between NETs and the vasculature. The proteases and histones of NETs kill endothelial cells (4). NETs stimulate type

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I interferon production, which reduces the numbers and function of restorative endothelial progenitors (5). Furthermore, NET-derived DNA triggers coagulation, while histones activate platelets (6).

In studies from our group and others, NETs have been observed to play a role in the thrombotic complications of APS. Neutrophils isolated from patients with APS have a diminished threshold for spontaneous NET formation, while neutrophils from healthy volunteers can be activated to release NETs by exposure to APS serum or purified antiphospholipid antibodies (7). In mouse models of antiphospholipid antibody–accelerated large vein thrombosis, treatments that counteract NETs such as neutrophil depletion (8), administration of intravenous deoxyribonuclease (8), agonism of neutrophil adenosine A_{2A} receptors (9), boosting neutrophil cyclic AMP (cAMP) levels (10), and interfering with adhesive interactions between neutrophils and the endothelium (11) are all protective.

Defibrotide is a mixture of polyanionic phosphodiester oligonucleotides isolated from porcine intestinal mucosa cells. Defibrotide is approved for the treatment of patients who have venooclusive disease (VOD) with hepatic, renal, or pulmonary dysfunction complications developing following hematopoietic stem cell transplantation (HSCT) (12,13). Defibrotide is considered a multitarget compound, and is best known for its ability to limit endothelial cell activation (14). At the same time, some older literature demonstrates anti-leukocyte and anti-neutrophil properties of defibrotide (15), with that research mostly completed prior to the first descriptions of NETs in 2004 (2). Almost 20 years ago, defibrotide was first suggested as a possible treatment for APS, especially the life-threatening microangiopathic variant known as catastrophic APS (CAPS) (16). However, this possibility has not been investigated in trials, nor have possible mechanisms been explored in the laboratory. Here, we hypothesized that defibrotide might act to mitigate APS-relevant NET formation in vitro and in mouse models.

MATERIALS AND METHODS

Isolation of human IgG. A protein G agarose kit (Pierce) was used to isolate IgG from the sera of patients with APS and healthy controls. This was done according to the manufacturer's instructions (Pierce) as we have reported previously (7,9).

Human neutrophil isolation and NET formation assays. Neutrophils were isolated from human blood as previously described by our group (7,9). NET formation was monitored using an assay that quantifies nuclease-liberated myeloperoxidase (MPO) activity. Neutrophils were cultured in RPMI medium (Gibco) supplemented with 0.5% heat-inactivated fetal bovine serum (Gibco) and 0.5% bovine serum albumin (Sigma) at 37°C. Neutrophils were seeded into 96-well plates at a density of 1×10^5/well. Stimulation was for 3 hours with 100 n*M* of phorbol 12-myristate 13-acetate (PMA; Sigma) or 10 μg/ml of IgG isolated from patients with APS (which was pooled from 5 primary APS patients). In some cases, cultures were also supplemented with different concentrations (1–40 μg/ml) of defibrotide (Jazz Pharmaceuticals), 10 μ*M* KT5720 (protein kinase A [PKA] inhibitor; Tocris), 10 μ*M* 8-cyclopentyltheophylline (adenosine A_1 receptor antagonist; Tocris), or 10 μ*M* SCH442416 (adenosine A_{2A} receptor antagonist; Tocris).

After stimulation, the culture medium was discarded, and the plate was gently emptied over a paper towel (to remove residual culture medium containing soluble MPO). Discarded medium was immediately replaced with RPMI medium alone or RPMI medium + 10 units/ml micrococcal nuclease (Thermo Fischer Scientific). The samples incubated with RPMI medium alone (without nuclease) were used to account for any NET-independent background signal. EDTA (10 m*M*) was used to stop the digestion of NETs after 10 minutes at 37°C. Supernatants were next transferred into a V-shaped 96-well plate, which was centrifuged at 350*g* to remove debris. MPO activity was then measured in a fresh plate by adding an equal volume of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (1 mg/ml; Thermo Fischer Scientific). The reaction was stopped 10 minutes later by the addition of 1 m*M* sulfuric acid (50 μl). Finally, a Cytation 5 Cell Imaging Multi-Mode Reader was used to measure absorbance at 450 nm.

Qualitative immunofluorescence microscopy. Neutrophils were seeded onto poly-L-lysine–(Sigma) coated coverslips. After fixing with 4% paraformaldehyde for 15 minutes, blocking was done with 1% bovine serum albumin overnight. Neutrophil elastase was labeled with a primary antibody (Abcam product no. 21595, diluted 1:100). The primary antibody was detected with a fluorescein isothiocyanate (FITC)–conjugated secondary antibody (SouthernBiotech product no. 4052-02, diluted 1:250). Hoechst 33342 (Invitrogen) was used to stain DNA. A Cytation 5 Cell Imaging Multi-Mode Reader was used to capture images.

Measurement of intracellular cAMP. Neutrophils were incubated for 30 minutes at room temperature with 1 μg/ml of defibrotide or 1 μ*M* CGS21680 (adenosine A_{2A} receptor agonist; Tocris). Other neutrophils were incubated for 10 minutes with 100 μ*M* forskolin (adenylyl cyclase activator; Tocris). Levels of cAMP were then measured using the Bridge-It cAMP Designer fluorescence assay kit (Mediomics catalog no. 122934) as instructed by the manufacturer and as we have done previously (10).

Animal housing and treatment. Mice were fed standard chow and housed in a specific pathogen–free facility. The

University of Michigan Institutional Animal Care and Use Committee approved all protocols. Male C57BL/6 mice were obtained from The Jackson Laboratory.

Adenosine A_{2A} receptor–knockout mice. We introduced a conditional knockout of the adenosine A_{2A} receptor in murine neutrophils (and other myeloid-lineage cells such as macrophages) using the Cre/loxP system. Mice with a "floxed" adenosine A_{2A} receptor gene (Adora2a$^{+/fl}$) on the C57BL/6 genetic background were purchased from The Jackson Laboratory (product no. 010687). Adora2a$^{+/fl}$ mice were bred to obtain homozygous Adora2a$^{fl/fl}$ mice. The Adora2a$^{fl/fl}$ mice were then crossed with hemizygote MRP8-Cre^{+} mice (purchased from The Jackson Laboratory; product no. 021614). The offspring (Adora2a$^{+/fl}$ MRP8-Cre^{+}) were then crossed with Adora2a$^{fl/fl}$ mice to obtain the experimental mice of interest: Adora2a$^{fl/fl}$ MRP8-Cre^{+} and Adora2a$^{fl/fl}$ MRP8-Cre^{-} (for the description of the breeding scheme see Supplementary Figure 1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.42017/abstract).

In vivo induction of venous thrombosis in mice. We used an electrolytic inferior vena cava (IVC) model that has been used previously by our group (9,10). After exposure of the IVC of mice, any lateral branches were ligated using 7-0 Prolene sutures. These side branches remained ligated for the duration of the experiment. Most animals had 1 or 2 side branches, but some animals had none (in which case no ligatures were placed). A 30-gauge silver-coated copper wire (KY-30-1-GRN; Electrospec) was placed inside a 25-gauge needle and inserted into the IVC. The wire was positioned against the anterior wall of the IVC where exposed copper wire at its end functioned as the anode. Meanwhile, a needle implanted subcutaneously completed the circuit and functioned as the cathode. For 15 minutes, a constant current of 250 μA was applied. The needle was then removed, and the abdomen was closed. Before recovery from the anesthetized state, the mice were intravenously injected with IgG from either healthy controls or patients with APS (500 μg); the IgG from APS patients was pooled from 3 patients experiencing an episode of CAPS. After 24 hours, mice were euthanized, and thrombus length was determined. Defibrotide sodium was diluted in saline and administered by retroorbital intravenous injection. Two injections were given; the first 24 hours prior to surgery and the second at the time of thrombus induction.

Quantification of MPO–DNA complexes. Serum was collected from the mice for MPO–DNA testing at the time of venous thrombus harvesting. MPO–DNA complexes were quantified as described previously (9,10). The protocol uses reagents from the Cell Death Detection ELISA kit (Roche) as well as an anti-MPO antibody (Bio-Rad0400-0002) that reacts with both human and mouse MPO.

RESULTS

Inhibition of NET formation by defibrotide in cultures of neutrophils with PMA or APS patient antibodies. We first tested the ability of defibrotide to suppress NET formation when control neutrophils were activated with PMA. We found that defibrotide significantly reduced PMA-triggered NET formation at concentrations as low as 1 μg/ml (Figure 1A). Beyond PMA stimulation, we reasoned that defibrotide might also prevent antiphospholipid antibody–mediated NET formation. Indeed, at concentrations as low as 1 μg/ml, defibrotide suppressed NET formation elicited from control neutrophils stimulated with IgG isolated from APS patients (pooled from 5 patients with primary APS) (Figure 1B). Defibrotide also suppressed NET formation by neutrophils isolated from patients with clinical features of APS who were "triple positive" for anticardiolipin antibodies, anti-β_2GPI antibodies, and lupus anticoagulant (Supplementary Figure 2, available at http://onlinelibrary.wiley.com/doi/10.1002/art.42017/abstract).

The impact of defibrotide on APS IgG–mediated NET formation was also assessed by immunofluorescence microscopy, with similar results (Figure 1C). In contrast to IgG from APS patients, IgG isolated from heterologous healthy controls did not increase NET formation by control neutrophils (Supplementary Figure 3, available at http://onlinelibrary.wiley.com/doi/10.1002/art.42017/abstract).

Elevation of cAMP levels and mitigation of antiphospholipid antibody–mediated NET formation by defibrotide through adenosine A_{2A} receptor agonism. Defibrotide has been reported to act as an adenosine receptor agonist in some settings (17–19), and we recently found that adenosine receptor agonism protects against both NET formation and venous thrombosis in APS (9). We therefore hypothesized that the inhibitory activity of defibrotide might be mediated through activation of adenosine A_{2A} receptors. In neutrophils, we found that defibrotide increased the level of intracellular cAMP in a manner similar to the synthetic adenosine A_{2A} receptor agonist CGS21680 and the adenylate cyclase activator forskolin (Figure 1D); defibrotide did not significantly increase cAMP levels in peripheral blood mononuclear cells (Supplementary Figure 4, available at http://onlinelibrary.wiley.com/doi/10.1002/art.42017/abstract).

We next considered that inhibiting the key cAMP-dependent kinase PKA might reverse the effects of defibrotide. Indeed, the ability of defibrotide to suppress NET formation was neutralized by a PKA inhibitor (Figure 1E). Finally, we also found that the ability of defibrotide to suppress NET formation could be partially

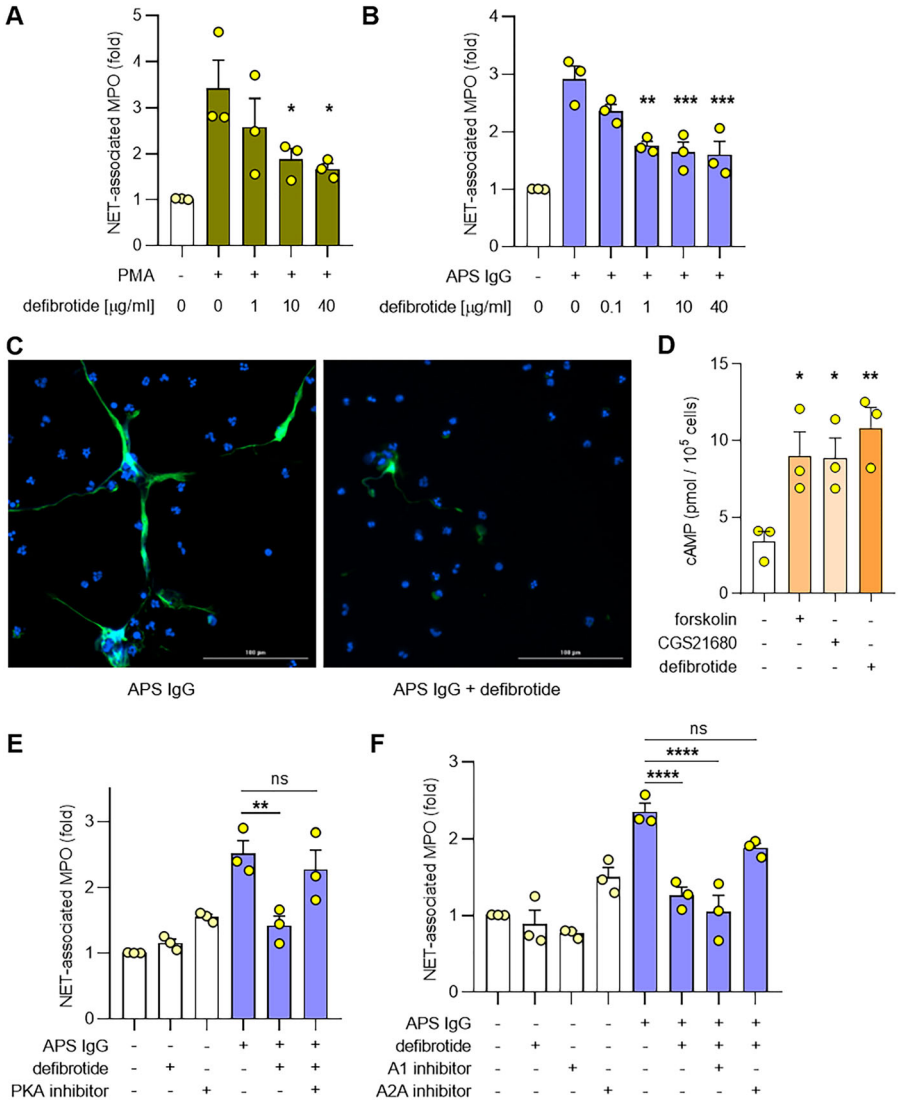

Figure 1. Defibrotide suppresses neutrophil extracellular trap (NET) formation in response to various stimuli through adenosine A_{2A} receptor agonism. **A** and **B**, Human neutrophils were isolated from healthy volunteers and then treated without or with phorbol 12-myristate 13-acetate (PMA) (**A**) or IgG from the sera of patients with antiphospholipid syndrome (APS) (**B**) for 3 hours in the presence or absence of different concentrations of defibrotide. NET formation was quantified by measuring the enzymatic activity of nuclease-liberated myeloperoxidase (MPO). **C**, NET formation in cultures of neutrophils treated with APS IgG in the presence or absence of defibrotide (1 μg/ml) was assessed qualitatively by immunofluorescence microscopy. Representative images are shown. Green = extracellular neutrophil elastase; blue = DNA. **D**, Human neutrophils were treated without or with forskolin (10 minutes), CGS21680 (30 minutes), or defibrotide (30 minutes), and cyclic AMP (cAMP) levels were measured. **E** and **F**, Neutrophils were treated with APS IgG in the presence or absence of defibrotide (1 μg/ml). Some samples were additionally treated with a protein kinase A (PKA) inhibitor (10 μ*M*), an adenosine A_1 receptor antagonist (10 μ*M*), or an adenosine A_{2A} receptor antagonist (10 μ*M*). NET formation was quantified by measuring the enzymatic activity of nuclease-liberated MPO. In **A**, **B**, **D**, **E**, and **F**, values are relative to untreated controls. Circles represent 1 of 3 independent experiments; bars show the mean ± SEM. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; **** = $P < 0.0001$ by one-way analysis of variance, corrected with Dunnett's test. NS = not significant.

reversed by blocking adenosine A_{2A} (but not adenosine A_1) receptors (Figure 1F). Notably, adenosine receptor antagonists had no effect on APS IgG–mediated NET formation in the absence of defibrotide (Supplementary Figure 5). Taken together, these data demonstrate that defibrotide can suppress NET formation and that this suppression is at least in part attributable to the activation of adenosine A_{2A} receptors.

Attenuation of antiphospholipid antibody–mediated venous thrombosis by defibrotide in wild-type mice but not adenosine A_{2A} receptor–knockout mice. Since defibrotide suppressed antiphospholipid antibody–mediated NET formation in vitro, we were interested in whether it might also mitigate antiphospholipid antibody–accelerated NET formation and thrombosis in mice. To test this, we utilized an electrolytic IVC

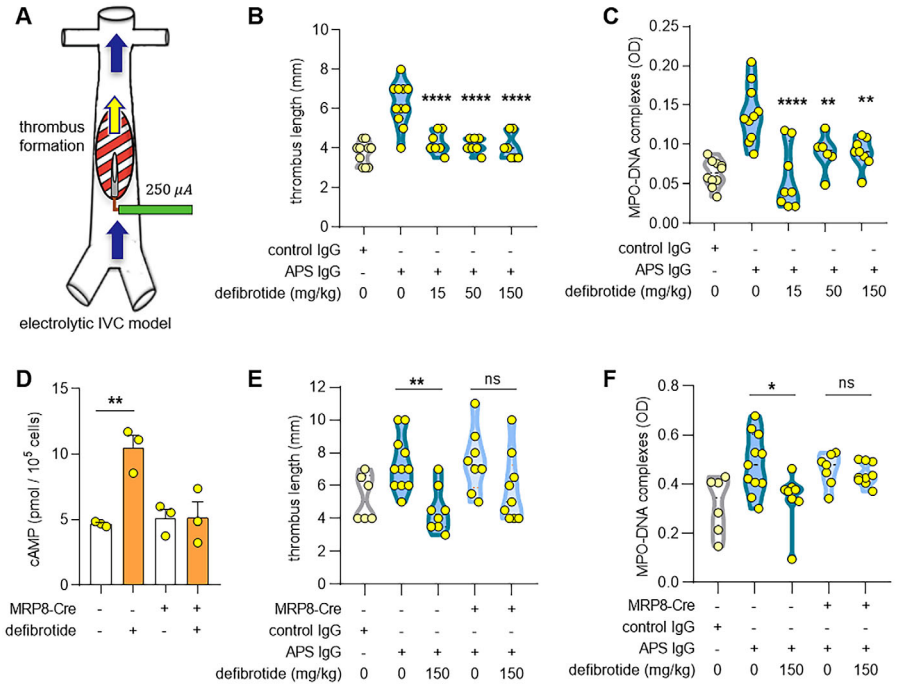

Figure 2. Defibrotide prevents antiphospholipid antibody–mediated venous thrombosis in wild-type mice but not in adenosine A_{2A} receptor–knockout mice. **A**, Schematic diagram depicting the electrolytic inferior vena cava (IVC) model of venous thrombosis. The application of direct current to a copper wire results in the release of free radicals. This activates endothelial cells and triggers a thrombogenic environment. Blood flow remains constant. **B** and **C**, C57BL/6J mice were treated without or with control IgG or APS IgG in the presence or absence of defibrotide. Thrombus formation was determined at 24 hours. Thrombus length (**B**) and MPO–DNA complexes (**C**) were quantified in the mouse serum. **D**, Cyclic AMP levels were determined in neutrophils isolated from Adora2a$^{fl/fl}$ MRP8-Cre^{+} mice compared with Adora2a$^{fl/fl}$ MRP8-Cre^{-} mice in the presence or absence of defibrotide (1 μg/ml) for 30 minutes. Circles represent 1 of 3 independent experiments; bars show the mean ± SEM. **E** and **F**, Adora2a$^{fl/fl}$ MRP8-Cre^{+} or Adora2a$^{fl/fl}$ MRP8-Cre^{-} mice were treated without or with control IgG or APS IgG in the presence or absence of defibrotide. Thrombus formation was assessed at 24 hours. Thrombus length (**E**) and MPO–DNA complexes (**F**) were quantified in the mouse serum. Circles in **B**, **C**, **E**, and **F** represent individual mice. * = $P < 0.05$; ** = $P < 0.01$; **** = $P < 0.0001$ by one-way analysis of variance, corrected with Dunnett's test. See Figure 1 for other definitions. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.42017/abstract.

model to induce large-vein thrombosis (Figure 2A) (9,10). Administration of IgG isolated from patients with APS (pooled from 3 patients with CAPS), but not control IgG, increased thrombus length in C57BL/6 mice, which returned to baseline levels when defibrotide was administered at doses as low as 15 mg/kg (Figure 2B). As expected, administration of antiphospholipid antibodies increased a surrogate marker of NETs in serum (MPO–DNA complexes), which again returned to baseline when mice were treated with defibrotide (Figure 2C).

Having demonstrated in vitro that the suppressive effects of defibrotide on NET formation could be partially reversed by blocking adenosine A_{2A} receptors, we considered that the suppressive effects of defibrotide on venous thrombosis might be reversed in myeloid-specific adenosine A_{2A} receptor–knockout mice. We first confirmed that neutrophils isolated from these mice were resistant to the ability of defibrotide to boost intracellular cAMP levels (Figure 2D). We then found that defibrotide was not able to prevent venous thrombosis (Figure 2E) or NET formation (Figure 2F) in adenosine A_{2A} receptor–knockout mice. Taken together, these data suggest that defibrotide mediates its antithrombotic effects at least partially through adenosine A_{2A} receptors.

DISCUSSION

This study is the first to demonstrate a mechanism by which defibrotide prevents disease-relevant NET formation. Defibrotide is indicated for the treatment of patients who have VOD associated with hepatic, renal, or pulmonary dysfunction following HSCT. In those settings, the therapeutic dosage of defibrotide is 6.25 mg/kg given intravenously every 6 hours (for a total dosage of 25 mg/kg/day). The drug is typically infused over several weeks and may continue up to a maximum of 60 days. Given that the role of neutrophils in VOD has yet to receive significant attention, we can speculate that the anti-neutrophil properties of defibrotide may play a protective role in VOD. This is certainly an area that we hope will be investigated in the coming years by our group and others.

The data presented here suggest that adenosine A_{2A} receptor agonism is at least part of the mechanism by which defibrotide reduces NET formation. Several reports suggest that defibrotide mediates its effects by targeting multiple adenosine receptors (for example, both A_1 and A_2) (17–19). In the present study,

blocking adenosine A_1 receptors did not interfere with the ability of defibrotide to suppress NET formation. These findings are similar to those of a previous study in which the effects of defibrotide were abolished by a dual adenosine A_1/A_2 receptor antagonist, but not by a selective adenosine A_1 receptor antagonist (18). It is worth noting that adenosine A_{2A} receptors are more abundantly expressed by neutrophils than are adenosine A_1 receptors (20). The extent to which complementary defibrotide-mediated mechanisms may be at play in mitigating NET formation and thrombosis is certainly an area deserving of future research.

In conclusion, these preclinical data support the possibility of defibrotide as a repurposed drug candidate for APS. Given a dearth of effective therapies for patients with the microvascular variant of APS, one can consider whether defibrotide warrants systematic study in such individuals, who in many cases will be receiving therapy in the inpatient setting where administration of defibrotide would be quite feasible.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Knight had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Ali, Erkan, Knight.
Acquisition of data. Ali, Estes, Gandhi, Yalavarthi, Hoy, Shi, Zuo.
Analysis and interpretation of data. Ali, Estes, Gandhi, Yalavarthi, Hoy, Shi, Zuo, Erkan, Knight.

ROLE OF THE STUDY SPONSOR

Jazz Pharmaceuticals provided funding for these investigator-initiated preclinical experiments but did not have input regarding the experimental design, nor did they participate in the data analysis. The authors independently collected the data, interpreted the results, and had the final decision to submit the manuscript for publication. Publication of this article was not contingent upon approval by Jazz Pharmaceuticals.

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